Environmental DNA Detection for Endangered Freshwater Mussels Choctawhatchee River Basin

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Executive Summary

The Office of Environmental Management (OEM) of the Florida Department of Transportation (FDOT) contracted with Stantec Consulting Inc. (Stantec) to develop and implement an environmental DNA (eDNA) pilot study to facilitate detection of freshwater mussels. The eDNA methodology implemented here is a relatively new science and has the potential to supplement the Endangered Species Act (ESA) Section 7 consultation process specific to transportation projects, and/or projects which interact with watercourse locations with potential to host protected freshwater mussel species. This study explores the efficiency of eDNA as compared to conventional mussel surveys (i.e., tactile searches) to address ESA consultations, provide mussel assemblage data, reduce overall costs of projects, expedite time-frames for project delivery, and reduce safety concerns. The efficacy of eDNA as a survey method for mussels was evaluated by comparing results to data generated from state and federal prescribed tactile survey methods, which in wadeable streams typically includes snorkel and tactile searching.

Environmental DNA surveys occurred during October of 2022 at six stream bridge crossings within the Choctawhatchee River basin and resulted in the detection of 13 mussel species, including 11 species likewise observed during tactile surveys. Both tactile and eDNA surveys detected four federally protected species (Southern Sandshell [*Hamiota australis*], Choctaw Bean [*Obovaria choctawensis*], Tapered Pigtoe [*Fusconaia burkei*], and Fuzzy Pigtoe [*Pleurobema strodeanum*]).

On 33 of 41 occasions when a species was observed during the tactile surveys it's eDNA was likewise detected. When a species was observed during tactile surveys but not detected with eDNA, it was typically at low numerical abundance (<4 individuals). On 10 of 43 occasions a species was detected with eDNA but not observed during tactile surveys. On these occasions, the detections were at low eDNA abundance. Therefore, species that were detected with only one survey method were often the rarest species within any given bridge location. Additionally, the observed abundance across species and between bridges was strongly correlated with that of the eDNA abundance.

This study simulated eDNA transport via stocking of dead fish at an upstream location. The observed transport distance was often greater than 600 m; however, eDNA abundance metrics from the mussel community assessment provided an indicator for the true presence of a species in close proximity to a surveyed bridge. When a species occurs at low eDNA abundance, it is difficult to differentiate if it is truly present but rare from if the detection was a result of eDNA transport originating from an upstream source.

Across the four bridge locations within Holmes Creek and Tenmile Creek, the tactile surveys required on average 15.5 person hours of search, while the eDNA required on average 6.67 person hours. Environmental DNA surveys will not replace the physical relocation of mussels nor can it provide an assessment of organism and population health (recruitment of juveniles); however, this work demonstrates the practicality of incorporating eDNA as a qualitative-based survey methodology to describe diverse mussel communities including the detection of federally protected species. Environmental DNA provided comparable information to a tactile qualitative survey, while the eDNA collection can be completed in a faster amount of time with a smaller field crew, and in safer conditions.

1.0 INTRODUCTION

North America is comprised of the greatest freshwater unionid mussel richness (bivalve mollusks belonging to the order Unionida), encompassing 303 of 840 global species (Graf & Cummings 2007, Williams et al. 2017, FMCS 2021). However, >70% of North American mussel species are protected and deemed endangered, threatened, or species of concern (Haag & Williams 2014, Williams et al. 2017), mainly due to decreasing water quality from various pollution sources, habitat loss, reduced population-size of host fishes, and/or the introduction of invasive species (Williams et al. 1993, Ricciardi et al. 1998). Mussels play a major ecological role in maintaining freshwater ecosystems through water filtration, nutrient cycling, habitat modification, and serving as food resources for other animals (Vaughn & Hakenkamp 2001, Gutiérrez et al. 2003, Vaughn 2018). Due to their ecological importance and their continuing population declines, the monitoring and management of unionid populations is of high priority. For example, of the 303 currently recognized mussel species within the U.S. (FMCS 2021), nearly one third are federally endangered (77 species) or federally threatened (18 species) (USFWS 2023). Due to their rarity and the fact that unionids burrow into the benthic substrate, tactile surveys are time-consuming and require expert taxonomists. Additionally, tactile surveys require removing the organism from the environment, increasing risk of mortality and resulting in negative effects on health and reproduction. Therefore, novel methods to improve the efficiency and accuracy of surveys could be beneficial for unionid mussel species.

The analysis of environmental DNA (eDNA, which is genetic material released from urine, waste, mucus, or sloughed cells), is becoming increasingly integrated into natural resource surveys designed to detect the presence of special status species and to describe entire assemblages (Beng & Corlett 2020, Deiner et al. 2021). Sampling for eDNA has the potential to provide information on multiple taxa and is therefore a powerful tool for detecting presence of multiple species (Deiner et al. 2017). As tactile mussel surveys can be difficult to conduct and require considerable expertise, eDNA methodology can be more sensitive, less costly, less intrusive on the environment, identify cryptic speciation, and provide sampling capabilities for challenging and remote habitats (Evans et al. 2017, Jerde 2021).

Analysis of eDNA using single-species quantitative Polymerase Chain Reaction (qPCR) methodology has been developed for many groups of freshwater bivalves, such as invasive dreissenid mussels (Gingera et al. 2017, Shogren et al. 2019, Marshall et al. 2021, 2022a), invasive Corbicula clams (Cowart et al. 2018), and numerous unionid mussel species (Cho et al. 2016, Sansom & Sassoubre 2017, Currier et al. 2018, Gasparini et al. 2020, Lor et al. 2020).Given the high diversity of unionid communities within North American waterways, the use of single-species qPCR analysis is inefficient for community-level description of potentially diverse mussel beds. Therefore, an eDNA metabarcoding approach, which can simultaneously identify multiple species, is an alternative approach that has been successfully used to detect bivalve communities in river systems (Klymus et al. 2021, Prié et al. 2021, Coghlan et al. 2021, Marshall et al. 2022b). Metabarcoding can describe diverse mussel beds, including the detection of threatened and endangered species, aiding in environmental impact assessments and support conservation efforts of freshwater mussels.

1.1 PROJECT OBJECTIVES

The goal of this pilot study was to demonstrate the efficacy of eDNA for mussel surveys by comparing data generated from previous tactile surveys to that from eDNA methods. Efforts to repair, maintain, and expand road and bridge networks by the Florida Department of Transportation (FDOT) within the Choctawhatchee River Basin require Section 7 or 10 Endangered Species Act (ESA) consultation with the FWS. Specifically, Section 7 of the ESA states, no public or private entity, can "take" an endangered species of fish or wildlife. "Take" is broadly defined as impacts to federally protected species, such as harass, harm, pursue, hunt, shoot, wound, kill, trap, capture or collect; in addition this may include acts that result in habitat modification or degradation of that for a protected species (USFWS & NMFS 1998). While Section 10 of the ESA may permit an otherwise unlawful "take" if it is incidental to an otherwise lawful activity and the permit applicant has devised an acceptable habitat conservation plan (HCP). Fundamental elements of the ESA consultation process include site specific presence/absence surveys and mussel salvage and relocation efforts. Both activities use formally described methods defined within the "Freshwater Mussel Survey Protocol for the Southeastern Atlantic Slope and Northeastern Gulf Drainages in Florida and Georgia" (GDOT 2018).

The Choctawhatchee River basin is a ~280 km river in the southern United States, flowing through southeast Alabama and the Panhandle of Florida before emptying into Choctawhatchee Bay (Figure 1). In Florida, the river flows for 140 km, draining 10,101 km^2 across five counties: Bay, Holmes, Jackson, Walton, and Washington. The state of Florida supports a diverse assemblage of native freshwater mussels, including 69 species, of which 16 are listed for protection under the federal ESA (see Attachment A). The Choctawhatchee River basin is comprised of 25 mussel species historically, including six federally protected species (Southern Sandshell [*Hamiota australis*], Alabama Moccasinshell [*Medionidus acutissimus*], Choctaw Bean [*Obovaria choctawensis*], Southern Kidneyshell [*Ptychobranchus jonesi*], Tapered Pigtoe [*Fusconaia burkei*], Fuzzy Pigtoe [*Pleurobema strodeanum*]) (see Attachment A) (Blalock-Herod et al. 2005).

The Office of Environmental Management (OEM) of FDOT contracted Stantec Consulting Inc. (Stantec) to develop and implement an eDNA pilot study to facilitate detection of freshwater mussels. This work is anticipated to support the advancement of scientific methodologies for the ESA Section 7 consultation process for specific projects which interact with watercourse locations hosting freshwater mussel species. The main goal of this pilot study was to evaluate the efficacy of eDNA surveys for mussels by comparing data generated to that from tactile survey methods. The **objectives** to achieve this goal included:

1. Coordinate with mussel researchers, agency partners, and those individuals or institutes that may have archived mussel DNA;
    a. Compile a genetic library for use in future projects;
2. Optimize eDNA sample location, sampling intensity, and replication;
    a. Refine field and laboratory techniques for sample collection and processing for use in Florida waters;
3. Compare the results of conventional and eDNA results for mussel presence/ probable absence;
    a. Assess eDNA performance over representative sites of routine conventional mussel projects;
4. Conduct creek system-specific assessment of eDNA transport distances; and
5. Assess costs and benefits of conventional and eDNA sampling approaches.

2.0 METHODS

To evaluate the validity and applicability of eDNA methods for aiding in the ESA consultation process, a Study Plan was developed in coordination with FWS (Attachment B). This Study Plan designed a protocol to sample eDNA from six bridge locations. Four of these bridges were previously surveyed as part of the consultation process using visual and tactile mussel surveys following protocols prescribed in GDOT (2018), while the other two bridge locations were surveyed by FWS malacologists following the same prescribed protocols. These bridge locations provide a unique opportunity to directly compare a consultation framework based on current tactile survey protocols to that designed for an eDNA approach.

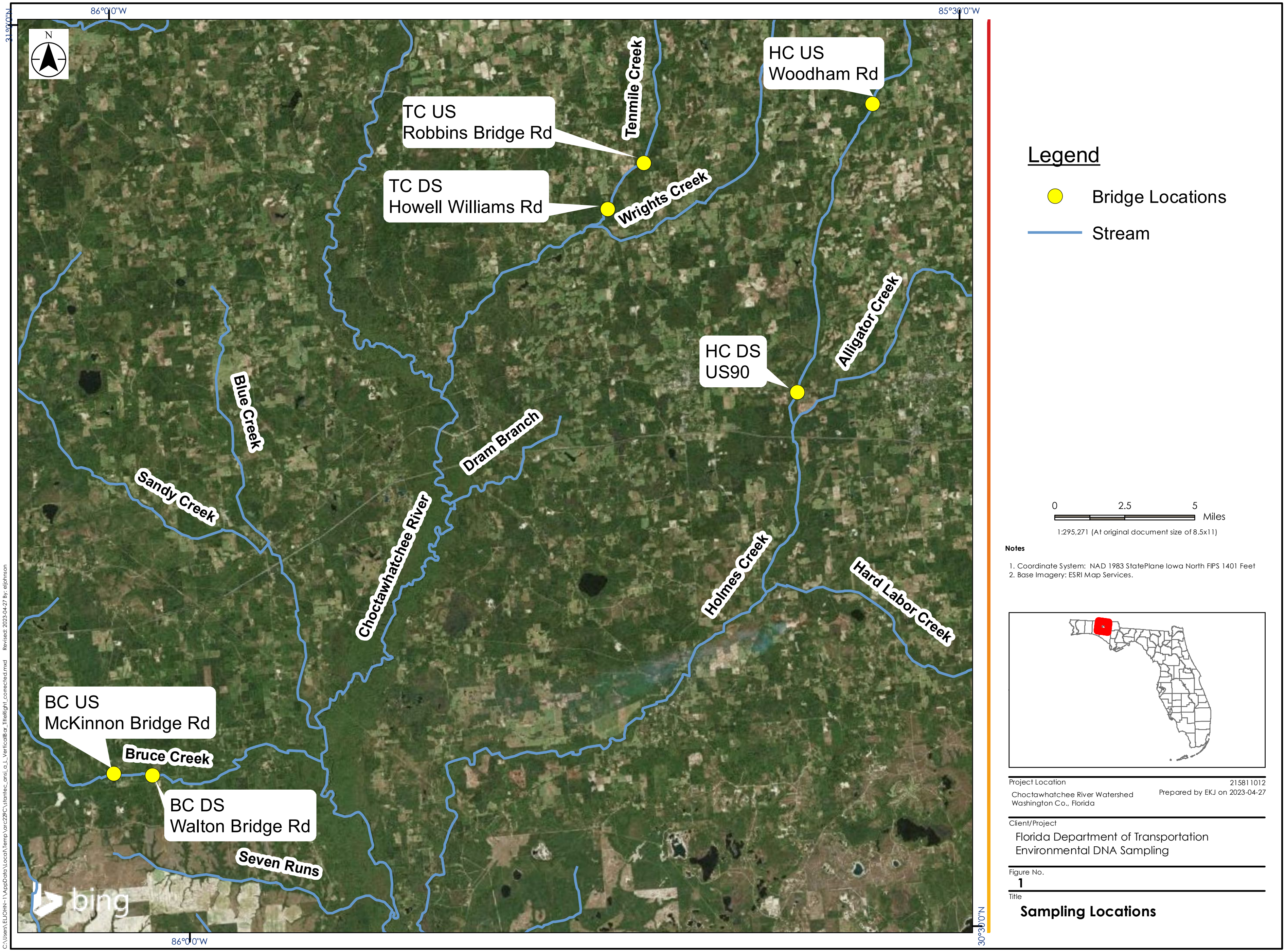
2.1 TACTILE SURVEY DATA

Six bridge locations within the Choctawhatchee River basin were chosen for mussel eDNA sampling (Table 1, Attachment C). These six bridge locations occur in three creek systems (Tenmile Creek, Holmes Creek, and Bruce Creek) (Table 1, Figures 1 – 4). Data were compiled from tactile mussel surveys conducted from 2019 to 2023 within each of these six bridge locations (Table 1). Qualitative surveys as part of a consultation process were conducted in Holmes Creek (May and October 2019) and Ten Mile Creek (October 2019 and August 2022). Biologists from FWS conducted qualitative surveys within Bruce Creek in May of 2023. The methodology used for these surveys was a site-specific modification to the formally described freshwater mussel protocol (Carlson et al. 2008, GDOT 2018). These surveys were conducted during periods of suitable flow with medium to low turbidity. The length of the survey area was variable ranging from 60 m (40 m downstream to 20 m upstream) to 400 m (300 m downstream to 100 m upstream) (Table 1). The length of the survey varied based on an adaptive study plan within the field, allowing the biologist to focus on locations of high mussel density. Time was recorded over the entire survey area to calculate a catch per unit effort (CPUE) for each creek reach, which ranged from 1.73 to 86.36 (Table 1).

Tactile surveys proceeded from the downstream end of the survey area and moved upstream to minimize turbidity. Habitat in the search area was assessed and all represented habitat types were searched using visual and tactile means (hand-grubbing 1-2 inches into substrate to search for deeply buried mussels). All mussels found were sorted by species, placed in mesh bags, and photographed in groups. Out of water time was minimized to limit handling stress. Federally protected species were carefully placed back into the sediment in a posterior-up position where they were originally found. A federally permitted collector was required to be present on site at all surveys.

Table 1 Bridge locations for eDNA sampling conducted in the Choctawhatchee River basin.

Creek	Bridge	Site Name	Latitude	Longitude	Year of Tactile Survey	Search Length	Person Hours	CPUE
Tenmile Creek	Robbins Bridge	TC US	30.902	-85.693	2019	250m	15	1.73
Tenmile Creek	Howell Williams	TC DS	30.880	-85.717	2022	150 m	15	22.00
Holmes Creek	Woodham	HC US	30.921	-85.556	2019	200 m	18	6.72
Holmes Creek	US90	HC DS	30.779	-85.616	2019	60 m	14	86.36
Bruce Creek	McKinnon Bridge	BC US	30.617	-86.036	2023	400 m	16	27.13
Bruce Creek	Walton Bridge	BC DS	30.614	-86.014	2023	400 m	16	23.38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85°30'0"W
31°0'0"N
30°30'0"N
N
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Woodham Rd
TC US
Robbins Bridge Rd
TC DS
Howell Williams Rd
HC DS
US90
BC US
McKinnon Bridge Rd
BC DS
Walton Bridge Rd
Tenmile Creek
Wrights Creek
Alligator Creek
Blue Creek
Dram Branch
Sandy Creek
Choctawhatchee River
Holmes Creek
Hard Labor Creek
Bruce Creek
Seven Runs
Legend
Bridge Locations
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2. Base Imagery: ESRI Map Services.
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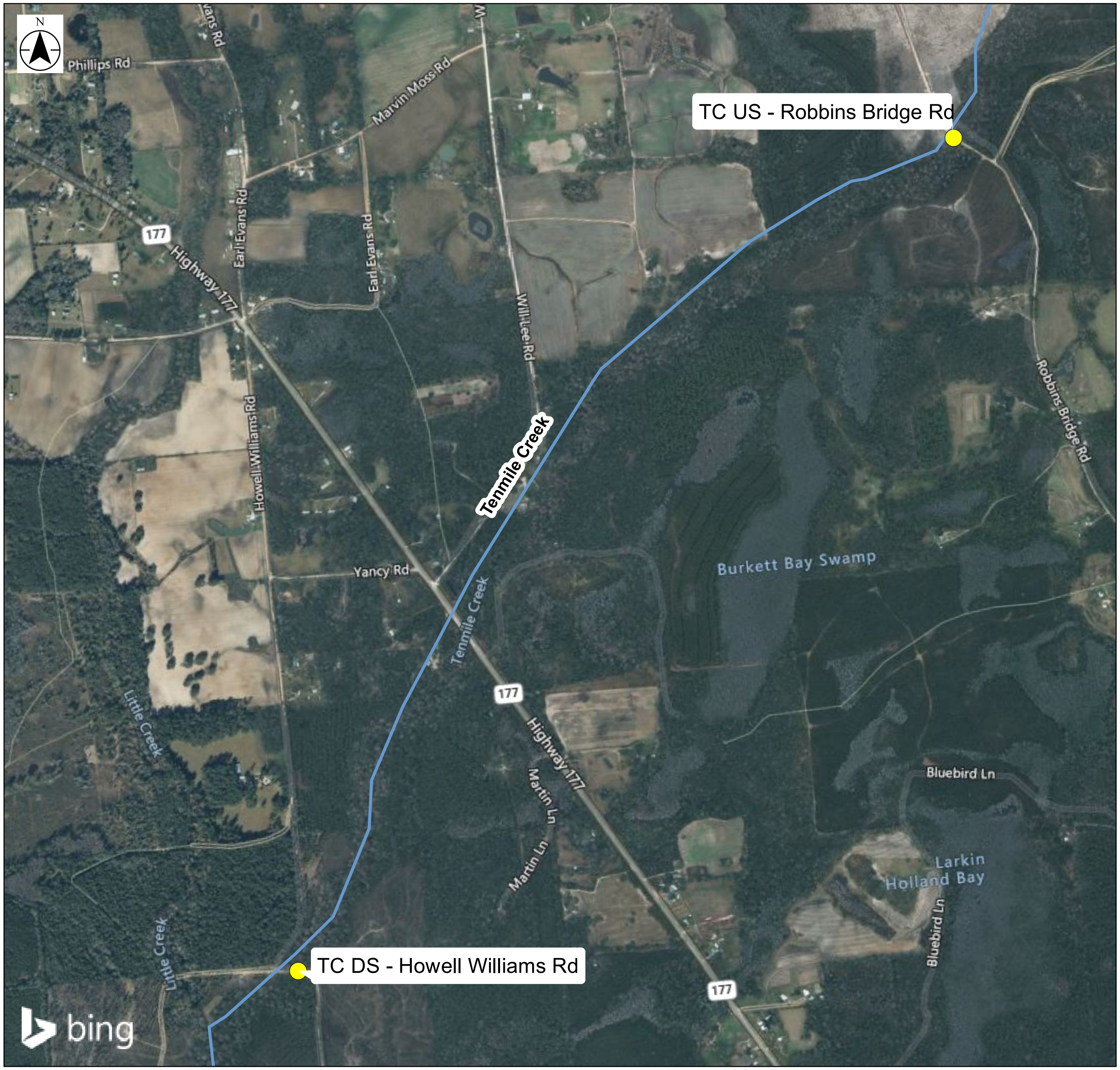
Choctawhatchee River Watershed
Washington Co., Florida
Prepared by EKJ on 2023-04-27
Client/Project
Florida Department of Transportation
Environmental DNA Sampling
Figure No.
1
Title
Sampling Locations



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- Bridge Locations
- Stream

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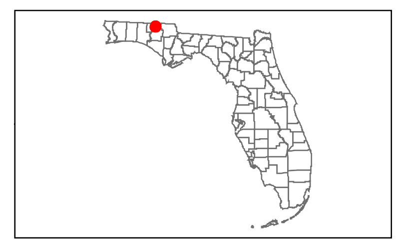

Project Location
Choctawhatchee River Watershed
Washington Co., Florida

215811012
Prepared by EKJ on 2023-04-27

Client/Project
Florida Department of Transportation
Environmental DNA Sampling

Figure No.
2

Title
Tenmile Creek - Sampling Locations

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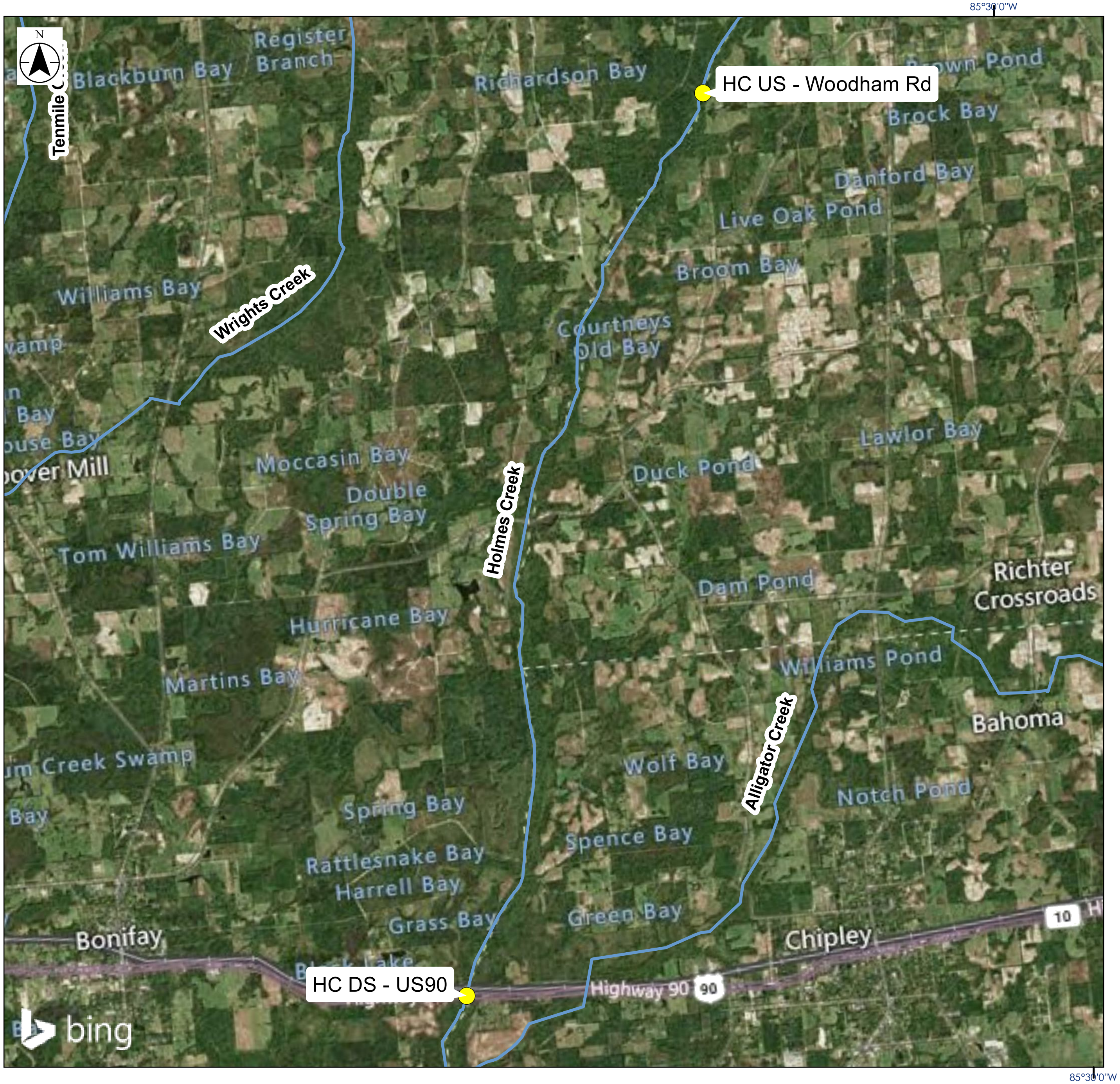

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- Bridge Locations
- Stream

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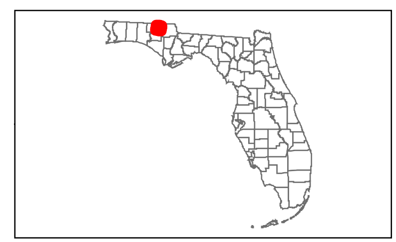

Project Location 215811012

Choctawhatchee River Watershed
Washington Co., Florida

Prepared by EKJ on 2023-04-27

Client/Project

Florida Department of Transportation
Environmental DNA Sampling

Figure No.

3

Title

Holmes Creek - Sampling Locations

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Legend

Bridge Locations

Stream

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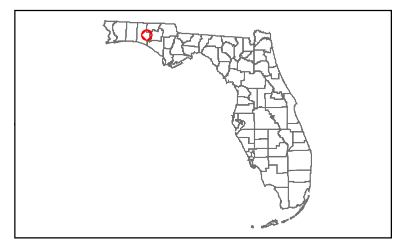

Project Location
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Choctawhatchee River Watershed
Washington Co., Florida
Prepared by EKJ on 2023-04-27

Client/Project
Florida Department of Transportation
Environmental DNA Sampling

Figure No.
4

Title
Bruce Creek - Sampling Locations

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2.1.1 Mussel eDNA Collection

The eDNA survey design included three levels of sampling, which resulted in 18 water samples per bridge.

(1) Six **bridge locations** – two bridges in each of three creeks;
(2) Six **sampling sites** per bridge location – spaced 100 m apart; and
(3) Triplicate 1 L **water samples** per sampling site.

Within each of the six bridge locations, six sampling sites were spaced evenly 100 m apart, spanning from 300 m downstream of the bridge to 200 m upstream of the bridge (Figure 5). Triplicate water samples were collected from each of the six sampling sites per bridge location, for 18 total eDNA water samples. At each sampling site, 1000 mL water samples were collected from near the substrate using a peristaltic pump with a polypropylene filter holder attached to a painter's pole. eDNA was collected along a transect spanning the width of the river for each replicate sample. Once the first sample was collected, the surveyor took the next sample ~2 m upstream, and continued this for three replicates per s site. Samples were filtered on a 47-mm-diameter glass microfiber filter GF/C (nominal pore size 1.2 µm; GE Healthcare Life Science, MA, USA). Filters were preserved by placing them into separate coin envelopes, which were then placed in Ziploc bags with silicone desiccant beads and stored in a freezer (-20°C). At each bridge location, the water temperature, pH, dissolved oxygen, and conductivity were recorded (Table 2).

2.1.2 Fixed-point eDNA Transport Setup

Within flowing systems, eDNA detection may be driven by water flow and hydrological dynamics of the river. To estimate the potential distance an eDNA molecule may be transported, we measured the eDNA concentration of a fixed-point source at increasing distances downstream. These concentrations were then modeled to characterize the effect of distance on eDNA concentration and detection (Espe et al. 2022). To characterize eDNA dispersal, fixed-point DNA source experiments were completed at one bridge location within each creek system in conjunction with sample collection (Table 2). A controlled eDNA source was implemented at 300 m upstream of the bridge location by tethering 12 individuals of the commercially available frozen bait Ballyhoo (*Hemiramphus brasiliensis*) in a laundry bag that was tied to a post in the midsection of the creek (Figure 5, see Attachment C). Ballyhoo is a marine fish species that does not occur in the study area. The sampling distance intervals ranged from 0 to 600 m away from the eDNA source and were collected on 100 m intervals (Figure 5). At the location of the Ballyhoo, the creek flow rate was estimated by averaging three measurements of flow using a Global Water Portable Flow Probe (Table 2). Next, the amount of time required for water to travel a distance of 600 m (the farthest sampling point downstream) was calculated. The first sample was collected from the downstream 600 m site after the calculated time had passed. Triplicate water samples were collected at each distance interval in Tenmile and Holmes Creek using 1.2 µm GF/C filters.

Nagler et al. (2022) has suggested that small fractions of eDNA (e.g., cell organelles, extracellular pieces of DNA) are likely to be transported larger distances than the large fraction of eDNA (e.g., pieces of tissue, full intact cells). Therefore, at the Bruce Creek Ballyhoo eDNA bridge location, a sampling approach was designed to test the impacts of filter-pore size on eDNA concentration with increasing distance from the source. From each sampling site, duplicate water samples were collected each from a 1.2 µm GF/C filter and an 8.0 µm polycarbonate filter.

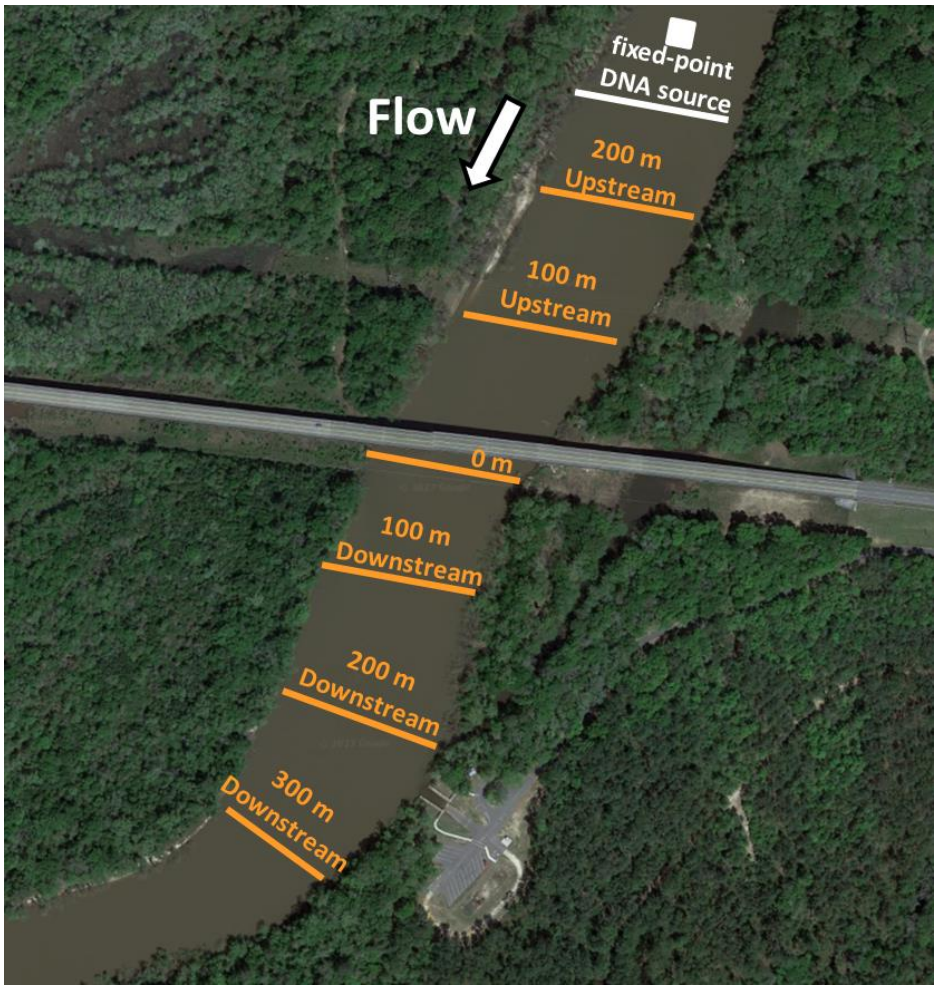

Figure 5 Sampling design at each bridge location with six sites spanning from 300 m downstream to 200 m upstream of a bridge. A fixed-point DNA source (300 m upstream) was included at one bridge location within each creek system.

2.1.3 Field Controls

On each sampling day after the first bridge location was surveyed, 500 mL of distilled water was poured into a clean Nalgene bottle in the field and filtered using peristaltic pump system to act as a field negative control. All collection equipment was decontaminated with 10% bleach solution after each day of sampling. The eDNA surveyor wore new sterile nitrile gloves at each site and when handling eDNA equipment. All filters were shipped on ice to Cramer Fish Science Genidaqs (Sacramento, California, USA, https://genidaqs.com) for DNA extraction and metabarcoding using Illumina MiSeq processing.

Table 2 Water quality and fixed-point environmental DNA sites for each of the six bridge locations within the Choctawhatchee River basin.

Creek	Bridge Name	Date	Temperature °F (°C)	pH	DO (mg/L)	DO (%)	Conductivity (µS/cm)	Fixed-point Ballyhoo	Flow (ft/s)	Wait time before sampling (mins)
Tenmile Creek	Robbins Bridge	Oct 18, 2022	63 (17.2)	7.64	9.37	98.6	250			
Tenmile Creek	Howell Williams	Oct 20, 2022	57 (13.9)	8.14	10.66	99.6	207	Yes	0.30-0.65	66
Holmes Creek	Woodham	Oct 19, 2022	59 (14.8)	7.48	8.22	82.8	235	Yes	0.50-0.70	60
Holmes Creek	US90	Oct 19 & 20, 2022	60/53 (15.5/11.9)	6.8	9.19	92.9	226			
Bruce Creek	McKinnon Bridge	Oct 18, 2022	64 (17.7)	8.5	9.1	96.7	34	Yes	0.70-0.90	50
Bruce Creek	Walton Bridge	Oct 19, 2022	57 (14.0)	7.85	10.7	102.1	37			

2.2 LABORATORY AND DATA PROCESSING

Environmental DNA metabarcoding consists of multiple steps in the laboratory sample and bioinformatic data processing workflow (Figure 6) (see Pawlowski et al. 2018)). Laboratory processing of an eDNA water sample includes (1) the extraction and isolation of DNA fragments, (2) PCR amplification using a genetic assay targeting the taxonomic community of interest (e.g., freshwater mussels), and (3) high-throughput sequencing (HTS) (e.g., Illumina MiSeq) to obtain the DNA sequences of the amplified DNA (Figure 6). A MiSeq Illumina dataset typically results in >10,000,000 DNA sequence reads, and bioinformatic data processing steps are required to quality filter the sequences and to cluster the millions of DNA sequence reads into unique representative units. This process is completed by (4) quality filtering to reduce sequencing error and dereplicating identical sequences to obtain a representative sequence list, (5) further clustering the sequences into Molecular Operational Taxonomic Units (MOTUs) to account for intraspecific variation, and (6) assigning taxonomic identification based on a genetic voucher database (Figure 6).

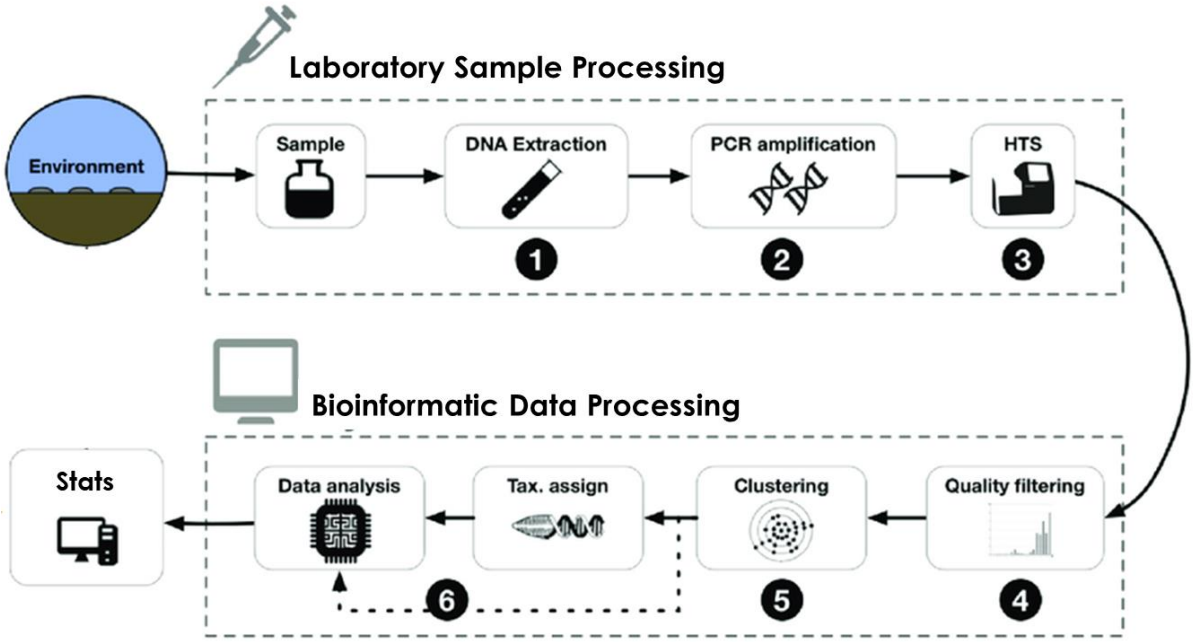

Figure 6 Schematic depicting the workflow for laboratory sample and bioinformatic data processing in an environment DNA metabarcoding study. Adopted from Pawlowski et al. (2018).

2.2.1 Sample DNA Extraction

The DNA was extracted from each filter using a modified Qiagen DNeasy® Blood and Tissue Kit protocol. In brief, each filter was processed overnight at 56°C in 540 µl ATL and 60 µl Proteinase K. The resulting supernatant was passed through a Qiashredder spin column, mixed with 600 µl AL and incubated at 70ºC for 10min. After adding 600 µl ethanol, the resulting mixture was loaded onto a DNeasy Spin column following manufacture's protocol, with a final elution volume of 100 µl. The DNA was further processed with a Zymo Research One Step PCR Inhibitor Removal kit (Zymo Research, Irvine, California, USA). A negative control was simultaneously extracted to test for possible laboratory contamination.

2.2.2 Polymerase Chain Reaction – DNA Amplification

To analyze the DNA mixture within each collected water sample, a specific DNA region of interest is targeted and copied millions of times through a process known as PCR. For freshwater mussel analysis, each water sample was amplified for a ~175 bp fragment of the 16S gene region which has previously been tested for amplification of unionid mussels from eDNA samples (Marshall et al. 2022b, Prié et al. 2021). Mussel eDNA was sequenced with MiSeq Illumina metabarcoding as previously described in Marshall et al. (2022b) (See Attachment D for more information).

From bridge locations that included a collection of Ballyhoo eDNA, samples were analyzed for presence of Ballyhoo via qPCR (Genidaqs; unpublished assay). All filters were processed as mentioned above. DNA extractions were analyzed with three technical qPCR replicates for a total of nine data points per distance in Holmes Creek and Tenmile Creek and six data points per filter type at each distance in Bruce Creek. A qPCR replicate was considered positive if amplification occurred prior to amplification cycle 40, and the eDNA concentration (ng/µL) was quantified using a log-dilution standard curve consisting of DNA extracted from Ballyhoo tissue.

2.2.3 Bioinformatic Processing & Taxonomic Identification

The data was processed following a bioinformatic pipeline previously described in Marshall et al. (2022b) (See Attachment D for more information). Many species within the geographic sampling region are currently missing available genetic vouchers for the 16S gene region. Therefore, many of the current taxonomic identifications are based on sequence similarity to close relatives and the known mussel assemblage within these three creeks. Updated voucher sequences have been cross referenced with collaboration from Dr. N. Johnson's mussel genetics laboratory (United States Geological Survey (USGS) Wetland and Aquatic Research Center) for accurate identification. This updated data provided new sequences for mussel populations present within the Choctawhatchee River basin, thereby providing the most accurate database to incorporate potential genetic variation between populations.

2.3 STATISTICAL ANALYSIS

To compare the species richness detected with each survey, a Welch two sample t-test was used to compare observed richness within each of the six bridge locations between the two methods. To compare estimates of eDNA abundance with observed mussel abundance, the eDNA sequence read abundance (defined as the mean count that a DNA sequence appears across all water replicates within a sampling site) was compared to the total observed mussel abundance at each bridge location for each species. The eDNA sequence read abundance was calculated by averaging the sequence read counts across the triplicate water samples within each site. The sequence read abundance for each species was further averaged across the six sampling sites per bridge to obtain an abundance estimate per bridge. Linear regression was used to compare abundances for each species at all six bridge locations between the two survey methods.

The mean mussel abundance (log-transformed) was compared between species successfully detected with eDNA to those undetected with eDNA using a Welch two sample t-test. Finally, to evaluate mussel presence when a species was undetected with the tactile survey but detected with eDNA, the mean eDNA sequence

read abundance (log-transformed) was compared between species detected with both methods to those undetected with the tactile survey using a Welch two sample t-test.

Occupancy estimation is a model-based approach to estimate the probability of species presence in an area while accounting for the imperfect detection probabilities that are inherent in most sampling methods (MacKenzie et al. 2002). Using the eDNA data, single-season occupancy models were evaluated for all species to estimate the mean occupancy (ψ) (i.e., the estimated number of occupied sites) and the mean detection probability (*p*) (i.e., the probability of successful eDNA detection of a species within a replicate environmental sample). Next, the survey design was evaluated by calculating the cumulative site-level detection probability (*p**) for each species: $p^* = 1-(1-p)^n$ where *p* is the estimated detection for a single replicate environmental sample and n is the total number of replicates (Hagler et al. 2011). Occupancy models were analyzed in the R package 'ednaoccupancy' (Dorazio & Erickson 2018). To evaluate the eDNA protocol, the estimated probability of detection for each species was further compared to the observed species abundance during the tactile surveys.

3.0 RESULTS

3.1 BIOINFRORMATIC PROCESSING

The MiSeq data resulted in 13,920,277 raw sequence reads, of which 5,936,653 were filtered through the bioinformatic pipeline and retained for final analysis. On average each sample retained 68,237.39 (80,916.84 Standard Deviation). (See Attachment D for more information). The downstream bridge sites at all three creeks provided the greatest number of mussel sequence abundances (Tenmile Creek –Robbins Bridge = 125,612 *vs* Howell Williams = 1,441,755; Holmes Creek – Woodham = 373,158 *vs* US90 = 1,825,415; Bruce Creek – McKinnon Bridge = 20,463 *vs* Walton Bridge = 2,148,012). Rarefaction curve analysis suggest sufficient sequence read depth across the samples to provide appropriate analysis of the mussel community assemblage.

3.1.1 Field Controls

Two of the three field negative controls yielded low measures of unionid sequences (Tenmile Creek – total 188 sequence reads and Holmes Creek – total 139 sequence reads). In both of these negative controls, the sequences corresponded to two species not presumed present within sampling region (Wabash Pigtoe [*Fusconaia flava*] and Kidneyshell [*Ptychobranchus fasciolaris*]). Incorporating a sequence read threshold (<10 reads) and a sample replicate threshold (present in >1 replicate per sampling site) removed the presence of these sequences from any sample that they appeared.

3.1.2 Taxonomic Identification

The MiSeq data from the six bridge locations resulted in 13 taxa assigned as a unionid female mitotype spanning across four unionid tribes (Table 3). The identified taxa range from 85-100% identity to a species within the curated genetic database (see Attachment E for more information).

3.2 TACTILE SURVEY COMPARED TO ENVIRONMENTAL DNA SURVEY

3.2.1 Detection within the Choctawhatchee River basin

Results of tactile surveys conducted from 2019 to 2023 at the six bridge locations collected 2,712 mussels representing 12 species, including one federally endangered species (Choctaw Bean) and three federally threatened species (Southern Sandshell, Tapered Pigtoe, and Fuzzy Pigtoe) (Table 3). The eDNA survey detected 13 species across the six bridge locations, 11 of which were found during the tactile surveys (Table 3). The four federally protected species observed during tactile surveys were also detected with the eDNA survey (Table 3). Only a single species were observed in the tactile survey but absent from the eDNA survey. This was fourth least abundant species within the tactile surveys across all sites, at 10 individuals of Purple Pigtoe (*Cyclonaias succissa*) (Table 3). In the eDNA survey, three samples did provide sequence reads for this species; however, all detections of Purple Pigtoe were removed from the dataset as it was inconsistently detected across replicates and with low sequence read abundance, and therefore was considered to be below the threshold set for positive detection.

Table 3 Mussel abundance found during tactile surveys and corresponding environmental DNA detection from all six bridge locations within the Choctawhatchee River basin.

Tribe	Species	Common Name	Tactile	eDNA
Anodontini	*Strophitus williamsi*	Flatwoods Creekshell	11	X
Lampsilini	*Hamiota australis* FT	Southern Sandshell	61	X
	Lampsilis straminea	Rough Fatmucket	8	X
	Obovaria choctawensis FE	Choctaw Bean	7	X
	Toxolasma corvunculus	Southern Purple Lilliput	23	X
	Toxolasma sp.		–	X
	Leaunio lienosus	Little Spectaclecase	801	X
	Leaunio sp.		–	X
	Villosa vibex	Southern Rainbow	765	X
Pleurobemini	*Elliptio* sp.	Gulf Spike	707	X
	Fusconaia burkei FT	Tapered Pigtoe	304	X
	Pleurobema strodeanum FT	Fuzzy Pigtoe	12	X
Quadrulini	*Cyclonaias succissa*	Purple Pigtoe	10	–
	Uniomerus columbensis	Apalachicola Pondhorn	3	X
		Total Species	*12*	*13*

FT = Federally Threatened, FE = Federally Endangered

X = Detected with eDNA

Red = Detected with tactile only, Orange = detected with eDNA only, Green = detected with both

3.2.2 Comparison of Species Abundance

The two survey methods provided similar estimates of species richness across the six bridge locations, (mean species richness in the tactile survey = 6.83, mean species richness in the eDNA survey = 7.17, t = 0.2187, df = 8.9391, p-value = 0.8318). Within the individual bridge locations, a mussel species was observed during tactile surveys 41 times (Figure 7A & 7B), of which eDNA successfully detected the same species on 33 of these occasions (80%). Of the eight times a species was not detected with eDNA, two occasions occurred with a federally protected species (Fuzzy Pigtoe from Walton Bridge in Bruce Creek and Choctaw Bean from McKinnon Bridge in Bruce Creek) (Figure 7A). On 10 occasions, a species was detected with eDNA but not observed at the same bridge location, of which two occasions occurred for the federally threatened Tapered Pigtoe and two occasions occurred for the federally threatened Southern Sandshell (Figure 7A). The abundance estimated for each species per bridge location was positively correlated between the tactile and eDNA surveys, with all bridges displaying a strong correlation with the exception of McKinnon Bridge in Bruce Creek (Spearman Correlation = 0.74, R^2 = 0.53, p < 0.001***) (Figure 7C).

The mean mussel abundance per species determined from the tactile survey did not differ between creeks (Figure 8A); however, the three downstream bridge locations displayed higher mean mussel abundance per species than the three upstream bridge locations (mean mussel abundance in upstream bridge locations = 8.72, mean mussel abundance in downstream bridge locations = 49.27, t = 3.26, df = 28.91, p-value = 0.003**) (Figure 8C). Similarly, the mean eDNA abundance per species did not differ between creeks (Figure 8B); however, the downstream bridge locations displayed higher mean eDNA abundance per species than the upstream bridge locations (mean eDNA abundance in upstream bridge locations = 279.57, mean eDNA abundance in downstream bridge locations = 4375.81, t = 2.95, df = 22.57, p-value = 0.007**) (Figure 8D).

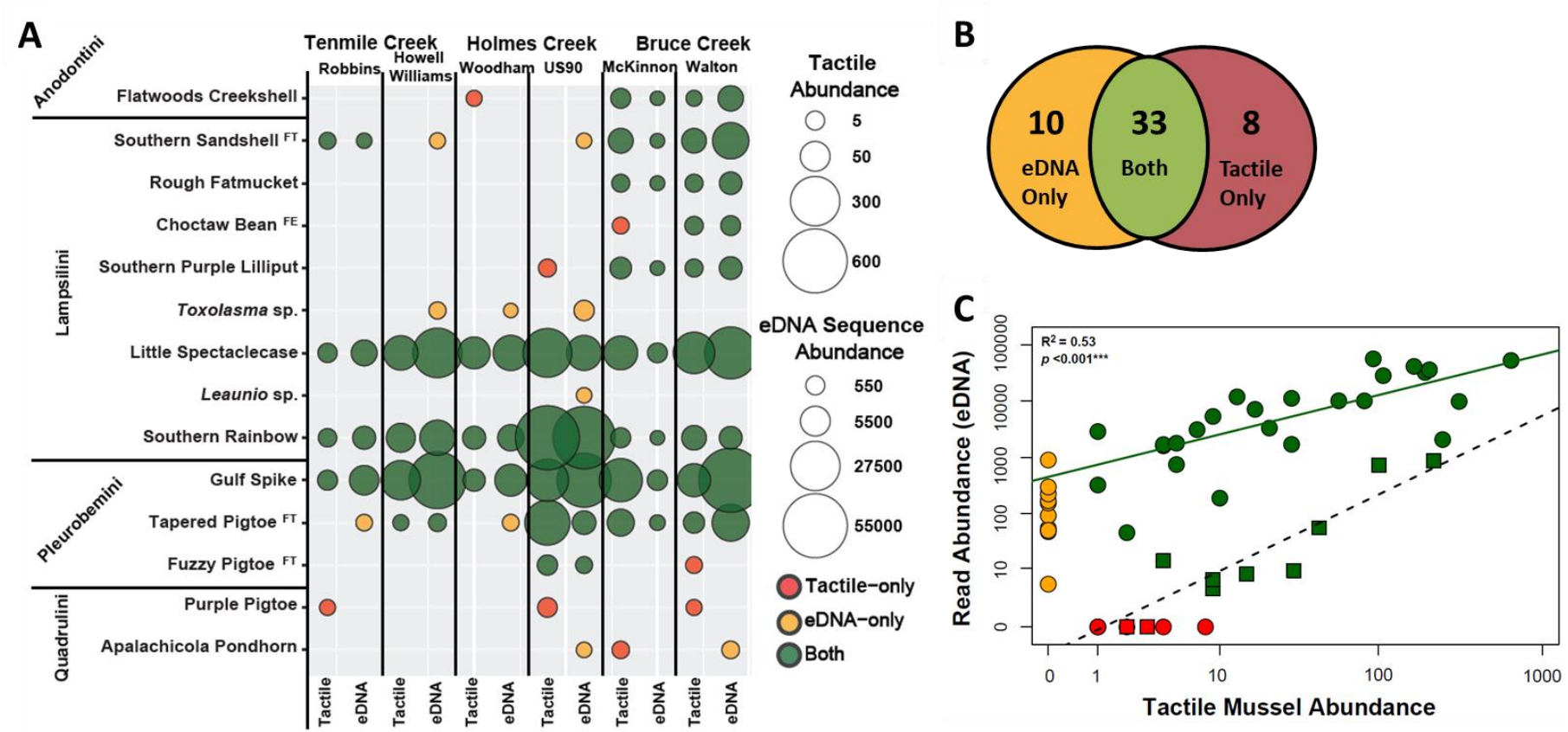

Figure 7 (A) Species detected and their abundance within the six bridge locations in the Choctawhatchee River basin determined from tactile or environmental DNA surveys. FT = Federally Threatened, FE = Federally Endangered. (B) Number of species detected with each survey method. (C) Relationship between the abundance per sepcies from each of the six bridge locations in the Choctawhatchee River basin determined from tactile or environmental DNA surveys. Squares represent outlier points from McKinnon Bridge in Bruce Creek.

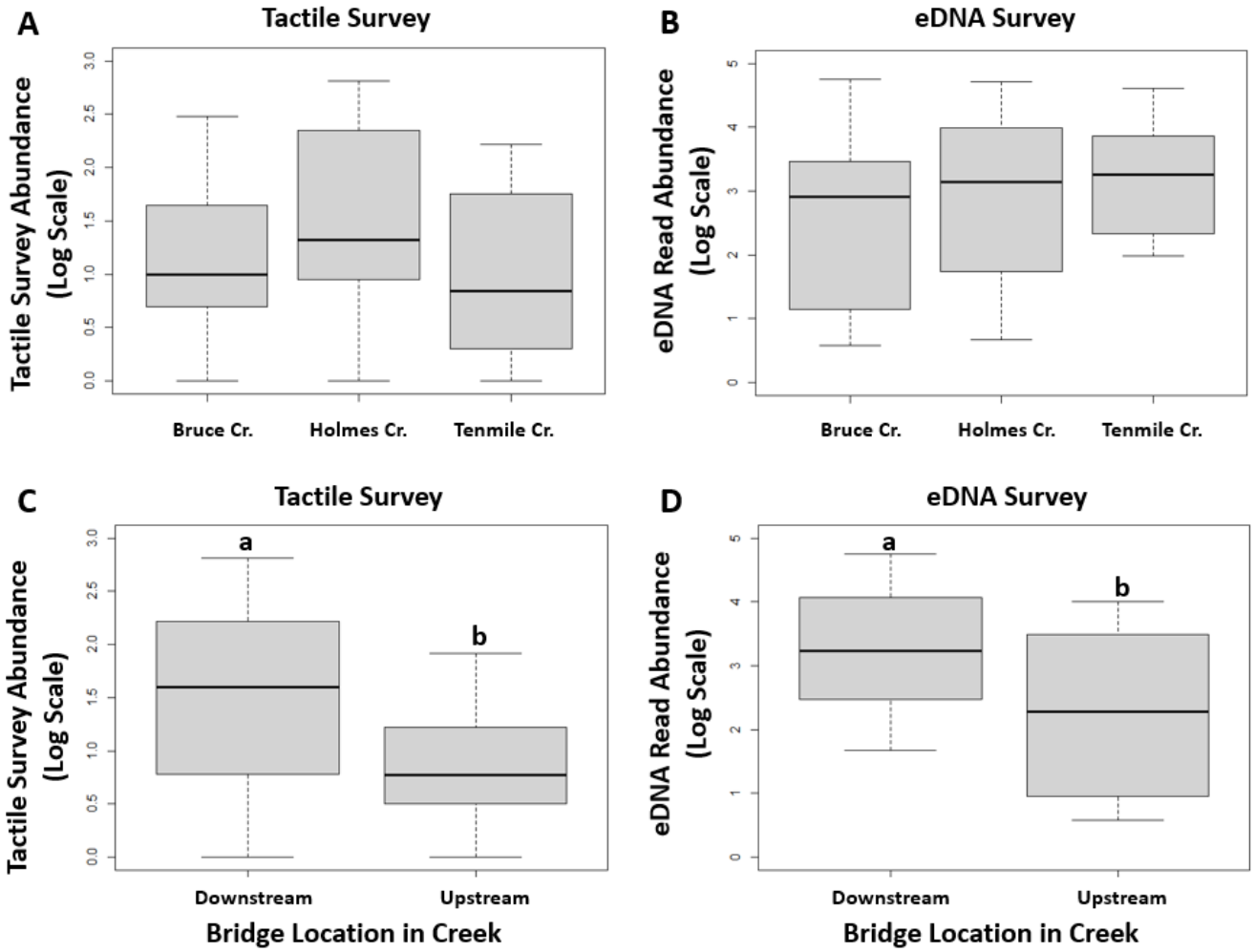

Figure 8 Mean ± standard error mussel abundance (A) and environmental DNA abundance (B) from each of the three creek systems within the Choctawhatchee River basin. Mean ± standard error mussel abundance (C) and environmental DNA abundance (D) based on the bridge location within the creek (upstream or downstream).

The center line indicates the median, and each box indicates the lower and upper quartiles. Ends of the whiskers indicate maximum and minimum values within the quartile ± 1.5 x interquartile spread. Open circles (if present) indicate values falling outside the quartile ± 1.5 x interquartile spread. Lowercase letters designate significant difference between groups (p<0.05).

3.2.3 Spatial Analysis at each Bridge

3.2.3.1 Tenmile Creek

Two federally protected species were detected with both surveys (Table 4); however, eDNA detected the two species at both bridge locations, while the tactile survey only observed two individuals of Southern Sandshell at the Robbins Bridge and one individual of Tapered Pigtoe at Howell Williams (Table 4). Additionally, eDNA detected an unknown *Toxolasma* sp. species at Howell Williams (Table 4). The tactile survey observed a single individual of Purple Pigtoe at Robbins Bridge which went undetected within the eDNA dataset (Table 4).

Table 4 Mussel abundance found during tactile surveys and corresponding environmental DNA detection from two bridge locations within Tenmile Creek.

			Tenmile Creek			
			Robbins Bridge		Howell Williams	
Tribe	Species	Common Name	Tactile	eDNA	Tactile	eDNA
Lampsilini	*Hamiota australis* FT	Southern Sandshell	2	X	–	X
	Toxolasma sp.		–	–	–	X
	Leaunio lienosus	Little Spectaclecase	7	X	107	X
	Villosa vibex	Southern Rainbow	5	X	57	X
Pleurobemini	*Elliptio* sp.	Gulf Spike	9	X	165	X
	Fusconaia burkei FT	Tapered Pigtoe	–	X	1	X
Quadrulini	*Cyclonaias succissa*	Purple Pigtoe	1	–	–	–
		Total Number of Species Detected	*5*	*5*	*4*	*6*

FT = Federally Threatened, FE = Federally Endangered
X = Detected with eDNA
Red = Detected with tactile only, Orange = detected with eDNA only, Green = detected with both

The eDNA sequence read abundance was greater at Howell Williams compared to Robbins Bridge (Figure 9A). Species richness and overlap between the two bridge locations was similar, with one more species detected in Howell Williams (the unknown *Toxolasma* sp. species) (Figure 9A). The relative abundance of the mussel community between eDNA sampling sites was similar within both Robbins Bridge and Howell Williams (Figure 9B). The relative mussel abundance estimated from eDNA was strongly correlated with that estimated from tactile surveys (Figure 9C).

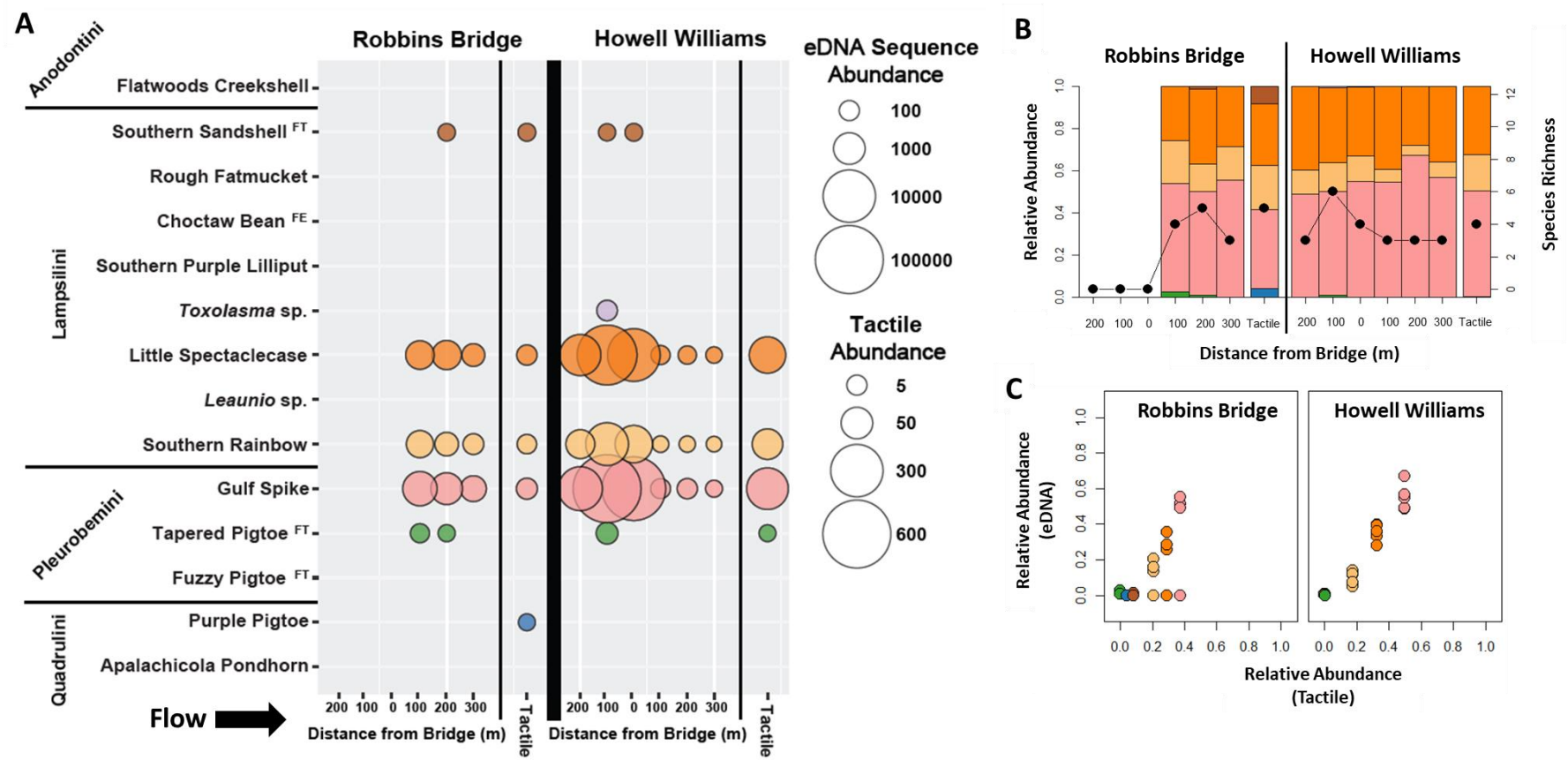

Figure 9 (A) Environmental DNA species detection and sequence read abundance within the two bridge locations in Tenmile Creek. FT = Federally Threatened, FE = Federally Endangered. (B) Species richness (black points) and releative abundance (bar plots) along the six eDNA sampling sites and the tactile survey within Tenmile Creek. (C) Comparitive relative abundance estimates from eDNA and tactile surveys within Tenmile Creek.

3.2.3.2 Holmes Creek

Two federally protected species were detected with the tactile survey, while the eDNA survey detected three federally protected species (Table 5). Tapered Pigtoe and Fuzzy Pigtoe were detected with both surveys, while the eDNA survey additionally detected Southern Sandshell at US90. While Southern Sandshell was not observed, it was detected in four of the six sampling sites with eDNA (Figure 10A). Environmental DNA detected an unknown *Toxolasma* sp. species and Apalachicola Pondhorn (*Uniomerus columbensis*) (Table 5), while the tactile survey observed a single individual of Flatwoods Creekshell (*Strophitus williamsi*) and four individuals of Southern Purple Lilliput (*Toxolasma corvunculus*) which went undetected with eDNA (Table 5). Additionally, eight individuals of Purple Pigtoe were observed at US90; however only a single eDNA replicate water sample provided low abundance of sequence reads for this species, and thus it was considered undetected with eDNA (Table 5).

Table 5 Mussel abundance found during tactile surveys and corresponding environmental DNA detection from two bridge locations within Holmes Creek.

			Holmes Creek			
			Woodham		US90	
Tribe	Species	Common Name	Tactile	eDNA	Tactile	eDNA
Anodontini	*Strophitus williamsi*	Flatwoods Creekshell	1	–	–	–
Lampsilini	*Hamiota australis* FT	Southern Sandshell	–	–	–	X
	Toxolasma corvunculus	Southern Purple Lilliput	–	–	4	–
	Toxolasma sp.		–	X	–	X
	Leaunio lienosus	Little Spectaclecase	82	X	311	X
	Leaunio sp.		–	–	–	X
	Villosa vibex	Southern Rainbow	21	X	644	X
Pleurobemini	*Elliptio* sp.		17	X	205	X
	Fusconaia burkei FT	Tapered Pigtoe	–	X	247	X
	Pleurobema strodeanum FT	Fuzzy Pigtoe	–	–	10	X
Quadrulini	*Cyclonaias succissa*	Purple Pigtoe	–	–	8	–
	Uniomerus columbensis	Apalachicola Pondhorn	–	–	–	X
		Total Number of Species Detected	*4*	*5*	*7*	*9*

FT = Federally Threatened, FE = Federally Endangered

X = Detected with eDNA

Red = Detected with tactile only, Orange = detected with eDNA only, Green = detected with both

The eDNA sequence read abundance was greater at US90 compared to Woodham (Figure 10A). Species richness was greater at US90, with four species only detected in this location (Figure 10A). The relative abundance of the mussel community between eDNA sampling sites was similar within both Woodham and US90 (Figure 10B). The relative mussel abundance estimated from eDNA was strongly correlated with that estimated from tactile surveys (Figure 10C).

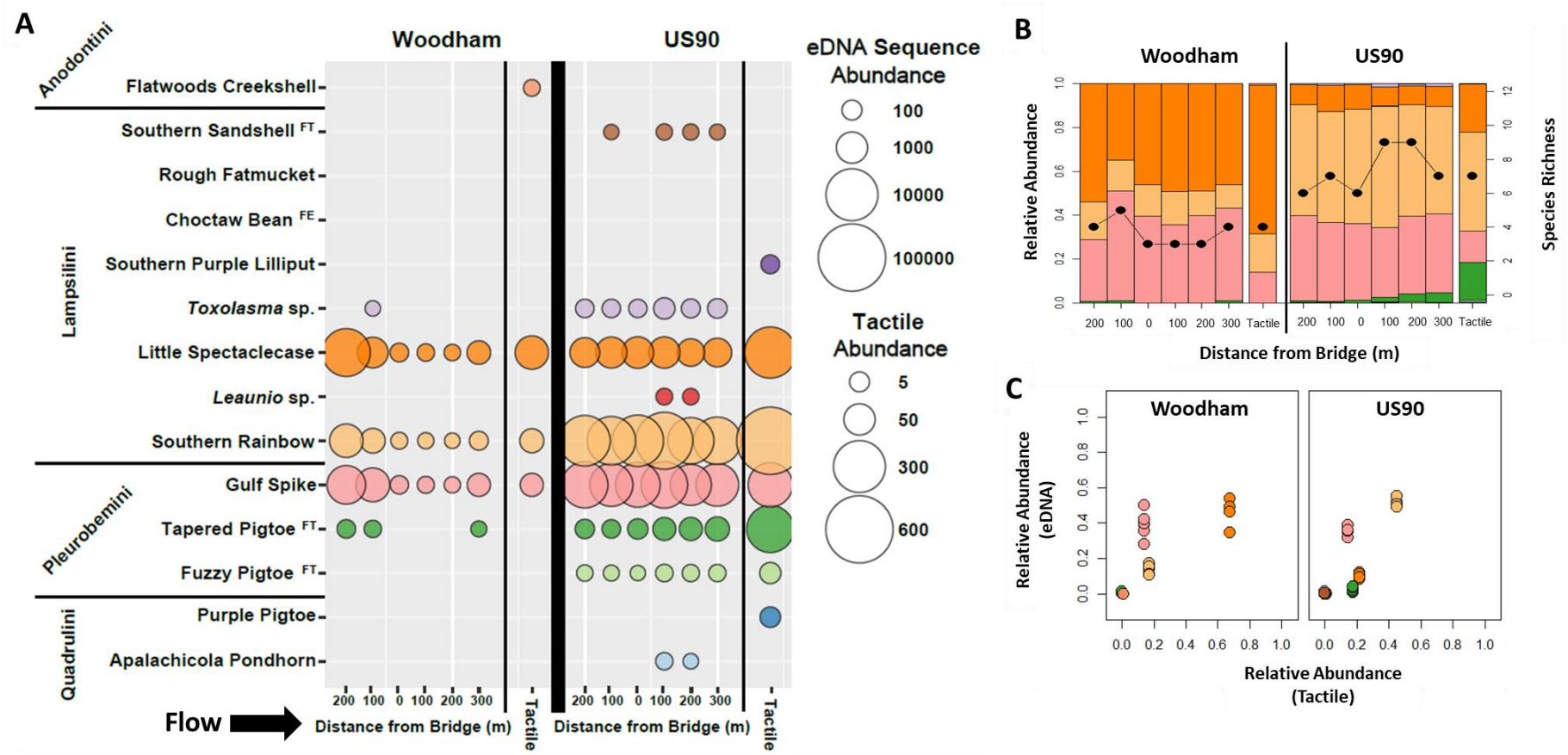

Figure 10 (A) Environmental DNA species detection and sequence read abundance within the two bridge locations in Holmes Creek. FT = Federally Threatened, FE = Federally Endangered. (B) Species richness (black points) and releative abundance (bar plots) along the six eDNA sampling sites and the tactile survey within Holmes Creek. (C) Comparitive relative abundance estimates from eDNA and tactile surveys within Holmes Creek.

3.2.3.3 Bruce Creek

Four federally protected species were detected with the tactile survey, while the eDNA survey detected three federally protected species (Table 6). The two surveys matched in distribution of these federally protected species, with the exception of the tactile survey observing two individuals of Fuzzy Pigtoe that went undetected with eDNA at Walton Bridge and two individuals of Choctaw Bean undetected with eDNA at McKinnon Bridge. Additionally, one individual of Purple Pigtoe was observed at Walton Bridge; however only a single eDNA replicate water sample provided low abundance of sequence reads for this species, and thus it was considered undetected with eDNA (Table 6). Environmental DNA detected Apalachicola Pondhorn throughout Walton Bridge (Table 6).

Table 6 Mussel abundance found during tactile surveys and corresponding environmental DNA detection from two bridge locations within Bruce Creek.

			Bruce Creek			
			McKinnon Bridge		Walton Bridge	
Tribe	Species	Common Name	Tactile	eDNA	Tactile	eDNA
Anodontini	*Strophitus williamsi*	Flatwoods Creekshell	9	X	1	X
Lampsilini	*Hamiota australis* FT	Southern Sandshell	30	X	29	X
	Lampsilis straminea	Rough Fatmucket	4	X	4	X
	Obovaria choctawensis FE	Choctaw Bean	2	–	5	X
	Toxolasma corvunculus	Southern Purple Lilliput	15	X	4	X
	Leaunio lienosus	Little Spectaclecase	101	X	193	X
	Villosa vibex	Southern Rainbow	9	X	29	X
Pleurobemini	*Elliptio* sp.		218	X	93	X
	Fusconaia burkei FT	Tapered Pigtoe	43	X	13	X
	Pleurobema strodeanum FT	Fuzzy Pigtoe	–	–	2	–
Quadrulini	*Cyclonaias succissa*	Purple Pigtoe	–	–	1	–
	Uniomerus columbensis	Apalachicola Pondhorn	3	–	–	X
		Total Number of Species Detected	*10*	*8*	*11*	*10*

FT = Federally Threatened, FE = Federally Endangered
X = Detected with eDNA
Red = Detected with tactile only, Orange = detected with eDNA only, Green = detected with both

The eDNA sequence read abundance was greater at Walton Bridge compared to McKinnon Bridge (Figure 11A). Species richness was greater at Walton Bridge, with two species only detected in this location (Figure 11A). The relative abundance of the mussel community between eDNA sampling sites was similar within both McKinnon Bridge and Walton Bridge (Figure 11B). The relative mussel abundance estimated from eDNA was strongly correlated with that estimated from tactile surveys at Walton Bridge, and weakly correlated at McKinnon Bridge (Figure 11C).

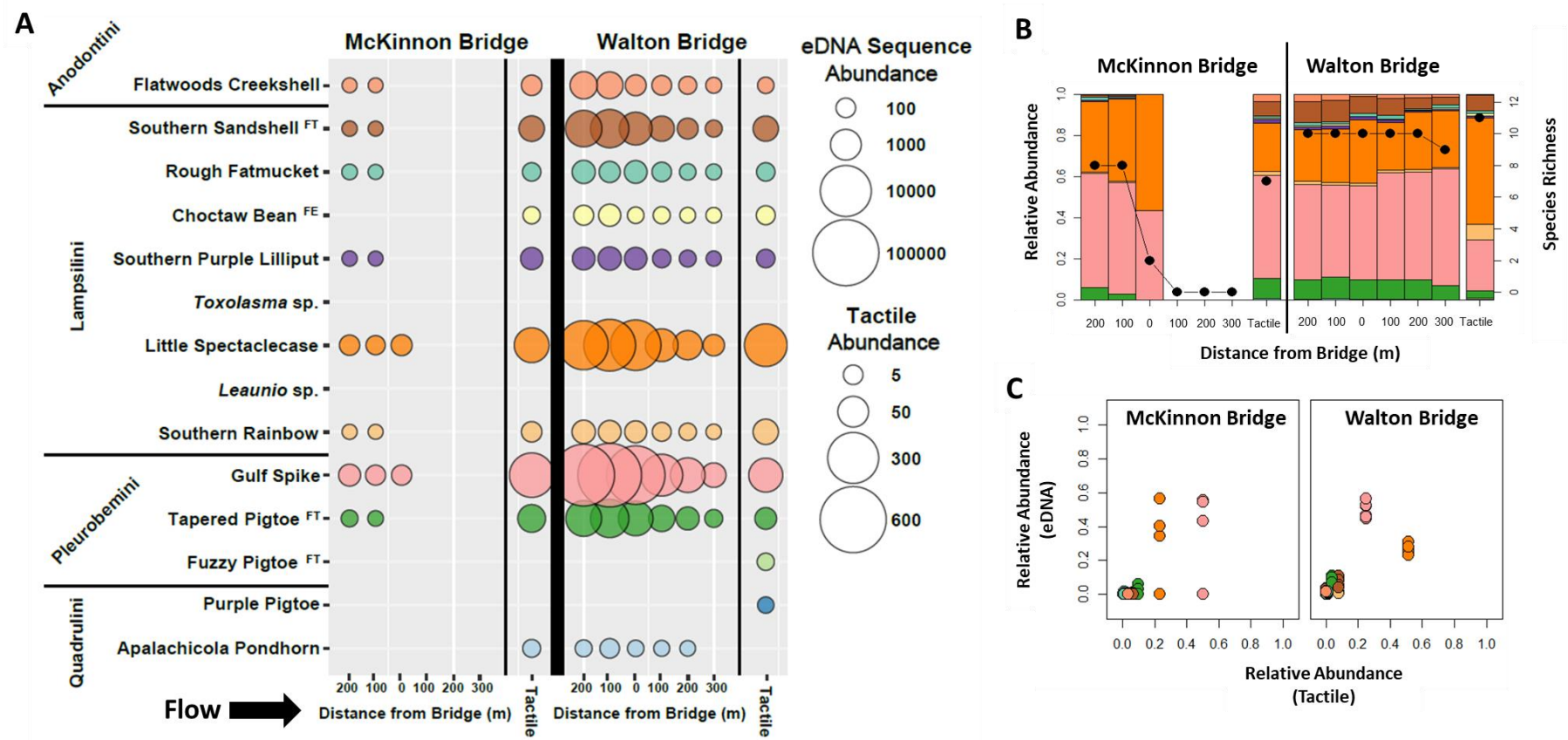

Figure 11 (A) Environmental DNA species detection and sequence read abundance within the two bridge locations in Bruce Creek. FT = Federally Threatened, FE = Federally Endangered. (B) Species richness (black points) and releative abundance (bar plots) along the six eDNA sampling sites and the tactile survey within Bruce Creek. (C) Comparitive relative abundance estimates from eDNA and tactile surveys within Bruce Creek.

3.2.4 Evaluating eDNA Detection Confidence

Species that went undetected with eDNA were found at lower mussel abundance than those detected with both surveys (mean mussel abundance when detection with both methods = 24.1, mean mussel abundance when non-detection with eDNA = 2.1, t = 6.06, df = 26.75, $p < 0.001$***) (Figure 12A). Species that went undetected with tactile survey were found at lower eDNA abundance than those detected with both surveys (mean eDNA abundance when detection with both methods = 1,342.7, mean eDNA abundance when non-detection with tactile = 99.4, t = 3.89, df = 31.66, $p < 0.001$***) (Figure 12B).

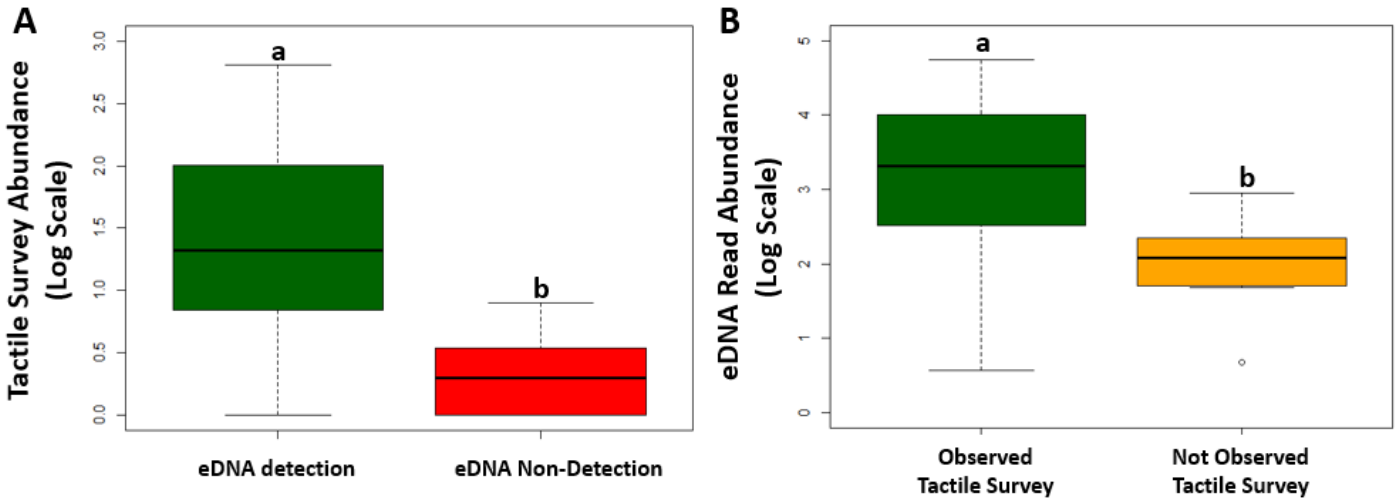

Figure 12 (A) Mean ± standard error mussel abundance for species that were undetected with environmental DNA compared to those detected with environmental DNA. (B) Mean ± standard error environmental DNA sequence read abundance for species that were observed compared to those that were not observed.
The center line indicates the median, and each box indicates the lower and upper quartiles. Ends of the whiskers indicate maximum and minimum values within the quartile ± 1.5 x interquartile spread. Open circles (if present) indicate values falling outside the quartile ± 1.5 x interquartile spread. Lowercase letters designate significant difference between groups (p<0.05).

The probability of eDNA detection for a species increased with mussel abundance (Figure 13). Species that occurred in greater density at a bridge displayed higher detection probabilities (Figure 13).

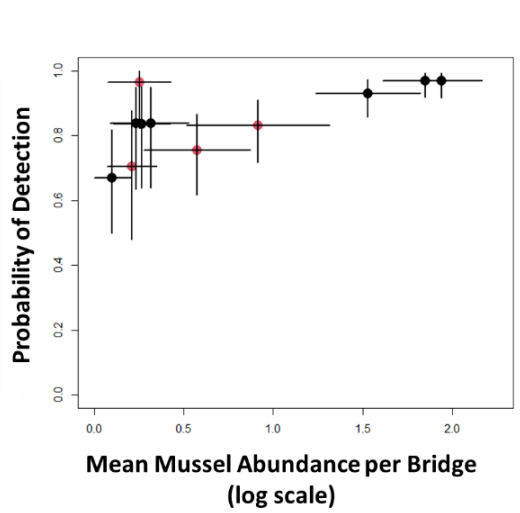

Figure 13 Estimated probability of eDNA detection compared to the mean mussel abundance for each species. Bars indicate standard error. Red points indicate federally protected species.

3.2.5 Estimating Fixed-point Ballyhoo eDNA Transport Distances

Ballyhoo eDNA was detected within each sampling site spanning 600 m from the fish source within each of the three creek systems (Figure 14). With the 1.2 µm pore-size filters, the eDNA concentration at distance 0 m was the same across the three creek systems (Figure 14). The eDNA concentration peaked at 600 m for Tenmile Creek, 500 m for Bruce Creek, and 300 m for Holmes Creek. Within Bruce creek, the 1.2 µm pore-size filter collected on average higher concentrations than the 8.0 µm pore-size filter; however, the trends in concentration with distance from the fixed-point source were similar (Figure 14).

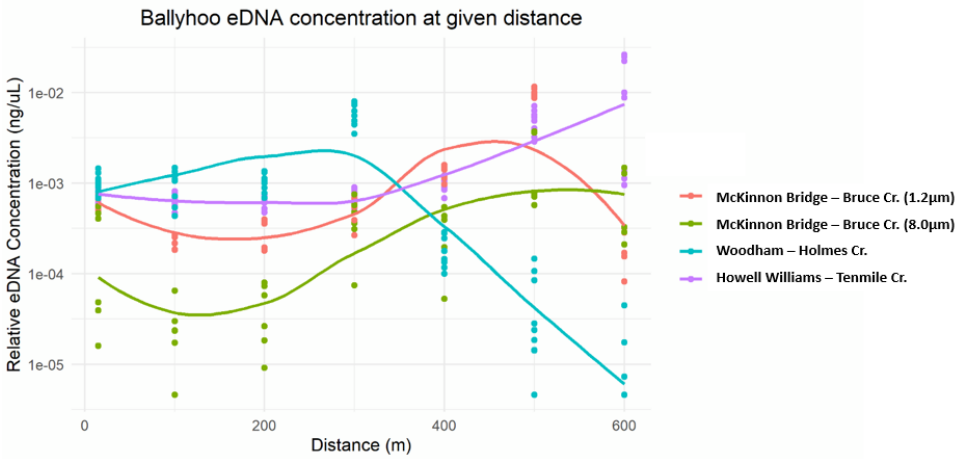

Figure 14 Relative Ballyhoo environmental DNA concentrations in Bruce Creek, Holmes Creek and Ten Mile Creek at a given sampling distance downstream from the fixed-point Ballyhoo.

4.0 DISCUSSION

4.1 EVALUATING ENVIRONMENTAL DNA

The two survey methods provided close agreement between species detections at the six bridge locations, with eDNA providing a positive detection on 33 of the 41 occasions a species was observed. The high-level of agreement between the two surveys is similar to Prié et al. (2021) and Marshall et al. (2022b), who used the same metabarcoding assay and found that eDNA detected as many or more species than tactile surveys. On two occasions a federally protected species was observed but not detected with eDNA; both occurring in Bruce Creek where two individuals of Fuzzy Pigtoe were observed at Walton Bridge and two individuals of Choctaw Bean were observed at McKinnon Bridge. When a species was observed but not detected with eDNA, its mussel abundance was on average two individuals or less (Figure 12A). Additionally, the probability of successfully detecting a species with eDNA increased with mussel abundance (Figure 13). This suggests when species are common at a bridge location, they are easily detected with eDNA (i.e., detected in high eDNA abundance and across many sampling sites), while the rarest of species have lower likelihood of detection.

On 10 of 43 occasions a species was detected with eDNA but not observed with the tactile survey. This may indicate occasions in which a species went undetected by the surveyors, or instances in which a mussel was actually present upstream of the surveyed area. In order for eDNA surveys to fit into a regulatory framework, a greater understanding of potential eDNA transport distance is required. This distinction is particularly important when interpreting presence of federally protected species, for example, on two occasions each the federally protected Southern Sandshell and Tapered Pigtoe had eDNA detections from bridge locations where they were not observed. Such detections may result in important environmental management decisions, which may ultimately impact consultation decisions. On the occasions where a species was detected with only eDNA at a bridge location, sequence read abundance was typically low, accounting for on average <150 reads per sampling site compared to an average of >1300 reads per sampling site when a species presence was confirmed (Figure 12B). While high eDNA sequence read abundance was indicative of species true presence, there were seven occasions in which a species was observed at <10 individuals and its eDNA was detected on average <150 reads per sampling site. Therefore, species at low eDNA abundance may be indicative of a species presence at low abundance (<10 individuals) or potentially eDNA transported from upstream.

4.2 ESTIMATING MUSSEL ABUNDANCE

The eDNA survey provided similar patterns of relative mussel abundance to that observed in the tactile survey. Results from both survey methods suggest there was no difference in the mean mussel abundance or eDNA sequence read abundance between the three creek systems; however, there was significantly greater mussel abundance and eDNA abundance at downstream bridge locations compared to upstream bridge locations (Figure 8).

Obtaining abundance estimates from eDNA metabarcoding datasets is challenging due to differences in species-specific eDNA shedding rates (e.g., differences in size ranges, life-histories and spawning times, and metabolic activity), biological behavior, habitat differences, and PCR-based biases (e.g., differential primer annealing and amplification) (Ruppert et al. 2019). However, a metanalysis of eDNA metabarcoding studies suggests eDNA sequence read abundance is often correlated with estimates of species abundance or biomass (Keck et al. 2022). In the current study, the observed abundance of each species during tactile surveys was found to be positively correlated with eDNA sequence read abundance within each of the six sampled bridge sites (Figures 9C, 10C, & 11C). Similarly, previous work specifically targeting populations of freshwater mussels has shown eDNA was strongly correlated to abundance estimates (Marshall et al. 2022b). This has also been shown in several studies of freshwater fish communities (Di Muri et al. 2020, Zhang et al. 2020, Boivin-Delisle et al. 2021, Blabolil et al. 2022, Cantera et al. 2022, Skelton et al. 2022).

The ability to not only detect mussel species, but to also gather information about abundances provides valuable information to track federally protected species. In most cases, moderate to high abundant species are easily distinguishable from low abundant species; however, it is typically difficult to predict abundance ranks across species that occur at low abundance (Skelton et al. 2022). In the current study, detections occurring at moderate to high eDNA sequence read abundance provided strong evidence that the species was truly present at the location (Figure 12B), while detections occurring at low eDNA sequence read abundance typically occurred when a species was rare or even undetected within the tactile survey. Therefore, the eDNA protocol used here provides a framework to estimate a range of abundances for which a species occurs at a site. For example, an eDNA detection occurring at low sequence read abundance typically represented a mussel population of less than 10 individuals (see further discussion in Section 4.5). It is important to note that the current surveys occurred within small streams that represent low discharge systems, and more work is required to understand the variation and limitations surrounding abundance estimates in larger systems.

The eDNA abundance was additionally correlated with mussel abundance between the bridge sites, with the exception of McKinnon Bridge in Bruce Creek (Figure 7). The ability to use eDNA concentrations to compare mussel abundance between sampling sites provides an opportunity to estimate species abundance at sites of unknown mussel density. However, the exception of McKinnon Bridge in Bruce Creek suggests more work is required to develop a method for utilizing eDNA for standardized abundance estimates. Estimating species abundance from eDNA may run into issues when inhibitor compounds are present within a sample. Inhibitors may be present within the environment from humic, phytic, and tannic acids, leaf litter, algae, and sediments. These compounds reduce the efficiency and accuracy of the PCR (Lance & Guan 2020). Additionally, high levels of total suspended solids and high concentrations of disturbed sediment may reduce eDNA quantification and increase presence of PCR inhibitors (Stoeckle et al. 2017). Considering a portion of the eDNA samples collected at McKinnon Bridge failed to produce mussel sequences suggests environmental inhibitors may have been present within Bruce Creek which interfered with estimating mussel abundance. A more quantitative metabarcoding technique is currently being developed which would reduce biases resulting from inhibitor compounds (Stoeckle et al. 2022).

4.3 ENVIRONMENTAL DNA TRANSPORT DISTANCES

Within flowing systems, eDNA detection is driven by water flow and hydrological dynamics of the river. The potential transport distance of eDNA is strongly associated with river discharge (Wilcox et al. 2016, Van Driessche et al. 2022). Considering bivalve eDNA has been reported to transport >1 km in some river systems (Deiner & Altermatt 2014, Shogren et al. 2019, Stoeckle et al. 2021), it is likely that eDNA transport had some effect on the eDNA detections. It is difficult to estimate the exact upstream origin distance of an eDNA detection. In the current study, assessment of potential eDNA transport distances within the three creek systems estimated peak eDNA concentration occurred between 300 m to >600 m downstream of the source (Figure 14). This follows patterns of eDNA plume dynamics that have been reported in other studies (Van Driessche et al. 2023b). Due to these eDNA plume dynamics, detections that occur at low sequence read abundance may be difficult to differentiate if the species is truly present at low mussel density or if the eDNA signal is a result from an upstream mussel bed.

Through qPCR analysis of Ballyhoo eDNA, the results here indicate an eDNA signal had the potential to originate from >600 m upstream within all three creek systems. Similarly, previous qPCR-based studies have suggested eDNA transport distances can exceed >1 km from the source (Deiner & Altermatt 2014, Wood et al. 2021, Shea et al. 2022). However, metabarcoding studies for the description of fish communities have suggested particle transport is less influential in metabarcoding-based approaches (Cantera et al. 2022, Laporte et al. 2022). In metabarcoding analysis, the eDNA signal may become swamped out by the local eDNA pool as distance from source increases. Consequently, the qPCR-based ballyhoo technique, being species-specific and more sensitive, may not be directly comparable to the metabarcoding technique used for mussels in assessing transport distances.

4.4 ESTIMATES OF SURVEY EFFORT

The main goal of this pilot study was to evaluate the efficacy of eDNA surveys for mussels by comparing data generated to that from tactile survey methods. Overall, there was high correspondence between the two survey methods. A tactile survey requires greater person-hours within the field compared to that needed to complete an eDNA survey. Across the four bridge locations within Holmes Creek and Tenmile Creek, the tactile surveys required on average 15.5 person hours of search (Table 1). Additionally, the updated mussel protocol specifies a minimum effort of 16 person hours of search must be conducted within streams greater than 15 m in width (GDOT 2018). The eDNA collection could be completed in less time, requiring on average 6.7 person hours per bridge. Additionally, tactile surveys require a permit holder with demonstrated expertise in tactile identification of mussels, including survey experience within the drainage basin and with the mussel assemblage potentially present. Tactile surveys are not infallible, and successful collection and identification can be dependent on expertise of the survey team, clarity of water, and site conditions. Tactile surveys may occur in deep and fast flowing waters, which require scuba-based searches and introduce safety issues that may constrain the timeframe in which a tactile survey may be conducted. Therefore, eDNA applications present an opportunity to perform qualitative mussel surveys to expediate the consultation process.

The eDNA survey requires an additional cost for molecular laboratory analysis. There are several molecular laboratories that offer eDNA metabarcoding services, and they can widely vary in the cost per sample (from $100 to $450 per sample). The study implemented here procured the services of Genidaqs which currently provides a commercial rate for metabarcoding of ~$117 per sample for the analysis of 100 eDNA samples. By following the current protocol of collecting 18 eDNA samples per bridge location (triplicate water replicates from six sampling sites per bridge), an eDNA study across six bridge locations can be accomplished with 108 eDNA samples.

4.5 A FRAMEWORK FOR ENVIRONMENAL DNA SURVEYS

Based on the freshwater mussel survey protocol for northeastern gulf drainages (Carlson et al. 2008, GDOT 2018), qualitative tactile surveys are recommended at all project sites with perennial streams, and a follow-up quantitative survey may be requested if federally protected species are found within the project area. A tactile qualitative survey includes dividing the creek into eight segments of 50 m each, spanning a minimum survey length of 100 m (~300 ft) upstream and 300 m (~900 ft) downstream of the proposed project. We implemented an eDNA survey in a similar range, spanning from 200 m upstream to 300 m downstream of each bridge location.

For the outlined visual and tactile protocol, the purpose of conducting qualitative surveys is to provide resource agencies with **(1)** presence/ probable absence data, **(2)** species richness, **(3)** estimates of relative abundances, and **(4)** information on recruitment (Carlson et al. 2008, GDOT 2018). In the current study, eDNA surveys produced similar data for **(1)** presence/ probable absence assessments, **(2)** species richness, and **(3)** estimates of relative abundances within these three creek systems occurring in the Choctawhatchee River basin. Tactile mussel survey reports submitted to FWS additionally require **(4)** measuring recruitment success by listing age-classes for the species surveyed within a project area. This is a piece of information that FWS uses in part of the consultation process for evaluating mussel population health status, of which eDNA is not currently capable of providing similar measurements of recruitment. Still, this work suggests eDNA surveys can be an additional management tool to provide qualitative-based survey information on mussel communities relating to species presence and abundance.

We present a framework in which eDNA can be used as a qualitative survey to inform the ESA consultation process (Figure 15). This framework is based on data analyzed here from three tributaries of the Choctawhatchee River Drainage, and consequently further studies need to evaluate how this framework applies at bridge locations that cross systems of larger discharge (e.g., the Choctawhatchee River mainstem). During the current regulatory process, a qualitative tactile survey is performed as a low effort evaluation of the species inventory. This information is used to consider the next steps needed to reduce impacts on any federally protected species that are present within the project area. In this study, eDNA produced similar data to these initial qualitative surveys. In this framework, the initial steps include performing a desktop review of the historical mussel assemblage. This will help identify what federally protected species may be present in the project area. Additionally, this information will be necessary to determine if the current eDNA assays are applicable for assemblage present within the project area.

Next, an eDNA qualitative survey can be conducted to determine if mussels are present, and if special status or protected species are present. If protected species are detected within the eDNA samples, it may be possible to differentiate low abundant species from moderate to high abundant species (see Figures 7 & 9-11). These surveys are informative on whether to move to a quantitative survey and/or relocation effort based on interpretations of the mussel assemblage and estimates of abundance for state and federally protected species. Within the current eDNA approach, it was possible to determine when populations occurred at low density, providing a potential framework to estimate occasions when there is probability of a low-level take (e.g., less than 10 individuals). For example, the federally threatened Southern Sandshell was not found during tactile surveys at Howell Williams bridge in Tenmile Creek; however, it was detected at a very low eDNA abundance. This suggested the species may be present at very low abundance surrounding the bridge site and provided a recommendation for a low-level of take for the project.

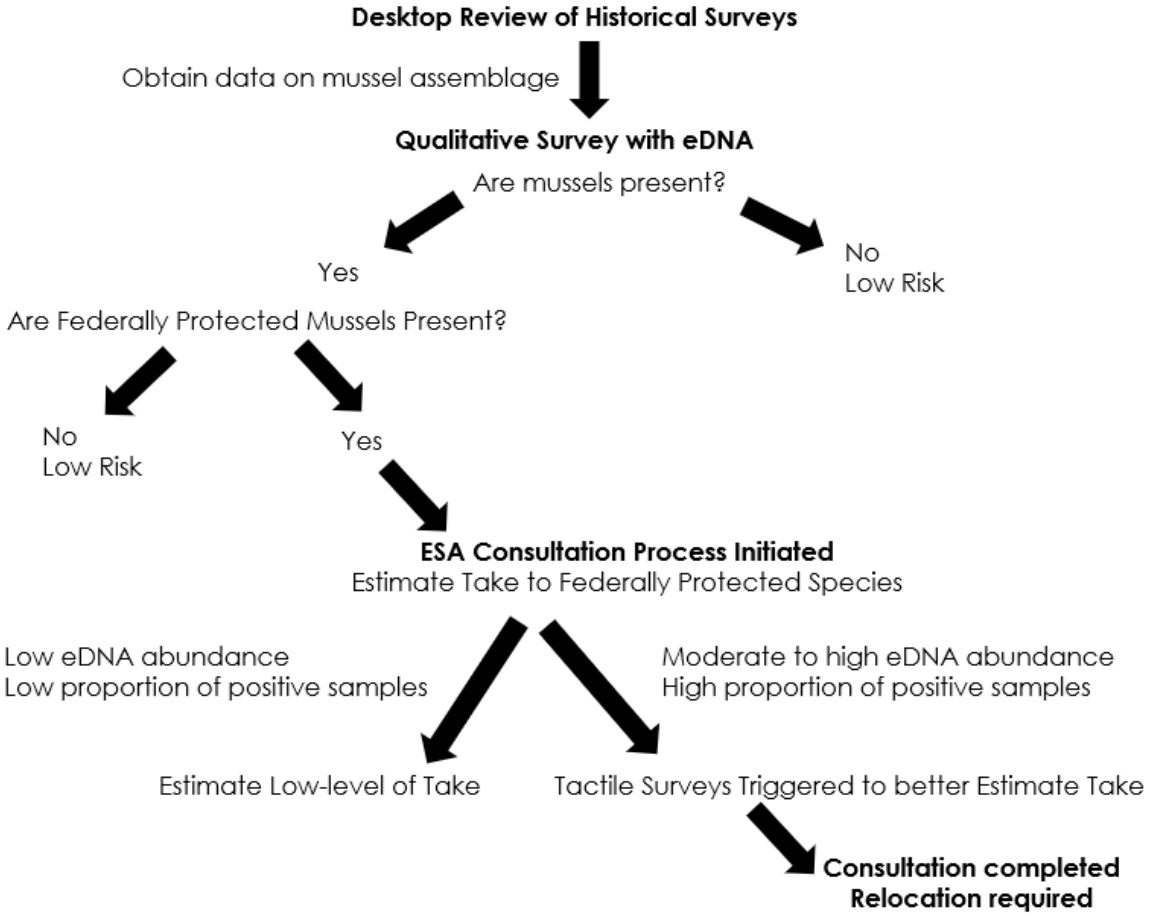

Figure 15 Decision framework of consultation process for incorporating environmental DNA to evaluate presence, species richness, and estimate of relative abundances.

4.6 CURRENT LIMITATIONS IN TAXONOMIC IDENTIFICATION

This pilot study demonstrates the efficacy of eDNA surveys at detecting protected mussel species and differentiating between many closely related species. The Choctawhatchee River basin is comprised of 25 mussel species historically, including six federally protected species (Southern Sandshell, Alabama Moccasinshell, Choctaw Bean, Southern Kidneyshell, Tapered Pigtoe, Fuzzy Pigtoe) (see Attachment A) (Blalock-Herod et al. 2005). The current tactile and eDNA surveys detected four of these protected species, with Alabama Moccasinshell and Southern Kidneyshell not detected at any bridge locations. The Southern Kidneyshell has been restricted to the headwater portions of the Choctawhatchee River within Georgia, and thus is only considered present well upstream of the current surveyed streams (Blalock-Herod et al. 2005). While Alabama Moccasinshell has previously been recorded within Holmes Creek, it was not found anywhere within the Choctawhatchee River in an extensive 2005 survey and may be extirpated from the basin (Blalock-Herod et al. 2005). Genetic data for the 16S gene region for Tapered Pigtoe cannot be differentiated from that of Narrow Pigtoe (*Fusconaia escambia*), and therefore the current eDNA data cannot definitively distinguish detections between Tapered Pigtoe and Narrow Pigtoe. However, only Tapered Pigtoe is considered present within the Choctawhatchee River basin, and thus sequences for this complex were considered true detections of this species.

The Choctawhatchee River basin has been regarded as containing unique genetic variants compared to other Florida river basins, with genetic tools identifying unique *Villosa* and *Strophitus* variants within the basin (Williams et al. 2014, Smith et al. 2018). This is an important consideration when comparing conventional survey methods with eDNA surveys. The eDNA data detected three species within the *Villosa* complex, which included 100% matches to *Leaunio lienosus* and *Villosa vibex*, both of which were observed during tactile surveys. The tactile survey at the Holmes Creek upstream bridge location identified a single individual as Rayed Creekshell. However, recent work has demonstrated that the Rayed Creekshell species complex is actually comprised of four different species, with Flatwoods Creekshell considered the correct nomenclature for individuals within the Choctawhatchee River basin (Smith et al. 2018). This species was confirmed within the eDNA through a voucher sequence from individuals collected within the basin.

Throughout all bridge locations, *Elliptio pullata* was observed at high abundances; however, there is likely a complex of many *Elliptio spp*. present throughout the basin (Blalock-Herod et al. 2005, Williams et al. 2014). Additionally, taxonomic classifications within this genus are considered to be one of the most problematic for species assignment (Williams et al. 2014, Perkins et al. 2017). This is largely due to vast plastic morphological ecophenotypes, which has resulted in improperly assigned taxa and over-estimates of species diversity (see Williams et al. 2014). Within the Choctawhatchee River basin, four species within the *Elliptio* genus are considered present (Attachment A), with all four of these species having >97.5% genetic similarity to the *Elliptio* eDNA sequence identified in this study. Therefore, it is possible that this eDNA sequence actually represents multiple species within the *Elliptio* complex. Uncertainty in nomenclature based on morphological identifications remains a limitation for accurate taxonomic identification from eDNA surveys. Proper and accurate nomenclature is required for the collection of accurate voucher specimens that are ultimately required for appropriate curation of genetic sequences. Current efforts to improve genetic databases are rapidly underway and DNA analysis is often being used to resolve taxonomic uncertainty for many freshwater mussel genera.

5.0 CONCLUSIONS

Environmental DNA presents several advantages over tactile surveys, including:

- eDNA can provide comparable information to a tactile qualitative survey;
- eDNA field data collection can be completed in a faster amount of time;
    - However, results are dependent on the lag-time for laboratory processing;
- eDNA field data collection can typically be collected with a smaller field crew and in safer conditions;
- Can be implemented in environments challenging for tactile surveys; and
- Can be completed without a federal mussel permit holder.

The current eDNA survey within the Choctawhatchee River basin resulted in the detection of 13 mussel species, including 11 species likewise observed during tactile surveys. On 33 of 41 occasions when a species was observed it's eDNA was also detected. Furthermore, species that were visually observed at a bridge were detected in high eDNA sequence read abundance.

On 10 of 43 occasions a species was detected with eDNA but not visually observed at a bridge. When this occurred, the species was typically found in low eDNA sequence read abundance. Information on eDNA sequence read abundance was informative on providing high confidence that a species was truly present in close proximity to the sampled bridge. However, when a species occurs at low eDNA sequence read abundance, it is difficult to differentiate if it is present and at low mussel abundance or if it is signal from eDNA transport. For example, this study suggests eDNA can be transported >600 m even in these small creek systems.

While eDNA cannot act as an all-out replacement of tactile surveys, (e.g., the physical rescue and relocation of mussels, assessment of recruitment, or organism health), this study demonstrates the practicality of incorporating eDNA metabarcoding as a supplemental sampling method for a qualitative-based survey to describe mussel assemblage and improve the detection of federally protected species. Environmental DNA has the potential to provide decision support for consultation on FDOT projects, at a lower cost with comparable survey data to tactile surveys. We developed a decision framework in which eDNA applications can be implemented into the consultation process. Current challenges remain regarding the ability to accurately estimate population abundances, especially when scaling to sample larger river systems. Still, eDNA metabarcoding shows promise as a survey method to provide comparable data to tactile surveys in creeks and small to medium rivers within the Choctawhatchee River Drainage.

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Attachment A – HISTORICAL MUSSEL ASSEMBLAGE

Table A1 Mussel species historically found throughout the state of Florida and within the Choctawhatchee River basin, their federal status, if they have available DNA reference sequences, and their historical presence within the Choctawhatchee River basin. E=endangered; T=threatened, PT=Proposed Threatened.

Tribe	Species	Common name	Status[1]	DNA reference Sequences[2]	Choctawhatchee River Basin[3,4]
Margaritiferidae	*Margaritifera marrianae*	Alabama Pearlshell		2	
Amblemini	*Amblema neislerii*	Fat Threeridge	E	1	
Amblemini	*Amblema plicata*	Threeridge		6	X
Amblemini	*Reginaia apalachicola*	Apalachicola Ebonyshell		0	
Amblemini	*Reginaia rotulata*	Round Ebonyshell	E	3	
Anodontini	*Alasmidonta triangulata*	Southern Elktoe	PT	2	
Amblemini	*Plectomerus dombeyanus*	Bankclimber		2	
Anodontini	*Alasmidonta wrightiana*	Ochlockonee Arcmussel		0	
Anodontini	*Lasmigona subviridis*	Green Floater		1	
Anodontini	*Pyganodon cataracta*	Eastern Floater		3	
Anodontini	*Pyganodon grandis*	Giant Floater		48	X
Anodontini	*Strophitus radiatus*	Rayed Creekshell		1	
Anodontini	*Strophitus williamsi*	Flatwoods Creekshell		0	X
Anodontini	*Utterbackia imbecillis*	Paper Pondshell		21	X
Anodontini	*Utterbackia peggyae*	Florida Floater		1	X
Anodontini	*Utterbackia peninsularis*	Peninsular Floater		3	
Anodontini	*Utterbackiana couperiana*	Barrel Floater		1	
Anodontini	*Utterbackiana hartfieldorum*	Cypress Floater		0	
Anodontini	*Utterbackiana heardi*	Apalachicola Floater		0	
Anodontini	*Utterbackiana suborbiculata*	Flat Floater		0	
Lampsilini	*Glebula rotundata*	Round Pearlshell		2	X
Lampsilini	*Hamiota australis*	Southern Sandshell	T	6	X
Lampsilini	*Hamiota subangulata*	Shinyrayed Pocketbook	E	6	
Lampsilini	*Lampsilis binominata*	Lined Pocketbook		0	
Lampsilini	*Lampsilis floridensis*	Florida Sandshell		0	X
Lampsilini	*Lampsilis ornata*	Southern Pocketbook		9	
Lampsilini	*Lampsilis straminea*	Southern Fatmucket		2	X
Lampsilini	*Lampsilis teres*	Yellow Sandshell		8	
Lampsilini	*Medionidus acutissimus*	Alabama Moccasinshell	T	1	X
Lampsilini	*Medionidus penicillatus*	Gulf Moccasinshell	E	0	
Lampsilini	*Medionidus simpsonianus*	Ochlockonee Moccasinshell	E	0	
Lampsilini	*Medionidus walkeri*	Suwannee Moccasinshell	T	0	

Tribe	Species	Common name	Status[1]	DNA reference Sequences[2]	Choctawhatchee River Basin[3,4]
Lampsilini	*Obovaria choctawensis*	Choctaw Bean	E	0	X
Lampsilini	*Obovaria haddletoni*	Haddleton Lampmussel		0	X
Lampsilini	*Ptychobranchus jonesi*	Southern Kidneyshell	E	2	X
Lampsilini	*Toxolasma parvum*	Lilliput		3	
Lampsilini	*Toxolasma paulum*	Iridescent Lilliput		0	
Lampsilini	*Toxolasma corvunculus*	Southern Purple Lilliput		0	X
Lampsilini	*Villosa amygadalum*	Florida Rainbow		0	
Lampsilini	*Leaunio lienosa*	Little Spectaclecase		3	X
Lampsilini	*Villosa vibex*	Southern Rainbow		0	X
Lampsilini	*Villosa villosa*	Downy Rainbow		2	X
Pleurobemini	*Elliptio ahenea*	Southern Lance		0	
Pleurobemini	*Elliptio arctata*	Delicate Spike		3	
Pleurobemini	*Elliptio chipolaensis*	Chipola Slabshell	T	0	
Pleurobemini	*Elliptio complanata*	Eastern elliptio		4	
Pleurobemini	*Elliptio crassidens*	Elephantear		5	X
Pleurobemini	*Elliptio fraterna*	Brother Spike		1	
Pleurobemini	*Elliptio fumata*	Gulf Slabshell		6	
Pleurobemini	*Elliptio icterina*	Variable Spike		6	
Pleurobemini	*Elliptio jayensis*	Florida Spike		3	
Pleurobemini	*Elliptio mcmichaeli*	Fluted Elephantear		3	X
Pleurobemini	*Elliptio monroensis*	St. Johns Elephantear		0	
Pleurobemini	*Elliptio nigella*	Winged Spike		0	
Pleurobemini	*Elliptio occulta*	Hidden Spike		0	
Pleurobemini	*Elliptio pullata*	Gulf Spike		3	X
Pleurobemini	*Elliptio purpurella*	Inflated Spike		3	X
Pleurobemini	*Elliptoideus sloatianus*	Purple Bankclimber	T	3	
Pleurobemini	*Fusconaia burkei*	Tapered Pigtoe	T	3	X
Pleurobemini	*Fusconaia escambia*	Narrow Pigtoe	T	3	
Pleurobemini	*Pleurobema pyriforme*	Oval Pigtoe	E	1	
Pleurobemini	*Pleurobema strodeanum*	Fuzzy Pigtoe	T	2	X
Quadrulini	*Cyclonaias infucata*	Sculptured Pigtoe		5	
Quadrulini	*Cyclonaias kleiniana*	Florida Mapleleaf		1	
Quadrulini	*Cyclonaias succissa*	Purple Pigtoe		4	X
Quadrulini	*Megalonaias nervosa*	Washboard		4	
Quadrulini	*Uniomerus carolinianus*	Eastern Pondhorn		4	
Quadrulini	*Uniomerus columbensis*	Apalachicola Pondhorn		0	X
Quadrulini	*Uniomerus tetralasmus*	Pondhorn		1	

1. USFWS 2023
2. National Center for Biotechnology Information - GenBank (https://www.ncbi.nlm.nih.gov/)
3. Blalock-Herod et al. 2005
4. Williams et al. 2014

Attachment B – FINAL EDNA STUDY PLAN

Stantec Consulting Services Inc.
11687 Lebanon Road, Cincinnati OH 45241-2012

August 15, 2022
File: FM #: 407085-1-32-04 – CA944 Task Work Order # 15, eDNA Pilot Study

Attention: Katasha Cornwell
State Environmental Process Administrator
Office of Environmental Management
Florida Department of Transportation
605 Suwannee Street, MS-37
Tallahassee, FL 32399

Dear Katasha,

Reference: Draft eDNA Pilot Study Design / Plan

This letter provides a draft for review of the study design for the environmental DNA (eDNA) pilot study to be conducted within the Choctawhatchee River drainage in September 2022. We welcome your review, input and comments by August 26th so that we can finalize the study design and make preparations to implement the survey.

The Florida Department of Transportation (FDOT) and the Office of Environmental Management (OEM), has contracted Stantec Consulting Inc. (Stantec) to develop and implement and eDNA pilot study to facilitate detection (presence / absence) of freshwater mussels and support the permitting process for transportation projects that interact with watercourse locations that may host protected freshwater mussel species.

Background

The analysis of environmental DNA (eDNA - genetic material released from urine, waste, mucus, or sloughed cells) is increasingly integrated into natural resource surveys designed to detect the presence of special status species and describe entire community assemblages (Beng & Corlett 2020, Deiner et al. 2021). Use of eDNA can provide information on multiple taxa and is therefore a powerful tool for studying species and community dynamics (Deiner et al. 2017).

As conventional mussel surveys can be difficult to conduct, necessitate permits, and require considerable expertise, eDNA methodology can enhance detection, is typically less costly and less intrusive on the environment, can identify cryptic species, and facilitate mussel surveys in challenging and remote habitats (Evans et al. 2017, Jerde 2021).

A DNA metabarcoding approach, which can simultaneously identify multiple taxa within a single sample, has been used to detect bivalve communities in river systems (Klymus et al. 2021 Prié et al. 2020, Coghlan et al. 2021, Marshall et al. 2022). Metabarcoding can characterize assemblages in diverse mussel beds, including the detection of threatened and endangered species, aiding in environmental impact assessments, and evaluating conservation efforts of freshwater mussels (Marshall et al. 2022). Still, a robust comparison of data generated by conventional surveys (e.g., field and dive surveys) and eDNA across different aquatic systems is lacking and remains a barrier to wider adoption of eDNA in regulatory processes (e.g., Clean Water Act Section 404/401 permitting, Endangered Species Act Section 7 & 10 Consultation).

The goal of this pilot study is to demonstrate the efficacy of eDNA for mussel surveys by comparing data generated by conventional survey methods following FDOT and U.S. Fish and Wildlife Service protocols to that of data generated using an eDNA approach.

Fifteen freshwater mussels in the State of Florida have protection under the federal Endangered Species Act (ESA). Efforts to repair, maintain, and expand road and bridge networks in the state result in regular consultations with the U.S. Fish and Wildlife Service (FWS) for mussels. Fundamental elements of these consultations are site specific presence/absence surveys and mussel salvage and relocation efforts. Both activities use methods prescribed by Carlson et al. (2008).

The objectives of this pilot study are to:

- Research mussel eDNA efforts to date, genetic assay availability, and consult with federal agencies about eDNA applications/limitations;
- Compile a genetic library for use in future projects;
- Optimize eDNA sample location, sampling intensity, and replication;
- Refine field and laboratory techniques for sample collection and processing for use in Florida waters;
- Compare the results of conventional and eDNA results for mussel presence;
- Assess eDNA performance over representative sites of routine conventional mussel projects;
- Conduct creek system-specific assessment of eDNA transport distances; and
- Assess costs and benefits of conventional and eDNA sampling approaches.

Technical Approach

The overarching approach for this study will be to conduct three tasks: 1) coordinate with FWS and U.S. Geological Survey (USGS) to design an eDNA pilot study that addresses survey concerns, 2) collect field samples for eDNA analysis, and 3) compare those results

to data from recent surveys using conventional field protocols. Stantec will work with subcontractor Genidaqs (https://genidaqs.com) for laboratory analysis of eDNA samples.

An overview of each task is provided below.

Study Design

Stantec personnel have reviewed relevant information provided by FDOT (OEM & District Three), FWS, and USGS regarding a list of presence/absence and/or salvage/relocation surveys conducted recently or planned for the coming survey season. Coordination for site selection has included a list of recent and upcoming mussel survey projects provided by District Three, discussions with Sandra Pursifull (FWS) and Mark Cantrell (FWS) about sites with recent mussel records, and discussions with Nate Johnson (USGS) about long-term monitoring sites. This list has resulted in the selection of six sites within three creek systems within the Choctawhatchee River drainage. These sites were chosen based on:

- having recent mussel survey data;
- having conventional survey data available for comparison with eDNA results in the pilot study;
- proximity to one another;
- ease of access to sampling site;
- presence of a diverse mussel assemblage;
- presence of threatened or endangered species.

Field Study Methods

The sampling design consists of six bridge sites, located within three different creek systems (**Table 1**). At one site in each creek system, a “live-car” experiment will be used to assess eDNA transport distances. Within each live-car, five dead individuals for a bait fish not found within Florida waters (e.g., Pacific salmon) will be placed in the stream in a flow through container within each river system (~250m upstream from the bridge site) at least 24-hours in advance of water sampling. The organism chosen will be based on discussions with Genidaqs to allow for reliable detection of chosen organism (e.g., an eDNA assay has been developed and is readily used within the lab). At each site, six eDNA transects will be sampled at fixed intervals of 100m, starting at 300m downstream from the bridge and moving in the upstream direction until reaching 200m upstream of the bridge (**Figure 1**).

Reference: **Draft Pilot Study Design Plan**

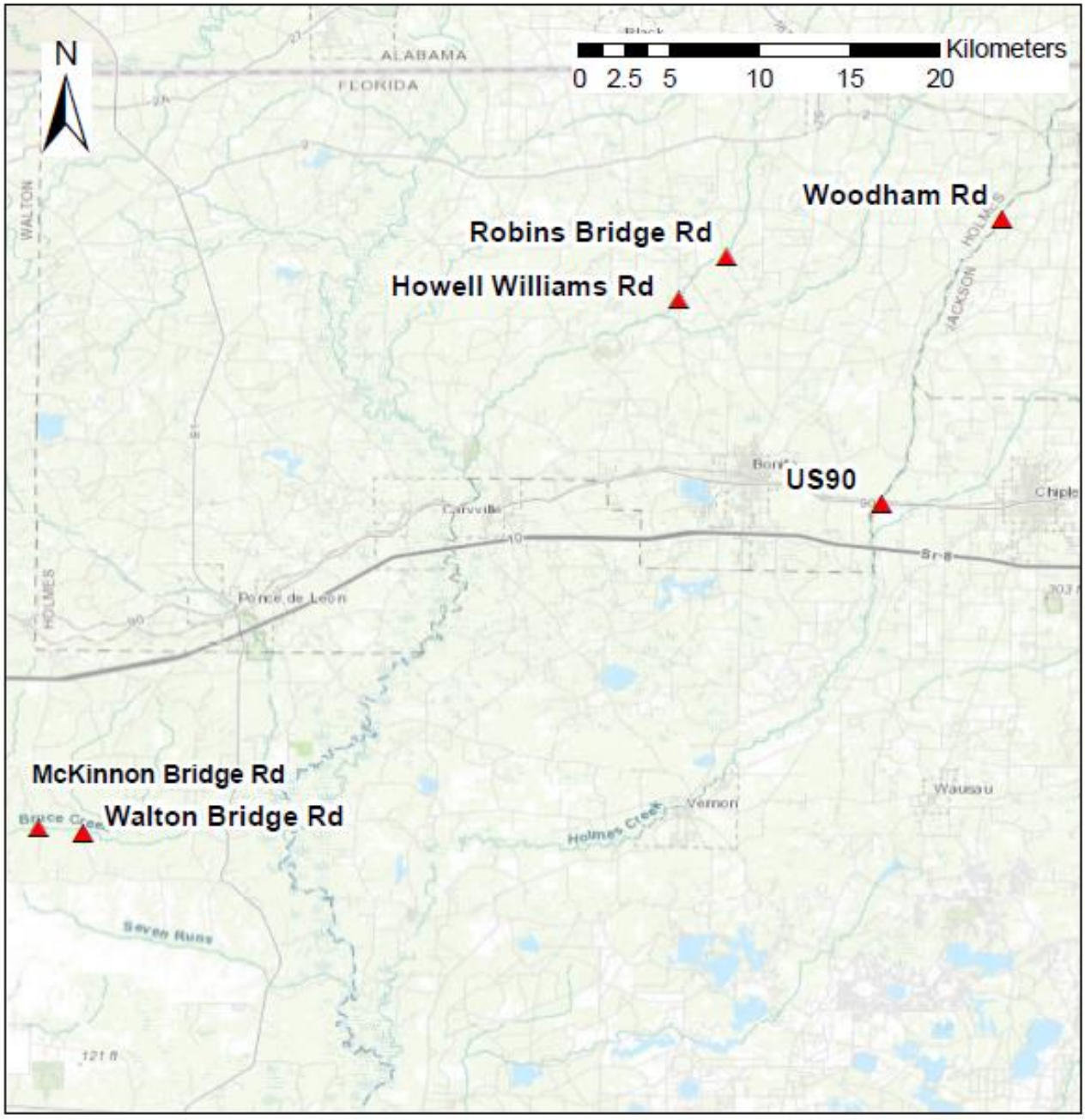

Figure 1: Sampling sites for eDNA collection within the Choctawhatchee River drainage in Florida. Map was generated in NAD83 geocentric datum and geographic coordinate system.

Reference: **Draft Pilot Study Design Plan**

Table 1: Sampling sites for eDNA within the Choctawhatchee River drainage basin.

Bridge	Creek	Latitude	Longitude	Year of mussel survey	Mussel Richness *(T&E)*	Mussel Abundance
US-90	Holmes	30.756	-85.619	2019	7 *(2)*	1209
Woodham Rd.	Holmes	30.921	-85.556	2019	4 *(0)*	121
Robbins Bridge Rd.	Tenmile	30.902	-85.693	2019	5 *(1)*	26
Howell Williams Rd.	Tenmile	30.88	-85.717	2022	-	-
McKinnon Bridge Rd.	Bruce	30.617	-86.036	2020	7 *(2)*	85
Walton Bridge Rd.	Bruce	30.614	-86.014	2017	11 *(4)*	176

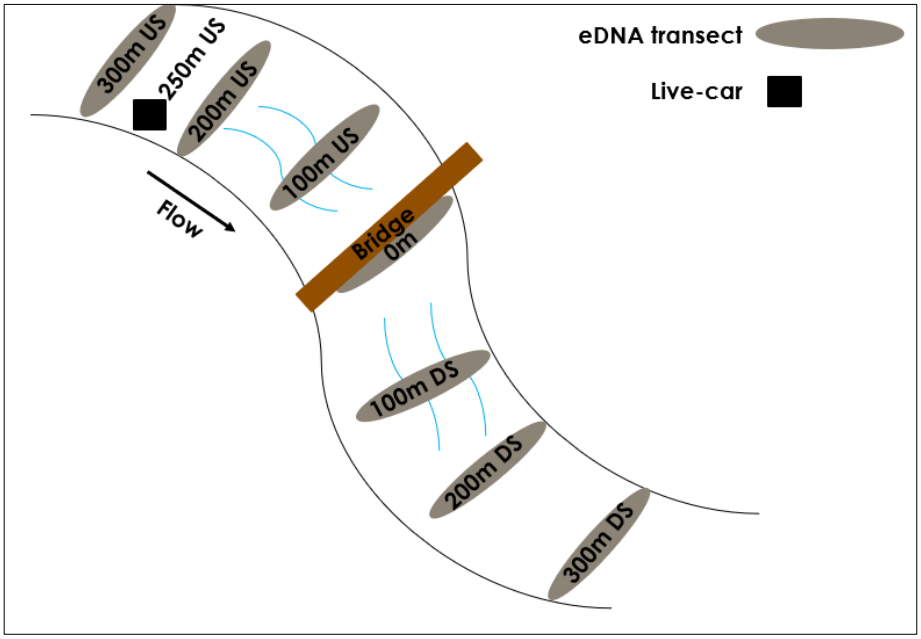

Figure 2: Schematic diagram depicting the eDNA sampling design for a site. Samples will be collected in 100m intervals starting 300m downstream (DS) from the bridge/crossing and ending 300 m upstream (US). At three of the sampling sites, a live-car experiment will be included to estimate eDNA transport distances.

Two sites will be sampled in each of the three creek systems (Holmes Creek, Tenmile Creek, and Bruce Creek). These three systems display a range of mussel community composition and mussel abundance. For example, surveys in 2019 found >1000 mussels at sites in Holmes Creek and <100 mussels at sites in Tenmile Creek. The mussel community composition is similar within the three systems with three federally threatened species surveyed; however, the federally endangered *Obovaria choctawensis* has only

been observed within Bruce Creek (**Table 2**). A live-car experiment will be used at one site in each system, to estimate eDNA transport and detectability related to flow and discharge.

Table 2: Mussel species found during surveys in Bruce Creek, Holmes Creek, and Tenmile Creek.

Common Name	Species name	Federal Status	Creek
Purple Pigtoe	*Cyclonaias succissa*		Bruce, Holmes, Tenmile
Gulf Spike	*Elliptio pullata*		Bruce, Holmes, Tenmile
Tapered Pigtoe	*Fusconaia burkei*	T	Bruce, Holmes
Southern Sandshell	*Hamiota australis*	T	Bruce, Tenmile
Southern Fatmucket	*Lampsilis straminea*		Bruce, Holmes
Choctaw Bean	*Obovaria choctawensis**	E	Bruce
Fuzzy Pigtoe	*Pleurobema strodeanum*	T	Bruce, Holmes
Rayed Creekshell	*Strophitus radiatus*		Holmes
Flatwoods Creekshell	*Strophitus williamsi**		Bruce
Gulf Lilliput	*Toxolasma sp. cf. corvunculus**		Bruce, Holmes
Little Spectaclecase	*Villosa lienosa*		Bruce, Holmes, Tenmile
Southern Rainbow	*Villosa vibex**		Bruce, Holmes, Tenmile
Downy Rainbow	*Villosa villosa*		Holmes

*Species with missing genetic data on public databases

Stantec staff will collect eDNA samples in the month of September due to estimated peaks in mussel eDNA concentrations associated with their biology and stream flows. Collection dates will be weather dependent. Survey methods include duplicate 500-1000mL water samples (volume dependent on turbidity) collected from near the benthos using a peristaltic pump with a polypropylene filter holder attached to a painter's pole. Water samples will be collected along a transect spanning the length of the river for each duplicate sample using a vacuum pump to draw water through a 47-mm-diameter glass microfiber filter GF/C (nominal pore size 1.2µm; GE Healthcare Life Science, MA, USA). Filters will be folded in half twice, then placed into separate coin envelopes, which are finally placed in Ziploc bags with silicone desiccant beads for sample desiccation and preservation. Samples will be kept in a cooler on ice within the field, and then placed in a non-frost-free freezer until shipment to Genidaqs (West Sacramento, CA) on ice via overnight courier.

All sample collection equipment will be decontaminated with 10% bleach solution after sampling at each site and rinsed with distilled water (DiH2O) prior to sample collection. Field staff for water sampling will follow best practices for eDNA collection, which includes wearing new sterile nitrile gloves at each site and when handling eDNA equipment, as well as sampling in the upstream direction to avoid contamination from boots and/or sediment. Furthermore, levels of field contamination will be assessed at two sites per day, where a sample of 500mL of DiH2O in a new container will be opened in the field and filtered to act as a field negative control to test for contamination during sample collection and handling.

Live-car study design will incorporate DNA of a deceased non-native organism into one selected site within each river system, to assess potential eDNA transport distances in each individual creek. This live car will target a non-native species with a pre-designed eDNA assay (e.g., Pacific Salmon). At the sites with a live-car experiment, the same water samples collected for analysis of mussel eDNA will additionally be analysed using a quantitative PCR (qPCR) for the detection of the live car species. The qPCR analysis will provide detection rates and quantitated concentrations of eDNA for the control species at 100m intervals from the live car (spanning from 50m to 550m).

The live-car results will be used to assess detection probability and eDNA concentration across distance from the source (i.e., cage) to understand eDNA transport within each river system. Recent work has suggested that small fractions of eDNA (e.g., organelles, extracellular pieces of DNA) are likely to be transported larger distances than the large fraction of eDNA (e.g., pieces of tissue, full intact cells) (Nagler et al. 2022). Therefore, a sampling approach that exclusively targets only large fractions of eDNA (e.g., the 10µm filter) may provide better localized detection rates of mussels.

At the three sites with a live-car, an additional set of samples will be collected using a larger filter pore-size (47-mm-diameter glass microfiber filter nominal pore size 10µm), to further assess eDNA transport related to particle size. The eDNA analysis with the live-car will assess how filter pore-size may reduce impact of transport distances and increase reliability of eDNA detections associated with a bridge site.

Analytical Methods

Stantec and Genidaqs have developed a method to extract and analyze eDNA samples to detect and quantify unionid mussel community composition and relative abundances using eDNA metabarcoding (Marshall et al. 2022). Using this same analytical method for the present pilot study, Stantec will compare unionidae (family of freshwater mussels, the bivalve molluscs sometimes known as river mussels, or simply as unionids) detections from conventional surveys and eDNA at each survey site. The rate of false negatives (resulting from lack of eDNA collection or amplification-bias against rare species) or potentially false

positives (resulting from eDNA transport down river) from eDNA sampling will be determined. The samples, along with the quality controls (i.e., negative controls with DiH2O, and positive controls with the live-car) will be amplicon sequenced on an Illumina MiSeq run using the already designed Prié et al. (2020) unionid-specific mitochondrial 16S rDNA primer pair. This metabarcoding primer pair was developed for Western Palearctic unionids and tested on environmental samples from France and Morocco. The primer pair appears to be universal across Unionidae and was used to successfully describe the diverse mussel assemblage (both community composition and relative abundances of >20 freshwater mussel species) within the Walhonding River in Warsaw, Ohio (Marshall et al. 2022). Therefore, this primer pair should be suitable for this pilot study in Florida and other North American mussel communities.

Gaps within a genetic reference database can lead to misidentification of eDNA data, and thus Stantec has compiled reference sequences for accurate eDNA identification. Stantec personnel have reviewed readily available databases (e.g., the National Center for Biotechnology Information - https://www.ncbi.nlm.nih.gov/ and the Barcode of Life Database - http://www.boldsystems.org/index.php/databases) and discussed genetic resources with agencies (e.g., U.S. Fish and Wildlife Service (USFWS) and U.S. Geological Survey (USGS)) to assemble a complete genetic database for species potentially occurring in the study area. It may be necessary to obtain tissue samples for the four species missing genetic data within the Choctawhatchee River drainage (**Table 2**). Tissue samples are available for the species with missing genetic data from Dr. Nathan Johnson (USGS). Photograph voucher images will be collected for each individual sampled for genetic reference data. Each tissue sample will be individually stored in RNAlater and shipped to Genidaqs for Sanger sequencing of the 16S gene region (Palumbi et al. 1991).

Reporting

The project team will prepare a report summarizing the results of the conventional mussel surveys and compare the results to that of the eDNA survey. Assemblage data will be presented by site and section of each creek. Following quality assurance and control from evaluating negative and positive controls, the species composition will be compared between duplicates within a sampling transect, and between eDNA and conventional surveys. The datasets will be compared within a bridge site, and across sampling sites. The metabarcoding read count data will be assessed to examine correlation with mussel abundance within each site. Finally, a table of listed taxa detected with eDNA will be compiled, including information on site localities and relative eDNA abundance measurements. The final report will include direct comparisons of labor effort (including both field and laboratory labor) and estimated timelines to complete each survey method. Budget estimates and effort for conventional surveys will be estimated based on state-wide

freshwater mussel protocols. These comparisons will be made within individual bridge sites and across the entire dataset.

Closure Statement

This document entitled “Draft eDNA Pilot Study Design / Plan” was prepared by Stantec Consulting Services Inc. (“Stantec”) for the account of Florida Department of Transportation (FDOT) and the Office of Environmental Management (OEM) (the “Client”). Any reliance on this document by any third party is strictly prohibited. The material in it reflects Stantec’s professional judgment in light of the scope, schedule and other limitations stated in the document and in the contract between Stantec and the Client. The opinions in the document are based on conditions and information existing at the time the document was published and do not take into account any subsequent changes. In preparing the document, Stantec did not verify information supplied to it by others. Any use which a third party makes of this document is the responsibility of such third party. Such third party agrees that Stantec shall not be responsible for costs or damages of any kind, if any, suffered by it or any other third party as a result of decisions made or actions taken based on this document.

Regards,

Stantec Consulting Services Inc.

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Mobile: 614 286-3131
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Attachment C – BRIDGE LOCATION PHOTOS

Photographic Log

Client:	**Project:**
Site Name:	**Site Location:**

Photograph ID: 1	
Photo Location: Bruce Creek	
Direction: McKinnon Bridge - Upstream Location	
Survey Date: 10/18/2022	
Comments:	

Photograph ID: 2	
Photo Location: Bruce Creek	
Direction: Walton Bridge - Downstream Location	
Survey Date: 10/19/2022	
Comments:	

Client:	**Project:**
Site Name:	**Site Location:**

Photograph ID: 3 **Photo Location:** Holmes Creek **Direction:** US90 - Downstream Location **Survey Date:** 10/20/2022 **Comments:**	
Photograph ID: 4 **Photo Location:** Holmes Creek **Direction:** Woodham Rd. - Upstream Location **Survey Date:** 10/19/2022 **Comments:**	

Client:	**Project:**
Site Name:	**Site Location:**

Photograph ID: 5 **Photo Location:** Tenmile Creek **Direction:** Howell Williams Rd. - Downstream Location **Survey Date:** 10/20/2022 **Comments:**	
Photograph ID: 6 **Photo Location:** Tenmile Creek **Direction:** Robbins Rd. - Upstream Location **Survey Date:** 10/18/2022 **Comments:**	

Client:	**Project:**
Site Name:	**Site Location:**

Photograph ID: 7 **Photo Location:** Tenmile Creek **Direction:** Howell Williams Rd. - Downstream Location **Survey Date:** 10/19/2022 **Comments:** Ballyhoo fixed-point eDNA	
Photograph ID: 8 **Photo Location:** Holmes Creek **Direction:** Woodham Rd. - Upstream Location **Survey Date:** 10/19/2022 **Comments:** Particulate collected from filtering 1L of water	

Attachment D – LABORATORY AND DATA PROCESSING

Laboratory Analysis of Mussel eDNA

Library preparation followed a three step PCR described in O'Donnell et al. (2016). Each sample was amplified for a ~175 bp fragment of the 16S gene region which has previously been tested for amplification of unionid mussels from eDNA samples (Marshall et al. 2022b, Prié et al. 2021). Initial PCR amplification was completed for each sample in triplicate with 10 µl PCR reactions containing 4 µl extracted eDNA, 0.4µM primer, and Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0. The amplifications started with an initial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 15s, 5% ramp down to 55ºC for 30s, and 72ºC for 30s. Triplicate PCR products were diluted 1:10 then pooled prior to starting the Illumina adaptor and barcoding PCR processes.

The MiSeq library dual indexed paired-end sample preparation was adapted as described in Miya et al. (2015) from '16S metagenomic sequencing library preparation: preparing 16S ribosomal gene amplicon for the Illumina MiSeq system (Illumina part no. 15044223 Rev. B, San Diego, California, USA). A PCR process initiated the incorporation of Illumina adaptors and multiplexing barcodes using Prié et al. (2021) forward and reverse primers containing 33 or 34 base pairs of 5' Illumina hanging tails to provide a priming site for a final PCR to incorporate barcodes and remaining base pairs of Illumina adaptors. The 12 µl PCR reaction contained 2 µl diluted pooled PCR product, 0.3 µM Illumina adaptor primers and 6 µl 1X Qiagen Plus Multiplex Master Mix. The PCR process denatured for 95ºC for 5 min, 5 cycles of 98ºC for 20s, 1% ramp down to 65ºC for 15s, and 72ºC for 15s., followed by 7 cycles of 98ºC for 20s, 5% ramp down to 65ºC for 15s, and 72ºC for 15s. PCR product was diluted 1:10 prior to use in the barcode adaptor PCR process.

The final PCR incorporated paired-end dual indices (eight base pair barcodes) that allowed samples to be identified in the raw read data, and the p5/p7 adaptor sequences to allow the sample to bind onto the Illumina MiSeq flow cell. This final 12µl PCR reaction contained 1µl diluted product from the previous PCR, 0.3 µM forward and reverse indexed primer and 6ul 1X KAPA HiFi HotStart Ready Mix PCR Kit (Roche Diagnostics, Indianapolis, Indiana, USA). Conditions were 3 minutes of initial denaturation at 95°C, followed by 10 cycles at 98°C for 20 s, 5% ramp down to 72°C for 15 s, with a final 5 min 72°C extension. All PCRs were completed on Bio-Rad C1000 Touch Thermal Cyclers. Illumina adapted PCR products were pooled with equal volumes, then size selected (target ~319bp) using 2% agarose gel electrophoresis. The final pool was sequenced with 2× 300 nt V3 Illumina MiSeq chemistry by loading 6.4 pmol library. An additional 20% PhiX DNA spike-in control was added to improve data quality of low base pair diversity samples. Additionally, a PCR no-template negative control was run for each library preparation step.

Bioinformatic Processing of Mussel eDNA

The forward and reverse primer sequences were removed from the demultiplexed reads using the cutadapt (Martin et al. 2011) plugin within QIIME 2 (Bolyen et al. 2019). Next, sequence reads were filtered and trimmed using the denoising DADA2 (Callahan et al. 2016) plugin within QIIME 2. Based on the quality scores from the forward and reverse read files, a "truncLen" was set to 120 for the forward and 110 for the

reverse read files. Using DADA2, error rates were estimated, sequences were merged and dereplicated, and any erroneous or chimeric sequences were removed. Unique sequences were then clustered into Molecular Operational Taxonomic Units (MOTUs) using the QIIME 2 vsearch de-novo with a 97.5% similarity threshold (Coghlan et al. 2021, Marshall et al. 2022b). MOTUs from unionid taxa were identified to the species-level using the Basic Local Alignment Search Tool (BLAST+, https://blast.ncbi.nlm.nih.gov/Blast.cgi; Camacho et al. 2009) against our custom database of both in-lab generated sequences and mt-16S sequences downloaded from NCBI GenBank. These MOTUs were further validated with comparisons against the complete NCBI nr database, to investigate alignment to mis-labeled sequences or species not historically within the sampling region. MOTUs that did not return a sequence match from the BLAST search were excluded, as they were considered not from unionid taxa. A species detection was retained for each sampling transect if it exceeded ten sequencing reads within a water sample replicate and was found in >1 water sample replicates per transect.

Due to potential high-throughout sequencing technology being prone to low levels of error resulting from tag-jumping or index-hopping (Bohmann et al. 2022), a detection threshold was implemented to remove any potential low-confidence detections that are resultant from low-levels of contamination or index-hopping. This was accomplished by compiling a final eDNA dataset that only included an eDNA detection if it occurred in greater than 10 sequence reads and in multiple water replicates per sampling site.

Unionids display a unique form of mitochondrial inheritance, termed doubly uniparental inheritance (DUI), in which males possess a paternal mitochondrial mitotype that is restricted to male gonads and gametes (Gusman et al. 2016). As the male mitotype is genetically distinct from the female mitotype (Curole & Kocher 2005), we only retained sequences determined to be the female mitotype for direct comparisons to the tactile survey. Sequences were assigned to a species if they met a threshold of >97.5% identity and 100% query coverage. Furthermore, sequences that assigned to multiple species with the same BLAST e-value score were inspected and a final decision was made based on known distribution and presence within Fish Creek drainage basin. Additionally, if multiple sequences assigned to the same taxonomy, they were inspected and removed or collapsed into a single MOTU to obtain a final matrix of read counts per taxa.

The MiSeq data resulted in 13,920,277 raw sequence reads, of which 8,252,596 (59.29%) were processed through the primer trimming and quality filtering steps. Of these sequence reads, 7,736,592 (93.75%) successfully merged in DADA2 and were considered a unionid mussel sequence, of which 5,936,653 (76.73%) were attributed to a female mitotype sequence (mean per sample = 68,237.39 ± 80,916.84 SD, range = 0 – 309,902), and 1,799,939 (23.27%) were attributed to a male mitotype sequence (mean per sample = 20,154.61 ± 33,872.05 SD, range = 0 – 150,100) (Figure D1). Within each of the six bridge locations, the proportion of male mitotype sequences was substantially higher within the Bruce Creek and Holmes Creek downstream bridges (mean proportion of male mitotype in Bruce Creek downstream = 0.34, Holmes Creek downstream = 0.35, all other locations <0.064).

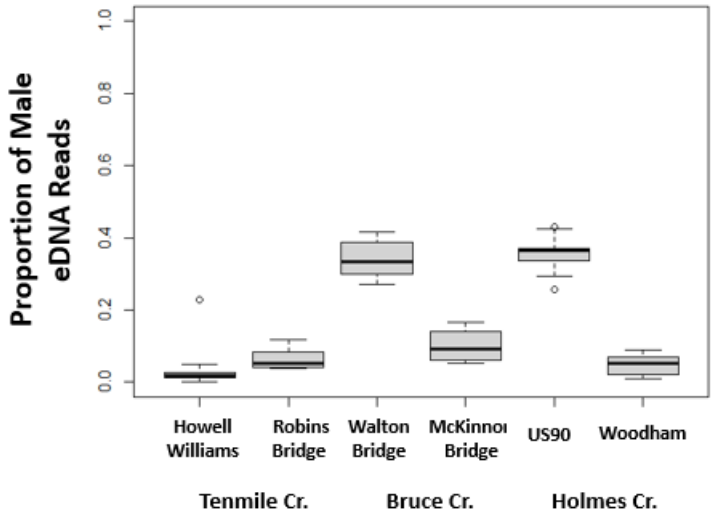

Figure D1 Mean ± standard error proportion of male unionid freshwater mussel environmental DNA from each of the six bridge locations sampled within the Choctawhatchee River basin.

The center line indicates the median, and each box indicates the lower and upper quartiles. Ends of the whiskers indicate maximum and minimum values within the quartile ± 1.5 x interquartile spread. Open circles (if present) indicate values falling outside the quartile ± 1.5 x interquartile spread.

Attachment E – TAXONOMIC IDENTIFICATION

For the federally protected species, Southern Sandshell and Tapered Pigtoe were identified with a 100% match and Fuzzy Pigtoe was identified with a 97.8% match to voucher sequences downloaded from NCBI Genbank (Table E.1, Figure E.1). The Choctaw Bean was identified with a 100% match to a voucher sequence obtained from N. Johnson (Table E.1, Figure E.1).

Flatwoods Creekshell (*Strophitus williamsi*), Southern Purple Lilliput (*Toxolasma corvunculus*), Southern Rainbow (*Villosa vibex*), and Little Spectaclecase (*Leaunio lienosus*) were all identified with 100% match to voucher sequences obtained from N. Johnson. The Apalachicola Pondhorn (*Uniomerus columbensis*) lacks a reference voucher sequence, and thus is currently identified based on genus-level similarity and known presence in the system.

Southern Fatmucket (*Lampsilis straminea*) has reference sequences, however, eDNA sequences were only found at a 95% match to available sequences on NCBI Genbank. This may represent large geographical genetic differences between populations within these species, and thus true identification requires voucher tissue specimens from this river drainage. In fact, the Choctawhatchee River basin has been regarded as containing unique genetic variants for some species compared to other eastern Florida river basins. For example, through genetic examination, unique *Villosa* and unique *Strophitus* genetic variants have been recognized (Williams et al. 2014, Smith et al. 2018). Therefore, true identification of the current eDNA sequences requires an updated curated genetic database based on correct morphological vouchers. Furthermore, two additional sequences were unable to be identified to the species-level with current genetic databases, an unknown *Toxolasma* sp. mussel and a third species within the *Villosa* complex that is a 97% match to Little Spectaclecase, but was only detected within the downstream bridge location on Holmes Creek.

Table E1 Taxonomic identification of the environmental DNA sequence data based on to species historically found within the Choctawhatchee River basin, their federal status, and if they have an available DNA reference sequence. E=endangered; T=threatened.

Tribe	Species	Common Name	16S Voucher Sequence	Percent Identification	Best Species Match
Anodontini	*Strophitus williamsi*	Flatwoods Creekshell	X	100.00	*Strophitus williamsi* (N. Johnson)
Lampsilini	*Hamiota australis* FT	Southern Sandshell	X	100.00	*Hamiota australis*
	Lampsilis straminea	Rough Fatmucket	X	94.82	*Lampsilis straminea*
	Obovaria choctawensis FE	Choctaw Bean	X	100.00	*Obovaria choctawensis* (N. Johnson)
	Toxolasma corvunculus	Southern Purple Lilliput	X	100.00	*Toxolasma corvunculus* (N. Johnson)
	Toxolasma sp.			87.00	Several *Lampsilini sp.*
	Leaunio lienosus	Little Spectaclecase	X	100.00	*Leaunio lienosus* (N. Johnson)
	Leaunio sp.			97.00	*Leaunio lienosus* (N. Johnson)
	Villosa vibex	Southern Rainbow	X	100.00	*Villosa vibex* (N. Johnson)
Pleurobemini	*Elliptio sp.*	Gulf Spike	X	100.00	Several *Elliptio* spp. >97.5%
	Fusconaia burkei FT	Tapered Pigtoe	X	100.00	*Fusconaia escambia, Fusconaia burkei*
	Pleurobema strodeanum FT	Fuzzy Pigtoe	X	97.81	*Pleurobema strodeanum*
Quadrulini	*Uniomerus columbensis*	Apalachicola Pondhorn		97.02	*Uniomerus declivus*

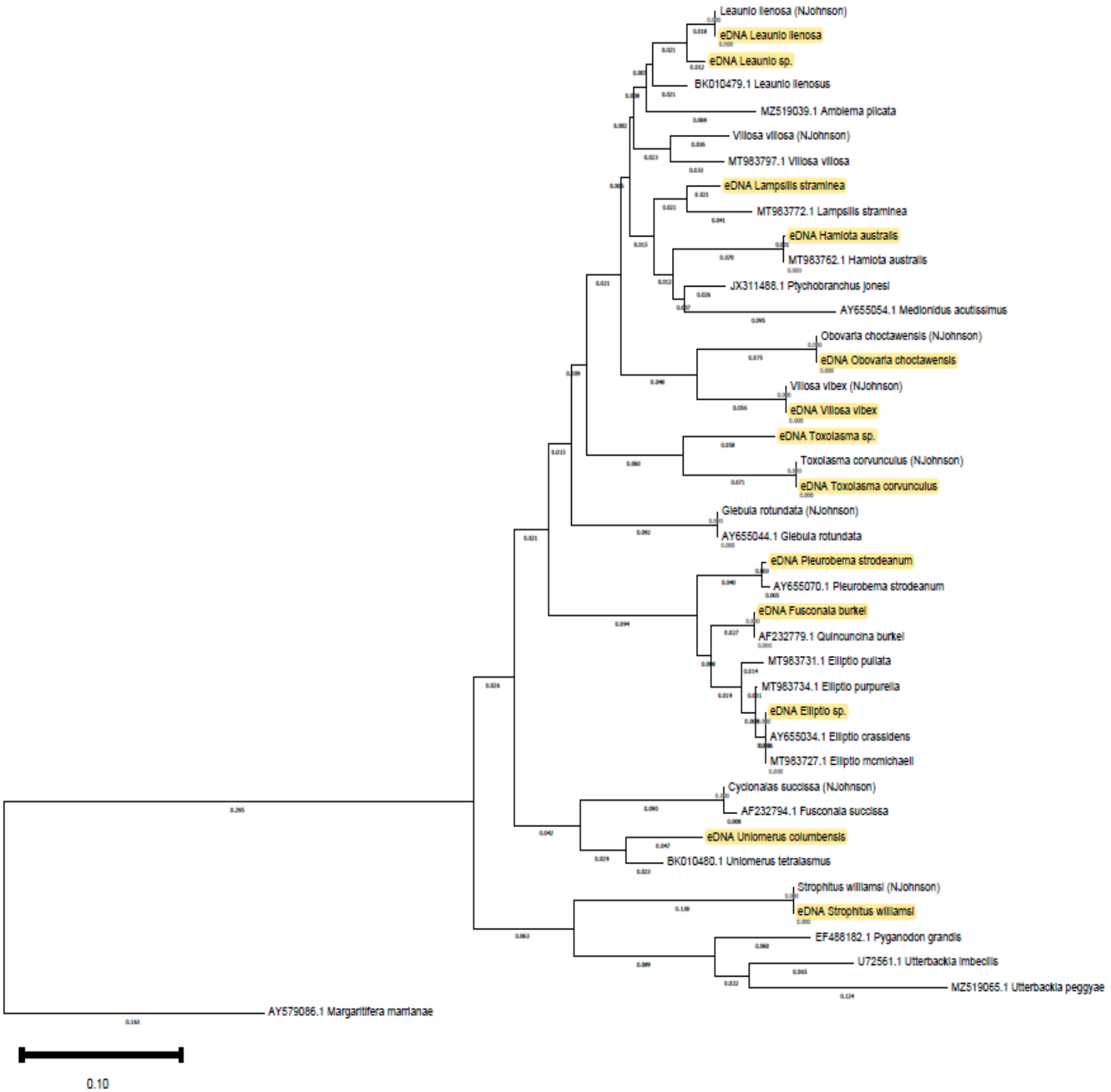

Figure E1 Phylogenetic tree based on neighbor-joining method using MEGA 7. The estimated genetic distance between taxa clusters are shown next to each branch. Highlighted names represent sequences detected with eDNA.