Field optimization of eDNA markers as an early detection tool for Grass Carp in the Sandusky River, OH.

Final Report

October 13, 2023

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Summary

Grass Carp represent a significant threat to many freshwater ecosystems where they compete with native species for food, they can significantly alter habitats through their foraging behavior, and they can reduce catches of commercial fish and damage commercial fishing gear. The Sandusky River, a tributary to western Lake Erie in Ohio, has a relatively high abundance of Grass Carp compared to other Great Lakes tributaries. Federal, State, and University biologists, researchers, and managers invest significant resources each year in controlling Grass Carp in the Sandusky River to prevent the spread and establishment of this species. Environmental DNA (eDNA) has been successfully used to monitor aquatic invasive species, including invasive carp, in many freshwater ecosystems and there was interest in exploring the use of eDNA as an additional monitoring tool for Grass Carp in the Great Lakes. The U. S. Fish and Wildlife Service's (USFWS) Whitney Genetics Lab recently developed new quantitative PCR (qPCR) assays for Grass Carp and validated these assays in multiple laboratories. In this study, we conducted a thorough field evaluation of these assays in the Sandusky River with the hope of developing an eDNA occurrence model. This model could then inform sampling plans and future Grass Carp eDNA monitoring efforts. We collected water samples at three sites in the Sandusky River a total of six times in 2019 and 2021. At two of these sites (Wightmans Grove and Brady's Island), we attempted to sample three distinct habitat types: main channel, side channel, and backwater. The third site near Tiffin, OH, had a single habitat type (main channel) and served as a control site where we did not expect to detect Grass Carp eDNA. We were able to detect Grass Carp eDNA during each season and in and in all habitat types. Overall detection rates were low, with an approximately 1.5% positive detection rate out of over 3,000 samples processed. The number of Grass Carp eDNA detections was roughly equal between Wightmans Grove and Brady's Island. Due to the lack of positive detections, eDNA occurrence models did not yield anticipated results. Models showed that the probability of Grass Carp eDNA presence (ψ) was highest in Summer and Fall sampling events. The probability of collecting eDNA when present (θ) was high and did not differ among sites or habitat types. The probability of molecular detection (ρ) with qPCR was essentially 0 across all sites and habitat types. Although Grass Carp were present in the Sandusky River during eDNA sampling events (based on telemetry data), the actual number of fish in the system was unknown and may have been too low for adequate eDNA detections necessary to facilitate model development. Sampling events may have also missed biological events (e.g., spawning) where detections could have been higher. This study demonstrated that these markers can be used to detect Grass Carp in the field; however, we do not have enough data to determine the optimal sampling scheme for a long-term eDNA monitoring program for Grass Carp. Future efforts may need to focus on areas where Grass Carp are more abundant in order to achieve higher detection probabilities. In the future, we hope to explore alternate eDNA collection strategies to determine if we can achieve higher detection probabilities.

Introduction

Grass Carp *Ctenopharyngodon idella* are a freshwater fish native to large rivers of eastern Asia, and one of four invasive carp species (including Bighead Carp *Hypophthalmichthys nobilis*, Black Carp *Mylopharyngodon piceus*, and Silver Carp *H. molitrix*) threatening the ecology of the Laurentian Great Lakes (Conover et al. 2007; Hayder 2019). Grass Carp were first imported to the U.S. in 1963 to evaluate their use as a potential biological control agent of nuisance aquatic vegetation in aquaculture facilities and farm ponds (Avault 1965; Mitchell and Kelly 2006). By the early 1970s, the potential for several commercial and trade applications (e.g., live food fish product; Guillory and Gasaway 1978) resulted in millions of fry being stocked nationally by private and public entities (Mitchell and Kelly 2006), despite concerns that Grass Carp would breed in the wild and become a nuisance (Cross 1969). Consequently, many diploid (i.e., fertile) Grass Carp have been introduced into the Missouri and Mississippi river basins through accidental or deliberate unauthorized release, leading to their establishment throughout much of North America (Pflieger 1978; Conover et al. 2007; Nico et al. 2020). Despite efforts to prevent further introductions and control the expansion of feral populations, Grass Carp have been introduced or captured in all the Great Lakes except Lake Superior (Chapman et al. 2021); however, managers do not consider these populations to be established (Cudmore et al. 2017).

Grass Carp have broad physiological tolerances (Guillory and Gasaway 1978) and advantageous life history traits (e.g., high fecundity, rapid growth, and long-life span; Shireman and Smith 1983) that have helped facilitate invasion success. High densities of Grass Carp can drastically alter aquatic habitats through feeding habits that remove macrophytes and reduce production potential (e.g., digging into banks and uprooting riparian vegetation; van der Lee et al. 2017), which in turn may result in increased alkalinity and turbidity, nutrient enrichment, and reduced dissolved oxygen (Lembi et al. 1978). Removal of macrophytes can also influence the abundance, diversity, and structure of invertebrate (e.g., plankton and benthic macroinvertebrates) and vertebrate (e.g., fish, mammals, and waterfowl) animal communities that rely on these plants for reproduction, refuge, and forage habitat (Chilton and Muoneke 1992; Cudmore et al. 2017). The presence of Grass Carp also has the potential to disrupt economic activities in the Great Lakes region and impose billions of dollars in costs over the coming decades if populations become established (Hayder 2019). For example, Grass Carp could negatively affect the commercial and recreational fishing industry by indirectly reducing landings of native fish species (e.g., Walleye *Sander vitreus* and Yellow Perch *Perca flavescens*), which increases operational costs and decreases revenue (Hayder 2014).

Historically, Grass Carp reported in Lake Erie were mostly bycatch in seines or trap nets during commercial fishing operations (Chapman et al. 2013). Other captures came from recreational angling and bowfishing, bycatch during agency surveys, and small-scale targeted agency Grass Carp sampling (Ohio DNR 2019). More recently, frequent captures of diploid Grass Carp, coupled with successful reproduction in the Sandusky and Maumee rivers of Ohio and suitable spawning conditions in other tributaries of concern, has prompted natural resource managers and researchers to work collaboratively through a structured decision-making process to develop an adaptive response strategy to protect the Great Lakes from Grass Carp (Embke et al. 2016; LEC 2018; Herbst et al. 2021; Robinson et al. 2021). The goal of this plan is to prevent Grass Carp from attaining densities capable of adversely affecting vegetated habitats, ecosystem functions, and associated fish communities in Lake Erie (LEC 2018). Progress towards this goal depends on results related to three objectives: 1) Improve the collective understanding of Grass

Carp population dynamics, behavior, and impacts in Lake Erie to inform effective management actions; 2) Implement control to minimize expansion of Grass Carp in Lake Erie; and 3) Minimize the likelihood of introduction and establishment of new breeding populations of Grass Carp in the tributaries and nearshore areas of Lake Erie and Lake St. Clair (LEC 2018). Successful suppression of the Grass Carp population in Lake Erie requires a comprehensive sampling approach that utilizes a variety of traditional and novel gears (e.g., boat electrofishing and trammel nets; Briggs et al. 2019; Fischer et al. 2022; Young et al. 2023) techniques (e.g., hydrological barriers; LEC 2018) for early detection, monitoring, and control of all life stages.

Grass Carp have been routinely observed and captured in the Sandusky River, which is believed to be the preferred habitat for Grass Carp in Western Lake Erie. Telemetry data suggest that fish reside in the Sandusky River throughout the year (Harris et al. 2021). Although Grass Carp abundance in the Sandusky River is estimated to be low (i.e., less than 200 fish at any time; Gouveia et al. 2023), capture numbers are consistently higher in the system than any other Lake Erie tributary. Information gathered during intensive monitoring (e.g., egg and larval sampling; Kocovsky et al. 2012) and control efforts (e.g., adult removals; Herbst et al. 2021) makes the Sandusky River a suitable location to refine and develop new tools to enhance Grass Carp surveillance. A recent study found that management of Grass Carp in the Sandusky River is crucial for effective control of the species in the Lake Erie Basin (Robinson et al. 2021).

Over the last decade, environmental DNA (eDNA) has become an increasingly popular tool for monitoring aquatic invasive species (AIS) (Rees et al. 2014b; Beng and Corlett 2020; NISC 2022), offering advantages that traditional sampling gear does not in several instances. This could include increased sensitivity and detection probabilities (Hinlo et al. 2018; George et al. 2021) the ability to efficiently sample across large landscapes (Rees et al. 2014a; Erickson et al. 2019; Mize et al. 2019), and the ability to efficiently survey for multiple invasive species simultaneously (Klymus et al. 2017). However, unlike traditional sampling approaches, eDNA does not provide organisms in hand, thus preventing collection of specific data, such as age structure, population size, sex ratios, etc. Because of this, eDNA is best used in conjunction with traditional sampling approaches in order to gain a complete picture of AIS invasions. Like all monitoring techniques, eDNA has an inherent level of uncertainty associated with it, thus managers must decide how much uncertainty is acceptable when implementing eDNA as a monitoring tool (Darling et al. 2021).

As the popularity of eDNA monitoring tools has increased, practitioners have come to recognize the need for robust validation procedures for eDNA markers in order to ensure high quality data collection (Thalinger et al. 2021). Thalinger et al. (2021) proposed a 5-step scale for validating eDNA markers and advocated that when eDNA markers are used for population monitoring, statistical models should be used to estimate detection probabilities and properly interpret detections vs. non-detections. Recently eDNA occupancy or occurrence models have become a popular tool for estimating the probability of detecting eDNA given different sampling conditions (time of year, habitat type, number of samples collected, etc.) (Akre et al. 2019; Erickson et al. 2019; Mize et al. 2019; Sutter and Kinziger 2019). These models help determine the probability that a species' eDNA is present when the probability of detection is less than 1.0. The framework for these models is similar to traditional occupancy models used to determine the probability a species is present in a given habitat (MacKenzie et al. 2002; Mackenzie and Royle 2005). However, eDNA occurrence models use a three-step hierarchy that includes the probability a species' eDNA is present at a sample site (ψ), the probability eDNA from the species can be collected (θ) (e.g., via filtering or water grab), and the probability that eDNA from

the species can be detected in the laboratory (ρ) (Erickson et al. 2019). It is important to note that when applied to eDNA detections, these models help estimate the probability a species' eDNA is present at a site and not the probability the species itself is present. This same framework has been previously used to develop detection probabilities and eDNA sampling regimes for Bighead and Silver carps in the Upper Mississippi River (Mize et al. 2019).

The Lake Erie Grass Carp working group was interested in utilizing eDNA as an additional monitoring tool. Recently, the Whitney Genetics Lab developed new eDNA markers for Grass Carp and conducted a round-robin laboratory validation and preliminary field trials of these markers. Prior to implementing an eDNA monitoring program for Grass Carp using these new markers, it was necessary to determine which habitat types to target for sampling, what time of year to collect samples, and how many samples to collect. In order to answer these questions, USFWS worked with partners from Ohio Department of Natural Resources (DNR), Michigan DNR, Michigan State University, and U.S. Geological Survey to design an eDNA occurrence model study for Grass Carp in the Sandusky River system. The theory was that information gained from the Sandusky River on Grass Carp eDNA occupancy could be used to inform monitoring efforts for the species across the Great Lakes and design a basin-wide eDNA monitoring program. Additionally, there was interest in using this data to refine USFWS monitoring programs for Bighead and Silver carps in the Great Lakes Basin given that existing monitoring efforts are based largely on data from the Upper Mississippi River Basin (Mize et al. 2019). Our specific objectives were:

1. Determine the detection probability of Grass Carp eDNA in the Sandusky River over different spatial and temporal scales.
2. Determine the limitations of Grass Carp eDNA detection in the Sandusky River.
3. Use data from Grass Carp in the Sandusky River to help refine eDNA monitoring efforts for Silver and Bighead carp in other Great Lakes tributaries.

Methods

Field Site Description

This study was modeled after a similar effort conducted by Mize et al. (2019) which examined habitats and timing of Bighead and Silver carp eDNA occupancy in the Upper Mississippi River (UMR). We examined two Sandusky River locations in the vicinity of Fremont, OH that had multiple habitat types (e.g., main channel, side channel, backwater) in close proximity to each other (Figure 1). Grass Carp had either been captured in these areas in the past or Grass Carp implanted with acoustic transmitters had been detected in these areas. One site at river kilometer 25 was near previously documented Grass Carp spawning sites in the Brady's Island area in Fremont, OH, downstream of the former Ballville Dam (Embke et al. 2016; Figure 1). A second site was downstream at river kilometer 7 in an area where Grass Carp were captured by commercial seine in the past near Wightmans Grove (Travis Brendan, Michigan State University, *personal communication*; Figure 1). There were multiple acoustic receivers in the vicinity of both locations that could detect implanted Grass Carp and previous years' telemetry data showed that Grass Carp utilize habitats at both locations (Harris et al. 2021). A control site was incorporated upstream of a low head dam in Tiffin, OH at river kilometer 70, where no or few Grass Carp were expected due the presence of the potential barrier (Figure 1). Only one habitat type, main channel, was present at the Tiffin control site.

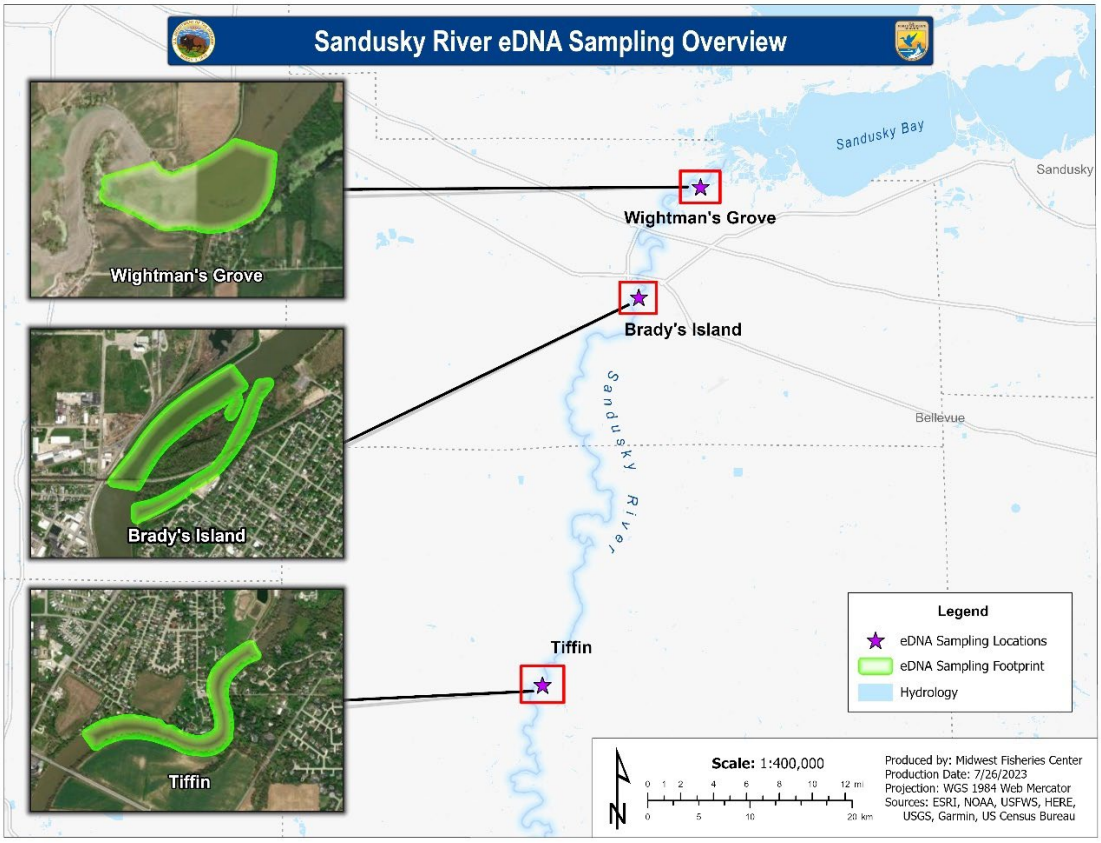

Figure 1. Study area, the Sandusky River in OH. eDNA sampling for this study was conducted at three sites: Wightmans Grove, Brady's Island, and a control site near the town of Tiffin.

We collected eDNA samples at four time periods over the course of this study: spring, summer, late summer, and late fall/early winter. The spring sampling event occurred in early March based on first reported commercial catch during this time period as well as telemetry data indicating greater movement by tagged fish during spring and summer months (Harris et al. 2021). The summer sampling event occurred in June targeting a late-May through mid-July period where spawning had previously been established near Fremont, OH (Embke et al. 2016). An additional sampling period in the late summer, September, was included to examine eDNA detections when previous telemetry data indicated that Grass Carp are moving more freely through the Sandusky River and are congregating less frequently in any particular area from August through October. The late fall sampling event occurred in late November based on telemetry data suggesting fish in the Sandusky River congregate in the area near Wightmans Grove (river kilometer 7) during fall and winter months.

Field efforts were coordinated with other researchers to minimize the presence of Grass Carp eDNA from non-fish sources (e.g., work boats, gill nets, etc.). We coordinated with other agencies to avoid the study areas 1 km upstream for one week prior to sampling and during sample collection. Communication was maintained to alert others at least four weeks in advance to when eDNA sampling would take place and once sample collection was finished.

Field Methods

Eighty samples, where one sample consisted of five, 50 ml conical tubes filled with surface water, were collected at each habitat type (main channel, side channel, backwater) for

Wightmans Grove and Brady's Island in Spring, Summer, Fall and Late Fall. Samples were only collected at one habitat type, main channel, at the Tiffin site, because that was the only habitat type present. All sampling and processing methods followed the USFWS Quality Assurance Project Plan (QAPP) for eDNA Monitoring of Bighead and Silver carps (USFWS 2023). Sampling sites within each habitat type were predetermined based on the area of the site and targeted number of samples for each site (Appendix I-III). An additional eight field blanks, consisting of one 50 ml conical tube of deionized water, were randomly incorporated with the 80 samples to provide field controls. Field blanks were opened and exposed to the open air at the sampling location, then re-sealed and treated like a regular sample. A total of 88 samples and controls were collected per habitat (i.e., main channel, side channel, backwater). Water depth and temperature were recorded for each eDNA sample collected.

Following 2021 water sampling at the habitat, a Vemco VR100 receiver was used to listen for Grass Carp implanted with acoustic tags. The receiver was used when the boat was stationary to listen for Grass Carp acoustic tag frequencies for 10 minutes at each of three locations across the sampling area for a total of 30 minutes listening per habitat type. In 2021 only, Water quality including water temperature (°C), dissolved oxygen (mg/l), dissolved oxygen (%), conductivity (µS/cm), pH, and turbidity (NTU) was measured at the three locations for each habitat type using a YSI DSS Pro meter.

All samples were transported on ice to a nearby field laboratory (eDNA processing trailer) where the DNA of each sample vial was concentrated via centrifugation. Samples were centrifuged at 4200 to 5000 rpm for 30 minutes in centrifuges set to 4° C. Following centrifugation, water was decanted from all samples, and samples were preserved with 20 ml of 99% isopropanol. Samples were then centrifuged again at 4200 to 5000 rpm for 10 minutes. Field sample collection and field processing of samples was conducted by the Alpena FWCO on location in Ohio. Samples were then transported to the Alpena FWCO office in Alpena, Michigan and shipped at ambient temperatures to the Whitney Genetics Laboratory in Onalaska, Wisconsin.

Laboratory Methods

Samples were processed following laboratory procedures outlined in the USFWS Invasive Carp QAPP (USFWS 2023) adapted for processing Grass Carp eDNA samples. Upon arrival at the laboratory, sample tubes were centrifuged again at 4700 rpm for five minutes. Samples were then stored at ambient temperatures for up to 3 weeks before isopropanol was decanted from each tube and tubes were placed in laminar flow hoods overnight to ensure all isopropanol was evaporated. Each hood also contained one "hood control (HC)" which was simply a 50 ml conical tube with the cap removed. These hood control samples were processed in the same manner as all other samples to monitor for any contamination that may have occurred during sample drying. Once samples were fully dried, they were stored at -80° C until DNA extraction. All of this initial sample processing occurred in a room dedicated to invasive carp eDNA sample processing (i.e., no DNA extraction or qPCR took place in this room).

DNA extractions were conducted in a separate room dedicated to invasive carp eDNA extraction. Samples were typically extracted in batches of 45 at a time. During DNA extraction, a single cotton swab was used to swab the inside bottom of all five 50 ml tubes that corresponded to a single 250 ml water sample. The tip of each swab was then broken off and placed into a Qiagen lyse and spin basket with a 350 µl of GSB buffer and 35 µl of proteinase K. Tubes were then incubated at 60° C for 30 minutes. Following incubation, DNA extractions were completed using a modified version of the IBI gMAX Mini Genomic DNA Kit blood protocol with a final

elution in 200 µl of elution buffer. Extracted DNA was stored at -20° C until quantitative PCR (qPCR). Each extraction batch also included an extraction positive ('EP'; diluted bluegill (*Lepomis macrochirus*) cells pipetted onto a cotton swab), to ensure the extraction process worked, as well as an extraction negative ('EN'; deionized water added onto a cotton swab), to monitor for contamination during the extraction process. EN and EP samples were processed in the same manner as eDNA samples.

qPCR master mix preparation occurred in a separate reagent room. All qPCR reactions were run in 20-µL volumes containing TaqMan Environmental MasterMix 2.0 (ThermoFisher Scientific), 0.5 µM of each primer, 0.125 µM of the probe, and 3 µL of template DNA. Master mix and template DNA were added to BioRad 384 well PCR plates using an Eppendorf EP Motion 5075 automated liquid handling robot. qPCR was run in octet for each sample. Temperature cycling began with an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 66°C for 1 minute. Each qPCR plate also contained two, six-point, five-fold standard curves with 10, 50, 250, 1250, 6250, and 31250 copies/µL of synthetic Gblock DNA (IDT), two PCR negatives ('PCRN'; 3 µL of deionized water), and two PCR positives ('PCRP'; 3 µL of extracted DNA from Grass Carp eyeballs). All qPCR was performed using Bio-Rad CFX384 Touch Real-Time PCR Thermocyclers and analyzed using CFX Manager 3.1 Software. Amplification plots were visually inspected by two separate laboratory personnel. The qPCR multiplex reaction consisted of 3 separate markers: GCTM10, GCTM22, and GCTM 32, all with separate probes (see Appendix IV for primer and probe sequences). Any qPCR reaction that crossed the background fluorescence threshold after 15 cycles was considered positive. A sample was considered positive if any of the eight molecular replicates showed positive amplification for any of the three assays.

Quality Control Analysis

As noted above, several field and lab controls were included in our workflow to monitor for any potential contamination and ensure data quality. After laboratory analysis, we checked all field blanks, HC, EP, EN, PCRP and PCRN samples to ensure results were consistent with expectations for these samples (e.g., no amplification detected for field blanks, positive amplification for PCRP). In the event that field or lab controls failed to meet QA/QC expectations defined in the USFWS QAPP (USFWS 2023), the range of samples associated with each control was discarded from data analysis. For example, if a hood control showed positive amplification, all samples associated with that control were discarded from analysis. All field and lab control samples were omitted from further statistical analysis (e.g., model development).

Occupancy Model Development

We analyzed our eDNA detection data using three-level occupancy models that considered each of three levels of detection: 1) the probability that Grass Carp eDNA is present at sampled sites, (ψ-psi); 2) the probability of eDNA sample capture and extraction, (θ-theta); and finally, 3) the probability of eDNA detection with qPCR using Grass Carp molecular assays, (*p;* Erickson et al. 2019). We first analyzed the influence of sampling month on detection probabilities by running a global model ("Model 1"), where we pooled occupancy models to generate ψ estimates for each site (Brady's Island and Wightmans Grove). This model then estimated θ for each site (Brady's Island and Wightmans Grove) during each month and lastly estimated *p* of each of the three qPCR assays for each site and sampling month. We used the R package occStan (Erickson and Peterman 2021) to fit all occupancy models in this study. This

package relies on Stan (Stan Development Team 2021) and RStan (Stan Development Team 2020) for estimating parameter posterior distributions using Bayesian methods.

Next, we analyzed a second model ("Model 2") where each site was split into the three habitat types: main channel, side channel, and backwater. We chose to exclude data from the control site near Tiffin because it contained only a single sampled habitat type (main channel) and the site was included as a negative control and we did not anticipate detecting Grass Carp eDNA. The probability of eDNA occupying Wightmans Grove and Brady's Island sites during each month, ψ, was independently estimated for each site_month unit (e.g., Wightmans_March). We then estimated the probability of eDNA sample capture, θ, for all habitat types at both sites during each month. The probability of molecular eDNA detection in a sample, *p*, was then estimated for each of the three Grass Carp assays at each habitat type for all site_month combinations. Models were fitted using the occStan R package (Erickson and Peterman 2021) as described above.

Results

Field Results

The study was initiated in mid-late 2019 with plans to be completed in early 2020. The 2019 sampling occurred in Fall (September 24-27) and Late Fall (November 4-7). The Covid-19 pandemic prevented sampling during 2020, and we decided to restart the study in 2021. The 2021 sampling occurred in Spring (March 21-25), Summer (June 21-25), Fall (September 28-October 1), and Late Fall (November 15-18).

A total of 3,450 water samples and field blanks were collected over the course of the study (2019 and 2021). Low water was an issue at the backwater sites, likely due to wind caused seiche effects that are common for Lake Erie tributaries. There was difficulty moving the boat to sample backwater habitats according to the predefined collection grid at Wightmans Grove and Brady's Island. We collected individual samples at one collection location but from different sides of the boat. This strategy allowed us to sample in areas that were relatively inaccessible due to low water. We were completely unable to collect samples due to extremely low water during Spring and Summer 2021 in the Wightmans Grove backwater and in Late Fall 2021 in the Brady's Island backwater.

Water temperature and depth were measured for every sample collected during the study. In 2021 only, a suite of other water quality data was recorded at one location in each sampled habitat for each site. Table 1 provides a summary of environmental data measured following water sampling. Wightmans Grove water temperatures ranged from 5.6-26.7 °C, Brady's Island water temperatures ranged from 6.1-24.4 °C, and Tiffin water temperatures ranged from 5.6-26.7 °C. Average water depth ranged from 0.54-3.04 m at Wightmans Grove, 0.38-1.55 m at Brady's Island, and 1.15-1.83 m at Tiffin. The pH values ranged from 7.57-8.23 at Wightmans Grove, 7.95-8.70 at Brady's Island, and 7.87-8.34 at Tiffin, and turbidity ranged from 34.31-57.12 NTU at Wightmans Grove, 13.69-91.57 NTU at Brady's Island, and 18.06-34.17 NTU at Tiffin. The average water depth varied across habitat types and was 1.91 m for main channel areas, 1.02 m for side channel areas, and was 0.55 m for backwater areas.

Table 1. Habitat and water quality summary for Grass Carp eDNA sampling in the Sandusky River during 2021. Habitat type abbreviations are as follows: Main Channel = MC, Side Channel = SC, Backwater = BW.

Location	Habitat	Season	Average Water Depth m*	Water Temperature °C*	pH	Water Turbidity NTU
Wightmans Grove	MC	Spring	2.96	6.6	7.97	NA
Wightmans Grove	MC	Summer	2.81	25.5	7.57	51.49
Wightmans Grove	MC	Fall	3.04	20.3	7.93	34.31
Wightmans Grove	MC	Late Fall	2.82	7.7	8.16	43.23
Wightmans Grove	SC	Spring	0.87	7.3	8.16	NA
Wightmans Grove	SC	Summer	0.80	23.5	7.95	57.12
Wightmans Grove	SC	Fall	0.98	20.7	8.13	38.48
Wightmans Grove	SC	Late Fall	0.86	7.2	8.15	36.17
Wightmans Grove	BW	Spring		Not sampled		
Wightmans Grove	BW	Summer		Not sampled		
Wightmans Grove	BW	Fall	0.74	20.4	7.79	52.54
Wightmans Grove	BW	Late Fall	0.54	7.4	8.23	39.87
Brady's Island	MC	Spring	1.30	11.0	8.05	NA
Brady's Island	MC	Summer	1.34	24.2	8.69	13.69
Brady's Island	MC	Fall	1.48	19.6	8.26	14.39
Brady's Island	MC	Late Fall	1.55	8.2	8.41	18.78
Brady's Island	SC	Spring	1.22	8.2	8.13	NA
Brady's Island	SC	Summer	1.14	22.4	8.66	22.16
Brady's Island	SC	Fall	1.15	21.8	8.32	18.09
Brady's Island	SC	Late Fall	1.12	6.9	8.40	17.22
Brady's Island	BW	Spring	0.38	10.7	8.02	NA
Brady's Island	BW	Summer	0.42	20.8	8.70	91.57
Brady's Island	BW	Fall	0.60	18.8	7.95	84.19
Brady's Island	BW	Late Fall	0.41	7.6	8.11	18.40
Tiffin - Control Site	MC	Spring	1.27	11.7	8.13	NA
Tiffin - Control Site	MC	Summer	1.83	21.9	8.34	34.17
Tiffin - Control Site	MC	Fall	1.15	18.7	7.87	19.51
Tiffin - Control Site	MC	Late Fall	1.33	7.4	8.10	18.06

*Represents the mean for all samples collected at this particular habitat and location combination.

Lab Results

Over the course of the study, we had a total of 44 positive detections (where a positive detection was at least one positive molecular replicate for any of the three qPCR markers) out of 3,138 field samples collected (approximately 1.40% positive; Table 2, Figure 2). Several of the collections (i.e., 80 samples from a habitat type) in our study had 0 positive detections (Figure 2; Table 2, Appendices I-III). Overall, we observed higher numbers of detections in the Summer and Fall sampling events and lower numbers of detections in the Spring and late Fall. The greatest number of detections were in main channel habitat (n = 20), and there were 12 detections each in the side channel and backwater habitats.

Table 2. eDNA detection summaries. Detections represent at least one positive molecular replicate for at least one of the three Grass Carp eDNA markers. Numerator is the number of positive detections, and the denominator represents the total number of samples collected at that site (e.g., "2/80" represents 2 positive detections out of 80 samples collected). "NA" refers to sites where no samples could be collected due to low water levels.

Site	Habitat Type	Fall 2019	Late Fall 2019	Spring 2021	Summer 2021	Fall 2021	Late Fall 2021
Brady's Island	Main Channel	2/80	0/80	1/80	2/80	0/80	6/80
Brady's Island	Side Channel	2/104	0/80	0/80	4/80	2/80	0/80
Brady's Island	Backwater	0/56	0/80	0/80	1/80	0/79	0/16
Wightmans Grove	Main Channel	3/80	1/80	0/80	1/80	1/80	0/80
Wightmans Grove	Side Channel	2/80	0/81	0/80	1/80	1/81	1/80
Wightmans Grove	Backwater	6/80	0/80	NA	NA	4/81	1/80
Tiffin (Control)	Main Channel	0/80	1/80	0/80	0/80	0/79	0/80

At the Wightmans Grove site, there were a total of 22 positive detections. The greatest number of detections at Wightmans Grove were in backwater habitat (n = 11), and the majority of those were in the Fall of 2019 (n = 6). There were six positive detections in the main channel at Wightmans Grove and five detections in the side channel. Nearly all of the detections at Wightmans Grove were in the Fall (n = 18), with the remaining detections observed in the Summer (n = 2) and Late Fall (n = 2). There were 21 Grass Carp eDNA detections total at Brady's Island. Twelve of these were in main channel habitat, eight were in side channel habitat, and one was in backwater habitat. There were eight positive detections in the Summer at Brady's Island, six each in the Fall and late Fall, and one positive detection in the Spring. There was one positive eDNA detection at the Tiffin control site. A sample collected in late Fall of 2019 had a single positive replicate at the GCTM10 assay. Maps of the complete detection results can be found in Appendices I-III.

None of the field blanks amplified at any of the three assays across the entire study indicating that there was no evidence of field contamination. One EN sample from the Fall 2021 did amplify for the Bluegill control assay we used. As per our standard protocols for processing

eDNA samples, all detection data associated with this associated with this control were omitted from the final dataset and were not reported. All other control samples showed expected results (i.e., positive controls amplified in all cases and negative controls showed no amplification).

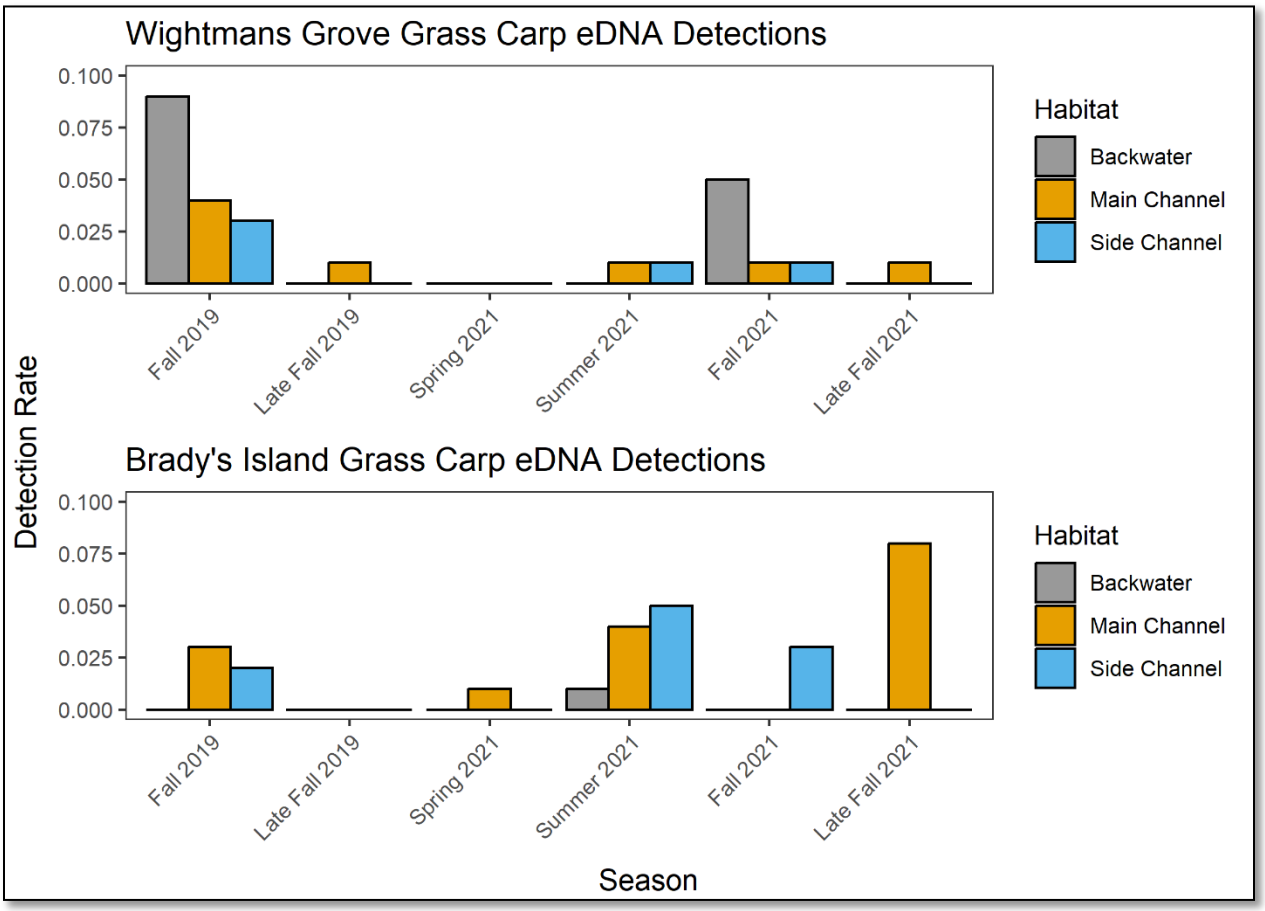

Figure 2 Grass Carp eDNA detection rates at Wightmans Grove (upper panel) and Brady's Island (lower panel). Detection rates represent the proportion of samples collected that had at least one positive replicate for at least one of the three Grass Carp eDNA assays.

Occupancy Model Results

Results by Site and Month (ψ)

The probability of Grass Carp eDNA being present at the sampling site was consistent across both models. The probability of Grass Carp eDNA presence was similar between sites but differed across months, with higher probabilities of eDNA presence occurring in summer and fall compared to spring and late fall (Figure 5, Appendix V, VI). The highest probability of Grass Carp eDNA being present at a given site and month occurred at Brady's Island in Summer (posterior distribution median = 0.72, 95% credible interval = 0.35 - 0.93) and was the only site with a median value above 0.7 (Wightmans Grove in Summer: median = 0.7, 95% CI 0.4 - 0.91). Wightmans Grove in late Fall had the lowest probability of Grass Carp eDNA being present (posterior distribution median = 0.42, 95% CI = 0.13 - 0.77).

Results by Site, Habitat, and Month (θ)

In site-habitat-month (unit level) combinations where Grass Carp eDNA was detected by qPCR, all models estimated a high probability of Grass Carp eDNA being detected at a site (all median θ estimates > 0.95) (Figures 3 & 6). Detections for habitats within sites did not change significantly between months or between sites. When Grass Carp eDNA was not detected, the models estimated θ to be 0.50 with wide confidence intervals (Appendix V, VI).

Results by qPCR Marker (ρ)

The probability of an individual sample containing eDNA when it was collected did not vary between individual Grass Carp assays or across months in Model 2. In site-habitat-month combinations where Grass Carp eDNA was detected by qPCR, both models predicted overall low probabilities of molecular detection (all median p estimates ≤0.01; Figures 4 & 7) with narrow confidence intervals. This was not surprising given the low number of positive detections we observed (approximately 1.4%).

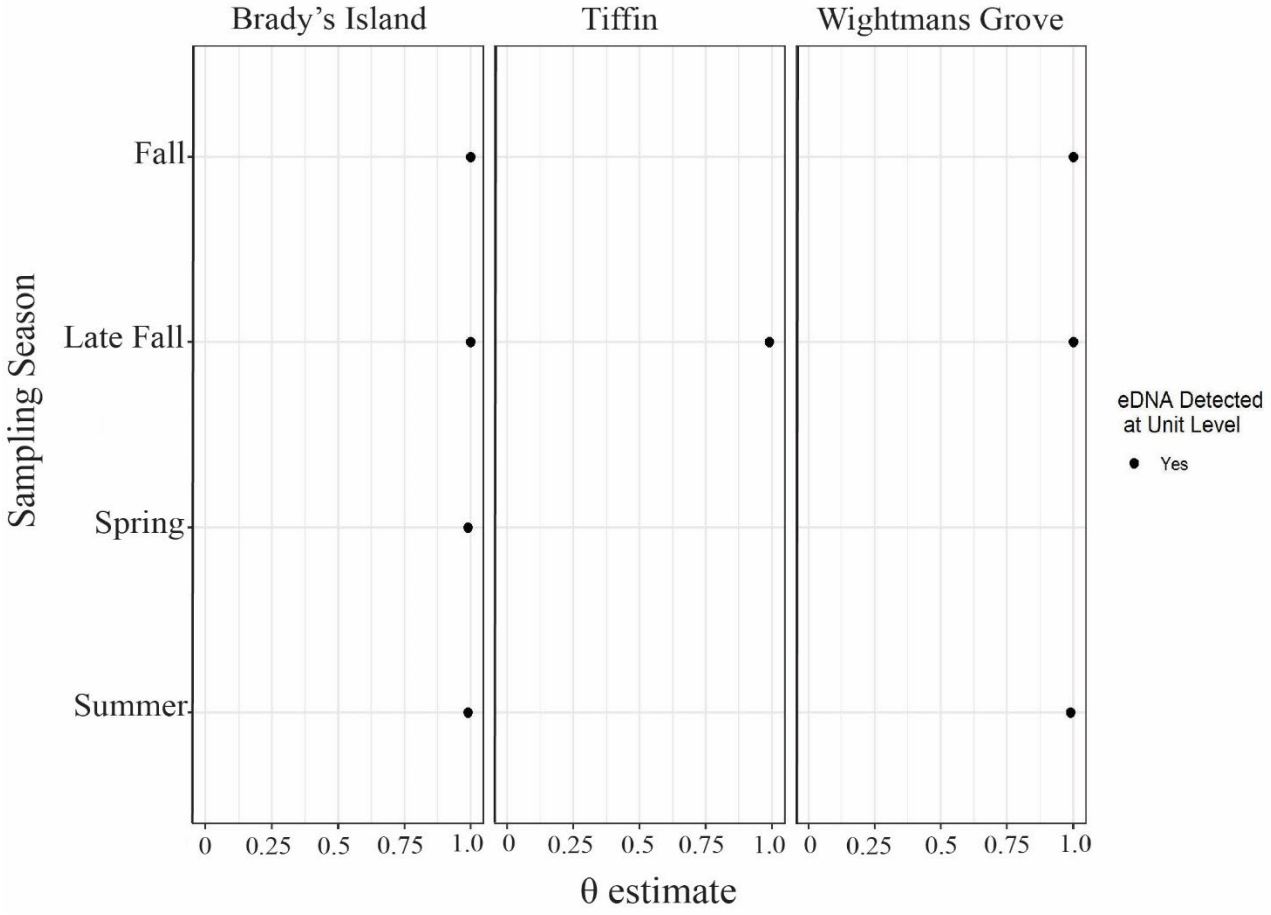

Figure 3. Occupancy Model 1 theta (θ) parameter estimates for each site and each month. Solid circles represent mean theta probability estimates in units with at least one positive eDNA detection.

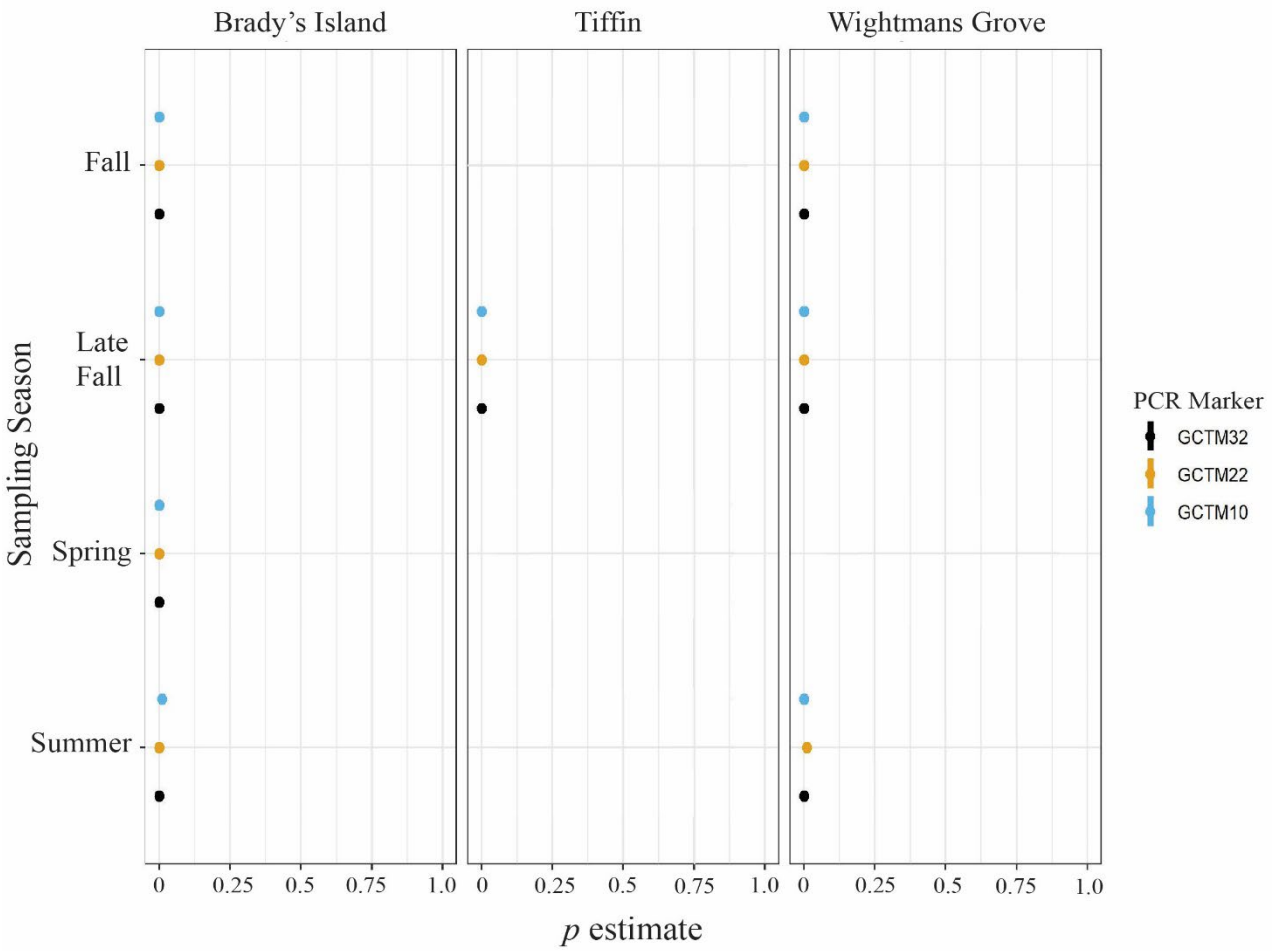

Figure 4. Occupancy Model 1 delta_p (*p)* parameter estimates for each assay across all sites and sampling months. Only units with at least one positive eDNA detection are displayed. Each color represents a unique Grass Carp qPCR assay. Solid circles represent mean delta_p probability estimates.

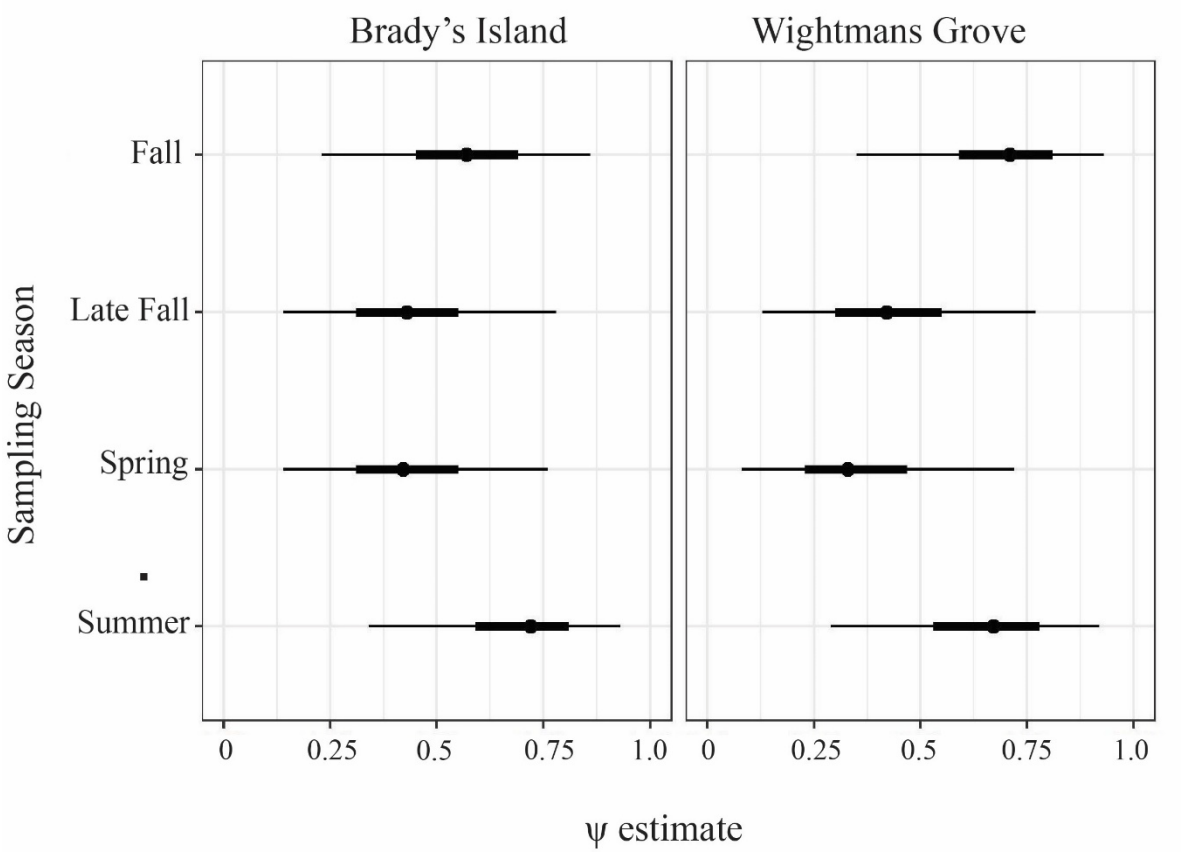

Figure 5. Occupancy Model 2 beta_psi (ψ) parameter estimates for each site and all sampling months. Psi mean probability estimates are displayed as solid circles with solid bold bars representing upper and lower 80% confidence intervals and solid thin lines representing upper and lower 95% confidence intervals.

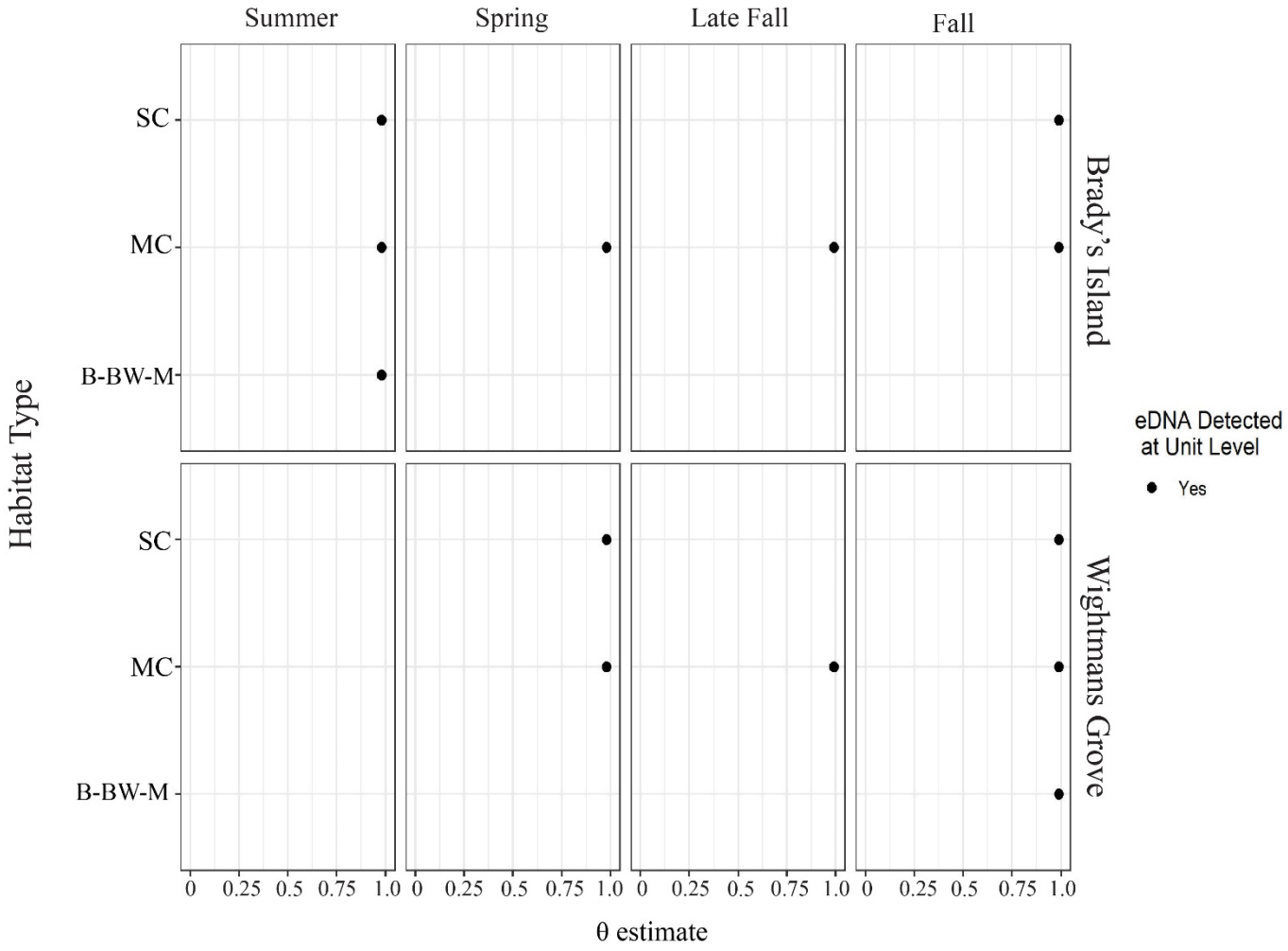

Figure 6. Occupancy Model 2 theta (θ) parameter estimates for three grouped habitat types for each site and each month. Solid circles represent mean theta probability estimates in units with at least one positive eDNA detection. Habitat type abbreviations are as follows: SC = side channel, MC = main channel, B-BW-M = backwater.

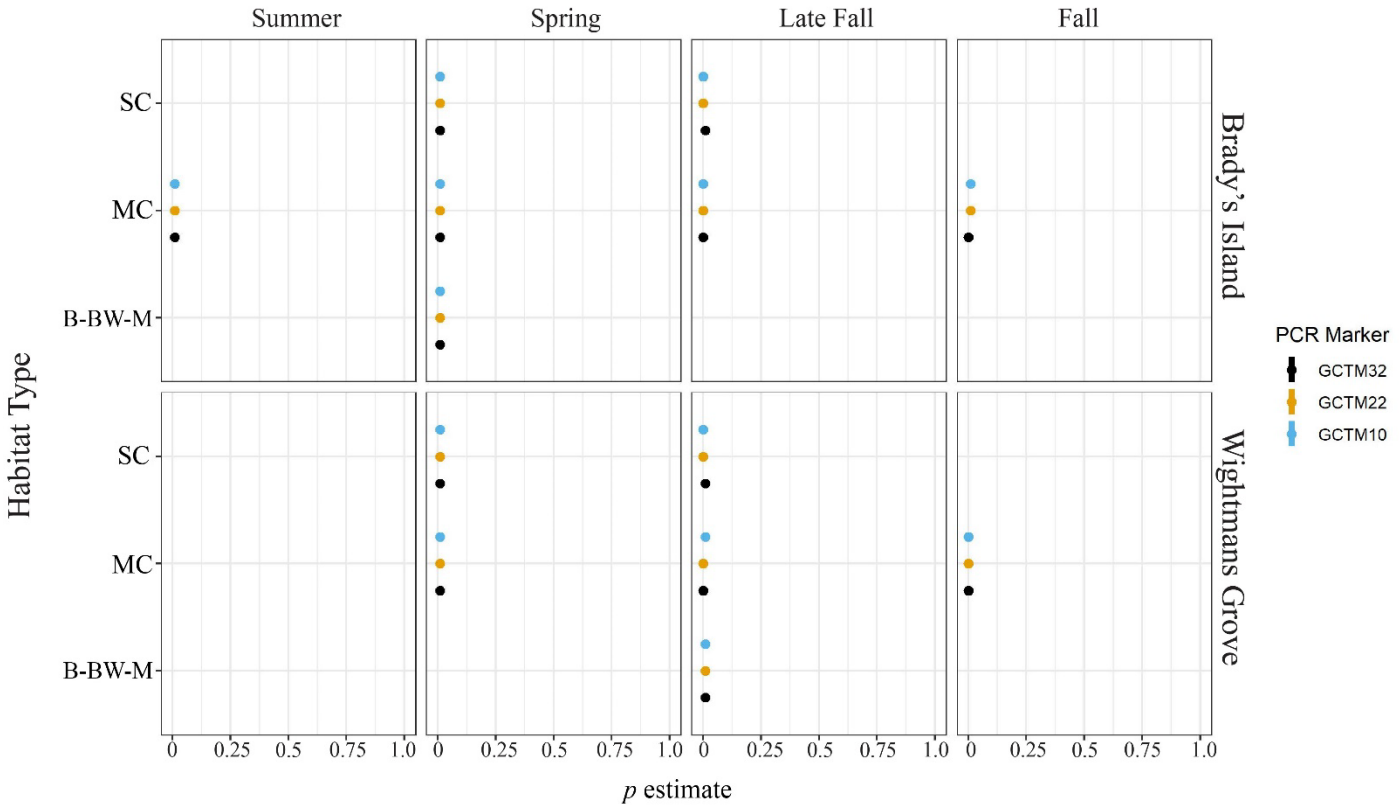

Figure 7. Occupancy Model 2 delta_p (*p)* parameter estimates for each assay across grouped habitat types for each site and each month. Only units with at least one positive eDNA detection are displayed. Each color represents a unique Grass Carp qPCR assay. Solid circles represent mean delta_p probability estimates. Habitat type abbreviations are as follows: SC = side channel, MC = main channel, B-BW-M = backwater.

Discussion

The objectives of this study were to: 1) Determine the detection probability of Grass Carp eDNA in the Sandusky River over different spatial and temporal scales; 2) Determine the limitations of Grass Carp eDNA detection in the Sandusky River: and 3) Use data from Grass Carp in the Sandusky River to help refine eDNA monitoring efforts for Silver and Bighead carps in other Great Lakes tributaries. We were able to detect Grass Carp eDNA in the Sandusky River at multiple sampling sites and multiple seasons over the course of the study. The overall number of detections was relatively low, however (i.e., 1.4% overall detection rate), and subsequently the detection probabilities we observed were very low (mean $p = 0.01$). This limited our ability to identify the limitations of Grass Carp eDNA detections in the Sandusky River and we could not use this data to make inferences related to other invasive carp species in the Great Lakes.

Previously we conducted preliminary, small-scale field trials of these three markers in ponds containing Grass Carp and using limited samples from the Sandusky River. The present study served as a larger-scale field validation effort for these three markers. Positive eDNA detections in this study suggest that these three markers can indeed be used to detect Grass Carp eDNA when the species is present. An informal comparison between our 2021 eDNA detection data and telemetry data showed that there were radio-tagged Grass Carp present in the Sandusky River up to two days prior to when eDNA samples were collected for this study and 2 days post eDNA collections (J. Bopp, Michigan State University, *personal communication*). Although we do not know the longevity of shed Grass Carp eDNA in the environment, previous studies suggest that eDNA from other carp species can persist in the environment for multiple days and perhaps multiple weeks (Barnes et al. 2014; Lance et al. 2017). eDNA detection data coupled with telemetry data suggest that detections we observed were at least in part due to the presence of live Grass Carp in the Sandusky River. It is important to note that eDNA detections do not always indicate the presence of live organisms, and there are many secondary vectors that can transport eDNA into a study area including: carcasses, boats, avian predators, recreational anglers, and researchers/field gear (Merkes et al. 2014; Barnes and Turner 2016; Homel et al. 2021).

Our results also showed that detection rates varied temporally and spatially. Higher numbers of detections in the summer and fall sampling events may coincide with spawning behavior in the early Summer and downstream movements observed in the Fall. Grass Carp eDNA detections were split nearly equally between Brady's Island and Wightmans Grove, but we did observe some spatial variation in detections within those sites with greater numbers of detections in main channel habitat at Brady's Island and more detections in backwater habitat at Wightmans Grove. Spatial detection data should be interpreted cautiously however since there were fewer eDNA samples collected in backwater habitats due to limited access to these sites and samples that were collected were not spatially independent since they were often collected from different sides of the boat at the same location.

The majority of our field and lab controls showed anticipated results suggesting that no contamination occurred. We did observe one positive detection from the Tiffin Control site in the Fall 2019 samples. Although Grass Carp are not regularly observed in this area above a presumed barrier, it is possible that fish could access this area, and there are also other vectors for transporting eDNA to the study site as we note above. All other samples at the Tiffin negative control site were blank and all field controls were blank, giving us confidence that we were not getting false positive detections in our study. We also had one failed DNA extraction control in

the laboratory at the Tiffin site in Fall of 2021, and positive detections associated with this control were omitted from further analysis.

Although we can detect Grass Carp eDNA using these markers, detection probabilities from the eDNA occurrence models were very low. This should not be surprising given the low number of detections we observed and used to inform our models. There are several possible explanations for the low number of detections in our study. First, the number of fish in the Sandusky River may have been too low and there was not large amounts of eDNA present in the study areas. Numerous studies have documented a positive relationship between fish abundance and positive eDNA detections (Yates et al. 2019; Rourke et al. 2022). Data for the Sandusky River suggest that there are approximately 200 fish in the river year-round based on electrofishing removal efforts (Gouveia et al. 2023), and these fish can disperse over a large area. Although these densities are relatively high for Grass Carp, they are much lower than densities of other invasive carp species in other watersheds (e.g., Bighead and Silver carps in the Illinois River) where much higher positive eDNA detection rates and higher detection probabilities have been observed (USFWS and USGS, *unpublished data*). Furthermore, the Sandusky River is an open system and fish can leave the system at any point, which could further reduce fish density and the amount of eDNA in the water. Additionally, Grass Carp were targeted and removed from the Sandusky River over the course of the study as part of ongoing management actions. Although we have capture and telemetry data that indicate fish were indeed in the Sandusky River during our sampling periods, the exact number of fish present during these periods is unknown.

Collection methods can have a large influence on eDNA detections (Schabacker et al. 2020; Bessey et al. 2022; Bockrath et al. 2022) and may be another reason we had low detection rates in our study. We used a single collection and preservation method; surface water collection followed by centrifugation and isopropanol preservation. This method was chosen because it has been successful for use by USFWS for Silver and Bighead carp eDNA monitoring (Bockrath et al. 2022; USFWS 2023) and because previous efforts using filtration in the Sandusky River proved to be inefficient due to high turbidity in the system. Researchers in the Sandusky River and other watersheds have reported successful Grass Carp eDNA detections using other eDNA capture methods including filtration (K. Robinson, Michigan State University, *personal communication*, S. Spear, USGS, *personal communication*). With larger filter pore sizes and new filtering technology now available, different filtering methods may be another eDNA capture method to explore in this system.

Species biology and timing of sampling events also play a critical role in eDNA detections (de Souza et al. 2016; Tsuji and Shibata 2021) and may be another reason why we did not observe greater numbers of eDNA detections. Our sampling schedule was pre-determined and was based on when we thought that fish were most likely to be in the river and based around behaviors such as movements and spawning activity. It was impossible to predict exactly when behaviors associated with greatest eDNA shedding rates (e.g., feeding, spawning, etc.) would occur. We followed a pre-determined sampling schedule because we wanted to avoid periods when removal efforts and behavior experiments conducted by other researchers (e.g., bait and attractant studies) may have artificially increased the concentration of Grass Carp eDNA in the study areas and changed the species behavior. Grass Carp typically move upstream and spawn in response to high flow events (Conover et al. 2007; Embke et al. 2016; Hessler et al. 2023). An examination of the hydrograph for the Sandusky River in Summer 2021 suggests that Summer eDNA sampling occurred several weeks after major peak flows in May and between minor peak

flow events in June (Appendix VII). Any subsequent spawning that occurred in spring or summer of 2021 was likely outside of the window of our eDNA sampling events and eDNA released during spawning events may have exited the system by this point. It is also important to note that species shed eDNA at different rates (Barnes and Turner 2016; Sassoubre et al. 2016; Andruszkiewicz Allan et al. 2021) and as noted above, there is no data on Grass Carp eDNA shedding rates. A recent study of another invasive carp, Black Carp, suggested that eDNA was difficult to detect in some habitats where Black Carp were presumed present (Guan et al. 2019) and the same may be true for Grass Carp in this study.

Conclusions and Next Steps

eDNA occurrence models help answer questions including how many samples to collect, when to collect them, and where to collect them in order to maximize eDNA detections for species and population monitoring (Erickson et al. 2019). Although we did get positive eDNA detections in the Sandusky River, and supplemental data suggests these were from live fish, the limited number of detections in our study and subsequent low detection probabilities from our model limit our ability to answer some of the questions necessary for developing a robust population monitoring program using eDNA (e.g., how many samples to collect, what time of year to sample, etc.). These markers do show promise and we feel there are multiple next steps worth exploring. Increased filter pore sizes and advances in filtering technology that facilitate on-the-water processing may make collecting eDNA with filters a viable option in the Sandusky River. Comparisons between filters and standard centrifuging methods could be one area to explore in the future. Fish abundance and behavior can change considerably from one year to the next and adding additional temporal data points to the study using the existing methods could also prove useful. We hope to be able to explore some of these additional options in the future, and the data collected during this study (e.g., higher detection rates in summer and fall) could help to inform future sampling efforts.

Acknowledgements

Funding for this work was provided by the USFWS Region 3 Fisheries Program and the Great Lakes Restoration Initiative We would like to thank several individuals who helped us design this study including Jenna Bloomfield, Scott Koproski, Kelly Robinson, Patrick Kocovsky, Lucas Nathan and Travis Brenden. We would also like to thank Anton Gereau II, Matthew McLean, Jessica Kosiara, Robert Barta, Michael Schindler, and Janine Lajavic of the Alpena FWCO and Steven Gambicki of the USDA-Natural Resources Conservation Service in Gaylord, MI for assisting with eDNA sample collections; and Aaron Johnson, Katie Bockrath, Maren Tuttle-Lau, Nick Grueneis, Joel Wils, and Zeb Woiak for laboratory assistance. Justin Bopp shared Grass Carp radio telemetry data from the Sandusky River which was referenced in this report. Ross Rhueman from the USFWS Midwest Fisheries Center produced the maps in this report. Richie Erickson (USGS) provided comments on an earlier version of this report. Data from this study have been archived on the USFWS ServCat data repository and are publicly available (USFWS ServCat reference number 160208). The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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Appendix I.

Grass Carp eDNA detection maps for Wightmans Grove.

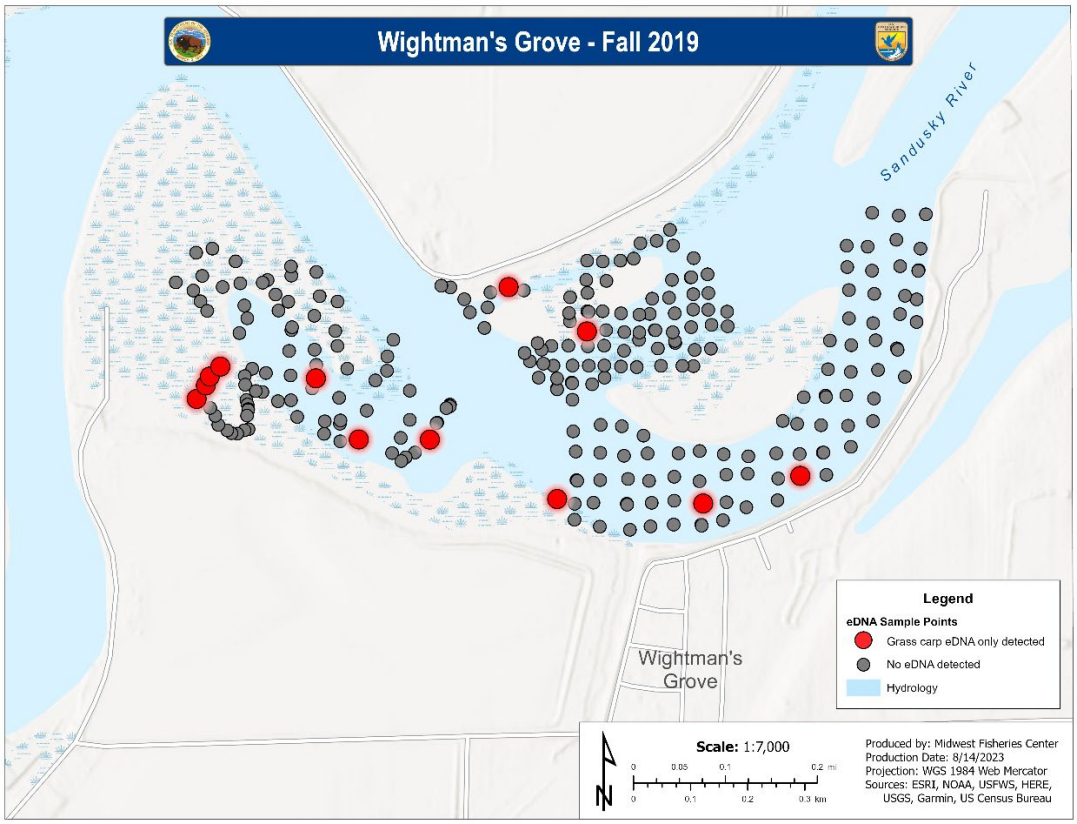

Figure AI-1. Fall 2019 eDNA detections for Wightmans Grove. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

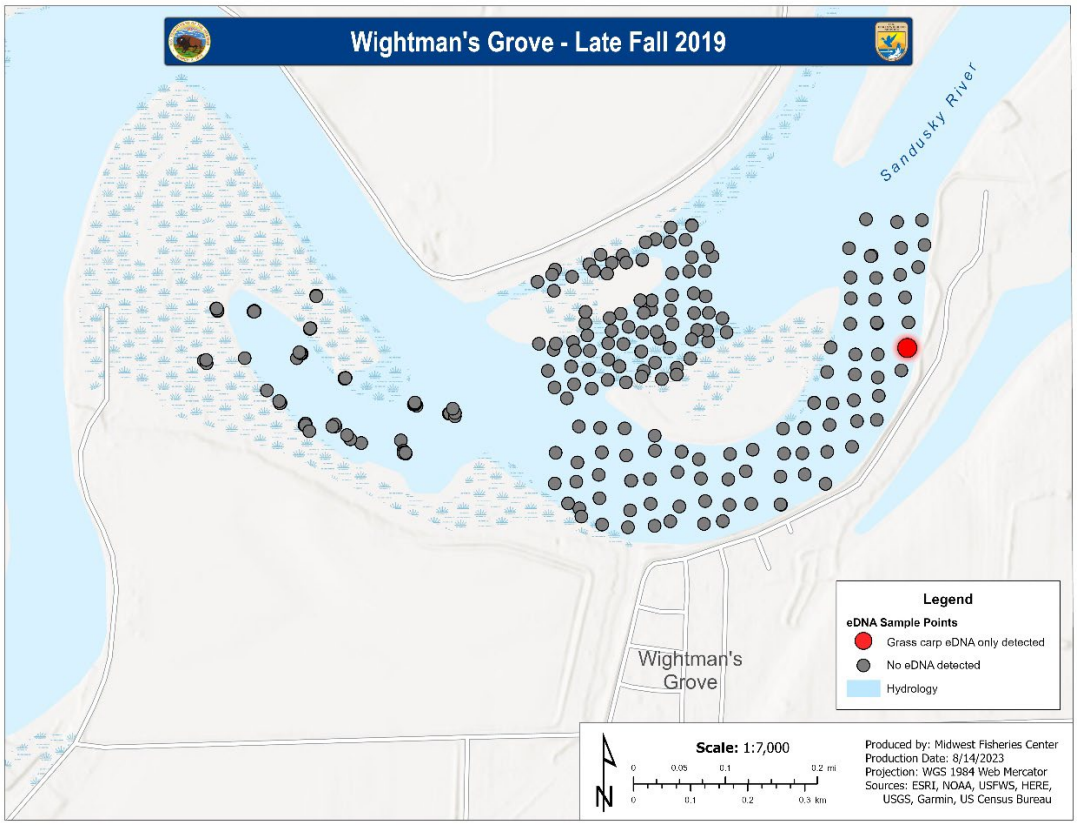

Figure AI-2. Late Fall 2019 eDNA detections for Wightmans Grove. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

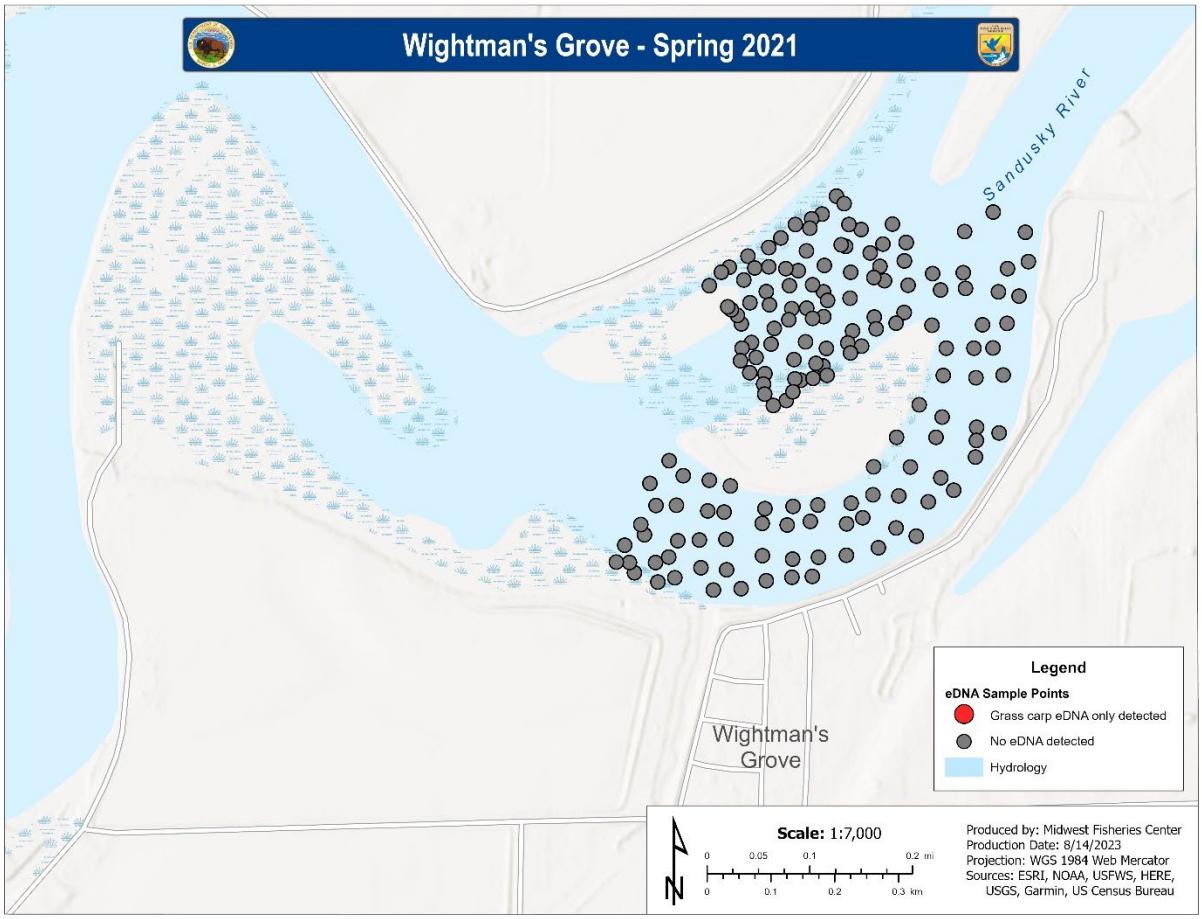

Figure AI-3. Spring 2021 eDNA detections for Wightmans Grove. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

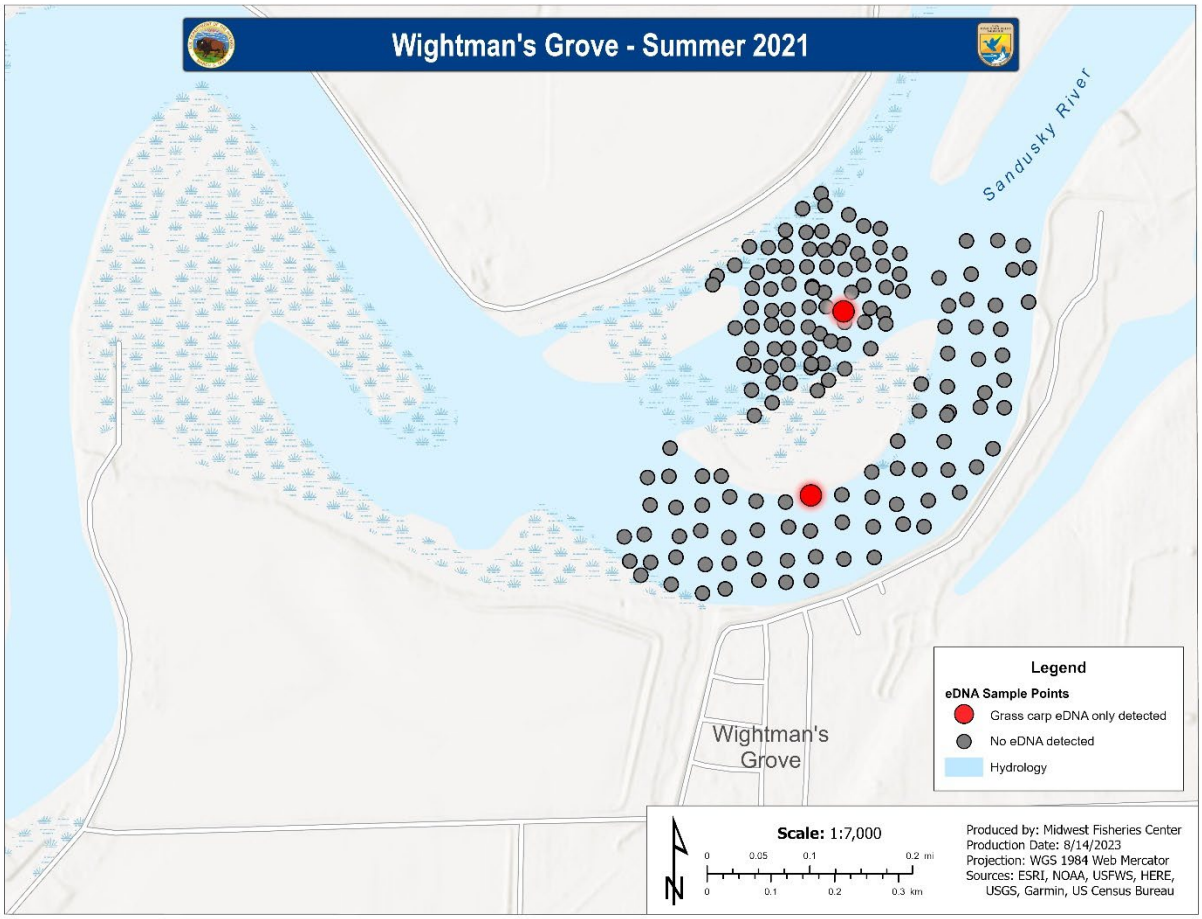

Figure AI-4. Summer 2021 eDNA detections for Wightmans Grove. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

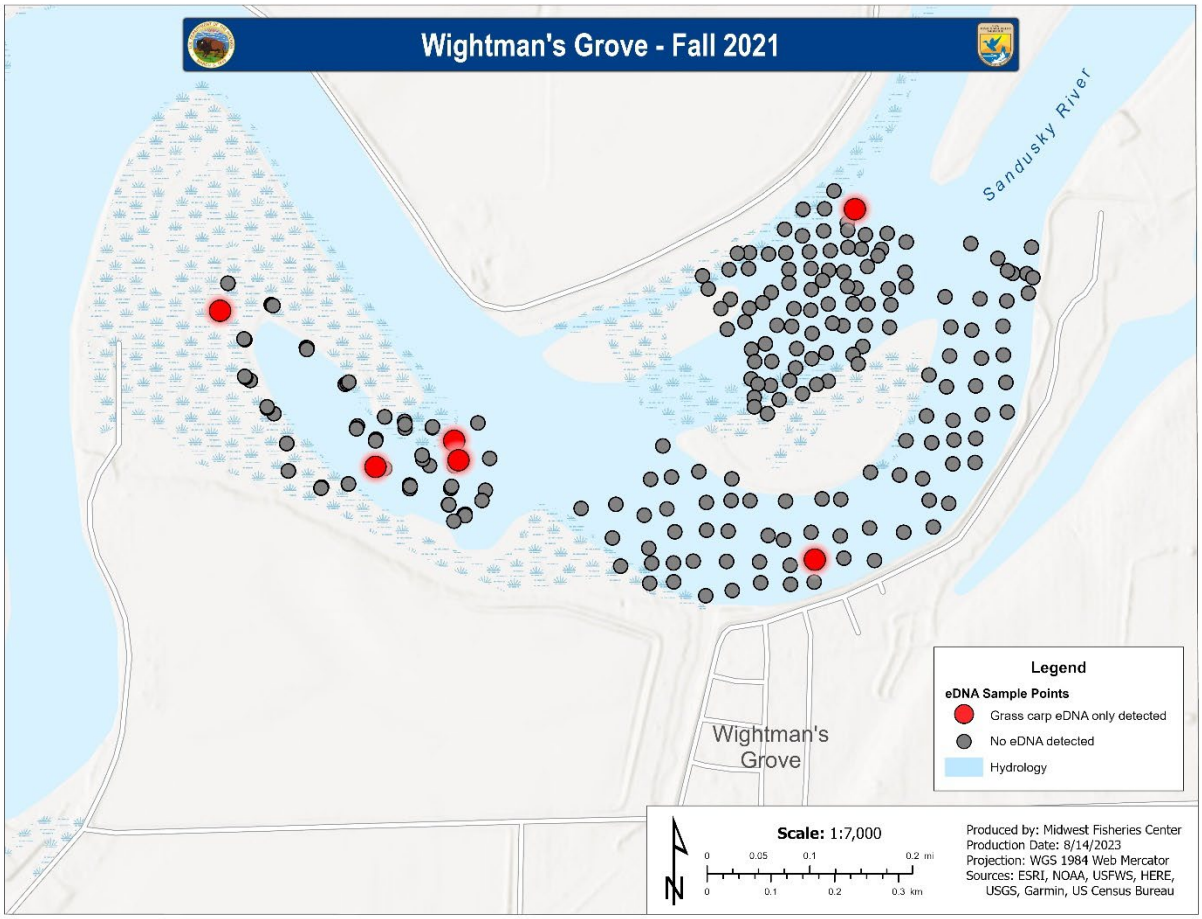

Figure AI-5. Fall 2021 eDNA detections for Wightmans Grove. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

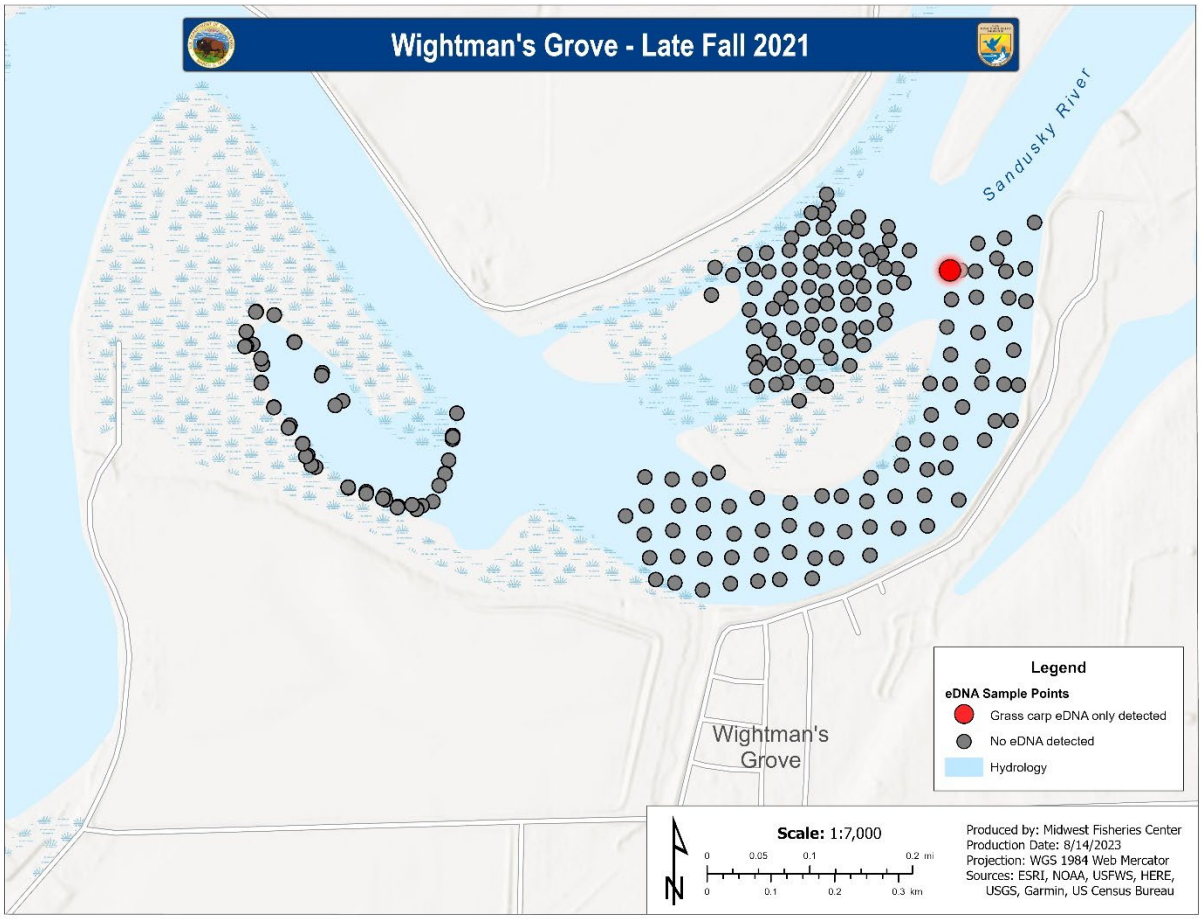

Figure AI-6. Late Fall 2021 eDNA detections for Wightmans Grove. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

Appendix II.

Grass Carp eDNA detection maps for Brady's Island.

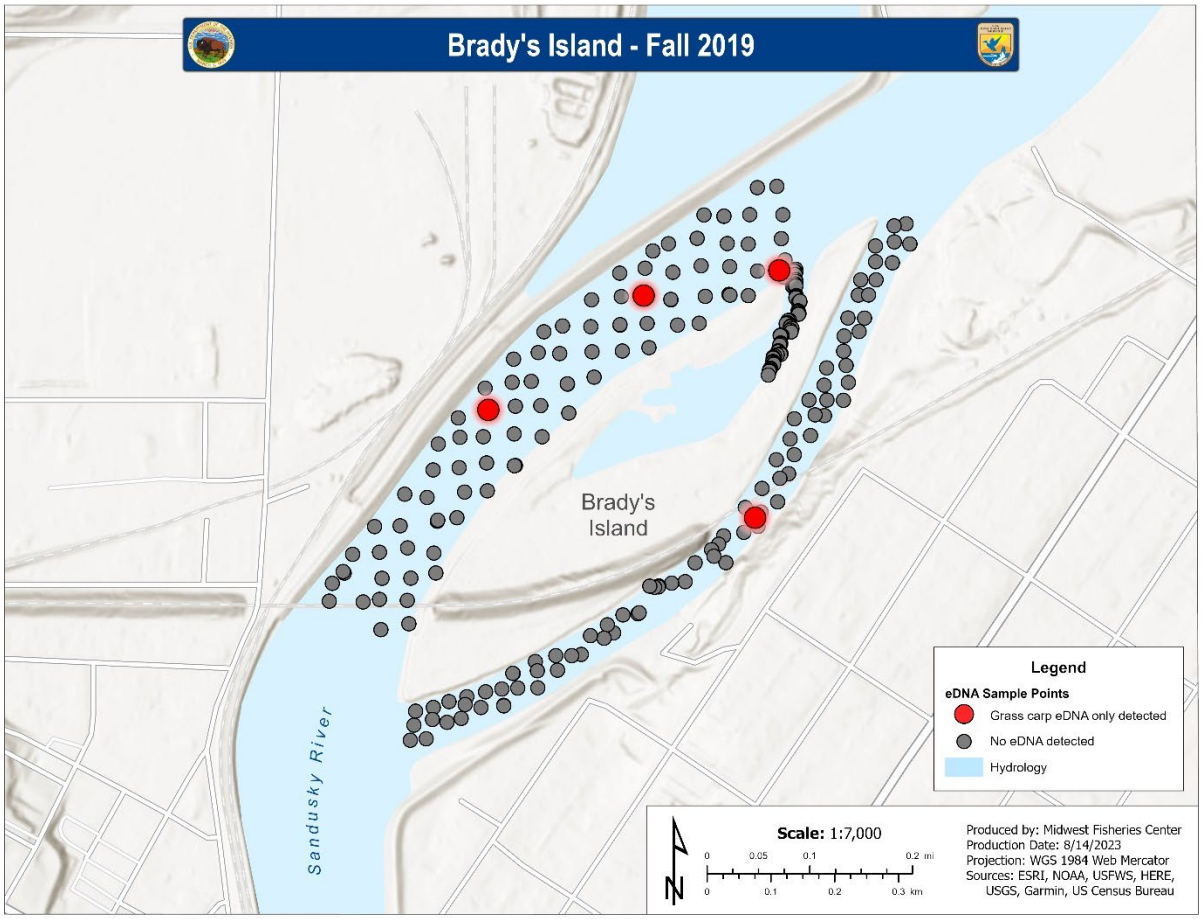

Figure AII-1. Fall 2019 eDNA detections for Brady's Island. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

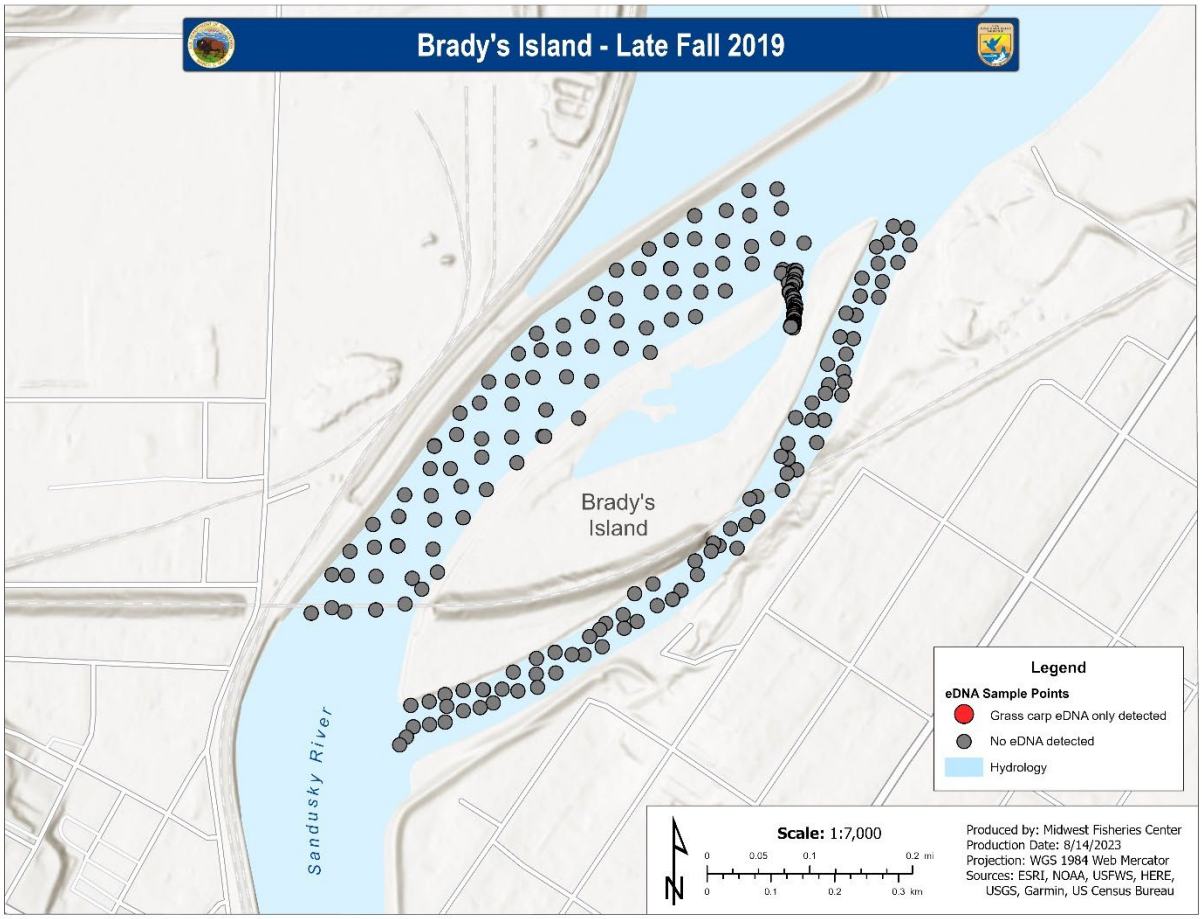

Figure AII-2. Late Fall 2019 eDNA detections for Brady's Island. Gray dots indicate sampling locations where no Grass Carp eDNA was detected.

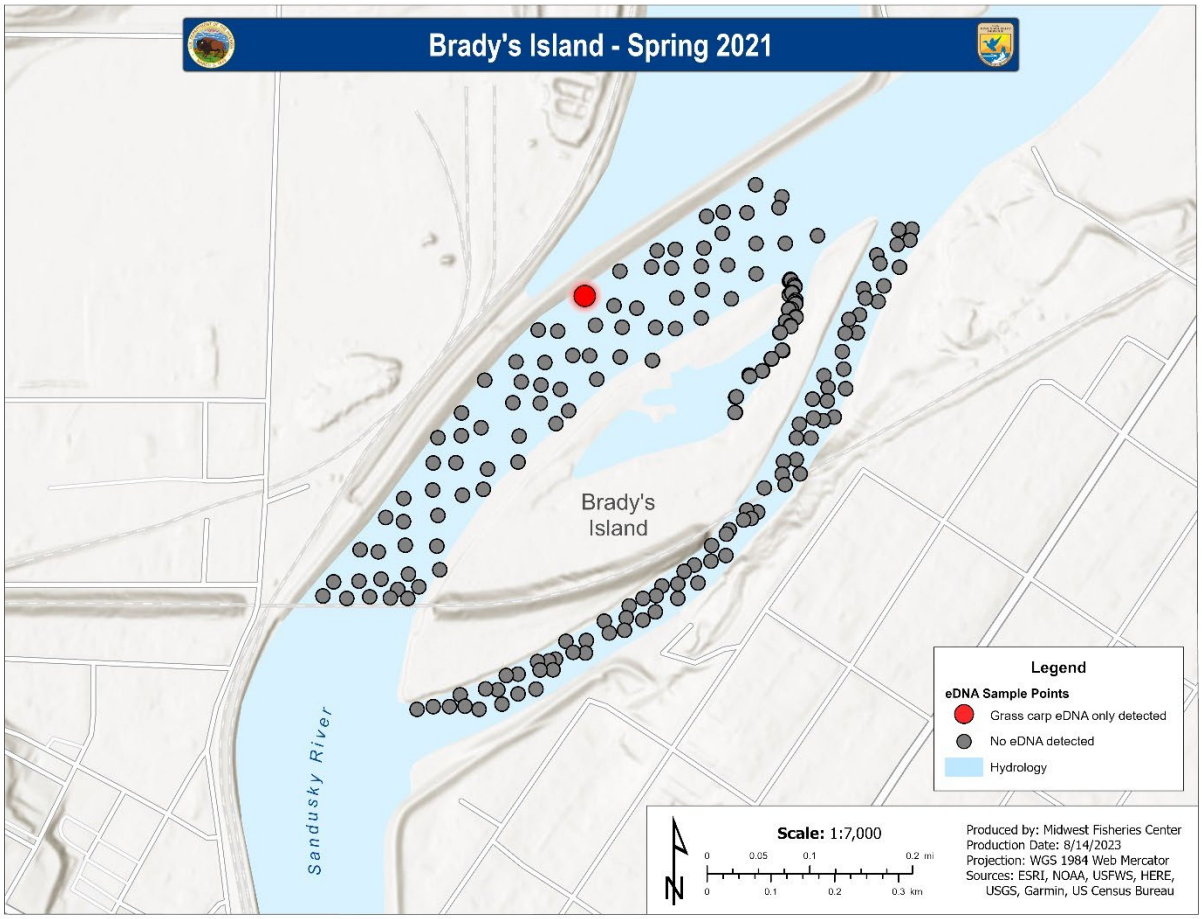

Figure AII-3. Spring 2021 eDNA detections for Brady's Island. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

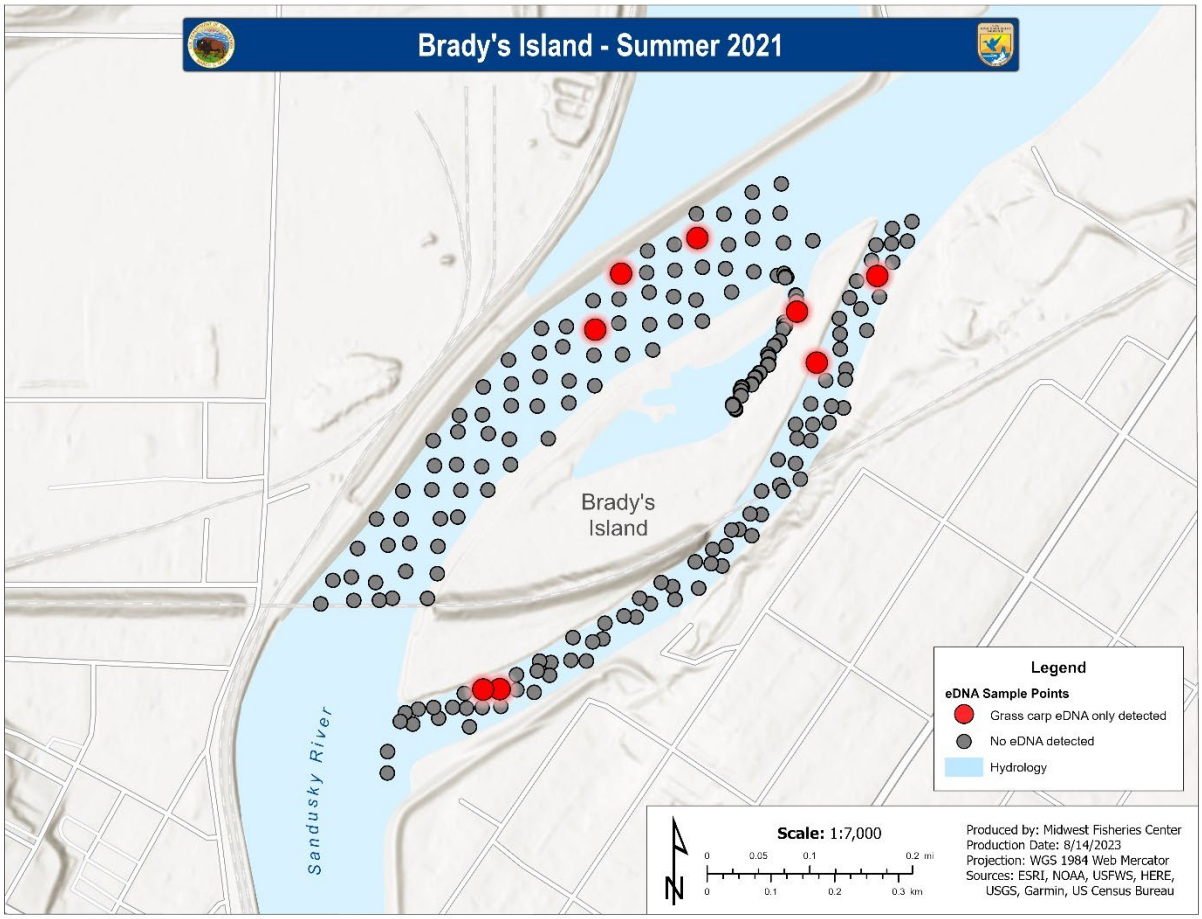

Figure AII-4. Summer 2021 eDNA detections for Brady's Island. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

Figure AII-5. Fall 2021 eDNA detections for Brady's Island. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

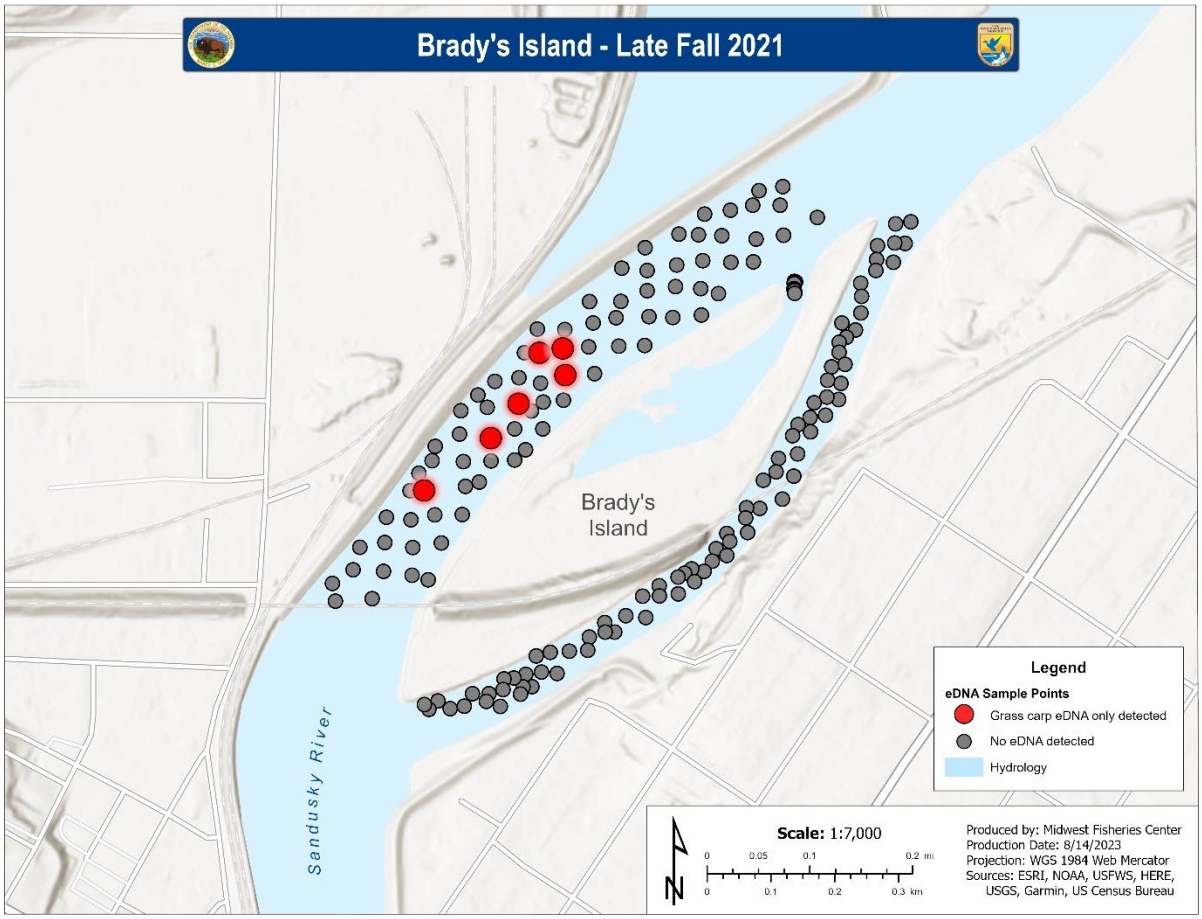

Figure AII-6. Late Fall 2021 eDNA detections for Brady's Island. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

Appendix III.

Grass Carp eDNA detection maps for Tiffin control site.

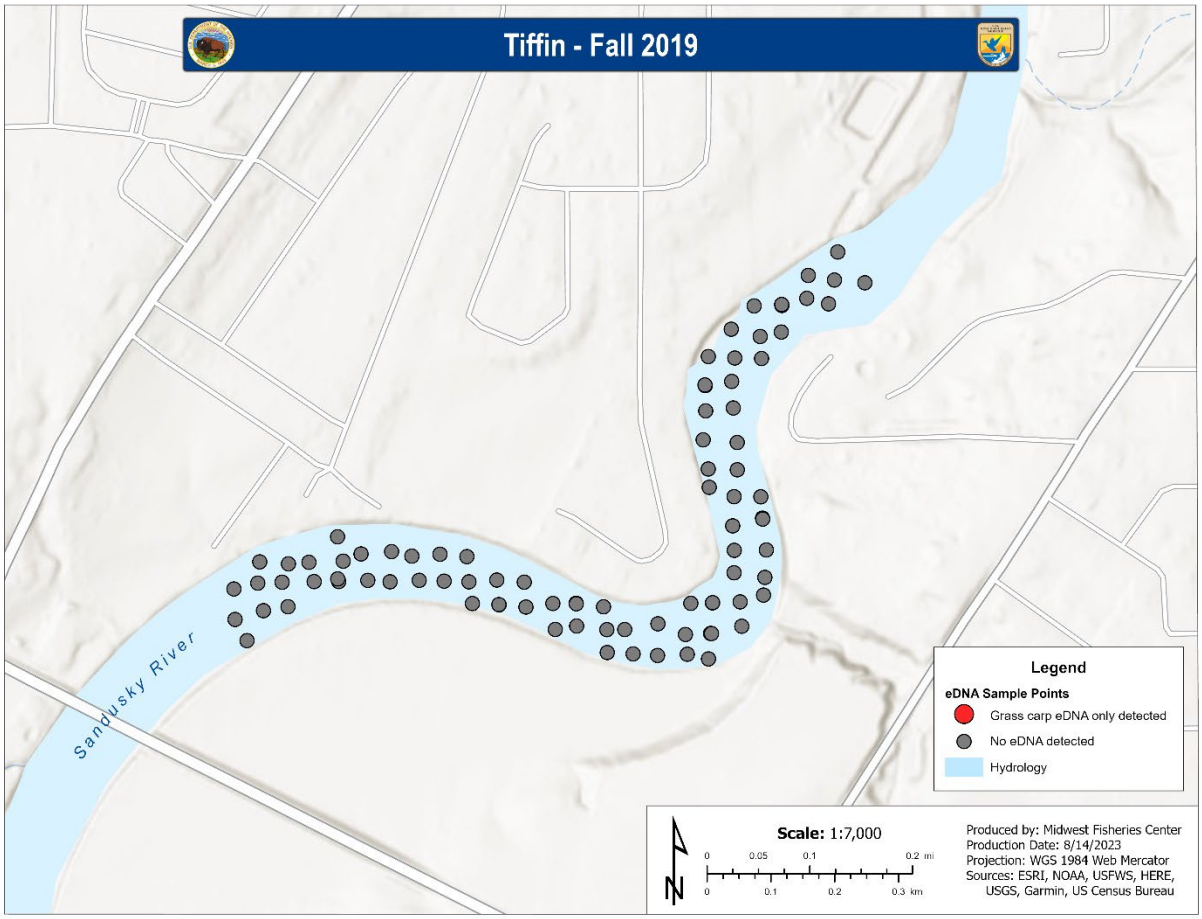

Figure AIII-1. Fall 2019 eDNA detections for Tiffin. Gray dots indicate sampling locations where no Grass Carp eDNA was detected.

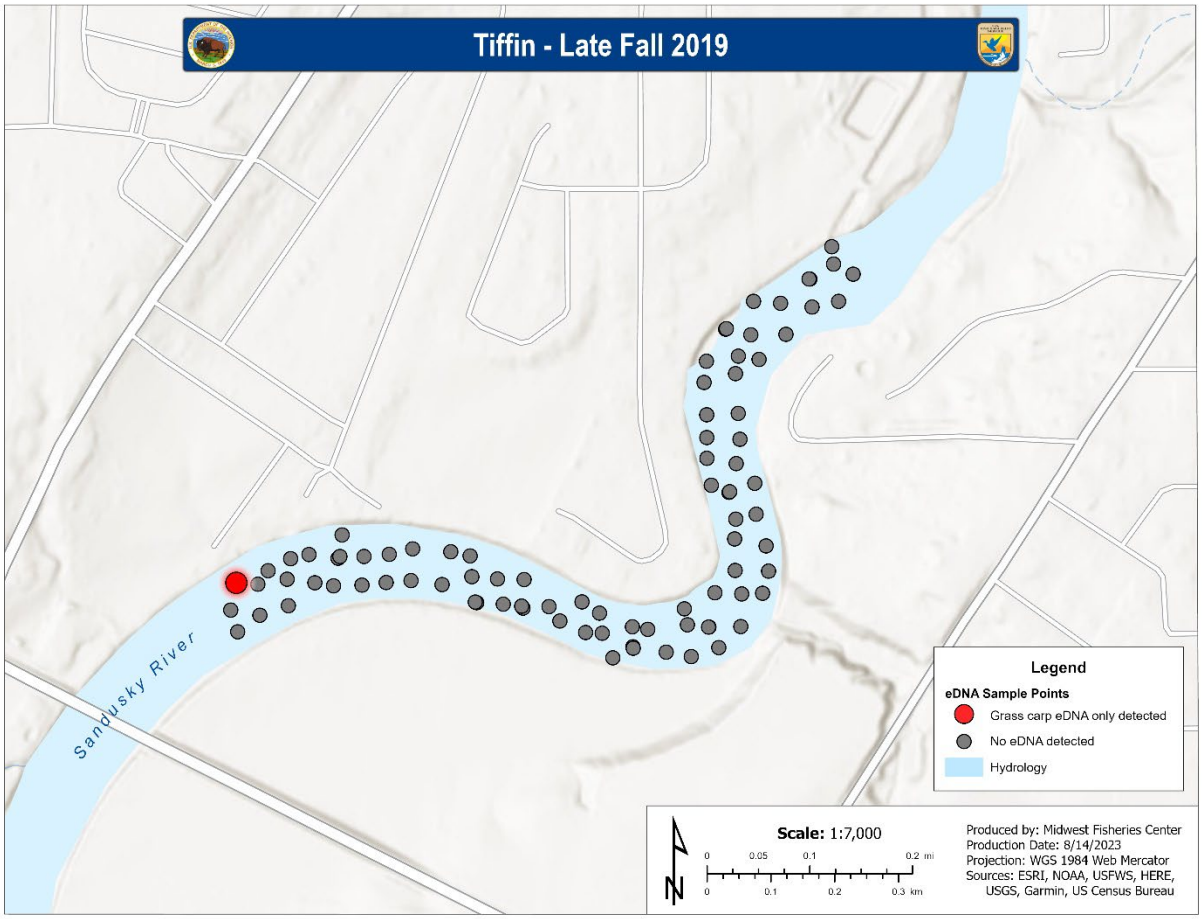

Figure AIII-2. Late Fall 2019 eDNA detections for Tiffin. Red dots indicate sampling locations where Grass Carp eDNA was detected and gray dots indicate sampling locations where no Grass Carp eDNA was detected.

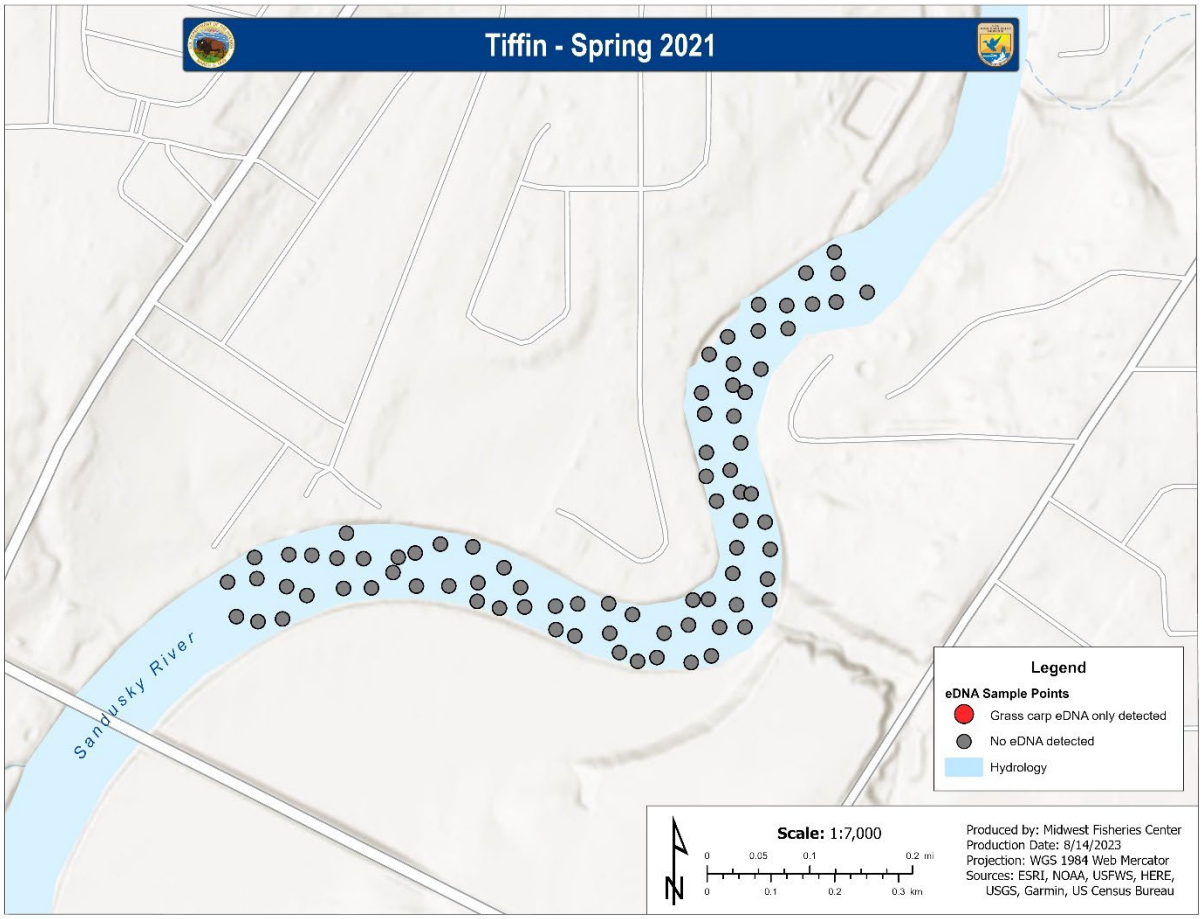

Figure AIII-3. Spring 2021 eDNA detections for Tiffin. Gray dots indicate sampling locations where no Grass Carp eDNA was detected.

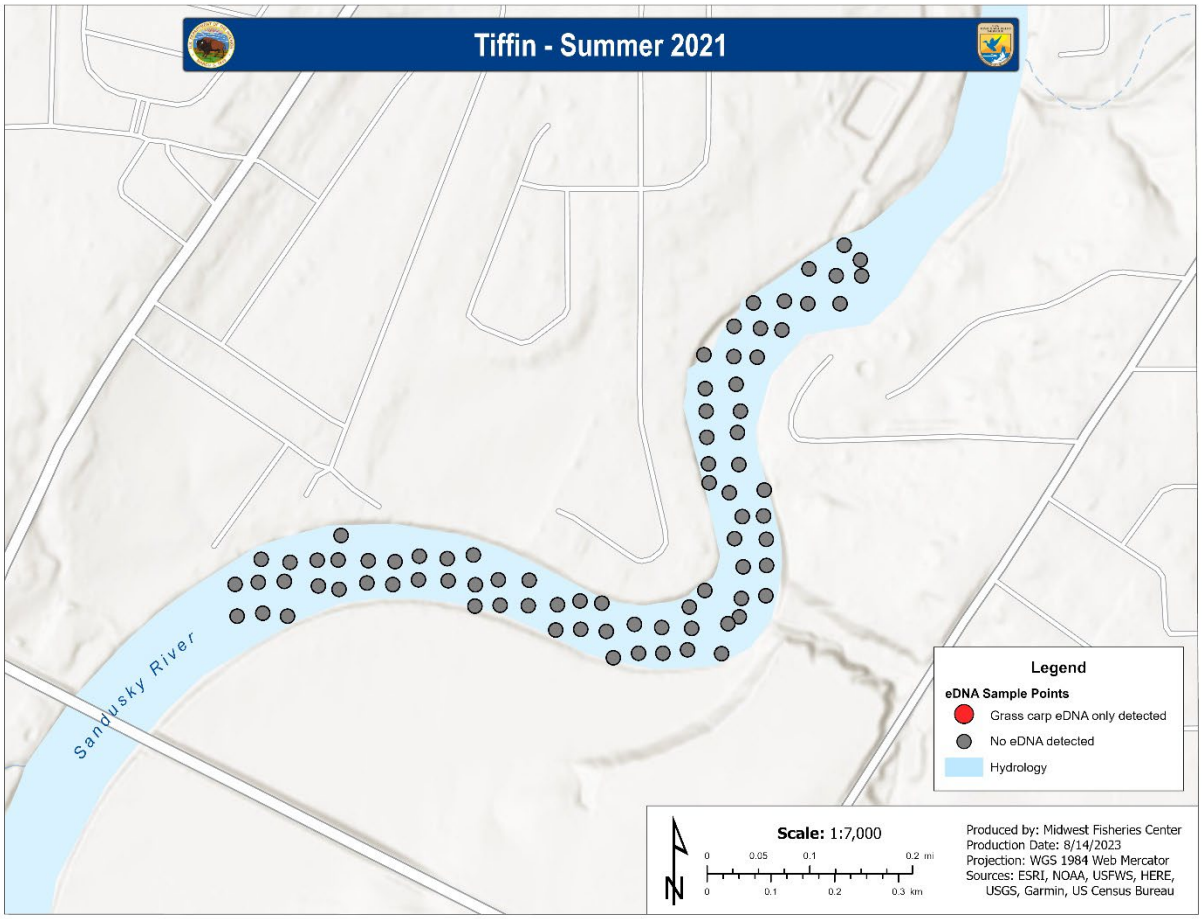

Figure AIII-4. Summer 2021 eDNA detections for Tiffin. Gray dots indicate sampling locations where no Grass Carp eDNA was detected.

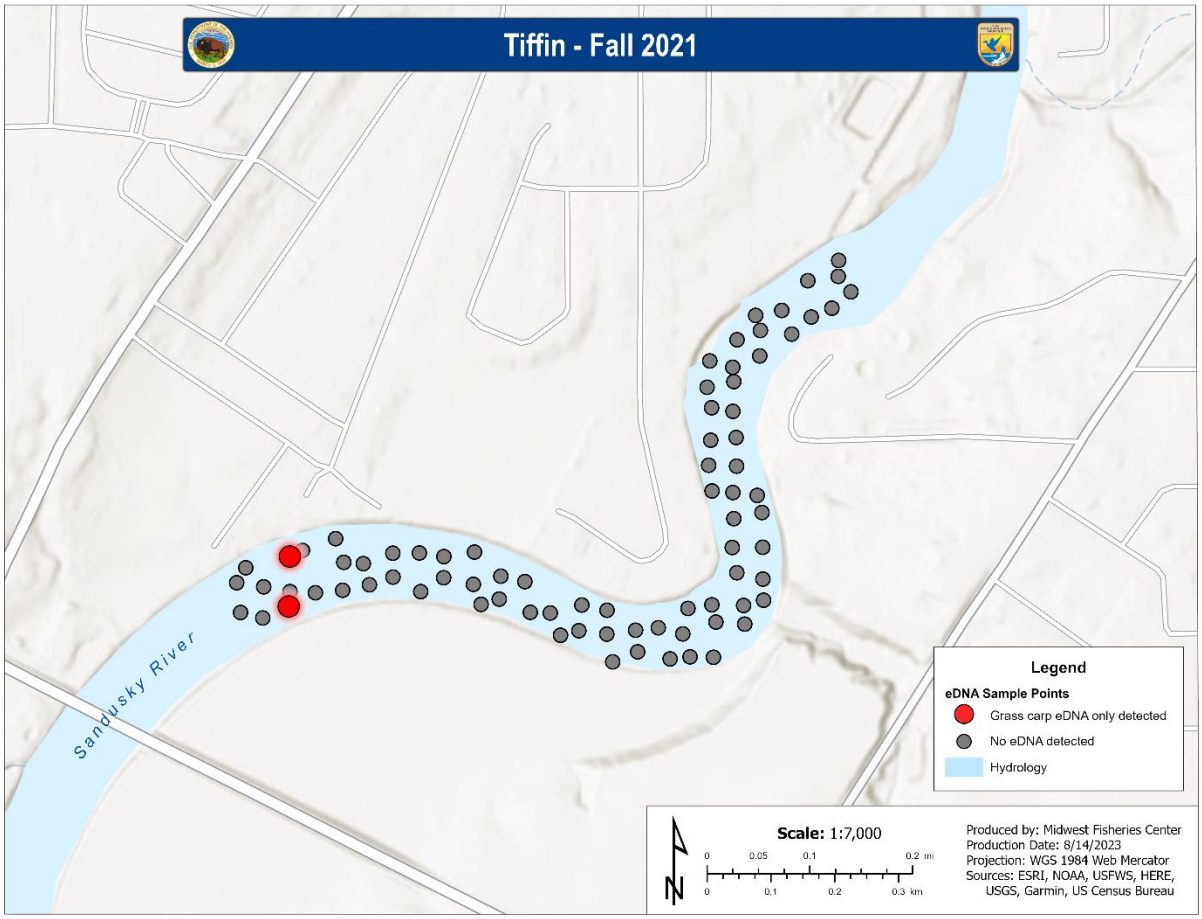

Figure AIII-5. Fall 2021 eDNA detections for Tiffin. Red dots indicate sampling locations where Grass Carp eDNA was detected. Gray dots indicate sampling locations where no Grass Carp eDNA was detected.

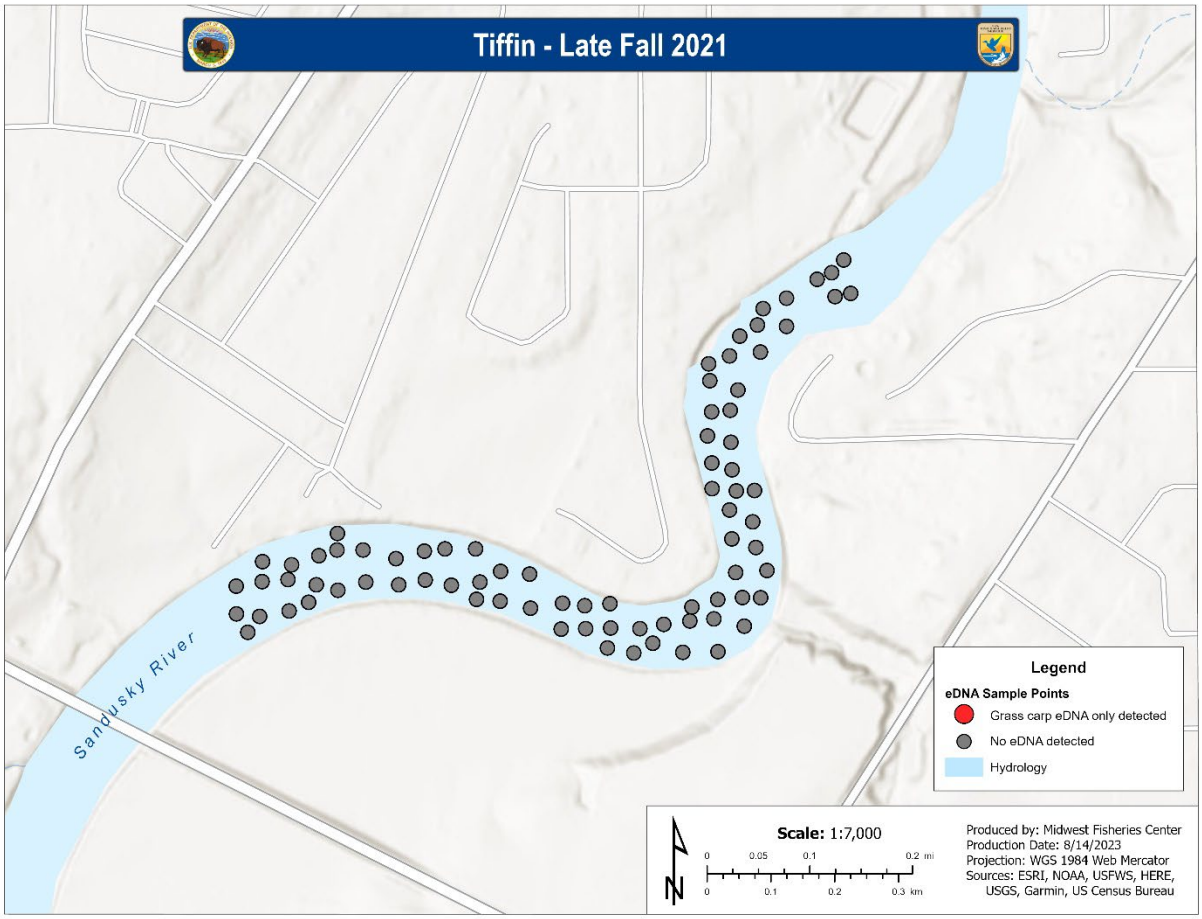

Figure AIII-6. Late Fall 2021 eDNA detections for Tiffin. Gray dots indicate sampling locations where no Grass Carp eDNA was detected.

Appendix IV.

Primer and probe sequences for the three qPCR assays used in this study.

Table IV-1. Grass Carp eDNA qPCR Assay Primers

mtDNA Gene Region	Assay Name	Forward/Reverse	Sequence
ND2	GCTM 10F	Forward	CCYTACGTACTCGCAATTCTAC
ND2	GCTM 10R	Reverse	GTGGTGGTGTTGGGCTATTA
COII	GCTM 22F	Forward	CCGACTCCTAGAAACAGATCAC
COII	GCTM 22R	Reverse	GGGACAGCTCAGGAATGTAATA
COIII	GCTM 32F	Forward	CCACGGACTACACGTCATTATT
COIII	GCTM 32R	Reverse	GATGTTCGGATGTAAAGTGGTATTG

Table IV-2. Grass Carp eDNA qPCR Assay Probes

Probe	5' Modification	Sequence	3' Modification
GCTM10-HEX	HEX	ACCCTAACCTTTGCTAGCTCCCAC	IABkFQ
GCTM 22-FAM	6-FAM	CCAGTTCGTGTCCTAGTATCTGCCGA	IABkFQ
GCTM 32-Cy5	Cy5	TTCCTAGCTGTTTGCCTTCTCCGT	IAbRQSp

Appendix V.

Grass Carp eDNA occurrence Model 1 probability summaries.

Unit	Parameter	Mean	Lower 95% C.I.	Upper 95% C.I.	eDNA Detections
Brady's Island_June	alpha_theta	0.99	0.99	1.00	8
Brady's Island_March	alpha_theta	0.99	0.99	1.00	1
Brady's Island_November	alpha_theta	1.00	0.99	1.00	7
Brady's Island_September	alpha_theta	1.00	0.99	1.00	8
Tiffin Lowhead Dam_November	alpha_theta	0.99	0.98	1.00	1
Wightmans Grove_June	alpha_theta	0.99	0.98	1.00	2
Wightmans Grove_November	alpha_theta	0.99	0.99	1.00	2
Wightmans Grove_September	alpha_theta	0.99	0.99	1.00	27
Brady's Island_June_GCTM10	delta_p	0.01	0.00	0.01	1
Brady's Island_June_GCTM22	delta_p	0.01	0.00	0.01	1
Brady's Island_June_GCTM32	delta_p	0.01	0.00	0.01	6
Brady's Island_March_GCTM32	delta_p	0.01	0.00	0.01	1
Brady's Island_November_GCTM22	delta_p	0.01	0.00	0.01	2
Brady's Island_November_GCTM32	delta_p	0.01	0.00	0.01	5
Brady's Island_September_GCTM10	delta_p	0.01	0.00	0.01	4
Brady's Island_September_GCTM22	delta_p	0.01	0.00	0.01	2
Brady's Island_September_GCTM32	delta_p	0.01	0.00	0.01	2
Tiffin Lowhead Dam_November_GCTM10	delta_p	0.01	0.00	0.01	1
Wightmans Grove_June_GCTM22	delta_p	0.01	0.00	0.01	2
Wightmans Grove_November_GCTM10	delta_p	0.01	0.00	0.01	1
Wightmans Grove_November_GCTM32	delta_p	0.01	0.00	0.01	1
Wightmans Grove_September_GCTM10	delta_p	0.01	0.00	0.01	10
Wightmans Grove_September_GCTM22	delta_p	0.01	0.00	0.01	9
Wightmans Grove_September_GCTM32	delta_p	0.01	0.00	0.01	8

Appendix VI.

Grass Carp eDNA occurrence Model 2 probability summaries.

Unit	Parameter	Mean	Lower 95% C.I.	Upper 95% C.I.	eDNA Detections
Brady's Island_March	beta_psi	0.43	0.14	0.55	1
Brady's Island_June	beta_psi	0.72	0.34	0.93	8
Brady's Island_September	beta_psi	0.57	0.23	0.86	8
Brady's Island_November	beta_psi	0.43	0.14	0.78	7
Wightmans Grove_June	beta_psi	0.67	0.29	0.92	2
Wightmans Grove_September	beta_psi	0.72	0.35	0.93	27
Wightmans Grove_November	beta_psi	0.42	0.13	0.77	2
Brady's Island_June_B-BW-M	alpha_theta	0.98	0.97	0.99	1
Brady's Island_June_MC	alpha_theta	0.98	0.96	0.99	3
Brady's Island_June_SC	alpha_theta	0.98	0.97	0.99	4
Brady's Island_March_MC	alpha_theta	0.98	0.97	0.99	1
Brady's Island_November_MC	alpha_theta	0.99	0.98	1.00	7
Brady's Island_September_MC	alpha_theta	0.99	0.98	1.00	2
Brady's Island_September_SC	alpha_theta	0.99	0.98	1.00	6
Wightmans Grove_March_MC	alpha_theta	0.98	0.96	0.99	1
Wightmans Grove_March_SC	alpha_theta	0.98	0.96	0.99	1
Wightmans Grove_November_MC	alpha_theta	0.99	0.98	1.00	2
Wightmans Grove_September_B-BW-M	alpha_theta	0.99	0.98	1.00	20
Wightmans Grove_September_MC	alpha_theta	0.99	0.98	1.00	4
Wightmans Grove_September_SC	alpha_theta	0.99	0.98	1.00	3
Brady's Island_June_MC_GCTM22	delta_p	0.01	0.00	0.01	1
Brady's Island_June_MC_GCTM32	delta_p	0.01	0.00	0.02	2
Brady's Island_June_SC_GCTM32	delta_p	0.01	0.00	0.02	4
Brady's Island_March_MC_GCTM32	delta_p	0.00	0.00	0.01	1
Brady's Island_November_MC_GCTM22	delta_p	1.00	0.00	0.01	2
Brady's Island_November_MC_GCTM32	delta_p	0.01	0.00	0.01	5
Brady's Island_September_MC_GCTM10	delta_p	0.01	0.00	0.01	1
Brady's Island_September_MC_GCTM22	delta_p	0.01	0.00	0.01	1
Brady's Island_September_SC_GCTM10	delta_p	0.01	0.00	0.01	3
Brady's Island_September_SC_GCTM22	delta_p	0.01	0.00	0.01	1
Brady's Island_September_SC_GCTM32	delta_p	0.01	0.00	0.01	2
Wightman's Grove_June_MC_GCTM22	delta_p	0.01	0.00	0.01	1
Wightman's Grove_June_SC_GCTM22	delta_p	0.01	0.00	0.01	1
Wightman's Grove_November_MC_GCTM10	delta_p	0.01	0.00	0.01	1
Wightman's Grove_November_MC_GCTM32	delta_p	0.01	0.00	0.01	1
Wightman's Grove_September_B-BW-M_GCTM10	delta_p	0.01	0.00	0.02	7
Wightman's Grove_September_B-BW-M_GCTM22	delta_p	0.01	0.00	0.02	7
Wightman's Grove_September_B-BW-M_GCTM32	delta_p	0.01	0.00	0.02	6
Wightman's Grove_September_MC_GCTM10	delta_p	0.01	0.00	0.01	1
Wightman's Grove_September_MC_GCTM22	delta_p	0.01	0.00	0.01	1
Wightman's Grove_September_MC_GCTM32	delta_p	0.01	0.00	0.02	2
Wightman's Grove_September_SC_GCTM10	delta_p	0.01	0.00	0.02	2
Wightman's Grove_September_SC_GCTM22	delta_p	0.01	0.00	0.02	1

Appendix VII

Sandusky River flows from May 1, 2021 to August 1, 2021. June 2021 eDNA sampling event is denoted on the graph and occurred June 21 to 25.

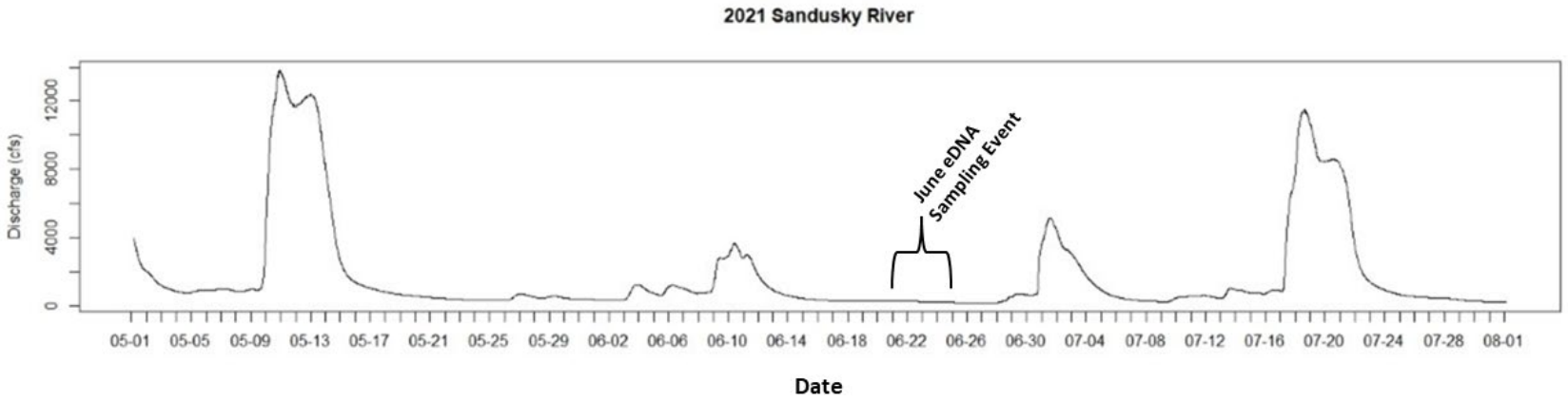

Figure VII-1. Sandusky River flows from May 1, 2021 to August 1, 2021. June 2021 eDNA sampling event is denoted on the graph and occurred June 21 to 25.