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Environmental DNA metabarcoding of fish in the Central Arizona Project canal for detection of non-native species

Gila River Basin Native Fishes Conservation Program
Lower Colorado Region
EcoLab-LCUAS-2023-02

U.S. Department of the Interior **July 2022**

Mission Statements

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The mission of the Bureau of Reclamation is to manage, develop, and protect water and related resources in an environmentally and economically sound manner in the interest of the American public.

Cover Photo – eDNA sample collection from the steep side wall of the CAP canal

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U.S. Department of the Interior **April 2023**

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Prepared by: Yale Passamaneck, Ph.D., Ecological Research Laboratory, Hydraulic Investigations and Laboratory Services, Technical Service Center, Bureau of Reclamation

Peer Review Certification

This section has been reviewed and is believed to be in accordance with the service agreement and standards of the profession.

JACQUE KEELE

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Acronyms and Abbreviations

CAP	Central Arizona Project
DNA	deoxyribonucleic acid
ESA	Endangered Species Act
eDNA	environmental DNA
PCR	polymerase chain reaction
Reclamation	Bureau of Reclamation
USFWS	U.S. Fish and Wildlife Service

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I. Introduction

The Central Arizona Project (CAP) canal is a 336-mile aqueduct which carries water from the Colorado River to central and southern Arizona. The CAP includes 14 pumping plants, which lift the water over 2,900 feet from the inlet in Lake Havasu to the terminus of the system near Tucson, Arizona. The system also includes a large storage reservoir (Lake Pleasant) which is operated by a hydroelectric pump/generating plant at New Waddell Dam. Typically, CAP water is pumped into Lake Pleasant during fall and winter, whereas water is released from the reservoir during spring and summer. The Bureau of Reclamation ('Reclamation' hereafter) initiated construction of the CAP in 1973, with water deliveries beginning in 1985 and construction being substantially completed in 1993.

Under Section 7 of the Endangered Species Act (ESA), Reclamation entered into formal consultation with the U.S. Fish and Wildlife Service (USFWS) over the potential for CAP water operations to impact federally listed species. Given that the CAP transports water between sub-basins of the Colorado River (from the Lower Colorado River basin to Gila River basin), concerns were raised regarding the potential of the CAP to transport non-native fishes between sub-basins which could in-turn travel upstream into waters inhabited by threatened and endangered native fishes. In 1994, USFWS issued a Biological Opinion (USFWS, 1994) and determined that the CAP jeopardized the existence of spikedace (*Meda fulgida*), loach minnow (*Tiaroga cobitis*), Gila topminnow (*Poeciliopsis occidentalis*), and razorback sucker (*Xyrauchen texanus*), and could adversely modify designated critical habitat of spikedace, loach minnow, and razorback sucker. Later revisions in 2001 and 2008, added Gila chub (*Gila intermedia*) and Chiricahua leopard frog (*Lithobates chiricahuensis*) as additional listed species affected by CAP operations (USFWS 2001, USFWS 2008).

In the 1994 Biological Opinion, the USFWS identified several reasonable and prude alternatives (RPAs) to remove jeopardy to these species – Reclamation later adopted these RPAs as Conservation Measures in the 2001 and 2008 revised Biological Opinions (USFWS, 2001; USWFS, 2008). One of the RPAs required Reclamation to develop and implement a long-term monitoring program to assess the presence and distribution of non-native fish in the CAP and its primary connected waters (canals and major streams) throughout the expected 100-year life of the CAP.

The long-term monitoring of the CAP and its primary connect waters was initiated in 1995, although pre-Opinion monitoring of the CAP occurred as early as 1986 (Mueller, 1996). Monitoring was conducted annually from 1995 through 2010; however, in recent years emphasis shifted towards monitoring wild populations of listed fishes in the Gila River basin. The CAP and its primary connected waters are now monitored once every 5 years according to Clarkson et al. (2011).

Prior to the present study, all monitoring efforts in the CAP canal have been conducted using traditional fish survey methods that depend upon the capture of fish from the sampled

environment for identification and counts. The primary gear type for traditional sampling has been a boat electrofisher. Secondary gear types have included trammel net, minnow trap, trotline, and angling.

Since the initial monitoring surveys in 1986, 23 species of fish have been detected in the CAP canal, including 3 native species, razorback sucker, desert sucker (*Catostomus clarkii*), Sonora sucker (*C. insignis*), as well as 20 species of non-native fish (Appendix A). In addition, two species of non-native aquatic reptile, spiny softshell turtle (*Apalone spinfera*), and red-ear slider (*Trachemys scripta*), have also been captured during sampling.

Traditional surveys, as discussed above, are widely used for environmental monitoring; however, they do present challenges. Over the years, fish sampling in the CAP has proven to be both intensive and a logistical challenge. The CAP features high water velocities (between 2,000 – 3,000 cfs at max capacity), steep banks, and limited access (no boat ramps; crane/special equipment required to place boat in canal). In addition, sampling in the CAP is focused on the detection of all potential non-native fish in the canal which requires a large array of fish sampling equipment reduce size-based and species biases. Sampling is also conducted primarily in pumping plant forebays during planned periods of reduced flow (approximately 500 cfs or less) or dry-ups; however, coordination issues and equipment failures have resulted in sites not being sampled during some years due to missed low-flow opportunities and unsafe sampling conditions.

Although fish monitoring in the CAP and its primary connect waters has been successful in detecting invasions of new non-native species in the Gila River basin, power analyses were conducted to evaluate the ability of the current monitoring regime to detect species, describe trends, and build density estimates (Wilson 1996, Allison 2000). These analyses primarily focused on the larger river sites (i.e., Gila River and San Pedro River); however, results are likely applicable to the CAP monitoring where sampling conditions can be equally variable and sub-optimal. Overall, the results indicated that the detection of new species or species that occur in low densities is problematic (Allison 2000). Researchers also found that it was difficult to detect annual trends for species that were less common or whose abundances were highly variable among monitoring reaches (Allison 2000).

In the past decade a novel approach for environmental surveys has been developed, termed environmental DNA (eDNA) metabarcoding. eDNA metabarcoding relies upon three complementary techniques and technologies. The first piece of this approach is DNA barcoding, which uses ‘universal’ primers that can are designed to be able to amplify a homologous fragment of a gene from all members of a taxon of interest by means of the polymerase chain reaction (PCR). The region of DNA between these primers is targeted to have sufficient variation such that the sequence may be diagnostic for a specific species. Based upon this, the DNA sequence for the fragment can be recovered from an unknown sample, and a species identification can be made based on comparison to a curated reference library of sequences of known origin.

The second piece of eDNA metabarcoding, is environmental DNA (eDNA). While traditional DNA techniques rely upon sampling directly from the tissue of an individual, eDNA relies upon

sampling of the environment (either water, soil, or air). Nearly all multicellular organisms shed DNA into the environment as a function of their physiology. Sources of eDNA include excretions such as mucus and saliva, release of feces and other waste products, and sluffing or shedding of ectodermal tissues including skin cells, scales, and feathers. All these functions deposit extra-organismal traces of DNA that can be collected through sampling of the environment in proximity to where the organism of interest is or has been.

The third piece of metabarcoding is the rapid development in DNA sequencing technologies in the last two decades. In recent years technologies for high-throughput sequencing have become widely available and the price of sequencing has dropped precipitously. Whereas traditional Sanger sequencing allowed only for the collection of a single DNA sequence per reaction, high-throughput sequencing allows for the sequencing of millions, or billions, of DNA fragments in parallel.

Taken together, these approaches allow for the sampling of DNA from the environment, sequencing of diagnostic DNA specifically from taxa of interest, and species identification through comparison of resultant sequences to those of known origin in a reference library. This approach of eDNA metabarcoding can facilitate broad surveys of organism with reduced effort in the field and relatively minimal impacts to the sampled environment.

The goal of the present study was to test the applicability of eDNA metabarcoding to detect the presence and distribution of native and non-native fish in the CAP canal. To this end two sampling strategies were selected. The first strategy was to collect samples at a relatively fine geographic scale along the length of the canal to determine the presence and distribution of fish eDNA. A sampling frequency of approximately one sample collection site each five river miles was selected based upon cost, practicability of sample collection and analysis, and data from current literature in the field regarding the distance from a source at which eDNA may be detected. The second sampling approach was to collect eDNA samples in parallel with traditional sampling surveys at pumping plant forebays along the CAP canal. The goal of this second strategy was to generate paired datasets that evidence the ability of eDNA metabarcoding to detect the species captured in traditional surveys, and to detect eDNA from species not captured in the surveys.

II. Methods

A. Sample collection

Sample collection was based on the U.S. Forest Service protocol for eDNA collection from streams (Carim et al., 2016). Samples were collected by filtering water through Whatman glass microfiber filters, grade 934-AH, with a nominal particle retention size of 1.5 microns. Filters were placed in single use analytical filter funnels. Prior to filed collections, filters were individually packaged in sampling kits, along with nitrile gloves, sterile disposable forceps, and plastic baggies containing desiccant beads, for sample handling and storage. In the field the filter assemblies were attached to flexible hosing and a battery-powered peristaltic pump. At each sample site, the filter assembly was submerged in the sampled water and the pump was run until the targeted volume of filtrate (generally 2 liters) was collected in an outflow bucket. Following filtration, the filter assembly was recovered, and the filter was removed using gloved hands and sterile forceps. Each filter was placed in a desiccant baggie for preservation during storage and shipment. At each sampling site a field blank was collected, with one liter of distilled water filtered through the filter assembly, before the field samples were collected at the site. Three field samples were collected at each site. At pumping plants the samples were collected from three separate locations: The top (at the escape ladder upstream in the canal closest to the pumping plant; approximately 100 to 300 meters upstream of the pumping plant intakes depending on the site), bottom-right (at the escape ladder river-right in the forebay; approximately 10 meters from the pumping plant intakes), and bottom-left (at the escape ladder river-left in the forebay; approximately 10 meters from the pumping plant intakes) of the forebay.

Three rounds of eDNA sample collection were performed for the study. In February 2021, sampling was conducted along the full length of the CAP canal (Appendix 1). Sampling was conducted at 83 sites, including the forebays of 12 pumping plants along the canal (Figure 1; Appendix B), and at an additional 71 sites spaced at approximately 5-mile intervals between the pumping plants (Figure 2; Appendix B). This sampling covered 318 river miles, from Lake Havasu adjacent to the forebay of the Mark Wilmer Pumping Plant (MARK_PP [CM 0.0] [canal mile (CM) 0.0]) to the San Xavier Pumping Plant (SANX_PP [CM 318.4]), just outside Tucson, AZ. The two additional rounds of eDNA sampling were conducted at pumping plant forebays in conjunction with traditional surveys of fish populations. In November 2020, samples were collected from the forebays of Brady (BRADY_PP [CM 253.8]), Red Rock (REDROCK_PP [CM 276.6]), and SANX_PP [CM 318.4], in parallel with traditional sampling surveys of fish populations at these sites. In July 2021, sampling was conducted at the Bouse Hills (BOUSE_PP [CM 25.0]), Little Harquahala (LHARQ_PP [CM 58.7]), and Hassayampa (HASSA_PP [CM 120.5]) Pumping Plants.

Traditional surveys employed boat-mounted electrofishing, minnow trapping, and trammel netting were conducted at all sites (Shollenberger et al., 2021). Spin-cast angling was also conducted at SANX_PP [CM 318.4].

Filters were processed for eDNA extraction in Reclamation's Ecological Research Laboratory (EcoLab) in Denver, CO. For each sample, half of the filter was processed for DNA extraction and purification, and the other half of the filter was stored at -80° C for subsequent analysis. All DNA extractions were performed using the Qiagen DNAeasy Blood & Tissue Kit. The proteinase K lysis was performed in Qiagen Investigator Lyse & Spin columns. Following an overnight incubation at 55° C, the lysate was recovered by centrifugation prior to further processing with the DNAeasy Blood & Tissue Kit. Following DNA extraction, the samples were purified using Zymo OneStep PCR Inhibitor Removal columns.

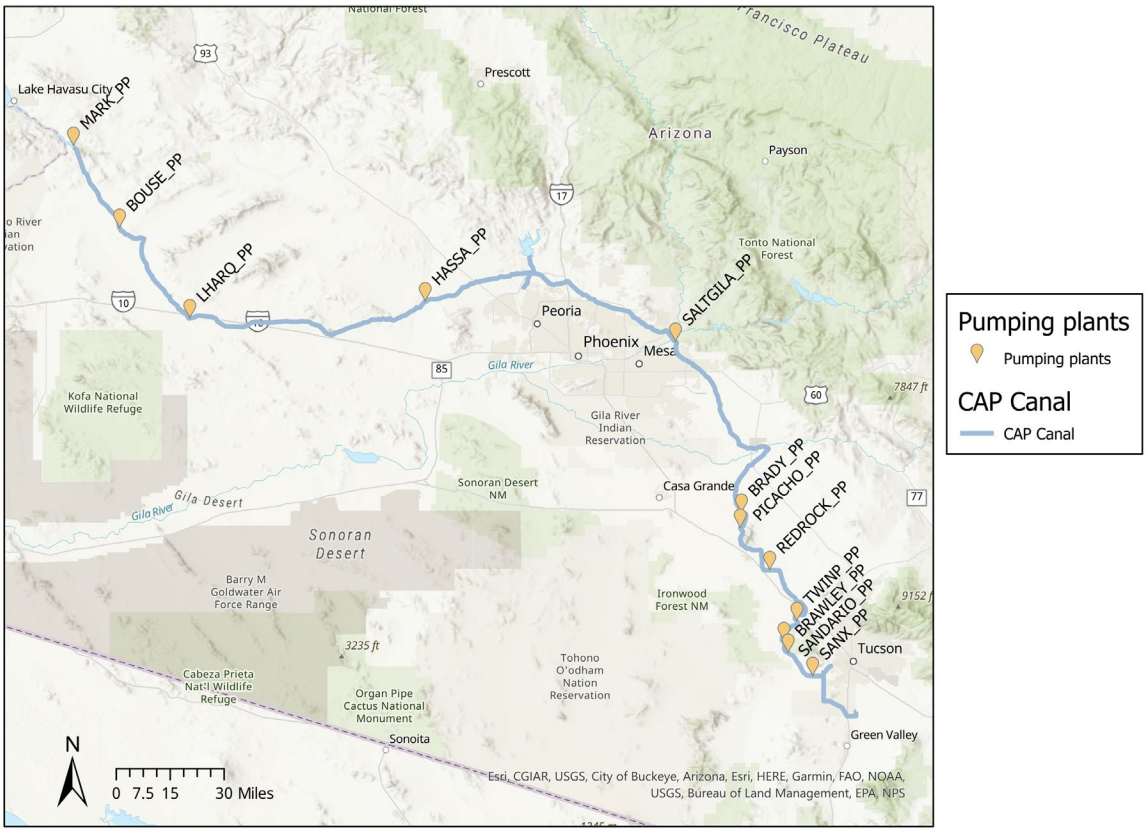

Figure 1: Locations of sampled pumping plant sites along the CAP canal.

Figure 2: Non-pumping plant sampling sites along the CAP canal. Note that not all sites are named in the figure due to space constraints.

B. PCR amplification

Polymerase chain reaction (PCR) amplification of DNA fragments was performed using the MiFish-U primers (Miya et al., 2015) which amplify a fragment of the 12S rRNA mitochondrial gene which is approximately 180 base pairs (bp) in length. For all samples, a first round PCR was performed using the MiFish-U primers. For samples intended for Illumina HiSeq sequencing, a second round of PCR was performed with MiFish-U primers labeled with unique 10 pb index sequences at the 5' end of the primer, to facilitate demultiplexing of DNA sequencing data. For samples intended for Amplicon-EZ sequencing, only the first round of PCR amplification with MiFish-U primers was performed. For all samples, PCR amplification was performed in four replicate reactions, with the replicates pooled prior to DNA sequencing. PCR reaction quality was checked by agarose gel electrophoresis. Reactions that did not show amplification were repeated to ensure the four replicates per sample were obtained. In some cases, sample dilution was adjusted to achieve amplification. All PCR amplifications were performed using Platinum SuperFi II Green MasterMix (Life Technologies). Following PCR amplification and agarose gel validation, PCR products were purified using the Zymo DNA Clean & Concentrator-5 kit.

C. Negative controls

Field negative controls were collected at each site prior to the collection of field samples. Field blanks were collected by filtering one liter of distilled water through a glass microfiber filter. Field blank filter samples were processed as described above for field samples. Extraction negative controls were also collected, consisting of unused glass microfiber filters, which were processed in parallel with field samples for DNA extraction. During PCR amplification, no template control reactions were included in all sets of PCR reaction. If any no template control reaction showed detectable product on the agarose gel, the entire set of reactions were discarded and rerun.

D. Mock communities

Mock communities were generated to validate the fidelity of PCR amplification and DNA sequencing. Samples of seven marine fish: Albacore tuna (*Thunnus alalunga*), Pacific cod (*Gadus macrocephalus*), halibut (*Hippoglossus stenolepis*), sablefish (*Anoplopoma fimbria*), Chinook salmon (*Oncorhynchus tshawytscha*), Coho salmon (*Oncorhynchus kisutch*), and sockeye salmon (*Oncorhynchus nerka*). DNA was extracted from tissue samples using the Qiagen DNeasy Blood and Tissue Kit, and a fragment of the 12S rRNA gene was amplified using the MiFish-U primers as described above.

E. Sequencing

All sequencing was performed by Genewiz, Inc. For all samples collected in November 2020 and February 2021, sequencing was performed with Illumina HiSeq 2 x 150 bp paired-end (PE) sequencing. Samples were divided across two runs, each with a targeted output of 350 million reads. Prior to sequencing, sample PCR products were pooled, with an equivalent mass of product for each sample added. For field and laboratory blanks that did not show amplification, and equivalent volume of the PCR reaction was added to the pooled mixture. For samples collected in July 2021, sequencing was performed using Genewiz's Amplicon-EZ service, which produces 2 x 250 bp PE data, with a targeted output of 50,000 reads per run. Each sample was sequenced individually to ensure sufficient read depth.

F. Analysis

1. DNA sequence data processing

DNA sequencing data were initially trimmed and demultiplexed using cutadapt (Martin, 2011) to orient all reads in the forward direction. Further data processing was then performed in QIIME2 (Boylen et al., 2019) to denoise sequences, identify amplified sequence variants (ASVs), and

quantify the number of ASVs for each sample. Denoising and ASVs identification in QIIME2 was performed with the dada2 plugin.

G. Taxonomic assignment

Taxonomic assignment of ASVs was initially performed using the BLAST and sklearn algorithms in QIIME2, using the Mitohelper dataset (Lim and Thompson, 2021). These methods provided limited support for taxonomic assignment of most ASVs, with only a small proportion of ASVs identified to the level of genus or species.

Subsequently, BLAST analysis was combined with phylogenetic reconstruction to identify the taxa to which sequences matched most closely. Briefly, all ASVs were searched against the GenBank non-redundant (nr) database using the BLASTN tool. A reference sequence library was constructed with the top BLAST hit results for each ASV, as well as 12S rRNA gene sequences for congeners of the species with the best hit. All native and non-native fish known occur in Arizona, for which 12S rRNA gene sequence data were available, were also included in the library. Sequences in the resultant library and the ASVs were aligned using MAFFT (Katoh and Standley, 2013). Phylogenetic. P reconstruction was then performed on the alignment using MrBayes version 3.2.7a (Ronquist et al, 2012) and RAxML-NG (Kozlov et al., 2019). Taxonomic assignment was determined based upon the clustering of ASVs and reference sequences in monophyletic groups in the resultant trees.

III. Results

A. CAP eDNA sampling – February 2021

Sequencing and analysis of samples collected from 83 sites along the CAP in February 2021 resulted in 293 unique ASVs being identified. Taxonomic assignment of these ASVs identified 201 sequences that matched most closely to fish sequences. Across sites the mean total number of sequence reads matched to fish reference sequences was 361,150.6 reads. The minimum number of sequence reads from a single site was 112,122 reads from SGBR_012 [CM 242.1] (Figure 3). The maximum number of sequence reads from a single site was 770,784 reads from SGBR_002 [CM 195.7] (Figure 3).

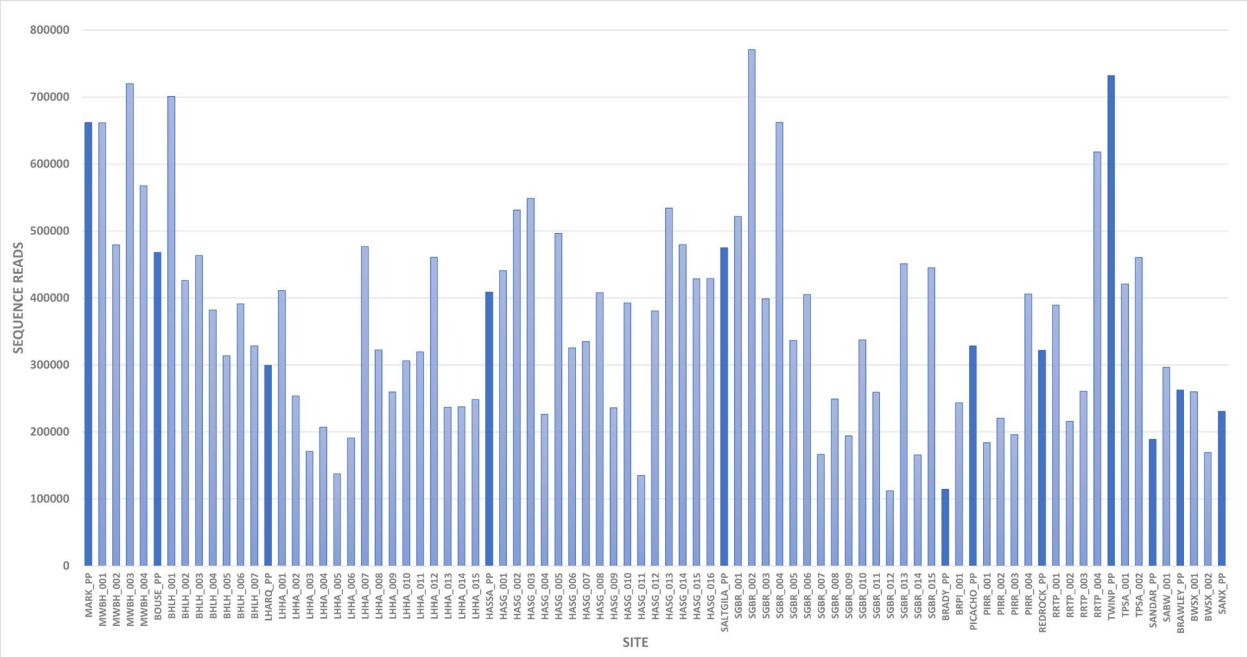

Figure 3: Total number of sequence reads per site. Sites are presented in order along the CAP canal, with the MARK_PP [CM 0.0] site to the left. Data from sites located in pumping plant forebays are highlighted in dark blue.

Based on BLAST hits and phylogenetic reconstruction, these ASVs were found to cluster into 25 distinct groups, interpreted as each corresponding to a single species of origin. The mean number of species detected across sites was 6.8 species. The maximum number of species detected from a single site was 15 species at MARK_PP [CM 0.0] (Figure 4). Note that this site is located in Lake Havasu, rather than in the CAP canal itself. The maximum number of species detected from a site within the CAP canal was 12 species at BOUSE_PP [CM 25.0] and SANX_PP [CM 318.4] (Figure 4). Overall, sites located at pumping plant forebays tended to yield a higher number of species detections, with a mean of 9.4 species detected per pumping plan site (8.6 species excluding MARK_PP [CM 0.0]) versus a mean of 6.5 species for canal sites between pumping plants. The numbers of species detected at canal sites proximate to pumping plants were slightly higher than the mean for all canal sites, with a mean of 7.1 species for sites

upstream of pumping plants, and a mean of 6.9 species for sites downstream of pumping plants. Patterns for individual species detected are discussed below.

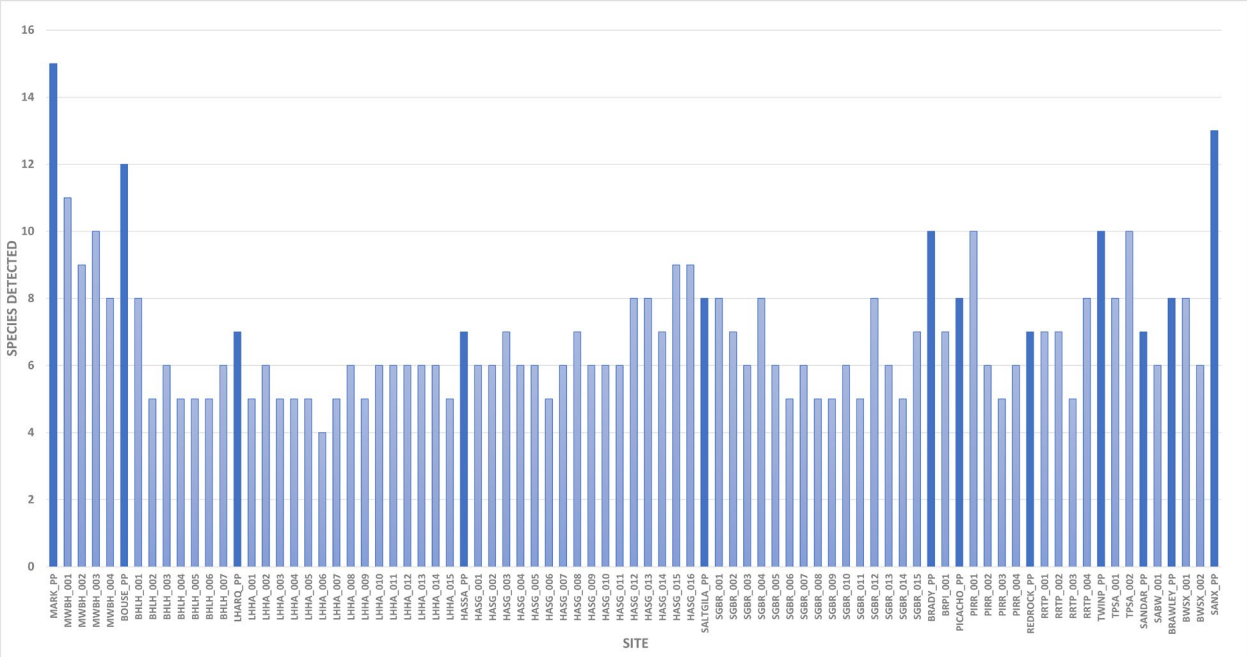

Figure 4: Total number of species detected per site. Sites are presented in order along the CAP canal, with the MARK_PP [CM 0.0] site to the left. Data from sites located in pumping plant forebays are highlighted in dark blue.

2. Grass carp (Ctenopharyngodon idella) and common carp (Cyprinus carpio)

DNA sequence reads matching to grass carp (*C. idella*) and common carp (*C. carpio*) reference sequences were detected in samples from all sites. Sequences for these two species represented the majority of sequences in samples from 72 sites (Figure 5 and Figure 6), with an average of 77.8% of reads per sample across sites matching to either grass carp or common carp. Grass carp had a minimum percentage of reads per site of 0.13% and a maximum percentage of reads per site of 85.7%, with a median value of 32.1%. Common carp had a minimum percentage of reads per site of 0.99% and a maximum percentage of reads per site of 95.2%, with a median value of 43.7%.

Across the sampled region of the CAP canal the two species displayed largely complementary patterns of read abundance (Figure 7). Grass carp represented the majority of reads in regions between sites MWBH_004 [CM 22] to LHHA_005 [CM 74.8], HASSA_PP [CM 120.5] to HASG_008 [CM 150.7], and PIRR_002 [CM 265.2] to RRTP_001 [CM 278.3]. Common carp represented the majority of reads in regions between sites LHHA_006 [CM 79.7] to LHHA_015 [CM 118.5], HASG_009 [CM 155.3] to PIRR_001 [CM 260.2], and RRTP_002 [CM 283.2] to SANX_PP [CM 318.4].

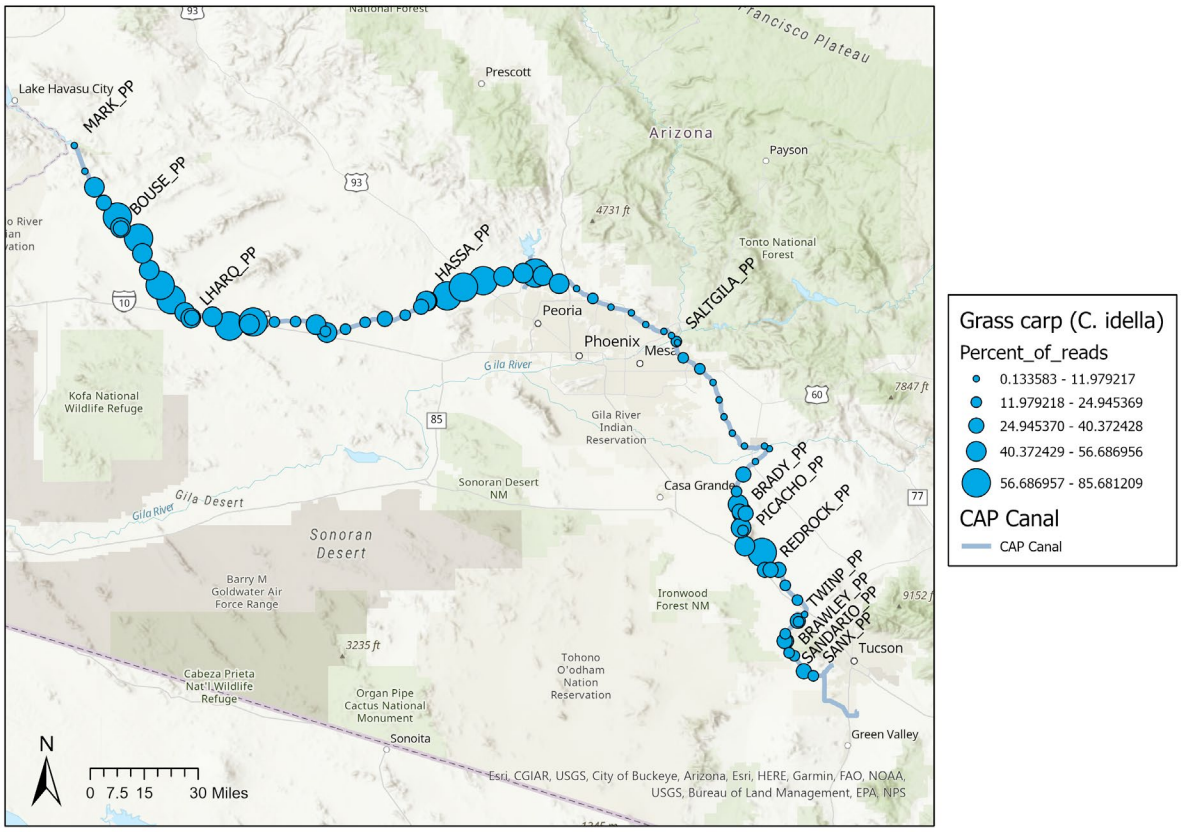

Figure 5: Distribution of grass carp (C. idella) sequence detections along the CAP canal.

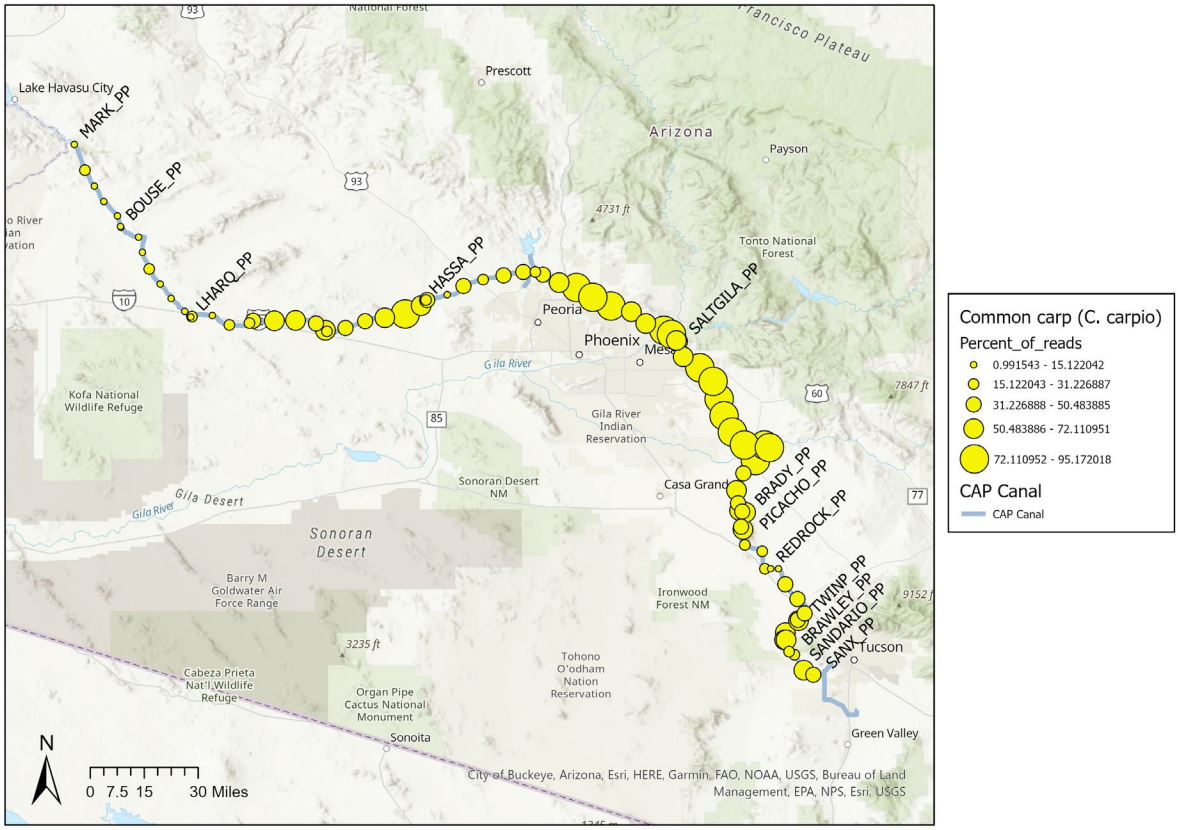

Figure 6: Distribution of common carp (C. carpio) sequence detections along the CAP canal.

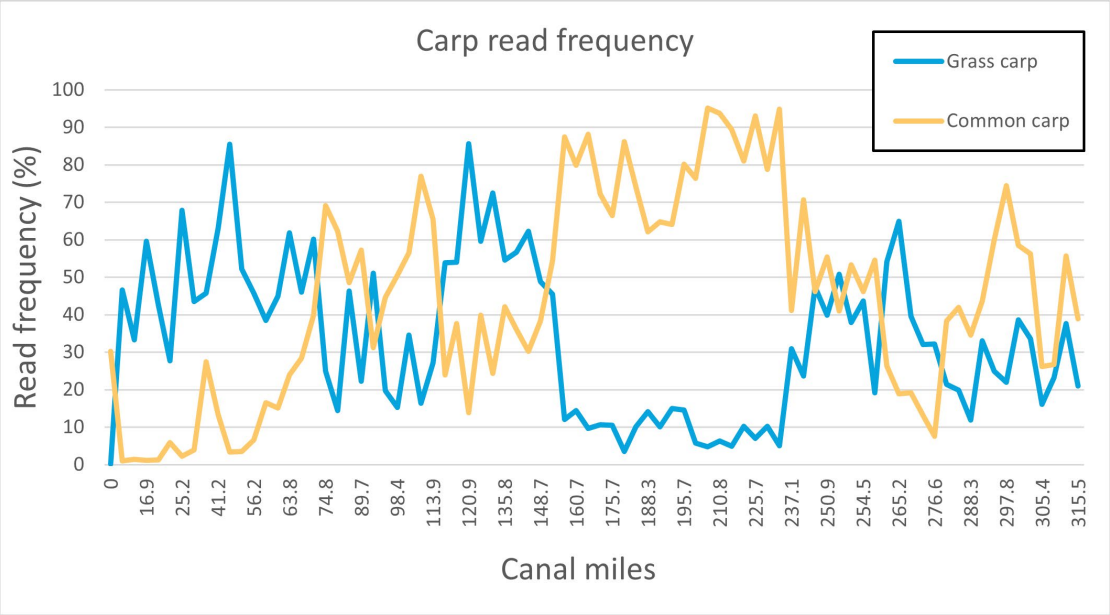

Figure 7: Percent read frequency of grass carp and common carp sequences plotted along the sampled length of the CAP canal.

3. Striped bass (*Morone saxatilis*) and White bass (*Morone chrysops*)

DNA sequences matching to striped bass (*M. saxatilis*) were found in samples from 78 sites (Figure 8). Read abundance ranged from 0.0005% to 66.4% of reads per site. At six sites, reads for striped bass represented the majority of reads. Reads for striped bass were most abundant in the upper region of the CAP canal, from MARK_PP [CM 0.0] to LHHA_002 [CM 63.8], and in the lower region of the CAP canal, from PIRR_004 [CM 275.2] to BWSX_001 [CM 310.6].

DNA sequences matching to white bass (*M. chrysops*), a congener of stiped bass, were detected in samples from only one site, TWINP_PP [CM 297.5], where it represented 1.06% of the reads (Figure 8).

Figure 8: Distribution of striped bass (M. saxatilis) and white bass (M. chrysops) sequence detections along the CAP canal.

4. Black crappie (Pomoxis nigromaculatus)

DNA sequences matching to black crappie (*P. nigromaculatus*) were detected in detected in samples from all 83 sites (Figure 9). Sequence abundance was very low across all sites, ranging from 0.003% to 0.02% of reads per site, with a median value of 0.006%.

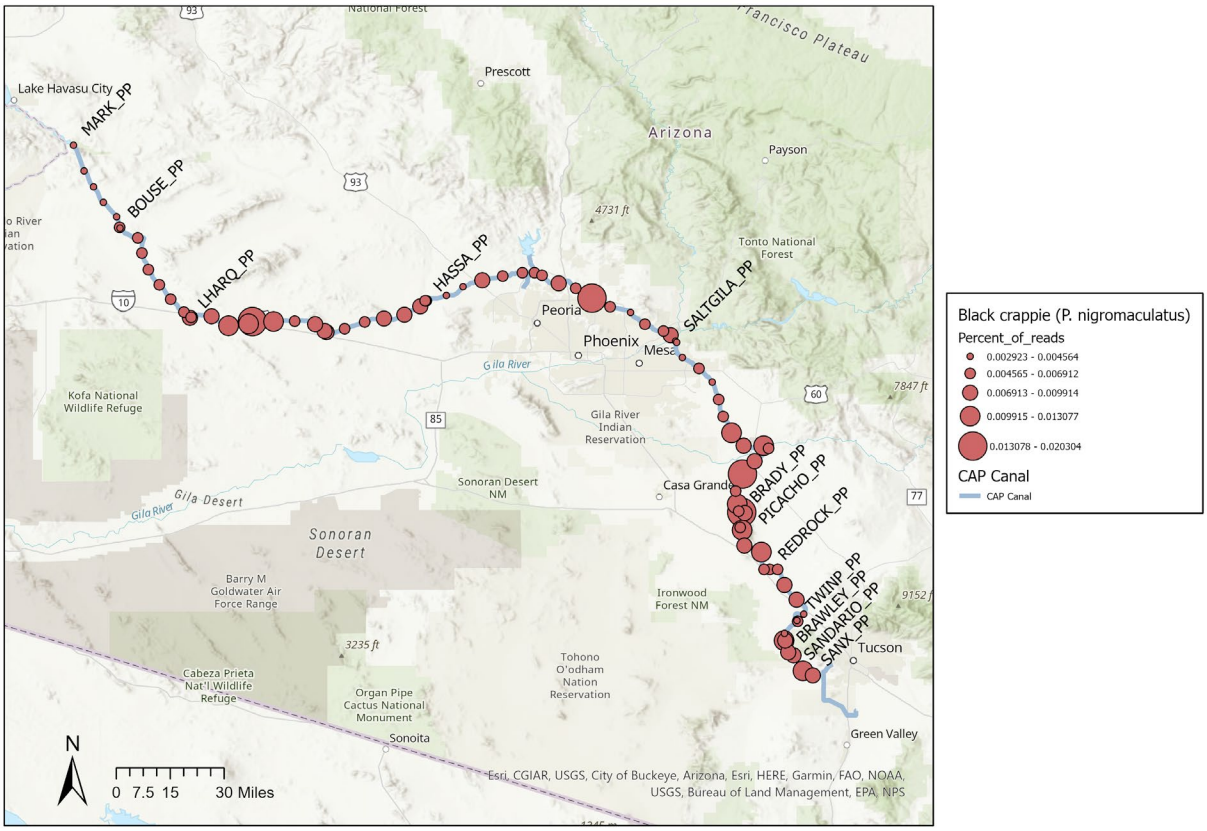

Figure 9: Distribution of black crappie (P. nigromaculatus) sequence detections along the CAP canal.

5. Channel catfish (*Ictalurus punctatus*) and Blue catfish (*Ictalurus furcatus*)

DNA sequences matching to channel catfish (*I. punctatus*) were detected in samples from 71 sites (Figure 10). Read abundance ranged from 0.0007% to 4.38% of reads per site, with a median value of 0.24%. No reads were detected from the three upper-most sampling sites, MARK_PP [CM 0.0], MWBH_001 [CM 6.9], and MWBH_002 [CM 11.9], although channel catfish has been reported from Lake Havasu in the vicinity of the Mark Wilmer Pumping Plant and the adjacent Bill Williams River (https://nas.er.usgs.gov/queries/SpecimenViewer.aspx?SpecimenID=164091).

DNA sequences matching to the congeneric blue catfish (*I. furcatus*) was detected in samples from four sites (Figure 11). One detection was in the upper region of the canal, at site BHLH_003 [CM 36.3], while the other three detections were in the Phoenix metropolitan area at sites HASG_012 [CM 170.6], SALTGILA_PP [CM 190.6], and SGBR_002 [CM 195.7]. Read abundance ranged from 0.005% to 0.30% of reads per site, with a median value of 0.04%.

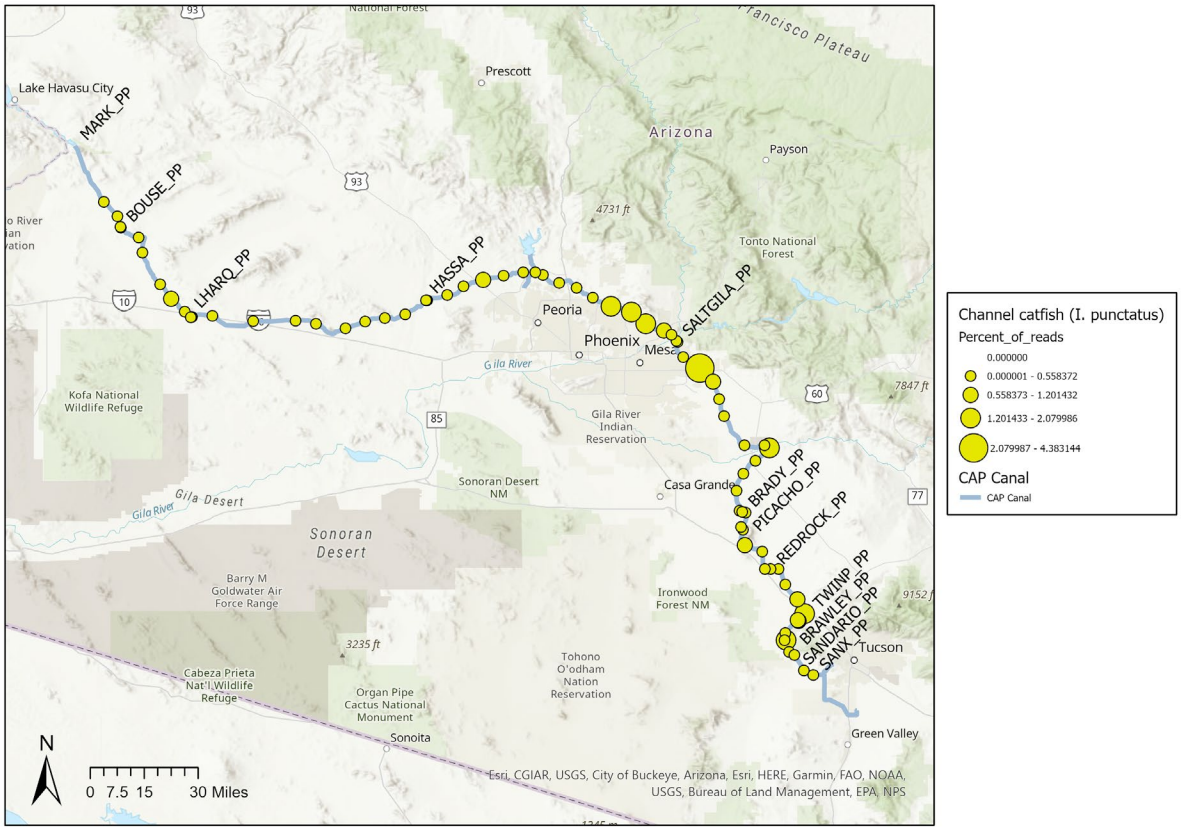

Figure 10: Distribution of channel catfish (I. punctatus) sequence detections along the CAP canal.

Figure 11: Distribution of blue catfish (I. furcatus) sequence detections along the CAP canal.

6. Green sunfish (*Lepomis cyanellus*), Bluegill (*Lepomis macrochirus*), and Redear sunfish (*Lepomis microlophus*)

DNA sequences for three distinct species of sunfish in the genus *Lepomis*, green sunfish (*L. cyanellus*), bluegill (*L. macrochirus*), and redear sunfish (*L. microlophus*), were detected from the CAP samples.

DNA sequences matching to green sunfish were detected in samples from 23 sites (Figure 12). Sequence was detected along the sampled length of the CAP canal, with sequence read abundances ranging from 0.0005% to 55.7% of reads per site, with a median value of 1.67%. The highest proportion of reads was from samples at site MARK_PP [CM 0.0] at the upper end of the CAP canal.

DNA sequences matching to bluegill were also detected in samples from 23 sites (Figure 13). Read abundances ranged from 0.0003% to 12.3% per site, with a median value of 1.78%. Sequence reads were detected in the upper region of the CAP canal from MARK_PP [CM 0.0] to BOUSE PP, and in the lower portion of the CAP canal from HASG_015 [CM 185.7] to SANX_PP [CM 318.4].

DNA sequences matching to redear sunfish were detected in samples from 13 sites (Figure 14). Read abundances for redear sunfish ranged from 0.0001% to 25.6% per site, with a median value of 1.60%. Sequence was detected in the upper region of the CAP canal from MARK_PP [CM 0.0] to LHHA_004 [CM 73.7], and in the lower region of the CAP canal, from SGBR_010 [CM 232.3] to SANX_PP [CM 318.4].

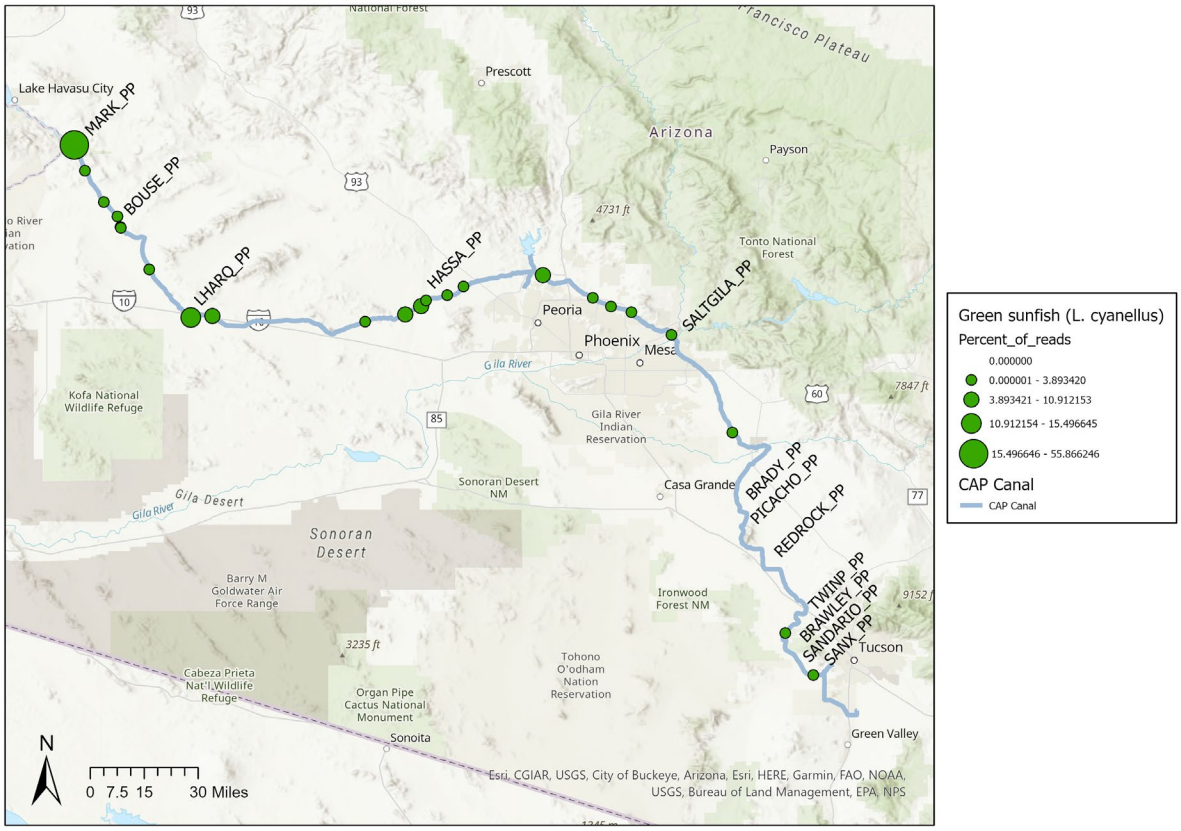

Figure 12: Distribution of green sunfish (L. cyanellus) sequence detections along the CAP canal.

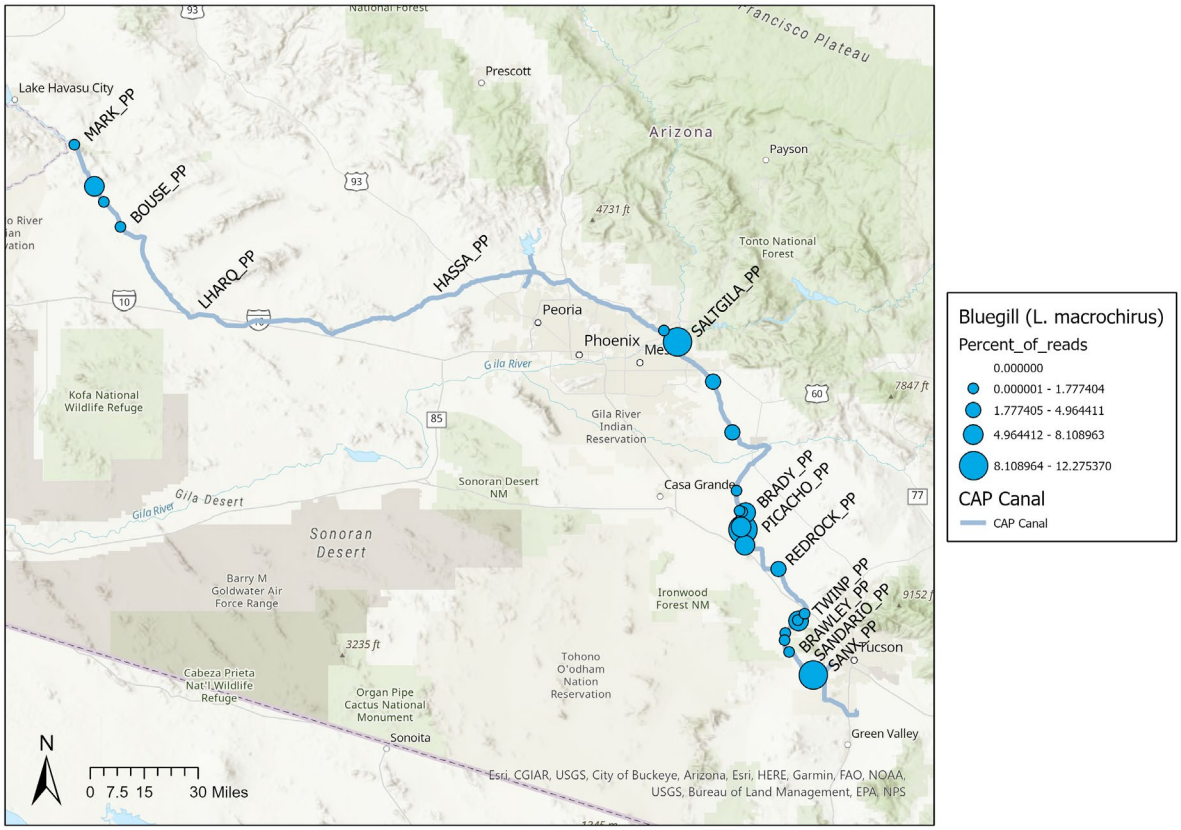

Figure 13: Distribution of bluegill (L. macrochirus) sequence detections along the CAP canal.

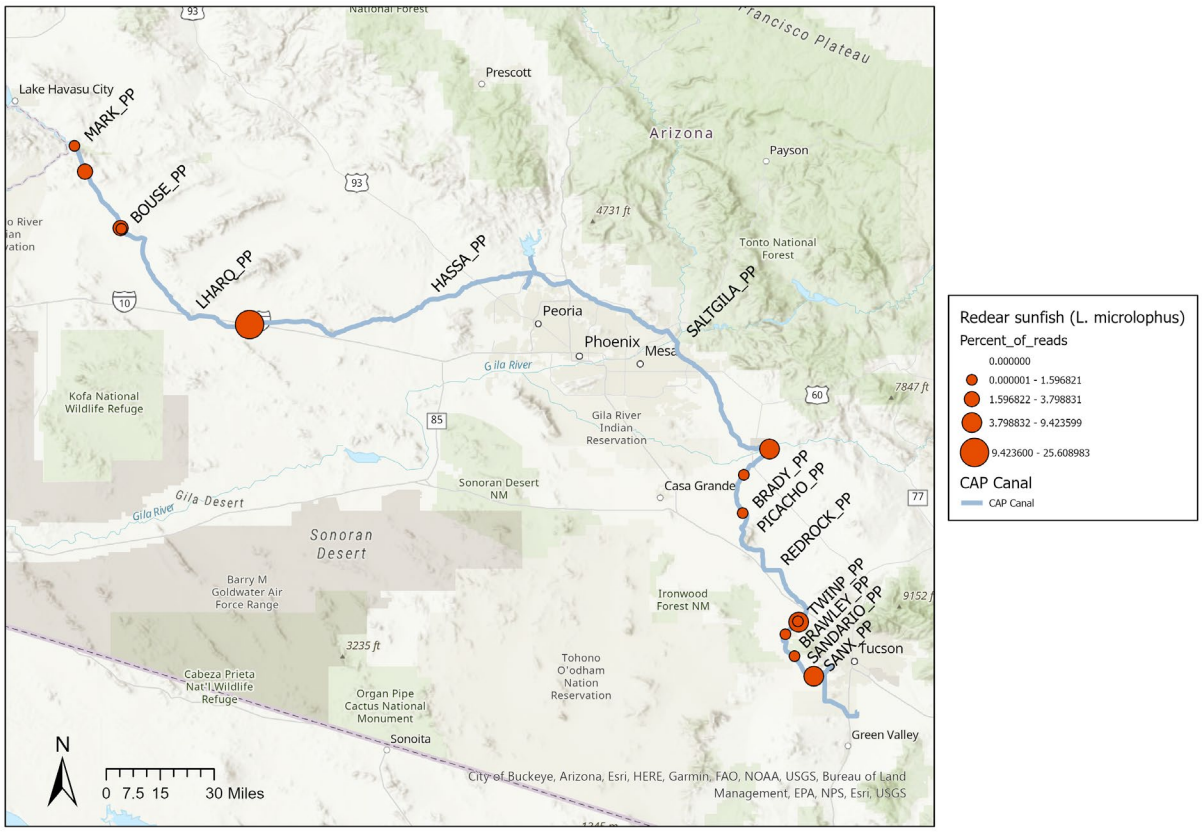

Figure 14: Distribution of redear sunfish (L. microlophus) sequence detections along the CAP canal.

7. Smallmouth bass (*Micropterus dolomieu*) and Largemouth bass (*Micropterus salmoides*)

Sequences matching to two species of the genus *Micropterus*, smallmouth bass (*M. dolomieu*) and largemouth bass (*M. salmoides*) were detected in samples from the CAP canal.

Sequences matching to smallmouth bass were detected in samples from 22 sites (Figure 15). Read abundances ranged from 0.0008% to 18.7% per site, with a median value of 1.81%. Sequence reads were detected from sites through most of the CAP canal, from MARK_PP [CM 0.0] to RRTP_002 [CM 283.2].

Sequences matching to largemouth bass were detected in samples from 15 sites (Figure 16). Read abundances ranged from 0.0007% to 17.7% per site, with a median value of 1.40%. Sequence reads were detected from sites in the upper region of the CAP canal, from MARK_PP [CM 0.0] to BHLB_007, and in the lower portion of the CAP canal, from HASG_015 [CM 185.7] to SANX_PP [CM 318.4].

It should be noted that sequences for 3 ASVs, identified from six sites, matched most closely to Florida bass (*M. floridanus*). Largemouth bass and Florida bass are not distinguished during traditional surveys in the CAP canal, and to simplicity all sequences from both species have been combined and reported as largemouth bass throughout this report.

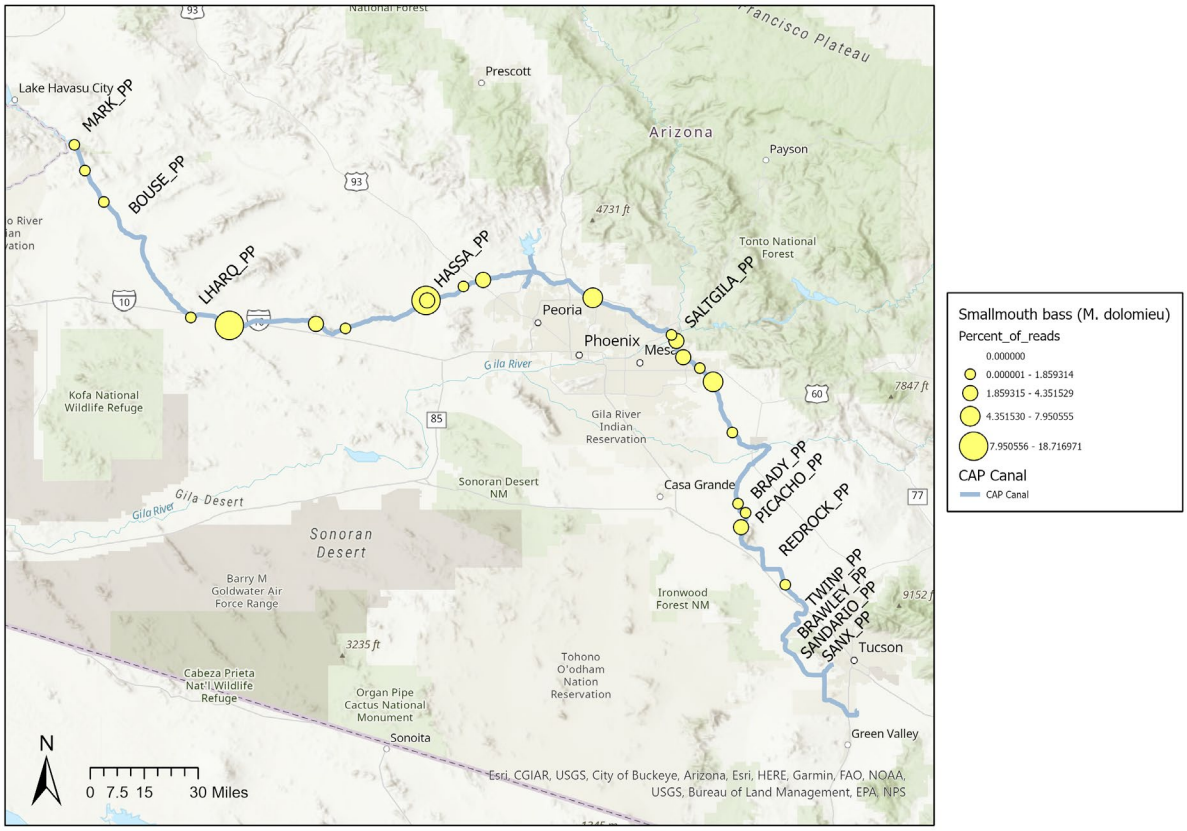

Figure 15: Distribution of smallmouth bass (M. dolomieu) sequence detections along the CAP canal.

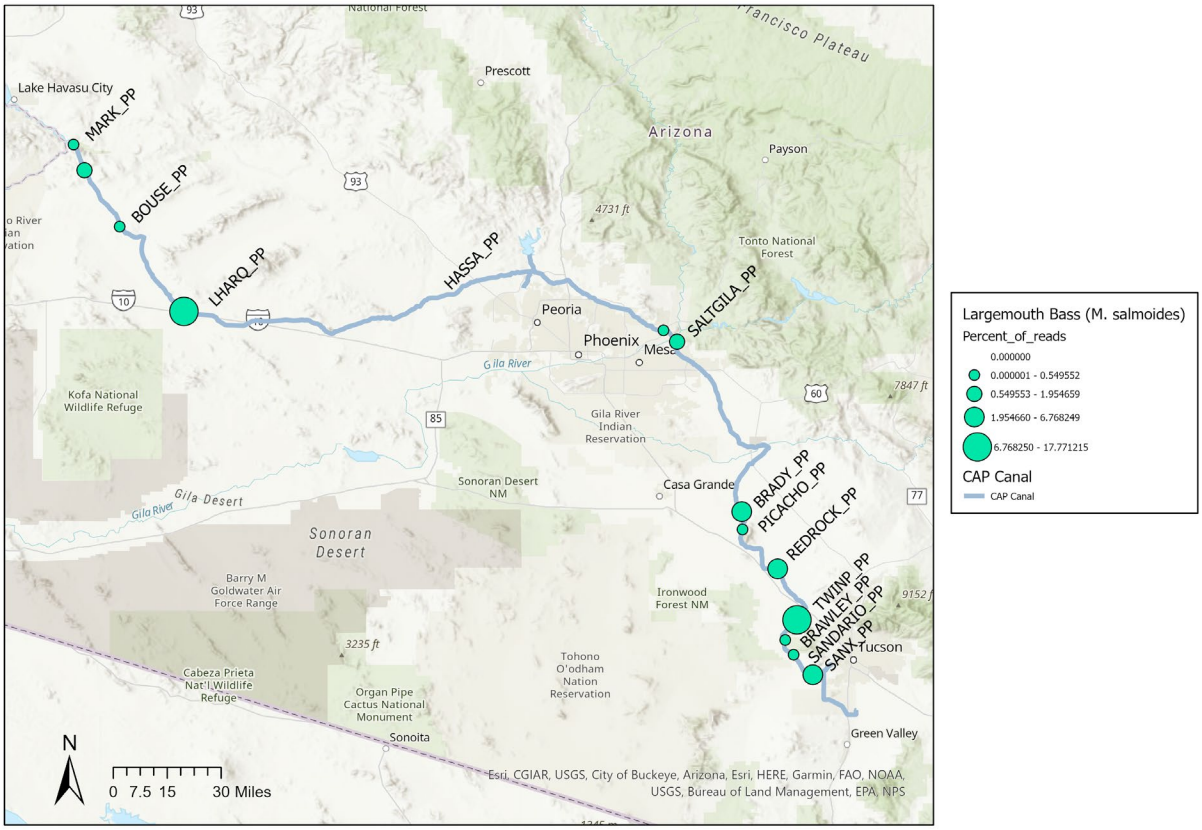

Figure 16: Distribution of largemouth bass (M. salmoides) sequence detections along the CAP canal.

8. Gizzard shad (*Dorosoma cepedianum*) and Threadfin shad (*Dorosoma petenense*)

Sequences matching to two species of the genus *Dorosoma*, gizzard shad (*D. cepedianum*) and threadfin shad (*D. petenense*), were detected in samples from the CAP canal.

Sequences matching to gizzard shad (*D. cepedianum*) were detected in samples from 10 sites (Figure 17). Read abundances ranged from 0.001% to 27.4% per site, with a median value of 3.67%. Sequence reads were detected in the upper region of the CAP canal, from MARK_PP [CM 0.0] to BOUSE_PP [CM 25], at LHHA_008 [CM 89.7], and in the lower region of the CAP canal from REDROCK_PP [CM 276.6] to SANX_PP [CM 318.4].

Sequences matching to and threadfin shad (*D. petenense*) were detected in samples from 6 sites (Figure 18). Read abundances ranged from 0.01% to 3.81% per site, with a median value of 0.60%. Sequences were detected in the upper region of the CAP canal, from MARK_PP [CM 0.0] to BHLH_001 [CM 25.2], and at HASG_016 [CM 188.3].

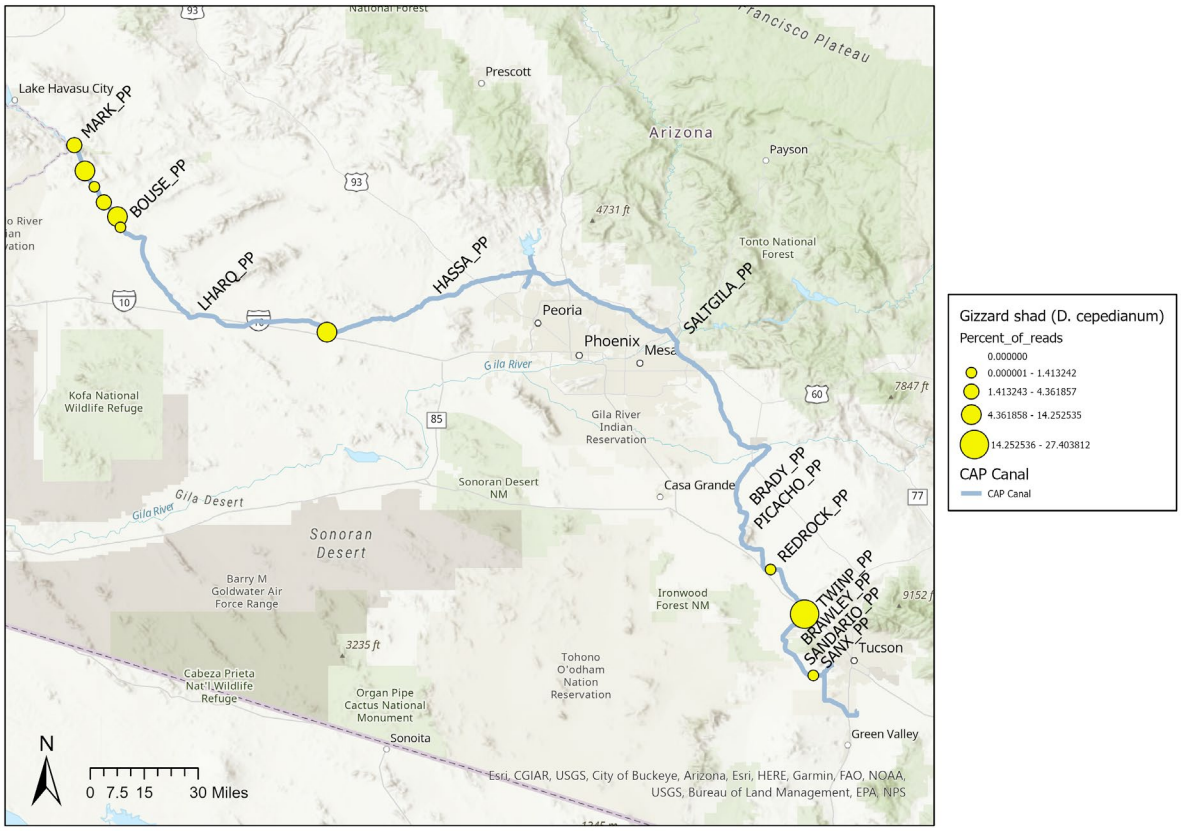

Figure 17: Distribution of gizzard shad (D. cepedianum) sequence detections along the CAP canal.

Figure 18: Distribution of threadfin shad (D. petenense) sequence detections along the CAP canal.

9. Red shiner (Cyprinella lutrensis)

Sequences matching to red shiner (*C. lutrensis*) were detected is samples from 4 sites (Figure 19). Read abundances ranged from 0.14% to 3.78% per site, with a median value of 0.62%. All detections were in the lower region of the CAP canal, from PIRR_004 [CM 275.2] to SANX_004.

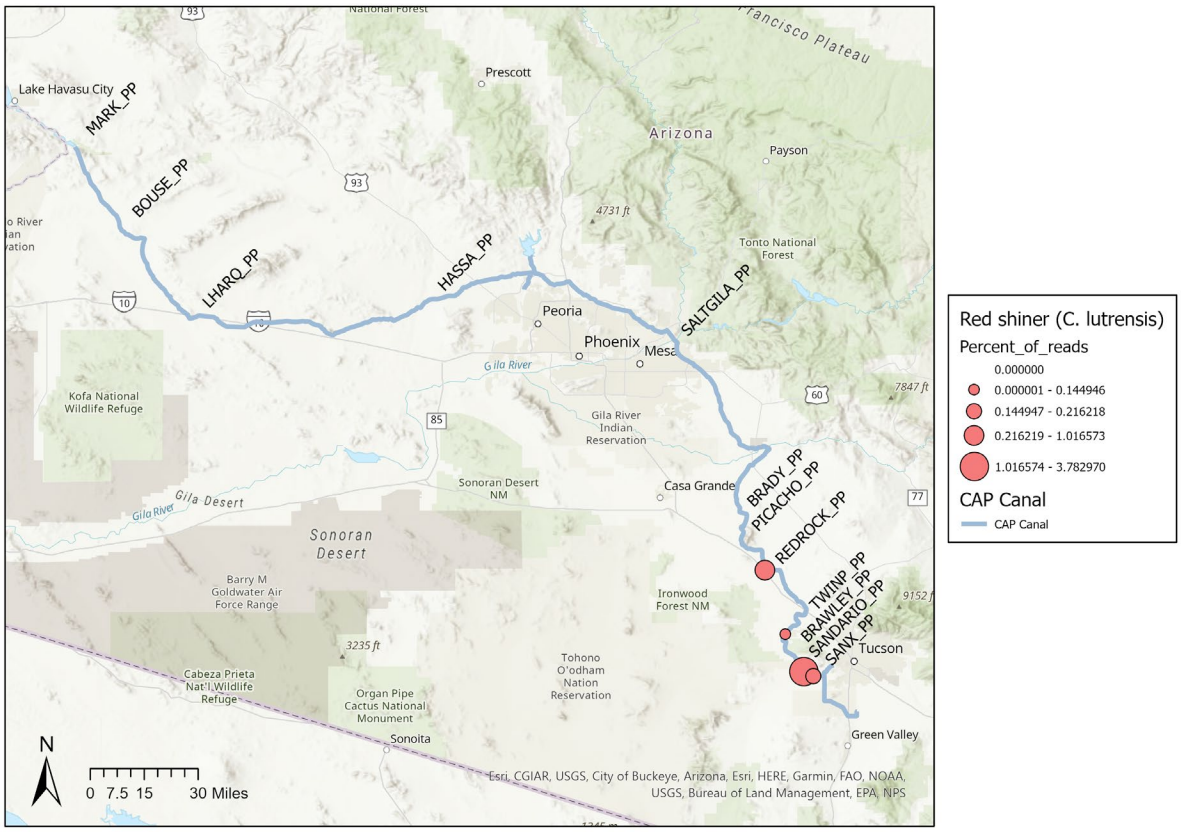

Figure 19: Distribution of red shiner (C. lutrensis) sequence detections along the CAP canal.

10. Western mosquitofish (*Gambusia affinis*)

Sequences matching to Western mosquitofish (*G. affinis*) were detected in samples from 32 sites (Figure 20). Read abundances ranged from 0.0003% to 20.14% per site, with a median value of 1.81%. Sequence reads were detected along the length of the CAP canal, with a cluster of sites with relatively high read abundances observed between HASG_005 [CM 140.8] and SGBR_001 [CM 190.9], in the Phoenix metropolitan area.

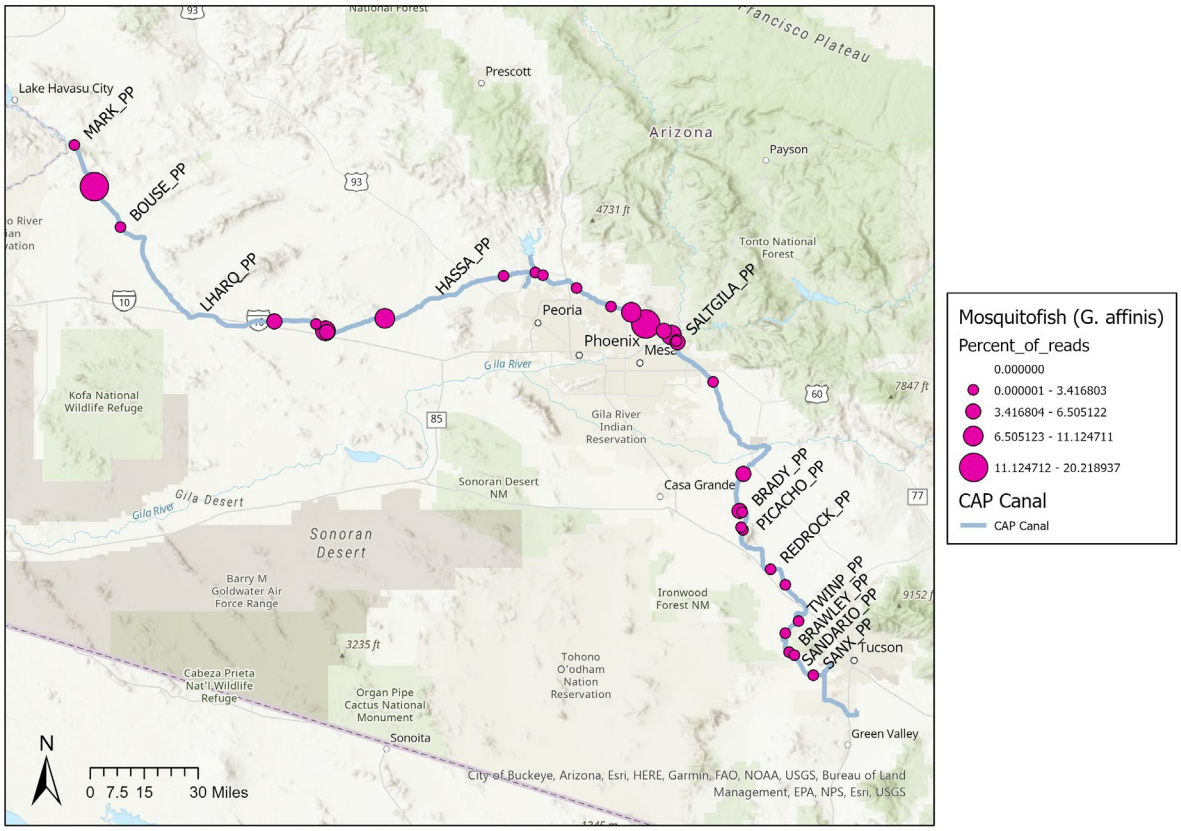

Figure 20: Distribution of Western mosquitofish (G. affinis) sequence detections along the CAP canal

11. Blue tilapia (*Oreochromis aureus*) and Nile tilapia (*Oreochromis niloticus*)

Sequences matching to two species of tilapia, blue tilapia (*O. aureus*) and Nile tilapia (*O. niloticus*) were detected in samples from the CAP canal. Sequences matching to blue tilapia were detected in samples from two sites at the upper end of the CAP canal, MARK_PP [CM 0.0] and MWBH_001 [CM 6.9] (Figure 21). Read abundances ranged from 0.48% to 0.81% per site, with a median value of 0.65%. Sequences matching to Nile tilapia were detected from a single site in the lower region of the CAP, BRADY_PP [CM 253.8], with a read abundance of 2.93% (Figure 21).

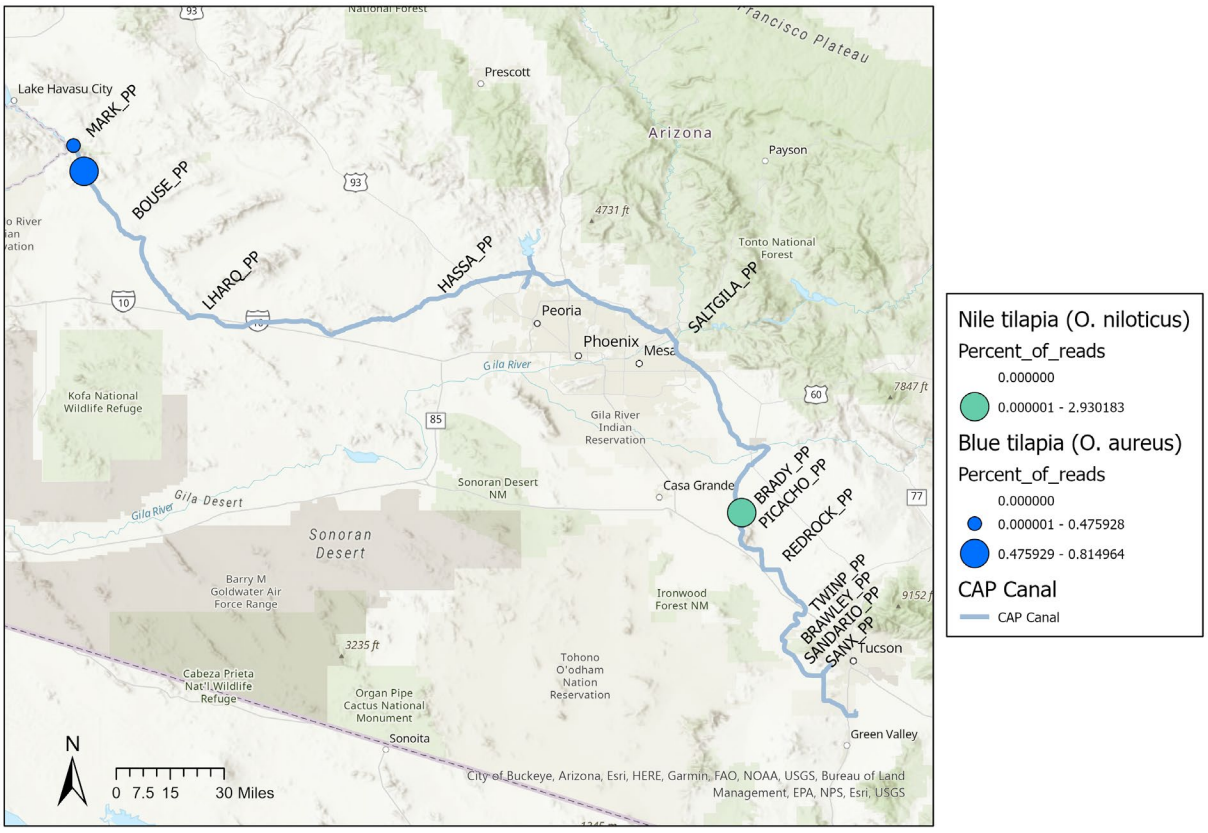

Figure 21: Distribution of blue tilapia (O. aureus) and Nile tilapia (O. niloticus) sequence detections along the CAP canal.

12. Flathead catfish (*Pylodictis olivaris*) and Shining catfish (*Tachysurus nitidus*; AKA shining catfish)

Along with the channel catfish (*I. punctatus*) and blue catfish (*I. furcatus*) discussed above, DNA sequences matching to two additional species of catfish, flathead catfish (*P. olivaris*) and shining catfish (*T. nitidus*) were also detected.

Sequences matching to flathead catfish were detected is samples from two sites (Figure 22). Read abundances ranged from 0.005% to 0.033% per site, with a median value of 0.017%. The two sites with detections were MARK_PP [CM 0.0] and HASG_009 [CM 155.3].

Sequences matching to shining catfish were detected is samples from two sites (Figure 22). Read abundances ranged from 0.0006% to 0.005% per site, with a median value of 0.003%. The two sites with detections were in the lower region of the CAP canal, at SGBR_005 [CM 210.8] and TWINP_PP [CM 297.5]. Shining catfish is a member of the speciose Bagridae family native to Asia and Africa. Shining catfish is native to East Asia, including Korea, China, and Russia. This species has not previously been reported from North America. This species is reported to be commercially important in its native range, suggesting that the presence of DNA matching to this species could be related to its use as a foodstuff. Detected DNA could have derived from intentional or accidental release of live specimens, or it could be due to introduction of

environmental DNA from tissue of a dead animal. If the source is from tissue of a dead individual, the detection at TWINP_PP [CM 297.5] is somewhat surprising, as this site is at least 80 miles along the CAP canal from possible sources at international markets or restaurants in the Phoenix metropolitan area. However, the source of the sequence is uncertain, as to our knowledge this fish is not imported to the United States. Additional testing of samples would be needed to further confirm the presence of DNA matching to *T. nitidus* in these and other samples.

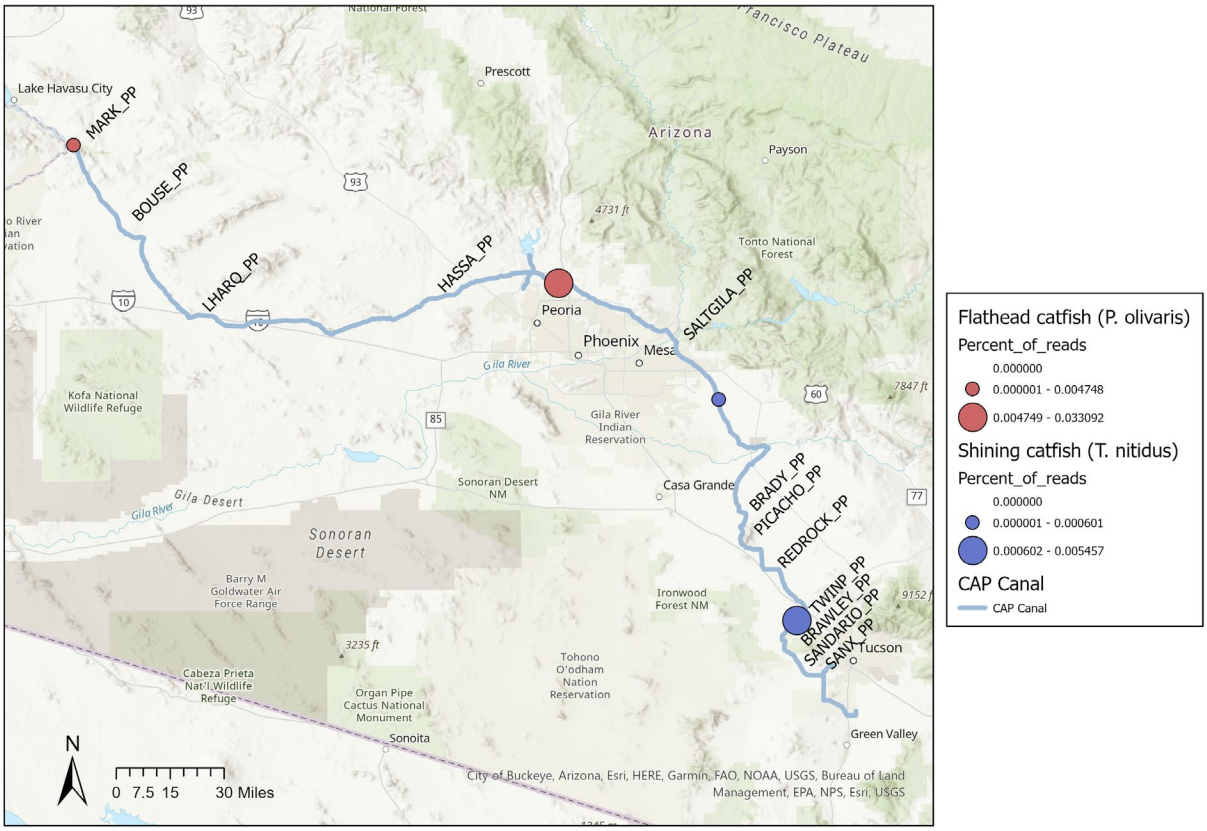

Figure 22: Distribution of flathead catfish (P. olivaris) and shining catfish (T. nitidus) sequence detections along the CAP canal.

13. Marine and anadromous species: Northern anchovy (*Engraulis mordax*), Japanese amberjack (*Seriola quinqueradiata*), Coho salmon (Oncorhynchus kisutch), Pacific sandlance (*Ammodytes personatus*), and Atlantic salmon (*Salmo salar*)

Sequences matching to five marine or anadromous species, northern anchovy (*E. mordax*), Japanese amberjack (*S. quinqueradiata*), Pacific sandlance (*A. personatus*), Atlantic salmon (*S. salar*), and coho salmon (*O. kisutch*), were detected from various sites along the CAP canal.

Sequences matching to northern anchovy were detected in samples from three sites in the upper region of the CAP canal, MARK_PP [CM 0.0], MWBH_002 [CM 11.9], and MWBH_004 [CM 22] (Figure 23). Read abundances ranged from 2.16% to 14.5% per site, with a median value of 5.86%. The northern anchovy is a marine species and would not be expected to survive in the CAP canal. However, it is a popular bait fish, and frozen anchovies are widely available in fishing supply stores in Lake Havasu City, NV, which is on the shore of Lake Havasu and close to the inlet of the CAP canal at Mark Wilmer Pumping Plant. Such bait is therefore the likely source of northern anchovy sequences in the CAP canal samples set. Given that recreational fishing is unlikely to be occurring along the CAP canal, Lake Havasu may reasonable be considered the originating source for all reads matching this species. This suggests that eDNA may be detectable quite a distance from the point source, as sequence was detected as far down the CAP canal as BOUSE_PP [CM 25.0], which is approximately 25 miles from the inlet of the canal at Mark Wilmer Pumping Plant. Alternatively, it may be that tissue (and associated DNA) from bait fish is more likely to be carried into the canal, effectively moving point sources for eDNA into the canal itself.

Sequences matching to Japanese amberjack were detected from three adjacent sites, HASG_013 [CM 175.7], HASG_014 [CM 180.6], and HASG_015 [CM 185.7], all of which are in the Phoenix metropolitan area (Figure 23). Read abundances ranged from 1.32% to 6.60% per site, with a median value of 4.95%. Like, northern anchovy, Japanese amberjack is a marine species that would not be expected to survive in the CAP canal. Japanese amberjack is widely used a foodstuff, particularly in Japanese restaurants, where it is often marketed as “yellowtail” or “hamachi,” and is frequently served raw in sushi. Detection of sequences matching to Japanese amberjack is most likely due to releases from restaurants or international markets.

Sequences matching to Pacific sandlance were detected from a single site in the lower region of the CAP canal, at PIRR_001 [CM 260.2], with a read abundance of 0.40% (Figure 23). The origin of these sequences is uncertain, as Pacific sandlance is a marine species that would not be expected to survive in the CAP canal. In addition, Pacific sandlance is not known to be widely used as a foodstuff or as bait (at least outside of coastal areas). Further complicating the interpretation of an anthropogenic origin, the single site where detection occurred, PIRR_001 [CM 260.2], is a considerable distance from any human habitations. Eloy, AZ (population 18,666), the closest community to PIRR_001 [CM 260.2], is approximately 10 miles from the sampling site. One possibility is that the detected sequences represent contamination from the marine samples used in study for mock communities. This seems unlikely for several reasons. First, Pacific sandlance was the only marine species detected in the PIRR_001 [CM 260.2] samples. Second, while Pacific sandlance sequence was detected in one of the seven mock samples, it occurred at a much lower frequency in the moth than in the PIRR_001 [CM 260.2] samples (<0.001% in the mock versus 0.04% in PIRR_001 [CM 260.2]). Pacific sandlance was not one of the marine species used to generate mock communities, and its tissue was not handled in the laboratory. Finally, mock samples were processed after field samples in the laboratory, suggesting any cross-contamination would most likely have moved from the field samples to the mock samples, rather than the converse.

Sequences matching to Atlantic salmon were detected from a single site in the lower region of the CAP canal, at BRAWLEY_PP [CM 309.2], with a read abundance of 3.60% (Figure 23). The

origin of these sequences is uncertain. Atlantic salmon is anadromous, but no reported records were identified for its introduction to Arizona, and stocking efforts in neighboring states and the 1800s and early 1900s all appear to have failed (www.nas.usgs.gov). Atlantic salmon is widely used as a foodstuff, and so the detection could well have been sourced from human consumption. There is small residential community, Avra Foothills Estates, in close proximity to BRAWLEY_PP [CM 309.2]. The western edge of the Tucson metropolitan area is approximately 10 miles from BRAWLEY_PP [CM 309.2], however Tucson is downstream of the site and thus would not be expected to be a direct source of introduction. No sequences matching to Atlantic salmon were observed in any of the mock community samples.

Sequences matching to coho salmon were detected from a single site in the lower region of the CAP canal, at SGBR_012 [CM 242.1], with a read abundance of 3.60% (Figure 23). Given that coho salmon was included in the mock communities, the sequences recovered from the one of the three SGBR_012 [CM 242.1] may be attributable to cross-contamination. As with the Atlantic salmon, coho salmon in unlikely to be living in the CAP canal system. Although coho salmon were stocked in the Lower Colorado River and in reservoirs of the Salt River basin in the 1960s and 1970s (www.nas.usgs.gov), there is no evidence for extant populations there or in other parts of Arizona. The detection could be attributable to human consumption, given that the closest habitation, Florence, AZ, is in close proximity to the CAP canal, and approximately five miles upstream of the SGBR_012 [CM 242.1] sampling site.

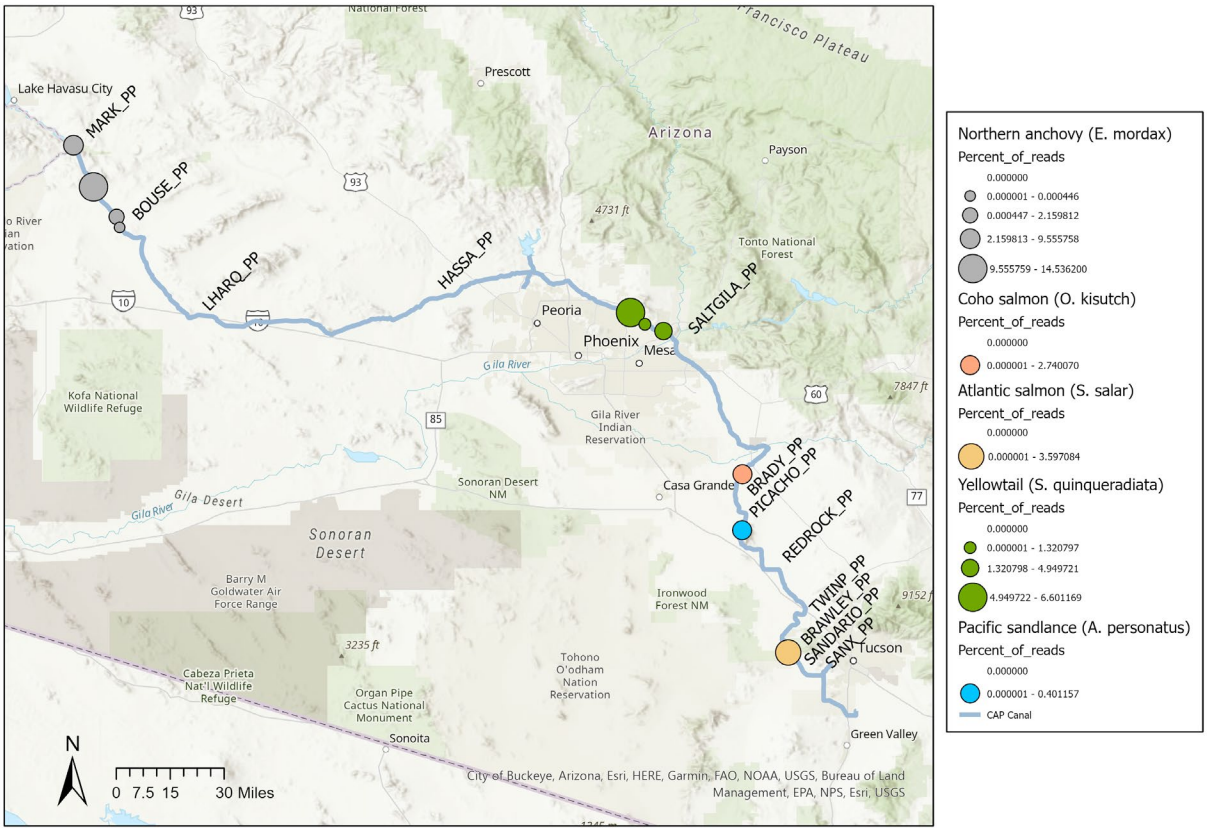

Figure 23: Distribution of northern anchovy (E. mordax), Japanese amberjack (S. quinqueradiata), Pacific sandlance (A. personatus), Atlantic salmon (S. salar), and coho salmon (O. kisutch) sequence detections along the CAP canal.

14. Non-fish sequences

Although the MiFish primers are designed to be specific to fish, they did display some cross-reactivity with non-fish species. Eighty-one of the 322 recovered ASVs did not to match to any available reference sequence for fish species. These non-fish ASVs accounted for 4.89% of the total reads. These sequences were analyzed separately by BLAST search against the GenBank nr/nt databases. One of these ASVs matched to the reference sequence for the softshell spiny turtle (*Apalone spinifera*), which was caught during a traditional survey of Brady Pumping Plant (BRADY_PP [CM 253.8]) in 2002 (Appendix 7). The matching ASV was detected from LHHA_014 [CM 113.9] and comprised 0.0016% of the total reads from the site. Thirty-nine of the non-fish ASVs matched to reference sequences for bird, primarily waterfowl. Twenty-five of the non-fish ASVs matched to reference sequences for mammals, including pig, cattle, sheep, goat, dog/coyote, domestic cat, beaver, and bats. One ASVs appeared to be bacterial in origin. The remaining 15 ASVs could not be reliably matched to sequences in the GenBank nr/nt databases.

B. Parallel fish surveys and eDNA sampling

For six sites located in pumping plant forebays eDNA sampling was conducted twice, once during the sampling along the length of the canal in February 2021, and a second time in conjunction with traditional fish surveys. Data for both eDNA and traditional surveys at these sites are presented below.

1. Bouse Pumping Plant

The Bouse Pumping Plant forebay (site BOUSE_PP [CM 25]) was surveyed for fish populations in July 2021 using traditional survey methods and eDNA sample collection for metabarcoding. Traditional surveys captured four species of fish, largemouth bass, striped bass, bluegill, and unidentified sunfish (*Lepomis* sp., all young of the year) (Figure 24). eDNA metabarcoding detected sequences matching to nine species. Three species, largemouth bass, striped bass, and bluegill, were detected by eDNA and captured by traditional surveys. Four species, common carp, mosquitofish, channel catfish, and smallmouth bass, were detected by eDNA but were not captured or observed by traditional surveys. Grass carp, which where the second most abundant reads (19.77% of reads) in the eDNA dataset, were confirmed to be present during visual surveys but were not captured. Seventeen young-of-year sunfish captured in the traditional surveys could not be identified to species and were recorded as "*Lepomis* sp." This classification was not applied to eDNA reads, but 72.88% of the read sequences matched to reference sequences for green sunfish. These reads may have originated from the unidentified sunfish captured in the traditional surveys.

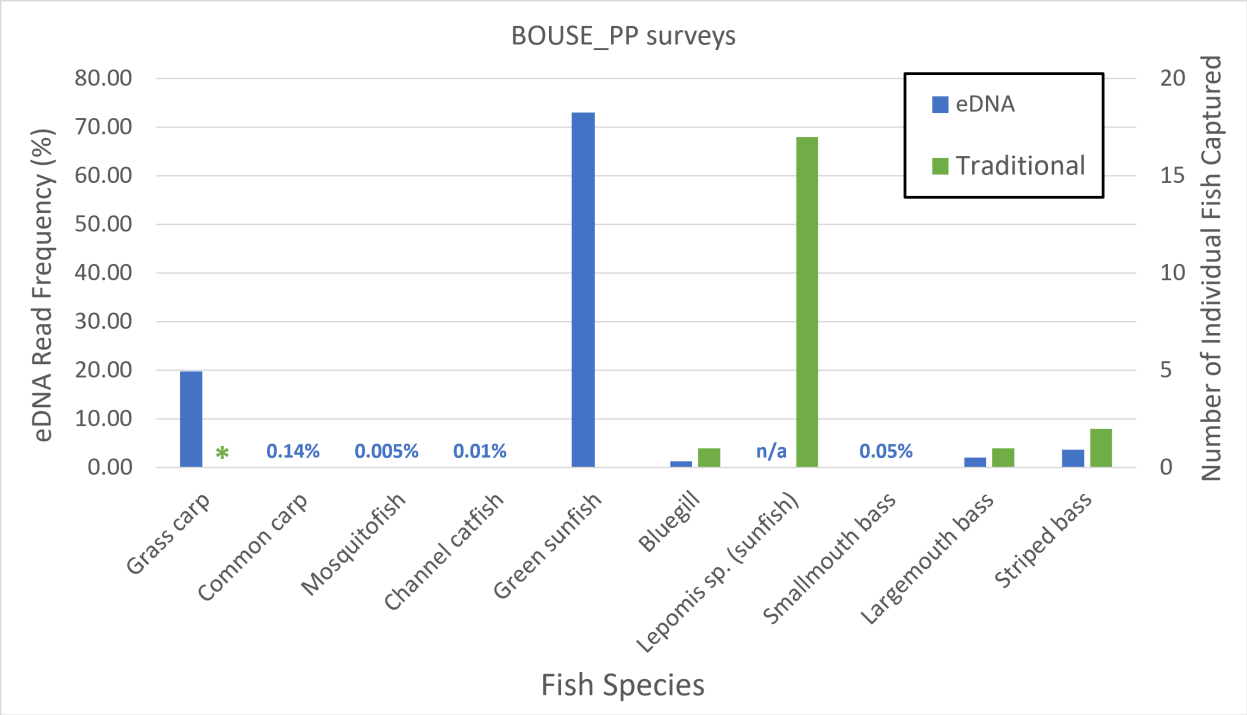

Figure 24: Survey results from eDNA metabarcoding and traditional surveys at Bouse Pumping Plant (BOUSE_PP [CM 25]). Percent read frequencies from eDNA metabarcoding are displayed in blue, with values shown on the left-hand vertical axis. For species where the read frequency was below 1%, the value is displayed above the species name in blue text. Counts for the numbers of individuals captured by traditional methods are shown in green, with values shown on the right-hand vertical axis. Grass carp were observed by visual survey during the traditional surveys, but were not captured, as denoted by an asterisk (*).

2. Little Harquahala Pumping Plant

The Little Harquahala Pumping Plant (site LHARQ_PP [CM 58.7]) was surveyed for fish populations in July 2021 using traditional survey methods and eDNA sample collection for metabarcoding. Traditional surveys identified four species of fish, grass carp, common carp, largemouth bass, and smallmouth bass (Figure 25). eDNA metabarcoding detected sequences matching to eight species. Three species, grass carp, common carp, and smallmouth bass, were detected by eDNA and captured by traditional surveys. Five species, mosquitofish, channel catfish, green sunfish, striped bass, and flathead catfish, were detected by eDNA but were not captured or observed by traditional surveys. Largemouth bass sequences were not detected in the eDNA metabarcoding data from samples collected contemporaneously with traditional sampling. The species was also not detected in the LHARQ_PP [CM 58.7] samples collected during the CAP canal-wide eDNA survey in February 2021.

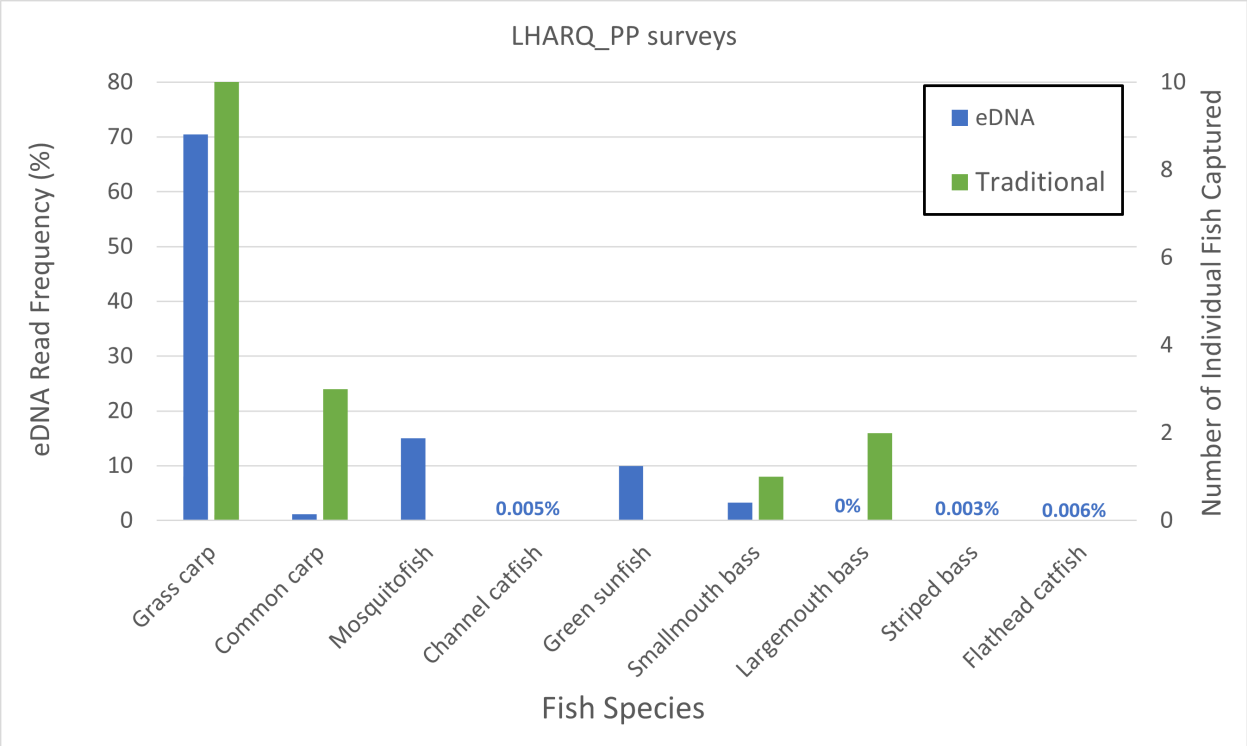

Figure 25: Survey results from eDNA metabarcoding and traditional surveys at Little Harquahala Pumping Plant (site LHARQ_PP [CM 58.7]). Percent read frequencies from eDNA metabarcoding are displayed in blue, with values shown on the left-hand vertical axis. For species where the read frequency was below 1%, the value is displayed above the species name in blue text. Counts for the numbers of individuals captured by traditional methods are shown in green, with values shown on the right-hand vertical axis.

3. Hassayampa Pumping Plant

The Hassayampa Pumping Plant (site HASSA_PP [CM 120.5]) was surveyed for fish populations in July 2021 using traditional survey methods and eDNA sample collection for metabarcoding. Traditional surveys captured five species of fish, grass carp, common carp, channel catfish, smallmouth bass, and striped bass (Figure 26). eDNA metabarcoding detected sequences matching to seven species. Five species, grass carp, common carp, channel catfish, smallmouth bass, and striped bass, were detected by eDNA and captured by traditional surveys. Two species, mosquitofish and green sunfish, were detected by eDNA but were not captured or observed by traditional surveys.

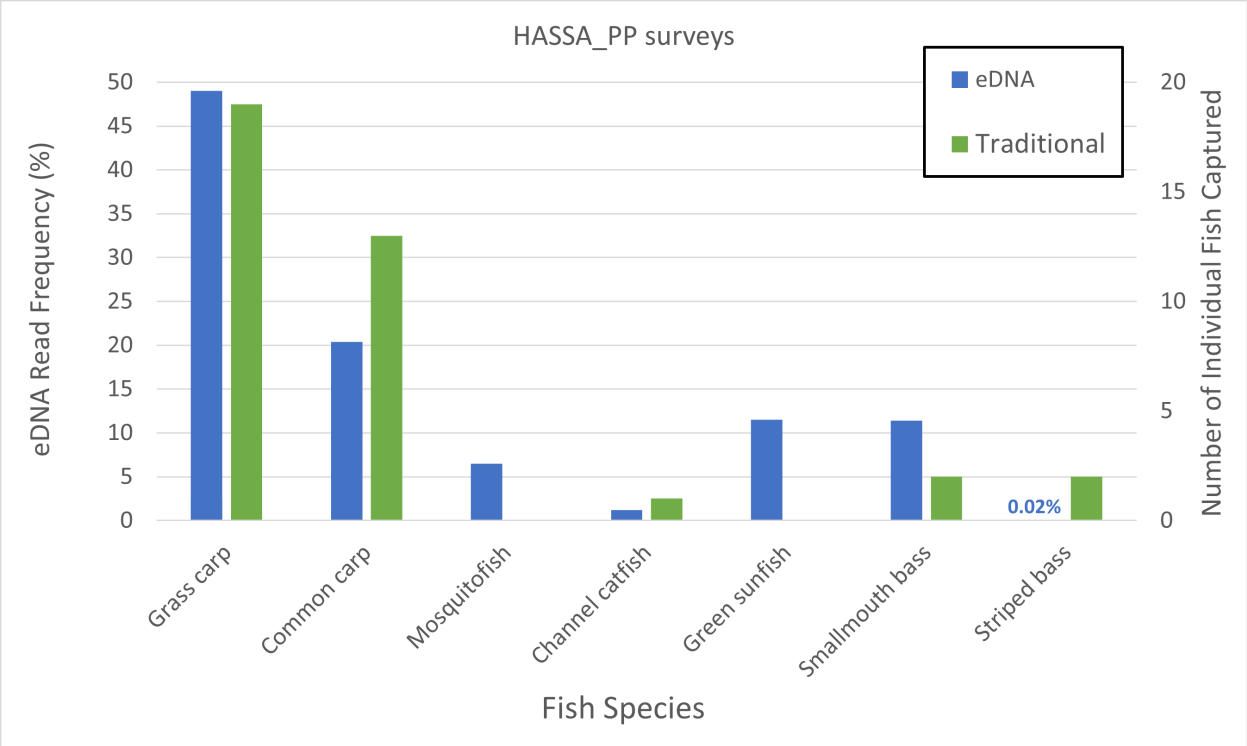

Figure 26: Survey results from eDNA metabarcoding and traditional surveys at Hassayampa Pumping Plant (site HASSA_PP [CM 120.5]). Percent read frequencies from eDNA metabarcoding are displayed in blue, with values shown on the left-hand vertical axis. For species where the read frequency was below 1%, the value is displayed above the species name in blue text. Counts for the numbers of individuals captured by traditional methods are shown in green, with values shown on the right-hand vertical axis.

4. Brady Pumping Plant

The Brady Pumping Plant (site BRADY_PP [CM 253.8]) was surveyed for fish populations in November 2020 using traditional survey methods and eDNA sample collection for metabarcoding. Traditional surveys captured five species of fish, grass carp, common carp, bluegill, largemouth bass, and striped bass (Figure 27). eDNA metabarcoding detected sequences matching to seven species. Four species, grass carp, common carp, bluegill, and striped bass, were detected by eDNA and captured by traditional surveys. Two species, mosquitofish and smallmouth bass, were detected by eDNA but were not captured or observed by traditional surveys. Largemouth bass sequences were not detected in the eDNA data; however, largemouth bass eDNA was detected in samples collected from BRADY_PP [CM 253.8] during the CAP canal-wide eDNA survey in February 2021.

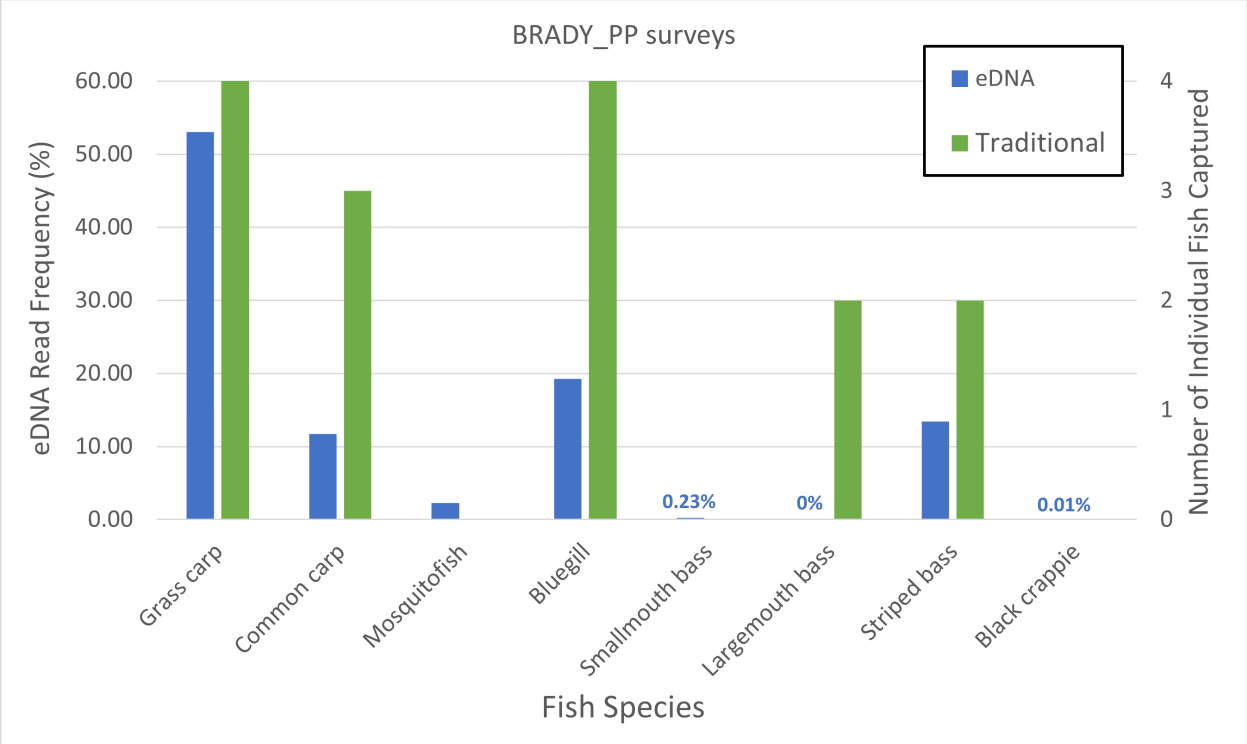

Figure 27: Survey results from eDNA metabarcoding and traditional surveys at Brady Pumping Plant (site BRADY_PP [CM 253.8]). Percent read frequencies from eDNA metabarcoding are displayed in blue, with values shown on the left-hand vertical axis. For species where the read frequency was below 1%, the value is displayed above the species name in blue text. Counts for the numbers of individuals captured by traditional methods are shown in green, with values shown on the right-hand vertical axis.

5. Red Rock Pumping Plant

The Red Rock Pumping Plant (site REDROCK_PP [CM 276.6]) was surveyed for fish populations in November 2020 using traditional survey methods and eDNA sample collection for metabarcoding. Traditional surveys captured two species of fish, largemouth bass and striped bass (Figure 28). eDNA metabarcoding detected sequences matching to 12 species, including largemouth bass and striped bass. Ten species, grass carp, common carp, red shiner, gizzard shad, mosquitofish, channel catfish, green sunfish, bluegill, redear sunfish, largemouth bass, striped bass, and black crappie, were detected by eDNA but were not captured or observed by traditional surveys.

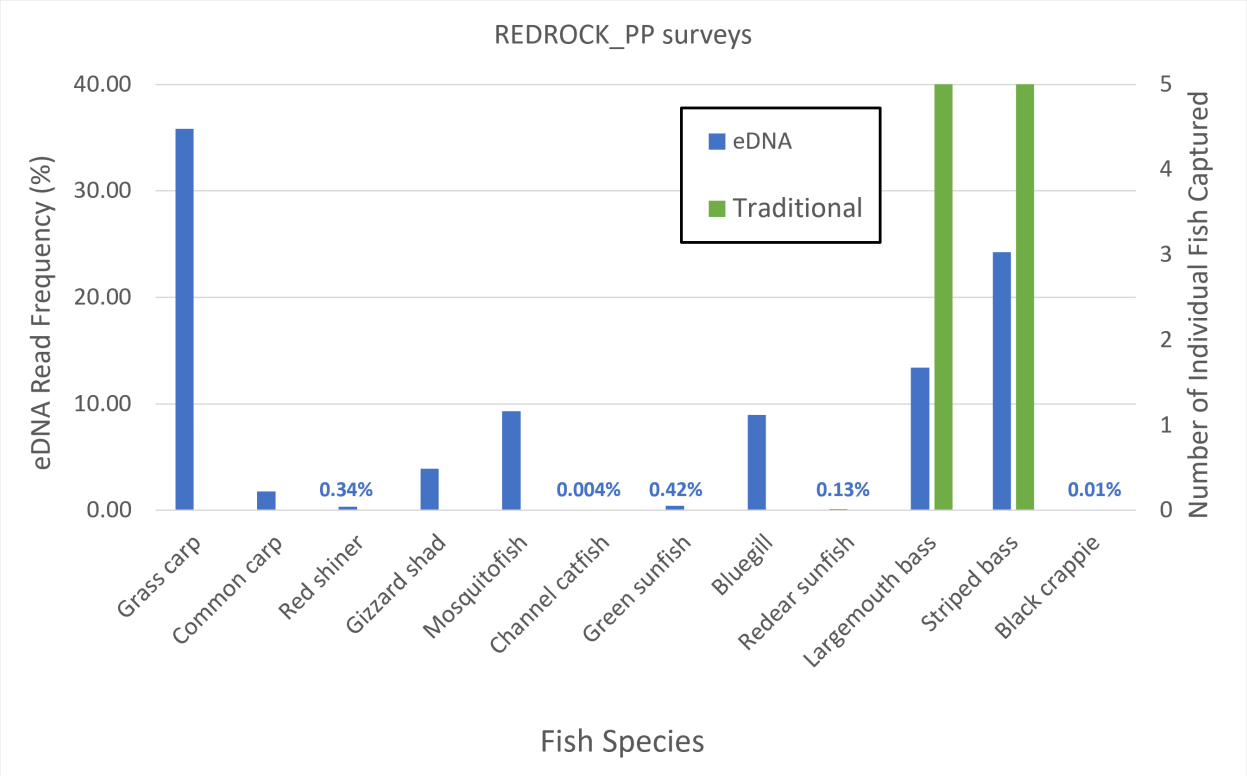

Figure 28: Survey results from eDNA metabarcoding and traditional surveys at Red Rock Pumping Plant (site REDROCK_PP [CM 276.6]). Percent read frequencies from eDNA metabarcoding are displayed in blue, with values shown on the left-hand vertical axis. For species where the read frequency was below 1%, the value is displayed above the species name in blue text. Counts for the numbers of individuals captured by traditional methods are shown in green, with values shown on the right-hand vertical axis.

6. San Xavier Pumping Plant

The San Xavier Pumping Plant (site SANX_PP [CM 318.4]) was surveyed for fish populations in November 2020 using traditional survey methods and eDNA sample collection for metabarcoding. Traditional surveys captured six species of fish, grass carp, common carp, channel catfish, bluegill, redear sunfish, and largemouth bass (Figure 29). eDNA metabarcoding detected sequences matching to 11 species, including all six of the species captured in traditional surveys. Five species, gizzard shad, mosquitofish, green sunfish, striped bass, and black crappie, were detected by eDNA but were not captured or observed by traditional surveys.

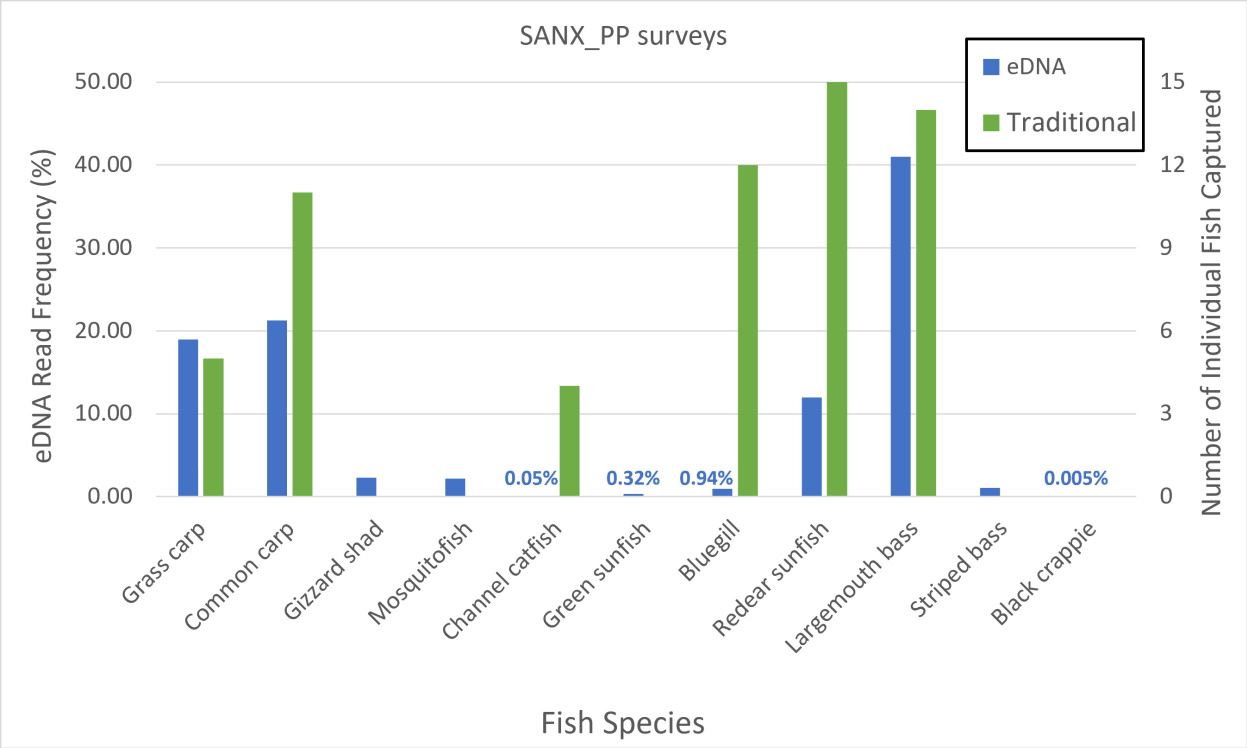

Figure 29: Survey results from eDNA metabarcoding and traditional surveys at San Xavier Pumping Plant (site SANX_PP [CM 318.4]). Percent read frequencies from eDNA metabarcoding are displayed in blue, with values shown on the left-hand vertical axis. For species where the read frequency was below 1%, the value is displayed above the species name in blue text. Counts for the numbers of individuals captured by traditional methods are shown in green, with values shown on the right-hand vertical axis.

IV. Discussion

A. Fish detection with eDNA metabarcoding

eDNA metabarcoding analysis of samples collected along the CAP canal detected sequences matching to reference sequences for 25 unique species of fish. Among these were sequences matching to 15 of the 23 fish species that have been detected in the CAP canal since monitoring was initiated in 1986. Of the remaining 8 species that have been captured in the CAP canal and were not detected by eDNA, none were captured during traditional surveys performed contemporaneously with eDNA sample collection. Two of these species, Sonora sucker (*Catostomus insignis*) and inland silverside (*Menidia beryllina*), were captured from the Salt-Gila Pumping Plant during the 2020 survey year. These fish were captured in October 2019, prior to the initiation of the eDNA sampling effort. It is important to note that reference sequences are not currently available for Sonora sucker (or for desert sucker). However, none of the recovered ASVs showed similarity to reference sequences available for closely related congener species, as would be expected if they had derived from either sucker.

eDNA metabarcoding sequences were also found to match to an additional 10 species that have not previously been described from the CAP canal. The five freshwater species detected, included gizzard shad, blue catfish, blue tilapia, Nile tilapia, and shining catfish. In the case of blue tilapia DNA was detected from only two sites, MARK_PP [CM 0.0], which was sampled within Lake Havasu, and MWBH_001 [CM 6.9], which was canal sampling site most proximate to Lake Havasu. It is likely that the sequences detected at MWBH_001 [CM 6.9] originated not from fish within the CAP canal itself, but from fish residing in Lake Havasu upstream of the CAP canal. Gizzard shad have not previously been reported from the CAP canal but were captured from the Gila River and from the Florence-Casa Grande canal during traditional surveys in the 2020 season. The origins of DNA matching other freshwater species not previously detected in the CAP canal (Nile tilapia, blue catfish, and shining catfish) are less certain.

Sequences matching to five marine or anadromous species, northern anchovy, Japanese amberjack, Pacific sandlance, Atlantic salmon, and coho salmon, were also detected from the eDNA metabarcoding survey of the canal. As discussed above, the northern anchovy sequences are likely due to widespread use of this fish as bait in the Lake Havasu. The detection of coho salmon could be due to its use as one of the species included in mock communities sequenced in parallel with the field samples. The source of sequences matching to the other three species is more uncertain, although the presence of sequences matching to Japanese amberjack may be related to human consumption in the Phoenix metropolitan area.

B. Sampling sites: pumping plants versus canal

Looking across the canal, detected species abundance from eDNA metabarcoding tended to be as high or higher in samples from pumping plant forebays as compared to samples from along the canal. Sequences matching to each of 21 freshwater species were detected from at least one of the pumping plant sampling sites. Taken together, these results support that focusing eDNA metabarcoding sampling on pumping plant forebays appears to be the most worthwhile expenditure of survey effort. Whether elevated detections from forebays was a result of these sites representing preferred habitat for fish or because hydrodynamic conditions at these sites contribute to an accumulation of eDNA could not be distinguished in this study.

C. Seasonality and flow rates

Six pumping plant forebays (BOUSE_PP [CM 25], LHARQ_PP [CM 58.7], HASSA_PP [CM 120.5], BRADY_PP [CM 253.8], REDROCK_PP [CM 276.6], and SANX_PP [CM 318.4]) were sampled for eDNA metabarcoding during both the CAP canal-wide survey in February 2021 and in conjunction with traditional surveys. Traditional surveys with paired eDNA sampling were conducted in November 2020 for BRADY_PP [CM 253.8], REDROCK_PP [CM 276.6], and SANX_PP [CM 318.4], and in July 2021 for BOUSE_PP [CM 25], LHARQ_PP [CM 58.7], and HASSA_PP [CM 120.5]. Data from these sites provide the opportunity to evaluate the impacts of seasonality and/or flow regime, as the CAP canal-wide survey was conducted during normal flow regimes, while traditional surveys (and eDNA sampling) were conducted during flow outages (i.e. zero or low flow).

Comparing datasets, no clear pattern emerged with respect to flow regime or sampling season (Figure 30). With regards to flow regime, three sites had higher numbers of species detections from collections during normal flow regimes in the CAP canal-wide survey, as compared with under flow outage conditions during the traditional surveys. Two sites had a lower number of species detections during normal flow regimes, and one site had an equal number of detections from the normal flow regime and the flow outage samples. Although seasonality is not directly comparable, given that half the traditional survey samples were collected in November, and the other half in July, there was likewise no discernible pattern in the number of species detected (Figure 30). A more focused sampling effort would be required to determine whether the timing of sampling, with respect to either flow regime or seasonality, has an impact on species detection.

As mentioned previously, CAP water typically flows into Lake Pleasant in fall and winter, and is released from the reservoir in spring and summer. During the canal-wide collections and November traditional sampling (which included pumping plant forebays downstream of Lake Pleasant only), CAP water was being pumped into Lake Pleasant. It is unknown if species detections via eDNA in the CAP would be different when Lake Pleasant is releasing water. As

such, additional eDNA samples were collected during August/September 2022 in Lake Pleasant, the Waddell Canal, and in the CAP upstream and downstream of the Waddell Canal turnout when flows were exiting Lake Pleasant. These samples are currently being analyzed and comparison results will be reported in a separate document

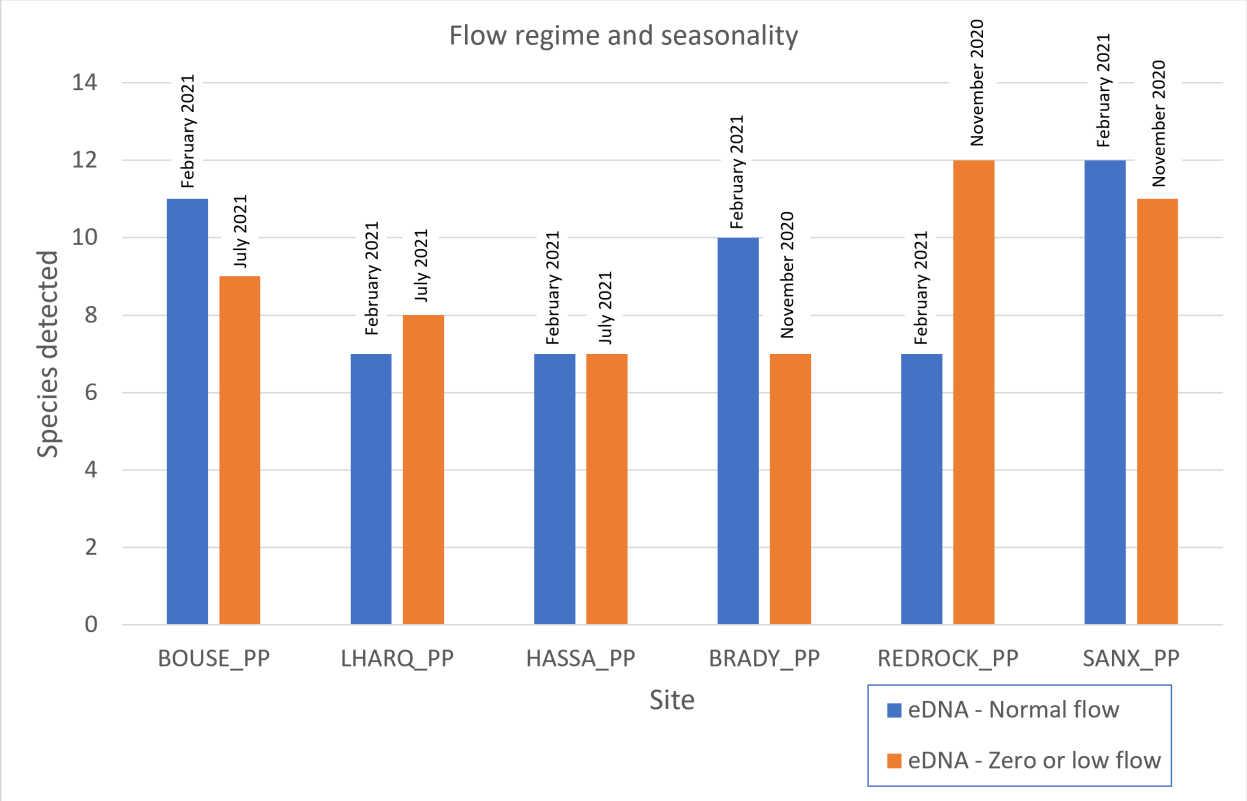

Figure 30: Comparisons of eDNA metabarcoding species detections at pumping plant forebays sampled during normal flow conditions in February 2021 (blue bars) and during flow outage conditions in conjunction with traditional surveys (orange bars). The timing of the traditional surveys is listed above the data bars.

D. eDNA metabarcoding versus traditional surveys

Comparing species detections from eDNA metabarcoding with captures from traditional surveys, where the two sampling methods were conducted in conjunction, eDNA metabarcoding consistently identified the presence of more species across all six sampling sites (Figure 31). As discussed above, all species captured in traditional surveys were also detected by eDNA metabarcoding, with the exception of largemouth bass, which was captured at LHARQ_PP [CM 58.7] and BRADY_PP [CM 253.8], but not detected in eDNA data from these sites. A bioinformatic analysis of the MiFish primers and reference sequences revealed a single nucleotide mismatch between the MiFish_U_F primer and available largemouth bass sequences. It is possible that this created a competitive disadvantage in PCR reactions, such that amplification of other sequences was favored over that of largemouth bass. This should be

evaluated further to determine if changes to primers would improve representative sequence recovery and detection.

Comparing historical survey data, more species have been detected cumulatively by traditional surveys between survey years 1995 and 2020 than were detected by eDNA barcoding in, at all sites except REDROCK_PP [CM 276.6] (Figure 31. It should be noted that some species had not been captured by traditional surveys in more than a decade and may no longer be present in the CAP canal (Appendices 1, 4-9). While eDNA did not detect some species that have been captured in recent surveys, such as the inland surveys, it did detect some species that have not been captured in decades. For example, black crappie eDNA appeared to be ubiquitous at low levels throughout the CAP canal, but the species has not been captured since initial surveys were conducted in 1986. This suggests that eDNA may be useful or the detection of species not easily captured in traditional surveys due either to their rarity or their behavior. The two turtle species that have been captured in traditional surveys (one in 2020) were not expected to have been detected by eDNA barcoding based on primer specificity, which is targeted to fish. Softshell spiny turtle eDNA was detected from one site, however a more accurate assessment would require primers designed to match the species or taxon.

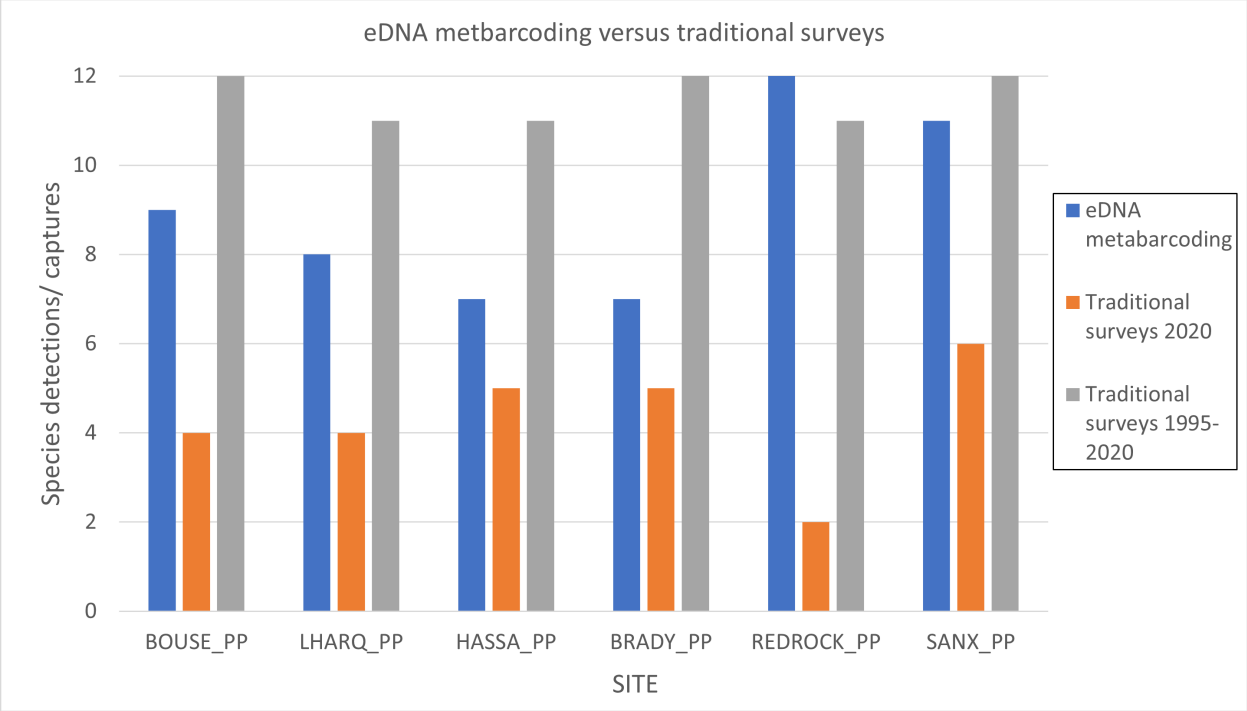

Figure 31: Comparison of the number of species detected by eDNA metabarcoding and from traditional surveys. The number of species detected by eDNA per site are shown in blue. The number of species captured per site by traditional surveys employed in conjunction with eDNA sampling are shown in orange The cumulative number of species captured per site by traditional surveys conducted between sampling years 1995 and 2020 are shown in grey. Sampling at HASSA_PP [CM 120.5] was conducted in survey years 1997 through 2020.

Comparison between the number of individuals captured from a forebay and the eDNA metabarcoding read frequency for eDNA sequences matching that species showed and positive relationship, although the relationship was relatively weak, with a linear trendline for the data having an R-squared value of 0.4532 (Figure 32). Although the data in the current study are

limited, they are suggestive that read frequencies may be correlative with species abundance or total biomass. Such correlations have been demonstrated for fish in other systems (Rouke et al., 2022), and further validation in the CAP system could extend the utility of eDNA studies.

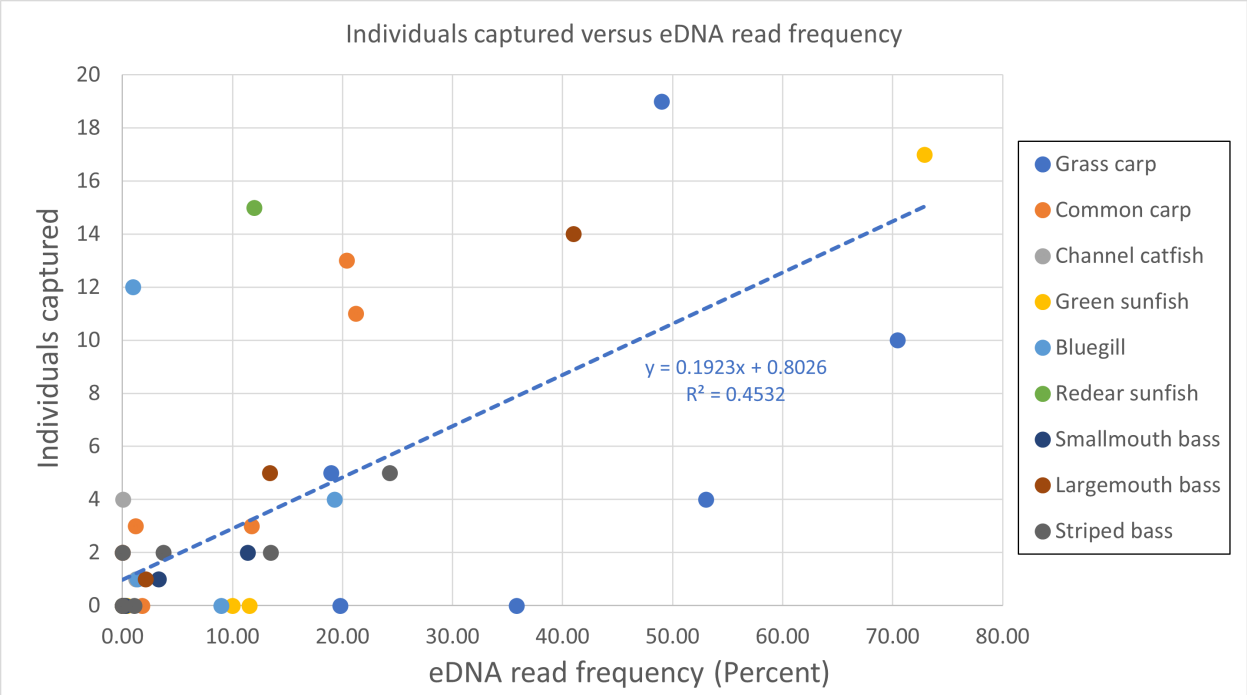

Figure 32: Dot plot of the number of individuals captured versus the eDNA read frequency for sequences matching the species from sampling at pumping plant forebays. Each dot represents a paired data for agiven species from one forebay. Juvenile Lepomis sp. individuals caught at BOUSE_PP [CM 25] were paired with eDNA sequence data from green sunfish for this comparison (as discussed above).A linear trendline for the data is shown as a blue dashed line. Species that were identified from eDNA data but that were not camptured at any forebay were excluded from the chart.

E. Final considerations

The current study has demonstrated the potential value of eDNA metabarcoding as a tool for detecting the presence of non-native fish in the CAP canal. In total, DNA sequences matching to 21 freshwater fish species and 5 marine or anadromous species were detected from eDNA metabarcoding data. This included two-thirds of species captured from the CAP canal since traditional surveys were initiated 37 years ago (Appendix 1 and Appendix 3). In addition, in paired sampling events, eDNA metabarcoding identified nearly all the fish captured at each sampling site (with the exception of largemouth bass at two sites, as discussed above). eDNA metabarcoding also detected more species than were captured than traditional methods at each sampling location. Although the read frequency from eDNA metabarcoding does not directly correlate to the number of fish captured from traditional measures, the overall appearance of comparable trends in the two datasets suggest that further investigation might yield a quantifiable and reliable metric of abundance from eDNA metabarcoding data. These results, together with the fact that eDNA metabarcoding sampling collection is much faster and simpler that traditional sampling, suggest that eDNA metabarcoding has the potential to serve as a valuable complement to traditional sampling approaches for accessing the presence of non-native fish in the CAP canal, and in connected and proximate waterways.

Cost is often a consideration of ecological surveys, and cost-efficiency have frequently been discussed as an advantage in the adoption of eDNA based approaches. eDNA sample collection can generally be conducted with fewer personnel and in less time as compared with additional surveys. Although equipment costs for laboratory processing of samples are significant, these are generally factored into the capital costs of the facility and amortized across multiple projects and/or an extended timeframe. For the current project, Reclamation's Phoenix Area Office provided a budget of $84,604.14 to the Technical Service Center's Ecological Research Laboratory for sampling kits, sample processing, DNA sequencing, and data analysis. Exclusive of labor for sample collection, this equates to approximately $950 per site. Budgeting for the current project benefited from the large number of samples analyzed, as economies of scale for DNA sequencing significantly reduced the per sample cost of DNA sequencing. For smaller scale projects it is anticipated that a cost of less than $1,500 per site (including three replicate field samples, a field blank, and appropriate laboratory controls) can be realized.

Based on the results of this study, one strategy to use eDNA most efficiently would be to sample at, above, and below pump plants. The number of species detected was highest at pumping plants and surrounding sites, and all freshwater species were detected from at least one pumping plant.

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Appendix 1

Table of fish and turtle species detected in traditional surveys of the CAP canal

Scientific name	Common name	First capture	Most recent capture	eDNA surveys*	Ecology
Ameiurus melas	Black bullhead	1995	2007	No	Freshwater - non-native
Ameiurus natalis	Yellow bullhead	1988	2004	No	Freshwater - non-native
Apalone spinifera	Spiny softshell turtle	2002	2002	Yes§	Freshwater - non-native
Carassius auratus	Goldfish	1988	2004	No	Freshwater - non-native
Catostomus clarkii	Desert sucker	1988	1988	No^	Freshwater - native
Catostomus insignis	Sonora sucker	1988	2020	No^	Freshwater - native
Colossoma sp.	Pacu	2006	2006	No	Freshwater - non-native
Ctenopharyngodon idella	Grass carp	1995	2020	Yes	Freshwater - non-native
Cyprinella lutrensis	Red shiner	1995	2015	Yes	Freshwater - non-native
Cyprinus carpio	Common carp	1986	2020	Yes	Freshwater - non-native
Dorosoma petenense	Threadfin shad	1986	2020	Yes	Freshwater - non-native
Gambusia affinis	Western mosquitofish	1988	2020	Yes	Freshwater - non-native
Ictalurus punctatus	Channel catfish	1986	2020	Yes	Freshwater - non-native
Lepomis cyanellus	Green sunfish	1986	2015	Yes	Freshwater - non-native
Lepomis macrochirus	Bluegill	1986	2020	Yes	Freshwater - non-native
Lepomis microlophus	Redear sunfish	1986	2020	Yes	Freshwater - non-native
Lepomis sp.	Sunfish, undetermined or hybrid	1995	2020	No+	Freshwater - non-native
Menidia beryllina	Inland silverside	2020	2020	No	Freshwater - non-native
Micropterus dolomieu	Smallmouth bass	2004	2020	Yes	Freshwater - non-native
Micropterus salmoides	Largemouth Bass	1986	2020	Yes	Freshwater - non-native
Morone chrysops	White bass	1995	1995	Yes	Freshwater - non-native

Morone saxatilis	Striped bass	1986	2020	Yes	Freshwater - non-native
Pomoxis nigromaculatus	Black crappie	1986	1986	Yes	Freshwater - non-native
Pylodictis olivaris	Flathead Catfish	1988	2004	Yes	Freshwater - non-native
Trachemys scripta	Red-eared slider	2020	2020	No[§]	Freshwater - non-native
Xyrauchen texanus	Razorback sucker	1986	1986	No	Freshwater - native

* Denotes whether the species was detected from eDNA metabarcoding data

[§] Turtle species. Detection from eDNA metabarcoding data was not predicted based on primer specificity to fish. *Apalone spinifera* sequence was detected from one site.

^ Reference DNA sequences were not available for Desert sucker or Sonora sucker

[+] *Lepomis* sp. Was not included as a category in the eDNA reference library. All *Lepomis* eDNA sequences were matched to species. See discussion of green sunfish in text.

Appendix 2

Table of site information for canal-wide eDNA metabarcoding survey

Site	Latitude	Longitude	Mileage *	Site type	Reads §
MARK_PP	34.29227	-114.106	0.0	Pumping plant forebay	662094
MWBH_001	34.18886	-114.063	6.9	Canal	661738
MWBH_002	34.1264	-114.025	11.9	Canal	479275
MWBH_003	34.06302	-113.987	16.9	Canal	720106
MWBH_004	34.00507	-113.933	22.0	Canal	567617
BOUSE_PP	33.96279	-113.921	25.0	Pumping plant forebay	468085
BHLH_001	33.95954	-113.919	25.2	Canal	701057
BHLH_002	33.92037	-113.848	30.5	Canal	426021
BHLH_003	33.85995	-113.832	36.3	Canal	463271
BHLH_004	33.79257	-113.805	41.2	Canal	382002
BHLH_005	33.73131	-113.761	46.3	Canal	313668
BHLH_006	33.6742	-113.716	51.2	Canal	391158
BHLH_007	33.62234	-113.662	56.2	Canal	328687
LHARQ_PP	33.59955	-113.638	58.7	Pumping plant forebay	299233
LHHA_001	33.60069	-113.633	58.8	Canal	411352
LHHA_002	33.60563	-113.551	63.8	Canal	253798
LHHA_003	33.56696	-113.482	68.8	Canal	170841
LHHA_004	33.57516	-113.402	73.7	Canal	207067
LHHA_005	33.58376	-113.386	74.8	Canal	137359
LHHA_006	33.58375	-113.302	79.7	Canal	190933
LHHA_007	33.58556	-113.216	84.7	Canal	476901
LHHA_008	33.57356	-113.134	89.7	Canal	322236
LHHA_009	33.54681	-113.097	92.8	Canal	259631
LHHA_010	33.54083	-113.089	93.4	Canal	306087
LHHA_011	33.55476	-113.015	98.4	Canal	319451
LHHA_012	33.58324	-112.936	103.4	Canal	460668
LHHA_013	33.597	-112.857	108.4	Canal	236907
LHHA_014	33.61092	-112.775	113.9	Canal	237498
LHHA_015	33.64448	-112.711	118.5	Canal	248298
HASSA_PP	33.66732	-112.691	120.5	Pumping plant forebay	408971

HASG_001	33.66834	-112.686	120.9	Canal	441032
HASG_002	33.68883	-112.606	125.8	Canal	531529
HASG_003	33.7244	-112.54	130.8	Canal	548486
HASG_004	33.74981	-112.462	135.8	Canal	226218
HASG_005	33.76624	-112.379	140.8	Canal	496506
HASG_006	33.7798	-112.301	145.9	Canal	325545
HASG_007	33.78045	-112.252	148.7	Canal	334840
HASG_008	33.77045	-112.221	150.7	Canal	407810
HASG_009	33.73791	-112.155	155.3	Canal	235938
HASG_010	33.71767	-112.086	160.7	Canal	392835
HASG_011	33.67771	-112.021	165.6	Canal	134923
HASG_012	33.64411	-111.947	170.6	Canal	380611
HASG_013	33.62	-111.865	175.7	Canal	534031
HASG_014	33.57365	-111.808	180.6	Canal	479806
HASG_015	33.54615	-111.734	185.7	Canal	428516
HASG_016	33.52981	-111.705	188.3	Canal	428817
SALTGILA_PP	33.50519	-111.684	190.6	Pumping plant forebay	474983
SGBR_001	33.50031	-111.679	190.9	Canal	521577
SGBR_002	33.43898	-111.657	195.7	Canal	770784
SGBR_003	33.39565	-111.59	200.8	Canal	398787
SGBR_004	33.34012	-111.537	205.8	Canal	661980
SGBR_005	33.27129	-111.512	210.8	Canal	336413
SGBR_006	33.20248	-111.493	215.8	Canal	405054
SGBR_007	33.13684	-111.46	220.8	Canal	166416
SGBR_008	33.08515	-111.41	225.7	Canal	248955
SGBR_009	33.08554	-111.33	230.9	Canal	194181
SGBR_010	33.07444	-111.31	232.3	Canal	337390
SGBR_011	33.02342	-111.366	237.1	Canal	259167
SGBR_012	32.9705	-111.415	242.1	Canal	112122
SGBR_013	32.90221	-111.443	247.2	Canal	451204
SGBR_014	32.85058	-111.437	250.9	Canal	165684
SGBR_015	32.82266	-111.431	252.9	Canal	445088
BRADY_PP	32.81781	-111.419	253.8	Pumping plant forebay	114774
BRPI_001	32.81493	-111.407	254.5	Canal	243512
PICACHO_PP	32.75733	-111.425	259.3	Pumping plant forebay	328539

PIRR_001	32.7462	-111.416	260.2	Canal	183968
PIRR_002	32.68345	-111.408	265.2	Canal	220468
PIRR_003	32.6579	-111.339	270.2	Canal	195765
PIRR_004	32.58794	-111.328	275.2	Canal	406072
REDROCK_PP	32.58788	-111.305	276.6	Pumping plant forebay	321785
RRTP_001	32.58776	-111.274	278.3	Canal	389013
RRTP_002	32.52542	-111.246	283.2	Canal	215681
RRTP_003	32.46586	-111.198	288.3	Canal	260781
RRTP_004	32.40857	-111.168	293.4	Canal	617854
TWINP_PP	32.3829	-111.196	297.5	Pumping plant forebay	732102
TPSA_001	32.37862	-111.193	297.8	Canal	420898
TPSA_002	32.3302	-111.246	302.7	Canal	460174
SANDAR_PP	32.30228	-111.248	305.3	Pumping plant forebay	188720
SABW_001	32.30122	-111.244	305.4	Canal	296174
BRAWLEY_PP	32.25538	-111.231	309.2	Pumping plant forebay	262447
BWSX_001	32.24308	-111.21	310.6	Canal	259873
BWSX_002	32.17937	-111.172	315.5	Canal	168992
SANX_PP	32.16236	-111.133	318.4	Pumping plant forebay	230667

* Canal miles from the intake at MARK_PP [CM 0.0] in Lake Havasu

§ Total number of reads matched to a fish species

Appendix 3

Table of species identified from eDNA metabarcoding data

Scientific name	Common name	Traditional surveys*	Ecology	eDNA sites§
Ammodytes personatus	Pacific sandlance	No	Marine - non-native	1
Ctenopharyngodon idella	Grass carp	Yes	Freshwater - non-native	83
Cyprinella lutrensis	Red shiner	Yes	Freshwater - non-native	4
Cyprinus carpio	Common carp	Yes	Freshwater - non-native	83
Dorosoma cepedianum	Gizzard shad	No	Freshwater - non-native	10
Dorosoma petenense	Threadfin shad	Yes	Freshwater - non-native	6
Engraulis mordax	Northern anchovy	No	Marine - non-native	4
Gambusia affinis	Western mosquitofish	Yes	Freshwater - non-native	32
Ictalurus furcatus	Blue catfish	No	Freshwater - non-native	4
Ictalurus punctatus	Channel catfish	Yes	Freshwater - non-native	71
Lepomis cyanellus	Green sunfish	Yes	Freshwater - non-native	23
Lepomis macrochirus	Bluegill	Yes	Freshwater - non-native	23
Lepomis microlophus	Redear sunfish	Yes	Freshwater - non-native	13
Micropterus dolomieu	Smallmouth bass	Yes	Freshwater - non-native	22
Micropterus salmoides	Largemouth Bass	Yes	Freshwater - non-native	15
Morone chrysops	White bass	No	Freshwater - non-native	1
Morone saxatilis	Striped bass	Yes	Freshwater - non-native	78
Oncorhynchus kisutch	Coho salmon	No	Euryhaline - non-native	1
Oreochromis aureus	Blue tilapia	No	Freshwater - non-native	2
Oreochromis niloticus	Nile tilapia	No	Freshwater - non-native	1
Pomoxis nigromaculatus	Black crappie	Yes	Freshwater - non-native	83
Pylodictis olivaris	Flathead Catfish	Yes	Freshwater - non-native	2
Salmo salar	Atlantic salmon	No	Euryhaline - non-native	1
Seriola quinqueradiata	Japanese amberjack	No	Marine - non-native	3
Tachysurus nitidus	Shining catfish	No	Freshwater - non-native	2

* Denotes whether the species has been captured in traditional surveys conducted between 1986 and 2020

§ Number of sites from which reads matched to a fish species were detected

Appendix 4

Table of fish and turtle species detected in traditional surveys of BOUSE_PP [CM 25] for SY1995-2020

Scientific name	Common name	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2015	2020	eDNA
Ameiurus melas	Black bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ameiurus natalis	Yellow bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Carassius auratus	Goldfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Catostomus insignis	Sonora sucker	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Colossoma sp.	Pacu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ctenopharyngodon idella	Grass carp	0	**2**	0	0	0	0	**1**	**2**	**466**	0	0	0	0	0	0	**1**	**1**	0	**Yes**
Cyprinella lutrensis	Red shiner	0	0	0	0	0	0	0	0	**79**	0	0	0	0	0	0	0	0	0	**Yes**
Cyprinus carpio	Common carp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	No
Dorosoma petenense	Threadfin shad	**126**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Gambusia affinis	Mosquitofish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Ictalurus punctatus	Channel catfish	**6**	0	0	0	0	**9**	0	**4**	**52**	0	**2**	**1**	0	**2**	**7**	**6**	**7**	0	**Yes**
Lepomis cyanellus	Green sunfish	0	0	0	0	0	0	0	**9**	0	0	0	0	0	**2**	0	**4**	0	0	**Yes**
Lepomis macrochirus	Bluegill	**1**	**2**	**1**	**15**	**1**	0	**5**	0	0	0	**1**	**4**	0	0	**17**	**1**	0	**1**	**Yes**
Lepomis microlophus	Redear sunfish	**1**	0	0	0	0	0	**1**	0	0	0	0	**1**	0	0	0	**3**	**1**	0	No
Lepomis sp.	Undetermined or hybrid sunfish	0	0	0	**26**	0	0	0	0	0	0	**2**	**3**	0	0	0	**1**	0	**17**	No
Menidia beryllina	Inland Silverside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Micropterus dolomieui	Smallmouth bass	0	0	0	0	0	0	0	0	**2**	0	0	0	0	0	**1**	0	**1**	0	**Yes**

Micropterus salmoides	Largemouth bass	**1**	**2**	0	0	0	**2**	**4**	**6**	**1**	0	**7**	**6**	0	**1**	**3**	0	0	**1**	**Yes**
Morone chrysops	White bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Morone saxatilis	Striped bass	**17**	**29**	**3**	**6**	**3**	**3**	**5**	**4**	**1273**	0	**10**	**20**	0	**6**	**10**	**11**	**4**	**3**	No
Pylodictis olivaris	Flathead catfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Apalone spinifera	Spiny softshell turtle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Trachemys scripta elegans	Red-eared Slider	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Appendix 5

Table of fish and turtle species detected in traditional surveys of LHARQ_PP [CM 58.7] for SY1995-2020

Scientific name	Common name	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2015	2020	eDNA
Ameiurus melas	Black bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ameiurus natalis	Yellow bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Carassius auratus	Goldfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Catostomus insignis	Sonora sucker	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Colossoma sp.	Pacu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ctenopharyngodon idella	Grass carp	0	**16**	**1**	**3**	**1**	0	0	**6**	**1**	0	**4**	**1**	0	**10**	**4**	**7**	**4**	**10**	**Yes**
Cyprinus carpio	Common carp	**1**	**1**	0	**2**	0	**1**	0	**1**	0	0	**1**	0	0	**1**	**1**	0	0	**3**	**Yes**
Cyprinella lutrensis	Red shiner	0	0	0	**200**	0	**4**	0	0	0	0	0	0	0	0	0	0	0	0	No
Dorosoma petenense	Threadfin shad	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Gambusia affinis	Mosquitofish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Ictalurus punctatus	Channel catfish	**10**	**12**	**3**	**1**	**3**	**2**	0	**1**	0	0	**2**	**1**	0	0	**1**	**1**	**1**	0	**Yes**
Lepomis cyanellus	Green sunfish	0	0	**1**	**5**	**1**	0	0	**3**	0	0	0	0	0	**1**	0	**1**	0	0	**Yes**
Lepomis macrochirus	Bluegill	0	0	**2**	**17**	**2**	0	0	0	**3**	0	0	0	0	0	0	0	0	0	No
Lepomis microlophus	Redear sunfish	0	0	**1**	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Lepomis sp.	Undetermined or hybrid sunfish	0	0	**38**	**2**	**38**	**5**	0	0	0	0	**5**	**3**	0	0	0	0	0	0	No
Menidia beryllina	Inland Silverside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Micropterus dolomieui	Smallmouth bass	0	0	0	0	0	0	0	0	**4**	0	0	**1**	0	0	0	0	**1**	**1**	**Yes**

Micropterus salmoides	Largemouth bass	**7**	0	**1**	0	**1**	0	0	**1**	0	0	**6**	**3**	0	**1**	**1**	0	0	**2**	No
Morone chrysops	White bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Morone saxatilis	Striped bass	**3**	**3**	**2**	0	**2**	0	0	**1**	**1**	0	**1**	0	0	**3**	0	**1**	**2**	0	**Yes**
Pylodictis olivaris	Flathead catfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Apalone spinifera	Spiny softshell turtle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Trachemys scripta elegans	Red-eared Slider	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Appendix 6

Table of fish and turtle species detected in traditional surveys of HASSA_PP [CM 120.5] for SY1997-2020

Scientific name	Common name	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2015	2020	eDNA
Ameiurus melas	Black bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ameiurus natalis	Yellow bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Carassius auratus	Goldfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Catostomus insignis	Sonora sucker	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Colossoma sp.	Pacu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ctenopharyngodon idella	Grass carp	**2**	**1**	**16**	**1**	**7**	**8**	**6**	**5**	0	**30**	**1**	0	**7**	**4**	**9**	0	**19**	**Yes**
Cyprinus carpio	Common carp	**11**	**10**	**4**	**10**	**4**	**5**	**31**	**13**	0	**22**	**24**	0	**10**	**9**	**8**	0	**13**	**Yes**
Cyprinella lutrensis	Red shiner	**2**	0	**5**	0	0	**2**	0	**10**	0	0	0	0	0	0	0	0	0	No
Dorosoma petenense	Threadfin shad	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Gambusia affinis	Mosquitofish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Ictalurus punctatus	Channel catfish	**1**	0	**2**	0	**4**	**3**	**12**	**2**	0	**7**	**9**	0	**6**	**10**	**8**	0	**1**	**Yes**
Lepomis cyanellus	Green sunfish	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Lepomis macrochirus	Bluegill	0	**1**	0	**1**	0	0	0	**2**	0	0	0	0	0	0	0	0	0	No
Lepomis microlophus	Redear sunfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Lepomis sp.	Undetermined or hybrid sunfish	0	0	**2**	0	0	0	**6**	0	0	0	**5**	0	0	0	0	0	0	No
Menidia beryllina	Inland Silverside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Micropterus dolomieui	Smallmouth bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	**2**	**Yes**

Micropterus salmoides	Largemouth bass	**34**	**5**	**5**	**5**	**4**	0	**2**	**2**	0	**7**	**2**	0	**5**	**1**	**1**	0	0	No
Morone chrysops	White bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Morone saxatilis	Striped bass	**6**	**1**	0	**1**	0	0	**1**	**1**	0	**7**	0	0	0	0	**3**	0	**2**	**Yes**
Pylodictis olivaris	Flathead catfish	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	No
Apalone spinifera	Spiny softshell turtle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Trachemys scripta elegans	Red-eared Slider	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Appendix 7

Table of fish and turtle species detected in traditional surveys of BRADY_PP [CM 253.8] for SY1995-2020

Scientific name	Common name	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2015	2020	eDNA
Ameiurus melas	Black bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ameiurus natalis	Yellow bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Carassius auratus	Goldfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Catostomus insignis	Sonora sucker	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Colossoma sp.	Pacu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ctenopharyngodon idella	Grass carp	**2**	0	**1**	**3**	**1**	**1**	**3**	**3**	0	0	0	**3**	**6**	**2**	**5**	0	**2**	**4**	**Yes**
Cyprinus carpio	Common carp	**5**	0	**33**	**38**	**33**	**1**	**3**	**9**	**7**	**3**	**3**	**6**	**11**	**15**	**11**	0	**7**	**3**	**Yes**
Cyprinella lutrensis	Red shiner	**14**	0	**10**	0	**10**	0	0	**5**	0	**1**	**1**	**1**	0	0	0	0	**1**	0	No
Dorosoma petenense	Threadfin shad	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Gambusia affinis	Mosquitofish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Ictalurus punctatus	Channel catfish	0	0	0	0	0	0	0	0	**2**	0	**1**	**1**	**1**	0	0	0	**3**	0	No
Lepomis cyanellus	Green sunfish	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	No
Lepomis macrochirus	Bluegill	**1**	0	0	0	0	0	0	0	**1**	0	**2**	**5**	0	**1**	**1**	0	**2**	**4**	**Yes**
Lepomis microlophus	Redear sunfish	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	No
Lepomis sp.	Undetermined or hybrid sunfish	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Menidia beryllina	Inland Silverside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Micropterus dolomieui	Smallmouth bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**

Micropterus salmoides	Largemouth bass	**6**	**1**	0	0	0	**2**	**4**	**1**	**3**	0	**2**	**4**	**3**	**1**	0	0	**1**	**2**	No
Morone chrysops	White bass	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Morone saxatilis	Striped bass	**5**	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**8**	0	**3**	0	0	**5**	**15**	0	**2**	**1**	**Yes**
Pylodictis olivaris	Flathead catfish	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	No
Apalone spinifera	Spiny softshell turtle	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	No
Trachemys scripta elegans	Red-eared Slider	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Appendix 8

Table of fish and turtle species detected in traditional surveys of REDROCK_PP [CM 276.6] for SY1995-2020

Scientific name	Common name	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2015	2020	eDNA
Ameiurus melas	Black bullhead	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ameiurus natalis	Yellow bullhead	**5**	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Carassius auratus	Goldfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Catostomus insignis	Sonora sucker	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Colossoma sp.	Pacu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ctenopharyngodon idella	Grass carp	0	0	0	0	0	0	**10**	**1**	0	**3**	**5**	0	**1**	**1**	**3**	0	0	0	**Yes**
Cyprinus carpio	Common carp	**5**	0	**3**	0	**3**	0	**1**	0	0	**2**	**3**	**1**	**1**	0	0	0	0	0	**Yes**
Cyprinella lutrensis	Red shiner	**2**	**11**	0	**3**	0	0	0	0	0	0	0	0	0	0	0	0	**15**	0	**Yes**
Dorosoma petenense	Threadfin shad	0	**56**	0	**4**	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	No
Gambusia affinis	Mosquitofish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Ictalurus punctatus	Channel catfish	0	0	**1**	0	**1**	**3**	0	**1**	0	0	0	**2**	0	**1**	0	0	**3**	0	**Yes**
Lepomis cyanellus	Green sunfish	**59**	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Lepomis macrochirus	Bluegill	**21**	**9**	**3**	**15**	**3**	0	**3**	**7**	0	**14**	**9**	0	0	**4**	**1**	0	0	0	**Yes**
Lepomis microlophus	Redear sunfish	**89**	**11**	**19**	**32**	**19**	**7**	**21**	0	0	**11**	**1**	**1**	**3**	**2**	0	0	0	0	**Yes**
Lepomis sp.	Undetermined or hybrid sunfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Menidia beryllina	Inland Silverside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Micropterus dolomieui	Smallmouth bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Micropterus salmoides	Largemouth bass	**26**	**4**	**9**	**11**	**9**	**1**	**4**	**6**	0	**7**	**6**	**3**	**2**	**8**	**10**	0	0	**5**	**Yes**
Morone chrysops	White bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Morone saxatilis	Striped bass	0	**1**	**1**	0	**1**	0	**6**	**11**	0	**4**	**6**	**1**	0	**2**	**3**	0	**2**	**5**	**Yes**
Pylodictis olivaris	Flathead catfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Apalone spinifera	Spiny softshell turtle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Trachemys scripta elegans	Red-eared Slider	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Appendix 9

Table of fish and turtle species detected in traditional surveys of SANX_PP [CM 318.4] for SY1995-2020

Scientific name	Common name	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2015	2020	eDNA
Ameiurus melas	Black bullhead	**16**	**1**	0	**4**	0	0	**16**	**8**	**7**	**7**	0	**1**	0	0	0	0	0	0	No
Ameiurus natalis	Yellow bullhead	0	0	0	0	0	0	0	0	**2**	0	0	0	0	0	0	0	0	0	No
Carassius auratus	Goldfish	**6**	0	**1**	**2**	**1**	**3**	**4**	0	**2**	0	0	0	0	0	0	0	0	0	No
Catostomus insignis	Sonora sucker	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Colossoma sp.	Pacu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Ctenopharyngodon idella	Grass carp	**14**	0	**6**	**3**	**6**	**2**	0	**2**	**2**	**9**	**3**	**7**	**1**	**8**	0	0	**2**	**5**	**Yes**
Cyprinus carpio	Common carp	0	0	0	0	0	0	0	0	0	0	0	**3**	**13**	0	0	0	**15**	**11**	**Yes**
Cyprinella lutrensis	Red shiner	**10**	0	0	0	0	0	0	**1**	**28**	0	0	0	0	0	0	0	**5**	0	No
Dorosoma petenense	Threadfin shad	0	0	**1**	**7**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Gambusia affinis	Mosquitofish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**Yes**
Ictalurus punctatus	Channel catfish	0	0	0	0	0	0	**1**	**4**	**38**	**4**	**14**	**26**	**2**	**5**	**1**	0	**4**	**4**	**Yes**
Lepomis cyanellus	Green sunfish	**327**	**41**	0	0	0	**5**	**8**	**12**	0	0	0	0	0	0	0	0	0	0	**Yes**
Lepomis macrochirus	Bluegill	**318**	**367**	**243**	**3**	**243**	**25**	**20**	**95**	**19**	**13**	**12**	**48**	**1**	**5**	**4**	0	**3**	**12**	**Yes**
Lepomis microlophus	Redear sunfish	**11**	**28**	**1**	0	**1**	**28**	**58**	**136**	**28**	**16**	**19**	**204**	**46**	**87**	**17**	0	**3**	**15**	**Yes**
Lepomis sp.	Undetermined or hybrid sunfish	**5**	0	0	**574**	0	**13**	**67**	**18**	**2**	0	**2**	**1**	0	0	0	0	0	0	No
Menidia beryllina	Inland Silverside	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Micropterus dolomieui	Smallmouth bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No

Micropterus salmoides	Largemouth bass	**1**	0	0	0	0	0	**4**	**26**	**10**	**17**	**23**	**44**	**5**	**48**	**3**	0	**26**	**14**	**Yes**
Morone chrysops	White bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Morone saxatilis	Striped bass	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	**Yes**
Pylodictis olivaris	Flathead catfish	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Apalone spinifera	Spiny softshell turtle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No
Trachemys scripta elegans	Red-eared Slider	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	No