Optimizing Threatened Wood Turtle (*Glyptemys insculpta*) Environmental DNA Surveillance with Experimental Control Standardization.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

By

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Table of Contents

List of Tables

List of Figures

Abstract

OPTIMIZING WOOD TURTLE (*GLYPTEMYS INSCULPTA*) ENVIRONMENTAL DNA SURVEILLANCE WITH EXPERIMENTAL CONTROL STANDARDIZATION

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Turtles are experiencing declines global while their distributions are poorly understood by modern science. Environmental DNA (eDNA) has proven to be a valuable tool in rapid species detection. However, in such a rapidly growing field, standardized practices have not yet taken hold, and rigorous comparisons among methodologies have not been evaluated. This thesis consists of two chapters that seek to answer questions facing eDNA methodologies used for monitoring of cryptic and endangered turtles. Chapter 1 is a literature review developed to provide comparisons on the current methods used for turtle eDNA research. Among current literature, turtles represent the most well-represented group of reptiles to date and most studies have been conducted in the global north with slow growth in the number of publications conducted south of the equator which includes several areas of high turtle diversity. Taxonomic representation of eDNA work is also disproportionate with the family Emydidae being the focus of more than 53% of the studies found in the literature to date. There is also a wide diversity of

approaches in experimental design and the implementation of laboratory and field protocols. Factors such as sample type, genetic marker selection, and implementation of experimental controls are also highly variable between publications.

Chapter 2 is a research chapter focusing on a threatened North American species. Wood Turtle (*Glyptemys insculpta*) is one of many turtle species currently experiencing declines across their range. However, their species status is still not fully understood. eDNA has been demonstrated to be an effective and sensitive noninvasive surveillance approach for detecting turtles that can provide rapid information on detection and occupancy for conservation monitoring and management. The field of eDNA requires dealing with several challenges as it relies on rigorous validation of the results by carefully assessing the sources of error that produce false results (false positives and false negatives) when conducting work to determine species presence. This chapter focuses on the ongoing issue involving field blank false positive amplifications which led to the development of a new standardized protocol. This new protocol has led to a reduction in false positives in field blanks showcasing the importance of standard eDNA practices concerning control usage at every stage of sampling.

Chapter 1: A Literature Review of the Application of Environmental DNA to Monitor Turtle Species

Turtles are currently experiencing global declines due to detrimental human activities (Nordstrom et al. 2022). Rapid surveillance efforts are urgently needed to accurately estimate the distribution of threatened populations so that appropriate conservation management action plans can be implemented to preserve them. To date, 56% of reptile eDNA studies have focused on turtles, making them the most studied group of reptiles using eDNA technologies (Nordstrom et al. 2022). Two recent reviews (Adams, et al. 2019a, Nordstrom, et al. 2022) show that research utilizing eDNA to study reptiles is variable in experimental methodology, understudied compared to other vertebrate groups (fishes, amphibians, mammals), and the majority are conducted on North American species. Due to the rapid growth and implementation of eDNA technologies focused on turtles in the past two years, these two reviews no longer completely cover the current literature. Several recently published studies have introduced changes and include novel approaches such as the expansion of citizen science in turtle eDNA collection (Feng et al. 2023) and the development of turtle eDNA assays for previously understudied geographic regions (Yudha et al. 2023).

Previous reviews have focused broadly on reptiles with turtles being detected/analyzed in the majority of these eDNA studies. To date, there has not been a review of the literature that specifically focuses on eDNA being applied exclusively for turtles despite their global declines. Experimental design variation between different studies has not been thoroughly examined in areas such as selected genetic markers, differences in collection methods, and varying methods for sample preservation. A complete comparison of eDNA sampling methodology, genetic marker selection, and turtle taxonomic coverage has not been explored by the scientific community. This review would serve to standardize and compare different experimental set ups and sampling systems.

As a noninvasive approach, eDNA sampling has opened the door for rapid and sensitive surveillance of species that were previously shown to be challenging to monitor by other, traditional methods (Akre et al. 2019, Abrego et al 2018, Fukumoto et al. 2015, Hernandez et al. 2020). The use of eDNA technology as a significant method for the detection of cryptic and invasive species has seen rapid proliferation over the last three decades. Initial use of eDNA was conducted to detect aquatic microbial pathogens in the 1990s (Adams et al. 2019b, Díaz-Ferguson et al. 2014, Goldberg et al 2015, Goldberg et al. 2016, Thomsen and Willerslev 2015), and more recent studies have used this technology for the detection of vertebrate species, beginning in 2008 with the detection of bullfrogs in France (Ficetola et al. 2008). The first published work on detecting reptile eDNA was not until 2014 and was centered around invasive Burmese Pythons in the

United States (Hunter et al. 2015, Piaggio et al. 2014). The first publication of turtle eDNA detection quickly followed later that year and successfully detected sea turtles in a multiple species aquarium tank (Kelly et al. 2014). After the expansion of eDNA detection into most taxonomic groups, focus within the field turned towards improving the accuracy and effectiveness of detection rates (Adams et al. 2019a, Hutchins et al. 2022, Goldberg et al 2015, Goldberg et al. 2016, Sepulveda et al. 2020, Thomsen and Willerslev 2015). Variables such as volume of water, speed of water, air temperature, water temperature, UV radiation levels, pH of the water, time spent in the water column, and the composition of the local microbial community impact eDNA persistence and detectability (Akre et al. 2019, Harrison et al. 2019, Rees et al. 2015, Strickler et al. 2014). Other variables that are currently being addressed include the increase in reported false positives and false negatives which cause consistent issues in sample analysis (Hutchins et al. 2022, Ruppert et al. 2019, Sepulveda et al. 2020).

Work in the field has led to the development of the "shedding hypothesis" (Adams et al. 2019b). Where organisms that have a greater amount of interaction with their environment also tend to shed more eDNA making them easier to detect (Goldberg et al. 2016, Thomsen and Willerslev 2015). Freshwater vertebrates, such as fish and amphibians, spend more time in the water and release more DNA through shedding mucus, feces, cells, etc. (Goldberg et al 2015, Goldberg et al. 2016, Pont et al. 2018, Thomsen et al 2012). Mammals, reptiles, and birds tend to have less consistent interaction with the water system and detection takes a greater understanding of the

ecology of the target organism to effectively detect them regularly as well introducing requirements to account for other sources of eDNA introduction including soil, air, and feces from predators of target species (Foote, et al. 2012, Goldberg et al 2015, Lacoursière-Roussel et al. 2016).

With the impact of human activities increasing, many species are undergoing shifts in their population size and stability. In the face of a global biodiversity crisis, eDNA's applications in the study of species of conservation concern cannot be understated with its utility in detecting species through molecular methods and less disruptive effects as a non-invasive method. Threatened and invasive species have been the subject of more studies pushing the boundaries of eDNA detection (Barnes and Turner 2016, Beng et al. 2020, Bohmann et al. 2014, Katz et al. 2021). Examples of invasive species monitored through the use of eDNA include: American Bullfrogs in Europe (Ficetola et al. 2008), Burmese Pythons in the southern United States (Hunter et al. 2015, Piaggio et al. 2014), and Asian Carp in North America (Amberg et al. 2015, Klymus et al. 2015,). Both the first successful detection of vertebrates and the first successful detection of reptiles was accomplished while conducting eDNA studies of invasive species with applications broadening to threatened ones in similar taxa in subsequent work (Ficetola et al. 2008, Hunter et al. 2015, Piaggio et al. 2014).

There have been two reviews covering eDNA work focused on reptiles (Adams et al. 2019a, Nordstrom et al. 2022). Both found that reptile-focused work is not increasing at the same rate as other groups of vertebrates, but both reviews restricted their analysis

to general trends across all reptiles without looking at taxa specific data. The literature search conducted for this chapter was completed using ResearchGate and referenced with Google Scholar during July 2023. Search terms included combinations of "Turtle", "Testudines", "Chelonian", "Reptile", "Environmental DNA", "eDNA", and "Metabarcoding" with a total of 49 papers using eDNA to study turtle ecology selected for analysis in this review.

Species-Specific Targeted versus Metabarcoding Studies: eDNA studies have been categorized in two different technologies that differ in scope. Species-specific targeted eDNA work (targeted) is solely focused on a few choice species and involves a qPCR approach with species-specific markers for presence-absence determination with targeted regions often occurring within the mitochondrial genome (Kelly et al. 2014, Nordstrom et al. 2022). This specificity is accomplished through the development of gene expression assays that consist of primers to amplify the target gene and a species-specific probe that will provide measurable fluorescence only if the target species' DNA is present. These studies are primarily used for the detection of species or ecological analysis concerning species of conservation concern and non-native origin (Alfaro-Cordova et al. 2022, Akre et al. 2019, Hernandez et al. 2020, Kundu et al. 2018, Lam et al. 2020, Lam et al. 2022, Loeza-Quintana et al. 2020, Siler et al. 2021, Tarof et al. 2021, Villacorta-Rath et al. 2022).

Metabarcoding studies consist of research utilizing metagenomic approaches to sample entire community assemblages with a set of primers (focused on either one

genetic marker or several) to conduct DNA barcoding to identify individual species (Cannon et al. 2016, Rivera et al. 2018, West et al. 2021). The samples are run through an amplification step and then sequenced with the resulting data compared against published sequences (Kelly et al. 2014, Mojica et al. 2021, Rivera et al. 2018, West et al. 2021, West et al. 2023). Successful detection of turtles or turtle microbiota has been conducted including a range of possible targets from only testing for reptiles to including all possible organisms (Diaz-Abad et al. 2022, Mojica et al. 2021, Sigsgaard et al. 2020, West et al. 2021, West et al. 2023, Zhang et al. 2023). Metabarcoding projects on turtle ecology have used water filters, shell scrapings, and fecal analyses for eDNA detection and analysis (Ducotterd et al. 2020, Ducotterd et al. 2021, Kanjer et al. 2022, Kelly et al. 2014, Koizumi et al. 2016, Sarkis et al. 2022, Zhang et al. 2023).

As of July 2023, the majority of turtle eDNA studies found within this review have been conducted using targeted approaches, but metabarcoding studies have been proportionately increasing in recent years which matches the increase in the use of eDNA research with broader objectives than presence/absence determination.

Genetic Marker Selection – Genetic markers selection is a key aspect of eDNA protocol design and requires an understanding of species being detected. Across this review, the majority of publications focused on genetic markers within the mitochondrial genome due to the ease of detectability when compared to nuclear markers (Akre et al. 2019). When looking at a comparison of genetic markers between targeted and metabarcoding publications, targeted qPCR approaches had the most variation in genetic marker

selection. Eight different genes (12S, 16S, ATP6, CO1, CR, CytB, ND2, ND4) were identified to successfully detect turtle eDNA in both the metabarcoding and species-specific assays. This is a slight increase from the 7 genes identified in the most recent reptile eDNA review from 2022 (Nordstrom, et al. 2022). The newly tested gene, ATP6, was found to amplify eDNA from both diamondback terrapins (*Malaclemys terrapin*) and red-eared sliders (*Trachemys scripta)* in targeted controlled water samples, but the associated probes were not species-specific (Portnoy et al. 2023). CO1 and CytB were the most commonly used genetic markers for targeted qPCR research with 11 and 9 publications respectively (**Figure 1**).

Metabarcoding publications only covered 5 genetic markers (12S, 16S, CO1, CR, CytB) (**Figure 1**). 12S and 16S were the two most often used genetic markers with 7 and 6 publications. The lower number of genetic markers can be attributed to the need for metabarcoding genetic markers to be amplifiable across a wider range of taxa, constricting the number of usable markers (Nordstrom et al. 2022).

Availability of existing genomic resources for rare and threatened turtle species remains an issue for research focused on community composition and biodiversity in general. Several studies have solely focused on turtle primer design for an array of genes rather than actively testing for eDNA in the environment (Hernandez et al. 2020, Lam et al. 2020, Nordstrom et al. 2022, So et al. 2020). Fifty six percent (56%) of the studies reported developing their own primer assays with an increasingly diverse set of species covered. Five of the genes (12S, 16S, CO1, CytB, ND4) were tested in studies

undertaken only in a laboratory setting using controls such as tissue or blood samples (Hernandez 2020, Lam 2020, So 2020). Six of the genes (12S, 16S, CO1, CR, CytB, ND2) were tested within a controlled environment (ie: tanks or managed pools containing the target species). All eight genetic markers were tested and successfully detected turtle eDNA in the field through a targeted qPCR approach.

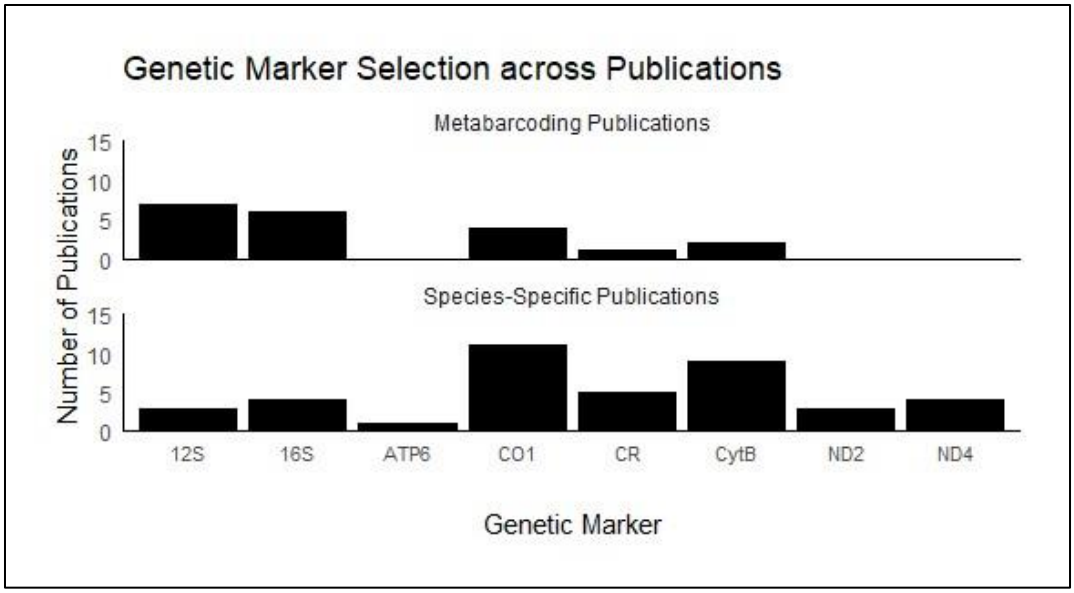

Figure 1: Genetic marker selection comparison between metabarcoding and species-specific approaches. Species-specific approaches are more prevalent with a wider variety of genetic markers used, while metabarcoding publications are still comparatively new and are increasing in number. Most recorded genetic markers used in eDNA research have been located within the mitochondrial genome.

Variation in Study Design – Study design remains variable across eDNA publications due to eDNA's usage through a variety of different sample types in different ecosystems (**Figure 2**). Studies focused on turtle ecology have utilized eDNA from freshwater (**Table 1A**) marine water (**Table 1B**) sand for species detection (**Table 1B**), fecal sampling for dietary and microbiome analyses (Table 1C), throat swabs for further dietary work (**Table 1C**), epibiotic (shell scraping and nest sediment samples) for local community testing (**Table 1C**), and tissue for primer development and testing (**Table 1D**). Fresh and marine

water samples were used primarily for species detection using water filtration systems across a variety of different turtle taxonomic groups (Adams et al. 2019, Akre et al. 2019, Davy et al. 2015, Feist et al. 2018, Feng et al. 2022, Kundu et al. 2018, Lam et al. 2022, Wang et al. 2023, Wilson et al 2018) or precipitation methods (Villacorta-Rath et al. 2022). Dietary, fecal, and epibiotic analyses were focused on a few select groups: invasive members of Emydidae and various species of Cheloniidae (Diaz-Abad et al. 2022, Ducotterd et al. 2020, Ducotterd et al. 2021, Kanjer et al 2022, Koizumi et al. 2016, Sarkis et al 2022, Salleh et a. 2022). Epibiotic sampling involving shell scraping was used to look at individual micro-community assemblages (Kanjer et al. 2022, Parks et al. 2020) and was even able to identify individuals based on their microbiota (Rivera, et al. 2018).

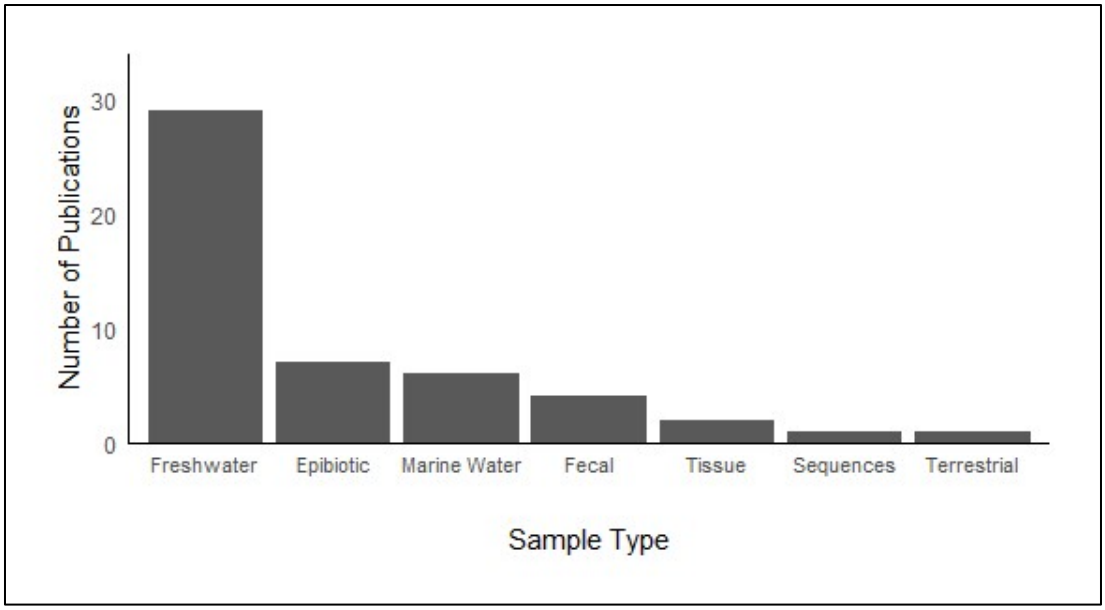

Figure 2: Sample type comparison used across the review. Freshwater sampling continues to be the most widely used followed by epibiotic and marine water sampling.

Table 1: Full list of surveyed papers by sample type concerning eDNA usage to study turtle ecological monitoring. Publications ordered by sample type A) freshwater, B) Marine and Sand, C) Epibiotic, Fecal, and Dietary, and D) Laboratory-based testing. Information on each publications' primary author/year, the turtle families detected, usage of species-specific or metabarcoding methods, location of sample origin, sample type used in the publication, and the conditions of sample collection and analysis.

Primary Author/Year	Family	Study Type (Species-Specific/Metabarcoding)	Location	Type of Sampling	Sample Origin
Table 1A	Freshwater Publications				
Adams 2019	Emydidae	Species-Specific	United States	Freshwater	Controlled
Akre 2019	Emydidae	Species-Specific	United States	Freshwater	Field
Cannon 2016	Emydidae	Metabarcoded	United States	Freshwater	Field
Davy 2015	Emydidae, Trionychidae, Chelydridae, Kinosternidae	Species-Specific	Canada	Freshwater	Controlled
de Souza 2016	Kinosternidae	Species-Specific	United States	Freshwater	Field
Engelstolft 2015	Emydidae	Species-Specific	United States	Freshwater	Field
Feist 2018	Chelydridae	Species-Specific	United States	Freshwater	Controlled/field
Feng 2022	Kinosternidae	Species-Specific	Canada	Freshwater	Field
Fyson 2021	Emydidae	Species-Specific	Canada	Freshwater	Field
Gleeson 2022	Emydidae	Species-Specific	Australia	Freshwater	Controlled/field
Gleeson 2022	Emydidae	Species-Specific	Australia	Freshwater	Controlled/field
Kakuda 2019	Emydidae	Species-Specific	Japan	Freshwater	Field
Kessler 2020	Chelydridae	Species-Specific	United States	Freshwater	Field
Kirtane 2019	Emydidae	Species-Specific	United States	Freshwater	Field
Kundu 2018	Trionychidae	Species-Specific	India	Freshwater	Field
Lacoursière-Roussel 2015	Emydidae	Species-Specific	Canada	Freshwater	Field

Lam 2022	Platysternidae	Species-Specific	China	Freshwater	Field
Loeza-Quintana 2020	Emydidae	Species-Specific	Canada	Freshwater	Field
Portnoy 2023	Emydidae	Species-Specific	United States	Freshwater	Controlled/field
Raemy 2018	Emydidae	Species-Specific	Switzerland	Freshwater	Field
Risti 2022	Emydidae	Species-Specific	Indonesia	Freshwater	Controlled
Tarof 2021	Emydidae	Species-Specific	Canada	Freshwater	Field
Siler 2021	Emydidae	Species-Specific	United States	Freshwater	Field
Villacorta-Rath 2022	Chelidae	Species-Specific	Australia	Freshwater	Field
Wang 2023	Carettochelyidae, Emydidae, Geoemydidae, Podocnemididae	Metabarcoded	China	Freshwater	Controlled
West 2021	Cheloniidae	Metabarcoded	Australia	Freshwater	Field
Wilson 2018	Geoemydidae	Species-Specific	Malaysia	Freshwater	Controlled/field
Yudha 2023	Geoemydidae	Metabarcoded	Indonesia	Freshwater	Field
Zhang 2023	Trionychidae, Emydidae	Metabarcoded	China	Freshwater	Field
Table 1B:	Marine Publications				
Alfaro-Cordova 2022	Cheloniidae	Metabarcoded	Peru	Marine	Field
Farrell 2022	Cheloniidae	Species-Specific	United States	Marine and Sand	Controlled/field
Harper 2020	Cheloniidae	Species-Specific	United States	Marine	Controlled/field
Kelly 2014	Cheloniidae	Metabarcoded/ Species-Specific	United States	Marine	Controlled
Sigsgaard 2020	Cheloniidae	Metabarcoded	Saudi Arabia	Marine	Field
West 2021	Cheloniidae	Metabarcoded	Australia	Marine	Field

Table 1C:	Epibiotic, Dietary, and Fecal Publications				
Diaz-Abad 2022	Cheloniidae	Metabarcoded	Portugal	Throat Swabs	Field
Ducotterd 2020	Emydidae	Metabarcoded	Switzerland	Faeces	Field
Ducotterd 2021	Emydidae	Metabarcoded	Switzerland	Faeces	Field
Kanjer 2022	Cheloniidae	Metabarcoded	Croatia	Scraping shell	Field
Koizumi 2016	Emydidae, Geoemydidae	Metabarcoded	Japan	Feces	Field
Martin 2021	Cheloniidae	Species-Specific	France	Microbiome	Field
Mojica 2021	Emydidae, Geoemydidae	Metabarcoded	Colombia	Feces	Field
Parks 2020	Emydidae	Metabarcoded	United States	Scraping shell	Field
Rivera 2018	Cheloniidae	Metabarcoded	France	Scraping shell	Field
Salleh 2022	Geoemydidae	Metabarcoded	Malaysia	Feces	Controlled
Sarkis 2022	Cheloniidae	Metabarcoded	United States	Throat Swabs	Field
Table 1D:	Laboratory Based Publications				
Hernandez 2020	Emydidae, Trionychidae,	Species-Specific	Canada	Online sequences	Lab
Lam 2020	Platysternidae, Emydidae, Trionychidae	Species-Specific	China	Tissue	Lab
So 2020	Geoemydidae, Platysternidae, Emydidae, Trionychidae	Species-Specific	China	Tissue	Lab

Water sampling was the most common method used with 28 publications using freshwater and 6 using marine water. Many of these studies used a filtration method with water samples being collected and filtered at the site (Akre et al. 2019, Feng et al. 2023, Fyson et al. 2021, Loeza-Quintana et al. 2020, Sigsgaard et al. 2020, Tarof et al. 2021) or being transported back to a testing facility for later filtration (De Souza et al. 2016, Harper et al. 2020, Kakuda et al. 2019, Kessler et al. 2020, Risti et al. 2022, Villacorta-Rath et al. 2022, West et al. 2021, Wilson et al. 2018). Pore size of these filters spanned 0.22 µm to 1.5 µm with the most common size being 0.45 µm (**Figure 3**). Marine water samples were sampled using smaller filter pore sizes while freshwater sampling was conducted with more variation in filter pore size. Sample volume also demonstrated differences across this survey with volumes used between 15 mL (Kundu et al. 2018) to 4 L (Engelstolft 2015, Yudha et al. 2023). However, the study using 15 mL was conducted inside a temple in a small pond that had a high density of turtle species. This experimental design in an artificial setting allowed them to successfully detect turtle eDNA at low volumes. Other studies had to adjust their water collection methods based on local hazards. A study conducted in Australia obtained water samples by remotely using extension poles to avoid conflict with local predators. This field sampling design has been utilized in many studies but is limited to sampling deeper water (Gleeson et al. 2022, Villacorta-Rath et al. 2022, West et al. 2021, West et al. 2023). An alternative to filtration-based sampling is precipitation though this method was used significantly less in turtle ecology eDNA work with only one study reported using this method (Villacorta-

Rath et al. 2022) which is most likely due to filtration's higher sensitivity when using smaller pore sizes (Cooper et al. 2022).

Other sampling types were not as widely spread in their usage (**Figure 2**). Amounts of samples collected and used such as tissue and fecal testing are not always recorded or all available material is used (Ducotterd et al. 2020, Ducotterd et al. 2021, Koizumi et al. 2017, Lam et al. 2020, So et al. 2020). Only one publication used terrestrial samples (sand) and suspended the samples in TE Solution, and the supernatant was then filtered to collect the eDNA sample (Farrell et al. 2022). Epibiotic sampling consisted of both shell scraping using sterile tools (ie: sterile toothbrushes) and skin samples (Kanjer et al. 2022, Parks et al. 2020, Rivera et al. 2018).

The lack of standard sample control usage has been highlighted as an ongoing issue in the field of eDNA research (Sepulveda et al. 2020), and this was reflected in this survey with high variability between study systems (**Figure 2**). Proposed standardized methods implement controls at three levels during the sampling and analysis phases: field controls collected on site of sample collection and stored with the samples, extraction controls added during the DNA extraction step with every extraction performed, and amplification controls during the DNA analysis phase (such as qPCR) (Hutchins et al. 2022). Field controls also consisted of "cooler blanks", containers of water that were brought to the site while collection was conducted and then stored with samples to detect any contamination during transport (De Souza et al. 2016). This method is useful for testing transportation contamination but fails to account for any direct sampling

contamination while in the field. Other variations included clean water samples filtered alongside field samples in the testing facility (Kakuda et al. 2019) and pond water samples from exclusionary pools (Raemy et al. 2018, Tarog et al. 2021). Control usage (**Figure 4**) was variable with amplification controls being the most widely used/reported in 51.02% of the studies while extraction controls were only used/reported in 30.61% of the studies. Field controls were the most variable due to either lack of reporting by authors and/or differing needs of specific sampling types. Lack of control standardization is not only a broader eDNA issue, but one present within this taxon-specific work as well.

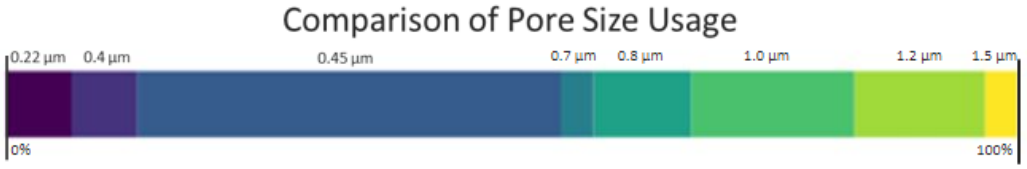

Figure 3: Filter pore size comparison across studies using eDNA obtained from water samples. 0.45 µm pores are the most widely used, followed by 1 µm and 1.2 µm pore sizes. Filters were used in freshwater, marine water, and terrestrial (samples were dissolved) sample set ups.

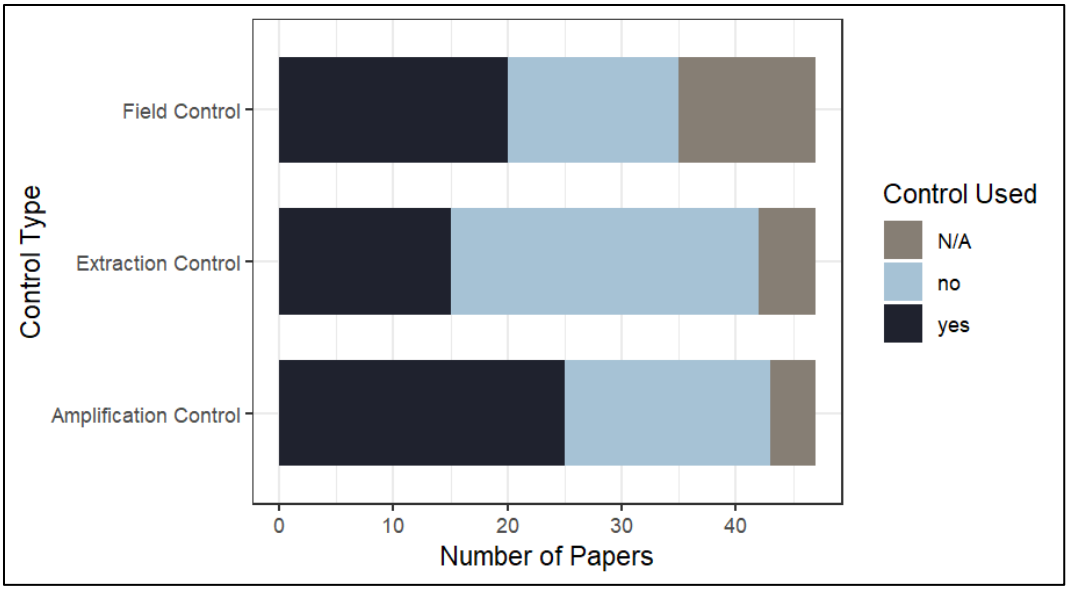

Figure 4: Implementation of experimental controls across eDNA sampling protocols. Publications that reported methods that required the corresponding controls and did report using controls for this step were categorized under "yes". Publications that reported methods that required corresponding controls but did not report using controls for this step were categorized as "no". Publications that reported methods that did not require controls at specific steps were categorized as "N/A".

Geographic Distribution of Studies: The locations of published turtle eDNA work echoes the trends seen in previous reptile eDNA reviews with a concentration of research in the Global North developed nations as this field continues to expand (Adams, et al. 2019a, Nordstrom, et al. 2022). The greatest percentage of turtle focused eDNA work has been published in North America with fifteen publications from the United States of America and four from Canada. The majority of papers were targeted approaches for local threatened species (Akre et al. 2019, Davy et al. 2015, De Souza et al. 2016, Feist et al.

2018, Feng et al. 2022, Fyson et al. 2021, Kessler et al. 2020, Lacoursière-Roussel et al. 2015, Loeza-Quintana et al. 2020, Siler et al. 2021, Tarof et al. 2021). These projects have been primarily aimed at validation of assays and developing rapid assessments for populations that are poorly understood and assumed to be declining due to challenges such as the increase of habitat development and vehicle collisions.

East and Southeast Asia have seen a significant increase in the detection of turtles using eDNA with an emphasis on metabarcoding detecting full community assemblages in a mix of ecosystems both rural and urban with turtles being a small fraction of the focus (Wang et al. 2023, Yudha et al. 2023, Zhang et al. 2023). When compared to a previous review in 2019, turtle eDNA detection has increased from two publications to eleven within the last four years (Adams et al. 2019a).

Australian eDNA turtle research (4 publications) continues the trend of metabarcoding alongside targeted studies, but instead of large community-based work, they have focused on specifically reptiles in different environments including marine, brackish, and freshwater (Villacorta-Rath et al. 2022, West et al. 2021, West et al. 2021b). South America had two recorded publications. Both being metabarcoding research with one marine publication and one freshwater publication (Alfaro-Cordova et al. 2022, Lozano Mojica et al. 2021). The Middle East, Central America, and Africa have one publication each with all four studies analyzing the family Cheloniidae (Alfaro-Cordova et al. 2022, Bezy et al. 2020, Diaz-Abad et al. 2022, Sigsgaard et al. 2020).

This publication record is not representative of the global diversity and location of turtle species. Studies specifically on invasive species were found across Europe and Asia and detected members of Emydidae (*Trachemys scripta* in Japan, China, France, Australia, and Indonesia) (Gleeson 2022, Kakuda 2019, Koizumi 2016, Lam 2020, Risti 2022, So 2020, Wang 2023, Zhang 2023) and Trionychidae (*Apalone ferox* was detected in China) (Zhang 2023).

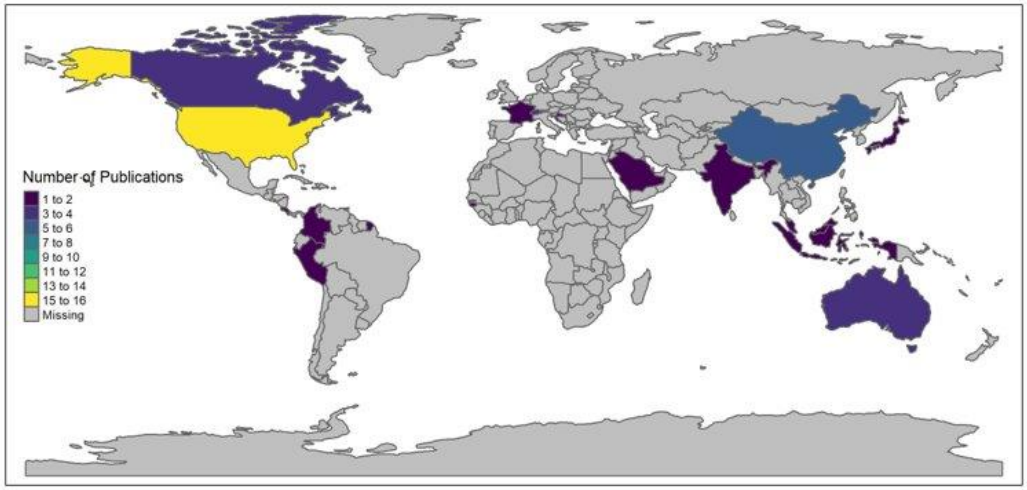

Figure 5: Geographic distribution and frequency of eDNA studies as of July 2023. There is a high concentration of publications and research being conducted in the global north and in North America. Recent publications have been expanding turtle focused eDNA work, but more is necessary for proper surveillance of global turtle populations.

Taxonomic Representation: Of the currently recognized 14 families of turtles, 10 have been successfully detected in eDNA research: Carettochelyidae, Chelidae, Cheloniidae, Chelydridae, Emydidae, Geoemydidae, Kinosternidae, Platysternidae, Podocnemididae, and Trionychidae (**Figure 6**) (Rhodin et al. 2017). Out of the approximately 356 turtle species recognized extant species, 33 species have been featured in eDNA research.

The most represented family was Emydidae with 26 primary publications featuring members of this group in both targeted and metabarcoding approaches, but most of the studies were focused on using water for detection (Adams et al. 2019, Akre et al. 2019, Cannon et al. 2016, Davy et al. 2015, Fyson et al. 2021, Kakuda et al. 2019, Kirtane et al. 2019, Lacoursière-Roussel 2015, Loeza-Quintana 2020, Lozano Mojica et al. 2021, Risti et al. 2022, Siler et al. 2021, Tarof et al. 2021). The next most commonly analyzed family was Cheloniidae with 13 studies (Farrell et al. 2022, Harper et la. 2020, Kelly et al. 2014, Sigsgaard et al. 2020, West et al 2021, West et al. 2023). Trionychidae was analyzed within 6 studies (Davy et al. 2015, Hernandez et al 2020, Kundu et al. 2018, Lam et al. 2020, So et al. 2020, Zhang et al. 2023), and Geoemydidae was analyzed in 7 studies (Koizumi et al. 2016, Lozano Mojica et al. 2021, Mohd Salleh et al. 2022, So et al. 2020, Wang et al. 2023, Wilson et al. 2018, Yudha et al. 2023). The rest of the represented families were analyzed in either 3 publications or less (**Figure 6**).

There is a clear taxonomic bias influenced by geographic location when it comes to the selection of species being analyzed with eDNA work. Emydidae is the most

common turtle family in North America which has led to its larger representation in eDNA research as well as containing several highly invasive species which led to detection in other regions (Spinks et al. 2016). Cheloniidae is also represented second most in eDNA research most likely due to the widespread distributions of its species (Duchene et al. 2012).

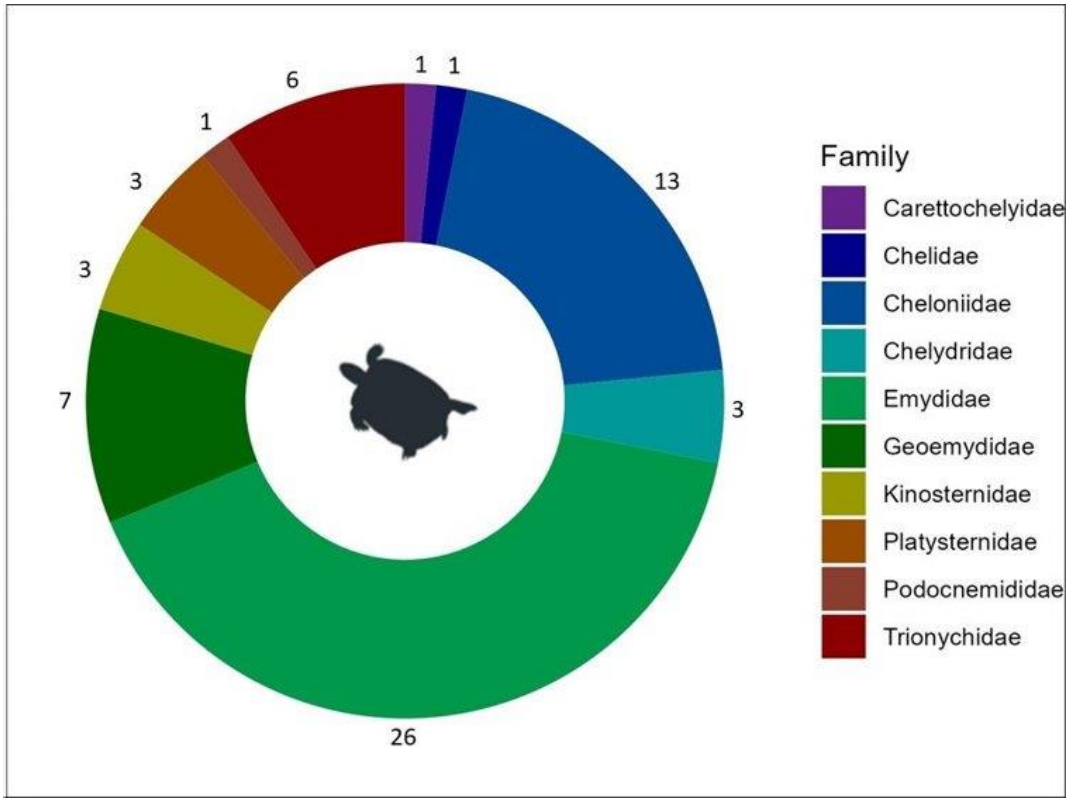

Figure 6: Turtle taxonomic representation in the literature by family. Emydidae was analyzed in the largest portion of studies along with Cheloniidae and Geoemydidae. This represents reported analyzed families detected using eDNA and/or studied using eDNA techniques in both species-specific and metabarcoding publications.

Conclusion of Chapter 1

This review shows the rapid expansion of published eDNA work centered around turtle ecology globally as eDNA techniques continue to spread and become more accessible. The high degree of variation in eDNA research leads to need for consistent and reviews to assess the full extent of eDNA usability as the field continues to progress (Beng and Corlett et al. 2020, Nordstrom et al. 2022). With this expansion, taxon specific reviews also rise in their importance to ensure the ease of accessibility of different methodologies and results across the field. Aquatic and semi-aquatic reptiles to date remain the most studied turtle groups with the majority of research being conducted in the northern and western hemispheres. These trends are changing with research expanding rapidly particularly in East Asia, but the global south still remains relatively understudied through the use of eDNA methods despite the high level of turtle biodiversity found there (Nordstrom et al. 2022). Standardization of control usage is also key to the future to ensure the accuracy of results and comparability between study designs (Hutchins et al. 2022, Sepulveda et al. 2020). Four families of testudines have not yet been analyzed using eDNA methods and represent groups that are threatened globally

with low diversity of species (Rhodin et al. 2017). This makes expanding eDNA surveillance to these groups both difficult and important. With turtles declining globally, eDNA applications are a key part for expanding our understanding of these rapidly disappearing animals.

Chapter 2: Refining Field Blank Protocols for Reliable Wood Turtle Environmental DNA Sampling

Wood turtles are an ideal species for conducting conservation focused eDNA surveillance due to their declining populations and preferred habitat. The rivers and streams that wood turtles inhabit have low amounts of inhibiting factors and water moving unidirectionally so the relative location of eDNA contributors can be more readily determined (Akre et al. 2019, Davy et al. 2015, Lacoursière-Roussel et al. 2015). It is crucial to determine the presence of the existing populations in their changing habitats, caused primarily by human development, for effective management and conservation efforts (Akre et al. 2019, Jones et al. 2015, Jones et al. 2018). The effectiveness of eDNA as a noninvasive sampling tool has been demonstrated in similar study systems, including species like *Emydoidea blandingii, Clemmys guttata, Apalone spinifera* in Ontario (Davy et al. 2015), as well as *Sternotherus depressus* in Alabama (De Souza et al. 2016). eDNA techniques have also successfully detected wood turtles both in Virginia and in Canada (Akre et al. 2019, Davy et al. 2015). In Canada, monitoring efforts for wood turtles have intensified over the last decade due to the species being listed under Committee on the Status of Endangered Wildlife in all four provinces where they occur (Jones et al. 2018). In the United States, ongoing efforts to monitor wood

turtles are taking place across their entire range. Despite being protected in all 17 states of their range and listed on the IUCN Red List, wood turtles in the United States have not yet received federal protection (Akre et al. 2019, Jones et al. 2018, van Dijk and Harding 2011, Virginia Department of Game and Inland Fisheries 2015). Increasing surveillance across their range using eDNA would significantly improve our understanding of their current species status and assist in the classification of their protection. The implementation of a reliable eDNA sampling protocol that accounts for issues such as false positives is essential in advancing the expansion of wood turtle eDNA surveillance across their range.

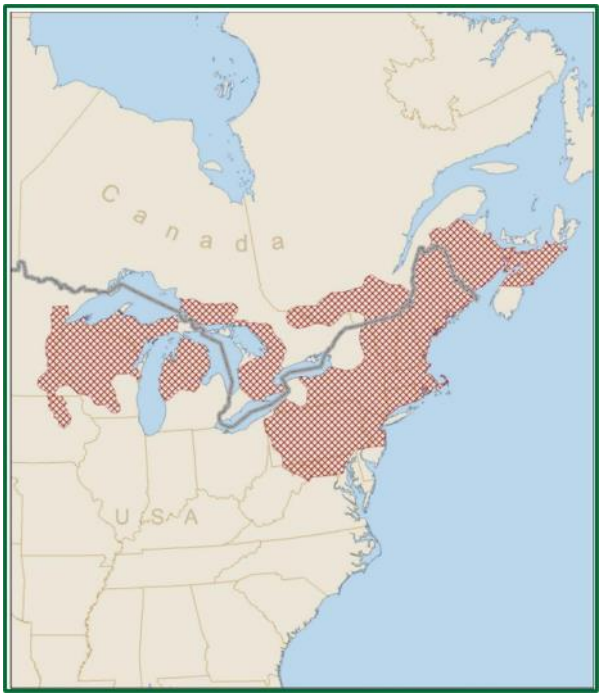

Figure 7: Map adapted from the Minnesota Department of Natural Resources displaying the approximate current geographic range of the wood turtle (*G. insculpta*) in 2008.

Implementation of eDNA techniques to assess threatened populations has seen a continuous expansion over the last decade with new challenges being presented concerning standardization of protocols rising in importance (Hutchins et al. 2022, Nordstrom et al. 2022).

Further research has been conducted to assess the likelihood of contamination at various stages of the eDNA sample collection and analysis process (Hutchins et al. 2022). Systemic contamination, which refers to contamination introduced consistently across the process (ie., contaminated reagents) can be detected with as few as five negative controls. Sporadic contamination, on the other hand, involves contamination occurring in an irregularly between samples pattern (ie., sample to sample crossover) and requires a greater number of negative controls for accurate detection. However, established workflows that prevent sample-to-sample contamination can effectively eliminate sporadic contamination (Hutchins et al. 2022). Resolving and preventing these contamination events is of paramount importance in the design of eDNA studies key to eDNA study design to ensure the reliability of the results. The primary objective of this research is to determine the minimum number of field controls required for the accurate detection of potential sources of contamination while also addressing possible causes of

contamination in previous sampling events to enhance the trustworthiness of this assay for future use.

Imperfect detection as a major challenge in the field of ecological surveillance is imperative to consider (Kery et al. 2009). Current ecological surveillance techniques often fail in capturing entire populations of organisms. This can lead to inaccurate estimations regarding the composition of the target populations (Kery et al. 2009). In eDNA study systems, it is crucial to address the confidence level associated with detecting the presence of the target organism within the study area. This is especially critical because false positive DNA amplifications can arise due to various factors, including equipment contamination during fieldwork and contamination between samples in the laboratory. To address these concerns, effectively monitoring and controlling contamination both in the field and in the lab are central objectives of this project.

The population of wood turtles inhabiting the Potomac Water Shed represents a unique genetic group within this species which, located at the southernmost extent of their range, is one of four such distinct genetic groups (Jones et al. 2018). In an effort to study and monitor these turtles, a specific eDNA surveillance assay and protocol was developed and validated for wood turtles with water filter samples from Virginia. This research also included a cost comparison analysis contrasting the eDNA surveillance method with the traditional Visual Encounter Surveys (VES) (Akre et al. 2019). The methodology employed in this work utilized a targeted PCR approach utilizing mitochondrial control region as the genetic marker and a hydrolyzed FAM probe drawing

on techniques and methodologies from various sources (Akre et al. 2019, Amato et al. 2008, Kelly et al. 2019, Untergasser et al. 2012, Veldhoen et al. 2016). The study's findings revealed that, in a wood turtle system, the eDNA approach was more cost-effective compared to VES. VES, while providing a higher degree of confidence (95%) in detecting wood turtle presence within just two samples, required more intensive training for technicians and effort completing surveys. In contrast, the eDNA method required four samples to reach the same level of confidence. To expand on these findings, four sampling seasons were conducted Fall 2021 through Spring 2023 utilizing a modified version of the protocol from the original publication (Akre et al. 2019). This initiative involved an expansion to an additional 49 sampling sites with collaborations with local and state agencies in Virginia, West Virginia, and Maryland.

During the Fall 2021 and Spring 2022 sampling seasons, ecological samples were collected in triplicate with a corresponding field blank at each sampling event. Subsequent genetic analysis was performed on a total of 378 filters resulting in qPCR amplification in 8 out of 77 field blanks. None of the PCR and extraction negatives controls displayed any amplification which suggested that contamination was not originating from the laboratory protocols but was instead linked to the eDNA water filter collection process. In the original instances of contamination detection during the Fall 2021 and Spring 2022 seasons, contaminated blanks were collected at two different times by two different teams of technicians, each using two different eDNA sampler backpacks supplied by Smith-Root Inc., a commercial fisheries science and technology company

(https://www.smith-root.com/). The occurrence of contamination in these different sampling events strongly indicated the presence of sporadic contamination issues in our sampling protocol (Hutchins et al. 2022).

Dealing with false positives and false negatives in eDNA methodology is a persistent issue that often goes unaddressed in contemporary eDNA studies (Hutchins et al. 2022, Sepulveda et al. 2020). In a recent paper summarizing two comprehensive literature reviews of eDNA publications, the first review found that out of the 156 papers analyzed, only 49% of targeted studies and 80% of metabarcoding studies reported having negative controls for laboratory procedures. The absence of controls in publications leads to a failure to account for instances of contamination in the field (Sepulveda et al. 2020). The second review, which examined a total of 659 studies, found that 30 targeted and 32 metabarcoding publications reported negative control amplification with no steps being taken to reduce these events. There was a noticeable lack of consensus on how to effectively address this issue, with many studies simply ignoring the potential effects of contamination on their analysis and conclusions (Sepulveda et al. 2020).

Protocol development to detect and control contamination of field blanks – To develop a protocol for detecting and controlling contamination in field blanks, both teams convened in August 2022 at the Center for Conservation Genomics (CCG) at the National Zoological Park. The primary objective was to determine likely sources of contamination. The teams conducted replications of field sampling and introduced three treatments, each

involving three filters taken from one of the two backpacks used, resulting in a total of eighteen filters processed. The first treatment included tap water sourced from a large retail provider which had served as the water source for previous field blank water samples (Akre et al. 2019). The second treatment utilized lab distilled water provided by CCG, which had been employed during the Spring 2022 season and was subsequently stored in one of the field team's vehicles for several months alongside equipment used in VES during that season. The third treatment featured lab distilled water provided by CCG, which had been exposed to soil from a high-density population site for wood turtles. Throughout the testing process, disposable gloves were consistently worn and changed between each of the three treatments. These filters were processed alongside negative extraction controls, and qPCR negative and positive controls, aligning with the protocol utilized during previous field seasons. This comprehensive approach allowed us to discern potential contamination sources and establish effective measures to address and control contamination in field blanks.

Out of the three treatments implemented, eDNA amplification occurred solely in the second treatment, which consisted of lab distilled water used during the previous season and stored in close proximity to equipment used during concurrent VES. In this specific treatment, four out of six filters from both backpacks amplified for wood turtle eDNA. No contamination was detected in the first or third treatments. As a result of these findings, it was concluded that the contamination likely originated from the proximity of the water storage containers to equipment that had previously come into contact with

wood turtles in enclosed spaces. During the testing period, the initial protocol allowed contact between the rim of the bottle and the water during the collection of the field blanks. This case of contamination prompted a proposed change in the protocols for both water storage and field blank collection (Refer to Figure 6) to mitigate the risk of contamination in future sampling efforts.

The contamination source testing conducted in August 2022 led to the development of a new protocol designed to prevent future field blank contamination. With the assistance of service technicians from Smith-Root Inc, it was determined that the primary source of contamination typically occurred during the step of pouring the field blank water over the filter during the collection process. Two key issues in the previous protocol were identified:

1. **Lack of Sterilization**: The water containers used for field blank water were not properly sterilized with bleach before sampling. This oversight increased the risk of introducing contaminants into the samples.

2. **Inadequate Glove Usage**: It was observed that disposable gloves were not consistently and correctly used for the specific task. For example, gloves were often put on early in the process, and technicians sometimes touched additional items unrelated to the protocol. This practice compromised the integrity of the field blank collection process.

In order to prevent contamination in future sampling events, the implementation of the new protocol (Protocol 3) involved several adjustments that required several weeks to put into practice. An intermediate protocol (Protocol 2) was utilized during this transitional period which required immediate changes such as:

1. **Independent Glove Packaging**: Pairs of disposable gloves were packaged independently for use during sampling.
2. **Storage Practices**: Water containers were stored in an eDNA clean space.
3. **Sanitation: S**pigots of water storage containers were wiped down between use with bleach wipes, and clearer guidelines were established for glove usage.

While utilizing Protocol 2 for a limited set of samplings, only a single low-level contamination event occurred in one PCR replicate in a single field blank. This provided valuable feedback for the refinement of Protocol 3. Protocol 3 implemented several important changes which included:

1. Further specification of when gloves should be used during the protocol.
2. Switching all field blank water to Type 3 laboratory grade milliQ RO water.
3. Utilizing the tripod to collect the field blanks for reduced handling of the filter cases.
4. Adopting wide-mouthed two-liter water containers so that field blank collection could mirror the process of ecological sample collection (ie., dipping the filter tube vertically, facing downward into the water).

Throughout the Fall of 2022, additional sampling was conducted using Protocol 3 and none of the 58 field blanks collected displayed wood turtle eDNA amplification. While this outcome was promising for the efficacy of Protocol 3, it is important to note that the sampling was conducted in sites where wood turtle densities are known to be low. Since the presence of wood turtles was not definitively confirmed in these sites, false negatives cannot be ruled out (Sepulveda et al. 2020) and further testing in high density locations was necessary.

The inconsistent use of field blank's in a standardized manner in eDNA assays underscores the importance of this validation study. It serves as an important step in establishing requirements that utilize protocols featuring the appropriate number of field controls, thereby providing a measure of confidence in the accuracy of ecological sampling (Akre et al. 2019, Hutchins et al. 2022, Sepulveda et al. 2020). This project was structured to measure and control for the possibility of contamination at various stages, including on site during sampling, between sites due to contaminated equipment, and throughout the entire process. The overarching goal of this research was to implement VES to determine wood turtle presence and density, which will serve as a validation of the eDNA results. This experimental design will enable the identification of the possible sources of false signals, allowing further refinement if needed. Ultimately, the aim was to enhance the accuracy and reliability of ecological sampling methods in the context of wood turtle conservation and monitoring.

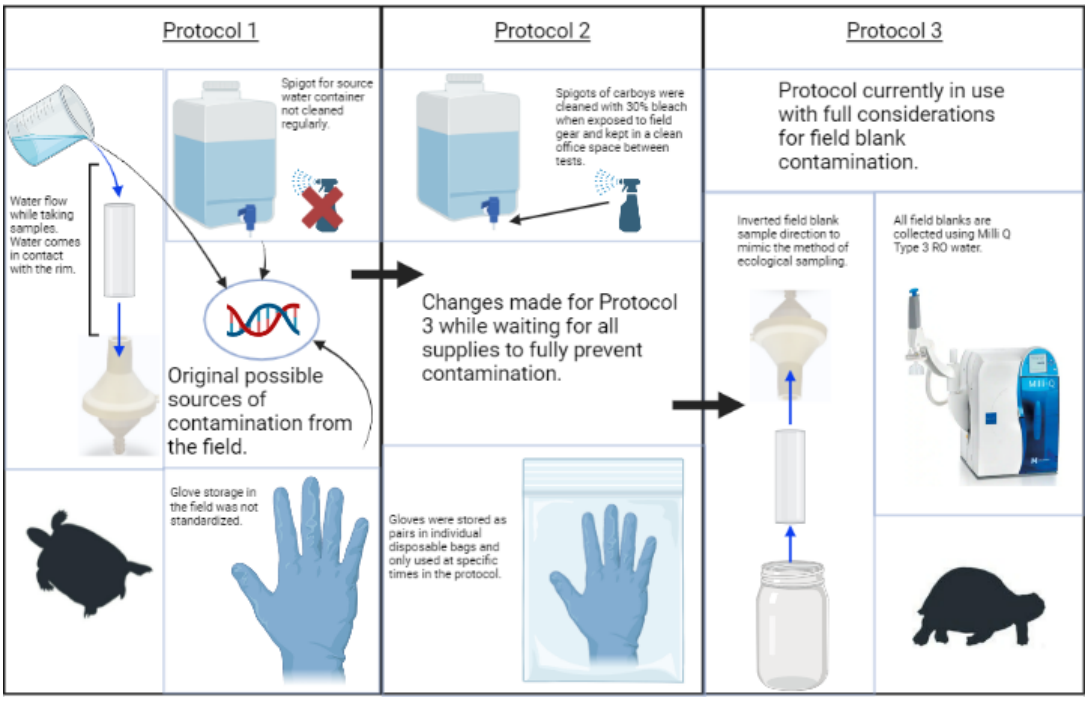

Figure 8: Possible sources of contamination introduced in protocol 1 with adjustments in the subsequent protocols. Protocol 2 contained immediately implemented changes that did not rely on specific equipment. Protocol 3 was the final methodology used for the conclusion of the study and showed a decrease in control contamination rates.

Methods and Materials

Field Sampling Methods. – In order to test this hypothesis, field sampling was conducted at seven different sites previously determined to have high-density wood turtle populations through recent Visual Encounter Surveys, which confirmed turtle presence. These sampling sites were selected to be situated one kilometer downstream from the VES start point. The selected sites primarily consisted of medium to small shallow

streams located within forested areas. Access to these sites was granted on both public lands and private properties, with the permission of the respective landowners (Akre et al. 2019).

Water filtration was conducted using the eDNA sampling backpack (Smith-Root Inc.) utilizing 5 µm Smith-Root eDNA filters. Three rounds of field sampling were completed with three filters being collected concurrently at each site. Additionally, three field blanks were filtered individually at each site, using one liter of Type 3 laboratory grade milliQ RO water. In total, 63 field blanks and 63 ecological samples were collected. A total of six liters of stream water was passed through the ecological filters (two liters per filter) (Akre et al. 2019). All filters were subsequently repackaged directly after sampling, stored at room temperature, and transported to the Center for Conservation Genomics at the National Zoo and Conservation Biology Institute to undergo genetic analysis.

Genetic Laboratory Methods. – The filters underwent DNA extraction in a designated room for pre-PCR preparations following a modified Qiagen DNEasy Blood and Tissue Kit. The modifications included increased volumes of various reagents, such as buffer ATL, proteinase K, ethanol, and buffer AL, to ensure complete coverage of the filters during the extraction process. An additional extraction control was included in the procedure to detect any potential contamination during the extraction process. With the modifications included increased volumes of various reagents identical to filter extractions.

We conducted qPCR analysis on the DNA extracts using specific primers (Forward Primer: 5'-ACAACGTTACCAGTTTCAGG-3' Reverse Primer: 5'-CATTAACCAGAGGCCTTTTA-3') for targeting the mtDNA Control Region. These primers were chosen for their conservative nature in wood turtles (Akre et al. 2019). We used a Luna Universal Probe qPCR Master Mix from New England Biolabs along with a hydrolyzed probe ordered from IDT (Integrated DNA Technologies) that uses a FAM dye to target a 3bp motif in the mtDNA control region (Akre et al. 2019). This motif is specific to this species and conserved across all populations (5'- /56 FAM/TTATAAGTG/ZEN/GCGTACATAACT -3'). To maximize the detection of low-concentration samples, qPCR of the DNA extracts was then run in triplicate. In each run, a positive control extracted from wood turtle tissue, and a negative control with type 3 laboratory water was also included to detect any potential PCR-based contamination. The highly specific nature of the primers and probe enabled us to confirm the presence of wood turtle eDNA in samples with positive amplification.

Table 2: qPCR protocol specifications for Luna Master Mix along with thermocycler specifications for qPCR runs. Plates were filled with 21 µL reactions and underwent 44 consecutive runs before analysis.

qPCR Reagent	Volume Required per Reaction (21 µL)
Luna Master Mix	10.0 µL
Forward Primer	0.8 µL
Reverse Primer	0.8 µL
Hydrolyzed Probe	0.4 µL
BSA	1.0 µL
Template	8.0 µL

Thermocycler Temperature	Time per Step
95°C	1:00
44x	---
95°C	0:15
55°C	0:30

Analysis. – With the assistance of Dr. Grant Connette, a Bayesian logistic regression analysis was employed to estimate the false positive rate from the previous sampling season. This analysis yielded a minimum requirement of 60 field blanks to achieve a 95% confidence interval regarding the effectiveness of Protocol 3 in successfully eliminating the sporadic contamination observed. After all of the filters were collected, extracted, and subjected to qPCR, the same analysis was employed to determine the false positive rate and determine Protocol 3's efficacy in minimizing contamination.

In order to address concerns related to imperfect detection, the Bayesian logistic regression analysis was chosen for this test to determine the false positive rate, using field blank amplification results as the response variable. Bayesian analysis was preferred over Maximum Likelihood (ML) due ML's inability to estimate a false positive rate in the absence of no false positives. An uninformative prior was used allowing the false positive rate to range between 0-100% to determine the probability of false positive presence in analyzed samples. This analysis was conducted within the R statistics environment (R Studio 2023.3.0+386) using the JagsUI package v1.5.2 for MCMC, particle filtering, and hierarchical modeling (Kellner and Meredith et al. 2022, R Core Team 2022).

Results

Field Collection Results: Filter collection was conducted from March 8, 2023, to April 14, 2023. I conducted sampling at the five sites located in Virginia, while a project field technician sampled the two sites in West Virginia. All 126 filters were carefully collected and delivered to the lab for analysis in their original packing within cardboard boxes kept at room temperature.

Lab Results: Out of the 63 ecological samples that were collected, 50 of these samples exhibited amplification in at least one of the qPCR replicates. 35 samples consistently showed qPCR amplification in all 3 replicates conducted. Among the 63 field blanks, two of them displayed a single amplification out of three replicates during the qPCR analysis. To determine if this was the result of plate contamination, both filters were run through an additional three rounds of qPCR, each of which produced one replicate with

contamination. It is noteworthy that neither the extraction controls nor the qPCR negative controls exhibited any amplification across all tests. Field controls had an overall amplification rate of 1.06%, while ecological samples yielded a significantly higher overall amplification rate of 66.67%.

Table 3: Record of qPCR amplification rate by site and visit number (values organized as site.visit). Site names were assigned numbers to mask locations due to the threatened conservation status of this species. The qPCR amplification per site is recorded as well as amplification rate per visit with three visits to each site. Both field blank and ecological amplification rates are recorded with Sites 1 and 4 having the highest ecological sample amplification rate. The only field blank amplification was detected from samples from the first visit to Site 1 and may have been due to less-than-optimal sampling conditions.

Site Number-Visit Number	Field Blank qPCR Amplification Rate	Ecological Sample qPCR Amplification Rate
1	7.4	92.59
1.1	22.2	88.8
1.2	0	100
1.3	0	88.9
2	0	29.62
2.1	0	11.1
2.2	0	11.1
2.3	0	66.7
3	0	62.96
3.1	0	44.4
3.2	0	44.4
3.3	0	100
4	0	96.29
4.1	0	100
4.2	0	88.9
4.3	0	100
5	0	70.37

5.1	0	55.6
5.2	0	55.6
5.3	0	100
6	0	48.15
6.1	0	0
6.2	0	77.8
6.3	0	66.7
7	0	66.67
7.1	0	88.9
7.2	0	11.1
7.3	0	100

Data Analysis: The data analyzed in this thesis is part of a greater series of wood turtle filter collections conducted throughout the Potomac Watershed. The samples collected for this study were analyzed within the context of this dataset. The Bayesian Logistic Regression was conducted in four stages, using data from four different categories (See Table 3, Chapter 2 Appendix). A total of 78 field blanks collected under protocol 1 (Old) were analyzed to determine the likely contamination rate associated with this protocol. These results were used to establish prior values that informed subsequent analyses. The estimated false positive rate for protocol 1 exhibited an upper 95% confidence interval of 18.99% and a median value of 12.19%. For protocol 2 (Revised), which included 16 samples, confidence intervals displayed an increased degree of variability due to the low number of field blanks collected under that protocol. The estimated false positive rate for

protocol 2 had a median value of 3.97% with an upper 95% confidence interval of 16.14%.

Protocol 3 (New) encompassed a total of 155 filters, which included the additional samples collected for the contamination rate analysis. The median estimated false positive rate for protocol 3 was 1.72% with an upper 95% confidence interval of 3.98%. Within the subset of 63 samples collected specifically for this thesis, the only contamination case emerged from a field blank collected under protocol 3. The median estimated false positive rate for this subset was 4.16% and an upper 95% confidence interval at 9.48% (See Table 3, Chapter 2 Appendix).

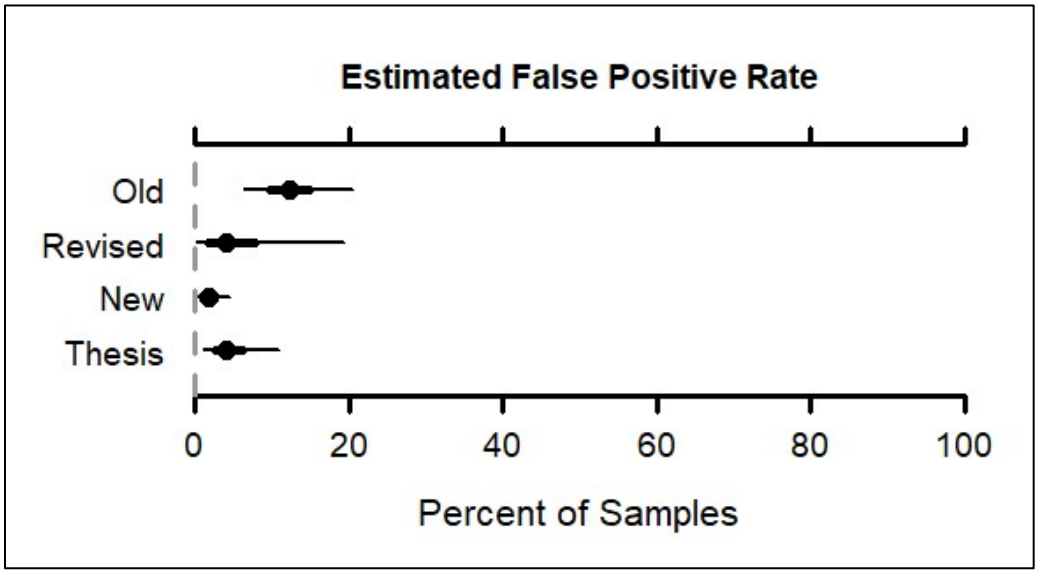

Figure 9: Estimated false positive rates in field blanks for each protocol. (Old = Protocol 1, Revised = Protocol 2, New = all filters collected under Protocol 3, Thesis = Subset of samples collected specifically for this study.

Discussion

The issue of the lack of discussion regarding contamination rates in eDNA research persists to date (Sepulveda et al. 2020). Accurate detection of target species is key for the reliability of this method, and in my thesis project I have demonstrated that we can implement steps to mitigate false positive rates in field sampling systems. This work aimed to quantify, determine, and mitigate the false positive rate in a sampling project that had been experiencing higher than expected false positive rates. Here, I have implemented a new protocol to address these issues, resulting in a dramatic reduction in estimated false positive rates across a substantial set of field blanks.

Protocol-Related Changes in Estimated False Positive Rates – The variance in the estimated false positive rate for Protocol 1 can be attributed to various factors, including non-standardized glove handling and storage, field blank collection methods, and water storage for field blanks before collection. Protocol 2 also displayed a highly variable estimated false positive rate. This protocol had fewer filter blanks than the other two due to it being used as a temporary nature before the full implementation of Protocol 3. Although there was one instance of possible contamination in the field blanks collected

using this protocol, the Cq value exceeded the 40-cycle mark, suggesting that this was the result of possible dimer formation (Yang et al. 2020). In contrast, Protocol 3, exhibited the lowest variation in the estimated false positive rate, indicating that the changes implemented in protocol 3 effectively reduced the false positive rate in field blank collection with the large sample size. However, it is essential to note that the subset of samples collected for this thesis did not match this reduction due to two contaminated filters from the initial sampling round. Protocol 3's single digit estimated false positive rates within the 95% confidence interval shows a definitive decrease in the likelihood of false positives occurring in field blanks through following this protocol.

Contamination within protocol 3 subset - The results of the analysis demonstrated an overall reduction in contamination rates with the implementation of Protocol 3. A single contamination event occurred during the sampling at Site #1 (Table 1). Both filters that exhibited qPCR amplification in the test had only one out of three replicates amplify. Subsequent tests with three additional replicates yielded the same result confirming low-level contamination rates. The presence of sediment on the exterior of the filter casing indicates that the contamination likely originated during field collection. Protocol 3 methodology mandates the use of clean gloves and careful filter handling to prevent filter cases from coming into contact with the environment. While sporadic contamination remains a possibility in field sampling (Hutchins et al. 2022), this level of contamination is considered acceptable for protocol contamination control validation.

Conclusion of Chapter 2

While sporadic contamination is difficult to eliminate entirely, standardized protocols and large sample sets are currently the most effective means of minimizing its impact on eDNA analysis (Hutchins et al. 2022). This work has successfully reduced the false positive amplification rate in field controls through protocol standardization and review, despite occasional false positives occurring in field blank analysis. As discussions on eDNA protocol standardizations continue to evolve, it is imperative to continue to expand control usage at all of the stages of the eDNA workflow to better account for and reduce false positives in the field of eDNA.

Chapter 2 Appendix

Table 4: Summary of Field Blank Bayesian Logistic Regression Analysis

	Number of Field Blanks	Median Values	Upper 90%	Upper 95%	Upper 99%
Old Protocol (Protocol 1)	78	12.19	17.39	18.99	22.41
Revised Protocol (Protocol 2)	16	3.97	12.62	16.14	23.73
New Protocol (Protocol 3)	155	1.72	3.37	3.98	5.30
Thesis Set of Samples	63	4.16	8.09	9.48	12.47

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Biography

Seanessy Lyons graduated from Community District 99 Downers Grove North High School, Downers Grove, Illinois in 2015. He received his Bachelor of Science degree from Southern Illinois University's Department of Zoology and was employed as a Roving Naturalist Intern in 2016. During his time at SIU, he was also a member of the SUPERB Scholars Program, recipient of the University Excellence Scholarship, section leader in the Marching Salukis, and President of the Epsilon Kappa Chapter of Phi Mu Alpha Sinfonia. He completed his undergraduate research on marsupial helminthic parasites in the Parasite Diversity Lab led by Dr. Agustin Jimenez. After graduation, he was employed as a research technician at SIU under PhD candidate Tiffanie Atherton and assisted in trapping the locally threatened wood rat population for genetic analysis. In the Fall of 2019, he accepted an internship at the Elephant Endotheliotropic Herpes Virus Lab at the National Zoological Park's Pathology Department under Erin Latimer. In February 2020, he was contracted as the Freezer Technician at the Center for Conservation Genomics at the National Zoological Park for 3 years. He received his Master's of Science degree at George Mason University in December 2023 with a thesis centered around wood turtle eDNA surveillance and acted as a graduate teaching assistant for the Department of Biology.