DEPARTMENT OF TRANSPORTATION

Assessing a New Tool for Early Detection of Endangered Turtles on Proposed Transportation Projects

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As turtle populations decline worldwide, there is an increased need for rapid and reliable monitoring of species to meet the need of increased regulatory burdens. The use of environmental DNA (eDNA) as a monitoring tool for rare species is becoming increasingly common, but there is a still a need to optimize methods in the laboratory and the field at a per assay level. Here, we evaluated the effectiveness of environmental DNA (eDNA) for detecting Blanding's turtles and wood turtles across Minnesota. We developed two highly sensitive eDNA assays, sampled aquatic habitats across multiple seasons aligned with key life history stages of the turtles, analyzed occupancy and detection probabilities based on these sampling efforts, and assessed the costs associated with implementing these surveys. Both of our assays are developed up to the "Operational" level on the Thalinger eDNA validation scale and can be used with confidence for detecting turtles. We found that Blanding's turtles are easier to detect in the late summer and early fall months and are more likely to occupy smaller bodies of water. Individual predictor for wood turtles were weakly supported and would benefit from increased replication targeting this species in future studies. We found that our per sample cost of this study was $79.38, however cost per detection varied with time of year and species. These eDNA assays are rapid, reliable tools for detection of Blanding's and wood turtles at a reasonable cost and provide potential for greatly improved methods of monitoring these rare species throughout Minnesota.

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Assessing a New Tool for Early Detection of Endangered Turtles on Proposed Transportation Projects

Final Report

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List of Acronyms

- AIC: Akaike information criterion
- CI: confidence interval
- COI: cytochrome *c* oxidase subunit I
- $C_{q:}$ quantification cycle
- CTAB: cetyltrimethylammonium bromide
- CV: coefficient of variation
- eDNA: environmental DNA
- gDNA: genomic DNA
- HUC: Hydrologic Unit Code
- LOD: limit of detection
- LOQ: limit of quantification
- NCBI: National Center for Biotechnology Information
- NCEI: National Centers for Environmental Information
- NHDPlus: National Hydrography Dataset Plus
- NRSA BMMI: National Rivers and Streams Assessment benthic invertebrate multimetric index
- NTC: no template control
- NOAA: National Oceanic and Atmospheric Administration
- qPCR: quantitative polymerase chain reaction
- RTE species: rare, threatened, or endangered species
- USGS: U.S. Geological Survey
- VES: visual encounter surveys

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Executive Summary

In this report, we detail a comprehensive assessment of environmental DNA (eDNA) technologies and methodologies for the detection of two threatened turtle species in Minnesota, the Blanding's (*Emydoidea blandingii*) and wood turtle (*Glyptemys insculpta*). Historically, monitoring freshwater turtles has been challenging, as conventional sampling is often time consuming, costly, and difficult to scale. Species rarity and natural history characteristics only exacerbate these challenges. However, emerging technologies and methodologies are providing additional opportunities for rapid, sensitive, cost-effective, and scalable monitoring for freshwater turtles. Environmental DNA is one such approach. Environmental DNA is DNA that is shed by organisms into the environment, where it can then be collected from bulk environmental samples and analyzed to assess biodiversity (both single species and whole communities). Environmental DNA biodiversity monitoring has shown remarkable promise in species that cut across the tree of life, and in a wide array of ecological contexts. However, success with freshwater turtles has been mixed, suggesting that adoption of eDNA approaches should proceed only after comprehensive, rigorous testing.

Chapter 1 provides an introduction to the history and development of environmental DNA in biodiversity monitoring. Initially developed for microbiology or environmental microbial ecology, eDNA technologies and methodologies have rapidly expanded to cut across the tree of life and extend across ecosystems. Here we detail the history of eDNA biodiversity monitoring, particularly for aquatic vertebrates, with an emphasis on freshwater turtle species in general, and Blanding's and wood turtles specifically.

Chapter 2 offers a comprehensive assessment of existing eDNA quantitative PCR (qPCR) assays for the detection of Blanding's and wood turtles. In the past, a number of assays were designed for these turtles and many other species, but over time the design, validation, and optimization of targeted assays has become more rigorous. We used the more rigorous *in silico*, *in vitro*, and *in situ* testing to validate existing assays, particularly for Minnesota-derived samples. We found that these previously designed assays did not amplify DNA from tissues derived from Minnesota turtles, had high non-target amplification rates, and/or reduced sensitivity, rendering them ineffective for eDNA surveys for these target taxa in Minnesota. Therefore, we designed, optimized, and validated novel assays that included DNA sequence data from both Minnesota Blanding's and wood turtles, as well as other individuals from throughout the species' respective ranges. These novel assays were species-specific, sensitive, and efficient, outperforming previously published assays. We then tested these assays on environmental DNA samples from Minnesota waters with known turtle presence, recovering consistent positive detections. At this point, the assays have achieved Level 4 (Substantial) on the Thalinger eDNA validation scale.

Chapter 3 details a systematic assessment of the use of eDNA to detect target species throughout their range in Minnesota waters to estimate detection probability and occupancy, to understand what biotic and abiotic covariates may impact those estimates, and to assess how they change seasonally. To achieve this, we sampled sites with known recent or historic records of both species, processed those

samples using optimized workflows, subjected them to qPCR using the novel assays described in Chapter 2, and conducted rigorous occupancy modeling. We found that:

- While we were able to successfully detect both turtle species in all four seasons of the year, summer and fall recovered considerably higher detection probabilities and indicating that the timing of sampling is critically important.
- There were no significant abiotic or biotic covariates associated with the top competing wood turtle models, however all candidate models outperformed the null model and fit the data moderately well.
- Blanding's turtle occupancy had a negative relationship with stream order, suggesting that turtles prefer smaller, slower moving water.
- Detection probability of Blanding's turtle eDNA is positively associated with Julian day. We were most likely to detect eDNA in the late summer/early fall.
- There are several factors that could potentially improve our ability to detect turtle eDNA, including considering weather conditions, time of year, and field methods.

Additionally, when combined with Chapter 2, these results bring the novel targeted eDNA assays to Level 5 (Operational) on the Thalinger eDNA validation scale, meaning that if the target species is detected via eDNA analysis, it is very likely present, and if it is not detected, it is likely absent, given appropriate sample timing and replication. In addition, based on these results we have developed an (attached) power analysis widget for both turtle species. This allows users to choose a calendar or Julian day of the year, add a user-defined number of field replicates for up to three sampling events in a year, and calculate a cumulative detection probability for each, that is then summed to a multi-event detection probability.

In Chapter 4, we calculated the cost to process each sample, including materials, supplies, travel, and personnel. Next, we developed a cost-per-detection for both Blanding's and wood turtles. Then, based on the detection probabilities estimated in Chapter 3, we calculated the cost for the seasonal sampling density necessary to achieve 95% confidence in detection that the target species is indeed present. Here we reveal that:

- Blanding's turtle costs were highest in winter, with a cost of $2,063.88 per detection, and lowest in fall, with a cost of $192.79.
- For Blanding's turtles in winter, sampling density to achieve 95% confidence in detection for a given site could require as many as 91 spatially segregated samples at a cost of $7,223.58, whereas fall sampling could require as few as 6 spatially segregated samples at a cost of $476.28.
- Wood turtle costs were similarly high in winter, with a cost of $1,270.08 per detection, and lowest in summer, with a cost of $343.98.
- For wood turtles in winter, sampling density to achieve 95% confidence in detection for a given site would require a total of 69 spatially segregated samples per site at a total cost of $5,477.22 per site, whereas summer sampling would require 6 spatially segregated samples per site at a cost of $476.28 per site.

- Differences in cost per detection between Blanding's and wood turtles were driven by two factors.
    - Blandings turtles were detected at more sites overall than wood turtles.
    - At sites where target species were detected, wood turtles yielded more positive detections in both field and technical replicates.

These per-sample cost estimates to also assess sampling costs can be merged with user-defined sampling schemes in the developed "widget" to generate cost assessments for potential sampling schemes. Ultimately, this will allow for informed decision-making on when, where, and how to develop sampling projects/programs.

In Chapter 5, we provide our recommendations for research benefits and subsequent implementation steps. We believe that this research has following key benefits:

- Highly sensitive, species-specific assays have been designed, optimized, and validated to successfully amplify Minnesota native Blanding's and wood turtles, and is likely to be successful throughout the species' ranges
- The novel assays have both achieved Level 5, "Operational," on the Thalinger validation scale
- A spatio-temporally robust sampling design applied extensively across the range of both species and in all four seasons of the year
- Both species were detected in spring, summer, fall, and winter, but with highest detections coinciding with low water conditions and/or critical points in species' phenology
- Blanding's turtles were broadly detected throughout their range in Minnesota, but with imperfect detection rates
- Wood turtles were consistently detected from historic sites in the northeastern extreme of the range, while detections were sparse in the southeast extent, which has known extant populations but at low abundances
- Occupancy of both target species was moderately well-modeled from field sampling but without strong effects of any individual predictor, except for a negative relationship between stream order and occupancy for Blanding's turtles
- Detection probability increased through the year for both species, but this effect was strongest for Blanding's turtles, again likely because more frequent detections of this species improved power in models
- Both Blanding's and wood turtles were most detectable in late summer or autumn with similar effort by field replicates, requiring approximately six samples per site to achieve a 95% probability of detection, and cost ($476.28 per site)
- Model-predicted detection probabilities by calendar or Julian date can be used to design a sampling program or allocate effort throughout the year for monitoring of both Blanding's and wood turtles by eDNA in Minnesota

We recommend the following implementation steps:

- Given sparse detections of wood turtles in the southeastern extent of the range, a comprehensive eDNA assessment of a site with known densities and, ideally, radio-telemetered individuals, is necessary to understand the relationship between eDNA detections and abundance.
- To optimize eDNA detection in practice, utilize more focused sampling effort. Efforts in the present study were largely confined to bridge crossings and near roads. Biologists should target sampling locations using knowledge of turtle life history and habitat preferences when possible.
- Ideally, pairing the above with conventional sampling and occupancy modeling would facilitate a direct cost-comparison between eDNA surveillance and conventional monitoring.
- Given the consistent performance of eDNA detections for Blanding's turtles, we recommend the development of a phased state-wide eDNA assessment of distributions and habitat associations for this species based on MN DoT taskings and MNDNR priorities.
- For any subsequent sampling efforts and potential Phase II activities, focused sampling effort (vs simply proximity to roads) is necessary to increase detection for both species, especially where populations are low and/or when they don't concentrate near roads.

Chapter 1: Introduction

1.1 Environmental DNA as an Emerging Tool for Rapid, Sensitive, and Cost-effective Biodiversity Monitoring

Despite covering less than 1% of the Earth's surface, freshwater ecosystems harbor nearly 6% of the world's known biodiversity (Dudgeon et al., 2006). In addition, freshwater ecosystems are also among the most anthropogenically impacted and imperiled global ecosystems (Sondergaard and Jeppsen, 2007; Carpenter et al., 2011; Dodds et al., 2013). Unfortunately, these stressors, including overexploitation, habitat degradation, invasive species introductions, nutrient loading, and other threats are driving freshwater biodiversity declines, species extirpations, and extinction (Dudgeon et al., 2006; Barnosky et al., 2011; Dirzo et al., 2014, Tickner et al. 2020). Consequently, rare, threatened, or endangered (RTE) species are increasingly receiving legal protections, which often necessitates intensive monitoring to inform conservation interventions (Campbell et al., 2002; Troyer and Gerber, 2015; Robinson et al., 2018). Yet successful monitoring can be costly, time consuming, logistically challenging, and, particularly for RTE species, with low detection probabilities (Lindenmayer et al., 2020; Morant et al., 2020).

These monitoring challenges are often exacerbated by the very ecology and natural history of the RTE species we seek to monitor. In addition to being numerically rare, they are often elusive, cryptic, and/or with high habitat specificity, both spatially and temporally (O'Grady et al., 2004; Kotiaho et al., 2005; Bohm et al., 2016). And while surveys aimed at estimating abundance and/or occupancy at a landscape scale provide vital information necessary for decision-making, mitigation, etc., challenges associated with conventional animal survey and monitoring techniques can be confounded by movement patterns or crypsis due to habitat use, ultimately depressing detection probabilities and increasing effort necessary to achieve high confidence in species presence at a given site (Gu & Swihart, 2004; Steen, 2010; Durso et al., 2011; Durso & Siegel, 2015; Katz et al., 2021; Sternhagen et al., 2024, Samuels et al., 2025a; Samuels et al., 2025b).

Given these challenges, technological and methodological innovations in biodiversity monitoring are revolutionizing how we approach biodiversity surveys in rapid, sensitive, and cost-effective ways (Pimm et al., 2015; Stephenson, 2020; Montfort and Magrath, 2021; Borja et al., 2024). Advances in bioacoustics (Mooney et al., 2020; Penar et al., 2020; Hoefer et al., 2023; Kohlberg et al., 2024; Arzberger et al., 2025), camera trapping (Rowcliffe, 2017; Wearn and Glover-Capfer, 2019; Oliver et al. 2023;), remote monitoring methods like radio detection and ranging (RADAR) and light detection and ranging (LiDAR) (Kerry et al., 2022), satellite imagery (Nagendra and Gadgil, 2005; Mairota et al., 2015; to Buhne and Pettorelli, 2017), autonomous vehicles (Koh and Wich, 2012; Bayat et al., 2017; Di Ciaccio and Troisi, 2021), and environmental DNA (Bohmann et al., 2014; Thomsen and Willerslev, 2015; Deiner et al., 2020;). These technological and methodological advances have shown promise in broadly advancing biodiversity monitoring at scale, often with improved efficiency and sensitivity at lower costs. Ultimately, the adoption of emergent technologies and methodologies hold great promise for advancing our ability to effectively monitor RTE species.

1.2 The environmental (e)DNA revolution

In 2008, the first study to explore the efficacy of DNA collected from bulk environmental samples (i.e., eDNA) to detect macroorganisms was published (Taberlet et al., 2018). In both controlled experiments and in natural settings, water samples were collected and screened for invasive American Bullfrog (*Lithobates catesbeianus*) via quantitative (q)PCR (Ficetola et al., 2008). In this study, a multi-sampling design yielded America Bullfrog detections in all contexts (both controlled and natural settings) and in all environments, even when the species was present at low densities. Since its publication, there has been an exponential proliferation of eDNA studies in the scientific literature (Jian and Yang, 2017; Sahu et al., 2025), culminating in the founding of a dedicated peer-reviewed journal, Environmental DNA, in 2019, and signifying the rise of eDNA as a viable and vibrant Conservation Genetics subdiscipline.

Environmental DNA is DNA shed by organisms into their environment, which can be collected via bulk environmental samples, including water (Ficetola et al. 2008; Thomsen et al. 2012), sediment (Yoccoz et al. 2012; Katz et al. 2021), and air (Johnson et al. 2021; Clare et al. 2022), and analyzed to determine the presence of a species without requiring observation or capture of physical specimens at the time of survey. In fact, these samples can then be processed and analyzed to identify either target taxa (via targeted quantitative (q)PCR assays) or whole communities (via community metabarcoding) (Ficetola et al., 2008; Bohmann et al., 2014; Rees et al., 2014). Environmental DNA assessments are increasingly considered a viable complement or, in some cases, alternative to conventional sampling (Smart et al., 2015; Shaw et al., 2016; Qu and Stewart 2019; Wineland et al., 2019; Fediajevaite et al., 2021; Plante et al., 2021; Moss et al., 2022; Johnson et al., 2023). Moreover, eDNA has shown promise in transcending taxonomic groups and ecological contexts (Ishige et al., 2017; Sigsgaard et al., 2017; Niemiller et al., 2018; Franklin et al., 2019; Allen et al., 2023; Harper et al., 2023; Johnson et al., 2023a; Johnson et al., 2025; Samuels et al., 2025a). Environmental DNA has been particularly effective in aquatic systems (Ficetola et al., 2008; Jerde et al., 2011; Thomsen et al., 2012; Tsuji et al., 2019; Picq et al., 2024). Finally, eDNA has shown utility in detecting species that occur at low abundance/density/biomass, including both invasive (Dougherty et al., 2016;) and threatened/endangered (Fukumoto et al., 2015; Pfleger et al., 2016; Bonfil et al. ,2021; Koda et al., 2023) species. Ultimately, eDNA analyses have emerged as a promising tool in the conservation biologist's toolbox.

However, eDNA approaches have not been universally successful. In some contexts, eDNA has not performed well (Adams et al., 2019; Baker et al., 2020; Ratsch et al., 2020). Environmental DNA can underperform or even fail for a variety of reasons, including sample handling and processing (Curtis et al., 2020), extraction (Garcia et al., 2024), environmental conditions (Pilliod et al., 2014; Stewart, 2019; Barnes et al., 2021; Kessler et al., 2020; Curtis et al, 2021), species phenology (de Souza et al., 2016; Matsuhashi et al., 2019; Herve et al., 2022; Yonago et al., 2024), and many other factors (Rees et al., 2014). Given the above, initiating a comprehensive eDNA biodiversity monitoring program at scale should be carefully considered and based on strong preliminary studies aimed at both optimizing methodological and technological aspects, and assessing the influence of various biotic and abiotic factors is necessary for a fair adjudication of the tool.

1.3 Environmental DNA and freshwater turtle biodiversity monitoring

Freshwater turtles are among the most imperiled segments of biodiversity on the vertebrate tree of life (Buhlmann et al., 2009; Rhodin et al., 2018). Moreover, they are notoriously difficult to effectively survey and monitor, due to myriad reasons with manifold effects (Adderley-Heron et al., 2024). Consequently, emerging technologies and methodologies, including eDNA, are being leveraged in an attempt to develop more rapid, sensitive, and cost-effective approaches to measuring and monitoring freshwater turtle biodiversity (Nordstrom et al., 2022).

Early turtle eDNA studies aimed at developing and validating novel targeted-assays for the detection of freshwater turtles (Davy et al., 2015; Lacoursiere-Roussel et al., 2016; Feist et al., 2018; Wilson et al., 2018; Kirtane et al., 2019; Lam et al., 2020), though we note that these assays predate the establishment of rigorous standards for assay design, optimization, and validation (Thalinger et al., 2021). And while novel assay design, optimization, and validation remains a key component of contemporary freshwater turtle eDNA research (Lam et al., 2022; Rohan et al., 2023; Fields et al., 2024; Nelson et al., 2025; Rishnan et al., 2025), the field has rapidly expanded to explore critical aspects of freshwater turtle biology and conservation. From establishing distributional area (Nordstrom et al., 2024), to identifying overwintering sites (Feng et al., 2020; Tarof et al., 2021), to comparing with conventional sampling methods (Akre et al., 2019; Fyson and Blouin-Demers, 2021; Sternhagen et al., 2024), understanding biotic and abiotic factors influencing freshwater turtle detection and occupancy (de Souza et al., 2016; Kessler et al., 2020; Feng and Lougheed, 2023; Hong et al., 2023), to rediscovering freshwater turtles thought to have been extirpated (Villacorta-Rath et al., 2022), to monitoring invasive freshwater turtle species (Kakuda et al., 2019; Rohan et al., 2023; Wei et al., 2024), eDNA applications for freshwater turtles have been rapidly advancing.

Within the broader context of the development of eDNA technologies and methodologies for freshwater turtle biodiversity monitoring and conservation, two species have received considerable scrutiny. With broad North American distributions, intense conservation concern, and particular challenges associated with rapid, sensitive, and cost-effective monitoring, Blandings' turtle (*Emydoidea blandingii*) and wood turtle (*Glyptemys insculpta*) have been the intensive focus of eDNA research. Targeted eDNA assays were designed early on for both species (Stedman, 2013; Davy et al., 2015; Hernandez et al., 2020), and were subsequently deployed to detect populations and assess occupancy (Lacoursiere-Roussel et al., 2016; Akre et al., 2019; Roehl, 2019; Schneider, 2020; Fyson, 2020; Fyson and Blouin-Demers, 2021; Tarof et al., 2021; Hickey, 2023; Ruppert et al., 2023; Adderly-Heron et al., 2024). And while some of these results may be rather equivocal, there is optimism that the continued development of eDNA monitoring for these species could complement and/or improve upon conventional methods of monitoring.

This is particularly true in Minnesota, because both Blanding's and wood turtles are currently under review for listing under the U.S. Endangered Species Act and are presently listed under the state of Minnesota's endangered species statute. These turtles occur in a variety of Minnesota's freshwater

habitats, including public waters such as lakes, ponds, and streams, as well as in ditches and in created wetlands used to manage stormwater. Due to their listed status, transportation projects and maintenance activities that impact habitats near documented observations of these species may be required to follow costly avoidance and minimization measures, even if turtle presence within the project area is unknown. Currently, one or both turtle species are known to occur in approximately 50 of 87 Minnesota counties. Transportation agencies must comply with a variety of environmental regulations, including state and federal endangered species regulations. Compliance can be costly and may delay projects due to limitations related to the timing of surveys and other investigations (i.e., wildlife surveys are often limited to summer months). Innovative tools can help overcome some limitations and potentially streamline regulatory approval processes for projects. Given the above, a rigorous assessment of the efficacy of eDNA technologies and methodologies to detect Blanding's and wood turtles in Minnesota's waters is necessary.

Chapter 2: Assessment of Existing Design, Optimization, and Validation of Targeted Assays for the Detection of Wood Turtles and Blanding's Turtles in Minnesota

2.1 Introduction

Accurately monitoring natural animal populations is inherently difficult (Martin et al., 2022). This can be particularly daunting for rare, threatened, or endangered (RTE) species that ostensibly persist at low densities throughout their range (Cunningham & Lindenmayer, 2005). However, there is an ever-present need to improve detection and monitoring techniques as certain taxonomic groups are experiencing drastic global population declines.

In addition to rarity, an animal's natural history may further exacerbate low detection rates (Katz et al. 2021). This is certainly the case for many turtle species (Tarof et al., 2021). Turtles and tortoises (Order: Testudines) are considered among the most "at-risk" vertebrates, with at least 50-60% of species classified as threatened or endangered worldwide (Lovich et al., 2018). Moreover, their often cryptic, highly aquatic lifestyles can render monitoring exceptionally difficult (Daigle & Jutras, 2005). Consequently, conventional methods for surveying turtles are typically expensive, time consuming, and often requiring multiple years (Chandler et al., 2024). These limitations reduce survey efficacy, frequently yielding low detection probabilities despite cost- and time-intensive survey effort (Sterrett et al., 2010).

In systems with similar challenges, environmental DNA (eDNA) analysis has been used in tandem with or as alternative to conventional survey methods (Taberlet et al., 2012). Environmental DNA is DNA naturally shed by organisms into their environment via feces, skin sloughing, or other secretions/excretions (Taberlet et al., 2012; Johnson et al., 2023a). Analysis of eDNA present in bulk environmental samples (e.g., water, soil, air) can be used to improve detection rates and estimate occupancy (Bohmann et al., 2014; Tetzlaff et al., 2024). However, the use of eDNA for detection of RTE species is not equally effective across the Tree of Life, with reptiles in general and turtles specifically proving particularly challenging (Raemy and Ursenbacher, 2018; Nordstrom et al., 2022; Rojahn et al., 2023; but see de Souza et al., 2016; Kessler et al., 2020; Sternhagen et al., 2024). Aquatic reptiles differ in several ways from other aquatic taxa where eDNA has been effectively used. They have scales instead of epithelial cells and produce less urine, both of which may drastically reduce the amount of DNA shed into the environment (Ficetola et al., 2019). Typically, two different genetic methods are used in eDNA studies, and they differ based on the intended goal. Leveraging universal primers, metabarcoding provides a broader, potentially community level perspective of an eDNA sample. On the other hand, targeted assays via quantitative polymerase chain reaction (qPCR) are used to isolate single species and often utilize fluorescent species-specific probes to increase sensitivity and specificity. This enhanced

specificity of qPCR assays typically allows for greater success in detecting species associated with low eDNA concentrations like many herpetofauna (Harper et al. 2018; Moss et al., 2022).

Yet, despite the specificity associated with qPCR assays, researchers should yield caution before adopting an assay at a broad scale. To minimize the chance of false negatives, as well as the chance of cross-amplification of closely related, geographically overlapping species, eDNA assays should be developed and validated to the geographic region of interest (Goldberg et al., 2016). Here we assess the efficacy of eDNA for detecting two species of North American turtles: Blanding's (*Emydoidea blandingii*, Holbrook, 1838), and wood turtles (*Glyptemys insculpta*, Le Conte, 1830). Blanding's and wood turtles are both considered "Endangered" on the IUCN Red List (IUCN 2024) and are afforded protections in numerous states and provinces (Table 1). Given the imperilment of these two species, targeted eDNA assays are vital to expanding rapid and sensitive monitoring.

A number of assays have been developed for both Blanding's and wood turtles, however several target a different gene region (or are proprietary) than the one that is of focus here, the mitochondrial cytochrome *c* oxidase subunit I (COI) region (Akre et al., 2019; Loeza-Quintana et al., 2021; Fyson & Blouin-Demers, 2021; Tarof et al., 2021). There are two COI assays for Blanding's and wood turtles; one that is probe-based (Hernandez et al., 2020) and the other is a primer-only assay (Davy et al., 2015). There is an additional assay for wood turtles (Lacoursiere-Roussel et al., 2016), however, this assay only tested against two non-target species during the development stage. We decided to compare the sensitivity and specificity of the novel assays we developed here for Blanding's and wood turtles against the probe-based Hernandez et al. (2020) and primer-only Davy et al. (2015) assays. The previously published assays do not report results for sensitivity testing, so we sought to test these values in our system and compare them to our primers and probes. Additionally, these assays did not account for cross-amplification of the full suite of turtle species that are known to occur in our regions of interest. Therefore, we sought to design, optimize, and validate, *in silico*, *in vitro*, and *in situ*, species-specific assays for Blanding's and wood turtles and compare our assays to previously published eDNA assays for these species.

2.2 Materials & Methods

2.2.1 Novel assay design and validation

We developed novel qPCR assays targeting the mitochondrial cytochrome *c* oxidase subunit I (COI) gene for both Blanding's and wood turtles (Table 2.1). Primers and probes were designed based on reference libraries constructed from National Center for Biotechnology Information (NCBI) downloaded sequences of Blanding's and wood turtles. We also supplemented the reference libraries with Blanding's (n=13) and wood (n=3) sequences that we generated from blood and tissue samples, including from Minnesota, Wisconsin, and Illinois (Table 2.2). Primer and TaqMan® MGB probe design was informed using Primer3 v 2.3.7 (Untergasser et al., 2012) implemented in Geneious Prime with COI sequences for each species available on NCBI GenBank.

Assay specificity was validated in silico against the full NCBI nucleotide (nr) database with NCBI Primer-BLAST (Ye et al., 2012) using forward and reverse primers. We further tested for potential qPCR cross-amplification with the eDNAssay AI webtool (Kronenberger et al., 2022) using an alignment containing sequences available on NCBI GenBank for all non-target turtle species known to occur in the Midwestern United States. Assays with non-target species assignment probabilities greater than 0.3 will require (1) all eDNA amplicons to be confirmed as target species via DNA sequencing or (2) in vitro specificity testing, as recommended by Kronenberger et al. (2022), by performing qPCR using non-target species tissue-derived genomic DNA (gDNA) extracts. Initial in silico testing resulted in multiple primer and probe combinations for each assay.

We validated our assays in vitro using 1) synthetic DNA (gBlocks® Gene Fragments, Integrated Gene Technologies, Coralville, IA, USA) of Blanding's and wood turtle DNA, 2) extracted DNA from blood and tissue of Blanding's and wood turtles, and 3) extracted DNA from blood and tissue of non-target turtle species that are genetically similar and/or co-occur with the target species. Target species gBlocks were created based on sequence alignments that included both NCBI downloaded sequences and sequences from extracted tissue of locally collected turtles. Multiple qPCR plates were run to optimize qPCR chemistry and primer and probe concentrations, as well as to choose which primer/probe combinations resulted in optimally performing assays. We conducted non-target testing by running plates including extracted DNA of both target and non-target turtle species to confirm specificity of the assay. If any non-targets did amplify, we looked to the quantification cycle (C_q) values to determine if the values between target and non-target samples were distinct from one another. The C_q value indicates the PCR cycle number at which the sample's reaction curve intersects with the threshold line and gives us an idea of the concentration of our target sequence. Lower C_q values indicate higher amounts of the target sequence. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for both species assays via a 7-fold dilution series of target species gBlocks (0.1–100,000 copies/µL). We considered the LOD as the lowest standard concentration with 95% detection using three PCR replicates and the LOQ as the lowest standard concentration with a coefficient of variation (CV) of C_q values below 35% (Klymus et al., 2020a). For our assays, each standard reaction was run in triplicate using the following conditions: 20 µL reaction volumes consisting of 3 µL of gBlocks template, 10 µL of TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 4 µL of sterile molecular grade water, and 1 µL of each primer and probe with optimized concentrations. Initial thermocycling conditions for all assays were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s. All qPCRs were conducted on a 96-well QuantStudio™ 3 Real-Time PCR system (Applied Biosystems) and amplification and standard curve analysis was performed using Thermo Fisher Connect™ online software with default settings. Depending on the results of the non-target testing, we adjusted the annealing temperature to enhance specificity to our target species. We calculated the LOD/LOQ for each assay using the LOD/LOQ calculator R script (Klymus et al., 2020a).

Table 2.1 Definitions of eDNA assay validation metrics

Assay validation metric	Definition
Coefficient of determination (**R^2**)	How well the data points fit the line in a standard curve using a dilution series of known concentrations
Efficiency	How effectively the PCR reaction duplicates the DNA every cycle
Limit of Detection (**LOD**)	The lowest standard of known concentration at which 95% of technical replicates amplify
Limit of Quantitation (**LOQ**)	The lowest standard concentration for which the coefficient of variation (CV) value is <35%

2.2.2 Assay Comparison

For both Blanding's and wood turtles we conducted in vitro testing on two different previously published assays; a probe-based and a primer only assay (Table 2.1). We ran the two published assays for each turtle species following the specifications described in their respective papers (Davy et al., 2015; Hernandez et al., 2020). To compare the efficacy of our assays against existing assays, we assessed both sensitivity toward target species and their ability to discriminate against non-target species. We ran qPCR plates for each assay that included extracted DNA from tissues of both target and non-target turtle species. Each sample was run in triplicate. We evaluated which non-target species amplified, as well as their C_q values compared to the C_q of the target extracts. Additionally, we compared the LOD/LOQ values, efficiency, and R2 between our developed assays and the existing assays using target gBlocks (Table 2.2).

2.3 Results

2.3.1 Novel eDNA assay design and validation

Assay specificity was validated *in silco* using NCBI Primer-BLAST, resulting in zero potential non-target matches for the Blanding's assays and only two potential non-target matches (i.e., *Clemmys guttata* and *Emydoidea blandingii*) for the wood turtle assay (Table 2.3). The minimum number of nucleotide mismatches among primers/probe and all non-target sequences ranged from 5–17 (Table 2.3). Specificity testing for each assay with eDNAssay software identified 1–3 potential non-target species that had assignment probabilities higher than 0.3 (Table 2.3), ranging from 0.322–0.507, indicating a need for target species DNA sequence confirmation and future in vitro specificity testing.

2.3.2 Non-target testing

For both Blanding's and wood turtles, both novel and previously published assays all recovered positive non-target turtle species amplifications. We adjusted the annealing temperature of our assays from 60°C every 1°C to 66°C to determine at which point the C_q values were both at their lowest for the target turtle species and resulted in the largest difference in C_q between target samples and non-target samples. While we found that the standard 60°C was optimal for Blanding's, our wood turtle assay

performed the best at an annealing temperature of 62°C. While all assays amplified non-target DNA, after optimization all assay's target species C_q values were distinctly lower than non-target C_q values.

Chapter 3: Comparison between assays

We validated three different assays for both turtle species to determine which are expected to perform the best on environmental samples collected from Minnesota's aquatic habitats. For Blanding's, the primer-only assay did not amplify tissue-derived genomic DNA nor Blanding's gBlocks® synthetic DNA fragments. The values for R^2 (0.985) and LOD (0.75 copies/µL) for our assay were both better than the Hernandez assay (R^2=0.961, LOD=1.8 copies/µL; Table 2.4). The LOQ was slightly lower for the Hernandez assay at 7.05 copies/µL compared to our assay at 13.9 copies/µL. Acceptable efficiency values range between 90% and 110%, with the optimal value at 100% efficiency. Values recovered for these assays were slightly higher than this range (120-130%), potentially due to human error associated with working at very low concentrations. Higher starting concentrations coupled with well-optimized assays generally yield efficiencies within the accepted range.

The published primer-only wood assay did successfully amplify tissue-derived DNA, as well as wood turtle gBlock DNA. Each of the validation metrics were within the acceptable ranges, except efficiency which was at 81%. Our new assay and the probe-based Hernandez assay for wood turtles yielded R^2, efficiency, LOD, and LOQ values all within acceptable ranges (Table 2.4).

3.1 Discussion

We developed and validated highly sensitive qPCR assays to detect Blanding's and wood turtles, two imperiled species of North American freshwater turtle communities, using recommended best practices for assay design and validation (Klymus et al., 2020a; Klymus et al., 2020b; Langlois et al., 2021; Thalinger et al., 2021). Additionally, we validated previously published assays for Blanding's and wood turtles using the same synthetic gBlock DNA fragments and regionally collected tissue-derived genomic DNA. For all assays we conducted rigorous *in silico*, *in vitro*, and *in situ* testing. Ultimately, these novel assays are specific, sensitive, and successful in detecting target species in natural settings.

Blanding's and wood turtles are of considerable conservation concern throughout their ranges, and in many cases are at risk of extirpation (van Dijk 2011; Jones et al., 2018; King et al., 2021). Their range-wide imperilment has precipitated listing decisions at the state/provincial and federal level, thereby necessitating a finer-grained assessment of their distribution throughout its historic range. Yet conventional methods are often time and labor intensive, and, when combined with the often-challenging habitats in which they persist (e.g. bogs, marshes, and sedge meadows), difficult to survey efficiently and effectively (Beaudry et al., 2009; Davy and Fenton 2013; Davy et al., 2015). Environmental DNA has shown considerable promise in rapid and sensitive detection of Blanding's and wood turtles (Davy et al., 2015; Lacoursiere-Roussel et al., 2017; Akre et al., 2019; Fyson et al., 2021; Tarof et al., 2021) but given their broad distributional area and general lack of sequence data available in online repositories, existing assays may only work in a portion of the range. Our comparison of existing assays suggests that unique genetic diversity may preclude detections in certain geographic contexts. Thus, we developed, optimized, and validated novel assays that included regional sequence data. These developed assays are sensitive, specific, and effective in field conditions (see Chapter 3).

After designing, optimizing, and validating novel assays for Blanding's and wood turtles and comparing them to the previously published assays, we determined that we would proceed with the novel assays for in situ testing of environmental samples. In general, they performed as well, if not better, than published assays, and within the generally accepted standards in the field (Klymus et al., 2020a). As for the primer-only assays, the Blanding's assay did not result in any positive amplification while the wood assay did, however, the wood assay generally did not perform as well as either probe-based assay. Our novel probe-based assays and the previously published probe-based assays performed similarly for both R^2, efficiency, LOD, and LOQ for both turtle species. Considering the comparable results, we will use the novel assays for subsequent in situ testing of environmental samples in our studies to limit the possibility of false negatives that could result because of geographic variation. We do, however, caution against the adoption of any eDNA assay, including ours, at-scale without rigorous *in vitro* testing to assure that regional variation is compatible with the designed assay to avoid the risk of false negatives and the conservation consequences that may stem from them. Additionally, there may be different problematic non-target species in their region of interest compared to the region/s used in the design phase (Thalinger et al., 2021). Therefore, researchers should determine 1) the sources of DNA sequences used in published primer/probe designs to see if sequences from their region of interest were utilized, and 2) what non-target sequences were incorporated in the *in silico* validation stages. If either step is unsatisfactory for their project, researchers should consider novel primer/probe design.

For a number of reasons, turtles are an ideal candidate for eDNA monitoring. Conventional survey methods are not universally effective across species, habitats, and time of year, among other reasons, that could result in false negatives (Mazerolle et al., 2007). Furthermore, rigorous and time-intensive surveys are often required to ensure high detection rates, which could drive up the cost of a project (Akre et al., 2019; Sternhagen et al., 2024). Therefore, there is a need for a more efficient and cheaper surveying method for aquatic turtles and eDNA may provide a solution. However, compared to other aquatic vertebrates (particularly fish and amphibians), eDNA monitoring of turtles and other non-avian reptiles has proven particularly challenging (i.e. Raemy & Ursenbacher, 2018; Nordstrom et al., 2022). For one, many species are often at low abundance in aquatic habitats leading to stochastic patterns in detection; potentially due to the rarity of the taxa to begin with or because many turtle species spend a significant amount of time on land depending on the season (Nordstrom et al., 2022; Ernst & Lovich, 2009; Vitt & Caldwell, 2013). Turtles do not release gametes into aquatic habitats, unlike many other more easily detectable taxa like fish (e.g., Tillotson et al., 2018). In fact, many species do not even build nests adjacent to waterways (Ernst & Lovich, 2009; Vitt & Caldwell, 2013). Lastly, turtle DNA may be in low concentrations in aquatic habitats because they have mostly keratinized skin and do not shed eDNA at the same rate or to the same degree as organisms with a mucus layer (e.g. amphibians and most fish; Andruszkiewicz et al., 2021).

Nevertheless, advances in targeted eDNA assay design, optimization, and validation have driven increasingly positive outcomes in monitoring rare, threatened, and/or endangered freshwater turtles (Kessler et al., 2020; Fyson & Blouin-Demers, 2021; Tarof et al., 2021; Villacorta-Rath et al., 2022; Nordstrom et al., 2024; Sternhagen et al., 2024). As more assays are developed and utilized in natural settings, we gain insight into what factors increase sensitivity to detection of eDNA. Our assays were

developed following current guidelines for *in silico*, *in vitro*, and *in situ* validation (Thalinger et al., 2021). For both targeted assays, we have completed *in silico* testing on both sympatric and allopatric turtle species, providing critical insights into the species specificity of the assays. We also completed *in vitro* validation with target species, as well as closely related and/or sympatric species. Moreover, we quantified both LOD and LOQ. Our assays also met the requirements of fourth level of validation, which not only indicates that we have had detections in environmental samples, but also that a LOD was determined and extensive field testing of environmental samples and *in vitro* testing on co-occurring non-target species has occurred. For all assays to achieve the fifth and final level of validation scientists will need to estimate detection probabilities and investigate what environmental and physical factors impact our ability to detect turtle eDNA, and we will explore our efforts to conduct occupancy modeling with concomitant detection probabilities for Blanding's and wood turtles in the next chapter.

Ultimately, freshwater turtle species are among the most imperiled species on the planet. Therefore, efforts to monitor populations are a conservation imperative. However, their natural history and habitat proclivities often render monitoring time intensive and cost prohibitive. Environmental DNA has shown considerable promise, broadly, in measuring and monitoring biodiversity, and particularly for freshwater turtles. Here we reported the design, validation, and optimization of targeted assays for two imperiled (i.e. Blanding's and wood) North American turtle species. These assays should provide expanded opportunities for eDNA assessments for these species, ultimately facilitating critical data and information to inform adaptive management strategies for these species.

Table 2.1 Forward and reverse primers, probes, and gBlocks sequences, including optimized primer/probe concentrations and the cytochrome c oxidase subunit 1 (COI) gene fragment amplicon size, for each qPCR assay.

Species/ Assay	Name	Type	Optimized conc. (µM)	Amplicon length (bp)	Sequence (5'–3')
Blanding's/ Novel	EMBL-157F	F Primer	12	94	CGCCTTCATCATAATCTTCTTC
	EMBL-206R	R Primer	4	94	TATATCTGGTGCTCCGATTATTAG
	EMBL-166P	Probe	4	94	CAATTTCCAAATCCGCCGATTATAATAGGT
Blanding's	EMBL-COI-GB	gBlocks			CCCTATACTTAATTTTCGGAGCTTGAGCAGGAATAGTAG GCACAGCATTAAGTCTACTAATCCGCGCAGAACTAAGTC AACCAGGAACCCTTTTAGGGGATGACCAGATCTATAATG TTATCGTTACAGCCCACGCCTTCATCATAATCTTCTTCATA GTCATACCTATTATAATCGGCGGATTTGGAAATTGACTTG TACCACTAATAATCGGAGCACCAGATATAGCATTTCCAC GTATAAATAATATAAGTTTCTGACTTTTACCACCATCCCT ACTACTACTTCTAGCATCATCAGGAATTGAAGCAGGGGC AGGCACAGGCTGAACTGTATATCCACCACTAGCTGGAAA CTTAGCCCACGCCGGTGCCTCTGTAGACCTAACTATCTTT TCCCTCCACCTAGCCGGTGTATCTTCAATTCTAGGGGCTA TTAACTTTATTACCACAGCAATTAACATAAAATCCCCAGC CATATCACAATACCAAACACCCCTGTTTGTCTGATCAGTA CTTATTACAGCTGTCCTATTACTATTATCATTACCAGTACT AGCTGCAGGTATCACAATACTACTTACAGACGAAACCTA AATACAACCTTCTTTGACCCTTCAGGAGGAGGAGACCCA ATCCTATACCAACACTTATTC
Blanding's/ Hernandez et al. 2020	EMBL_COIF	F Primer	10	179	ATCATCAGGAATTGAAGCAGGG
	EMBL_COIR	R Primer	10	179	GGGATTTTATGTTAATTGCTGTGGTAATA
	EMBL_COI_	Probe	10	179	CTGAACTGTATATCCACCACTA
Blanding's/ Davy et al. 2015	CO1-EBl-01-F	F Primer	0.2	216	ATCATAATCTTCTTCATAGTC
	CO1-EBl-01-R	R Primer	0.2	216	AGTTTCCAGCTAGTGGTGGA

Species/ Assay	Name	Type	Optimized conc. (µM)	Amplicon length (bp)	Sequence (5'–3')
Wood/ Novel	GLIN-COI-304F	F Primer	6	123	TTACTACTCCTAGCATCATCAGGG
	GLIN-COI-378R	R Primer	10		TAGAGAAAAGATGGTTAGGTCTACAG
	GLIN-COI-305P	Probe	8		TGTGCCTGCTCCTGCTTCAAC
Wood	GLIN-COI-GB	gBlocks			TATACTTAATTTTCGGAGCCTGAGCAGGAATAGTAGGCA CAGCATTAAGTCTACTAATCCGCGCAGAACTAAGTCAAC CAGGAGCTCTTTTAGGGGATGACCAAATCTATAATGTTA TCGTTACAGCCCATGCCTTCATTATAATTTTCTTTATAGTC ATGCCAGTCATAATCGGTGGATTTGGAAACTGACTTGTA CCATTAATAATCGGGGCACCAGATATAGCATTCCCACGT ATAAATAACATAAGTTTCTGACTTTTACCCCCATCCCTATT ACTACTCCTAGCATCATCAGGGGTTGAAGCAGGAGCAG GCACAGGCTGAACTGTATACCCTCCACTAGCTGGAAACT TAGCCCACGCCGGTGCCTCTGTAGACCTAACCATCTTTTC TCTACACCTGGCCGGTGTATCTTCAATCTTAGGGGCTATC AACTTCATTACCACAGCAATCAACATAAAATCCCCGGCCA TATCTCAATACCAAACACCCCTATTTGTATGATCAGTACT TATTACAGCTGTCCTATTACTATTATCATTACCTGTTCTAG CTGCAGGCATCACTATACTACTTACAGACCGAAACCTAA ATACAACCTTCTTTGACCCTTCAGGGGGAGGAGACCCAA TTCTATACCAACACCTGTTC
Wood/ Hernandez et al. 2020	GLIN_COIF	F Primer	10	173	CTGGCCGGTGTATCTTCAATCT
	GLIN_COIR	R Primer	10		AGTATAGTGATGCCTGCAGCTAGTACA
	GLIN_COI	Probe	10		CCGGCCATATCTCAATA
Wood/Davy et al. 2015	CO1-GIn-02-F	F Primer	0.2	155	GCCAGTCATAATCGGTGGA
	CO1-GIn-02-R	R Primer	0.2		CTGCTCCTGCTTCAACCCCT

Table 2.2 Sources of Blanding's and wood turtle tissue samples included in development of our assays and synthetic gBlock fragments

Turtle Species	Location	Source of Sample
Blanding's	Will Co., Illinois (n=4)	Illinois Natural History Survey (Sample IDs: 20196, 21196, 21197, 21713)
	Kenosha Co., Wisconsin (n=3)	Illinois Natural History Survey (Sample IDs: 20456, 20457, 20458)
	Cass Co., Minnesota (n=1)	United States Department of Defense – Fort Ripley (Sample ID: 102422)
	Wright Co., Minnesota (n=1)	St. Cloud State University
	Hennepin Co., Minnesota (n=1)	Minnesota Department of Natural Resources
	Washington Co., Minnesota (n=1)	Minnesota Department of Natural Resources
Wood	St. Louis Co., Minnesota (n=3)	Minnesota Department of Natural Resources

Table 2.3 Minimum number of nucleotide mismatches for forward primer, reverse primer, and probe, and maximum assignment probabilities for all non-target taxa for each turtle assay. Taxa in bold have assignment probabilities above the 0.3 threshold. Asterisks (*) denote taxa identified as potential non-targets via NCBI Primer-BLAST.

Assay	Non-target taxa	Mismatches (min)				Assignment probability (max)
		F	R	P	Total	
Blanding's assay	*Chelydra serpentina*	2	4	6	12	0.132
	Chrysemys picta	3	2	4	9	0.140
	Clemmys guttata	4	4	3	11	0.143
	Glyptemys insculpta	2	5	4	11	0.122
	Sternotherus odoratus	2	4	3	9	**0.322**
Wood assay	***Chelydra serpentina***	6	7	3	16	**0.322**
	Chrysemys picta	5	2	3	10	0.227
	****Clemmys guttata***	3	3	1	7	**0.507**
	**Emydoidea blandingii*	P	3	2	8	0.271

Table 2.4 Mean R-squared (R2), mean percent amplification efficiency, limit of detection (LOD), and limit of quantification (LOQ) estimates for each assay. The Blanding's Davy et al. 2015 assay did not amplify.

Assay	R^2	Efficiency	LOD copies/µL	LOQ copies/µL
Wood – Novel primers	0.996	103%	0.68	5
Wood – Hernandez et al. 2020 primers	0.993	105%	0.34	11
Wood – Davy et al. 2015 (primer only assay)	1.00	81%	0.99	1.14
Blanding's – Novel primers	0.985	130%	0.75	13.9
Blanding's – Hernandez et al. 2020 primers	0.961	122%	1.8	7.05
Blanding's - Davy et al. 2015 (primer only assay)	-	-	-	-

Chapter 4: Environmental DNA Occupancy Modeling Reveals Differential Habitat Proclivities for Two Imperiled North American Freshwater Turtle Species

4.1 Introduction

The use of environmental DNA (eDNA) has gained significant momentum as an efficient and non-invasive method for monitoring rare and invasive species in conservation management (Rees et al., 2014). Molecular techniques have rapidly advanced since the development of eDNA tools, and eDNA assays are now highly sensitive and effective for detecting species, even at very low abundances. While there are still efforts to optimize these techniques, there is an additional need to understand what biotic and abiotic factors in the field are associated with higher rates of eDNA detection.

Many of the variables that contribute to variation or uncertainty with traditional survey methods can similarly impact eDNA studies (Schmidt et al., 2013; de Souza et al., 2016). Given that conservation managers must understand not only where species occur but also the environmental factors driving their distribution, it is essential to consider how these factors may influence eDNA survey results to avoid misinformed management decisions. In this study, we applied that approach to investigate two threatened turtle species in Minnesota. Both Blanding's turtle (*Emydoidea blandingii*, Holbrook, 1838) and wood turtle (*Glyptemys insculpta*, Le Conte, 1830) are rare and threatened turtle species native to North America.

Here we used multilevel hierarchical occupancy models (MacKenzie et al., 2002; Schmidt et al. 2013) to estimate the probability that sites throughout Minnesota are occupied by Blanding's or wood turtles (i.e. occupancy, *psi*) and the probability that given a site is occupied, that eDNA of that species is detected (i.e. detection probability, *p*). We included variables related to land cover, stream quality, stream size, weather, and seasonality in our competing models to determine which were important for turtle occupancy and eDNA detection probability. The results of our study can guide managers in determining optimal timing for future eDNA surveys and offer insights into the environmental factors that influence turtle presence across the landscape.

4.2 Materials & Methods

4.2.1 eDNA field sampling

We collected water samples from lentic and lotic habitats throughout the native ranges of Blanding's and wood turtles in Minnesota. Between May 23rd, 2023, and April 11th, 2024, we collected eDNA samples during four different four-day sampling periods to target different periods of the turtle's life history. The May/June 2023 sampling was intended to overlap with the initiation of breeding and egg

laying, as well as travel for foraging (Ernst et al., 1994; Sajwaj and Lang, 2000). Sampling in September 2023 was intended to coincide with hatchling emergence and dispersal (Congdon et al., 1983; Ernst et al., 1994; Piepgras and Lang, 2000), as well as travel to seek refuge from drought conditions for Blanding's turtle. Winter sampling in January 2024 should correspond with turtles in overwintering sites (Ernst et al., 1994; Piepgras and Lang, 2000), where they were expected to remain with minimal movement until emerging in late March and April. Our final sampling trip in April 2024 was anticipated to coincide with this spring emergence. For our occupancy modeling analyses, we made sure to include sites with confirmed Blanding's and/or wood turtle populations in recent history (within the last five years), as well as sites with expected absences. We also included sites that represented historical presences (greater than five years) for each species, which could potentially help us understand the degree of sensitivity for our assays.

At each eDNA sampling site, three replicate water samples were collected at the right, left, and center of the water body in sterile, 1-L plastic bottles (3-L total). In lentic water bodies we conducted subsampling by collecting water every few meters as we walked along transects parallel to the shore. To access water during the January sampling trip, we drilled holes through the ice using an ice auger and dipped the sampling bottle in to collect water. To minimize the potential for field contamination, personnel always entered the water downstream of the sampling location and always collected samples upstream wearing gloves that were changed between sites. Waders/boots were decontaminated between sites using a 20% bleach solution. Additionally, we included a 1-L bottle of distilled water at each site to serve as a negative field control. All samples were stored in coolers prior to filtration to minimize the effects of high temperatures and UV radiation. Within 12 h of sampling (but typically less than 8; Curtis et al., 2020), water samples were filtered using a vacuum pump through 1.2-µm cellulose acetate filters. In cases where turbid water would cause the filters to clog, multiple filters were used to fully process the full 1-L sample. Filters were then stored in vials of cetyltrimethylammonium bromide (CTAB) buffer at room temperature for two weeks prior to DNA extraction.

4.2.2 eDNA extraction

All eDNA samples and field controls were extracted using a modified phenol-chloroform-isoamyl alcohol extraction (Renshaw et al., 2015; Garcia et al., 2024). DNA was eluted in 100 µL of TE buffer and was subsequently treated with the OneStepTM PCR Inhibitor Removal Kit (Zymo Research) to remove inhibitors before being stored at −20°C until qPCR reactions were run. Additionally, we included an extraction blank of molecular grade water as a negative control for each extraction event. All above steps were carried out using standard cleanroom procedures in laboratory spaces physically separate from locations where post-PCR products are handled.

4.2.3 qPCR

Each eDNA sample, extraction blank, field blank, and qPCR standard (gBlocks® of known DNA concentration) was run in triplicate in a qPCR reaction using our validated assays.

Any sample that was split between two filters was pooled after the inhibitor removal step and before qPCR. We used primers and probes that targeted different regions of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI). The Blanding's assay targeted a 94 bp fragment (Forward Primer: 5'-CGCCTTCATCATAATCTTCTTC–3', Reverse Primer: 5'-TATATCTGGTGCTCCGATTATTAG-3', Probe: 5'-CAATTTCCAAATCCGCCGATTATAATAGGT-3') and the wood assay targeted a 123 bp fragment (Forward Primer: 5'-TTACTACTCCTAGCATCATCAGGG-3', Reverse Primer: 5'-TAGAGAAAAGATGGTTAGGTCTACAG-3', Probe: 5'-TGTGCCTGCTCCTGCTTCAAC-3') The qPCR conditions were as follows: 20 µL reaction volumes consisting of 3 µL of eDNA extract, 10 µL of TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 4 µL of sterile molecular grade water, 1 µL of the forward primer (12 µM- Blanding's, 6 µM- wood), 1 µL of the reverse primer (4 µM- Blanding's, 10 µM- wood), and 1 µL of the probe (4 µM- Blanding's, 8 µM- wood). Initial thermocycling conditions for assays were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60 (or 62)°C for 60 s. The annealing temperature was optimized at 62°C for the wood turtle assay. We also included two columns of an 8-fold dilution series of target species gBlocks (0.1-1x10^6 copies/µL) as positive controls and three no template controls (NTCs) which included molecular grade water instead of DNA template. All qPCRs were conducted on a 96-well QuantStudio™ 3 Real-Time PCR system (Applied Biosystems) and amplification and standard curve analysis was performed using Thermo Fisher Connect™ online software with default settings. Each qPCR reaction used 3 µL of DNA product, following the conditions outlined above and included three no template controls (NTCs) which included molecular grade water instead of DNA template. A site was considered positive for a detection if at least one of the three replicates yielded an amplification and there was no evidence of contamination in field, extraction, or qPCR controls.

4.2.4 eDNA model predictors

For Blanding's and wood turtles we sought to estimate both occupancy and detection probabilities throughout their Minnesota ranges using a set of carefully chosen predictor variables (Table 3.1). We used several predictor variables from the United States Environmental Protection Agency's StreamCat database (https://www.epa.gov/national-aquatic-resource-surveys/streamcat-dataset; Hill et al., 2016) to estimate occupancy, including mean road density, percent forest cover, and the National Rivers and Streams Assessment (NRSA) benthic invertebrate multimetric index (BMMI). We ran all analyses using StreamCat variables at the catchment scale. For percent forest cover, we summed deciduous forest percentage, evergreen forest percentage, and mixed deciduous/evergreen forest percentage to get a single value (NLCD, 2021). Road density refers to the mean density of roads as a percentage within a catchment (US Census, 2010). The NRSA BMMI variable represents the predicted probability that a stream segment is considered to be in good biological condition (Hill et al., 2016). We also included other variables accessed outside of StreamCat. Stream order was derived from the U.S. Geological Survey (USGS) National Hydrography Dataset Plus (NHDPlus) flowline data. Total precipitation (cm; used for occupancy) and maximum air temperature (°C; used for detection probability) data were downloaded from the National Oceanic and Atmospheric Administration (NOAA) National Centers for Environmental Information (NCEI) database for the county in which the site was located. Total precipitation was calculated by summing precipitation (in cm) over a 72-hour period from the day before sampling date to the day after sampling. Maximum air temperature (°C) was the highest recorded

temperature on the day of sampling. Julian day was the numeric value representing the continuous count of days in a calendar year starting on January 1st.

4.2.5 Multilevel, hierarchical occupancy and detection probability models

We performed multilevel, hierarchical models of occupancy (*psi*) and detection probability (*p*) for both Blanding's and wood turtles using detections and non-detections from replicated field samples to quantify detection probability. We fit null and global models, where the global model included all occupancy and detection covariates, as well occupancy-only and detection-only models, and individual models specific to the biology of both Blanding's and wood turtles. All models that included detection probability had the same detection covariates; maximum air temperature (°C) and Julian day. In the Blanding's species-specific model, we included road density and the interaction of stream order and total precipitation (as a proxy for drought levels) in occupancy. Blanding's turtles are known to travel some of the farthest distances among turtle species for foraging and breeding efforts and road mortality is one of the major causes for their decline (Piepgras and Lang 2000). We used stream order and total precipitation as occupancy covariates because Blanding's turtles are known to seek drought refuge during drier months (Anthonysamy et al. 2013). In the wood turtle species-specific model, we included forest cover, macroinvertebrate index, and the interaction of stream order and total precipitation as occupancy covariates. We included forest cover because wood turtles are known to commonly travel between aquatic habitat and upland forests for foraging (Ross et al. 1991). Wood turtles are thought to rely on pristine waterways, making the use of the macroinvertebrate index as a proxy for stream health an effective approach to assess habitat quality. Lastly, stream order and precipitation were included because wood turtles tend to be found near larger streamways. We used single-season occupancy models for both species under the assumption that occupancy did not change for these long-lived species during our study period. We fit models using the *unmarked* package (Fiske & Chandler, 2011) in R version 4.4.0 (R Core Team Year), and selected top models using Akaike information criterion (AIC) (Burnham & Anderson, 2003). For both species, we performed model averaging on all models that had a ΔAIC <2 to determine our parameter estimates. To estimate the number of samples needed to yield a cumulative 95% detection probability of turtle eDNA, we used McArdle's (1990) cumulative probability equation on our most supported model.

Finally, using the outputs from the occupancy modeling, we developed a Microsoft Excel-based "widget," single-replicate detection probabilities for a calendar or Julian day are calculated (attached). The widget then fits a polynomial in Excel to the predicted detection probability for every day of the year over which we sampled (25 to 258) for both species. We note that we only included days in between the first and last sampling periods, and thus days 1-24 and 259-365 were not excluded (though we would predict declining detections after day 259). Using this polynomial relationship, the widget calculates a detection probability for a given day of the year, where all else (temperature, forest cover, stream order, etc.) should be interpreted as equal at a median or routine value. Next, the widget calculates a cumulative detection probability for the event using the cumulative detection probability equation (McArdle, 1990; Schmidt et al. 2013). Finally, the widget calculates a multi-event detection probability for a species by summing the three event cumulative detection probabilities.

4.3 Results

4.3.1 eDNA detection of Blanding's and wood turtles

We collected 492 eDNA samples (3 replicates per site) from a total of 78 unique sites across our four sampling periods. Most sites were sampled during multiple seasons; only 2 sites were sampled only once (Table 3.2).

We had at least a single detection for each turtle species in every sampling period (Table 3.2). In total we had 37 Blanding's and 15 wood turtle eDNA detections. The September 2023 period yielded the greatest number of detections for both species with 23 and 8 for Blanding's and wood turtle, respectively. On the other hand, there was only a single detection per species during the January 2024 period. None of our field blanks, extraction blanks, or NTCs yielded a detection, indicating undetectable levels of contamination across our field and laboratory processes.

The distribution of positive detections for Blanding's turtle was concentrated around east central Minnesota (Figure 1). Of the 21 United States Geological Survey (USGS) Hydrologic Unit Code 8 (HUC 8) watersheds that we sampled, we detected Blanding's eDNA in all but seven. Conversely, the highest concentration of wood turtle detections was in northeastern Minnesota, and we only had detections of wood turtle eDNA in five of the 21 HUC 8 watersheds that we sampled.

4.3.2 Occupancy modeling

For the wood turtle, all models except the detection-only model performed better than the null model (Table 3.3). The top performing model was the occupancy-only model, however, there were three alternative models that performed similarly. We conducted model averaging on these top four models for parameter estimation (Table 3.4). The top models had moderately good fit ($R^2 > 0.38$), but all covariates had 95% confidence intervals that overlapped zero, indicating weak individual effects of these variables.

All candidate Blanding's turtle models performed better than the null model (Table 3.5). The top performing model was the global model, which included all detection and occupancy covariates, but there were two equivalent models, which were all model averaged for parameter estimation (Table 3.6). We found that Blanding's turtle occupancy declined with increased stream order, and Blanding's turtle detection probability increased with Julian date, as both covariates had 95% confidence intervals that did not overlap zero (Table 3.6; Figure 3.2). Finally, using Julian day as the predictor in our cumulative detection probability model we determined that it would take six and four samples in late summer/early fall to detect wood and Blanding's turtles, respectively, with 95% confidence (see Tables 4.1 and 4.2). In the winter, 69 (wood turtle) and 91 (Blanding's turtle) samples would be required to achieve the same level of confidence, with spring and early summer sampling falling somewhere in between two extremes as far as number of samples necessary.

The model underlying the designed "widget" fits the data well ($R^2 = 0.9996$). For example, one might wish to sample both species on Calendar Days 30, 60, and 90, and with 4 field replicates at each

sampling period. For Blanding's turtles, this would yield a cumulative detection probability of 0.145, 0.171, and 0.231 for the 30-, 60-, and 90-day period, and a final, multi-event detection probability of 0.547. For wood turtles, this same sampling scheme would recover cumulative detection probabilities of 0.170, 0.248, and 0.368, and a final multi-event detection probability of 0.786. At the established cost per sample of $79.38, the cumulative cost of these sampling schemes would be $952.56 per site, with a likelihood of detection at approximately 55% for Blanding's turtle, and 79% for wood turtle.

4.4 Discussion

Using the novel targeted assays designed in Chapter 2, we successfully detected Blanding's and wood turtle eDNA in each of our four seasonal sampling periods. Freshwater samples were collected from rivers, streams, and wetlands during different times of the year to capture potential effects of seasonality. Repeated sampling was conducted at most, but not all sites, which is important for our analysis of occupancy and detection probability. Overall, we detected Blanding's turtle eDNA more frequently, over a broader geographic scale than wood turtle eDNA in Minnesota. We had 37 total Blanding's eDNA detections, compared to 15 wood turtle detections. Blanding's eDNA was detected in 14 out of 21 (66.67%) HUC 8 watersheds sampled, compared to 5 out of 21 (23.81%) for wood turtles. Accurately estimating freshwater turtle populations is challenging (Tesche and Hodges, 2015), and as a result, we lack precise information on the number of individuals or total biomass of each species across the Minnesota landscape. However, this difference in detection rate likely indicates a wider distribution and greater density of Blanding's turtles in Minnesota, as has been revealed in other freshwater turtle species (Kessler et al., 2020). While most detections for both species occurred at sites with a known turtle presence within the last five years, we were able to detect both Blanding's and wood turtle eDNA at sites with only historic records, highlighting the potential benefit of eDNA sampling to discover new populations.

With respect to seasonality, while overwintering sampling (Jan. 24) was the least effective, fall (Sept. 23) sampling yielded the highest number of positive detections. In our spring and early summer months, there were more Blanding's detections in April 2024 (n=9) than May/June 2023 (n=4), but less wood turtle detections in April (n=2) than in May/June 2023 (n=2). Both turtle species are in the process of emerging from overwintering sites during the month of April (though, wood turtles tend to emerge slightly later) so we would expect to detect eDNA with increased activity levels, as turtles move throughout the environment for basking and foraging (Ernst et al. 1994; Sajwaj and Lang 2002). We expected a noticeable increase in detections of both species during the May/June sampling period because of reproductive activity, however, this is one sampling point for this time of year and there are several environmental factors that could have influenced these results. We saw the greatest number of detections for both species in the fall, which corresponds with when juveniles are hatching and in the case for Blanding's turtle, they are traveling to certain sites for drought refugia (Anthonysamy et al. 2013). Ultimately, we expect increasing detection probability with increasing Julian date, peaking in late summer/early fall, and declining thereafter through late fall and into winter.

While the top-performing wood turtle models provided moderate fits to our data, none of the included covariates had strong relationships with occupancy or detection probability. This is not uncommon, as

models optimized for predictive ability by criteria like AIC can often return weak relationships of individual predictors variables (Tredennick et al. 2021). Although 95% confidence intervals for wood turtles always overlapped 0, our model-averaged coefficients suggest a negative associated between wood turtles' occupancy and road density, as well as a positive association between wood turtle detection probability and Julian date. Stronger inferences about wood turtle occupancy and eDNA detection probability relationships might be achieved by increasing sampling effort at habitats known or suspected to be occupied by this species.

We found that Blanding's turtle occupancy had a negative relationship to stream order and were more commonly associated with lower-order streams that tend to be narrower and shallower. This finding corroborates the habitat association of Blanding's turtles with smaller, but ephemeral waterways (Hamernick 2000; Piepgras and Lang 2000). We also found that detection probability of Blanding's turtle eDNA increased throughout the year. This is consistent with our very infrequent winter detections of turtle eDNA, increasing detections in the spring, and highest eDNA detections in the autumn. We did not sample between September and January so we cannot provide estimates of detection probability for this time period, but we would expect that probability would decline again as the year progressed and turtles traveled less frequently in colder months.

While we did detect both Blanding's and wood turtle eDNA through the ice, we only had one positive site replicate for each. Limited studies have addressed sampling eDNA overwintering freshwater turtles through ice (but see Feng et al., 2020; Tarof et al., 2021), however those that have, have yielded higher detection rates of overwintering turtle eDNA than those observed here. Two notable differences in those studies are 1) targeting sites with known occupancy from radio telemetry data, and 2) sampling further below the ice surface (up to 1.5m below), rather than just below the surface. While a handful of our sites have been monitored recently for telemetry-tagged turtles, this is the minority. For future studies in this system, we recommend following the practice outlined by Feng et al.(2020) of attaching collection bottles to 1.5 m poles to sample at greater depths below the ice or using grab samplers to sample at depth (Iacaruso et al., *In Review*). The ability to sample at depth, rather than just below the ice surface, could allow surveyors to either access water in closer proximity to where turtles are brumating, or sediment onto which eDNA may absorb (Ogram et al., 1988; Xue and Feng, 2018; Pietramellara et al., 2009).

Finally, occupancy modeling that includes estimates of detection probability elevates the assays designed in Chapter 2 to Level 5 ("Operational") on the Thalinger et al. (2021) eDNA assay validation scale (below). This means that our assays have completed every criterion of each validation level. In brief, our assays have successfully completed:

Table 3.1 Thalinger criterion scale for assay readiness with interpretation of results if detection of eDNA present.

Validation Level	Readiness Level	Criteria	Results Interpretation	
			Not Detected	Detected
1	Incomplete	In silico analysis Target tissue testing Target tissue PCR	NA	NA
2	Partial	Comprehensive PCR condition reporting Non-target in vitro testing	NA	NA
3	Essential	Extraction of environmental samples Concentration of eDNA from environmental samples Detection of target species from eDNA samples	NA	Likely Present
4	Substantial	Limit of detection Extensive field testing of environmental samples In vitro testing on co-occurring non-target species	Likely Absent	Very Likely Present
5	Operational	Comprehensive specificity testing Detection probability estimation Understanding physical and environmental factors influencing eDNA	Very Likely Absent	Very Likely Present

Thalinger et al. (2021) provide useful guidelines for how, then, to interpret results based on the validation. For example, at validation levels 1 and 2, it is impossible to determine if the target species are present or absent, as the assay has not yet received sufficient validation. At level 3, if the target is not detected from environmental samples, it is impossible to tell if the target is present or absent. However, if the target is detected, it is likely present, but only if field controls return negative, the work was conducted in an eDNA appropriate laboratory, and positive detections are sequenced. At levels 4 and 5, non-detections are interpreted as likely absent, assuming appropriate timing of sampling and replication of sampling, and detections suggest that the target is very likely present. Level 5 confers the added benefit that the probability of species presence can be estimated, despite negative results. Given the level 5 status of our targeted assays, we believe that they can now be confidently adopted and deployed at-scale for both Blanding's and wood turtles.

In closing, we have successfully demonstrated that eDNA can be utilized to detect Blanding's and wood turtles across time and space in Minnesota. The ability to detect eDNA varied between species. Blanding's turtles were more likely to occupy smaller bodies of water, and we were better able to detect their DNA in the environment during the late summer/fall sampling period. Models for wood turtles were predictive of both occupancy and detection probability with moderate fits, but individual predictors were weakly supported and would benefit from increased replication targeting this species in future studies. Our study was a proof-of-concept, and optimization of detection for both species could be improved in the future. The majority of our sampling was at bridge crossings. Therefore, many of these samples were collected some distance (often >50 meters) from the GPS point of an historic

confirmed element occurrence record. Field samples for eDNA collected closer to anticipated Blanding's or wood turtle habitat would likely improve detectability of these species. Julian Date was the only significant predictor variable for detection probability, illustrating seasonal trends in detection that are likely linked to species' behavior and phenology. Therefore, other variables (e.g. flow parameters) are not captured in the Widget. Nevertheless, past research has shown strong linkages between flow and detection probability, and therefore we would also recommend avoiding sampling after heavy precipitation events, as high flow events can dilute eDNA in streams and rivers (Curtis et al., 2021; Urycki et al., 2024).

Table 3.2 Predictor variables used in occupancy and detection probability modeling of Blanding's and wood turtle eDNA detections.

	Variable (Abbreviation)	Unit	Data source	Prediction
Occupancy (psi) covariates	Total % forest cover (SUMFORESTCAT)	Percent (0-100), summed values of deciduous, mixed deciduous/evergreen, and evergreen forest percentage within catchment area	StreamCat compiled using NLCD 2019	Wood turtles inhabit streams adjacent to forests, whereas Blanding's turtles are often found in wetland complexes and agricultural lands
	Mean road density (RDDENSCAT)	Percent (0-100), density of roads within catchment area	StreamCat compiled using US Census 2010	Negative relationship for both species. High density of roads increase likelihood of road mortality (Gibbs et al. 2002).
	Benthic invertebrate multimetric index (prG_BMMI)	Biological condition (0–1)	StreamCat compiled using NRSA data 2008–2009 (Hill et al. 2016; USEPA 2016)	Wood turtles are expected to inhabit streams in good condition, but Blanding's turtles occupy a larger variety of habitats including slightly disturbed areas
	Stream order (StreamOrde)	Measurement of relative stream size (1-12)	NHDPlus (Moore et al. 2019)	Wood turtles are expected to inhabit small- to medium-sized streams with fast-moving water. Blanding's turtles are expected to inhabit wetlands and shallow pools of water, and are less associated with medium-sized, fast-moving streams.
	Total precipitation (TotalPrecp)	cm, recorded at nearest stream gage over 72-hr period	NOAA NCEI	No relationship for wood turtle Negative relationship for Blanding's: As wetlands dry, there is less suitable habitat available for Blanding's turtles and they are more likely to occupy pools that are available

	Variable (Abbreviation)	Unit	Data source	Prediction
Detection (p) covariates	Maximum air temperature (MaxTemp)	(°C) Maximum air temperature recorded on day of sampling at nearest stream gage	NOAA NCEI	Detectability expected to increase with higher activity at warmer temperatures consistent with eDNA for other turtle species (de Souza et al. 2016).
	Julian day (julian_date)	Calendar Day (1–365 or 366)		Seasons of higher activity or reproduction will correspond to higher eDNA detection probabilities

Table 3.3 S Sampling sites with number of field replicates with positive detections for each sampling period for Blanding's ("B") turtle and wood ("W") turtle. Also included is the recency of visual encounters with the species. "Contemporary" accounts refer to detections within the last five years. Sites are named by county to protect sensitive data related to turtle locations. Bolded sites indicate that we had at least one site replicate detection within any sampling period.

Site	May/June 23 # Detections		Sept. 23 #. Detections		Jan. 24 # Detections		Apr. 24 # Detections		Blanding's Recency	Wood Recency
	B	W	B	W	B	W	B	W		
Anoka #1	1	0	1	0	NA	NA	0	0	Contemporary	None
Anoka #2	NA	NA	0	0	NA	NA	NA	NA	Contemporary	None
Brown #1	NA	NA	NA	NA	0	0	0	0	Historic	None
Brown #2	NA	NA	NA	NA	0	0	1	0	Historic	None
Carlton #1	0	0	0	0	NA	NA	0	0	None	Historic
Carlton #2	0	0	1	0	NA	NA	0	0	None	Historic
Cass #1	0	0	NA	NA	NA	NA	NA	NA	Historic	None
Cass #2	0	0	NA	NA	NA	NA	NA	NA	Historic	None

Site	May/June 23 # Detections		Sept. 23 #. Detections		Jan. 24 # Detections		Apr. 24 # Detections		Blanding's Recency	Wood Recency
	B	W	B	W	B	W	B	W		
Chisago #1	0	0	NA	NA	NA	NA	1	0	Historic	None
Chisago #2	0	0	NA	NA	NA	NA	0	0	Historic	None
Dakota #1	1	0	1	0	0	0	0	0	Contemporary	Contemporary
Dakota #2	0	0	2	0	NA	NA	0	0	Historic	None
Dakota #3	0	0	1	0	NA	NA	1	0	Historic	None
Dakota #4	0	0	0	0	NA	NA	0	0	Historic	None
Dodge #1	0	0	0	0	NA	NA	NA	NA	None	Historic
Freeborn #1	NA	NA	0	0	NA	NA	NA	NA	Historic	None
Freeborn #2	NA	NA	NA	NA	NA	NA	0	0	Contemporary	None
Freeborn #3	NA	NA	NA	NA	NA	NA	0	0	Contemporary	None
Isanti #1	0	0	NA	NA	0	0	1	0	Historic	None
Isanti #2	0	0	NA	NA	0	0	0	0	Historic	None
Martin #1	NA	NA	NA	NA	0	0	NA	NA	Contemporary	None
Martin #2	NA	NA	NA	NA	0	0	NA	NA	Contemporary	None
Martin #3	NA	NA	1	0	0	0	1	0	Contemporary	None

Site	May/June 23 # Detections		Sept. 23 #. Detections		Jan. 24 # Detections		Apr. 24 # Detections		Blanding's Recency	Wood Recency
	B	W	B	W	B	W	B	W		
Martin #4	0	0	1	0	NA	NA	0	0	Contemporary	None
Morrison #1	0	0	NA	NA	NA	NA	NA	NA	Contemporary	None
Morrison #2	NA	NA	0	0	NA	NA	NA	NA	None	None
Mower #1	NA	NA	0	0	NA	NA	NA	NA	Historic	Historic
Mower #2	NA	NA	0	0	NA	NA	0	0	Historic	Historic
Olmstead #1	1	0	0	0	0	0	NA	NA	Historic	Historic
Olmstead #2	NA	NA	NA	NA	NA	NA	0	0	Historic	Historic
Olmstead #3	NA	NA	0	0	NA	NA	NA	NA	Contemporary	Contemporary
Olmstead #4	NA	NA	0	0	0	0	0	0	Contemporary	Contemporary
Olmstead #5	NA	NA	0	0	NA	NA	NA	NA	Contemporary	Contemporary
Olmstead #6	NA	NA	0	1	0	0	0	0	Contemporary	Contemporary
Pine #1	NA	NA	1	0	0	0	0	0	Historic	Historic
Pine #2	NA	NA	0	1	0	0	0	0	None	Historic
Pine #3	NA	NA	2	0	0	0	0	0	Historic	Historic
Pipestone #1	NA	NA	NA	NA	0	0	0	0	Historic	None

Site	May/June 23 # Detections		Sept. 23 #. Detections		Jan. 24 # Detections		Apr. 24 # Detections		Blanding's Recency	Wood Recency
	B	W	B	W	B	W	B	W		
Pipestone #2	NA	NA	0	0	NA	NA	NA	NA	Historic	None
Pipestone #3	0	0	NA	NA	NA	NA	0	0	Historic	None
Pipestone #4	0	0	NA	NA	0	0	0	0	Historic	None
Pipestone #5	0	0	NA	NA	NA	NA	NA	NA	Historic	None
Rice #1	0	0	2	0	0	0	0	0	Contemporary	Contemporary
Rice #2	NA	NA	NA	NA	0	0	NA	NA	Contemporary	Contemporary
Rice #3	NA	NA	0	0	NA	NA	NA	NA	None	None
Rice #4	0	0	2	0	0	0	0	0	Contemporary	Historic
Sherburne #1	0	0	1	0	NA	NA	0	0	Historic	None
Sherburne #2	0	0	NA	NA	NA	NA	NA	NA	Contemporary	None
Sherburne #3	NA	NA	2	0	0	0	0	0	Contemporary	None
Sherburne #4	NA	NA	2	0	0	0	1	0	Contemporary	None
St. Louis #1	0	0	0	1	0	1	0	0	None	Contemporary
St. Louis #2	0	3	0	3	0	0	0	2	None	Contemporary
St. Louis #3	0	0	0	1	0	0	0	0	None	Contemporary

Site	May/June 23 # Detections		Sept. 23 #. Detections		Jan. 24 # Detections		Apr. 24 # Detections		Blanding's Recency	Wood Recency
	B	W	B	W	B	W	B	W		
St. Louis #4	0	1	0	0	0	0	0	0	None	Contemporary
Steele #1	NA	NA	0	0	NA	NA	0	0	None	Historic
Steele #2	0	0	0	0	NA	NA	NA	NA	None	Contemporary
Steele #3	NA	NA	NA	NA	0	0	0	0	None	Historic
Steele #4	NA	NA	0	0	NA	NA	0	0	None	Contemporary
Wabasha #1	NA	NA	NA	NA	1	0	NA	NA	Contemporary	None
Wabasha #2	NA	NA	0	0	0	0	0	0	Historic	None
Wabasha #3	NA	NA	1	0	0	0	3	0	Contemporary	None
Wabasha #4	NA	NA	NA	NA	0	0	NA	NA	Historic	None
Wabasha #5	0	0	0	0	NA	NA	NA	NA	None	Historic
Wabasha #6	NA	NA	NA	NA	NA	NA	0	0	None	Historic
Wabasha #7	0	0	NA	NA	NA	NA	NA	NA	Historic	None
Wabasha #8	0	0	0	0	NA	NA	0	0	None	Contemporary
Washington #1	NA	NA	NA	NA	0	0	0	0	Contemporary	None
Washington #2	0	0	NA	NA	0	0	NA	NA	Historic	None

Site	May/June 23 # Detections		Sept. 23 #. Detections		Jan. 24 # Detections		Apr. 24 # Detections		Blanding's Recency	Wood Recency
	B	W	B	W	B	W	B	W		
Watonwan #1	NA	NA	0	0	NA	NA	NA	NA	Historic	None
Watonwan #2	NA	NA	0	0	NA	NA	NA	NA	Historic	None
Watonwan #3	NA	NA	0	0	NA	NA	NA	NA	Historic	None
Watonwan #4	NA	NA	0	0	NA	NA	NA	NA	Historic	None
Wright #1	0	0	0	0	0	0	0	0	Contemporary	None
Wright #2	0	0	2	1	NA	NA	0	0	Contemporary	None
Wright #3	1	0	0	0	NA	NA	0	0	Contemporary	None
Wright #4	0	0	0	0	NA	NA	0	0	Contemporary	None

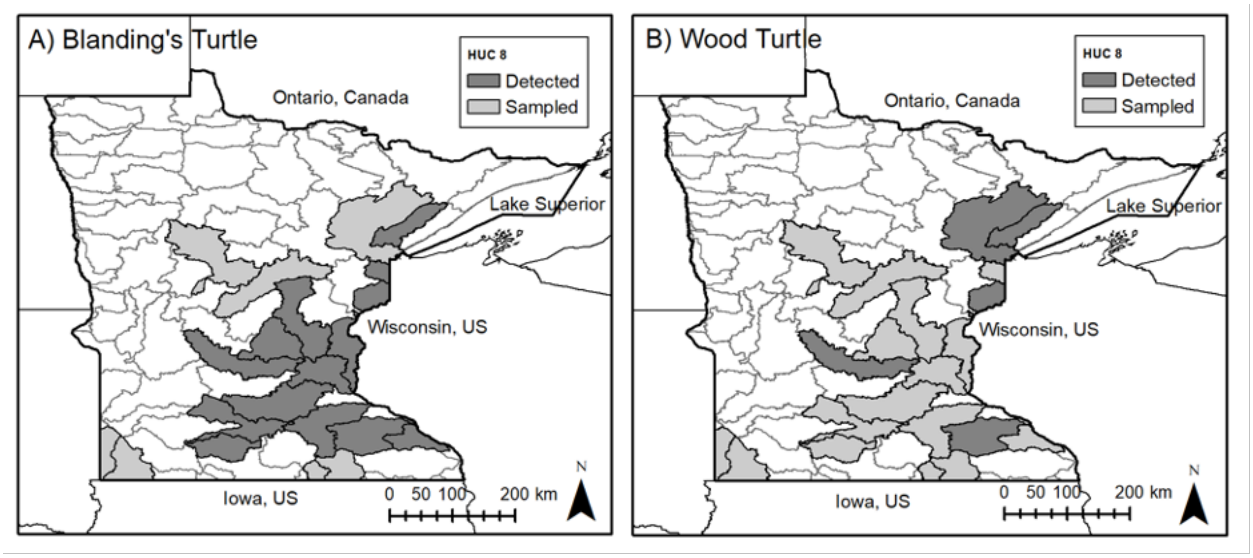

Figure 3.1 United States Geological Survey (USGS) Hydrologic Unit Code 8 (HUC 8) watersheds for the state of Minnesota where Blanding's turtle (A) and wood turtle (B) were sampled (light gray) or detected (dark gray) by environmental DNA (eDNA). Sampling location

Table 3.4 Candidate models for wood turtle eDNA occupancy and detection ranked by AIC.

Model name	Detection Parameters	Occupancy Parameters	k	AIC	ΔAIC	AICwt	R^2
Occupancy	p(.)	psi(SUMFORESTCAT + RDDENSCAT + prG_BMMI + StreamOrde*TotalPrecp)	8	86.83	0	0.41	0.4
Global	p(MaxTemp + julian_date)	psi(SUMFORESTCAT + RDDENSCAT + prG_BMMI + StreamOrde*TotalPrecp)	10	87.80	0.97	0.25	0.43
Wood turtle - specific	p(MaxTemp + julian_date)	psi(SUMFORESTCAT + StreamOrde*TotalPrecp + prG_BMMI)	9	88.48	1.65	0.18	0.41
Blanding's - specific	p(MaxTemp + julian_date)	psi(StreamOrde*TotalPrecp + RDDENSCAT)	8	88.81	1.98	0.15	0.38
Null	p(.)	psi(.)	2	111.39	24.56	0	0
Detection	p(MaxTemp + julian_date + TotalPrecp)	psi(.)	5	112.05	25.22	0	0.06

Table 3.5 Model averaged estimates and 95% confidence intervals (CI) for all parameters included in occupancy models for wood turtle eDNA.

Parameter	Estimate	95% CI	
		Lower	Upper
psi(Int)	-1.60	-8.83	5.62
psi(SUMFORESTCAT)	0.07	-1.48	0.19
psi(RDDENSCAT)	-1.73	-4.91	0.70
psi(prG_BMMI)	-9.37	-2.90	6.58
psi(StreamOrde)	0.33	-7.82	1.44
psi(TotalPrecp)	-4.85	-20.11	8.3
psi(StreamOrde:TotalPrecp)	5.96	-1.88	13.81
p(Int)	0.82	-3.03	4.67
p(MaxTemp)	-0.04	-1.71	0.01
p(julian_date)	0.01	-5.65	0.03

Table 3.5. Candidate models for Blanding's turtle eDNA occupancy and detection ranked by AIC.

Model name	**Detection Prarmeters**	Occupancy Parameters	**k**	**AIC**	**ΔAIC**	**AICwt**	**R^2**
Global	p(MaxTemp + julian_date)	psi(SUMFORESTCAT + RDDENSCAT + prG_BMMI + StreamOrde*TotalPrecp)	10	280.81	0	0.43	0.22
Wood turtle-specific	p(MaxTemp + julian_date)	psi(SUMFORESTCAT + StreamOrde*TotalPrecp + prG_BMMI)	9	281.10	0.29	0.37	0.21
Blanding's-specific	p(MaxTemp + julian_date)	psi(StreamOrde*TotalPrecp + RDDENSCAT)	8	282.27	1.47	0.20	0.19
Occupancy	p(.)	psi(SUMFORESTCAT + RDDENSCAT + prG_BMMI + StreamOrde*TotalPrecp)	8	294.45	13.64	0	0.12
Detection	p(MaxTemp + julian_date + TotalPrecp)	psi(.)	5	294.79	13.98	0	0.09
Null	p(.)	psi(.)	2	302.18	21.37	0	0

Table 3.6 Model averaged estimates and 95% confidence intervals (CI) for all parameters included in occupancy models for Blanding's turtle eDNA.

Parameter	Estimate	95% CI	
		Lower	Upper
psi(Int)	2.39	-2.05	6.83
psi(SUMFORESTCAT)	-0.03	-0.07	0.01
psi(RDDENSCAT)	0.22	-0.20	0.91
psi(prG_BMMI)	-0.91	-7.17	4.81
psi(StreamOrde)	-0.77	-1.41	-0.12
psi(TotalPrecp)	-0.92	-3.34	1.50
psi(StreamOrde:TotalPrecp)	0.45	-0.81	1.70
p(Int)	-2.34	-4.33	-0.34
p(MaxTemp)	-0.02	-0.05	0.01
p(julian_date)	0.01	0.01	0.02

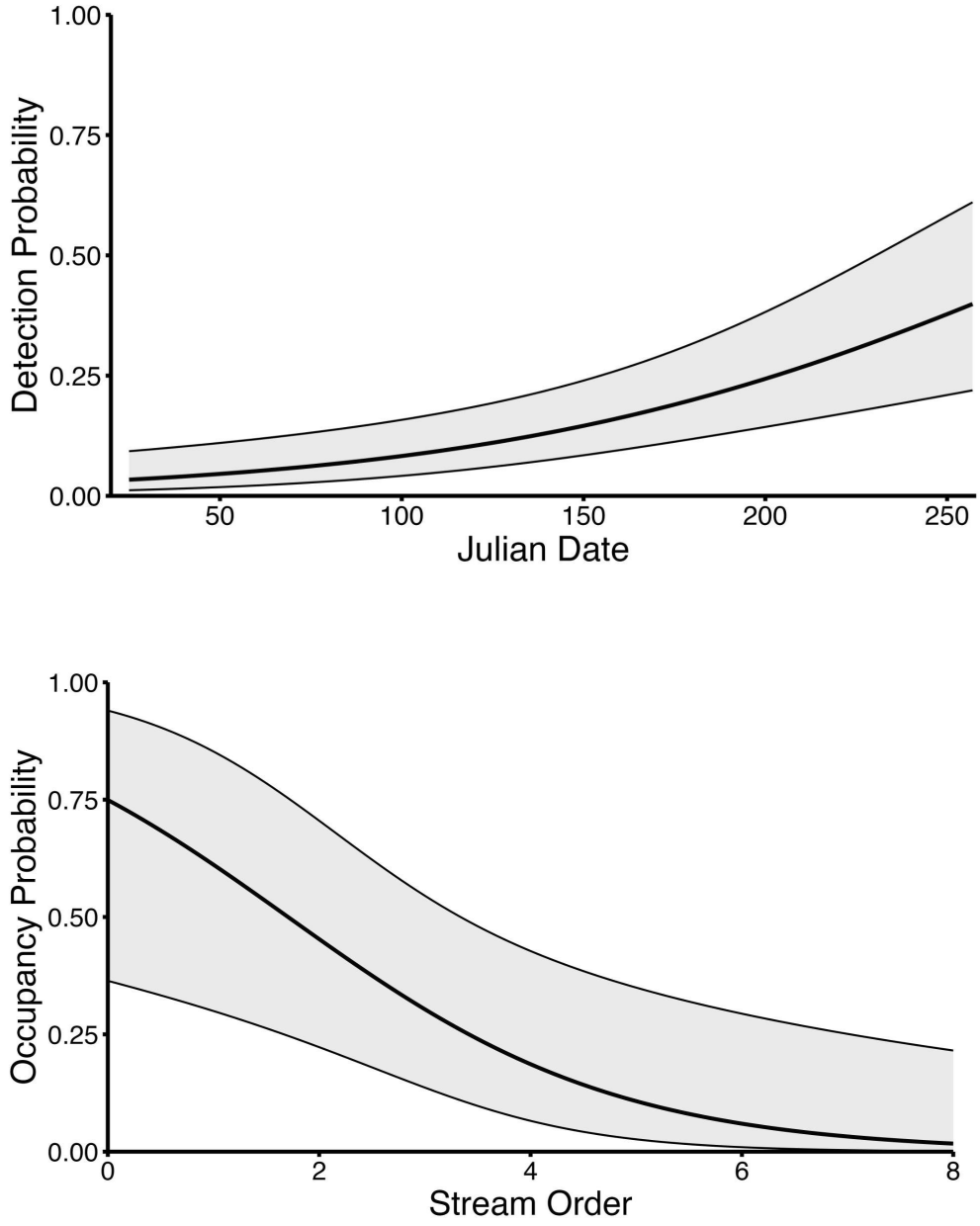

Figure 3.2 Predicted relationships from hierarchical modeling, with 95% confidence intervals, from the most supported Blanding's turtle model (Table 3.5). Julian date positively correlated with Blanding's detection probability (top) and stream order negatively correlated with Blanding's occupancy probability (bottom)

Chapter 5: Cost of eDNA Monitoring of Blanding's and Wood Turtles in Minnesota

5.1 Introduction

Environmental (e)DNA analysis has long promised a more sensitive, efficient, and cost- sensitive approach to biodiversity monitoring when compared to conventional approaches (Ficetola et al., 2008; Jerde et al., 2011; Thomsen et al., 2012, Tsuji et al., 2019). And while sensitivity and efficiency have consistently been assessed since the inception of eDNA analysis, cost assessments have been slower to follow. Those studies that have emerged have recovered somewhat equivocal results (Sigsgaard et al., 2015; Smart et al., 2015; Evans et al., 2017). These robust cost assessments are even more rare for freshwater turtles, with but two studies published to date (Akre et al., 2019; Sternhagen et al., 2024). Nevertheless, robust cost assessment is necessary for decision-making on if and when to adopt an eDNA monitoring program.

Here we conduct a cost assessment for two imperiled freshwater turtles, wood (*Glyptemys insculpta*) and Blanding's turtles (*Emydoidea blandingii*). Specifically, we sought to quantify the all-inclusive cost (in United States Dollars) of collecting, processing, and analyzing eDNA samples for both species, and then calculating the cost necessary to achieve 95% confidence in detection probability for a given hypothetical site occupied by the focal species. Using the framework we developed for alligator snapping turtle (*Macrolemmys temnickii*; Sternhagen et al., 2024). We also developed a Microsoft Excel-based "widget" that allows users choose a calendar or Julian day of the year and a number of field replicates for up to three sample events in a year, calculating a cumulative detection probability for each, that is summed to a multi-event detection probability.

5.2 Materials & Methods

To conduct a cost assessment, we recorded the time to collect three water samples plus a negative control (field blank), as well as time spent completing sample processing and analysis, including water filtration, eDNA extraction, and quantitative (q)PCR. Consumable supply costs were calculated for each sampling method based on published vendor supply prices via internet searches conducted in September of (2024) We did not include equipment costs (e.g. qPCR machines, digital dry baths, etc.) as they represent a single, up-front cost, and do not contribute to the ongoing costs of sampling and sample processing. Finally, we included travel time based on average daily distance traveled. Using this all-inclusive per-sample cost estimate, and leveraging occupancy modeling results, estimated the sampling density necessary for 95% confidence in detection if the species is present in each season, as well as the cost per detection, and, finally, the cost required for sampling at the 95% detection threshold for each season.

5.3 Results

Overall, the total per-sample cost, inclusive of field sampling, sample processing, and lab analysis was $79.38. For Blanding's turtles' cost per detection varied, with the highest cost per detection occurring in Winter ($2,063.88), and the lowest occurring in Fall ($192.78), due to seasonal variation in detection probability (Table 4.1). In this case, Blanding's turtle would require 91 samples per site in the winter, for a total cost of $7,223.58. In fall, 95% confidence in detection would be far less, at $476.28 per site. We also see the cost per detection decline over the course of the year, from $2063.88 in spring, to $192.78 in the fall, due to increasing detection probabilities over the course of the year.

Wood turtles exhibited a similar trend, wherein cost per detection varied, and the highest cost per detection occurring in Winter ($1,270), and the lowest occurring in Summer ($343.98), due to seasonal variation in detection probability (Table 4.2). Wood turtles would require 69 samples per site in the winter, for a total cost of $5,477.22. In summer, 95% confidence in detection would be far less, requiring 6 samples per site to achieve 95% confidence in detection at cost of $343.98 per site. We also see the cost per detection decline over the course of the year, from $5,477.22 in winter, to $381.24 in the fall.

5.4 Discussion

We find that the cost per detection and cost for implementing sampling density necessary to achieve 95% confident in detection decreased from winter to fall, with summer and fall sampling constituting the most cost-effective

Cost assessments in eDNA are rare in general, and particularly so for freshwater turtle species (Sternhagen et al., 2024). However, there has been some instructive work in both Blanding's and wood turtles. Davy et al. (2015) compared the cost of visual encounter surveys (VES), hoop nets, and eDNA, finding vast discrepancies in effort and cost. Particularly, VES and hoop nets both required high variability in both time (10-669 hours) and cost ($112.56-$7529.42 USD). In contrast, eDNA, from sampling to results, was estimated to take 12 hours, and cost $500 USD for a single species detection via targeted eDNA. In the interceding decade since this analysis, our results suggest that eDNA approaches for Blanding's turtles have improved and/or costs have declined, as our lowest cost per detection estimate (in fall) is $192.87). The results of both Davy et al. (2015) and our findings suggest that eDNA surveys are a cost-effective alternative.

For wood turtles, Akre et al. (2019) compared sampling costs and VES. Once again, the per-sample cost of eDNA ($42.60 USD) compared to VES ($275.30 USD) was much lower. Their per-sample cost of $42.60 is lower than our $79.38, however adjustment for inflation would raise the 2019 estimate to $53.29, and we note that mileage costs were not included in that estimate, likely more closely aligning the two estimates. However, our study does reveal that wood turtle eDNA monitoring may require further investigation. Detections are heavily weighted to sites in northeastern Minnesota. These sites consistently yielded detections across all seasons, whereas the few detections from southeastern Minnesota only occurred in fall sampling. As such, further investigation in southeastern Minnesota may be necessary to generate these estimates for this region, as turtle density is known to be much lower.

Overall, these results of this cost analysis suggest that eDNA monitoring for these two species may be a sensitive, efficient, and cost-effective means of sampling for these rare turtle species. Moreover, the developed "widget" can be used to fine tune sampling to maximize likelihood of detection and minimize costs. However, there remain a number of considerations to be mindful of. First, a comprehensive comparison between eDNA surveys with conventional sampling methods like visual encounter surveys (Davy et al., 2015; Akre et al., 2019) or hoop trapping (Davy et al., 2015; Sternhagen et al., 2024) was beyond the scope of this project. And so, a robust cost comparison with conventional means is lacking. Nevertheless, we suspect that eDNA would be competitive, if not superior on a cost-per-detection basis, using optimized sampling designs.

Second, we urge caution in guiding sampling design on the seasonal decrease in effort for 95% confidence in detection and concomitant decreases in cost. We speculate that the seasonal effect is driven by numerous factors, both biotic and abiotic, that are driving detection probabilities. For example, species' phenology likely plays a large role. For both Blanding's and wood turtles, they are largely dormant during the winter season, with depressed metabolism that may reduce eDNA deposition. Alternatively, when sampling in the fall of 2023 was conducted, Minnesota was under severe extreme drought conditions.

These conditions had reduced the amount of the water on the landscape, likely concentrating the turtles and, concomitantly, their eDNA. Moreover, wood turtles were actively mating during the sampling period (Larson et al., 2025), which has been shown to increase eDNA-based detection probabilities for turtles (de Souza et al., 2016). And so, considering things like species phenology, temperature (both water and air), recent precipitation, and other factors in deciding when to sample is vital, as these and other abiotic and biotic factors shape the likelihood of detection, with downstream impacts on overall cost. Finally, the results for wood turtle were, in large part, driven by consistently high detection rates at the northeastern extent of the range. These sites are in the highest population density portions of the range, and consistently recovered strong detection signals, both in terms of the number of replicates that were positive, and eDNA copy number, which can be indicative of population density or upstream biomass (Kessler et al., 2020). As densities are known to be much lower in the southeastern extent of the wood turtle range, a more robust sampling design and focused sampling effort may be necessary to better estimate the amount of effort and cost required to achieve 95% confidence in detecting the species where population sizes are increasingly low.

Table 4.1 Blanding's turtle sampling effort required for per-site 95% detection probability, cost per detection, and the cost for 95% detection were calculated. As the year progresses, sampling density necessary to achieve 95% confidence in detection at any given site.

Season	Sample Density (95% CI)	Number of Sites	Detections	Per Sample Cost (USD)	Cost per detection (USD)	Cost for 95%CI (USD)
Winter	91	26	1	$79.38	$2063.88	$7223.58
Spring	36	36	7	$79.38	$408.24	$2857.68
Summer	12	26	4	$79.38	$515.97	$952.56
Fall	6	34	14	$79.38	$192.78	$476.28

Table 4.2 Wood turtle sampling effort required for per-site 95% detection probability, cost per detection, and the cost for 95% detection was calculated. As the year progresses, sampling density necessary to achieve 95% confidence in detection at any given site. For both species, the per-site effort required for 95% confidence in detection decreased over the year, with lowest sample density for both species occurring in the fall. Concomitant with these decreases, the cost per detection decreases, as does per-site cost achieve 95% confidence in detection.

Season	Sample Density	Number of Sites	Detections	Per Sample Cost (USD)	Cost per detection (USD)	Cost for 95%CI (USD)
Winter	69	16	1	$79.38	$1270.08	$5477.22
Spring	23	19	1	$79.38	$1508.22	$1825.74
Summer	6	13	3	$79.38	$343.98	$476.28
Fall	4	24	5	$79.38	$381.024	$317.52

Chapter 6: Research Benefits & Implementation Strategy

6.1 Research Benefits

This research has yielded a comprehensive evaluation of eDNA technologies and methodologies as a new tool for the early detection of endangered turtles on proposed transportation projects. In the first phase, we critically evaluated existing targeted assays for eDNA detection of Blanding' and wood turtles. We found that these assays were likely to be unsuccessful in Minnesota, and so we designed, optimized and validated these assays to rigorous standards. The outcome of these efforts are highly sensitive, species-specific assays for both species that have achieved the "Operational Validation Level," meaning that non-detections can be interpreted as species' absence, while detections can be interpreted as the species' is likely to be present.

Next, we established a spatio-temporally robust experimental design that sampled extensively throughout the ranges of both species, and in all four seasons of the year. Moreover, we established a series of field and lab best practices for sampling collection and processing that has now been demonstrated to perform for both target species in Minnesota waters.

We conducted robust occupancy modeling for both species and find that our ability to detect Blanding's eDNA is significantly correlated with time of year. Detection rates were lowest in January during the turtles' overwintering period, then increased through spring and early summer as turtle activity rose, peaking in late summer and early fall with the emergence of hatchlings and adult turtles seeking refuge from drought conditions. Additionally, we found that Blanding's turtles were more likely to occupy smaller, slower moving waterways.

Additionally, our results were successful in detecting both target species in all seasons of the year, and in a variety of lentic and lotic systems throughout their respective Minnesota ranges. In the process, we reveal that strategically considering biotic and abiotic conditions relevant to the timing of sampling events can profoundly impact the likelihood of detection. Specifically, while both species were detected in spring, summer, fall, and winter, highest detections coincided with low water conditions and/or critical points in species' phenology rendered both species more likely to be detected with less effort later in the year. We designed a Microsoft Excel-based "widget" that end users can use to develop sampling schemes to maximize their likelihood of detection during multiple points in the year.

We developed a cost-assessment that when compared with other studies strongly suggest that eDNA is a cost-effective complement or alternative to conventional sampling. Moreover, this cost-assessment can be leveraged to determine the eDNA sampling density necessary to achieve 95% confidence in detecting target species if truly present. This information can be merged with the "widget" to inform decision-making on level of effort and cost to achieve desired outcomes with respect to detecting Blanding's and wood turtles in Minnesota.

6.2 Implementation Steps

Given the above, we recommend the following implementation steps:

- The development of a resource document for interested parties that may be interested conducting eDNA evaluations for Blanding's and wood turtles in Minnesota that includes
    - Detailed, step-by-step sampling instructions
    - Considerations for sampling design and density to achieve expected outcomes
    - How to interpret detections vs. non-detections
    - Communications plan for project partners to explain what detections/non-detections mean
- Given sparse detections of wood turtles in the southeastern extent of the range, a follow-up study that conducts a comprehensive eDNA assessment that:
    - Samples several sites with known densities and, ideally, radio-telemetered individuals, across multiple biologically relevant sampling periods
    - Is composed of a stratified sampling design that encompasses the entirety of the site, rather than focusing sampling at road crossings
    - Conduct a quantitative analysis on how eDNA detections relate to population density and/or upstream biomass
- Given consistent performance of eDNA detections for Blanding's turtles across contexts, we recommend the development of a phased (based on MN DoT taskings and MNDNR priorities) state-wide eDNA assessment
- For any subsequent sampling efforts and potential Phase II activities, focused sampling effort (vs simply proximity to roads) is necessary to increase detection for both species, especially where populations are low &/or when they don't concentrate near roads

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