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A pilot study to characterize aquatic biodiversity using eDNA metabarcoding on 13 National Wildlife Refuges in Oregon and Washington

2025 Progress Report

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On the cover is an image of Hardy Creek with Beacon Rock in the distance at Pierce National Wildlife Refuge. Photo credit: Joe Skalicky.

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PROGRESS REPORT

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Abstract

Aquatic ecosystems within National Wildlife Refuges (NWRs) are threatened by a host of factors, including the spread of invasive species, anthropogenic influences and the effects of climate change, which can result in the decline or loss of species and their unique habitats. Understanding the biodiversity in aquatic systems is essential for detecting changes in community structure and critical for developing effective recovery, conservation, and management strategies. However, accurately characterizing aquatic species richness using traditional methods (e.g., electrofishing, trapping, visual observations) can be challenging, given staffing and funding restrictions. Environmental DNA (eDNA) metabarcoding is becoming an increasingly popular tool for assessing biodiversity in aquatic systems because it is fast, noninvasive, and often more sensitive at detecting species than traditional survey techniques. We conducted a pilot study in the summer of 2023 to assess the feasibility of using eDNA as an assessment and surveillance tool for aquatic species detection on refuges. We conducted eDNA sampling at 80 aquatic habitats at 13 refuges in Oregon and Washington using PCR primers targeting the Cytochrome c Oxidase I (COI) and 12S rRNA (MiFish) genes to detect fish and other aquatic and semi-aquatic taxa. The goal of this study was to 1) describe species richness detected in aquatic habitats using eDNA metabarcoding, 2) evaluate the efficacy of eDNA metabarcoding for detecting different taxa, and 3) discuss the utility and limitations of eDNA metabarcoding as a management tool. The COI genetic marker identified 492 Operational Taxonomic Units (OTUs) belonging to 21 phyla, where the highest proportion of reads were assigned to phylum Arthropoda (32%). Fish predominated the vertebrate class detected by COI (24 unique species), followed by mammals (nine species) and amphibians (one species). The MiFish marker identified 58 OTUs, including 43 fish, nine mammals, four birds, and two amphibians. Among refuges, the highest species richness was detected at Billy Frank Jr Nisqually NWR (172 total species) with the COI marker and WL Finley and Ankeny NWRs with the Mifish marker (16 total species). Overall, the MiFish marker detected more unique fish species than the COI marker. However, the MiFish marker had a much lower taxonomic resolution than COI, resulting in a lower rate of species-level detections (43% versus 96%, respectively). The COI marker produced a greater number of reads and identified a wider range of taxonomic groups, but 40% of total reads were assigned to non-target taxa such as algae and fungi. In summary, this study provided valuable insights into fish diversity and aquatic species richness at refuges. It established a baseline that can be used to monitor changes in aquatic biodiversity over time in response to environmental factors and/or management practices. Based

on our results, we offer recommendations for future eDNA assessments on refuges derived from lessons learned during the implementation of the pilot study.

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Introduction

The mission of the National Wildlife Refuge System is to administer a national network of lands and waters for the conservation, management and, where appropriate, restoration of the fish, wildlife and plant resources and their habitats within the United States for the benefit of present and future generations of Americans. National Wildlife Refuges (NWRs) manage an extensive and diverse array of lands, each with specific habitats supporting aquatic, semi-aquatic, and terrestrial species. Freshwater species and ecosystems are increasingly threatened by many compounding factors, including the spread of invasive species, a changing climate, and numerous anthropogenic impacts that can lead to decreased biodiversity and loss of habitats. Understanding the biodiversity in aquatic systems is essential for detecting changes in community structure and critical for developing effective recovery, conservation, and management strategies. However, accurately characterizing aquatic species richness using traditional methods (e.g., electrofishing, trapping, visual observations, and many other methods) can be expensive, labor-intensive, time-consuming, and require extensive taxonomic expertise. Further, staffing and stagnant budgets have limited refuge staff from collecting, assessing and meeting their management goals. Environmental DNA (eDNA) metabarcoding is becoming an increasingly popular tool for assessing the biodiversity in aquatic systems because it is fast, noninvasive, and often more sensitive at detecting species than traditional survey techniques (Ruppert et al. 2019; McElroy et al. 2020; Keck et al. 2022) and may be a valuable tool for NWRs.

Environmental DNA is genetic material shed by an organism in the form of cells, gametes, mucus, urine, feces, hair, and other organic material. This genetic material is released continuously and remains present in an environment until it is diluted, degraded, or dispersed by the environment. Fragments of expelled DNA can be captured in an environmental sample (e.g., air, soil, sediment, or water) and extracted to confirm the presence of an organism without the need to capture or observe the organism directly. While early eDNA studies were often focused on the detection of single species in aquatic environments using species-specific PCR primers (Ficetola et al. 2008; Goldberg et al. 2011; Jerde et al. 2011; Goldberg et al. 2013; Piaggio et al. 2014), eDNA metabarcoding is quickly becoming the primary method for characterizing ecosystem-level biodiversity (Deiner et al. 2016; Lacoursière-Roussel et al. 2018; Lozano Mojica and Caballero 2020; Clusa et al. 2021; Macher et al. 2021; Ruppert et al. 2019). Environmental DNA metabarcoding uses universal PCR primer sets designed to amplify DNA from a specific taxonomic group (e.g., fish, mammals, insects, plants) or a broad range of organisms (e.g., all eukaryotes) within an environmental sample, allowing researchers to identify multiple species present by sequencing the amplified DNA using high-throughput next-generation sequencing and comparing the sequences to a reference database for taxonomic assignment.

While eDNA metabarcoding has proven effective in diverse ecosystems and for various species, there is a significant challenge in the lack of standardized sampling methods, making direct comparisons between research findings difficult. In general, the successful detection of species using eDNA relies on a complex interplay of factors, including where samples are collected, the number of sample replicates, volume of water filtered, filter material, filter pore size, DNA

extraction methods, selection of genetic markers and PCR primers, sequencing approach, and the bioinformatics analysis used to interpret the results. Additionally, environmental variables such as water temperature, discharge or flow rate, UV radiation, water chemistry, algae and bacteria density, organic material levels, and characteristics of the target organisms (e.g., species, size, abundance, distribution, eDNA shedding rate), can all impact the persistence and concentration of DNA present at a given sample location (Pilliod et al. 2013; Barnes et al. 2014; Herder et al. 2014; Strickler et al. 2014; Jane et al. 2015; Goldberg et al. 2016). Thus, a pilot study is a valuable way to test and refine eDNA sample methods and identify potential issues (e.g., filter clogging and sample inhibition) before committing to a larger, more expensive or long-term study.

The Columbia River Fish and Wildlife Conservation Office (CRFWCO) conducted an eDNA metabarcoding pilot study in the summer of 2023 to assess the feasibility of using eDNA as an assessment and surveillance tool for aquatic species detection on refuges. This study had the following objectives: 1) describe species richness detected in aquatic habitats using eDNA metabarcoding, 2) evaluate the efficacy of eDNA metabarcoding for detecting different taxa, and 3) assess the utility and limitations of eDNA metabarcoding as a species detection tool.

Methods

eDNA Site selection

We conducted eDNA sampling at 80 aquatic habitats at 13 NWRs in Oregon and Washington. Specific refuge selection for eDNA sampling was influenced by multiple factors, including distance from the CRFWCO in Vancouver, WA, having an adequate number of water bodies, and support from the respective refuge staff. Generally speaking, a refuge was selected if it was within a two to three-hour drive of the CRFWCO, had three to five relevant water bodies, and had refuge staff available to assist in the field and navigate to specific sites often behind locked gates. After selecting refuges, we met individually with refuge staff to review, assess, and select specific locations for sample collection at each water body.

For sample collection, the Refuge biologist or manager provided access and navigation to specific sites, often behind locked gates and without roads. At each sample site, locations were georeferenced, and a photograph was taken to document current physical habitat conditions. Baseline habitat characteristics (e.g., temperature, conductivity, turbidity, maximum water depth, dominant substrate type, dominant aquatic vegetation, percentage aquatic vegetation cover) were also recorded at each sample site using ArcGIS Survey123.

eDNA sample collection and filtration

Up to three eDNA samples were collected at each refuge water body depending on water quality (i.e., turbidity, presence of algae) and the size of the water body. Samples were collected using disinfected 1.0L or 2.0L Nalgene bottles and two-gallon Ziploc bags, or were filtered directly from the water body. Grab samples (i.e., Nalgene bottles and Ziploc bags) were collected from three different locations across the width of the water body (e.g., left bank, mid-channel, right bank) or spread evenly along the perimeter of the water body. Bottles and Ziplocs were triple rinsed with water at the site before water collection. All samples were collected from the surface, either by hand-dipping bottles/bags just below the surface of the water, holding the filter funnel

just below the water surface with an extension pole, or lowering the filter funnel into the water from a culvert or bridge. Water samples were filtered immediately on site using a 5.0µm cellulose nitrate filter and peristaltic pump. Water was filtered until a total of 5.0L was filtered, or until the filter was clogged (i.e., defined as a filtration flow rate of ~1 drop per second) and the volume of water passed was recorded. Filter membrane disks were folded and placed in a sterile 2.0ml vial with 100% ethanol and placed on ice in coolers until returning to the office. Samples were stored in a standard freezer (-18°C) until they were submitted to the Washington Department of Fish and Wildlife (WDFW) Molecular Genetics Lab for analysis. Ten field negative control samples were also collected throughout the duration of the study. Field controls were usually collected at the beginning of the day and consisted of filtering 5.0L of distilled water brought into the field. Control samples were processed in the field in the same manner as eDNA samples to assess potential for sample contamination associated with sample collection and handling or poor equipment disinfection techniques.

A general concern with the eDNA technique is the possibility of obtaining a false positive result due to field or lab contamination. In an effort to minimize this risk in the field, care was taken to remain out of the water or downstream of the filter funnel or sample bottle while acquiring water samples. In flowing water bodies, samples were collected from downstream to upstream sites. New and separate nitrile gloves were worn during sample collection, filtration and between sample sites. At the end of the day, equipment in direct contact with water samples (i.e., Nalgene bottles, forceps) were decontaminated by soaking in a 50% bleach solution for a minimum of one minute before rinsing and drying thoroughly. Other sampling components not in direct contact with water samples (i.e., silicone tubing, water outflow container) were soaked in a 10% bleach solution and rinsed daily. While we collected most eDNA samples without entering the water, waders and boots were disinfected in a 1% solution of Virkon Aquatic for a minimum of 30 minutes or sprayed with a bleach solution to prevent the spread of aquatic invasive species.

DNA extraction, PCR, and Library Preparation

All laboratory work was performed by the Washington Department of Fish and Wildlife's (WDFW) Molecular Genetics Lab in AirClean 600 Workstations (ISC Bioexpress) equipped with HEPA air filters and UV lights. All work surfaces were sterilized with 10% bleach and exposed to UV light for a minimum of one hour. DNA extractions were performed using the Qiagen DNeasy Blood & Tissue and Qiashredder kits (Qiagen, Inc.) per Pilliod et al. (2013). One extraction blank was processed per batch of 47 filters.

The mitochondrial cytochrome c oxidase subunit I (COI) and 12S rRNA (MiFish) genes were amplified in separate reactions. The COI gene region was amplified with the Leray-XT primers (Wangensteen et al. 2018), and the 12S gene region was amplified with the MiFish-U primers (Miya et al. 2015). The Leray-XT primers amplified a ~313bp gene fragment and included the forward primer mlCOIintF-XT 5′-GGWACWRGWTGRACWITITAYCCYCC-3′ (Wangensteen et al. 2018), modified from the original mlCOIintF primer developed by Leray et al. (2013), and the reverse primer jgHCO2198 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′ (Geller et al. 2013). The MiFish-U primers amplified a ~170bp gene fragment and included the forward primer 5′-GTCGGTAAAACTCGTGCCAGC-3′ and the reverse primer 5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′ (Miya et al. 2015).

PCRs were performed in 30µL volumes using the Multiplex PCR Kit (Qiagen). COI reactions contained 15µL (1X) master mix, 0.8µM of each primer, and 2µL of template DNA with thermal cycling conditions as follows: 95°C for 10min, 35 cycles of 95°C for 60sec, 50°C for 60sec, and 72°C for 60sec, and a final extension of 72°C for 5min. 12S reactions contained 15µL (1X) master mix, 2µM of each primer and 1µL of template DNA with thermal cycling conditions as follows: 95°C for 10min, 14 touchdown cycles of 94°C for 30sec, 69.5-50°C for 30sec, and 72°C for 90sec, 25 cycles of 94°C for 30sec, 50°C for 30sec, and 72°C for 45sec, and a final extension of 72°C for 10min. A PCR negative and positive control were included on each 96-well PCR plate. The negative control consisted of sterile molecular grade water in lieu of template DNA and kangaroo DNA was used as positive control template.

PCR products were size selected using Mag-Bind® TotalPure NGS (Omega Biotek) beads. A ratio of beads to product of 0.8X was used for the COI amplicon, and 1.2X was used for the 12S amplicon. Sample amplicons were then indexed with Nextera DNA unique dual (UD) indexes (IDT® for Illumina®), normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen), and each 96-well plate was subsequently pooled. Plate libraries were bead cleaned at a 0.8X bead ratio, quantified with a Qubit fluorometer (Invitrogen), and pooled by amplicon. Amplicon libraries were quantified and normalized to 4nM prior to loading at a 1:20 MiFish to COI ratio on the Illumina NextSeq™ 1000 platform. Sequencing was performed using the NextSeq™ 1000 P1 (600 cycles) Reagent Kit for paired end reads (Illumina).

Bioinformatic Processing

Amplicon sequence data were analyzed separately (COI and 12S) using either stand-alone QIIME 2 (Bolyen et al., 2019) and DADA2 (Callahan et al. 2016) or with Tourmaline (https://github.com/aomlomics/tourmaline), a Snakemake pipeline that wraps QIIME 2 and DADA2, providing reproducible metabarcoding analysis. Adapters and primers were trimmed from demultiplexed FASTQ reads using Cutadapt (Martin 2011). The program DADA2 was used to quality filter reads. Reads were truncated to a common length (200bp for the COI amplicon and 160bp for the 12S amplicon) with a maximum number of expected errors = 2, remove chimeras (consensus method), and export amplicon sequence variants (ASVs). To assign COI taxonomy, a custom reference database was generated to include the MIDORI database (Machida et al. 2017), containing over 580,000 nucleotide sequences and all mitochondrial COI sequences in the NCBI nucleotide database. To assign 12S taxonomy, a rCRUX (Curd et al. 2024) generated reference database was used that included all 12S sequences in the NCBI nucleotide database and an additional custom database comprised of all Actinopterygii mitogenomes (Gold et al. 2023). Global taxonomic alignments between query and reference reads were performed using the VSEARCH consensus taxonomy classifier (Rognes et al. 2016), and matches with ≥ 97% identity were retained. A table summarizing read counts for each operational taxonomic unit (OTU) per sample was generated. An OTU is a group of closely related individuals which are arranged together based on the similarity of specific sequences.

In order to remove potential false positives, contaminants or sequencing errors, the OTU table was filtered to remove all OTUs with less than 100 total reads as per guidance from the WDFW genetics laboratory. Read counts less than 100 are considered low reliability and may not be true indicators of presence.

Results

Cytochrome c Oxidase I (COI)

A total of 80 eDNA samples and 10 field negative control samples were collected across 13 NWRs (Figure 1; Table 1). The volume of water filtered for each eDNA sample ranged from 0.1-5.0 L (Mean 2.12 L). No contamination was detected in lab-negative controls from DNA extraction or PCR, however, DNA amplification occurred in eight of ten field-negative control samples with the COI genetic marker. Sequencing these negative controls resulted in 483,087 reads, of which 91% were assigned to three species: varied carpet beetle (*Anthrenus verbasci*; 232,797 reads), fungi (genus *Cladosporium*; 129,519 reads) and humans (*Homo Sapiens*; 75,869 reads) (Table 2). Read counts obtained from field negative controls were subtracted from each field sample processed on the same day, resulting in the removal of fungi detections in 17 sites, human detections in 3 sites, and golden algae detections (*Peduspumella encystans*) in 2 sites.

Eight COI samples did not pass quality filtering parameters (n = 7) or did not amplify DNA (n = 1). The remaining 72 samples produced a total of 38.42M sequencing reads, of which 33.16M were unassigned. Retaining only OTUs with reads ≥100 and removing read counts attributed to contamination in field negative controls, a total of 5.11M reads were assigned to the COI genetic marker (excluding unassigned reads). An average of 10,393 reads were obtained from each sample. COI analysis identified 492 OTUs belonging to 21 phyla, with the most significant proportion of reads assigning to phylum Arthropoda (32%; Figure 2). Of the 492 OTUs, 70% (342) were identified to species belonging to 216 families and 306 genera. The OTU with the highest cumulative reads was a diatom (*Melosira ambiqua*; 886,334 reads) detected in 14 sites. The OTU with the highest rate of occurrence was a plant pathogen of genus *Pythium* detected in 43 total sites.

Fish predominated the vertebrate class detected by COI, with 24 unique species belonging to 11 families and 15 genera (Figure 3). Fish from the family Cyprinidae (5 species) and Salmonidae (5 species) dominated the fish communities. At the species level, the most abundantly detected fish was the prickly sculpin (*Cottus asper*) detected at 17 sites, followed by cutthroat trout (*Oncorhynchus clarkii*) detected at 13 sites. The species with the highest read count was brown bullhead (*Ameiurus nebulosus*; 8,833 reads), followed by pink salmon (*Oncorhynchus gorbuscha*; 6,837 reads). Nonnative fish species (n = 10) accounted for 42% of all detected fish species, with the most abundant being the brown bullhead detected at six sites (Figure 3). COI also detected the presence of Olympic mudminnow (*Novumbra hubbsi*) at the Nisqually NWR (Dempsey Creek site 1), which is listed as a sensitive species in Washington.

A total of nine mammal species belonging to eight families were detected by COI . The American beaver (*Castor canadensis*) was detected at the most sites (20 sites) and cattle (*Bos taurus*) was the species with the highest read count (127,998 reads), followed by American beaver (27,804 reads). Unique mammal species detected by the COI marker included the Pacific jumping mouse, deer mouse, long-tailed vole, and North American river otter. The American bullfrog was the only amphibian species detected by COI (1,867 reads) at eight sites.

The Phylum Arthropoda was the dominant invertebrate taxa with 156 OTUs assigned to 85 species. Eighteen species belonged to the order Diptera (true flies), and 12 species belonged to

the order Diplostraca (water fleas). The mahogany dun mayfly (*Paraleptophlebia debilis*) was the invertebrate species detected in the most sites (14 sites), and the crustacean species, *Chydorus brevilabis* had the highest read count (466,156 reads) and was detected in 13 sites. Finally, COI detected 17 species of mollusks representing two classes, Gastropoda (n = 13) and Bivalvia (n = 4). Four mollusk taxa are considered nonnative/invasive in Oregon and Washington, including the Asian clam (Genus *Corbicula*) which were detected in nine sites, and the New Zealand mudsnail (*Potamopyrgus antipodarum*) which were detected in three sites (see Appendix for locations) and also had the highest read count of all mollusk species (20,415 reads). Eight marine species were detected at Nisqually NWR (four sites) and Willapa NWR (one site), including several species of sea slugs, manila clam and Pacific Blue mussel. Also of note was the detection of the native western pearlshell mussel in a single site at Willapa NWR (Bear River site 2). Though western pearlshell mussels (three sites), Oregon floater mussels (16 sites), and western ridged mussels (one site) were also detected at other locations, read counts were below the minimum threshold of ≥100 reads and were removed from the final OTU table.

MiFish_12S

Five field negative control samples (50%) amplified DNA with the MiFish genetic marker (Table 2). Sequencing these negative controls resulted in 11,070 reads of which 100% were assigned to *Homo sapiens*. Read counts obtained from field blanks were subtracted from each field sample processed on the same day, resulting in the removal of human detections in 5 samples.

Sixteen eDNA samples did not pass quality filtering parameters (n = 5) or did not amplify DNA (n = 11). The remaining 64 samples produced a total of 1.05M sequencing reads, of which 179,553 were unassigned. Retaining only OTUs with reads ≥100 and removing read counts attributed to contamination in field negative controls, a total of 859,026 reads were assigned to the MiFish genetic marker (excluding unassigned reads). An average of 14,559 reads were obtained from each sample (range 0 – 213,961 reads). The MiFish marker identified 58 OTUs from 35 unique families, including 43 fish, nine mammals, four birds, and two amphibian OTUs.

The most abundantly detected fish was the sculpin (genus *Cottus*), which was detected at 39, sites followed by cutthroat trout at 24 sites. The fish species with the highest cumulative read count was also sculpin (213,961 reads), followed by three-spined stickleback (*Gasterosteus aculeatus*; 96,095 reads) (Figure 4). Nonnative species accounted for 47% of all detected fish, with the most abundant being bluegill (*Lepomis macrochirus*), which were detected at 11 sites, and the pond loach detected at eight sites. Nonnative fish from the genus *Ameiurus* (bullhead) and family Cyprinidae (carp family) had higher site detection (14 and 13 sites respectively) and read counts (41,462 and 41,450 reads, respectively), but the OTUs were not identified to species (Figure 4). The MiFish marker detected seven marine fish species, including the Pacific staghorn sculpin (two sites), shiner perch (four sites), Pacific herring (one site), saddleback gunnel (one site), albacore tuna (3 sites) and a fish from the flounder family (Pleuronectidae) detected at two sites. The albacore tuna detection is intriguing because it occurred at three separate sites at Ankeny NWR (Sidney Power Ditch sites 1 and 2, Bashaw site 2) located in the Willamette Valley, OR nearly 100 miles from the Pacific Ocean. These may be false positive detections or could be the result of baited crayfish traps or other human-related causes. Also of interest was the detection of two species listed as sensitive species in Oregon and Washington. The Oregon

Chub (*Oregonichthys carmeri*) was detected at a single site at Ankeny NWR (Sidney Power Ditch site 2), and the Olympic mudminnow which was detected in two separate locations at Nisqually NWR (Dempsey Creek Site 1 and Unnamed Tributary).

A total of nine mammal OTUs were detected by the Mifish marker. American beavers and humans were the most abundant mammals detected at 16 and 14 sites, respectively. Cattle was the species with the highest read abundance (50,187 reads), followed by the American beaver (5,974 reads) and nutria (*Myocastor coypus*; 5,730 reads). Unique mammal species detected by the Mifish marker included raccoon *(Procyon lotor*), montane vole (*Microtus montanus*), mouse-eared bat (genus *Myotis*) and wild boar (*Sus scrofa*). The putative detection of wild boar is most likely explained by the detection of domestic pigs, which cannot be distinguished from wild boar based on the analyzed 12S gene region. The MiFish marker also detected three common bird taxa, including the wood duck (*Aix sponsa*), mallard duck (*Anas platyrhynchos*), and crow family (Corvidae), and two amphibians including the rough-skinned newt (*Taricha granulosa*) and coastal giant salamander (*Dicamptodon tenebrosus*).

COI & MiFish

Among Refuges, the highest species richness was detected at Nisqually NWR (172 total species) and Julia Butler Hansen NWR (108 total species) with the COI marker. Considering the MiFish marker, Finley and Ankeny NWRs had the highest species richness, with 16 OTUs identified to species. Baskett Slough NWR had the lowest species richness (four species COI; two species MiFish), where only one of three potential sites was sampled due to high turbidity conditions (Figure 5). Nisqually NWR had the most fish species detected by the COI marker (10), including four species of salmon and three unique species of sculpin (Figure 6). Ridgefield NWR had the highest number of unique fish detected by the MiFish marker (19) and the highest percentage of nonnative fish species among sampled refuges (63% nonnative species).

Only considering fish and mammals, a total of 44 unique fish and 13 mammal species were detected by COI and MiFish markers cumulatively. Fifteen fish and five mammals were detected by both markers (Figure 7; Figure 8). Nine fish and four mammals were unique to the COI marker, while 20 fish and four mammals were unique to the MiFish marker. Mammalian species detected by both genetic markers were either highly abundant (e.g., cattle, humans) or heavily reliant on the aquatic environment (e.g., American beaver and nutria). The MiFish marker detected 24% more fish species than the COI marker (including seven marine species), but only 43% of taxa were identified to species versus the COI marker which identified 96% of fish to species level.

A summary of eDNA results for each refuge can be found in Appendix A.

Discussion

Describing and monitoring species richness is an integral part of ecosystem management on NWRs. Environmental DNA metabarcoding is a powerful tool for assessing biodiversity because it can simultaneously detect multiple species (e.g., fish, bivalves, macroinvertebrates) within a single water

sample. In some cases, it may outperform traditional sampling methods (McColl-Gausden et al. 2020; Czeglédi et al. 2021; Gehri et al. 2021; Keck et al. 2022; Picq et al. 2024). The goal of this pilot study was to assess the utility of eDNA metabarcoding for characterizing aquatic biodiversity within various aquatic habitats (e.g., small streams, large rivers, sloughs, lakes) and enhance knowledge of native and nonnative species currently present at NWRs. A secondary goal was to provide recommendations to inform subsequent eDNA-based assessments (i.e., targeting specific taxa, rare or invasive species) at NWRs.

eDNA metabarcoding as a tool to monitor aquatic biodiversity

In eDNA metabarcoding, the choice of genetic marker and PCR primer pair can significantly impact the detection of specific species or taxonomic groups (Zhang et al. 2018; Harper et al. 2018; Pappalardo et al. 2021; Abidin et al. 2022; Fontes et al. 2024; Ferreira et al. 2024). Here, we used cytochrome c oxidase 1 (COI) and 12S rRNA (Mifish) genes to describe species richness in aquatic habitats across 13 NWRs. The COI marker can detect a diverse array of species within the eukaryotic kingdom, and the MiFish marker is primarily designed to detect fish species. However, it can often amplify the DNA of other vertebrates. Overall, the COI marker produced more reads and identified a wider range of taxonomic groups than the MiFish marker (Abidin et al. 2022; Baetscher et al. 2023). An impressive 492 OTUs (24 phylum, 342 species) were detected with the COI marker, compared to 58 total OTUs (1 phylum, 38 species) detected with the MiFish marker. The COI gene has conserved regions that allow primers to bind and amplify DNA across a wide range of taxa. However, because of this compatibility, the COI primers can also amplify DNA from species that are not the primary target of the study, potentially overwhelming or "masking" the signal from desired target species in the sample. (Collins et al. 2019; Kumar et al. 2022; Xu et al. 2024). Of the 5.11M total reads assigned to the COI marker (excluding unassigned reads), only 7% were assigned to chordates, and 40% were assigned to non-target taxa such as algae, bacteria, and fungi. For this reason, COI metabarcoding markers may be less suitable for use in waterbodies with high microbial and plankton densities and for projects targeting the detection of vertebrates (Collins et al. 2019; Jackman et al. 2021).

Among all vertebrate groups, fish were the most frequently detected by eDNA metabarcoding. Multiple studies have compared the performance of genetic markers or primer sets for detecting fish and found that the MiFish marker generally detects a greater diversity of fish species than COI (Collins et al. 2019; Shu et al. 2021; Abidin et al. 2022; Xu et al. 2024). We also found that the MiFish marker detected more unique fish species (35) than the COI marker (24). However, it had a much lower taxonomic resolution than COI, resulting in a lower rate of species-level detections. Only 43% of fish were identified to species level with the MiFish marker, while the COI marker identified 96% of fish to species level. For example, the MiFish marker could only identify sculpin to the genus level (*Cottus*). In contrast, COI was able to differentiate three different species of native sculpin (coastrange, inland riffle, and prickly sculpin). Additionally, the COI marker successfully identified pink salmon in the South Sound and Nisqually River (Nisqually NWR) and brook trout in Bird Creek (Conboy Lake NWR), which were both only identified to genus by the MiFish marker in the same locations.

Mammals were the next most represented group of vertebrates in terms of read proportions (240,309 cumulative reads) and species richness (13 unique species). Birds (three total species) and amphibians (three total species) were generally less represented. No reptiles were detected by COI and MiFish markers. The relative absence of birds and amphibians was notable but not unexpected, given similar findings in another study using the same genetic markers and primers (He et al., 2023).

Primer bias may have played a role in the infrequent detections of mammals, birds, and amphibians in our study. Metabarcoding primers may preferentially amplify DNA sequences from some species more than others (e.g., highly abundant species), leading to an underrepresentation of specific taxa in the sample. This may be caused by base pair mismatches between the primer and target DNA, an incomplete reference database, or low target DNA levels due to factors like low species abundance, poor eDNA shedding, DNA degradation, sample filter volume, or PCR inhibition (Kelly et al. 2014; Bylemans et al. 2019; Tsuji et al. 2022). Our results highlight the importance of using genetic markers and primers targeted to the taxonomic groups of interest to minimize detections of non-target species. While COI and MiFish are broadly used markers for eDNA metabarcoding, the primers used in this study were not optimized for all vertebrates other than for fish (MiFish). Using primers specifically targeted to amphibians (Sakata et al. 2022), birds (Ushio et al. 2018), and mammals (Ushio et al. 2017) could improve the detection rates of these groups.

Using multiple (i.e., two or more) genetic markers and primer pairs can reduce primer bias and provide a more comprehensive picture of biodiversity within a sample (Harper et al. 2018; Collins et al. 2019; Gehri et al. 2021; Pappalardo et al. 2021; Kumar et al. 2022; Wang et al. 2023; Jones et al. 2024; Ferreira et al. 2024; Xu et al. 2024). It can also reduce the loss of information if a sample fails to amplify successfully. In this study, there were 21 sites where an eDNA sample did not amplify DNA or did not pass quality filtering parameters. In 18 of these sites, at least one of the two genetic markers ran successfully, providing important diversity information that could have been lost. Using two genetic markers also improved the taxonomic resolution of ten fish species and increased our detection of unique fish species (29 total), providing a more comprehensive picture of fish diversity at NWRs. Further, common species identified by both genetic markers (15 fish species), helped reinforce our results' validity. While employing multiple markers increases the complexity and cost of the metabarcoding analysis, the potential gain in species detection often outweighs this factor, especially when studying diverse aquatic ecosystems. COI and MiFish genetic markers have merits that make them useful for species detection. The COI marker generally offers better species-level discrimination power. In contrast, the Mifish marker is better at capturing the depth of diversity within a specific group of taxa, such as fish. Managers should carefully consider the specific needs of their study when selecting the most appropriate genetic marker and primer set for a metabarcoding project.

Advantages and limitations of eDNA metabarcoding

Environmental DNA metabarcoding offers several advantages over traditional methods for assessing species richness, including increased sensitivity for detecting rare or elusive species, the ability to identify a broad range of taxa simultaneously, non-invasive sampling, higher taxonomic resolution, and collecting eDNA samples requires no taxonomic expertise (Ruppert et al. 2019; Rishan et al. 2023; Wee et al. 2023) or sampling permits, which can have limited application windows. Metabarcoding is often a faster and less labor-intensive method for studying aquatic biodiversity compared to traditional sampling techniques, allowing researchers to monitor species in challenging, sensitive, or difficult-to-access environments without capturing or disturbing an organism to obtain a positive detection. Environmental DNA metabarcoding is a powerful tool for ecological studies, including biodiversity monitoring, population dynamics, early detection of invasive species, conservation planning, and assessing how species presence and abundance may change over time in response to environmental shifts like climate change (Rishan et al. 2023; Wee et al. 2023). Also, eDNA samples can be archived and reanalyzed for species based on new research questions or study objectives many years after they were collected.

Several factors can limit the reliability of eDNA metabarcoding, including environmental conditions affecting DNA degradation and persistence (e.g., water temperature, UV exposure, water chemistry, water flow), uneven DNA shedding rates between species, spatial and temporal variability in eDNA distribution, incomplete or inaccurate reference databases, primer and PCR biases, the difficulty in detecting rare or low-abundance species (compared to species-specific qPCR) and contamination; all of which can lead to inaccurate or biased community composition assessments. In this study, our results were likely influenced by environmental conditions, community composition, and pilot study design. We collected eDNA samples in late summer when water levels were low. As a result, many sites (especially those with minimal water flow, such as lakes, ponds, and estuarine habitats) had high turbidity levels, significantly reducing the efficiency of sample filtration. Elevated turbidity can quickly clog filters, reducing eDNA recovery, potentially leading to an underestimation of species present in the water body. Large amounts of organic matter in turbid water can also introduce PCR inhibitors that interfere with the amplification of DNA in the lab, which can significantly lower the efficiency of detection or cause the complete failure of the analysis. Many eDNA studies indicate that filtering a larger volume of water leads to a higher yield of captured eDNA, thereby increasing the likelihood of detecting species present in the water sample (Blabolil et al. 2019; Muha et al. 2019; Bessey et al. 2020; Bruce et al. 2021; Takahashi et al. 2023). Additionally, a 0.45 µm pore size filter is commonly used for capturing eDNA because it effectively traps a broad spectrum of DNA fragments while maintaining a suitable water flow rate (Li et al. 2018; Rishan et al. 2023; Takahashi et al. 2023; Jackman et al. 2024). We attempted to use a 0.45 µm filter the first few days of the study but quickly switched to a 5.0 µm filter to minimize filter clogging and tearing due to high turbidity levels. While our goal was to filter 5.0L at each site, we only succeeded in filtering the full 5.0L volume in 20% of sites and could only filter one liter or less in 29% of sites. Using the 5.0 µm filters allowed for faster filtration and reduced clogging in turbid water but may have allowed smaller eDNA fragments to pass through the filter without being captured, potentially compromising the accuracy of species detection in the sample.

The diversity and abundance of species in a given habitat can also affect the probability of detecting their eDNA in a sample. Species with higher biomass or DNA shedding rates are more likely to be detected than less abundant species because their DNA is more prevalent in the environment. During PCR amplification, the DNA of highly abundant species may be preferentially amplified, generating a disproportionately large number of reads, saturating the sequencing data, and obscuring the presence of rare species. This 'masking effect' may result in missed detections and an underestimation of species diversity (Kelly et al. 2014; Harper et al. 2018; Gargan et al. 2022; He et al. 2023; Skelton et al. 2022; Millard-Martin et al. 2024). Many of our sample sites were highly eutrophic. Interpreting eDNA data from these locations is challenging because it is unclear whether our results accurately reflect the biodiversity present in the sample or were influenced by excessive algal growth.

Environmental, physical, and biological factors can vary significantly within a water body, leading to an uneven distribution and concentration of eDNA. Collecting eDNA samples from multiple locations within a waterbody (or collecting multiple sample replicates at a site) can help mitigate the spatial variability in DNA concentration, provide a more comprehensive picture of species composition, and potentially improve the detection of rare of low-abundance species (Bruce et al. 2021; Gehri et al. 2021; Blabolil et al. 2022; Cote et al. 2023). Collecting multiple eDNA sample replicates at a site also enables the use of occupancy modeling techniques, which allows researchers to estimate the probability of a species being present at a location (site occupancy) as well as the probability of detecting that species with their specific eDNA sampling method (detection probability), effectively accounting for imperfect detection that can occur in eDNA surveys (Schmidt

et al. 2013; McClenaghan et al. 2020; Burian et al. 2021; Fukaya et al. 2021). Environmental DNA persistence, distribution, and concentration can also fluctuate over time depending on environmental conditions (e.g., summer versus winter) and organismal activity (e.g., life stage, metabolic rate, reproduction events, migration patterns). Conducting repeated sampling at different time points (e.g., monthly, seasonally) is important to account for temporal variations in eDNA levels and capture representative data (Sevellec et al. 2025).

Contamination is a significant concern in eDNA metabarcoding because it can lead to false positive detections, potentially overestimating species diversity (Sepulveda et al. 2020; Rishan et al. 2023). Contamination can occur during any stage of the process, from sample collection and preservation to laboratory processing and analysis. Field sample collection is often considered the most challenging stage to prevent contamination due to the increased risk of picking up extraneous DNA sources from the surrounding environment. Due to the high sensitivity of eDNA metabarcoding, even trace amounts of contaminant DNA can be detected, making it crucial to manage potential contamination sources throughout the sampling process. In this study, we used multiple strategies to prevent sample contamination (see Methods) and collected a negative control at each NWR to monitor for potential contamination. While no amplification was detected in lab-negative controls from DNA extraction and PCR, DNA amplification was reported in eight of ten field control samples with the COI marker and five of ten field control samples with the MiFish marker. We used 'open' style filters exposed to the air during assembly and filtration. Most contaminant DNA in field control samples was from fungus/mold, insects, and humans, which likely entered the filter cup through the air during filter assembly in the lab or while processing the negative control samples outdoors in the field. Except for human DNA, there were no other vertebrate detections in negative control samples. This suggests that any other vertebrate DNA detected in our samples was from the environment and not contamination. These results highlight the extreme sensitivity of eDNA metabarcoding to detect aerosolized DNA while sampling, making it crucial to include negative controls to ensure that any detected DNA is from the environment being studied and not from contamination introduced during the sampling process.

False positive and false negative errors

Environmental DNA metabarcoding is susceptible to false positive and false negative errors that may influence the quality of information and, in turn, the reliability of results. False positives (detection of a species' DNA where it is not actually present) can occur through contamination during field sample collection or laboratory processing, downstream transport of eDNA into a site from upstream locations, poor primer specificity, PCR or sequencing errors, or misidentification of DNA sequences. False negative sampling errors (a species not detected where it is actually present) can also arise from various sources including inadequate sample collection methods (e.g., insufficient sample size, wrong sample location, inadequate sample volume), environmental factors affecting DNA degradation (e.g., water temperature, UV exposure, microbial activity, hydrodynamic factors), low eDNA concentrations (e.g., species is present in low abundance or sheds minimal DNA), PCR inhibition from substances in sample, primer and PCR biases, sequencing errors, or errors or omissions in reference databases. Understanding the sources of false positive and false negative errors and taking measures to minimize these risks (e.g., using standardized protocols, replicate sampling, negative controls, optimized primer design, and statistical modeling) ensures greater accuracy and reliability when interpreting eDNA results and is critical for making informed decisions regarding species conservation and management strategies.

Although the species assignments obtained from this pilot study are informative, they should be treated with caution given our limited study design and potential inaccuracies or omissions that can arise during DNA sequencing and bioinformatics. Despite the large number of taxa detected by COI and MiFish genetic markers, many were likely missed. Metabarcoding studies often use minimum sequence copy thresholds to filter out low abundance sequences that may be false positive detections from contamination or sequencing errors (McColl-Gausden et al. 2020; Drake et al. 2021). The challenge is choosing a threshold that is stringent enough to remove false positives while still capturing genuinely low abundance species. We opted to set a fixed minimum threshold of <100 reads based on guidance from WDFWs genetics laboratory. While this filtering likely removed several false positive detections, it may have also inadvertently removed true positive detections of rare organisms (false negative). For example, the COI genetic marker detected western ridged mussel in Muddy Creek at Finley NWR, but due to the low read count (eight total reads), it was omitted from the final dataset. A fish biologist with the USFWS confirmed that western ridged mussels were observed at WL Finley NWR during snorkel/tactile surveys a few years ago, so this was possibly a true positive detection. In another example, the nonnative mystery snail (*Cipangopaludina chinensis*) was observed in very high abundance within and along the shoreline of Morgan Lake at Baskett Slough NWR. However, it was not detected in the eDNA sample. The mystery snail is not included in the MiFish reference database, but it is part of the COI database and, thus, would be considered a false negative detection. This could be due to factors such as poor DNA preservation in the lake, limitations of the eDNA primer, or insufficient sampling effort. While the temperature in Morgan Lake was moderate (16◦C/61◦F), UV exposure was high, and we were only able to filter 1.0L of water due to high turbidity levels. It is possible that the snail DNA was too degraded to be amplified, the primers did not effectively amplify the DNA, or the sequencing data was too poor to be identified in the reference database. Given the high number of unassigned reads in the sample (369,048) compared to assigned reads (12,599 cumulative reads for six OTUs), it is possible the snail DNA is included in the unassigned reads but was too poor to be identified to species in the reference database. In this instance, the false negative detection could have been potentially avoided by collecting multiple samples around the lake at different locations or collecting samples in spring, when water quality would have been better. This example also highlights the importance of integrating eDNA metabarcoding with traditional methods (e.g., visual observation) to improve species detection and provide a more comprehensive picture of biodiversity in an ecosystem.

Conclusions and Recommendations

eDNA metabarcoding provided valuable insights into fish diversity and aquatic species richness on refuges. Although it likely did not capture the full extent of species diversity in aquatic habitats, we still detected hundreds of aquatic and semi-aquatic taxa including rare (e.g., Olympic mudminnow, Oregon chub), introduced (e.g., New Zealand mudsnail, Asian clam), and marine species. It is important to note that our results were likely influenced by our experimental approach (e.g., choice of genetic markers, primers, sample effort, habitat conditions) and specific lab protocols (e.g., extraction method, choice of primer, number of amplification cycles). Thus, our results should be interpreted with a degree of caution. This dataset captures a snapshot of current species composition at NWRs that can be compared to future surveys to assess changes in aquatic biodiversity over time in response to environmental factors or management practices. This pilot study served as a mechanism to identify the limitations and potential biases of our current sampling strategy. Therefore, we offer the following recommendations for future eDNA sample design based on lessons learned during the implementation of this pilot study.

- Closely review sequenced metabarcoding data (especially OTUs with low reads) to identify any potential false negative or false positive errors. Additionally, unexpected results (e.g., aquatic invasive species, questionable species, missing species) should be confirmed with traditional surveys or species specific qPCR analysis.

- When conducting eDNA metabarcoding, select genetic markers based on project objectives and targeted taxa.
    - The goal of this pilot study was to evaluate how well eDNA metabarcoding could identify the variety of species present in aquatic habitats on refuges. Although broad amplification is useful for biodiversity assessments, the COI genetic marker and Leray-XT primers generated a high percentage of unassigned reads (86% of all sequenced reads), and 40% of sequenced reads were assigned to algae, fungi and other microbial taxa. Although these non-target taxa represent an important source of biodiversity information, it was not necessarily the focus of our study. The COI marker provided high taxonomic resolution of sequenced reads, but it did not capture the breadth of diversity within any single group of taxa such as fish. The MiFish genetic marker was highly effective at capturing fish diversity at many sites, but only 43% of fish were identified to species. Overall, we would not recommend the COI marker for vertebrate focused studies or in habitats with high algal loads. We recommend using vertebrate-specific genetic markers or primers designed to target distinct groups such as amphibians and reptiles to minimize detection of non-target organisms (see Wang et al. 2023).

- Relying on a single genetic marker (e.g., COI) might miss certain species due to potential amplification biases or insufficient genetic variation in the marker. Using multiple markers from different genomic regions can provide a more comprehensive picture of species diversity, but this approach comes with increased costs.

- Use a rigorous eDNA sampling design to fully capture the diversity of species present in an ecosystem.
    - Collect three or more eDNA samples from different locations within a site (e.g., lake, river) and collect two to three eDNA replicates at each location to account for natural variation in eDNA concentration and distribution within a site, maximize the chance of detecting rare species, and provide a more reliable estimate of species richness. Collecting multiple sample replicates also allows for statistical analysis to assess the confidence in detection results.

- A single eDNA sampling protocol may not be effective across all aquatic habitats (e.g., wetland, pond, lake, river, stream, estuary) given differences in biological, physical, and chemical properties that can significantly impact how eDNA is distributed and preserved.
    - For example, collecting eDNA samples from ponds, lakes, or wetlands during the summer can be challenging because higher water temperatures often lead to increased bacterial and algal growth, which can significantly degrade or inhibit the detection of eDNA in the sample.

- Our results only capture a snapshot of the eDNA present at the exact time of sampling.
    - Environmental DNA samples should be collected monthly or seasonally to account for fluctuations in species presence throughout the year.

- To minimize the effects of turbidity in eDNA metabarcoding sampling, consider using a pre-filter with a larger pore size to remove large particles and debris before filtering the sample through a finer membrane filter. Additionally, consider sampling at a different time of year when turbidity levels may be lower.

- When the goal is to detect one or two specific species (e.g., rare, low abundance, threatened and endangered species), qPCR is generally preferred over eDNA metabarcoding due to its superior sensitivity in detecting targeted species and reduced likelihood or errors and biases arising from analyzing a large community of organisms. In contrast, when the primary goal is to identify a broad range of species, metabarcoding is considered more efficient than running multiple qPCR assays for each species.

- Archived eDNA metabarcoding samples from this pilot study can be re-run using qPCR to target specific species of interest.

- Combining eDNA metabarcoding with traditional sampling methods can offer a more complete understanding of species diversity because each technique captures different aspects of the aquatic community, filling in gaps that the other method might miss. Environmental DNA metabarcoding can often detect a wider range of species, particularly rare or elusive ones; whereas, traditional sampling can provide detailed information about specific species' behaviors, location, life stage, abundance, population structure, or habitat utilization.

- Although not the focus of this pilot study, comparing environmental variables (e.g., temperature, turbidity, habitat characteristics, volume of water filtered) to eDNA metabarcoding results (e.g., total OTUs or species detected) is beneficial to understand how environmental factors influence the detected biodiversity in a given area. This information can be used to predict how changes in environmental conditions may affect species composition and distribution in the future.

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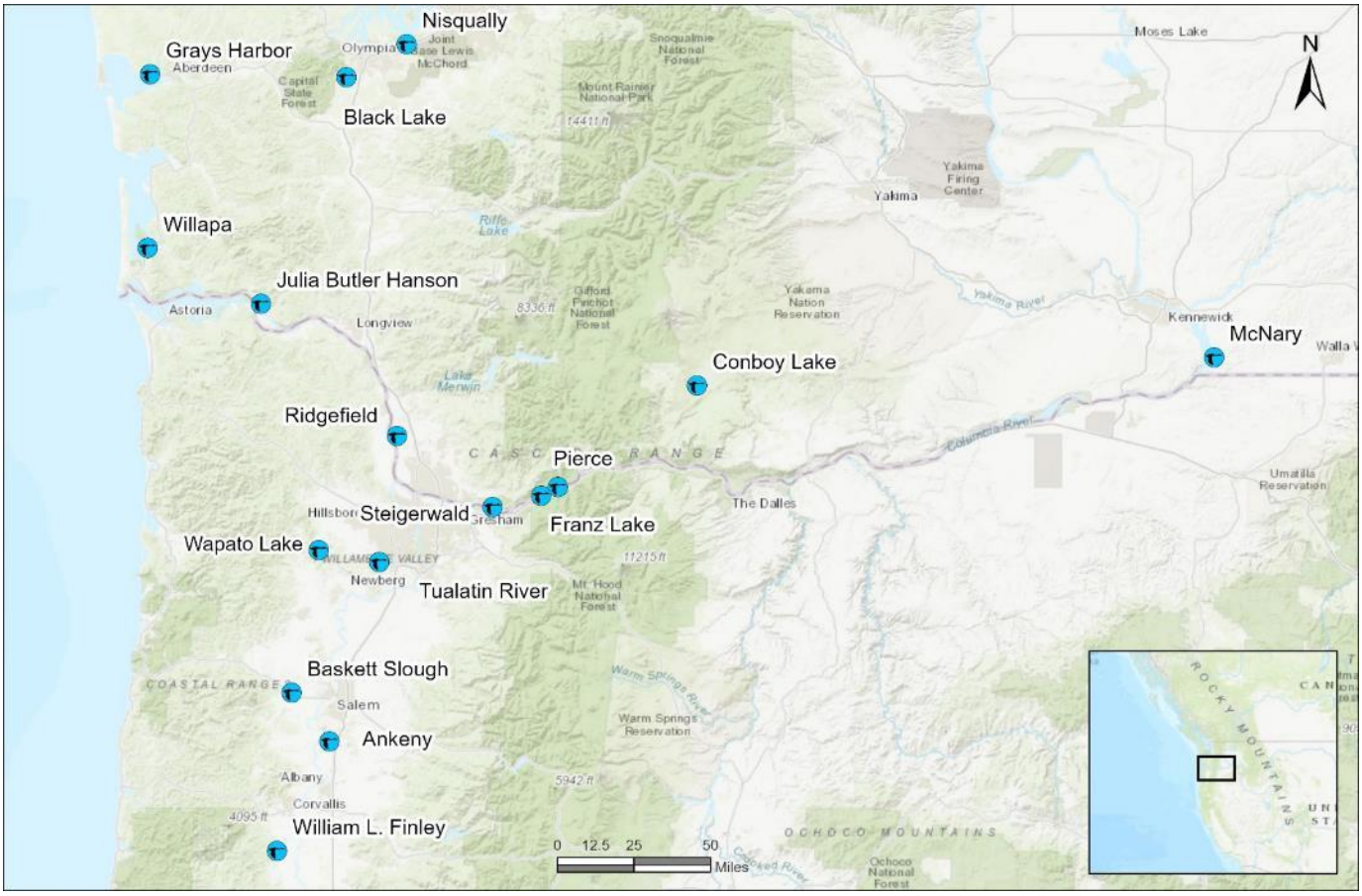

Figure 1. Location of 13 National Wildlife Refuges and refuge units where eDNA metabarcoding samples were collected in summer 2023. Note, Grays Harbor and Black Lake are included with Nisqually NWR and Wapato Lake is included with Tualatin River NWR in tables and figures.

Table 1. Summary of eDNA sample information, habitat characteristics and GPS coordinates of 80 eDNA sample locations and 10 field control samples collected at 13 NWRs, 2023.

Sample Number	Sample Date	NWR	Sample Location	Volume Filtered (L)	Habitat Type	Water Temp. (°C)	Conductivity (µS/cm)	Turbidity (JTU)	Dominant Substrate	Dominant Aquatic Vegetation	Veg. Cover (%)	X Coord.	Y Coord.
2023-001	8/17/2023	Steigerwald	**Field Control**	5.00									
2023-002	8/17/2023	Steigerwald	Gibbons Creek2	2.00	Flowing	19.6	57.1		Silt Clay Organic	Submerged	25	-122.3131736	45.56773017
2023-003	8/17/2023	Steigerwald	Lawton Creek	5.00	Flowing	17.5	56.8		Gravel	No veg	0	-122.2666835	45.56140972
2023-004	8/17/2023	Steigerwald	Gibbons Creek1	5.00	Flowing	17.1	58		Cobble	No veg	0	-122.3118908	45.57813616
2023-005	8/17/2023	Steigerwald	Campen Creek	2.80	Flowing	21.9	94.6		Silt Clay Organic	No veg	0	-122.315144	45.57709411
2023-006	8/17/2023	Steigerwald	Gibbons Creek3	0.30	Flowing	25.4	86.5		Silt Clay Organic	Submerged	10	-122.2959342	45.56082372
2023-007	8/18/2023	Pierce	**Field Control**	5.00									
2023-008	8/18/2023	Pierce	Lenas Lake	2.10	Still	12.1	29.8		Silt Clay Organic	Submerged	15	-122.0148767	45.62852769
2023-009	8/18/2023	Pierce	Lenas Lake outflow	5.00	Flowing	10.4	43.5		Silt Clay Organic	No veg	0	-122.0140278	45.62900655
2023-010	8/18/2023	Pierce	Hardy Creek3	0.75	Still	16.3	60.5		Silt Clay Organic	Submerged	80	-122.0057406	45.62912048
2023-011	8/18/2023	Pierce	Domestic Springs	0.50	Still	23.6	66.3		Silt Clay Organic	Emergent	40	-122.0069415	45.62986355
2023-012	8/18/2023	Pierce	Hardy Creek2	5.00	Flowing	18.7	36		Cobble	No veg	0	-121.9992654	45.63515527
2023-013	8/18/2023	Pierce	Hardy Creek1	4.00	Flowing	18.7	32.4		Boulder	No veg	0	-122.0034467	45.63485352
2023-014	8/24/2023	Tualatin	Wapato Creek1	0.50	Still	16.5	54	10	Silt Clay Organic	Floating	5	-123.1376175	45.43316582
2023-015	8/24/2023	Tualatin	Wapato Creek2	0.25	Still	18.9	59.8	40	Silt Clay Organic	Emergent	4	-123.1212576	45.41051026
2023-016	8/24/2023	Tualatin	**Field Control**	3.80									

2023-017	8/24/2023	Tualatin	Chicken Creek2	0.60	Flowing	17.5	151.3	12	Silt Clay Organic	Emergent	10	-122.8370519	45.38555614
2023-018	8/24/2023	Tualatin	Chicken Creek1	1.00	Flowing	17.5	151.3	5	Silt Clay Organic	Emergent	5	-122.849581	45.38221958
2023-019	8/24/2023	Tualatin	Tualatin River1	2.90	Flowing	21.1	239.4	2	Silt Clay Organic	Floating	3	-122.8464292	45.3889039
2023-020	8/24/2023	Tualatin	Rock Creek2	0.90	Still	19.3	259.1	5	Silt Clay Organic	Floating	100	-122.8321718	45.38496308
2023-021	8/24/2023	Tualatin	Rock Creek1	0.65	Still	17.4	232.1	5	Silt Clay Organic	Emergent	50	-122.8268495	45.37508338
2023-022	9/5/2023	JBH	**Field Control**	5.00									
2023-023	9/5/2023	JBH	Risk Creek2	0.65	Still	14.5	87.2	10	Silt Clay Organic	Submerged	50	-123.4014941	46.25163863
2023-024	9/5/2023	JBH	Elochoman2	5.00	Flowing	20	129.2	0	Silt Clay Organic	Emergent	10	-123.4182077	46.23959139
2023-025	9/5/2023	JBH	Elochoman1	2.55	Flowing	16.8	71.5	0	Silt Clay Organic	Submerged	15	-123.3925251	46.23226777
2023-026	9/5/2023	JBH	Alger Creek3	2.22	Flowing	19.8	122.6	5	Silt Clay Organic	Submerged	10	-123.4465447	46.26822843
2023-027	9/5/2023	JBH	Skamakawa Creek	4.50	Flowing	14.2	71.7	0	Silt Clay Organic	No veg	0	-123.4465105	46.28905019
2023-028	9/5/2023	JBH	Alger Creek1	2.50	Flowing	16	163.2	2.5	Silt Clay Organic	No veg	0	-123.4124133	46.26268825
2023-029	9/5/2023	JBH	Risk Creek1	1.00	Flowing	14.7	95.1	10	Silt Clay Organic	Emergent	10	-123.3976044	46.2518153
2023-030	9/18/2023	Nisqually	**Field Control**	5.00									
2023-031	9/18/2023	Nisqually	Black River1	4.00	Flowing			0	Sand	Submerged	5	-123.0196455	46.90899506
2023-032	9/18/2023	Nisqually	Dempsey Creek2	1.10	Flowing			7	Silt Clay Organic	Emergent	50	-123.0157441	46.96790325
2023-033	9/18/2023	Nisqually	Black River2	3.40	Flowing	13.6	86.2	2	Silt Clay Organic	No veg	0	-123.0019213	46.95387045
2023-034	9/18/2023	Nisqually	Dempsey Creek1	0.40	Flowing	12.7	64.4	15	Silt Clay Organic	Emergent	5	-123.0250435	46.95813353
2023-035	9/18/2023	Nisqually	Unnamed Trib.	2.10	Still	11.7	73.6	1	Silt Clay Organic	Floating	50	-123.0239431	46.92708883
2023-036	9/18/2023	Nisqually	Waddell Creek	5.00	Flowing	15.5	51	0		Submerged	25	-123.0515445	46.91395074
2023-037	9/18/2023	Nisqually	Black Lake	2.50	Still	20.8	91.8	2	Gravel	Emergent	5	-122.9751649	46.98294731

2023-038	9/18/2023	Nisqually	South Sound	2.30	Flowing	16	32350	4	Silt Clay Organic	No veg	0	-122.7264071	47.1003844
2023-039	9/19/2023	Nisqually	McCallister Creek1	4.00	Flowing	11.7	122	1	Silt Clay Organic	Emergent	70	-122.7261476	47.05004316
2023-040	9/19/2023	Nisqually	Nisqually River1	2.00	Flowing	14	55.4	10	Sand	No veg	0	-122.6922631	47.05783591
2023-041	9/19/2023	Nisqually	Parrotfeather Pond	2.00	Still	17	130.1	3	Silt Clay Organic	Floating	35	-122.6967058	47.07737059
2023-042	9/19/2023	Nisqually	Red Salmon Creek	5.00	Flowing	11.7	758	0	Sand	Submerged	60	-122.6894785	47.08077735
2023-043	9/19/2023	Nisqually	Hoquiam River	2.60	Flowing	17.6	32701	4	Silt Clay Organic	No veg	0	-123.8810511	46.97738047
2023-044	9/19/2023	Nisqually	Grays Harbor Trib.	1.80	Flowing	17.7	2277	5	Silt Clay Organic	Emergent	20	-123.9182978	46.98148007
2023-045	9/19/2023	Nisqually	Grays Harbor	0.90	Flowing	19.5	38919	12	Silt Clay Organic	Emergent	50	-123.9498278	46.98281006
2023-046	9/19/2023	Nisqually	Chehalis River	1.80	Flowing	18.2	25660	5	Silt Clay Organic	Emergent	10	-123.7707327	46.95737901
2023-047	9/25/2023	Willapa	**Field Control**	5.00									
2023-048	9/25/2023	Willapa	Willapa Bay	1.50	Flowing	16.2	36320	10	Silt Clay Organic	No veg	0	-123.9357894	46.41470619
2023-049	9/25/2023	Willapa	Cutthroat Creek	3.40	Flowing	12.7	60.1	3	Silt Clay Organic	Emergent	5	-123.9332916	46.41309424
2023-050	9/25/2023	Willapa	O'Meara Creek	5.00	Flowing	12.6	50.6	0	Gravel	No veg	0	-123.9496767	46.40240541
2023-051	9/25/2023	Willapa	North Creek	4.70	Flowing	13	59.4	0	Gravel	No veg	0	-123.9454723	46.3636856
2023-052	9/25/2023	Willapa	Chum Creek	3.50	Flowing	13	61	2	Gravel	No veg	0	-123.9408734	46.36015272
2023-053	9/25/2023	Willapa	Lewis Creek	1.50	Flowing	13	61.6	5	Gravel	Emergent	5	-123.9707021	46.35421117
2023-054	9/26/2023	Willapa	Dohman Creek	0.40	Flowing	14.3	94.8	2	Silt Clay Organic	Emergent	50	-123.9934543	46.3614724
2023-055	9/26/2023	Willapa	McCollum Creek	5.00	Flowing	53.2	53.2	0	Cobble	No veg	0	-123.9492519	46.3848951
2023-056	9/26/2023	Willapa	Bear River2	2.80	Flowing	12.7	54.2	1	Gravel	No veg	0	-123.9117332	46.32999016
2023-057	9/26/2023	Willapa	Bear River1	2.60	Flowing	12.7	56.6	4	Gravel	No veg	0	-123.8996521	46.31727475
2023-058	9/28/2023	Conboy	**Field Control**	5.00									
2023-059	9/28/2023	Conboy	Cold Springs Ditch	4.60	Flowing	10	61.3	1	Silt Clay Organic	Emergent	85	-121.3416185	45.96670684

2023-060	9/28/2023	Conboy	Bird Creek2	0.90	Still	10.4	25.3	8	Silt Clay Organic	Emergent	60	-121.3156969	45.97762788
2023-061	9/28/2023	Conboy	Bird Creek1	1.60	Still	9.4	24.1	4	Silt Clay Organic	Emergent	10	-121.315906	45.97763769
2023-062	9/28/2023	Conboy	Outlet Creek Section3	1.90	Still	13.3	67.1	3	Silt Clay Organic	Emergent	40	-121.2636029	45.98257488
2023-063	9/28/2023	Conboy	Mill Pond1	0.50	Still	14.4	55.9	15	Silt Clay Organic	No veg	0	-121.2197203	46.01515159
2023-064	9/28/2023	Conboy	Chapman Creek3	3.00	Still	13.6	88.2	3	Silt Clay Organic	Emergent	20	-121.3372829	45.9424345
2023-065	10/5/2023	Ridgefield	**Field Control**	5.00									
2023-066	10/5/2023	Ridgefield	Turtle Lake	1.20	Still	17.6	132	5	Sand	No veg	0	-122.7849467	45.81179862
2023-067	10/5/2023	Ridgefield	River S Expulsion	0.60	Still	17.8	194	7	Silt Clay Organic	Submerged	90	-122.7581072	45.81753131
2023-068	10/5/2023	Ridgefield	Campbell Lake Crossing	0.35	Still	19.4	191	30	Silt Clay Organic	No veg	0	-122.7538864	45.78255963
2023-069	10/5/2023	Ridgefield	Gee Creek1	2.00	Flowing	18.6	182	5	Silt Clay Organic	Submerged	80	-122.7472911	45.82634688
2023-070	10/5/2023	Ridgefield	Gee Creek2	0.75	Still	19.2	194	12.5	Silt Clay Organic	No veg	0	-122.7742606	45.84434909
2023-071	10/5/2023	Ridgefield	Whipple Creek	2.00	Flowing	19.5	214	5	Silt Clay Organic	Submerged	10	-122.7437765	45.7536329
2023-072	10/17/2023	Finley	**Field Control**	5.00									
2023-073	10/17/2023	Finley	Brown Creek	1.90	Still	11.3	63.7	5	Silt Clay Organic	Emergent	80	-123.3234207	44.42413436
2023-074	10/17/2023	Finley	Muddy Creek3	0.70	Still	13.6	56.1	15	Silt Clay Organic	Floating	5	-123.3194532	44.42046866
2023-075	10/17/2023	Finley	Grays Creek Beaver Pond	0.10	Still	14.7	41.8	70	Silt Clay Organic	Emergent	50	-123.3295852	44.39978471
2023-076	10/17/2023	Finley	Muddy Creek2	1.80	Still	13.7	57.9	5	Silt Clay Organic	Emergent	10	-123.3016104	44.38953105
2023-077	10/17/2023	Finley	McFadden Creek1	0.25	Still	14.6	82.6	15	Silt Clay Organic	Emergent	5	-123.2992753	44.38962544
2023-078	10/17/2023	Finley	Muddy Creek1	1.50	Still	14.1	58.6	5	Silt Clay Organic	Emergent	5	-123.2981472	44.37712291
2023-079	10/17/2023	Finley	Grays Creek2	2.00	Still	51.3	51.513	5	Silt Clay Organic	No veg	0	-123.3523526	44.39841077

2023-080	10/18/2023	Ankeny	Sydney Power Ditch2	1.15	Flowing	12.7	53.5	10	Silt Clay Organic	Floating	5	-123.0846393	44.79612138
2023-081	10/18/2023	Ankeny	Bashaw1	1.00	Still	12.4	53	10	Silt Clay Organic	Emergent	5	-123.0909205	44.77062716
2023-082	10/18/2023	Ankeny	Bashaw2	1.70	Flowing	12.5	56.1	5	Silt Clay Organic	Submerged	10	-123.0685892	44.77764537
2023-083	10/18/2023	Ankeny	Sydney Power Ditch1	1.30	Flowing	12.4	56.4	5	Silt Clay Organic	No veg	0	-123.0671905	44.77920402
2023-084	10/18/2023	Ankeny	Teal Marsh	1.50	Still	13.1	63.2	3	Silt Clay Organic	Emergent	90	-123.0742398	44.78805447
2023-085	10/18/2023	Baskett Slough	Morgan Lake1	1.00	Still	16	656	10	Silt Clay Organic	No veg	0	-123.2619484	44.98090935
2023-086	10/19/2023	McNary	Walla Walla River1	3.00	Flowing			0	Silt Clay Organic	Submerged	20	-118.840495	46.07375989
2023-087	10/19/2023	McNary	Walla Walla River2	1.00	Flowing			5	Silt Clay Organic	No veg	0	-118.9030817	46.06299636
2023-089	10/20/2023	Franz Lake	Franz Lake outlet	0.50	Flowing	14.4	51.3	15	Silt Clay Organic	No veg	0	-122.1042435	45.60033587
2023-090	10/25/2023	Franz Lake	**Field Control**	5.00									
2023-092	10/25/2023	Franz Lake	Indian Mary Creek	2.30	Flowing	9.3	40.3		Cobble	No veg	0	-122.073351	45.60628246

Table 2. Operational Taxonomic Units detected by COI and MiFish eDNA markers in ten field control samples collected at NWRs, 2023.

Sample Number	Refuge	eDNA Marker	Phylum	Class	Order	Family	Genus	Species	Common Name	Reads
2023-001	Steigerwald	COI	Chordata	Mammalia	Primates	Hominidae	Homo	sapiens	Human	302
2023-001	Steigerwald	COI		Chrysophyceae	Chromulinales	Chromulinaceae	Pedospumella	encystans	Golden algae	705
2023-007	Pierce	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium		Fungi	1547
2023-016	Tualatin	COI	Arthropoda	Insecta	Hemiptera	Cicadellidae	Dikrella	californica	Blackberry leafhopper	4487
2023-016	Tualatin	COI	Arthropoda	Insecta	Hemiptera				True bugs	10647
2023-016	Tualatin	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium		Fungi	5285
2023-016	Tualatin	COI	Chordata	Mammalia	Primates	Hominidae	Homo	sapiens	Human	4937
2023-016	Tualatin	COI		Chrysophyceae	Chromulinales	Chromulinaceae	Pedospumella	encystans	Golden algae	9666
2023-022	JBH	COI		Chrysophyceae	Chromulinales	Chromulinaceae	Pedospumella	encystans	Golden algae	2749
2023-047	Willapa	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium		Fungi	115643
2023-058	Conboy	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	herbarum	Fungi	1123
2023-058	Conboy	COI	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	lecanii	Black mold	670
2023-058	Conboy	COI	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	Malassezia	globosa	Yeast-like fungus	503
2023-058	Conboy	COI	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	Rhodotorula		Fungi	3319
2023-058	Conboy	COI	Chordata	Mammalia	Primates	Hominidae	Homo	sapiens	Human	62397
2023-058	Conboy	COI		Chrysophyceae	Chromulinales	Chromulinaceae	Pedospumella	encystans	Golden algae	5105
2023-058	Conboy	COI				Hartmannellidae	Vermamoeba	vermiformis	Amoeba	689
2023-065	Ridgefield	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium		Fungi	178
2023-065	Ridgefield	COI	Chordata	Mammalia	Primates	Hominidae	Homo	sapiens	Human	2200
2023-090	Franz Lake	COI	Arthropoda	Insecta	Coleoptera	Dermestidae	Anthrenus	verbasci	Varied carpet beetle	232797
2023-090	Franz Lake	COI	Arthropoda	Collembola	Entomobryomorpha	Entomobryidae	Entomobrya	intermedia	Slender springtail	902
2023-090	Franz Lake	COI	Arthropoda	Collembola	Poduromorpha	Hypogastruridae			Springtails	257

2023-090	Franz Lake	COI	Arthropoda	Insecta	Hemiptera				True bugs	2013
2023-090	Franz Lake	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium		Fungi	1814
2023-090	Franz Lake	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	bruhnei	Fungi	846
2023-090	Franz Lake	COI	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	herbarum	Fungi	3083
2023-090	Franz Lake	COI	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium		Fungi	366
2023-090	Franz Lake	COI	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	Rhodotorula		Fungi	1463
2023-090	Franz Lake	COI	Chordata	Mammalia	Primates	Hominidae	Homo	sapiens	Human	6033
2023-090	Franz Lake	COI	Nematoda	Enoplea	Dorylaimida	Longidoridae	Xiphinema	brevicollum	Dagger nematode	1361
2023-001	Steigerwald	MiFish	Chordata	Mammalia	Primates	Hominidae	Homo	Homo sapiens	Human	434
2023-016	Tualatin	MiFish	Chordata	Mammalia	Primates	Hominidae	Homo	Homo sapiens	Human	161
2023-058	Conboy	MiFish	Chordata	Mammalia	Primates	Hominidae	Homo	Homo sapiens	Human	8818
2023-065	Ridgefield	MiFish	Chordata	Mammalia	Primates	Hominidae	Homo	Homo sapiens	Human	1381
2023-072	Finley	MiFish	Chordata	Mammalia	Primates	Hominidae	Homo	Homo sapiens	Human	276
2023-030	Nisqually	N/A								

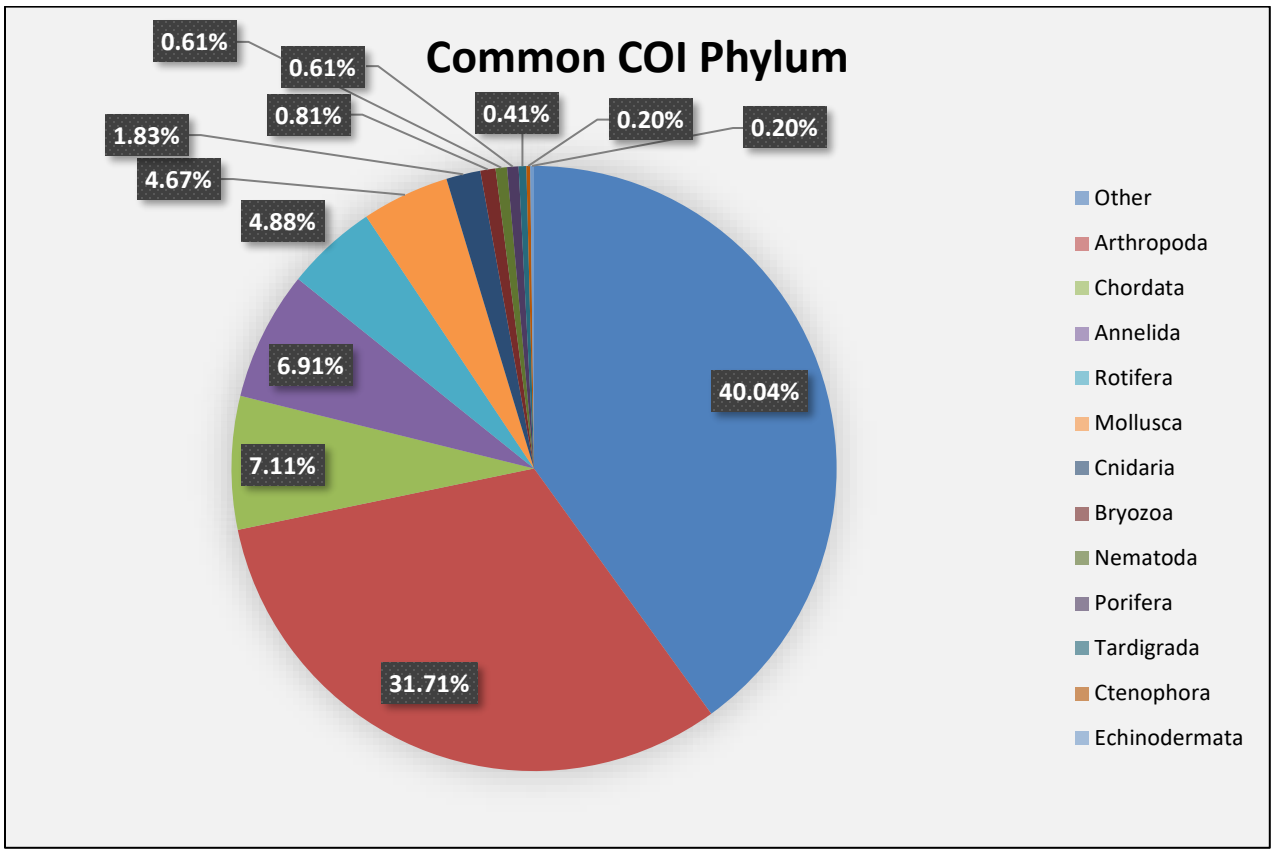

Figure 2. Proportion of common phylum identified by COI in 72 eDNA samples collected at NWRs. Other category includes nine phylum (Ascomycota, Bacillariophyta, Basidiomycota, Chlorophyta, Cryptophyta, Euglenida, Gastrotricha, Rhodophyta and Streptophyta; n=69 OTUs) and 128 other taxa not assigned to phylum such as algae, bacteria, fungi and amoebas.

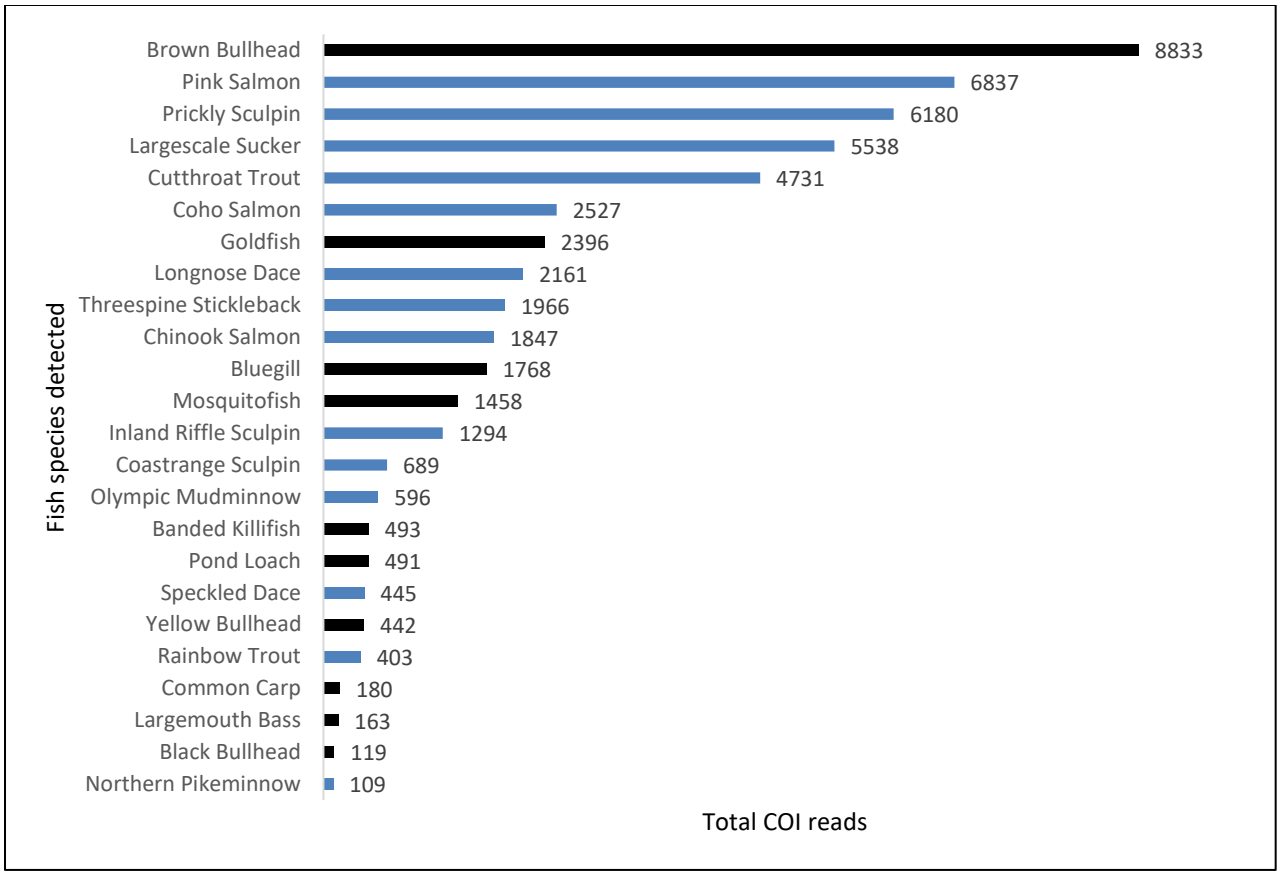

Figure 3. Cumulative read abundance of 24 fish species detected by the COI marker in 72 eDNA samples collected at NWRs. Dark bars depict nonnative species (n=10).

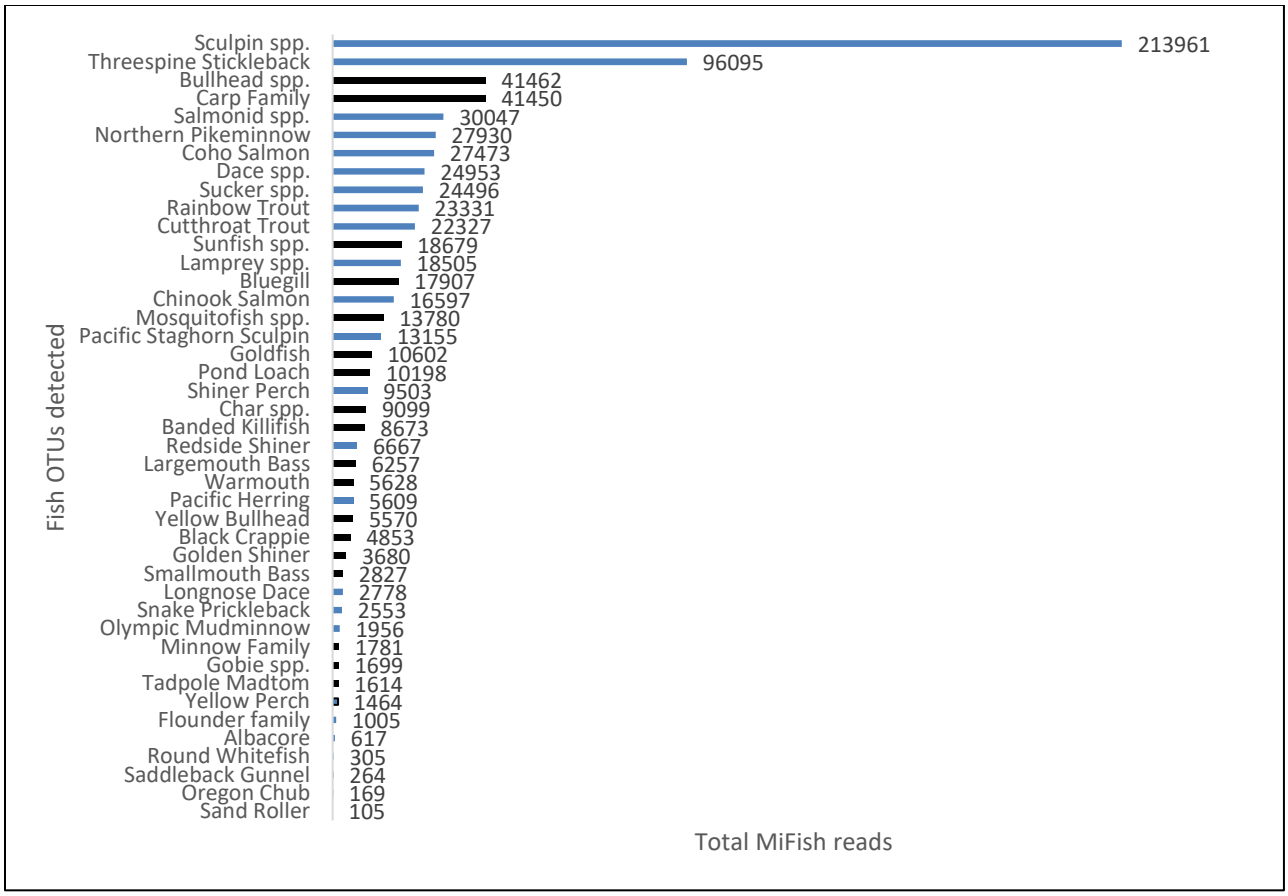

Figure 4. Cumulative read abundance of 43 fish OTUs detected by the MiFish marker in 64 eDNA samples collected at NWRs. Dark bars depict nonnative species (n=20).

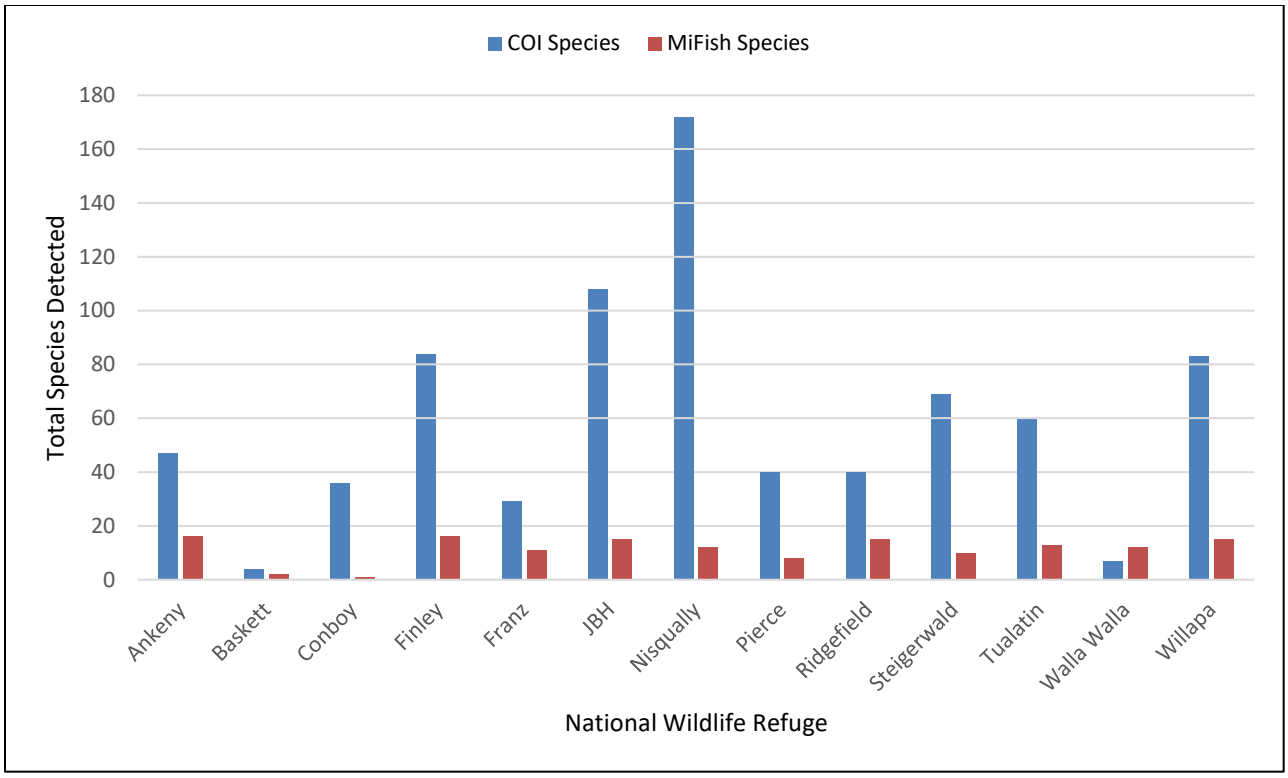

Figure 5. Total number of species detected by COI and MiFish markers at NWRs, 2023.

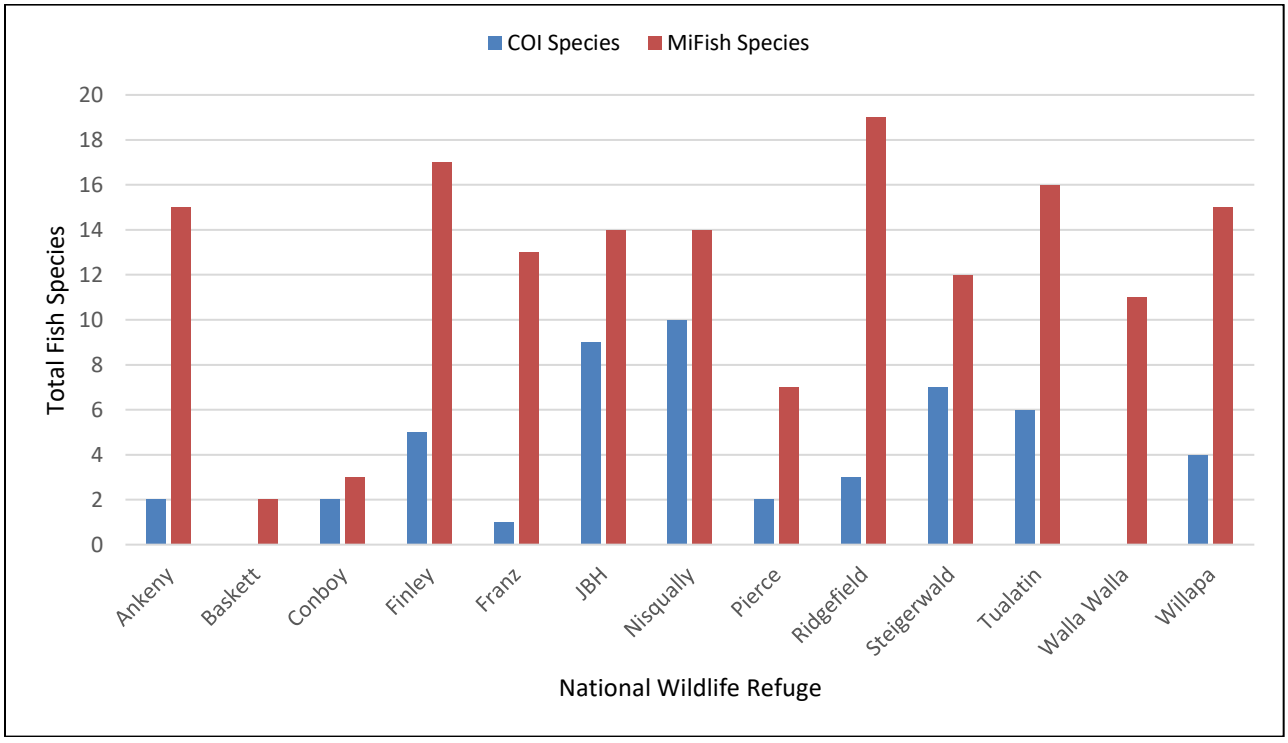

Figure 6. Total number of fish species detected by COI and MiFish markers at NWRs, 2023.

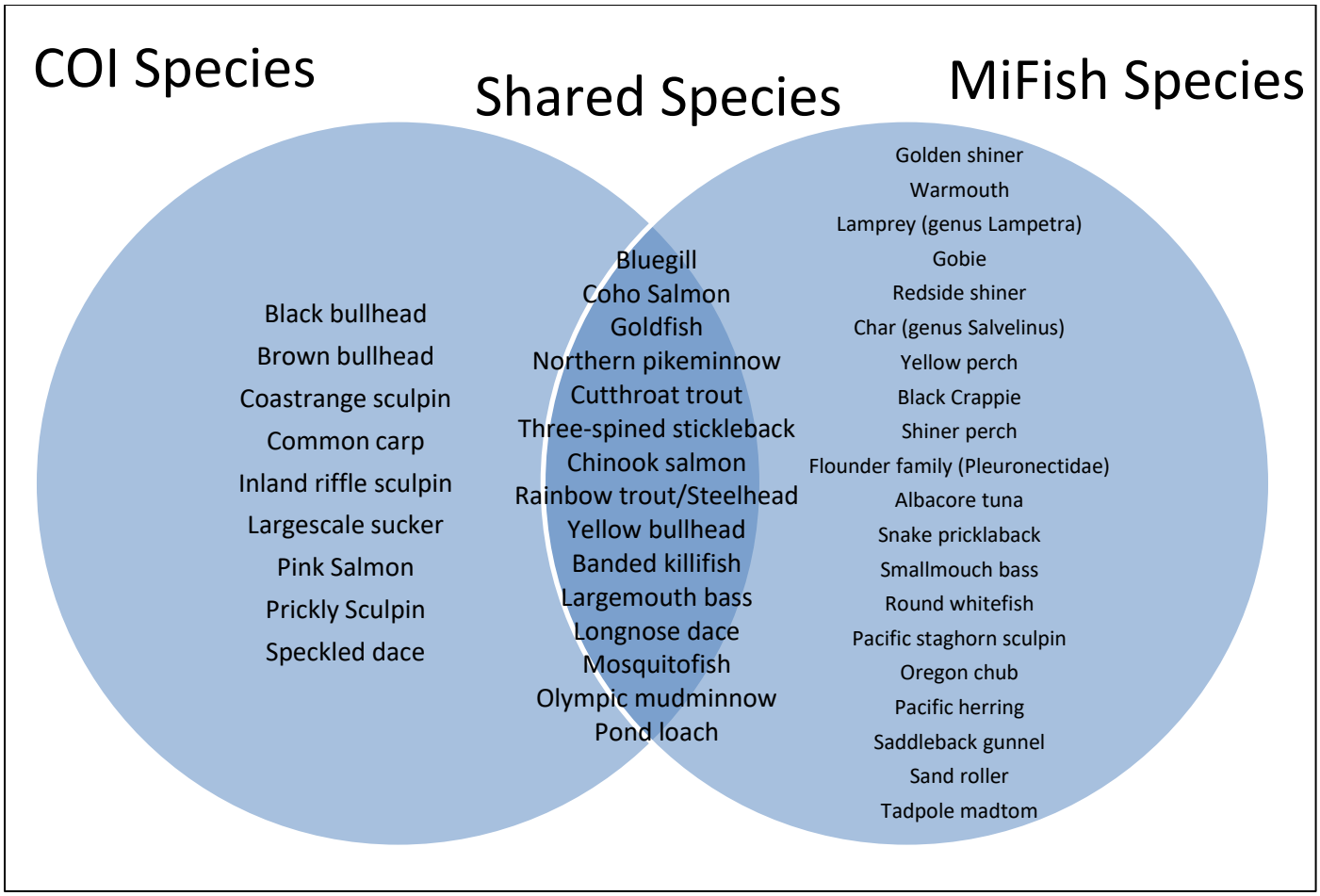

Figure 7. Venn diagram of unique and shared fish species detected by COI and MiFish markers at NWRs, 2023.

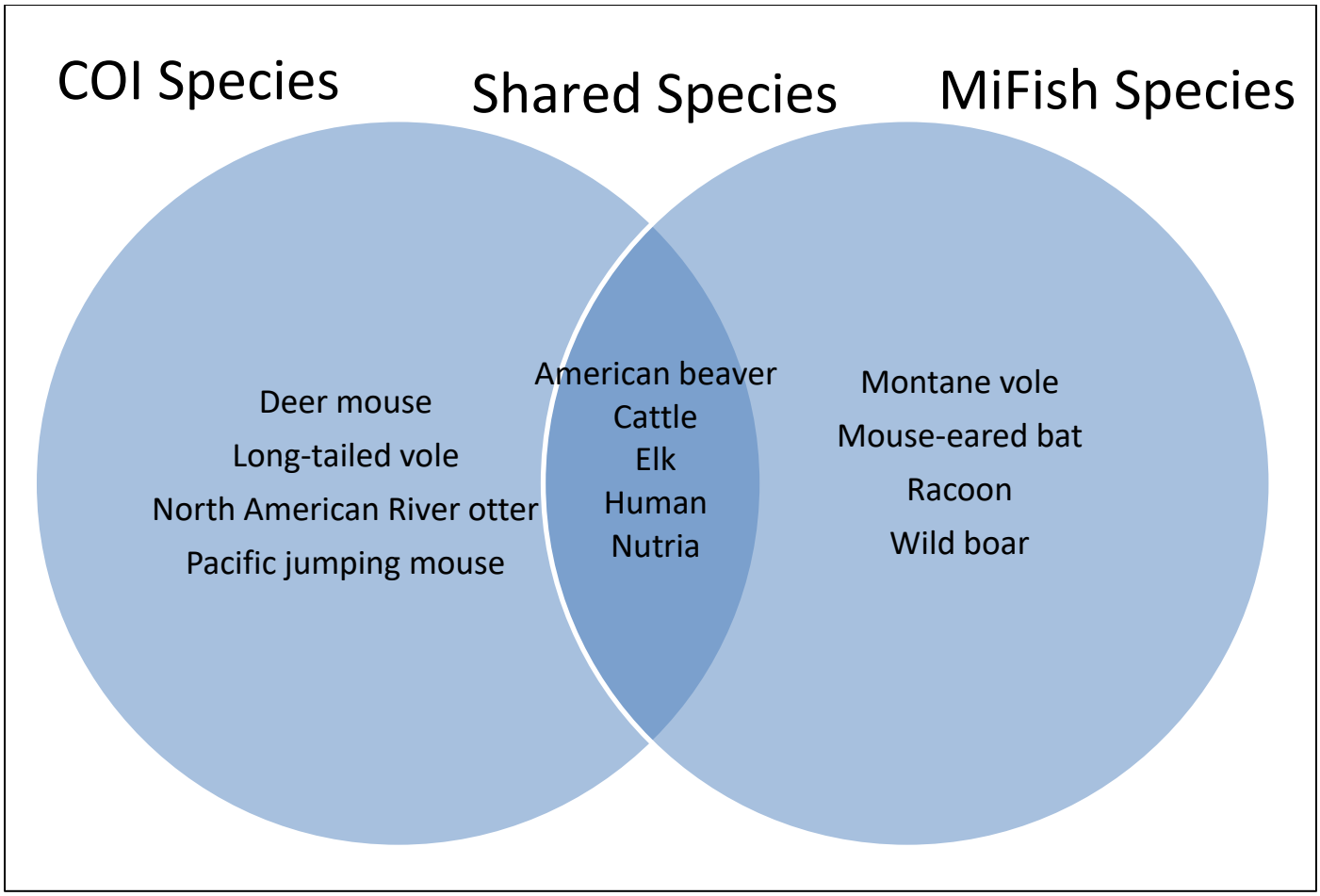

Figure 8. Venn Diagram of unique and shared mammal species detected by COI and MiFish markers at NWRs, 2023.

Appendix A

National Wildlife Refuge eDNA Summary

Ankeny NWR (COI)

Five sites were sampled at Ankeny NWR (Figure 9). The eDNA sample collected at Bashaw 1 did not pass quality filtering parameters. Collectively, COI detected 70 OTUs belonging to 11 phyla at the four sample locations (Figure 10). Of the 70 OTUs, 47 were successfully identified to species level (Figure 11), including two fish species (largescale sucker and prickly sculpin), four mammals (cattle, American beaver, deer mouse, and nutria), two mollusks (glassy juga and Asian clam) and one amphibian (American bullfrog). Western pearlshell mussels (*Margaritifera falcata*) and Oregon floater mussels (*Anodonta Oregonensis*) were detected at three locations at Ankeny NWR (Sydney Power Ditch 1&2 and Bashaw 2), but reads were below the minimum threshold of ≥100 (2-91 reads) and may not be reliable detections. The OTU with the highest cumulative reads was red algae (*Paralemanea annulata*) detected at three sites. Bashaw 2 had the highest number of OTUs (52) and species (34) detected at Ankeny NWR.

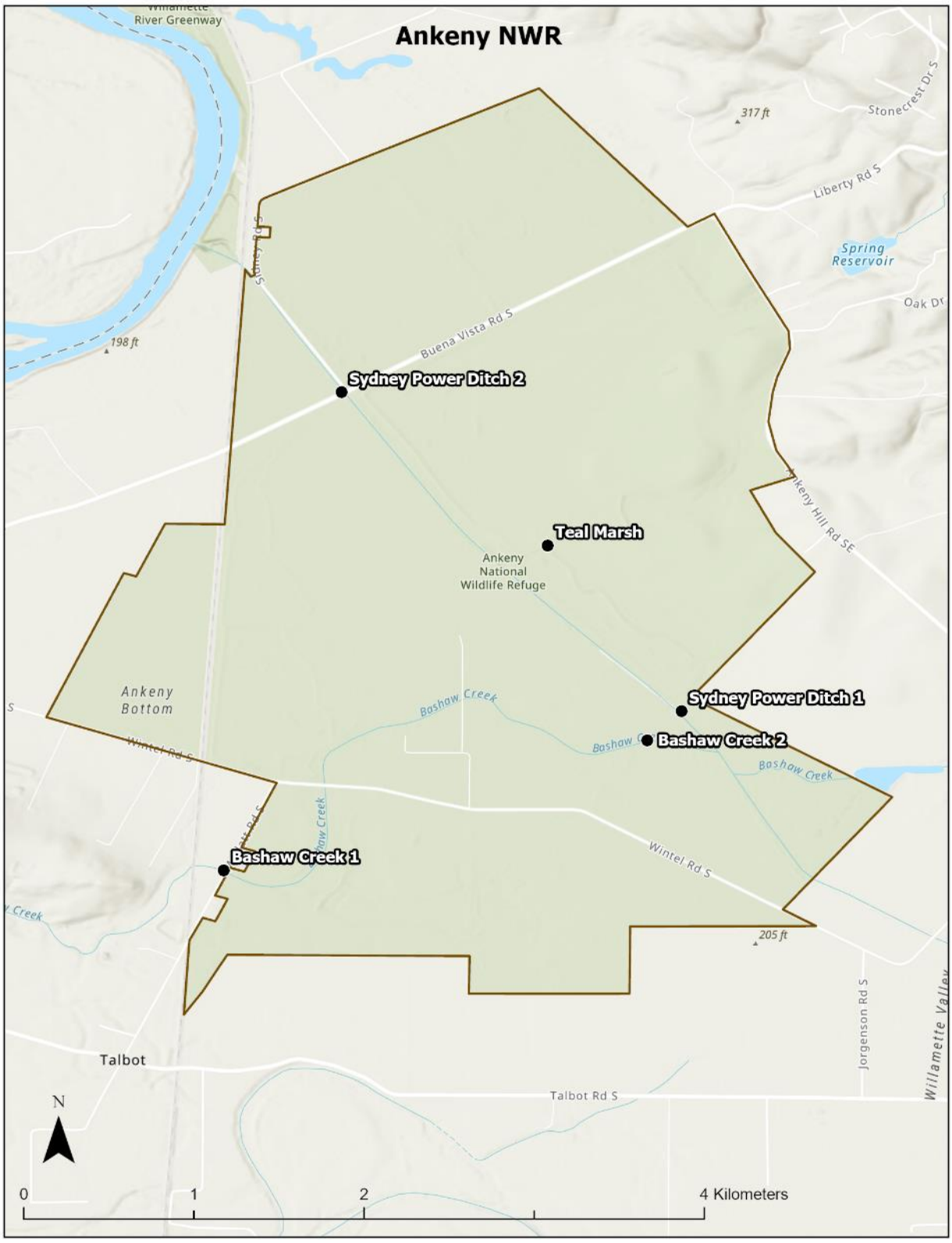

Figure 9. Environmental DNA sample locations at Ankeny NWR, 2023.

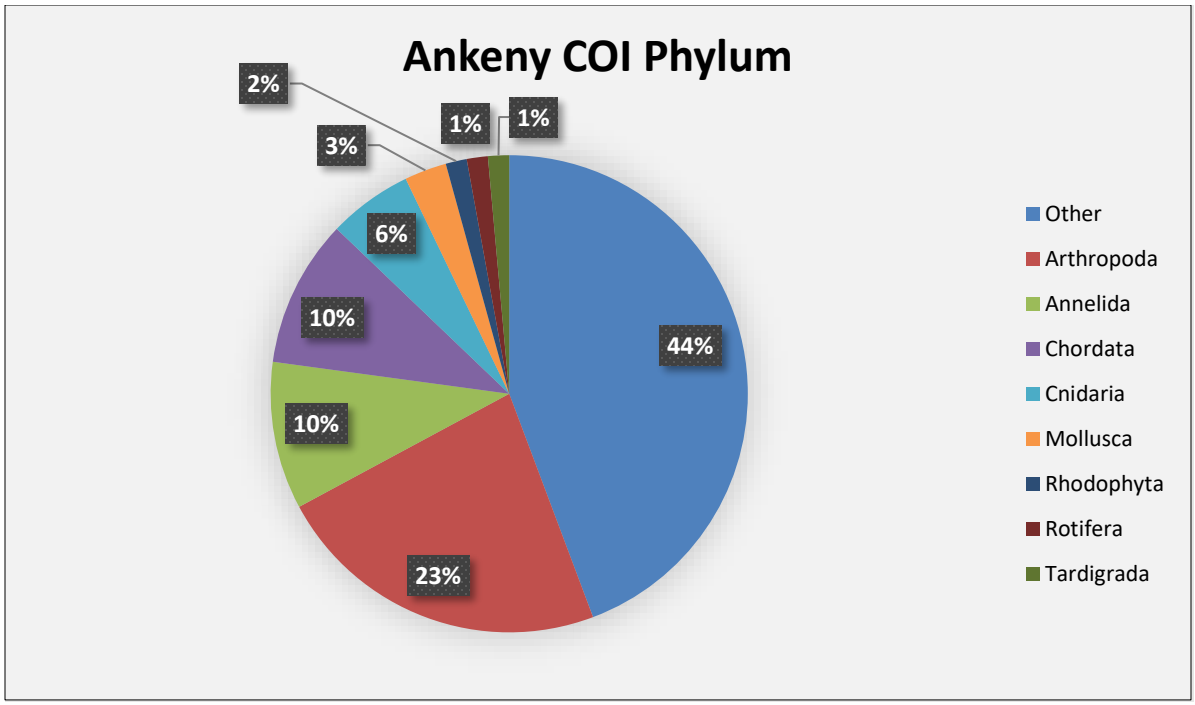

Figure 10. Proportion of common phylum identified by COI in four sample locations at Ankeny NWR. Other category includes: algae, bacteria, fungi, amoebas, etc.

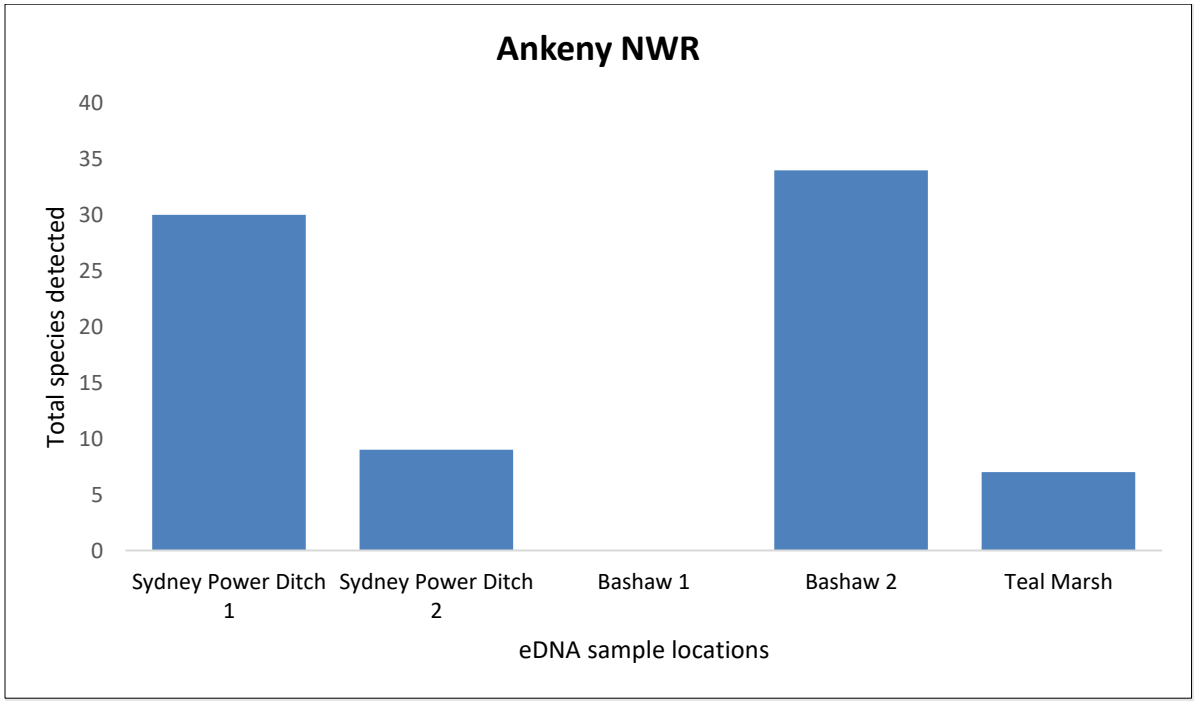

Figure 11. Total number of species detected by COI in four sample locations at Ankeny NWR, 2023.

Ankeny NWR (MiFish)

All sample locations ran successfully using the Mifish eDNA marker. A total of 23 OTUs were detected among the five sites including, 17 fish, four mammals (cattle, American beaver, nutria, and human), one amphibian (rough-skinned newt), and one bird OTU (mallard duck; Figure 12). Of note was the detection of Oregon chub in Sydney Power Ditch 2. Of the 17 fish OTUs detected, three are considered nonnative (largemouth bass, bluegill, and round whitefish). The OTU with the highest cumulative reads was sculpin, which was detected in four sites (Figure 13). Sydney Power Ditch 2 had the highest number of OTUs (20) and fish detected (16 OTUs) at Ankeny NWR.

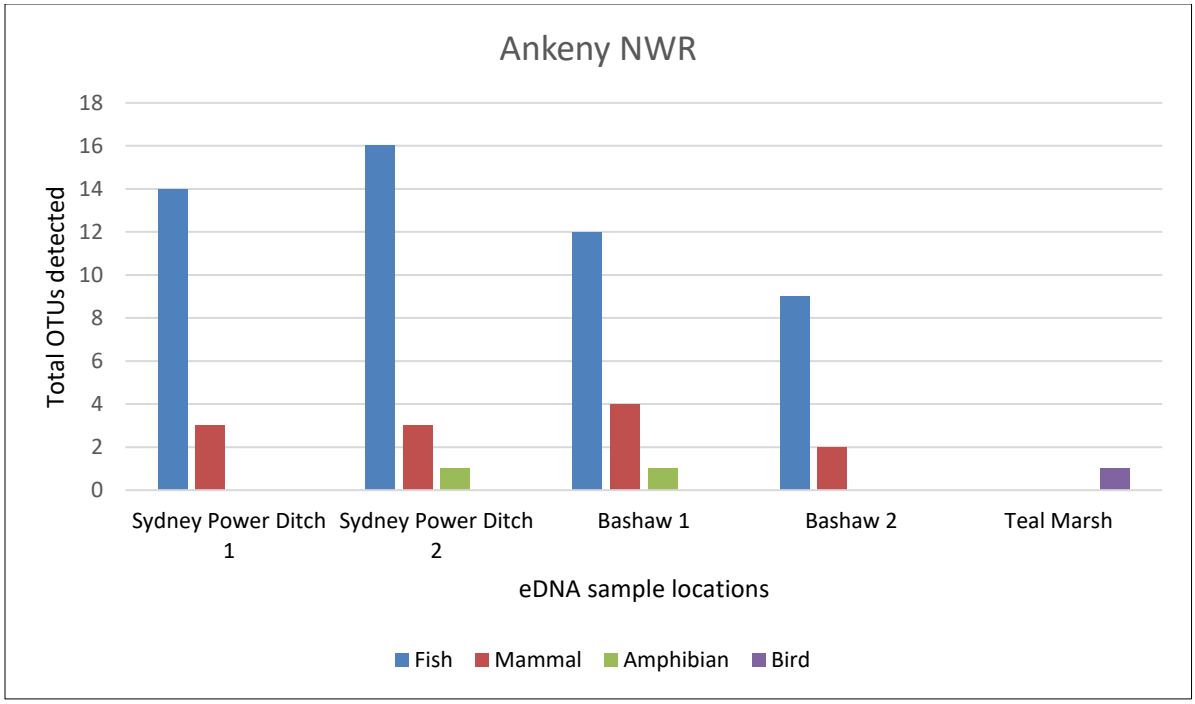

Figure 12. Fish, mammal, amphibian and bird OTUs detected by MiFish in five sample locations at Ankeny NWR, 2023.

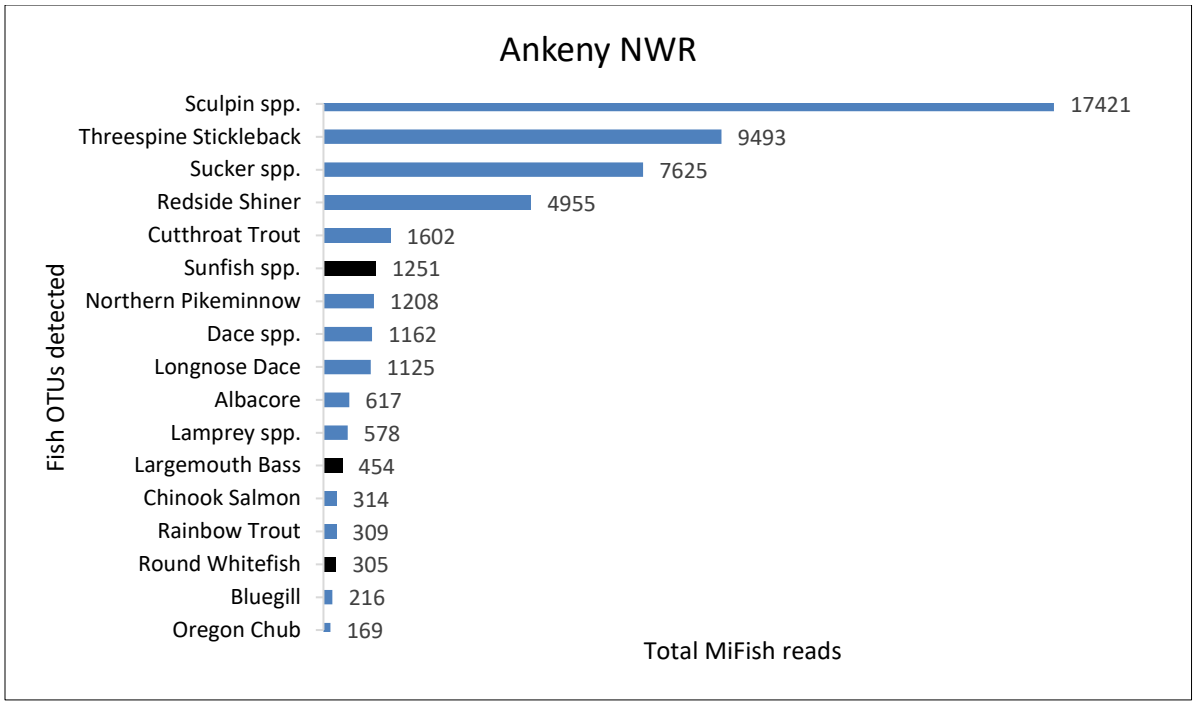

Figure 13. Cumulative read abundance of fish OTUs detected by the MiFish marker at five sample locations at Ankeny NWR, 2023. Dark bars represent nonnative species (n=3).

Baskett Slough NWR (COI)

Only one of three potential locations was sampled for eDNA at Baskett Slough NWR due to excessive water turbidity (Morgan Lake; Figure 14). Although sample sequencing resulted in 381,798 reads, over 96% were unassigned (i.e., not in the reference database). The remaining 12,599 reads were assigned to six OTUs belonging to four phyla: Arthropoda, Bacillariophyta (diatoms), Chlorophyta (green algae), and Rotifera. The OTU with the highest read count was a rotifer of genus *Polyarthra* with 9795 COI reads. Nonnative Chinese mystery snails (*Cipangopaludina chinensis*) were observed along the margin of Morgan Lake, but they were not detected in the eDNA sample (see Discussion).

Baskett Slough NWR (MiFish)

A total of four OTUs were detected in Morgan Lake: goldfish (genus *Carassius*), mosquitofish (genus *Gambusia*), mallard ducks (*Anas platyrhynchos*), and humans. Mosquitofish had the highest read count (4148 MiFish reads), followed by goldfish (574 MiFish reads).

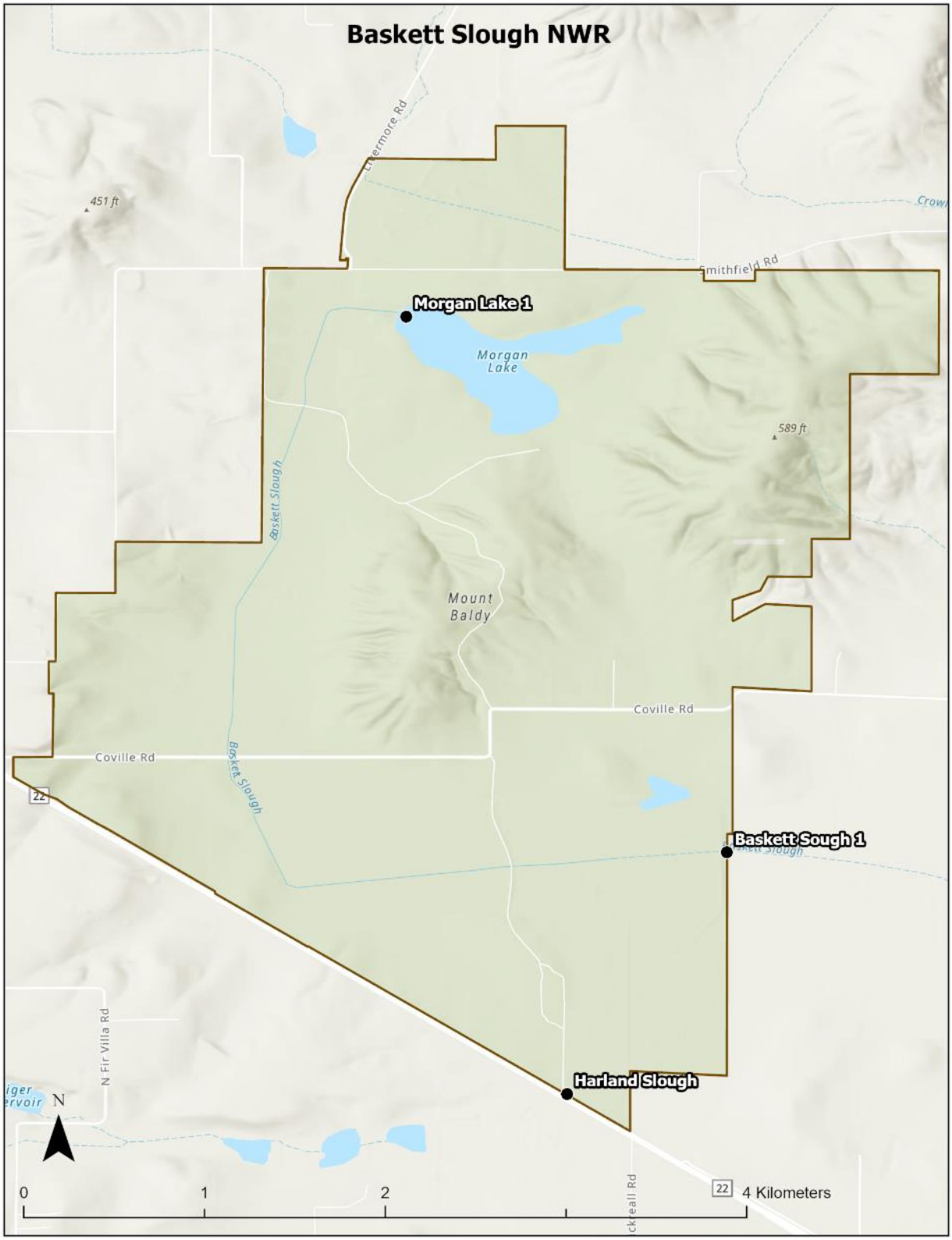

Figure 14. Environmental DNA sample locations at Baskett Slough NWR, 2023.

Conboy Lake NWR (COI)

Six locations were sampled for eDNA at Conboy Lake NWR (Figure 15). Samples collected at Chapman Creek and Cold Springs Ditch did not pass quality filtering parameters. The COI marker detected 63 OTUs belonging to 10 phyla in the four sample locations (Figure 16), including two fish (speckled dace and brown bullhead) and two mammal species (cattle and North American river otter). Oregon floater mussels were also detected at Outlet Creek 3, but read counts were below the minimum threshold of ≥100 reads (63 reads) and may not be reliable. The OTU with the highest cumulative read count was a fungus gnat from the family Sciaridae (99,128 COI reads), while the dinoflagellate *Alexandrium minutum*, was the only OTU detected in all four sample locations. Bird Creek 2 had the highest number of OTUs (42) and species (26) detected at Conboy Lake NWR (Figure 17).

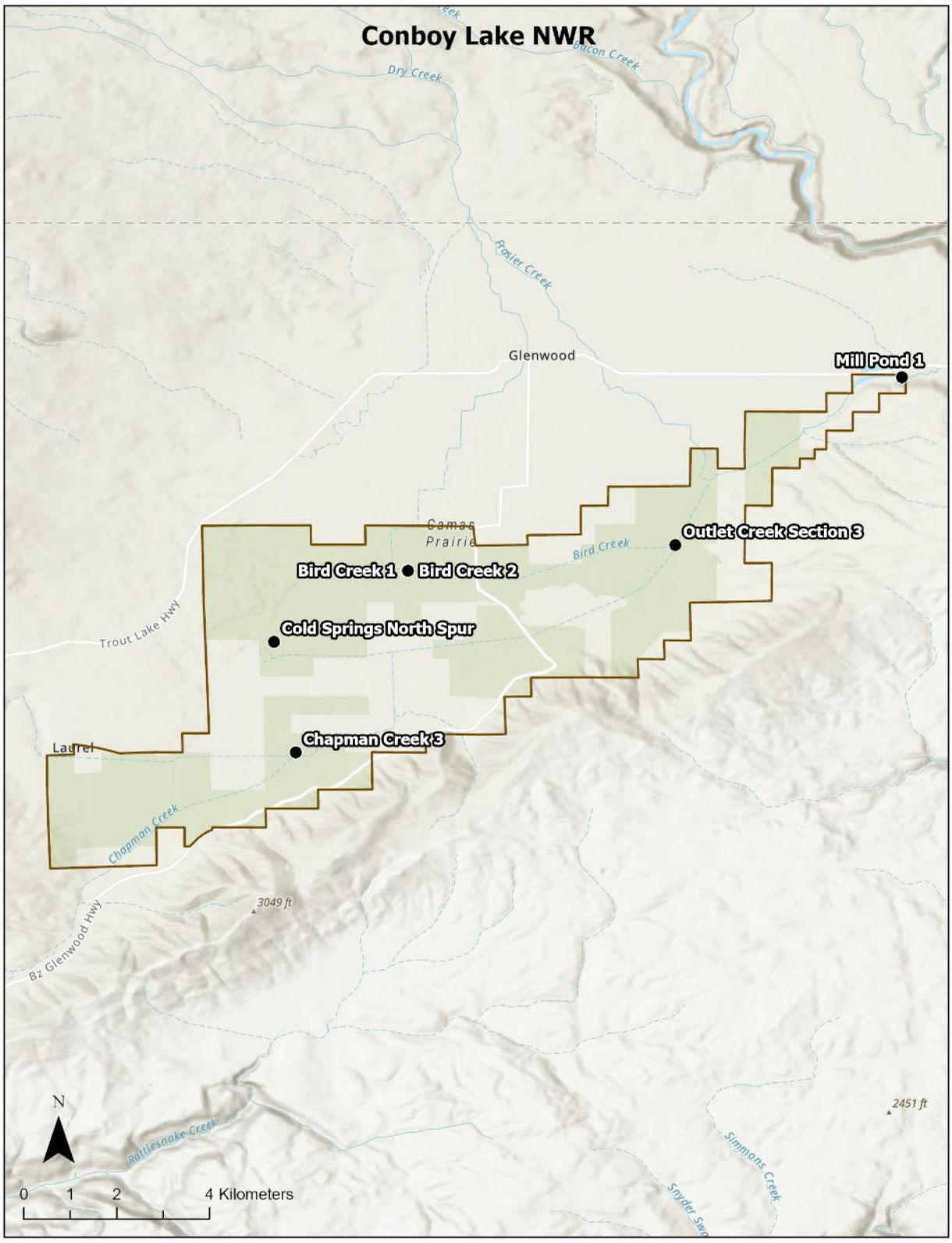

Figure 15. Environmental DNA sample locations at Conboy Lake NWR, 2023.

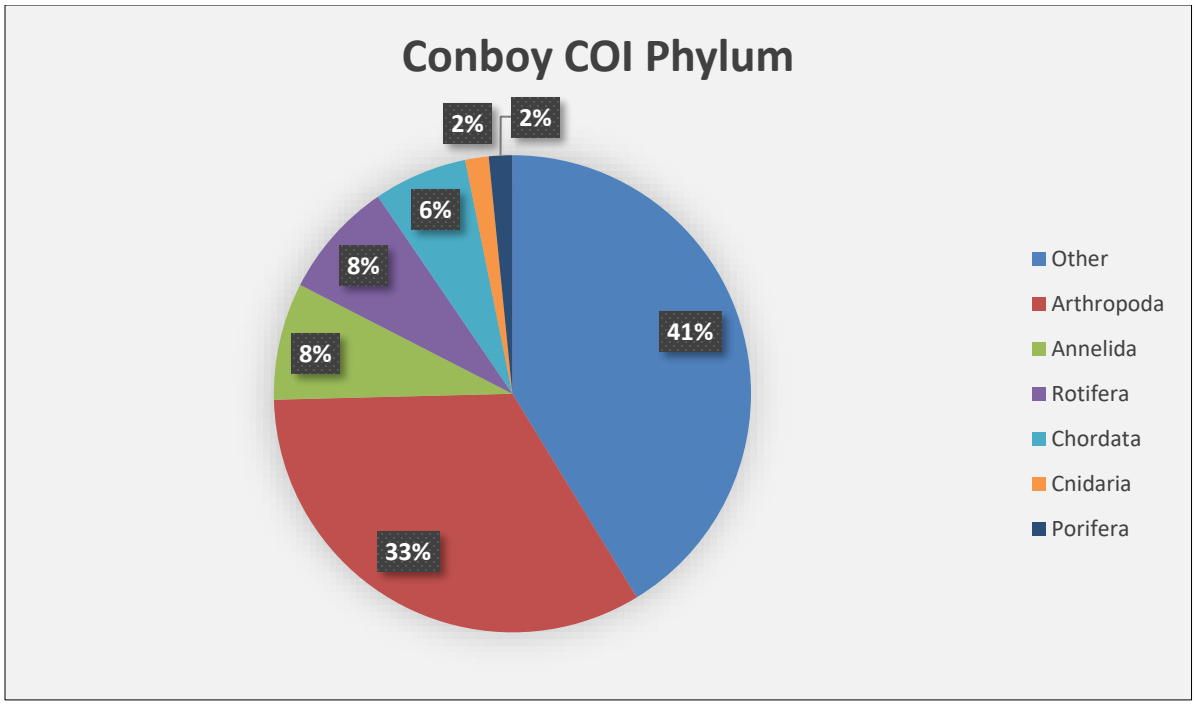

Figure 16. Proportion of common phylum identified by COI in four sample locations at Conboy Lake NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

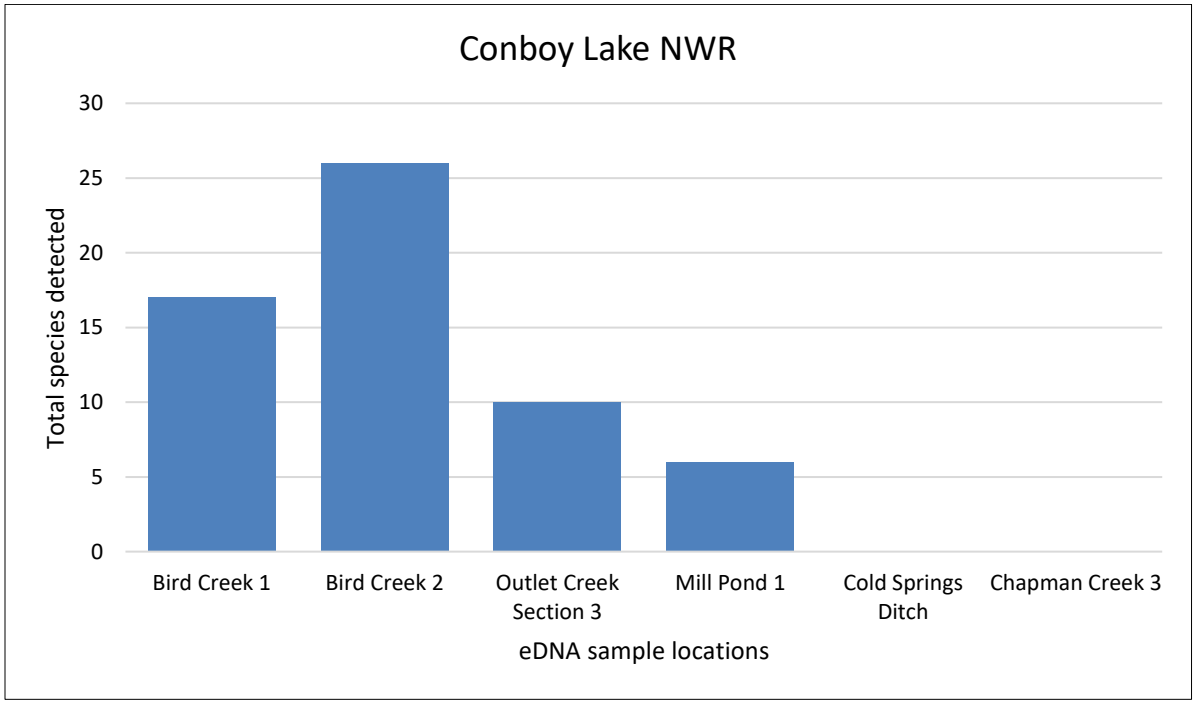

Figure 17. Total number of species detected by COI in four sample locations at Conboy Lake NWR, 2023.

Conboy Lake NWR (MiFish)

Two of the six sample locations (Cold Springs Ditch and Bird Creek 2) did not run with the MiFish marker. A total of four OTUs were detected among the four sample locations including three fish genera (bullhead, dace and char) and one mammal species (cattle). Although MiFish could not identify bullheads and dace to species, they were successfully identified as brown bullhead and speckled dace with the COI marker in the same sample locations. The fish genus *Salvelinus* was detected at two locations with the MiFish marker, but it's unclear whether the detections are for brook trout or bull trout. The COI marker detected brook trout to species at both sample locations in Bird Creek (below ≥100 read threshold), suggesting the MiFish *Salvelinus* detection may be brook trout at least in Bird creek. Cumulative OTU read counts were highest for bullhead (24,283 MiFish reads) and lowest for char (5,025 MiFish reads; Figure 18).

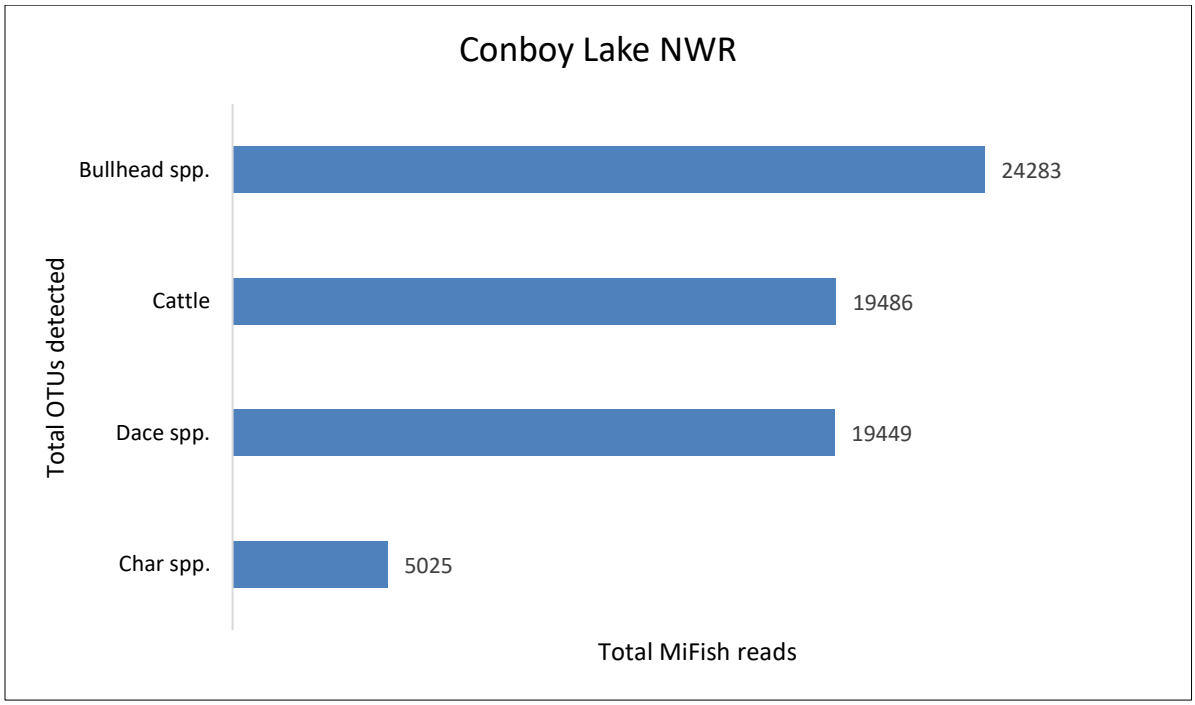

Figure 18. Cumulative read abundance of OTUs detected by the MiFish marker at four sample locations at Conboy Lake NWR, 2023.

Finley NWR (COI)

All seven sample locations ran successfully at Finley NWR (Figure 19). Collectively, COI detected 125 OTUs belonging to 15 phyla (Figure 20). Of the 125 OTUs, 84 were successfully identified to species level (Figure 21), including four fish (largescale sucker, island riffle sculpin, brown bullhead, and cutthroat trout), three mammals (cattle, American beaver, and humans), two mollusks (Asian clam and bladder snail) and one amphibian (American bullfrog). Western pearlshell mussels (one location), Oregon floater mussels (three locations), and western ridged mussels (one location) were each detected in Muddy Creek. However, total reads were below the minimum threshold of ≥100 (e.g., 8-50 reads) and may not be reliable detections. The OTU with the highest cumulative reads was *Chydorus brevalabris*, a freshwater crustacean or water flea (146,917 COI reads), and the OTU with the highest rate of occurrence was a plant pathogen of genus *Pythium* detected at six sites (21,810 COI reads). Grays Creek 2 had the highest number of OTUs (59), and Muddy Creek 2 had the highest number of unique species (38) detected at Finley NWR.

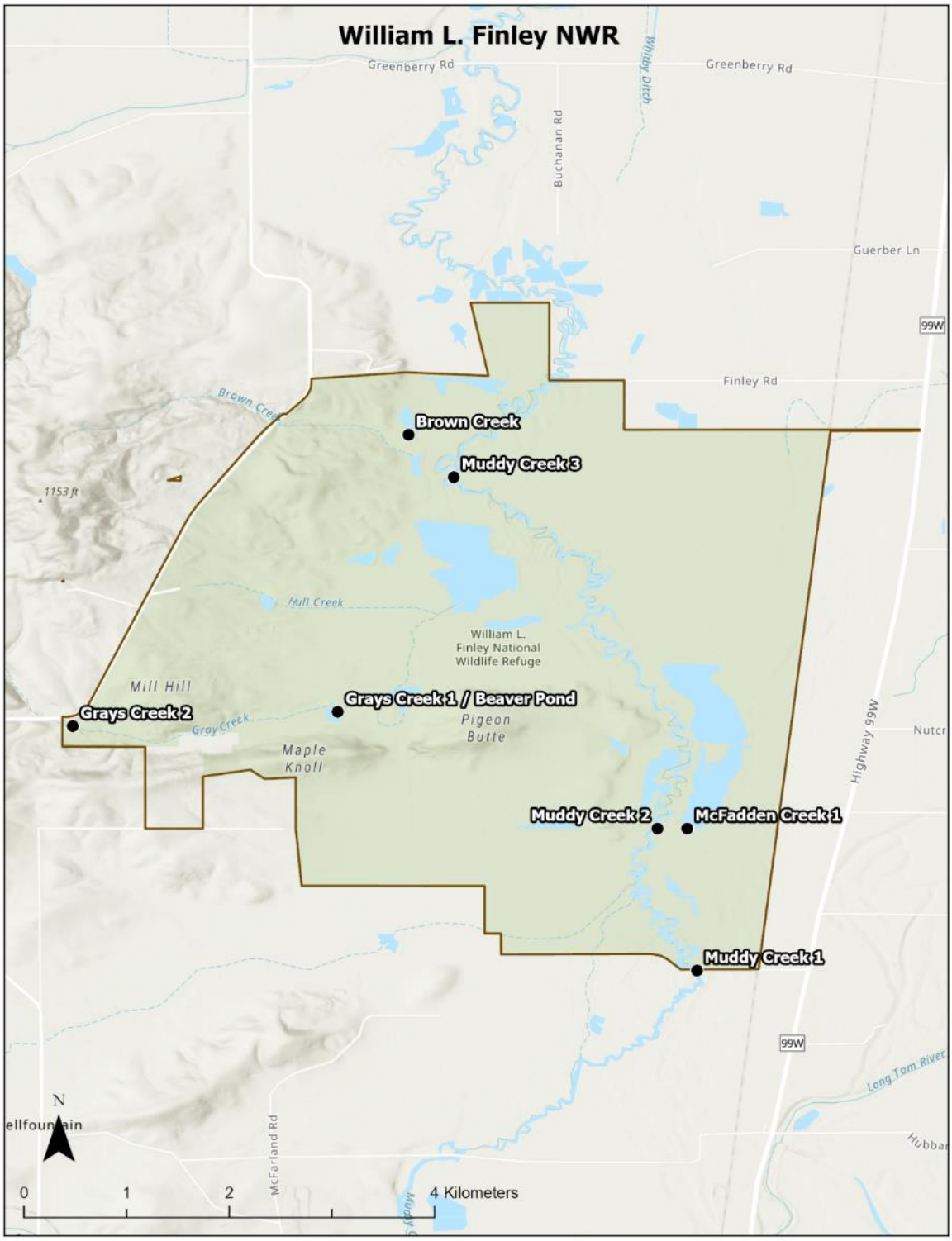

Figure 19. Environmental DNA sample locations at Finley NWR, 2023.

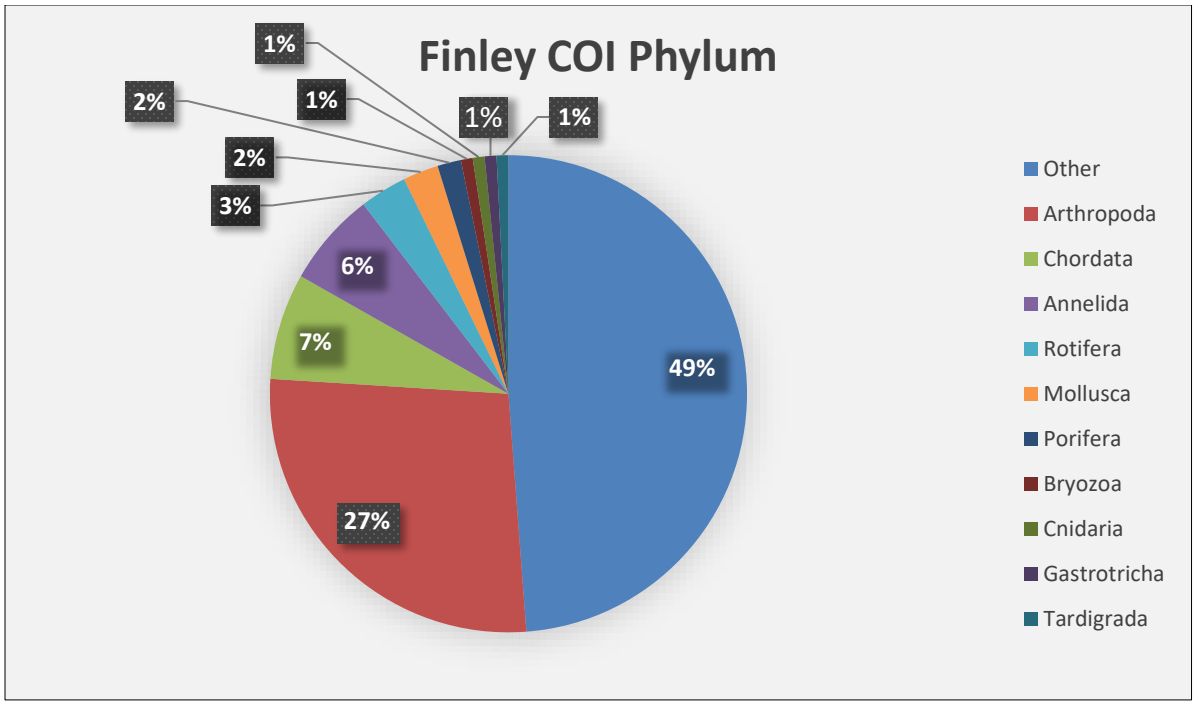

Figure 20. Proportion of common phylum identified by COI in seven sample locations at Finley NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

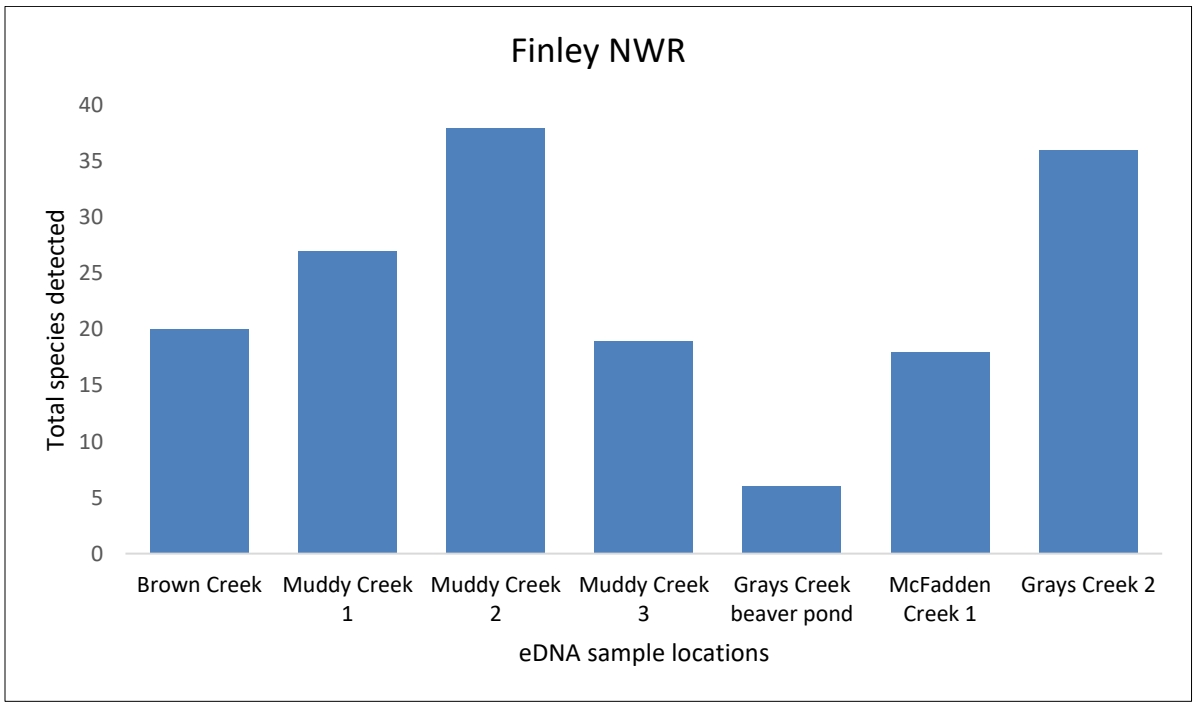

Figure 21. Total number of species detected by COI in seven sample locations at Finley NWR, 2023.

Finley NWR (MiFish)

All sample locations ran successfully using the Mifish eDNA marker. A total of 28 OTUs were detected among the seven sites, including 19 fish, three mammals (American beaver, nutria, and human), two amphibians (rough-skinned newt and coastal giant salamander), and two birds (wood duck and the corvid bird family; Figure 22). Ten of the 19 fish OTUs were nonnative species (Figure 23). The OTU with the highest cumulative reads was sculpin (26,250 reads; Figure 23), and OTUs with the highest rate of occurrence were sculpin, bullhead, and lamprey, each detected in five locations. Muddy Creek 3 had the highest number of OTUs (17) and fish (13 OTUs) detected at Finley NWR.

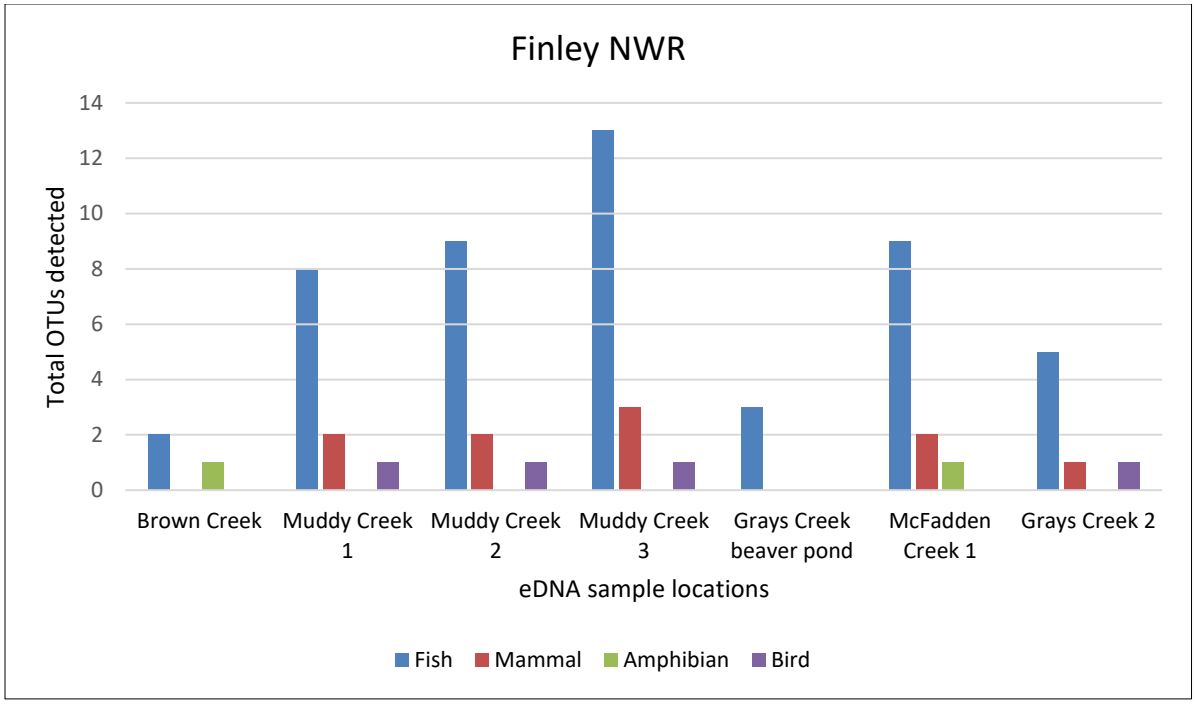

Figure 22. Fish, mammal, amphibian and bird OTUs detected by MiFish in seven sample locations at Finley NWR, 2023.

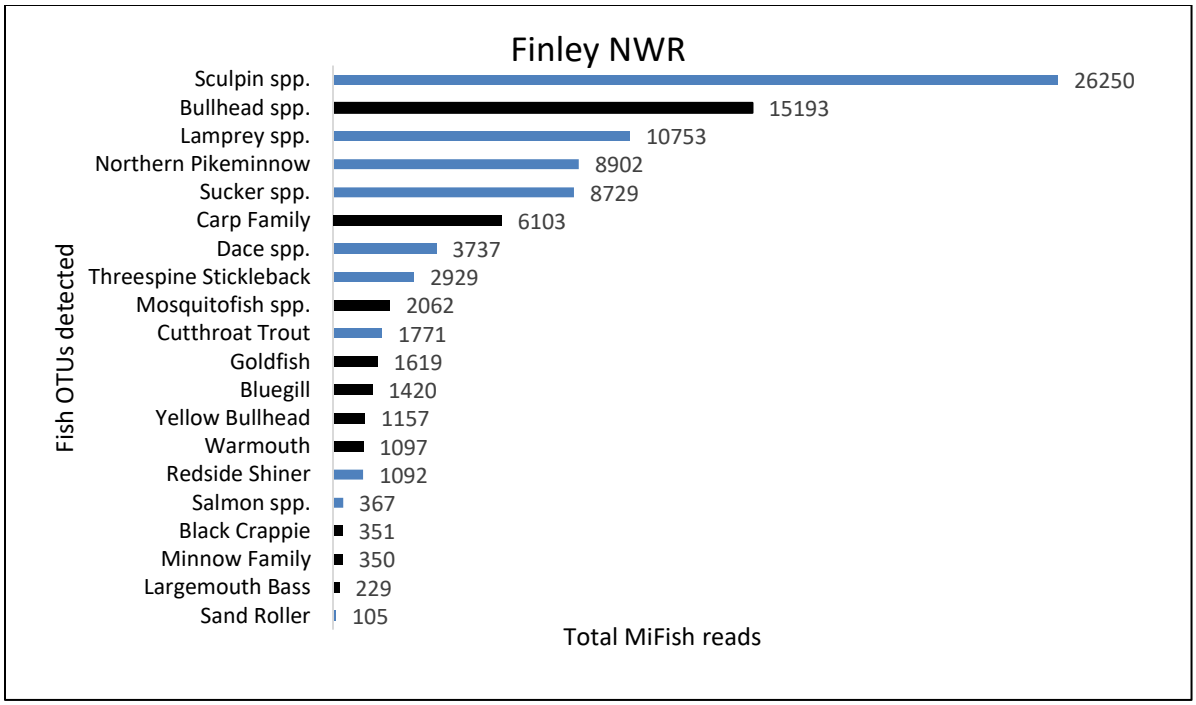

Figure 23. Cumulative read abundance of fish OTUs detected by the MiFish marker at seven sample locations at Finley NWR, 2023. Dark bars represent nonnative OTUs (n=10).

Franz Lake NWR (COI)

Two sites were sampled at Franz Lake NWR (Figure 24). The eDNA sample collected at Franz Lake outlet did not pass quality filtering parameters. The COI marker detected 43 OTUs belonging to 9 phyla in Indian Mary Creek (Figure 25). Thirty-one OTUs were identified to species level, including one fish (three-spined stickleback) and one mammal species (American beaver). The OTU with the highest cumulative read count was a diatom *Sellaphora minima*, with 10,120 reads.

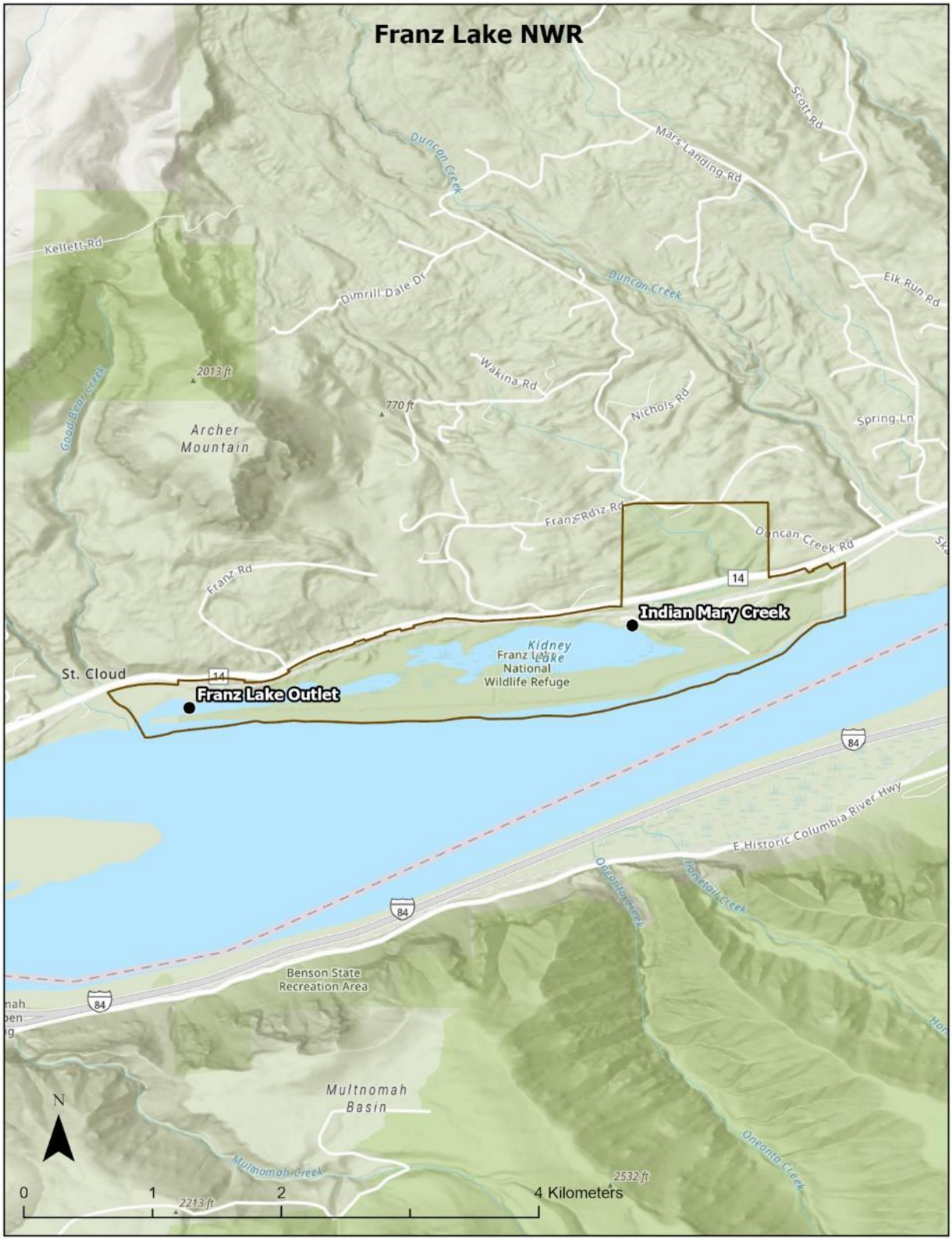

Figure 24. Environmental DNA sample locations at Franz Lake NWR, 2023.

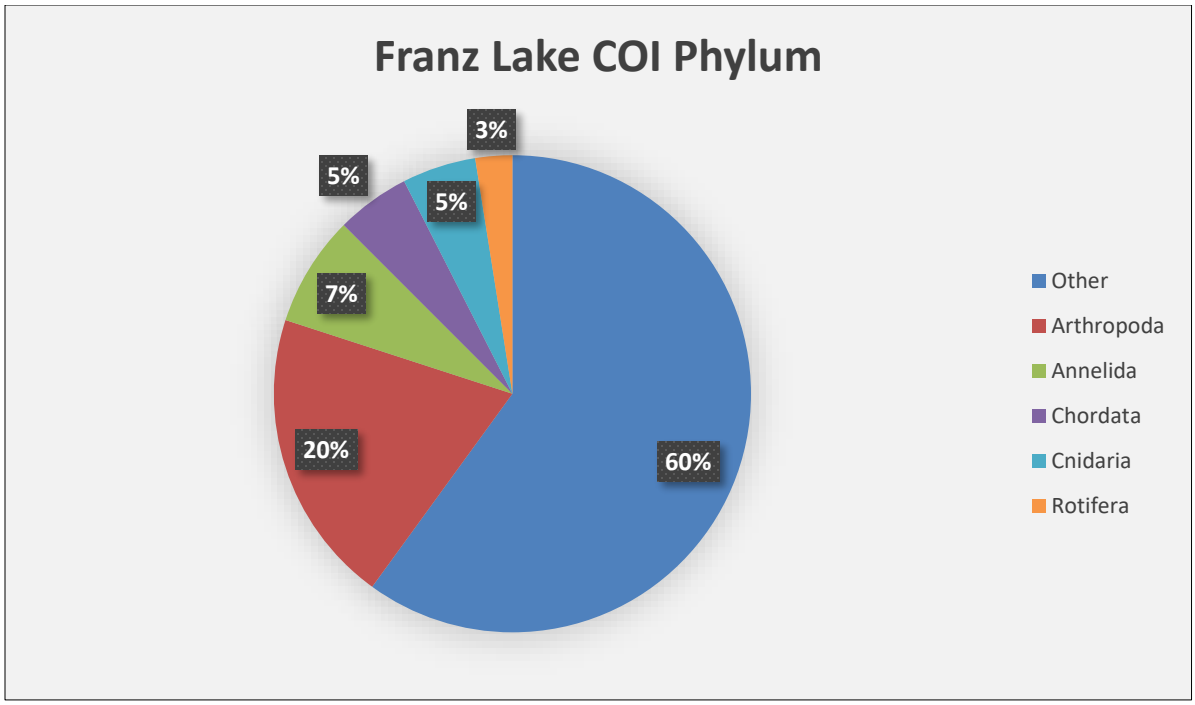

Figure 25. Proportion of common phylum identified by COI in Indian Mary Creek at Franz Lake NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

Franz Lake NWR (MiFish)

Both sample locations ran successfully using the MiFish eDNA marker. A total of 18 OTUs were detected at the two locations, including 15 fish, two mammals (American beaver and nutria), and one bird (mallard duck; Figure 26). Seven of the 15 fish OTUs were nonnative species. The OTUs with the highest cumulative read count were three-spined stickleback (17,925 reads) and sculpin (10,660 reads; Figure 27). Franz Lake outlet had the highest OTUs (11) and fish (9 OTUs) detected at Franz Lake NWR (Figure 27).

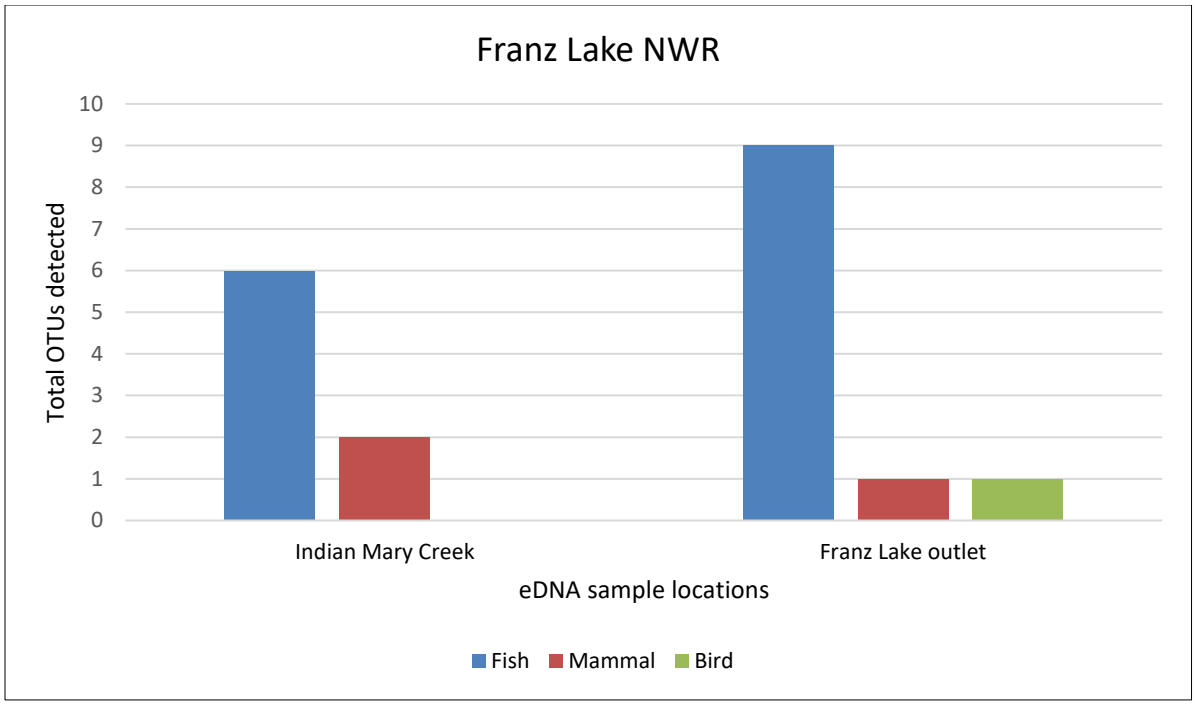

Figure 26. Fish, mammal and bird OTUs detected by MiFish in two sample locations at Franz Lake NWR, 2023.

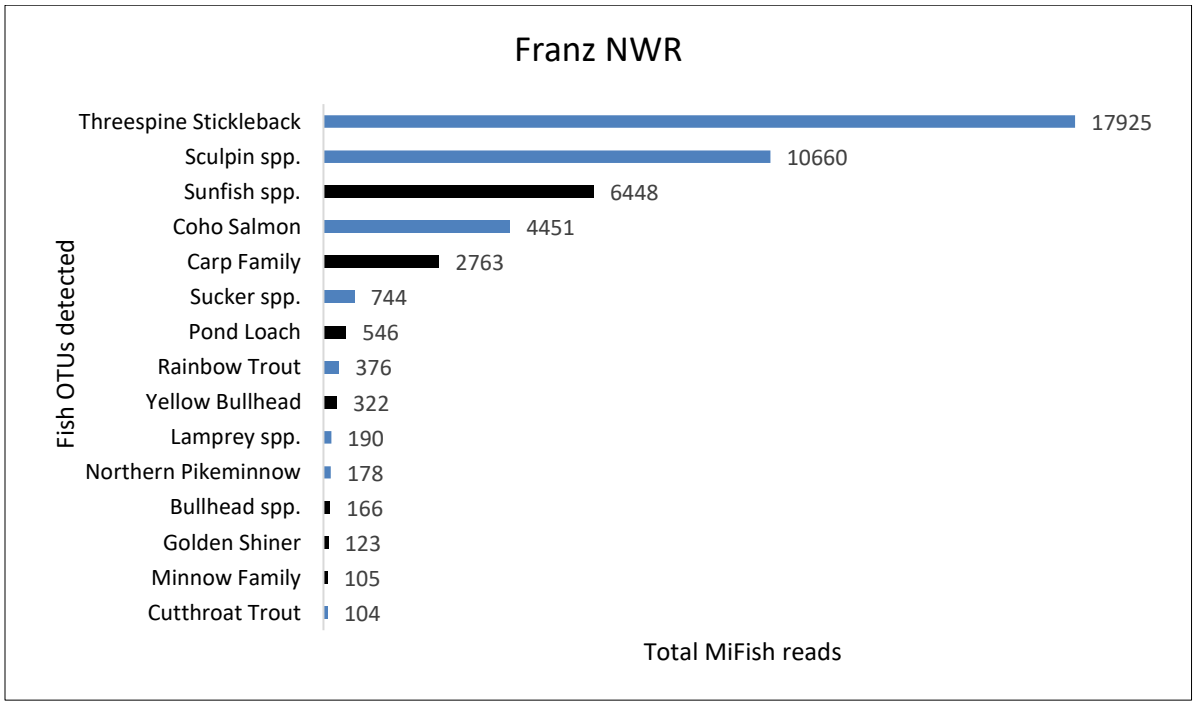

Figure 27. Cumulative read abundance of fish OTUs detected by the MiFish marker at two sample locations at Franz Lake NWR, 2023. Dark bars represent nonnative OTUs (n=7).

Julia Butler Hansen NWR (COI)

All seven sample locations ran successfully at Julia Butler Hansen (JBH) NWR (Figure 28). Collectively, COI detected 160 OTUs belonging to 12 phyla (Figure 29). Of the 160 OTUs, 108 were successfully identified to species level (Figure 30), including nine fish (largescale sucker, pond loach, coastrange sculpin, prickly sculpin, northern pikeminnow, banded killifish, cutthroat trout, coho salmon and Chinook salmon), four mammals (cattle, American beaver, Pacific jumping mouse and humans) and seven mollusks. Of concern was detecting invasive New Zealand mudsnail at Elochoman 2 and nonnative Asian clams at Elochoman 2 and Alger Creek 3. Oregon floater mussels were detected in Elochoman 2, but reads were below the minimum threshold of ≥100 reads (53 reads) and may not be a reliable detection. The OTU with the highest cumulative reads was *Pseudodiaptomus forbesi*, a copepod with 139,043 reads, and OTUs with the highest rate of occurrence were a plant pathogen of genus *Pythium* and fungi of genus *Cladosporium*, detected at six sites. Skamakawa Creek was the sample location with the highest number of OTUs (67) and unique species (48) detected at JBH NWR.

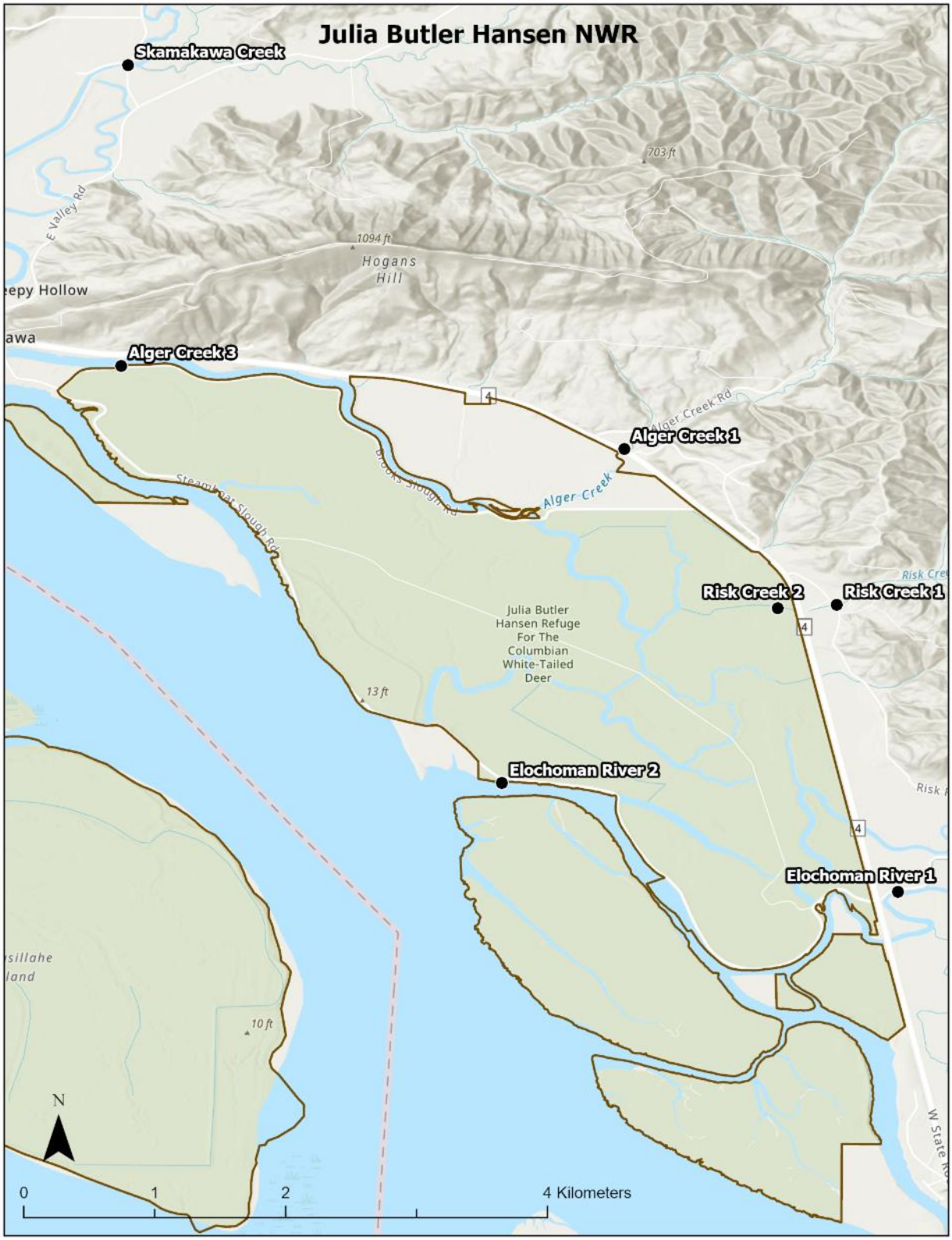

Figure 28. Environmental DNA sample locations at Julia Butler Hanson NWR, 2023.

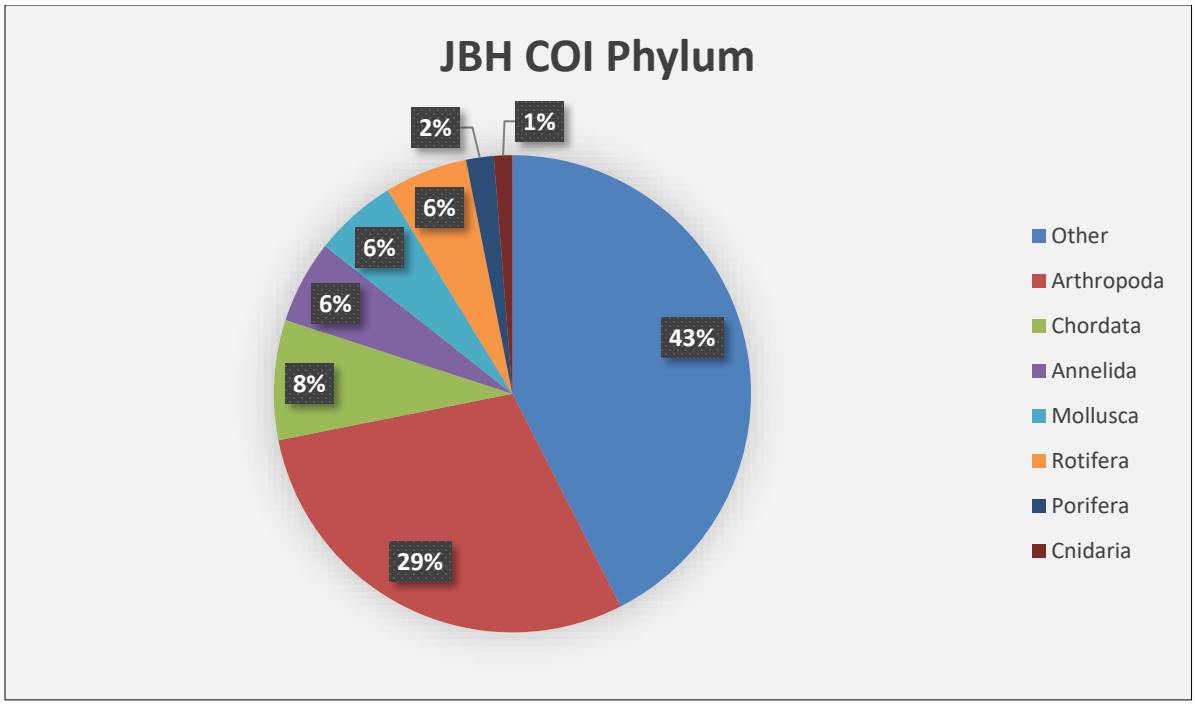

Figure 29. Proportion of common phylum identified by COI in seven sample locations at JBH NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

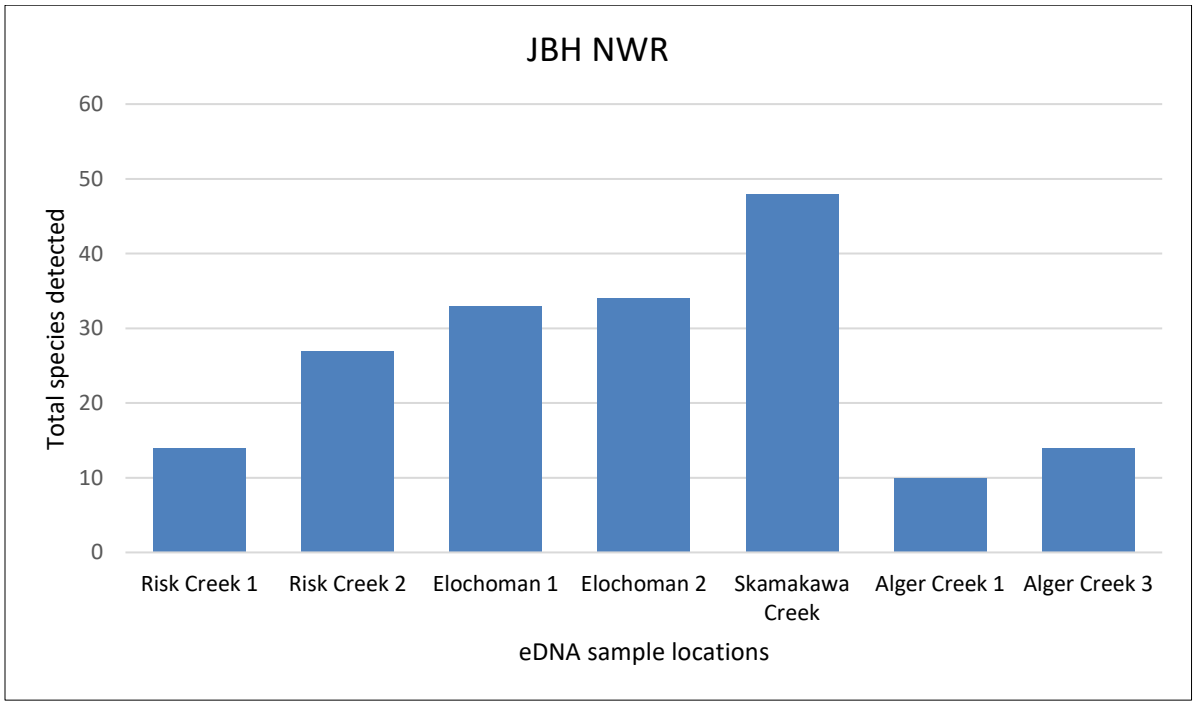

Figure 30. Total number of species detected by COI in seven sample locations at JBH NWR, 2023.

Julia Butler Hansen (MiFish)

Two of seven sites (Alger Creek 1 & 3) did not run with the MiFish eDNA marker (Figure 31). A total of 20 OTUs were detected, including 15 fish, three mammals (cattle, American beaver, human), one amphibian (rough-skinned newt), and 1 bird (mallard duck; Figure 32). The OTUs with the highest cumulative read count and occurrence were sculpin (18,516 reads) and northern pikeminnow (17,084 reads), both detected in four sample locations. Elochoman 2 had the highest number of OTUs (15) and fish (13 OTUs) detected at JBH NWR.

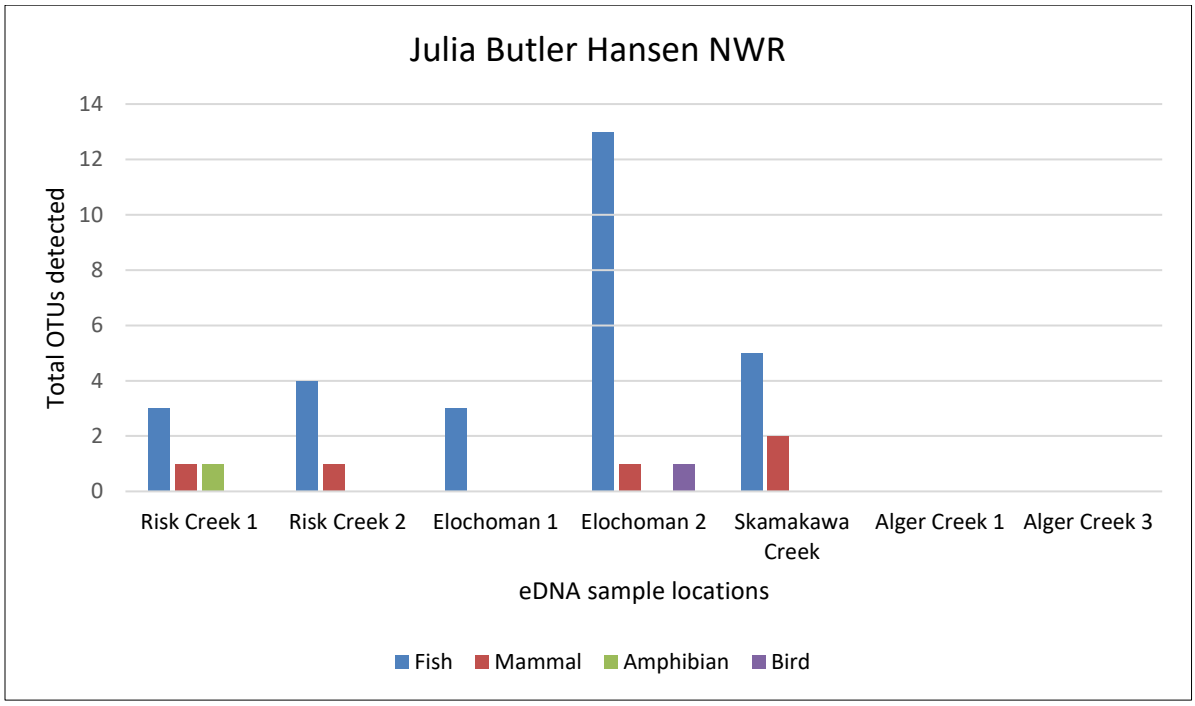

Figure 31. Fish, mammal, amphibian and bird OTUs detected by MiFish in five sample locations at JBH NWR, 2023.

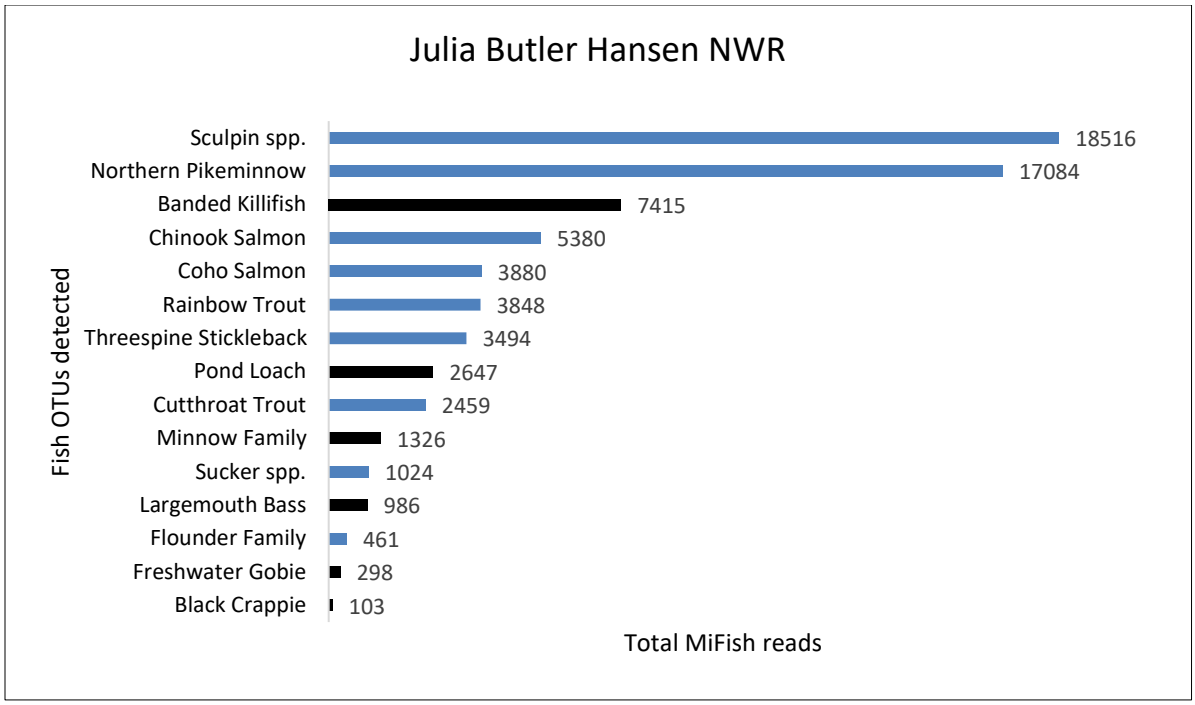

Figure 32. Cumulative read abundance of fish OTUs detected by the MiFish marker at five sample locations at JBH NWR, 2023. Dark bars represent nonnative OTUs (n=6).

Nisqually NWR (COI)

All 16 sample locations ran successfully at Nisqually NWR (Figure 33 and Figure 34). Collectively, COI detected 242 OTUs belonging to 16 phyla (Figure 35). Of the 242 OTUs, approximately 29% were marine taxa, and 172 were identified to species level, including 10 fish (Figure 35), three mammals (cattle, American Beaver, and human), one amphibian (American bullfrog), and 10 mollusks (Figure 35 and Figure 36). Of note was the detection of Olympic mudminnow at Dempsey Creek 1, listed as a sensitive species in Washington. The detection of invasive New Zealand mudsnail at McCallister Creek and Grays Harbor Tributary was of concern. Western pearlshell mussels were detected in Dempsey Creek 1, but reads were below the minimum ≥100 threshold (40 reads), so the detection may not be reliable. The OTU with the highest read count was *Melosira ambiqua*, a freshwater diatom, and the OTUs with the highest rate of occurrence were a dinoflagellate from genus *Peridinium* and non-biting midge from family Chironomidae, each detected at eight locations. The South Sound was the location with the highest OTUs (56) and total number of detected species (37) at Nisqually NWR (Figure 37 and Figure 38).

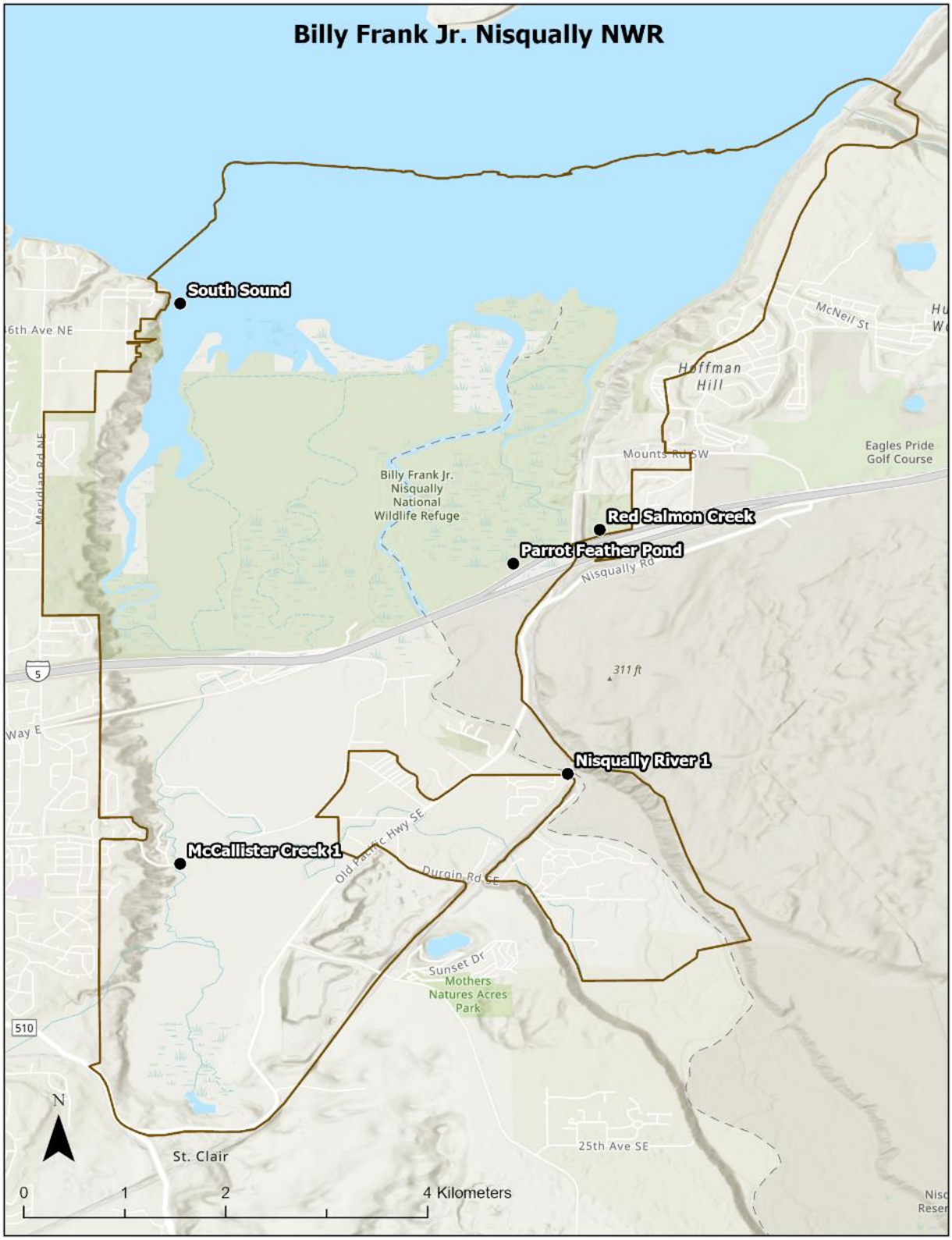

Figure 33. Environmental DNA sample locations at Nisqually NWR, 2023.

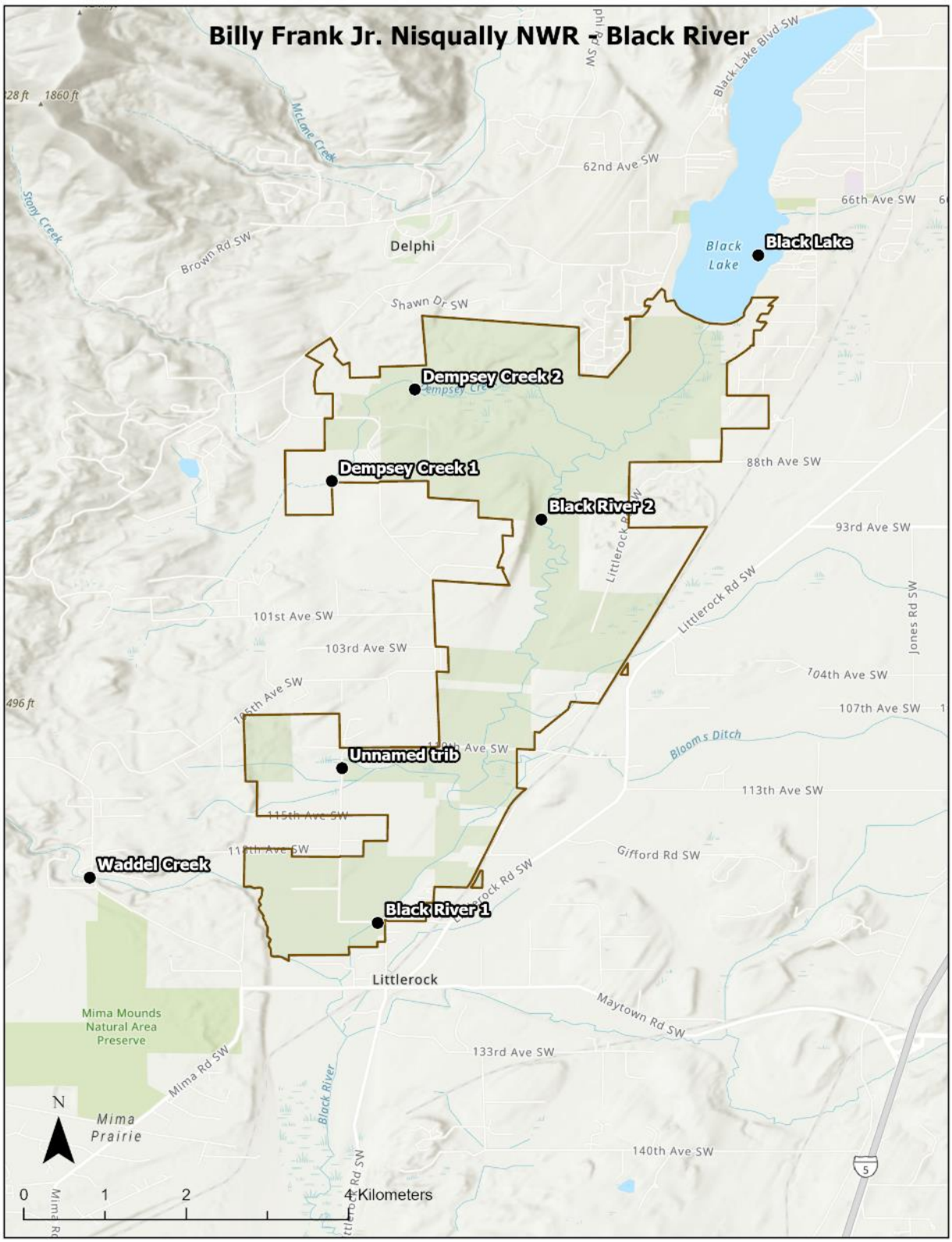

Figure 34. Environmental DNA sample locations at Nisqually-Black River NWR, 2023.

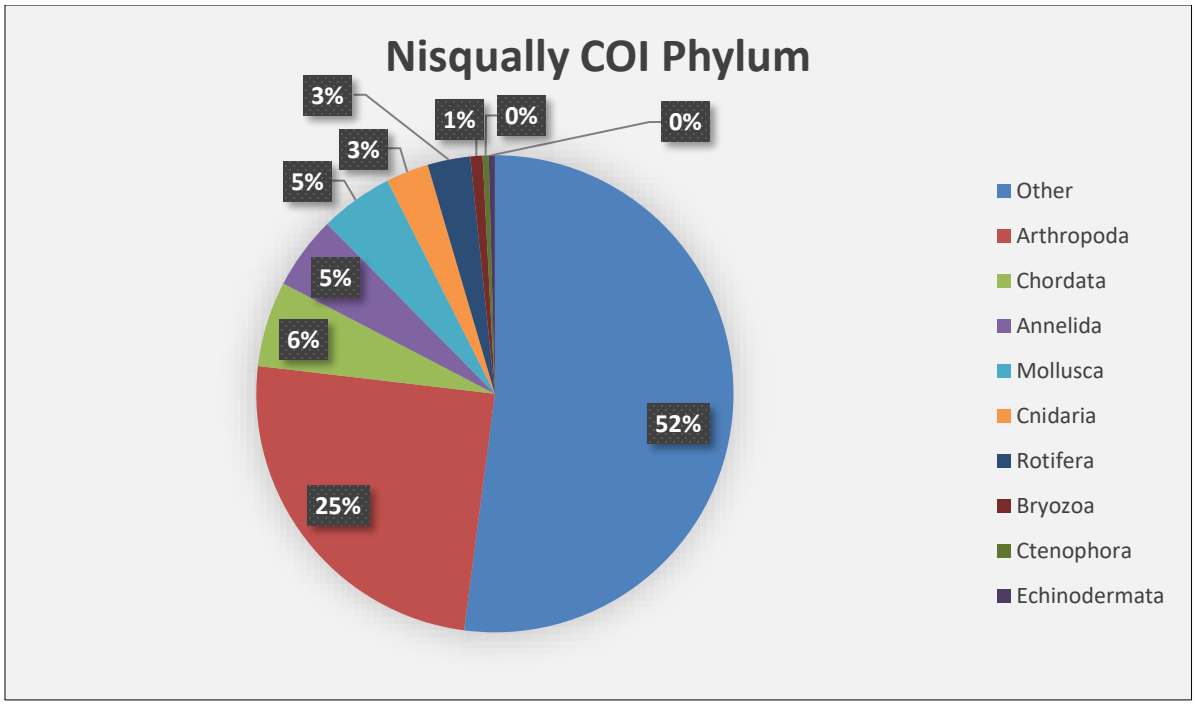

Figure 35. Proportion of common phylum identified by COI in 16 sample locations at Nisqually NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

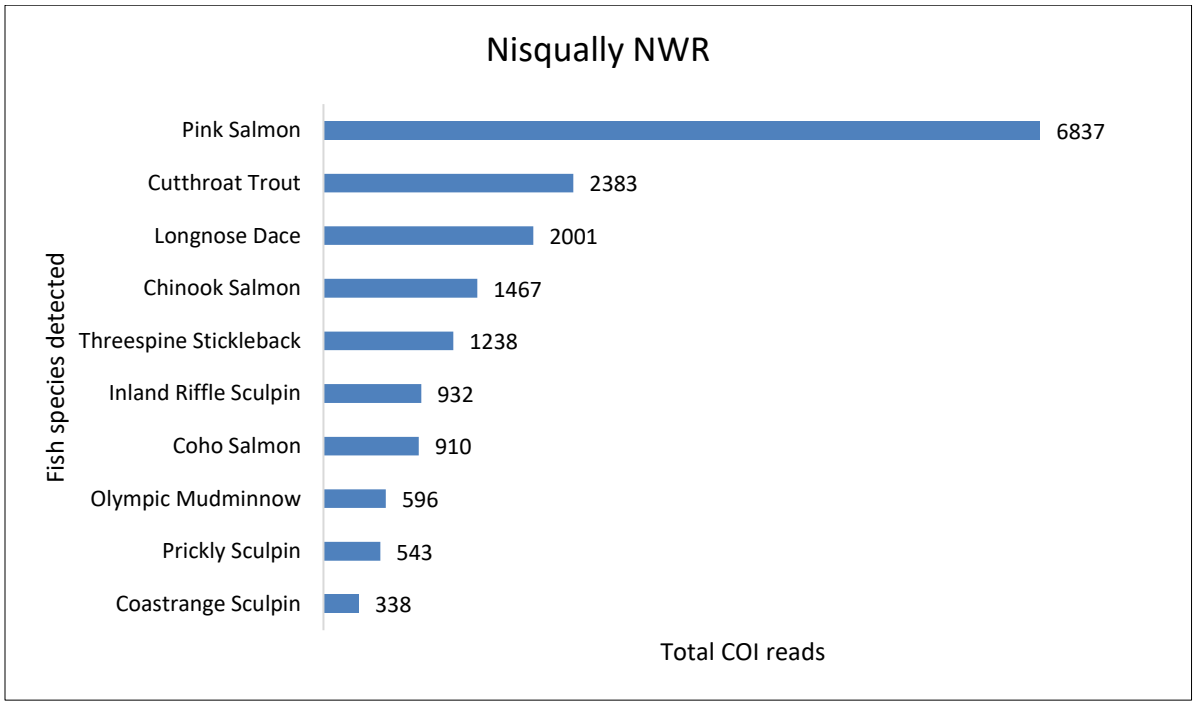

Figure 36. Cumulative read abundance of fish species detected by the COI marker at 16 sample locations at Nisqually NWR, 2023.

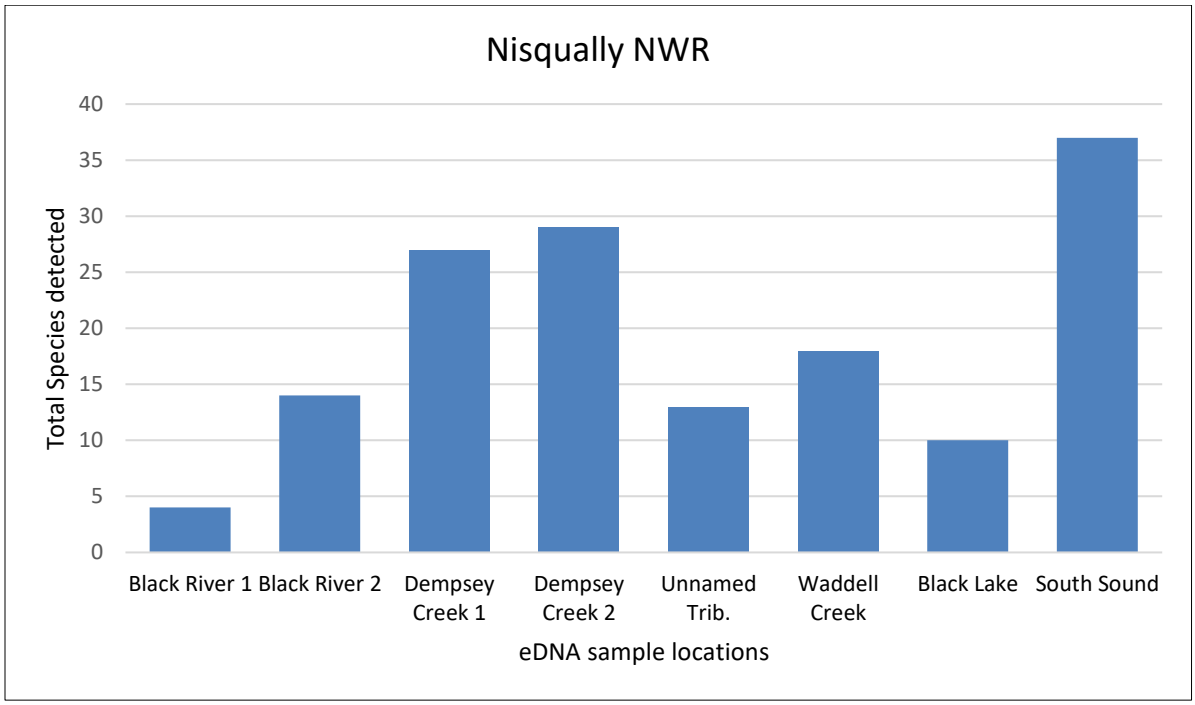

Figure 37. Total number of species detected by COI at eight sample locations at Nisqually NWR, 2023.

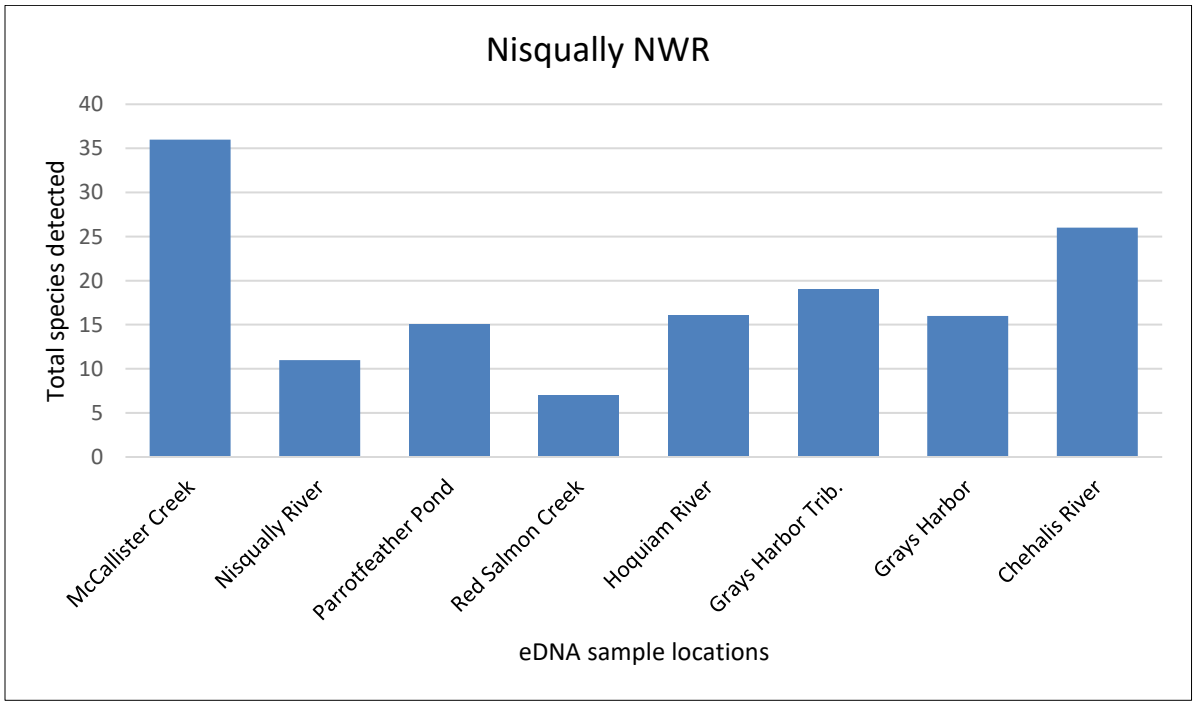

Figure 38. Total number of species detected by COI at eight additional sample locations at Nisqually NWR, 2023.

Nisqually NWR (MiFish)

Four of the 16 sample locations did not run with the MiFish eDNA marker, including Black River 1, Black Lake, Hoquiam River, and Waddell Creek (Figure 39). Six of the remaining Nine sites detected two or fewer species (Black River 2, Grays Harbor Tributary, Grays Harbor, Nisqually River 1, Parrotfeather Creek, and Red Salmon Creek). A total of 17 OTUs were detected using the MiFish marker, including 14 fish, two mammals (cattle and human), and one bird (family Atidae). Four of the fourteen fish detected were marine species (Pacific herring, Pacific staghorn sculpin, shiner perch, and snake prickleback), and one species was potentially nonnative. The fish genus *Salvelinus* was detected in Black River 2, but it is unknown if the detection was from a brook trout or bull trout. The fish genus *Oncorhynchus* was detected at two locations with the MiFish marker (Nisqually River 1 and South Sound), but the OTUs were not identified to species. The COI marker detected pink salmon at the same two locations (**Figure 41**), suggesting the MiFish *Oncorhynchus* detections may be pink salmon. Of note was the detection of Olympic mudminnow in two locations (Dempsey Creek 1 and Unnamed Tributary) at Nisqually NWR. The Olympic mudminnow was also detected in Dempsey Creek 1 with the COI marker. The OTU with the highest cumulative read count was three-spined stickleback (30,473 reads), which was detected in seven locations. Genus *Oncorhynchus* (presumably pink salmon) had the second highest read count (29,680 reads) and was detected in two locations. Dempsey Creek 1 was the sample location with the highest OTUs (7) and fish (6 OTUs) detected at Nisqually NWR.

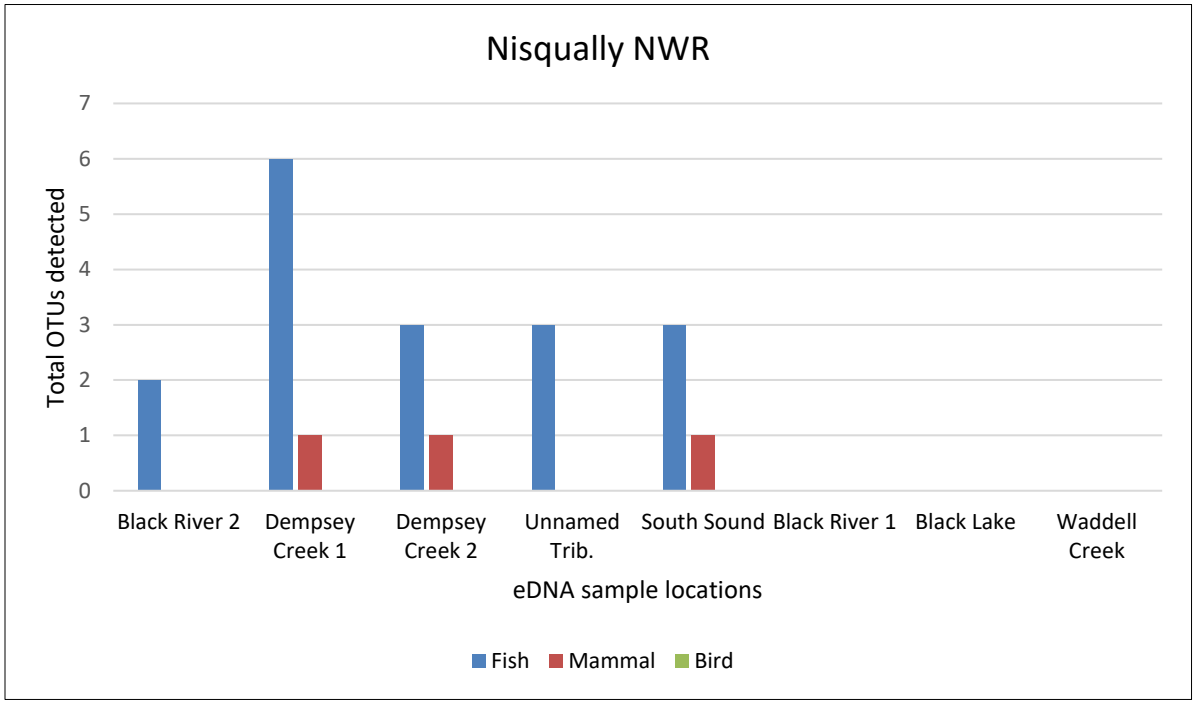

Figure 39. Fish, mammal and bird OTUs detected by MiFish in eight sample locations at Nisqually NWR, 2023.

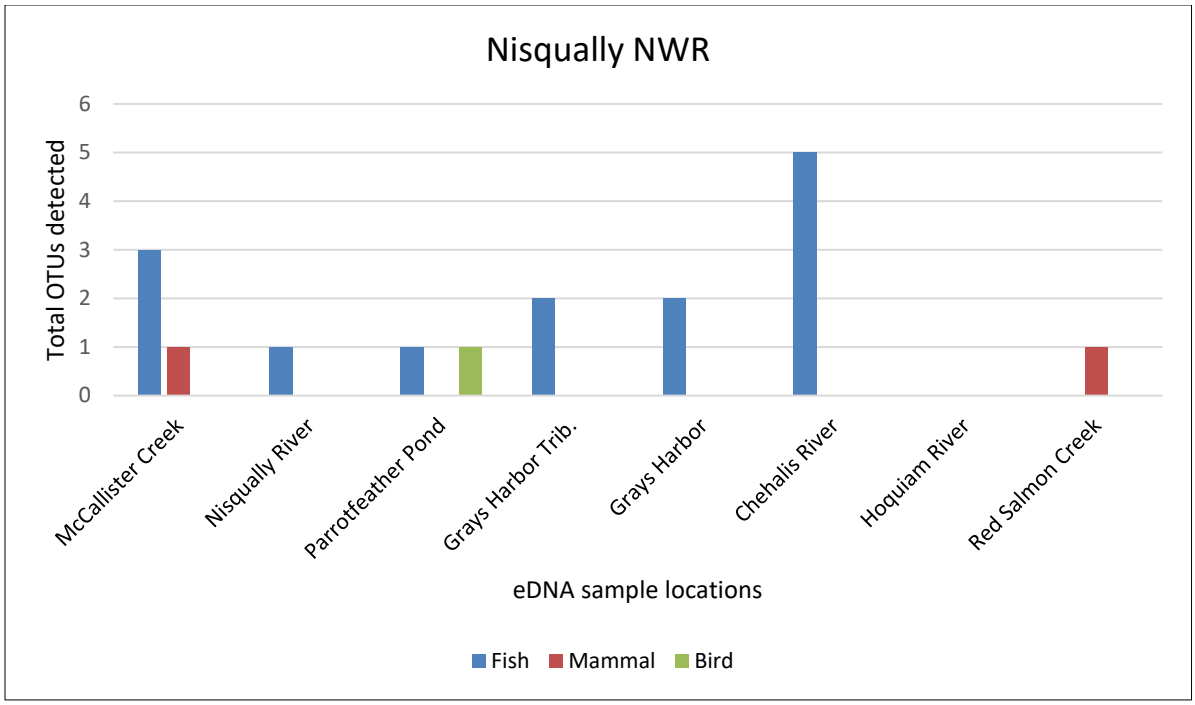

Figure 40. Fish, mammal and bird OTUs detected by MiFish in eight additional sample locations at Nisqually NWR, 2023.

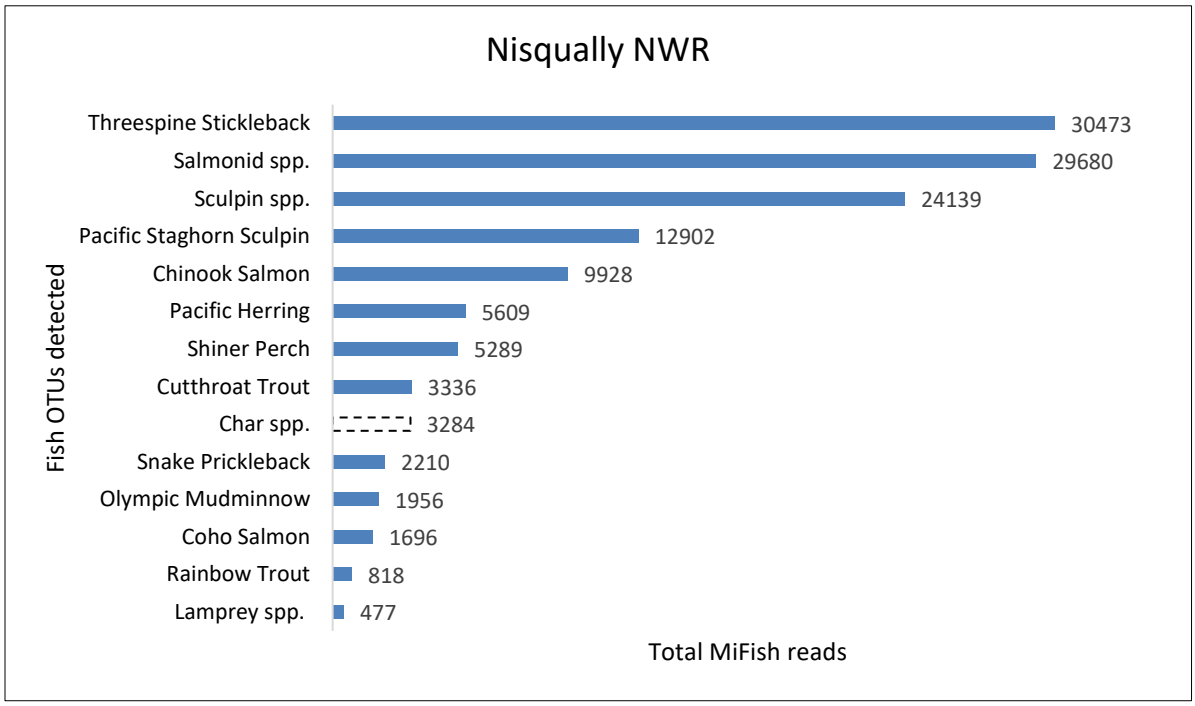

Figure 41. Cumulative read abundance of fish OTUs detected by the MiFish marker at 12 sample locations at Nisqually NWR, 2023. Black bar denotes potential nonnative species (n=1).

Pierce NWR (COI)

All 6 sample locations ran successfully at Pierce NWR (Figure 42). Collectively, COI detected 61 OTUs belonging to 11 phyla (Figure 43). Of the 61 OTUs, 40 were identified to species level, including two fish (three-spined stickleback and black bullhead) and one mammal (human). No freshwater mussels were detected at Pierce NWR. The OTU with the highest cumulative read count was *Chydorus brevilabris*, a freshwater crustacean with 110,351 reads, and the OTU with the highest rate of occurrence was a species of red algae (*Paralemanea annulate*) detected at four locations. Hardy Creek 3 was the location with the highest OTUs (26) and total number of detected species (17) at Pierce NWR (Figure 44).

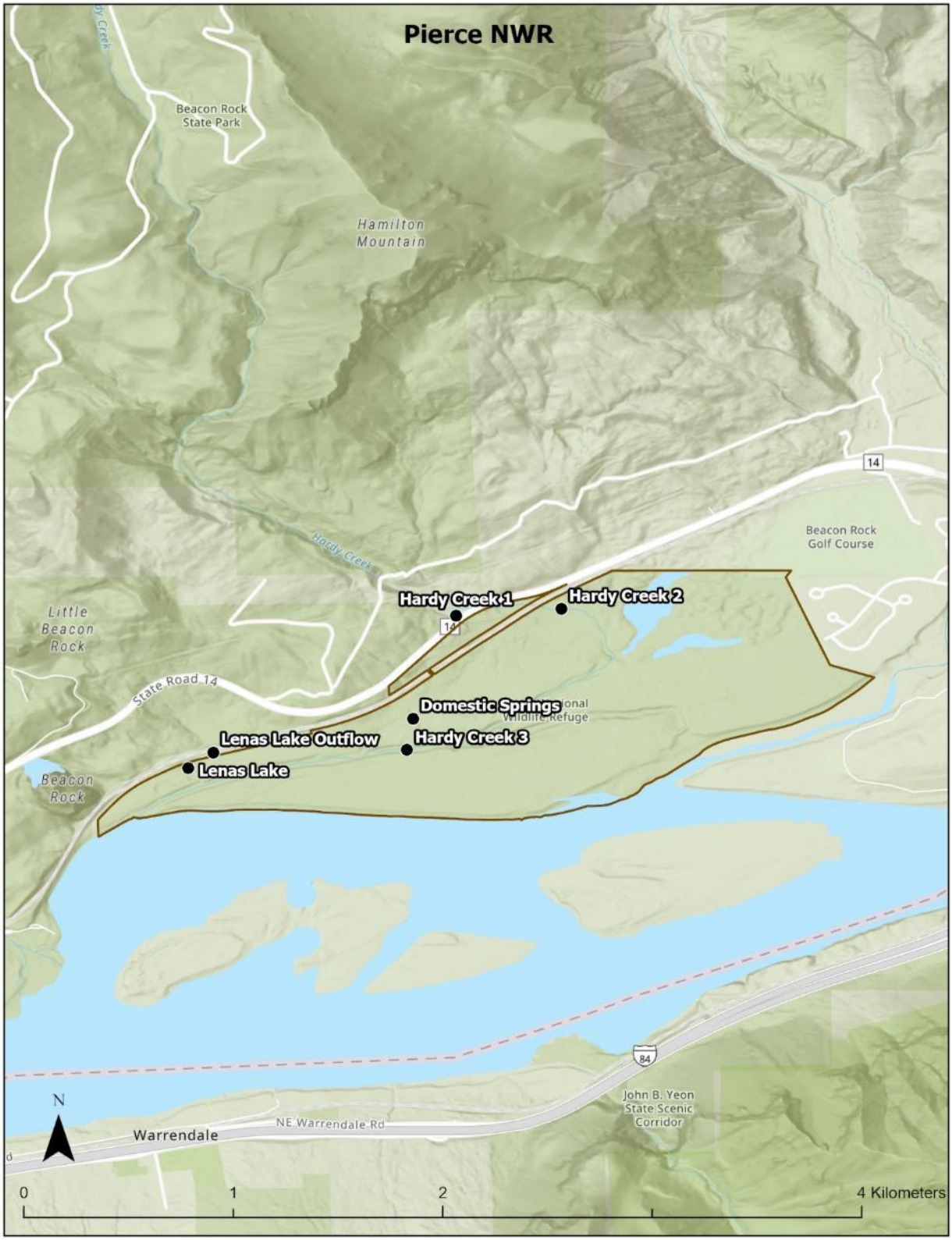

Figure 42. Environmental DNA sample locations at Pierce NWR, 2023.

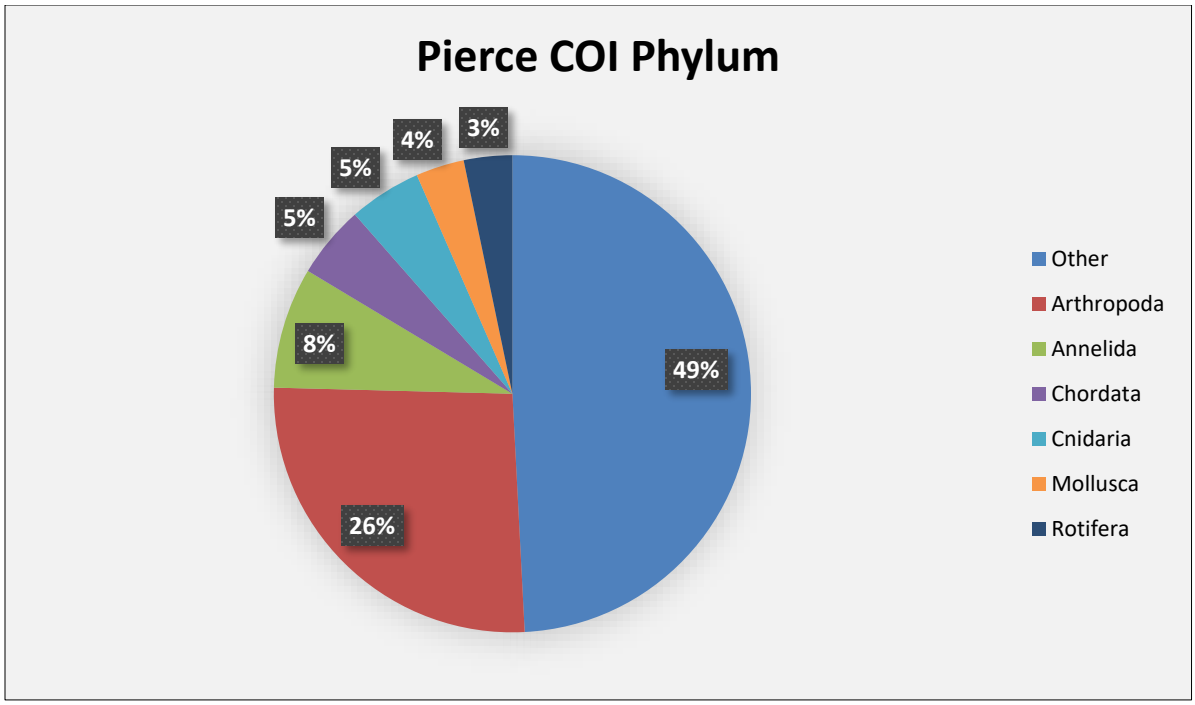

Figure 43. Proportion of common phylum identified by COI in six sample locations at Pierce NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

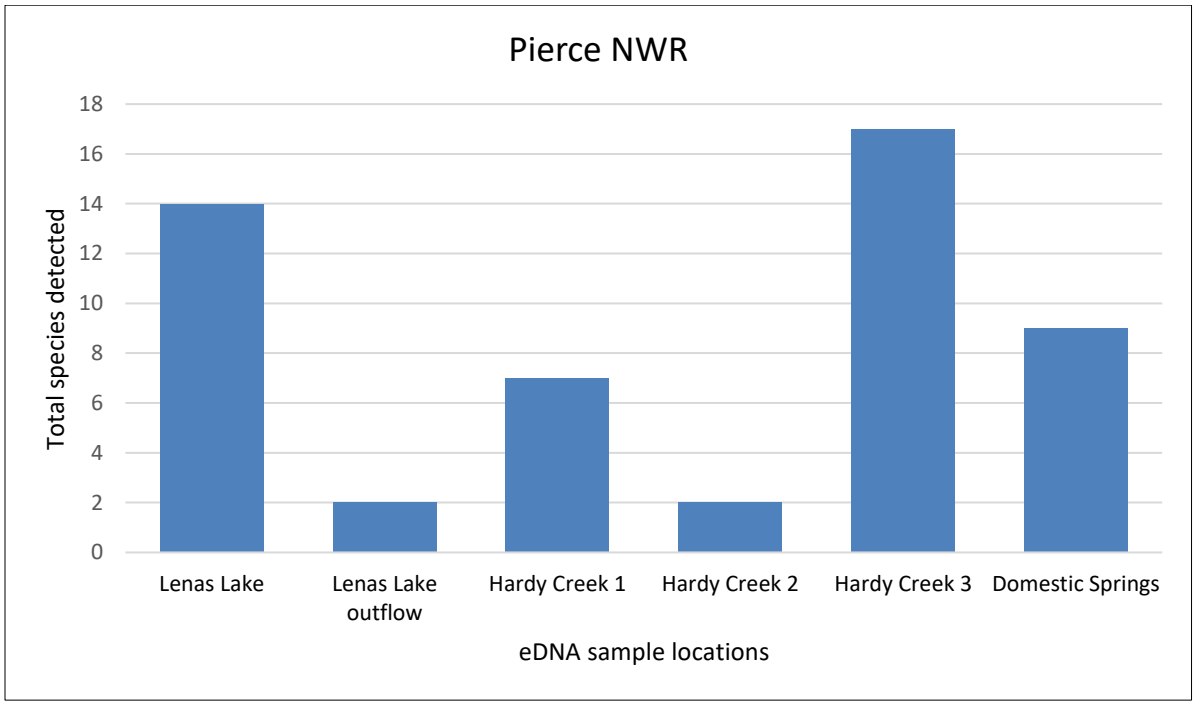

Figure 44. Total number of species detected by COI at six sample locations at Pierce NWR, 2023.

Pierce NWR (MiFish)

Two of the 6 sample locations did not run with the MiFish eDNA marker (Lenas Lake outflow and Hardy Creek 3; Figure 45). Three of the remaining four locations had two or fewer species detected by the MiFish marker (Hardy Creek 1, Hardy Creek 2, and Domestic Springs). A total of ten OTUs were detected, including seven fish, two mammals (mouse-eared bat and human), and one bird (mallard duck; Figure 46). Two of the seven fish detected were nonnative species (Figure 46). The OTU with the highest cumulative read count was rainbow trout/steelhead (14,506 reads), and sculpin were the only species detected at more than one location (two total). Lenas Lake was the sample location with the highest OTUs (6) and total fish (4 OTUs) detected at Pierce NWR.

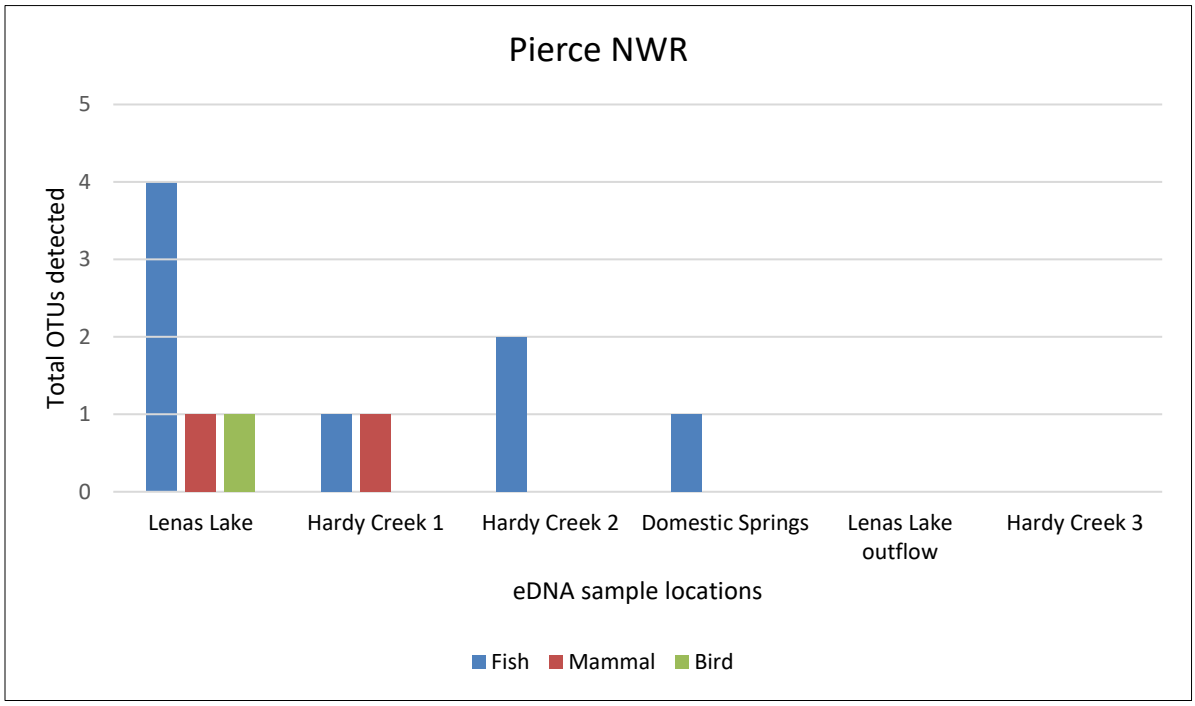

Figure 45. Fish, mammal and bird OTUs detected by MiFish in four sample locations at Pierce NWR, 2023.

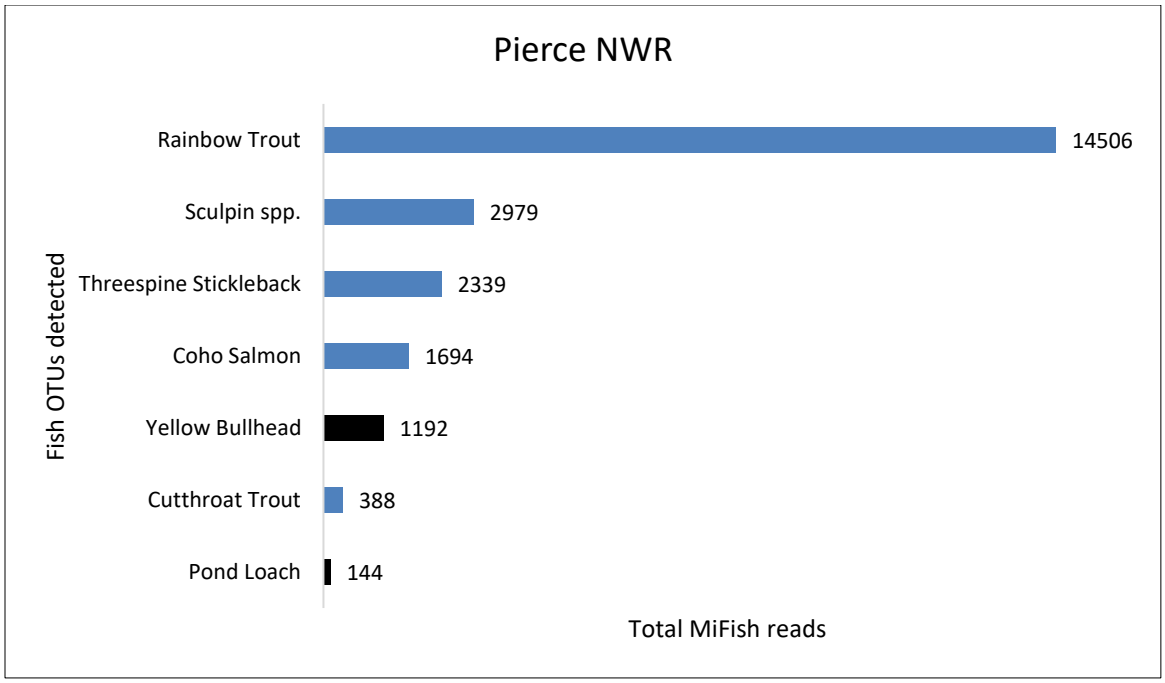

Figure 46. Cumulative read abundance of fish OTUs detected by the MiFish marker at four sample locations at Pierce NWR, 2023. Black bar denotes nonnative species (n=2).

Ridgefield NWR (COI)

Six locations were sampled at Ridgefield NWR (Figure 47). The eDNA sample collected at River S Expulsion did not pass quality filtering parameters. The COI marker detected 53 OTUs belonging to 12 phyla at the five locations (Figure 48). Of the 53 OTUs, 40 were identified to species level, including three fish (prickly sculpin, goldfish, and common carp), two mammals (American beaver and human), and one nonnative mollusk (Asian clam detected at Turtle Lake and Gee Creek 2). Oregon Floater mussels were detected at four locations (Turtle Lake, Campbell Lake Crossing, Gee Creek 1, and Gee Creek 2), and western pearlshell mussels were detected at a single location (Whipple Creek), but read counts were below the minimum threshold of ≥100 reads and may not be reliable detections. The OTU with the highest cumulative read count was *Melosira ambiqua*, a freshwater diatom with 458,254 reads. The OTU detected at most sample locations was also a freshwater diatom (*Nitzschia palea*) detected at four locations. Campbell Lake Crossing was the location with the highest OTUs (26) and total number of detected species (20) detected at Ridgefield NWR (Figure 49).

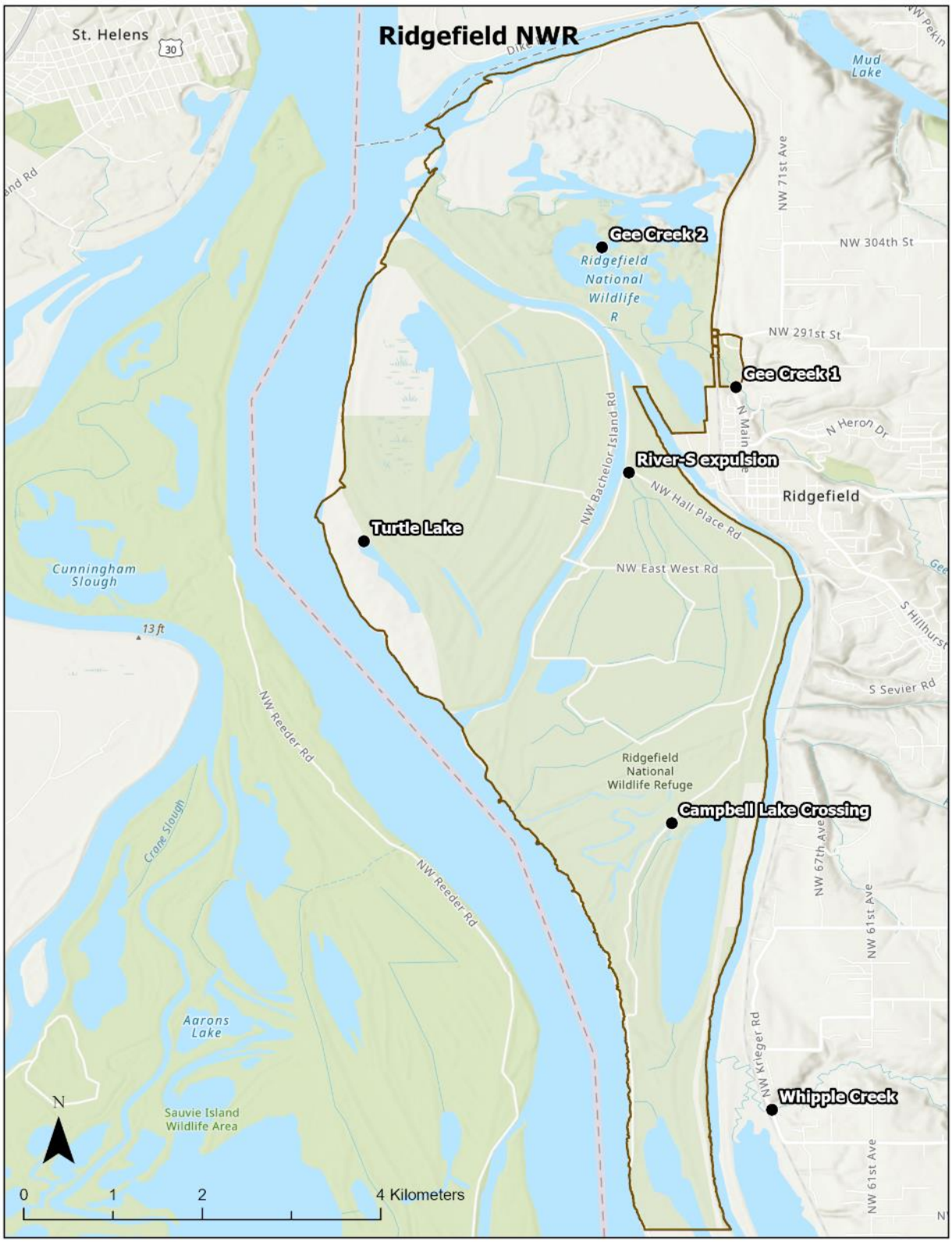

Figure 47. Environmental DNA sample locations at Ridgefield NWR, 2023.

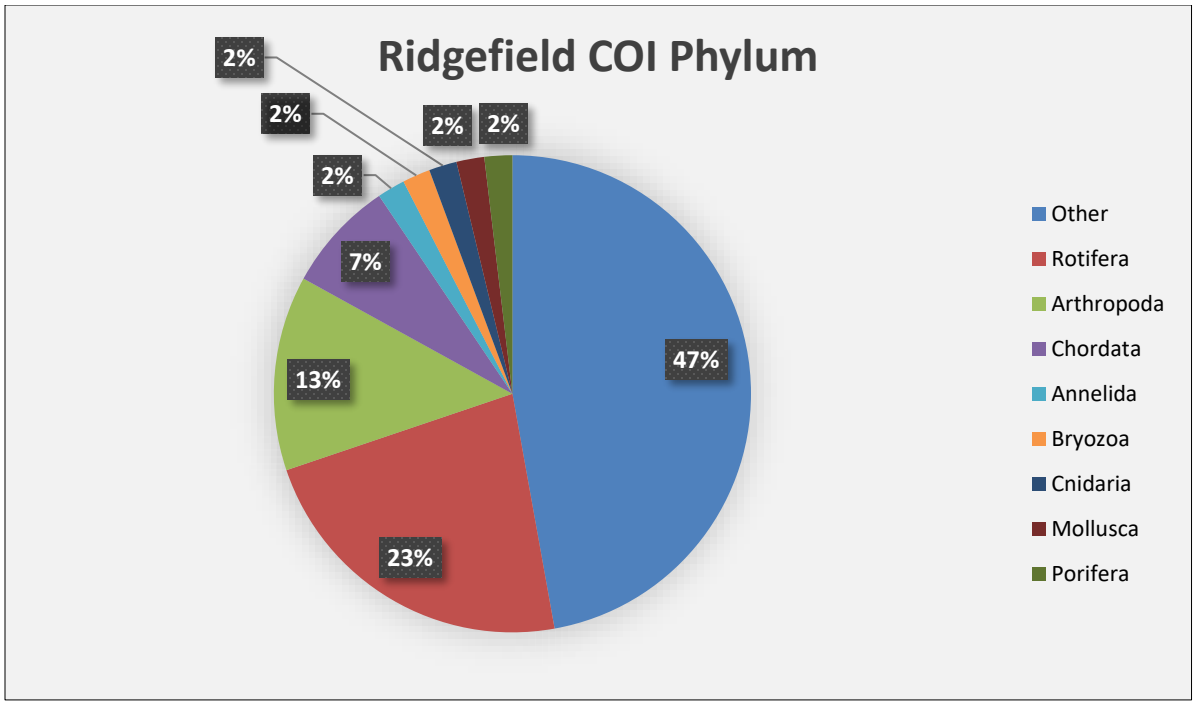

Figure 48. Proportion of common phylum identified by COI in five sample locations at Ridgefield NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

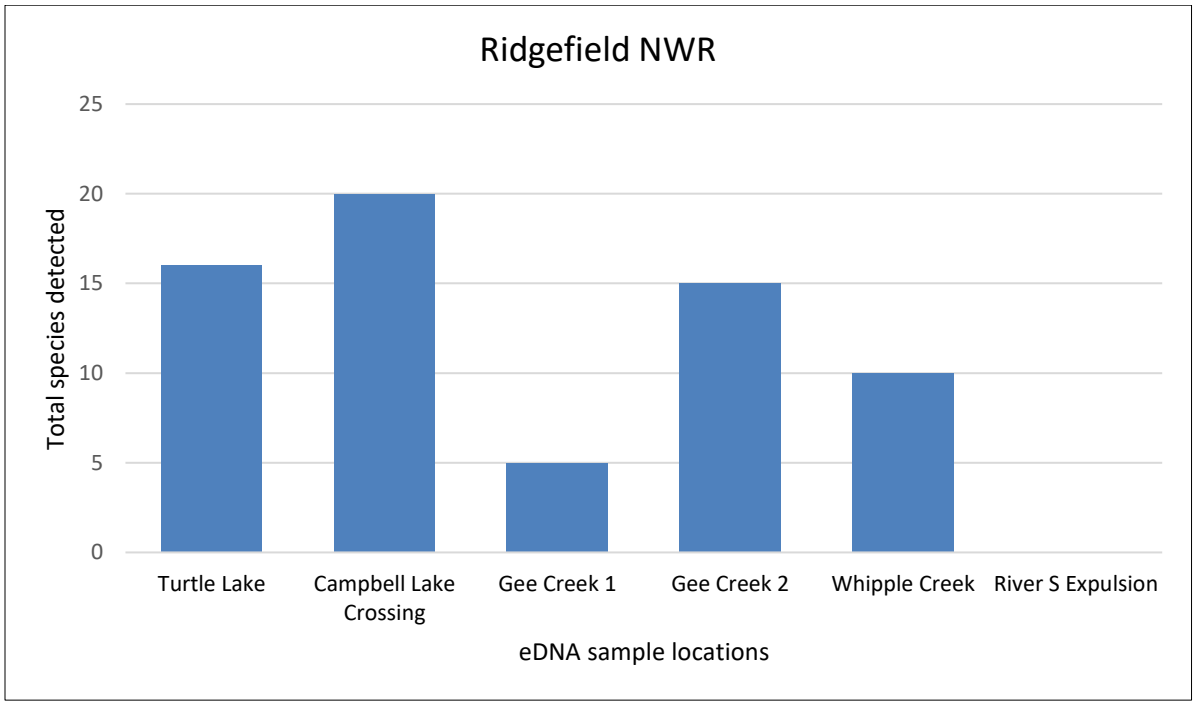

Figure 49. Total number of species detected by COI at five sample locations at Ridgefield NWR, 2023.

Ridgefield NWR (MiFish)

Two of the six locations did not run with the MiFish eDNA marker (Turtle Lake and River S Expulsion; Figure 50). A total of 24 OTUs were detected at four locations, including 21 fish, two mammals (American beaver, human), and two birds (mallard and wood ducks; Figure 51). Of the 21 fish OTUs, 67% were nonnative species (Figure 51). The OTUs with the highest cumulative read count were the carp family (16,208 reads) and sculpin (15,501 reads). The OTUs with the highest rate of occurrence were golden shiner, largemouth bass, pond loach, sunfish spp., and three-spined stickleback, each detected in three locations. Gee Creek 2 had the highest number of OTUs (14) and total fish (13 OTUs) detected at Ridgefield NWR.

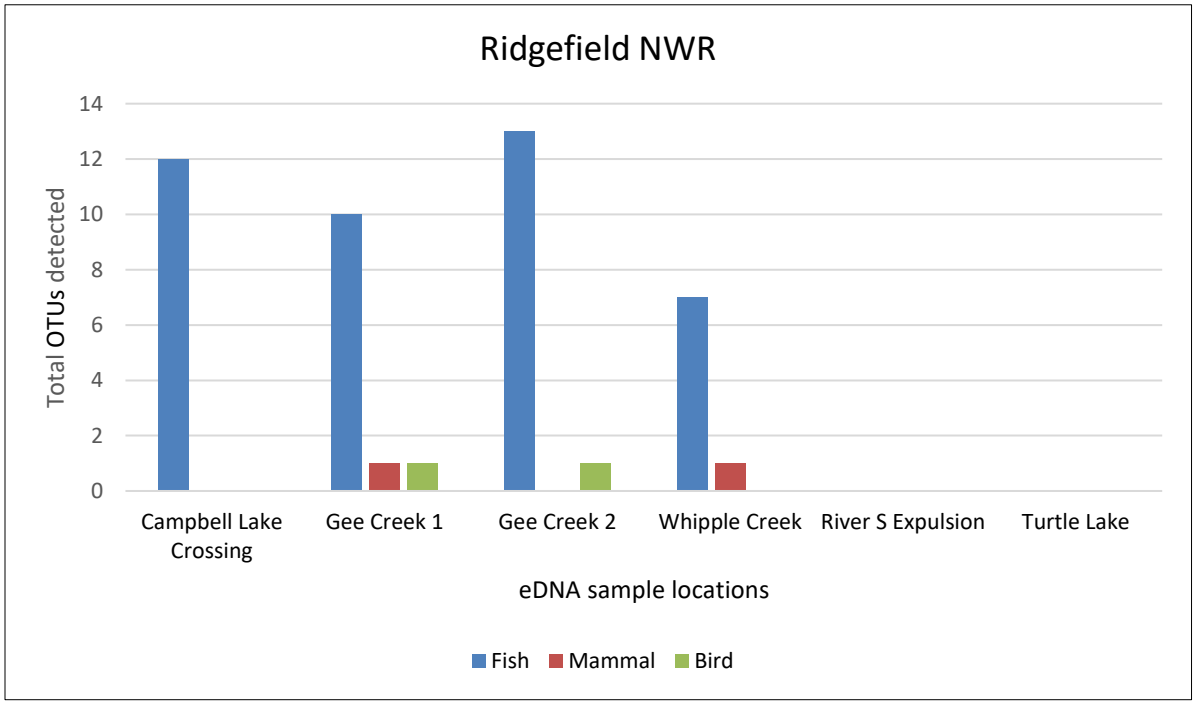

Figure 50. Fish, mammal and bird OTUs detected by MiFish in four sample locations at Ridgefield NWR, 2023.

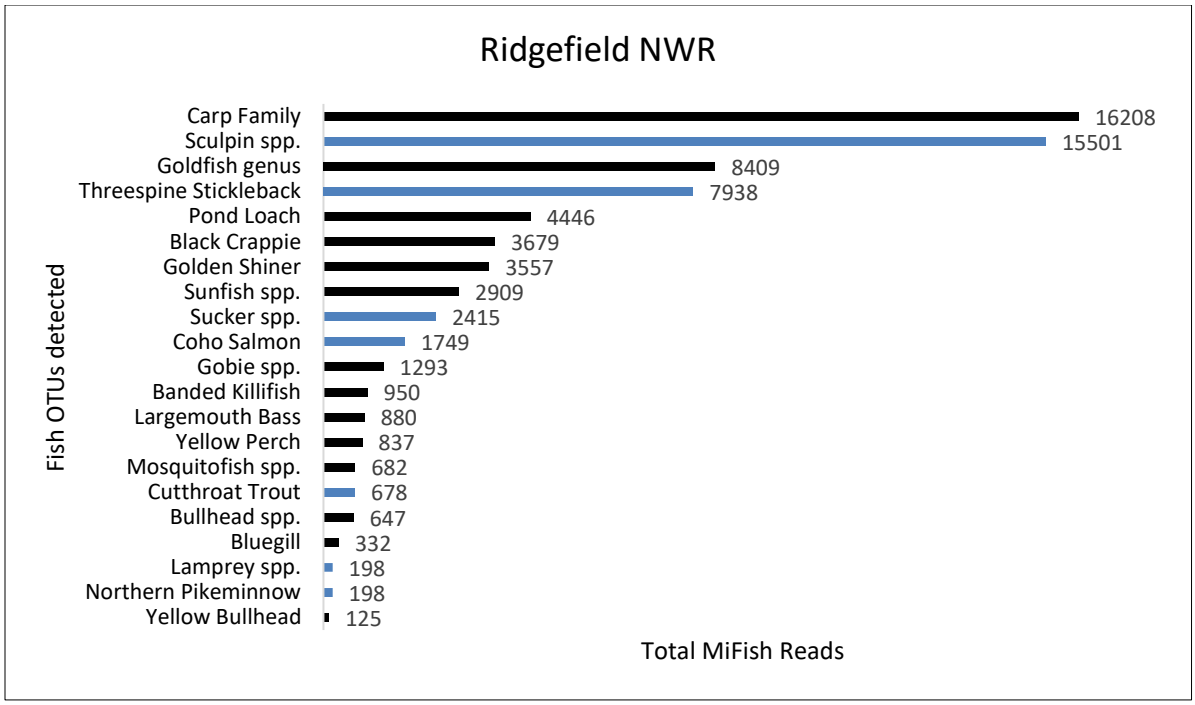

Figure 51. Cumulative read abundance of fish OTUs detected by the MiFish marker at four sample locations at Ridgefield NWR, 2023. Black bars denote nonnative species (n=14).

Steigerwald Lake NWR (COI)

All five eDNA samples collected at Steigerwald NWR ran successfully using the COI marker (Figure 52), though Gibbons 1 and Campen Creek had few detections. A total of 96 OTUs belonging to 11 phyla were detected (Figure 53). Of the 96 OTUs, 69 were identified to species level, including seven fish (largescale sucker, pond loach, prickly sculpin, goldfish, longnose dace, three-spined stickleback, and cutthroat trout), one mammal (American beaver), one amphibian (American bullfrog) and three mollusks (pond snail, bladder snail and glassy juga snail). No freshwater mussels were detected at Steigerwald NWR. The OTU with the highest read count was *Keratella cochlearis*, a rotifer with 59,049 reads and the OTU detected at the most sample locations was a freshwater polyp (*Hydra vulgaris*) detected at four locations. Gibbons Creek 2 was the location with the highest OTUs (62) and total number of species detected (42) at Pierce NWR (Figure 54).

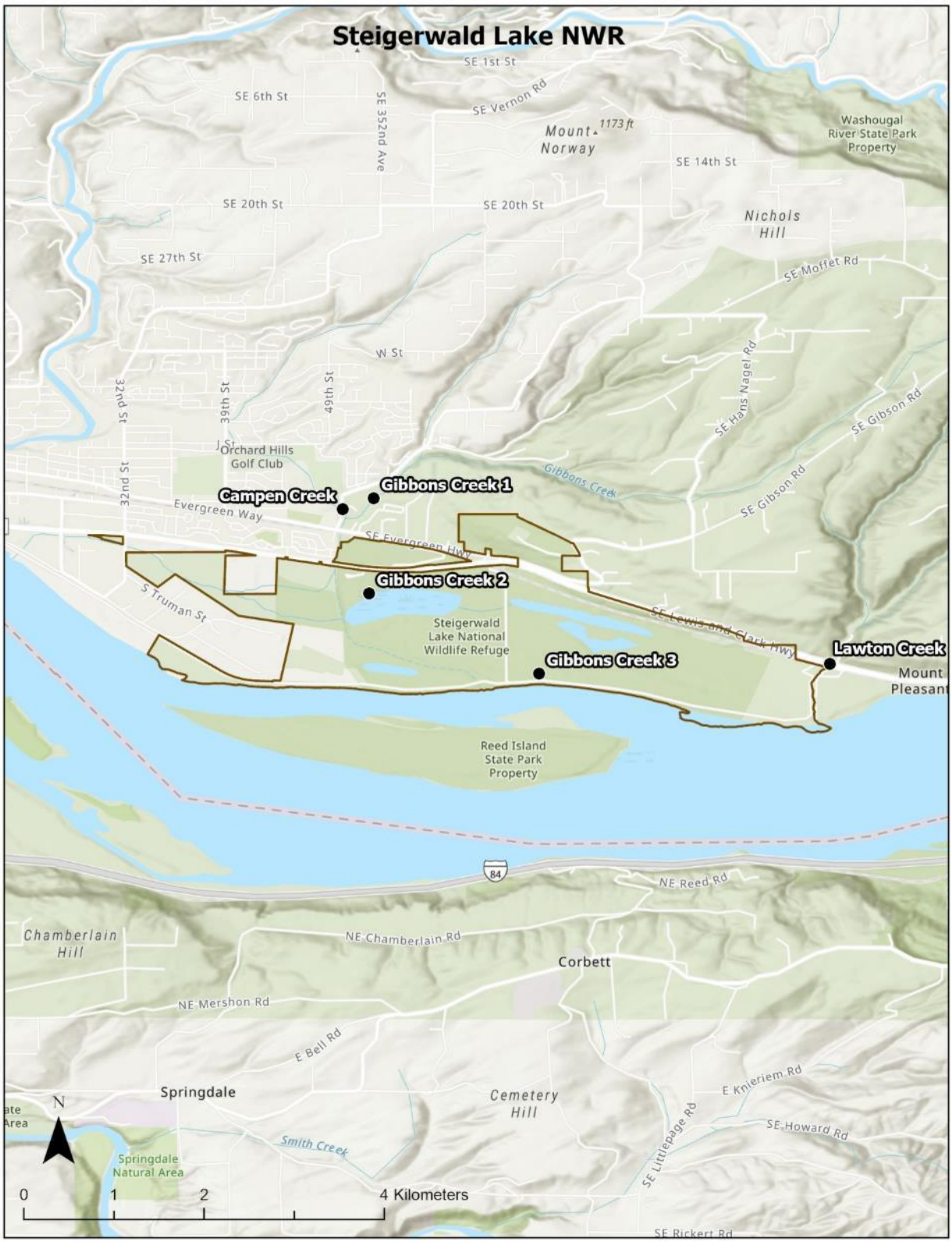

Figure 52. Environmental DNA sample locations at Steigerwald Lake NWR, 2023.

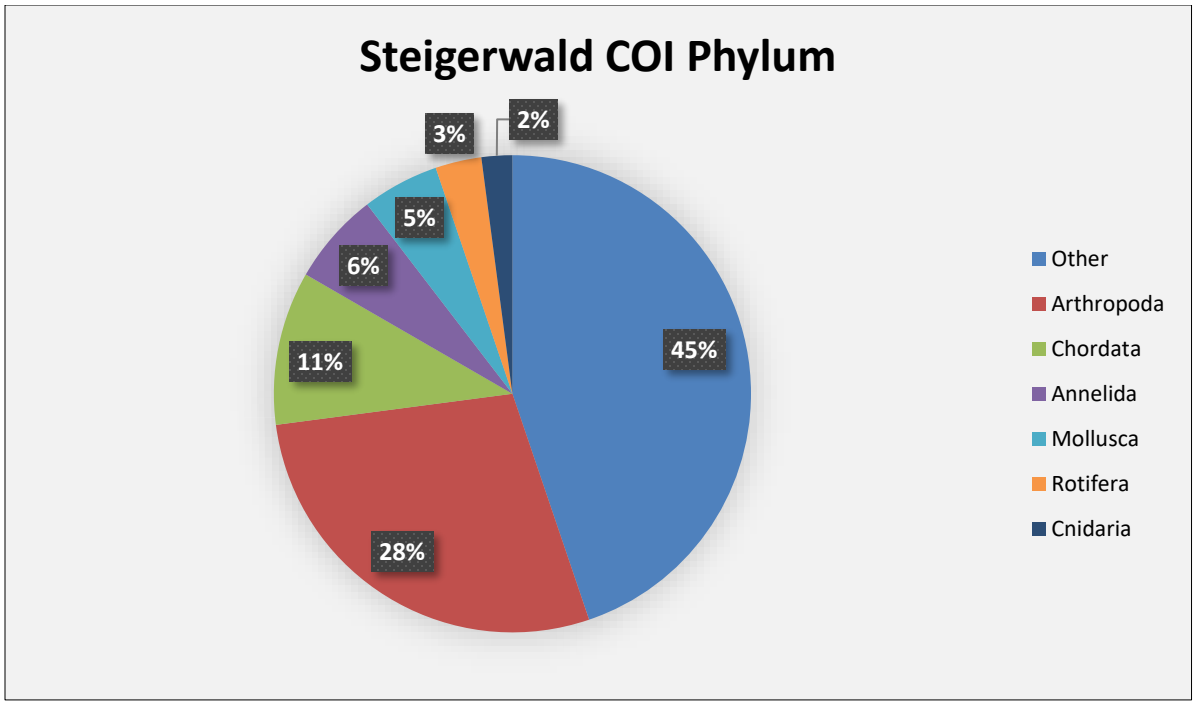

Figure 53. Proportion of common phylum identified by COI in five sample locations at Steigerwald NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

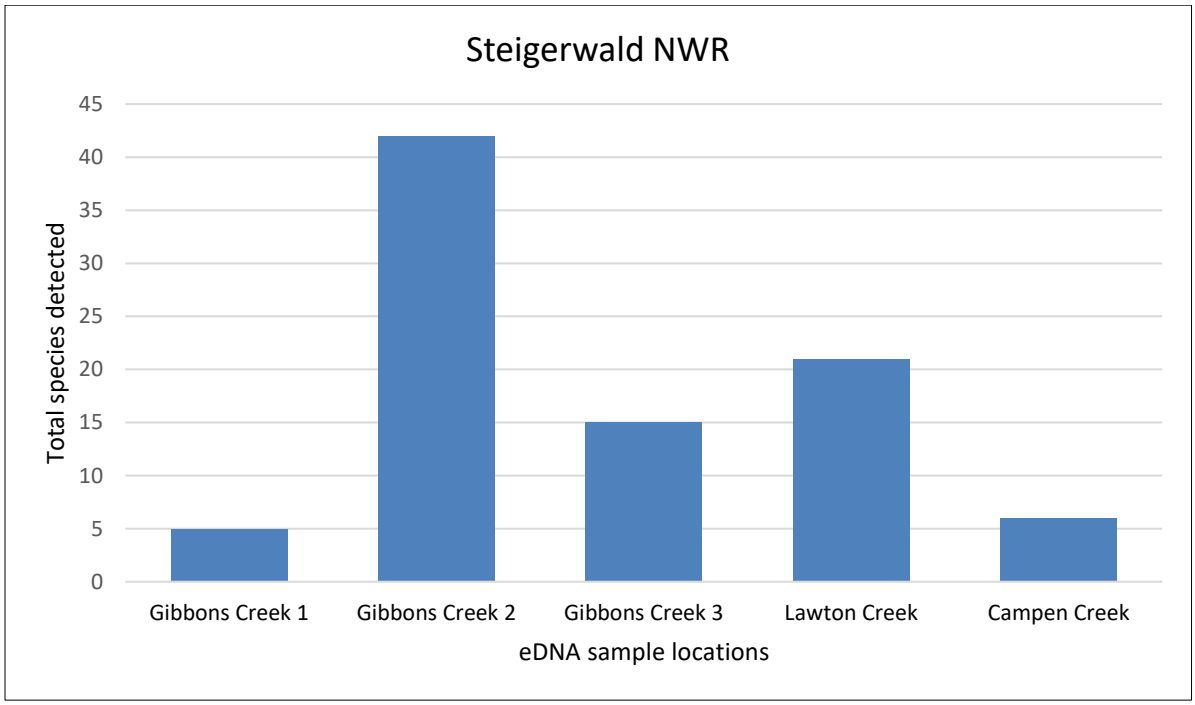

Figure 54. Total number of species detected by COI at five sample locations at Steigerwald NWR, 2023.

Steigerwald NWR (MiFish)

Three of five eDNA samples did not run with the MiFish marker (Gibbons Creek 1, Gibbons Creek 3, and Campen Creek; Figure 55). A total of 15 OTUs were detected, including 13 fish, one amphibian (coastal giant salamander), and one bird (mallard duck; Figure 56). Of the 13 fish OTUs, 5 were nonnative species. Five fish OTUs were detected in both sample locations (longnose dace, cutthroat trout, coho salmon, the minnow family, and sculpin), and sculpin was the OTU with the highest cumulative read count with 6,617 reads. Gibbons Creek 2 had the highest number of OTUs (11) and fish (10 OTUs) detected at Steigerwald NWR.

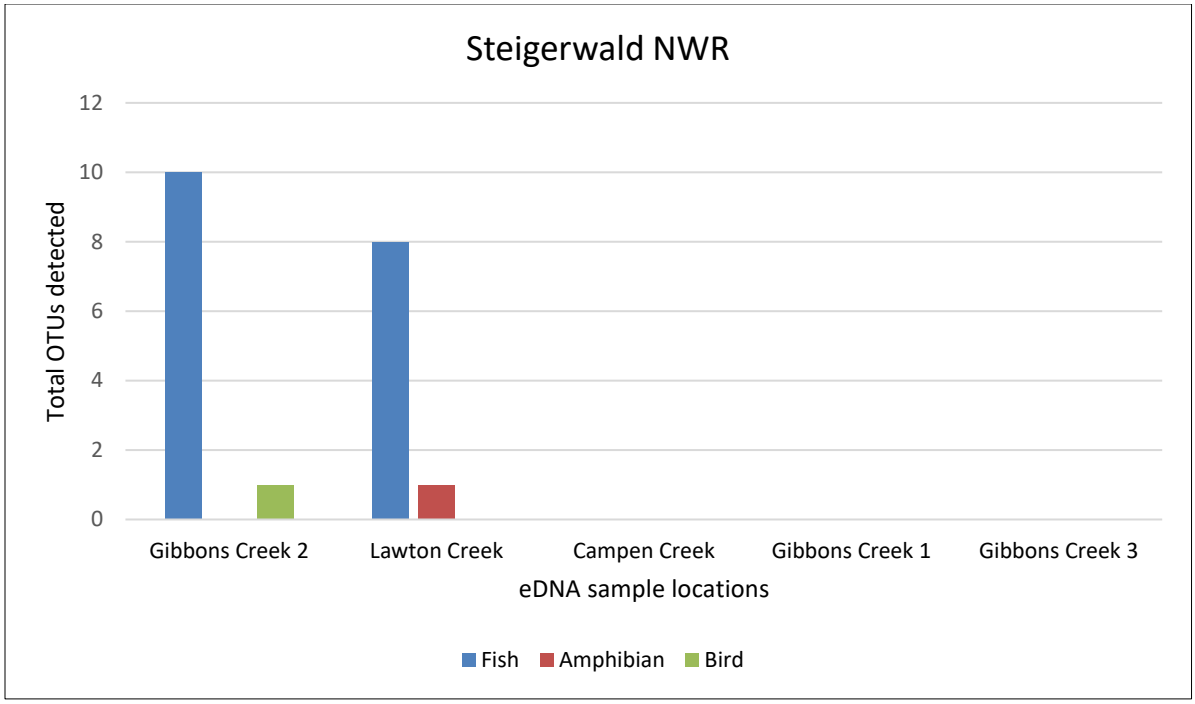

Figure 55. Fish, amphibian and bird OTUs detected by MiFish in two sample locations at Steigerwald NWR, 2023.

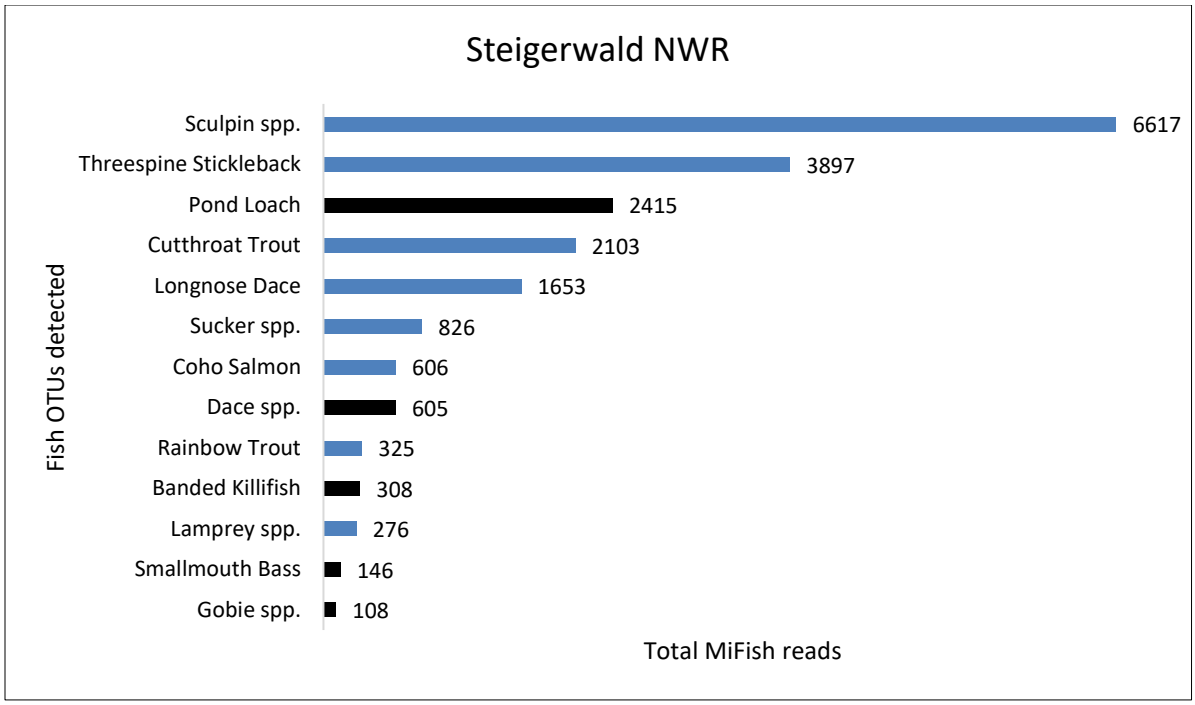

Figure 56. Cumulative read abundance of fish OTUs detected by the MiFish marker at two sample locations at Steigerwald NWR, 2023. Black bars denote nonnative species (n=5).

Tualatin NWR (COI)

All seven eDNA samples collected at Tualatin NWR ran successfully with the COI marker (Figure 57 and Figure 58). A total of 94 OTUs belonging to 11 phyla were detected (Figure 59). Of the 94 OTUs, 60 were identified to species level, including six fish (largescale sucker, bluegill, largemouth bass, yellow bullhead, mosquitofish, and cutthroat trout), two mammals (American beaver and nutria), one amphibian (American bullfrog), and two mollusks (glassy juga snail and nonnative Asian clam). Oregon floater mussels were detected at two locations (Wapato Creek 1 and Tualatin River). However, the read counts were below the minimum threshold of ≥100 reads (e.g., 57 and 21) and may not be reliable detections. The OTU with the highest read count was a rotifer of genus *Polyarthra* (126,142 reads), and the OTUs detected at the most sample locations were freshwater dinoflagellates (*Alexandrium minutum* and genus *Peridinium*), detected at six locations. Wapato Creek 1 was the location with the highest OTUs (39) and total number of species detected (25) at Tualatin NWR (Figure 60). Chicken Creek 2 also had 25 unique species detected by the COI marker.

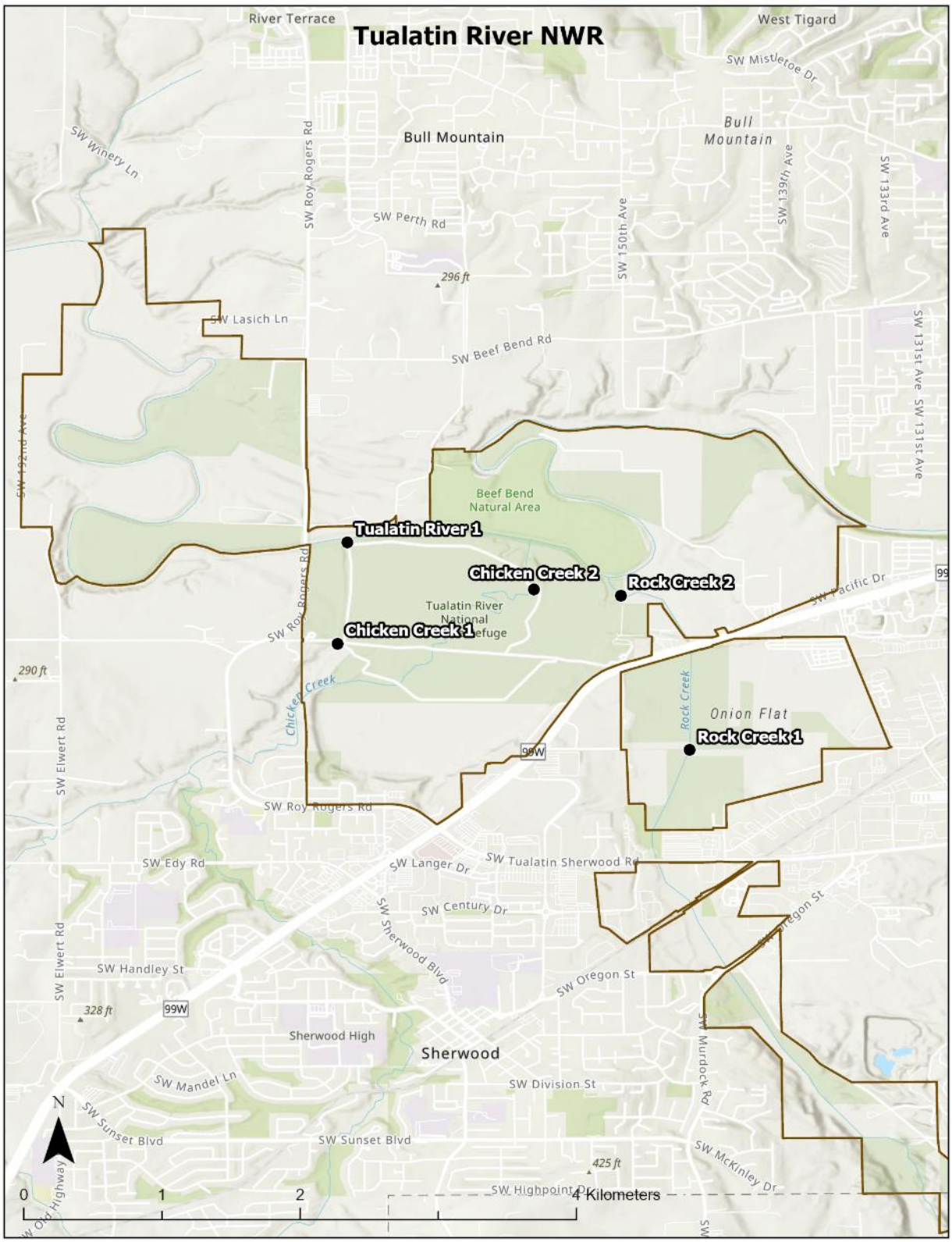

Figure 57. Environmental DNA sample locations at Tualatin River NWR, 2023.

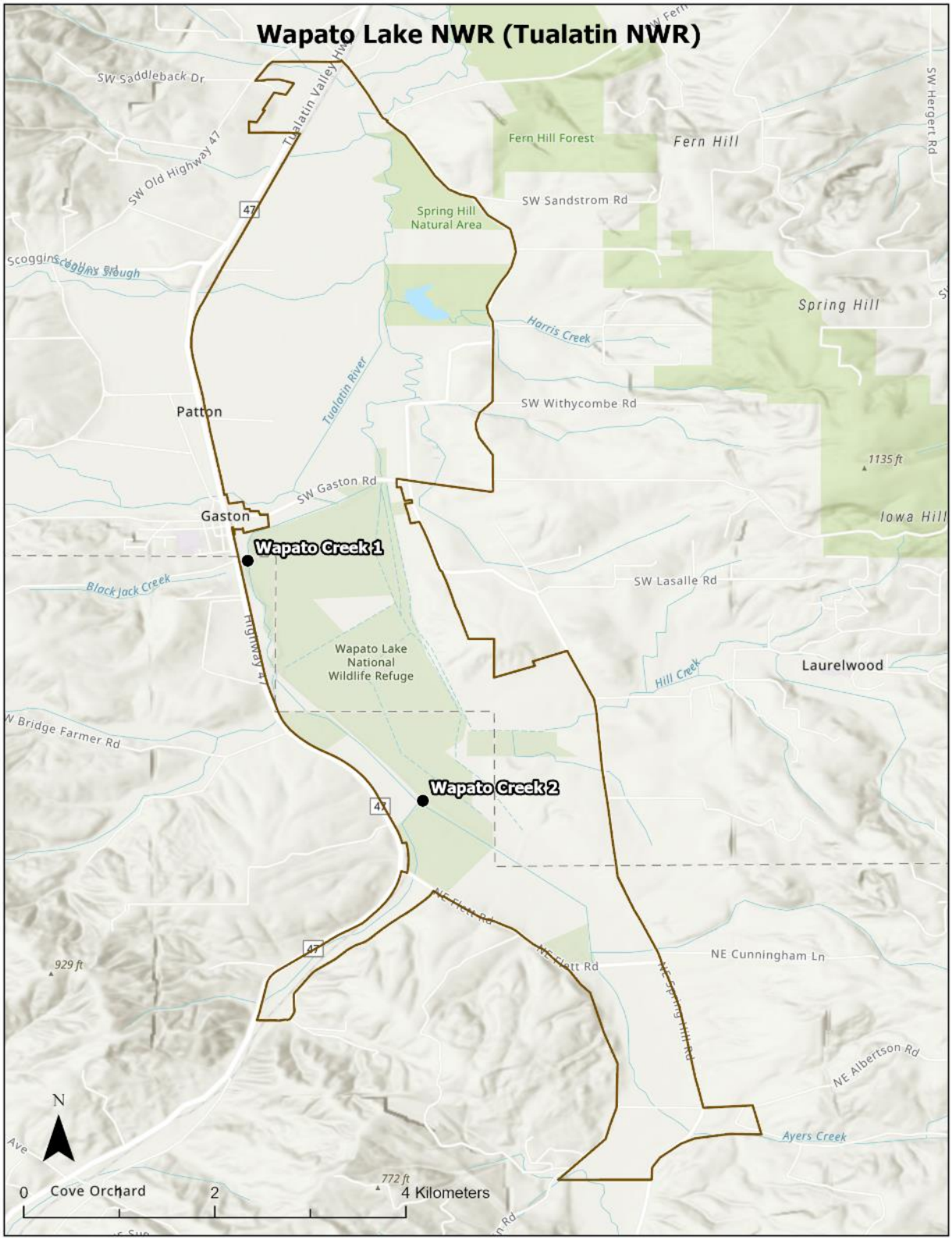

Figure 58. Environmental DNA sample locations at Wapato lake NWR, 2023.

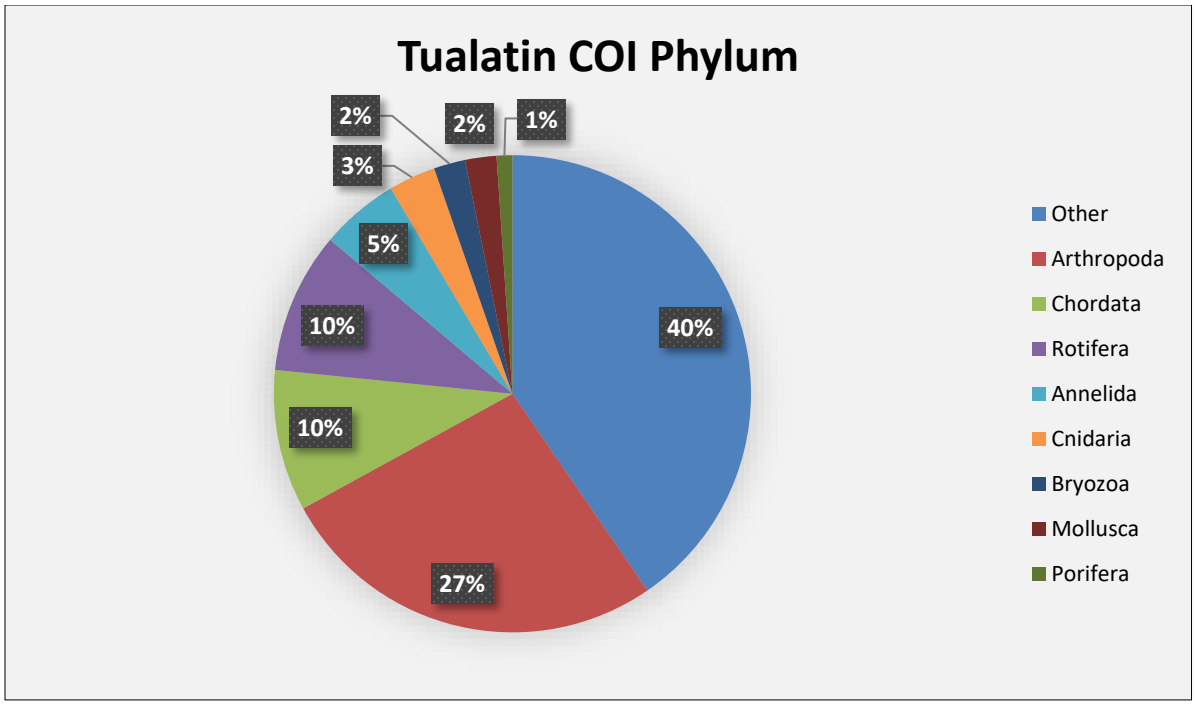

Figure 59. Proportion of common phylum identified by COI in seven sample locations at Tualatin NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

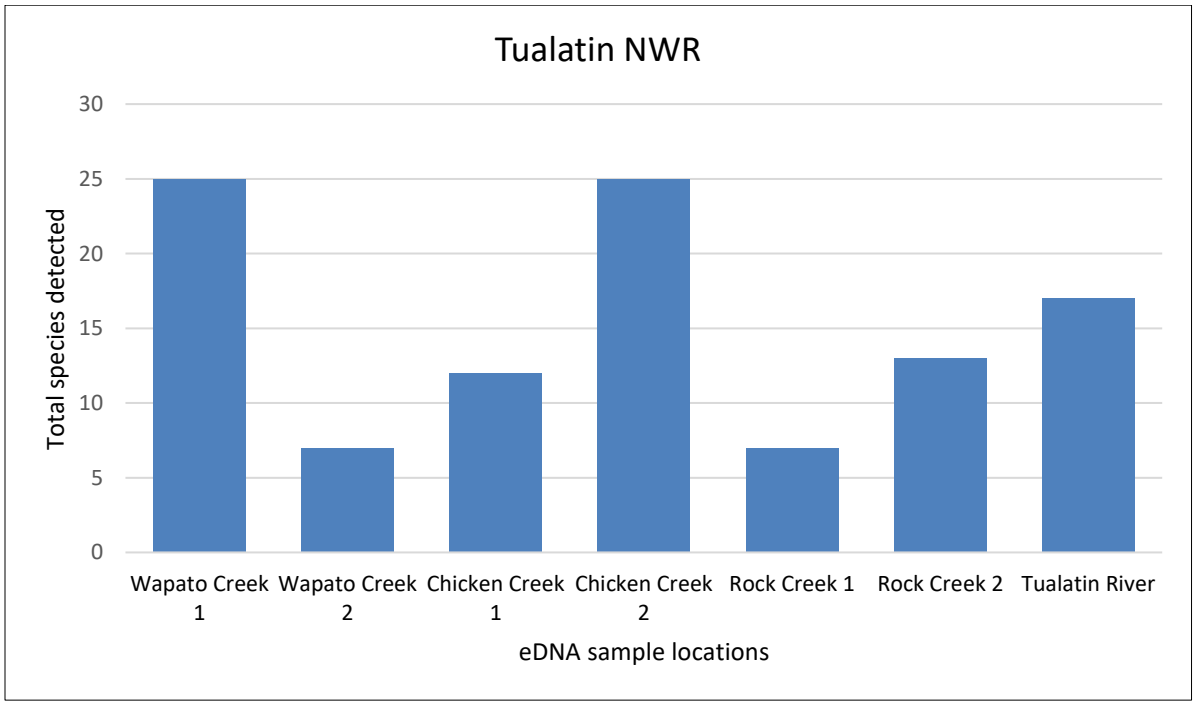

Figure 60. Total number of species detected by COI at seven sample locations at Tualatin NWR, 2023.

Tualatin NWR (MiFish)

All seven eDNA samples ran successfully with the MiFish marker (Figure 61). A total of 21 OTUs were detected, including 18 fish and three mammals (nutria, human, and wild boar). The putative detection of wild boar is most likely explained by the detection of domestic pig, which cannot be distinguished from wild boar based on the analyzed 12S gene region. Ten of the 18 fish OTUs detected were nonnative species (Figure 62). Three fish OTUs were detected in five locations (bluegill, carp family, three-spine stickleback, mosquitofish, and sculpin), with bluegill having the highest cumulative read count of all OTUs (15,709 reads; Figure 62). Wapato Creek 1 had the highest number of OTUs (11) and fish (10 OTUs) detected at Tualatin NWR.

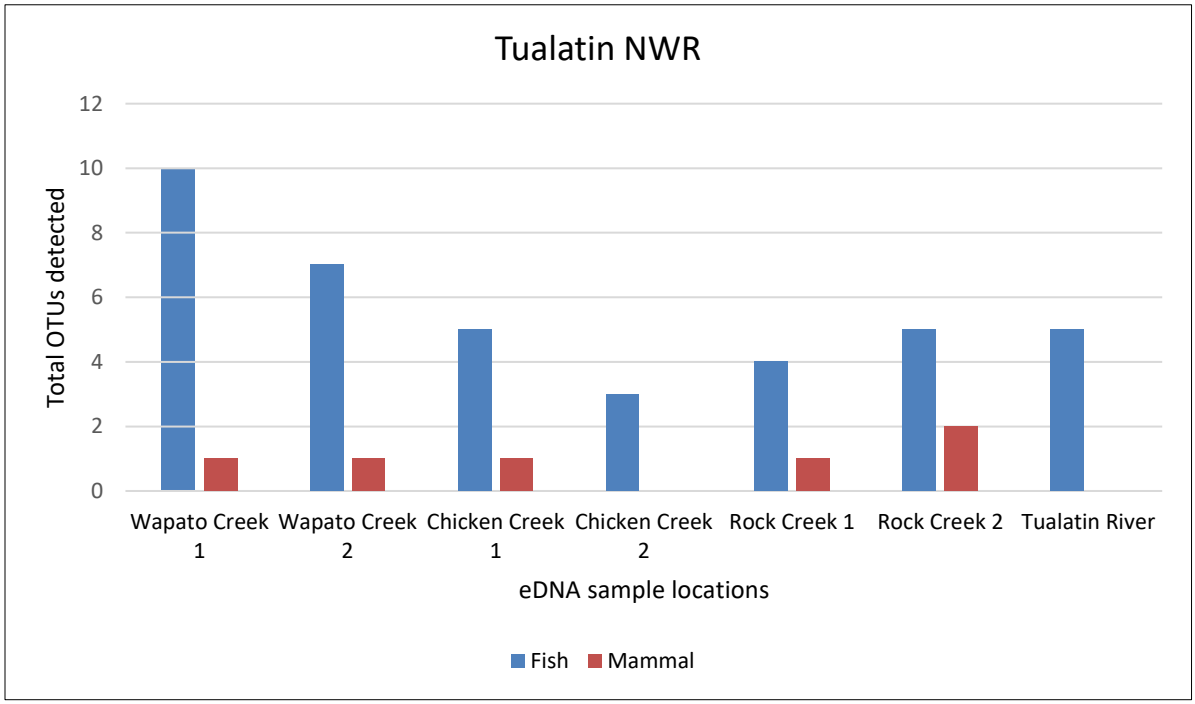

Figure 61. Fish and mammal OTUs detected by MiFish at seven sample locations at Tualatin NWR, 2023.

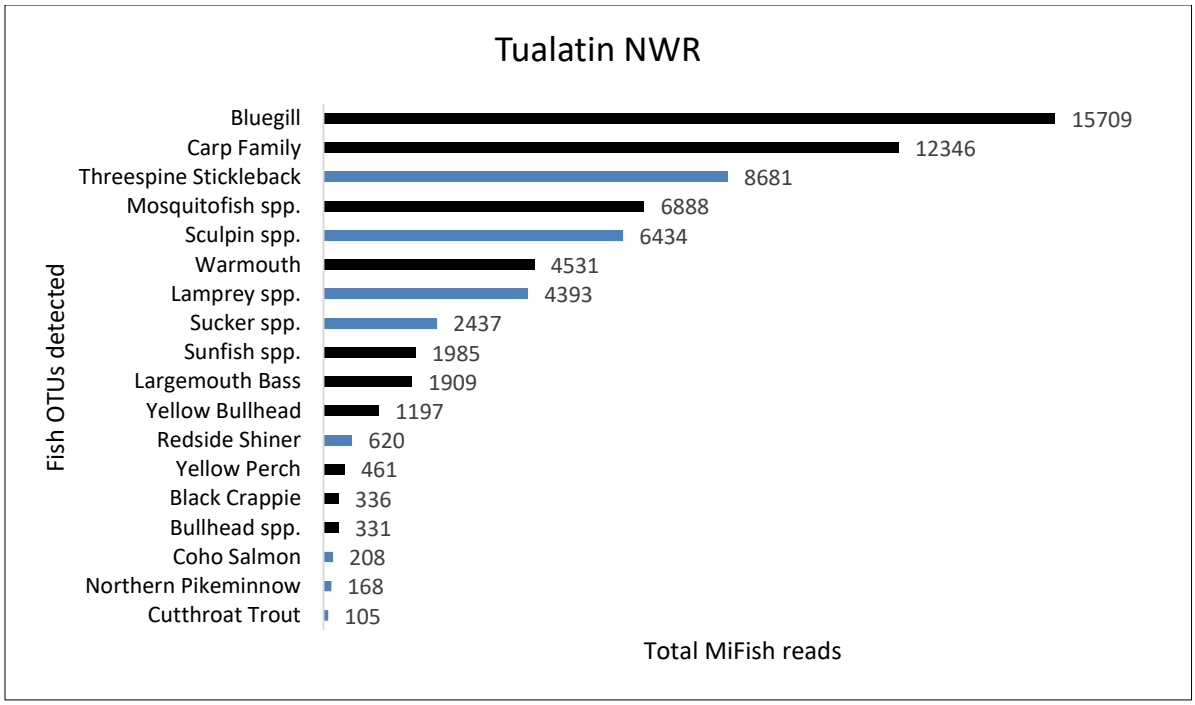

Figure 62. Cumulative read abundance of fish OTUs detected by the MiFish marker at seven sample locations at Tualatin NWR, 2023. Black bars denote nonnative species (n=10).

McNary NWR (COI)

Two sites were sampled at McNary NWR (Figure 63). Only one location ran successfully (Walla Walla 1). Twelve OTUs were detected with the COI marker belonging to 6 phyla (Figure 64). Seven OTUs were identified to species level, including one mammal (American beaver) and one mollusk (bladder snail) (Figure 65). No fish were detected with the COI marker. Although Oregon floater mussels were detected in Walla Walla 1, the read count was very low (5 reads), so the detection may be unreliable. The OTU with the highest reads was *Chydorus brevilabris*, a freshwater crustacean with 74,900 reads.

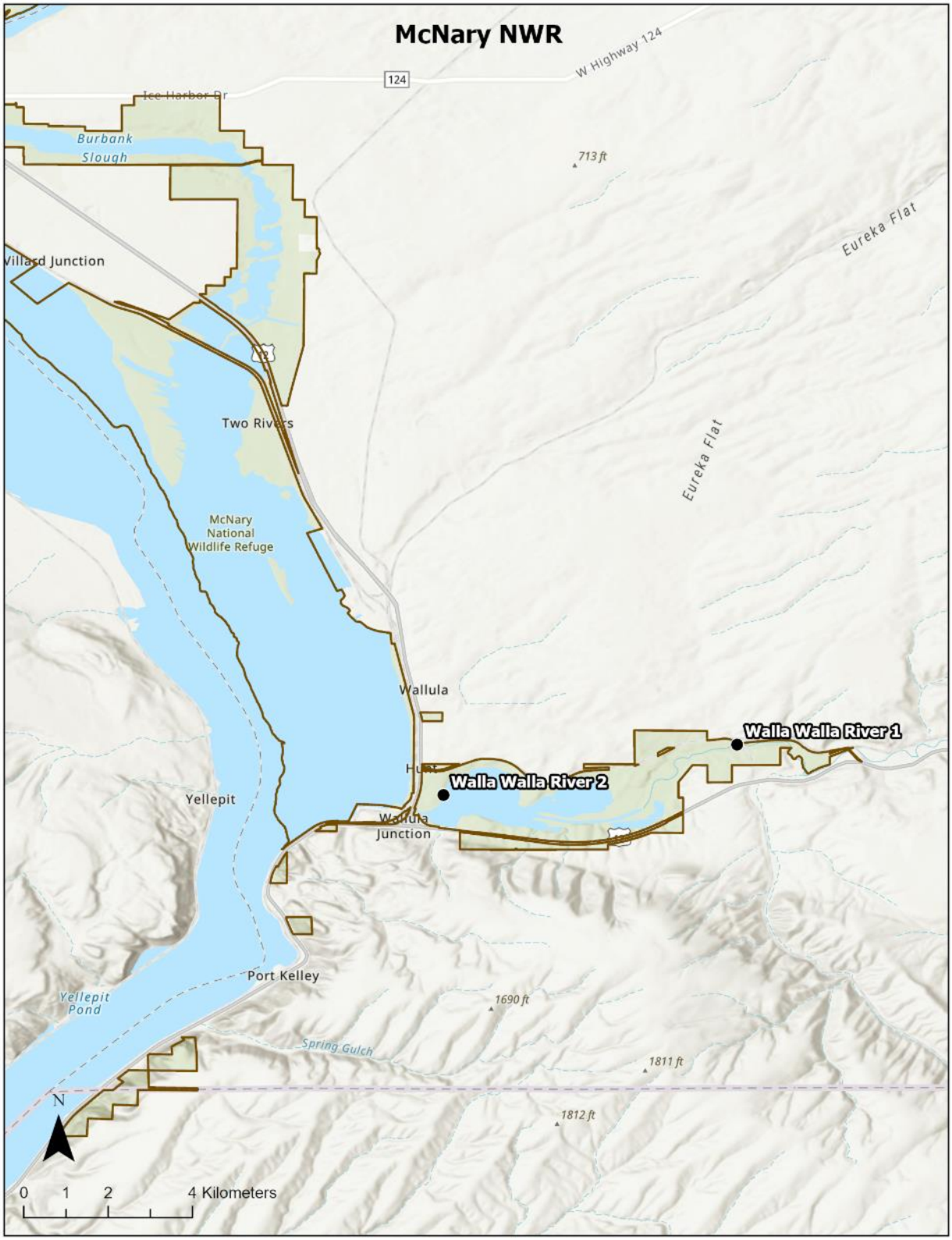

Figure 63. Environmental DNA sample locations at McNary NWR, 2023.

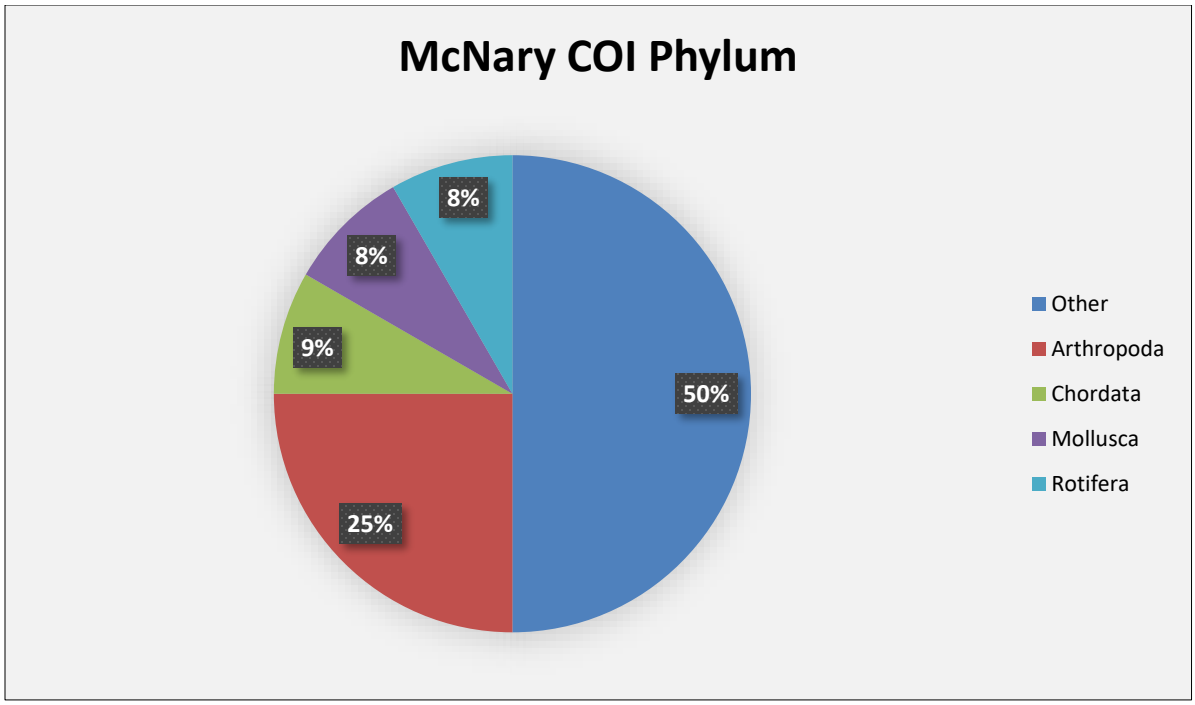

Figure 64. Proportion of common phylum identified by COI at a single sample location at McNary NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

McNary NWR (MiFish)

Only one of two locations ran successfully with the MiFish marker (Walla Walla 1). A total of 15 OTUs were detected including 12 fish, two mammals (American beaver and human) and one bird (mallard duck). All but three fish OTUs were nonnative species (Figure 65). The OTUs with the highest read count were the carp family (4,030 reads) and sunfish spp. (2,728 reads).

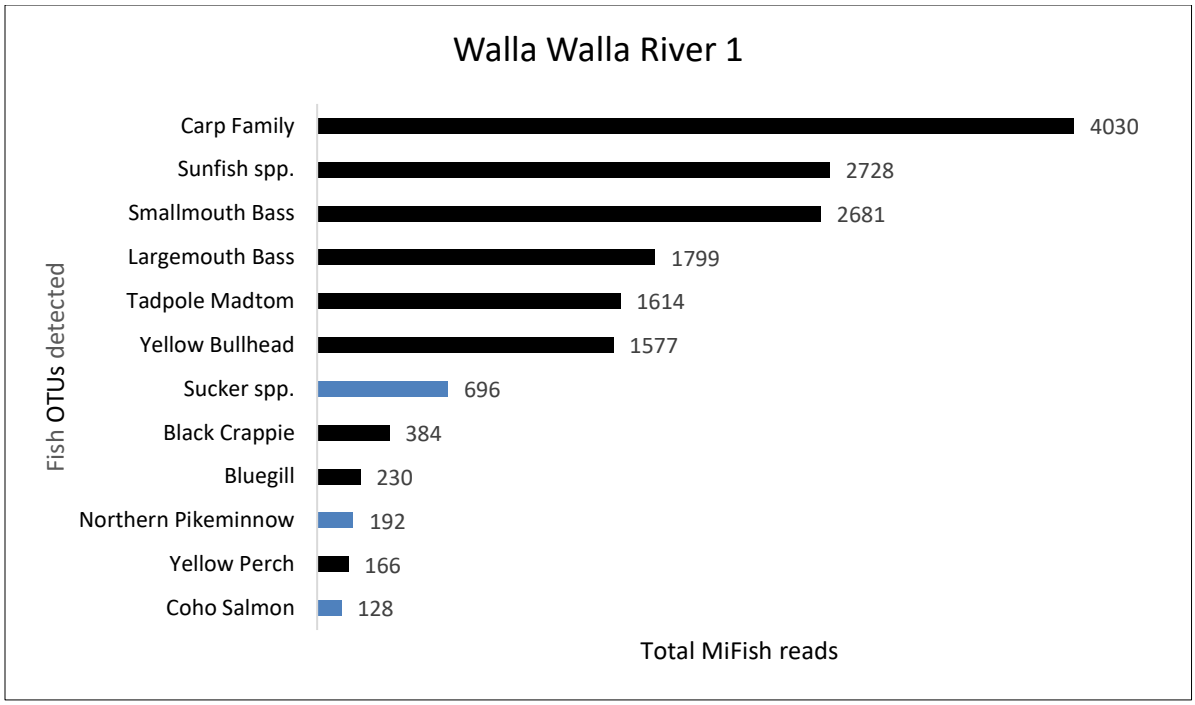

Figure 65. Cumulative read abundance of fish OTUs detected by the MiFish marker at a single sample location at McNary NWR, 2023. Black bars denote nonnative species (n=9).

Willapa NWR (COI)

Ten locations were sampled at Willapa NWR (Figure 66). Two sites (Cutthroat Creek and North Creek) did not run with the COI marker. Cumulatively, 124 OTUs belonging to 17 phyla were detected at Willapa NWR (Figure 67). Of the 124 OTUs, 83 were identified to species level, including four fish (prickly sculpin, cutthroat trout, coho salmon and rainbow trout/steelhead), four mammals (American beaver, elk, long-tailed vole and human) and four mollusks (Olive's sapsucker sea slug, minute sea slug, modest alderia sea slug and western pearlshell mussel (Bear River 2; Figure 68). Western pearlshell mussels were also detected in Bear River 1 (31 reads), and Oregon floater mussels were detected in Dohman Creek (5 reads), but read counts were below the minimum threshold of ≥100 reads and may not be reliable detections. The OTU with the highest cumulative reads was *Gonyostomum semen*, a freshwater algae with (163,071 reads), and the OTU with the highest occurrence was a nonbiting midge from the family Chironomidae detected at seven locations. Bear River 2 had the highest number of OTUs (77) and total number of species (49) detected with the COI marker at Willapa NWR.

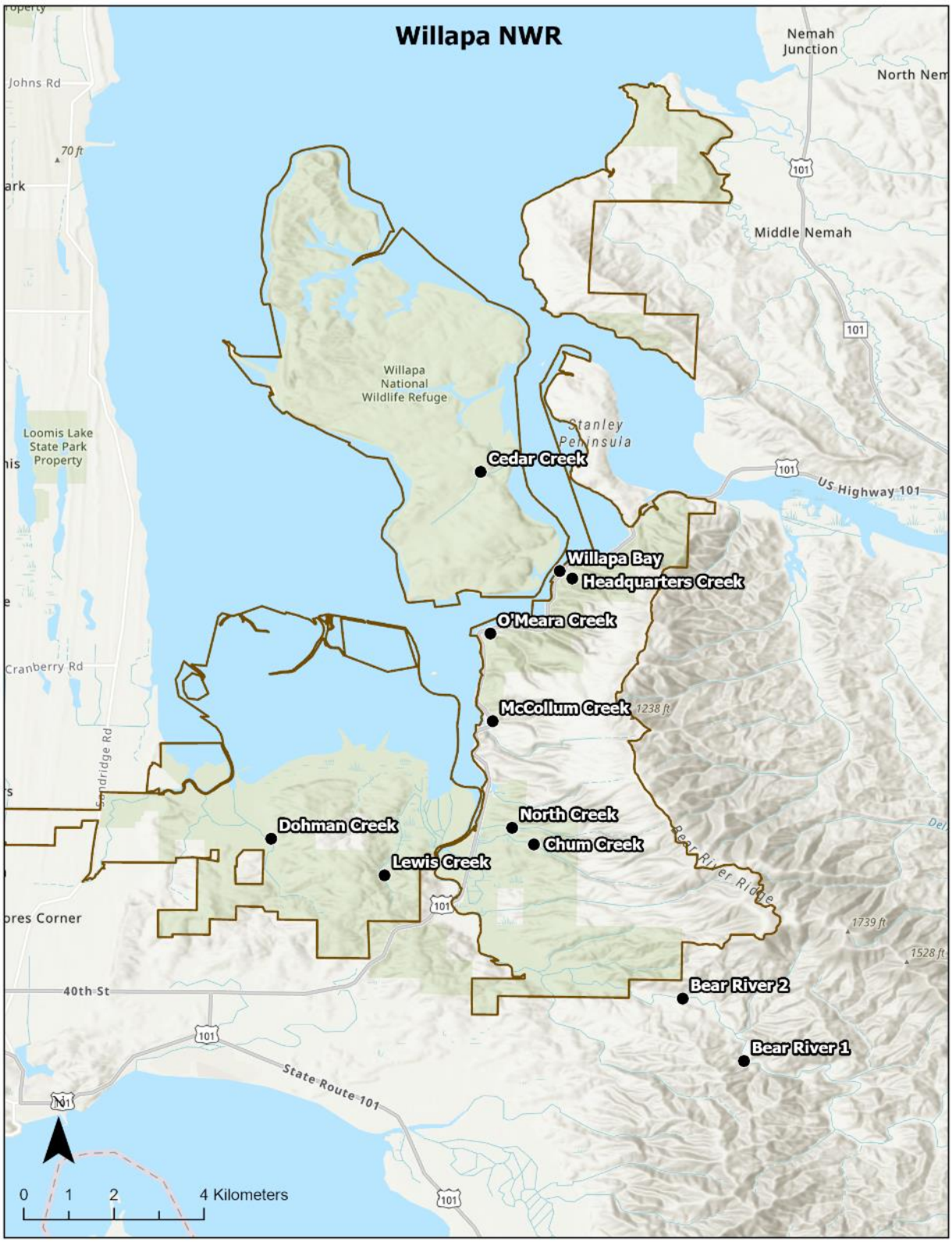

Figure 66. Environmental DNA sample locations at Willapa NWR, 2023.

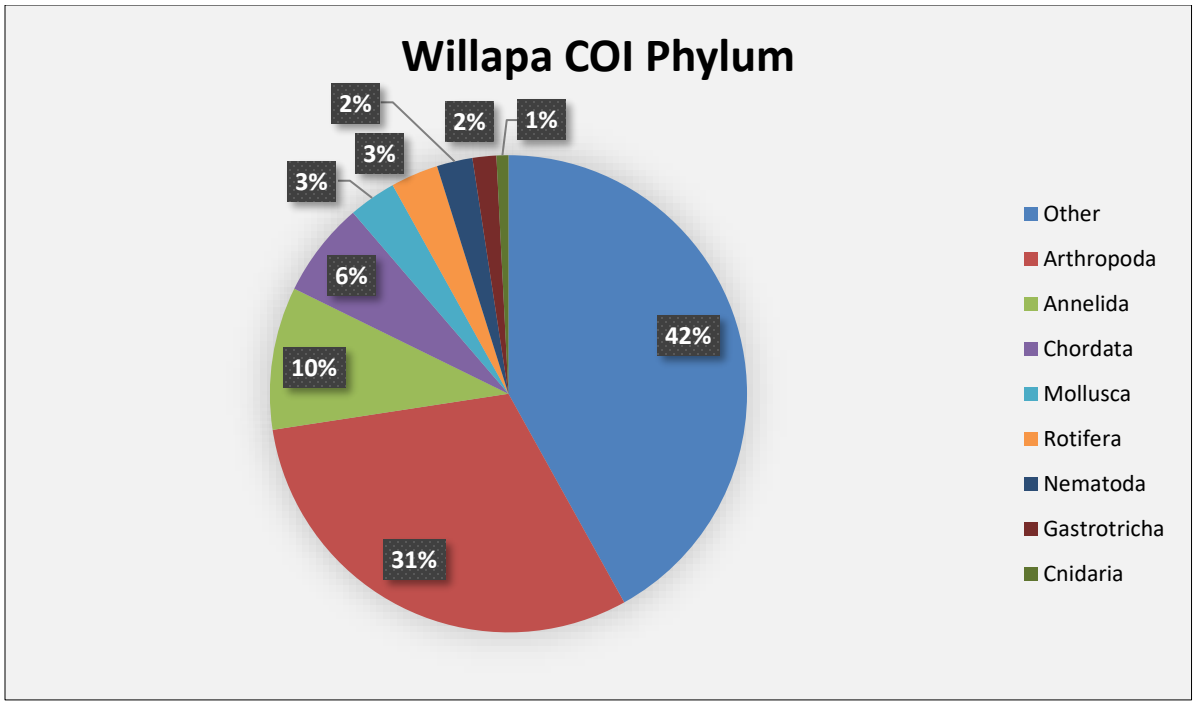

Figure 67. Proportion of common phylum identified by COI at eight sample locations at Willapa NWR. Other category includes: algae, bacteria, diatoms, fungi, amoebas, etc.

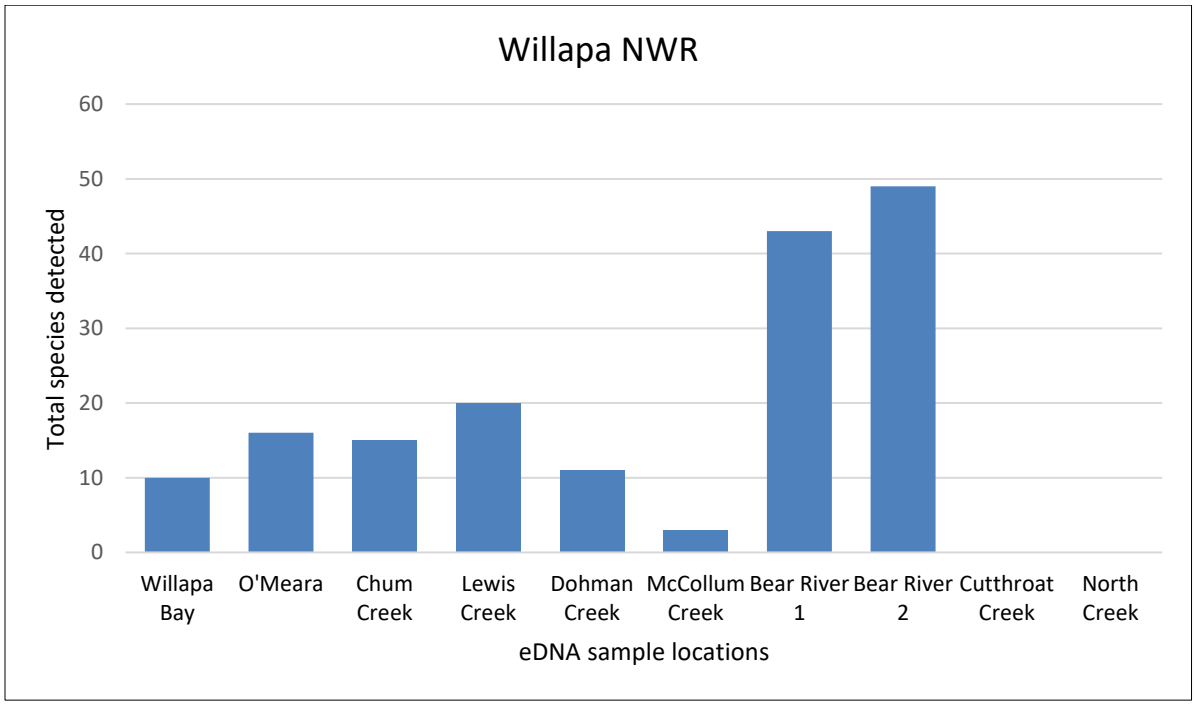

Figure 68. Total number of species detected by COI at eight sample locations at Willapa NWR, 2023.

Willapa NWR (MiFish)

All ten eDNA samples ran successfully with the MiFish marker, though only a single species (Sculpin) was detected at North Creek (Figure 69). A total of 23 OTUs were detected, including 15 fish, five mammals (American Beaver, elk, human, raccoon, and montane vole), two amphibians (coastal giant salamander and rough-skinned newt), and one bird (crow family). Five of the fish detected were marine species (Pacific staghorn sculpin, shiner perch, saddleback gunnel, flounder, snake prickleback), and three were potentially nonnative species (Figure 70). Genus *Salvelinus* was detected at two locations at Willapa NWR (McCollum Creek and Lewis Creek). However, it is unknown whether the detections are from native bull trout or nonnative brook trout. The OTUs detected at most locations were Sculpin (ten sites), cutthroat trout (eight sites), and coho salmon (eight sites). The OTU with the highest cumulative read count was Sculpin, with 85,444 reads (Figure 69). Dohman Creek had the highest OTUs (9) and Willapa Bay had the highest number of fish OTUs (7) detected at Willapa NWR.

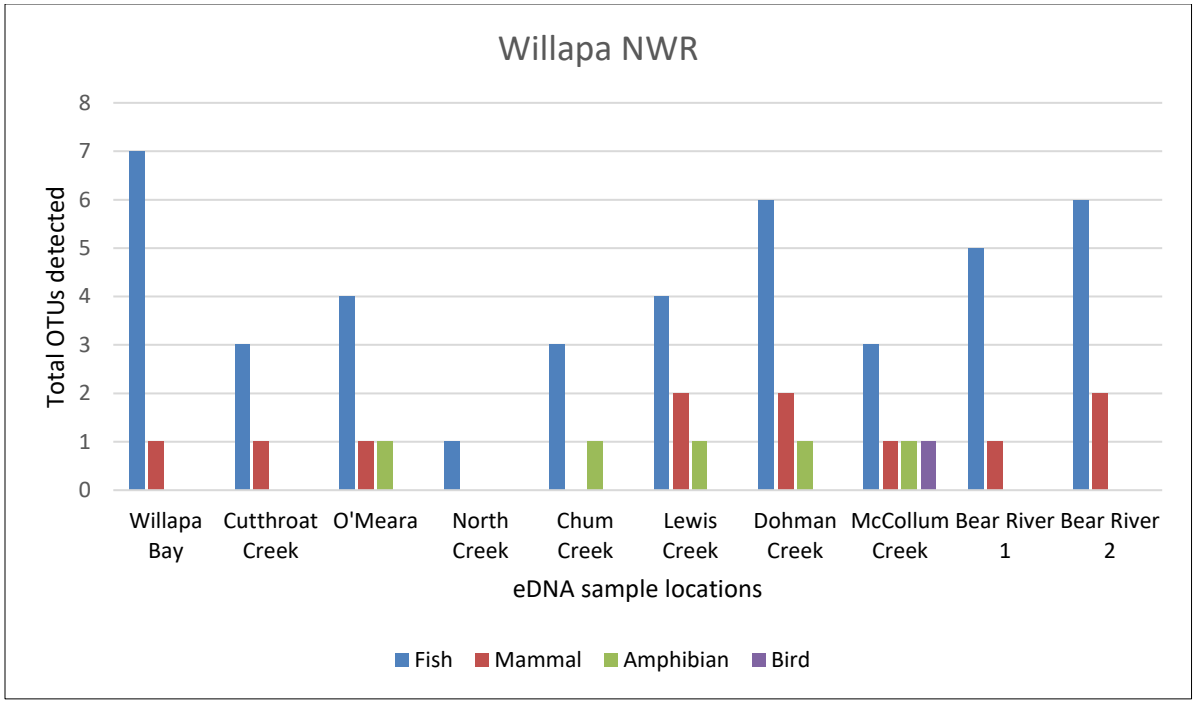

Figure 69. Fish, mammal, amphibian and bird OTUs detected by MiFish at ten sample locations at Willapa NWR, 2023.

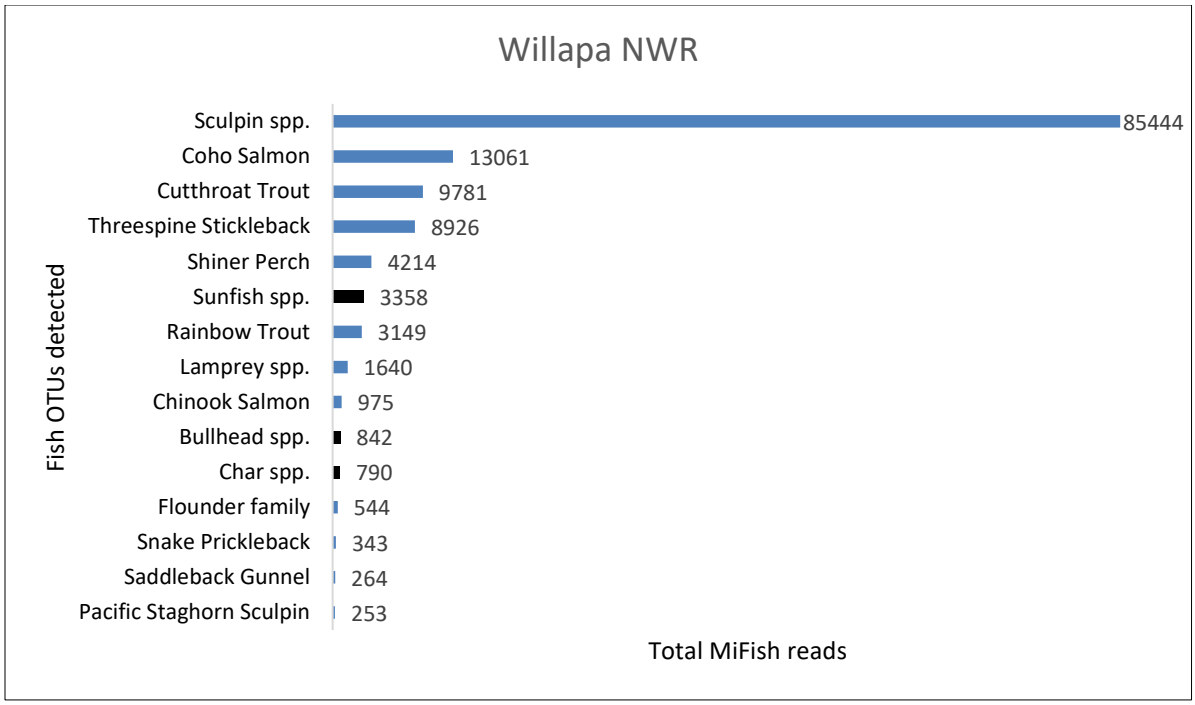

Figure 70. Cumulative read abundance of fish OTUs detected by the MiFish marker at ten sample locations at Willapa NWR, 2023. Black bars denotes potential nonnative species (n=3).

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