

Environmental DNA barcoding as a method of amphibian species detection compared to
conventional monitoring techniques in southern Ontario vernal pools

by

Cailyn Zamora

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Biology

Waterloo, Ontario, Canada, 2024

© Cailyn Zamora 2024

Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The content of this thesis was written and analyzed by the sole author, Cailyn M. Zamora, and submitted towards the completion of a Master's of Science from the University of Waterloo. The research presented in this thesis was supervised by Dr. Barb Katzenback who contributed to the project design, research input, intellectual contributions, review of thesis materials, and acquisition of funding for the project. Cailyn Zamora was the sole author of Chapters 1 and 3 containing an introduction of the topics in this thesis (Chapter 1) and a general conclusion (Chapter 3) which were not written with the intention of publication.

Chapter 2 was written with the intention of future publication and authorship is listed below.

Research presented in Chapter 2

Cailyn M. Zamora¹, Nathanael B. J. Harper¹, Maxwell P. Bui-Marinos¹, Paul M. Craig¹, Mark R. Servos¹, Andrew C. Doxey¹, John P. Giesy², Barbara A. Katzenback¹

¹ Dept. of Biology, University of Waterloo, Waterloo, ON, Canada, ² Dept. of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, SK, Canada

Nathanael B. J. Harper conducted field methods outlined in Sections 2.2.1, 2.2.2, and 2.2.3. These methods consisted of the fieldwork conducted in 2019 to collect conventional data, eDNA water samples, eDNA filtration, and eDNA extraction. Field methods were conducted in collaboration with *rare* Charitable research reserve. Additionally, field methods were conducted by Nathanael Harper alongside multiple field technicians including Jenna Quinn, Maxwell P. Bui-Marinos, Patrick Breadner, and Alexander J. Douglas. Nathanael B. J. Harper and Michael Lynch developed the DR-1 quality control assay used in Section 2.2.4.4. DNA extraction and initial eDNA quality control metrics (Section 2.2.4.4) were performed by Nathanael B. J. Harper and Maxwell P. Bui-Marinos, with quality control metrics rerun by Cailyn M. Zamora. Dr. Mark R. Servos and Leslie Bragg provided support including procurement of research supplies and contributed use of laboratory space and equipment for molecular biology methods. Dr. Mark R. Servos and Dr. Paul M. Craig provided intellectual

input as members of my Advisory Committee. Cailyn M. Zamora collected and analyzed the data and wrote the chapter.

Funding

This project was funded through the Canada First Research Excellence Fund – Global Waters Futures Grant acquired by Dr. John P Giesy, Dr. Mark R. Servos, Dr. Paul M. Craig, Dr. Barb Katzenback, and Dr. Andrew C. Doxey.

Cailyn M. Zamora was supported by the Natural Sciences and Engineering Research Council of Canada's Canadian Graduate Scholarship – Master's (NSERC CGS-M). Additional funding from the University of Waterloo included the University of Waterloo's President's Graduate Scholarship, the Science Graduate Award, the UW Graduate Scholarship, and UW Graduate Teaching Assistantships.

Abstract

Ongoing monitoring is vital for the conservation of amphibian species and is conducted through conventional auditory and visual surveys. A molecular method, termed environmental DNA (eDNA) barcoding, may offer a more sensitive method of species detection that negates the need for direct species observation. The research aims of this thesis were to conduct a comparative analysis of eDNA barcoding versus conventional (audio/visual) species detection methods for six amphibian species in southern Ontario. I hypothesized that eDNA barcoding would offer equal or greater species presence detections compared to the conventional methods. Conventional surveys and eDNA collections were conducted in three vernal pools from April-July 2019 in collaboration with *rare* Charitable Research Reserve (Cambridge, ON). Conventional methods included collection of daily audio files from acoustic song meters and weekly/biweekly visual encounter surveys. Audio data was analyzed using Kaleidoscope Pro. Alongside conventional surveys, duplicate water samples containing eDNA were collected at multiple sampling locations around three vernal pools. After water collection, eDNA was concentrated by filtration, extracted, and quality controlled. eDNA samples were processed using optimized eDNA barcoding assays using quantitative PCR. Comparative analysis between conventional methods and eDNA barcoding contradicts a one-size-fits-all model of amphibian monitoring. eDNA barcoding offered a reliable and effective method of species detection for five of the target amphibians especially for obligate vernal pool breeding species, however this method failed to accurately detect the spring peeper despite detections by passive acoustic surveys. I propose using eDNA barcoding alongside a conventional method of species detection to optimize detections across a spatiotemporal scale, however, this should be catered to the target species of interest. Future studies could implement a multi-year study as well as a comparison of eDNA barcoding to metabarcoding for Ontario amphibian species. eDNA barcoding offers a new method of species detection that could aid in ongoing amphibian monitoring and therefore conservation efforts of the declining taxa.

Acknowledgements

I am extremely grateful to my supervisor Dr. Barb Katzenback for the invaluable help throughout my journey as a graduate student. Thank you for the feedback throughout this process and for helping foster my success. I also want to thank the members of my advisory committee, Dr. Mark R. Servos, and Dr. Paul M. Craig, for their input and guidance throughout my research. I would not have been able to learn all that I have without wonderful scientists sharing their knowledge and experiences.

I would also like to extend my sincerest thanks to all the members of the Katzenback lab for making me feel welcome and providing a supportive environment. I want to extend a special thank you to Nathanael Harper for starting this project and teaching me so much throughout the last 2 years, I couldn't have done this without your help. I am so grateful to Drew Thompson for being my moral support, for the late-night work sessions, and for being my eternal coffee buddy. Our caffeine-induced rant sessions and the shared ups and downs throughout getting our degrees have honestly helped pull me through.

There were so many people in my personal life that without their support, I would not be here today. I have been so lucky to have the constant support of my amazing friends Jem and Nicole who have been with me since the beginning of my biology nerd journey. Thank you both for helping me keep my love of biology alive and understanding my insane obsessions. Love you both forever!

Indigo, I honestly don't know where to begin thanking you. The support you provided throughout my journey is indescribable. Your generosity and patience as I navigated this journey did not go unnoticed. I will be forever grateful for your ability to make me laugh so hard even when times were tough. You are amazing and I love you so much!

To my fantastic sisters, who have never wavered in their support and love for me. You both remember that little girl in the mud waiting for the frogs and instead of thinking I was the crazy swamp kid, you jumped on in with me. You both have made sure I never felt

alone through all the adventures and chances I have taken. Forever grateful to have been born into a family where I have two best friends for life.

Finally, I must thank my parents. There are truly no words to capture how grateful I am to you both. I am so proud to have the support of such strong and amazing people who nurtured my love of misunderstood creatures and encouraged me to follow my dreams, no matter where they swept me off to. “I love you to the moon and back,” said little nut brown hare.

Dedication

To Joan Zamora, a matriarch, a teacher, and a woman with enough grace to inspire all who knew her. Love you, and all shall be well.

Table of Contents

<i>Author's Declaration</i>	<i>ii</i>
<i>Statement of Contributions.....</i>	<i>iii</i>
<i>Abstract.....</i>	<i>v</i>
<i>Acknowledgements.....</i>	<i>vi</i>
<i>Dedication.....</i>	<i>viii</i>
<i>List of Figures</i>	<i>xiii</i>
<i>List of Tables</i>	<i>xiv</i>
<i>List of Abbreviations</i>	<i>xvi</i>
1 General Introduction.....	1
1.1 Global amphibian decline.....	1
1.2 Conventional monitoring techniques for amphibians.....	5
1.2.1 Trapping.....	5
1.2.2 Acoustic surveys	6
1.2.3 Visual encounter surveys	7
1.2.4 The current state of conventional amphibian monitoring.....	7
1.3 Environmental DNA	8
1.3.1 Introduction to environmental DNA	8
1.3.2 Species detection using eDNA.....	11
1.3.2.1 Metabarcoding.....	12
1.3.2.2 eDNA barcoding	12
1.3.3 eDNA processing standards for eDNA barcoding.....	14
1.3.4 Sampling design.....	16
1.3.5 Current use of eDNA barcoding for amphibian species detection	17
1.4 Target species.....	19
1.5 Objectives and Hypothesis.....	22

2	<i>Detection of amphibians using environmental DNA barcoding compared to conventional methodologies in southern Ontario vernal pools</i>	24
2.1	Introduction	24
2.2	Methods	29
2.2.1	Field Methods	29
2.2.1.1	Site Selection	29
2.2.2	Conventional amphibian monitoring	31
2.2.2.1	Acoustic surveys	31
2.2.2.2	Visual Surveys	34
2.2.3	eDNA sampling	34
2.2.4	eDNA sample filtration, extraction, and quality control	37
2.2.4.1	Decontamination for eDNA molecular processes	37
2.2.4.2	eDNA sample filtration	37
2.2.4.3	eDNA extraction	38
2.2.4.4	eDNA quality control	39
2.2.5	eDNA barcoding assay optimization	40
2.2.5.1	Generation of target sequence amplicons for use as target species qPCR standards	40
2.2.5.1.1	Tissue procurement	40
2.2.5.1.2	Genomic DNA extraction	41
2.2.5.1.3	DNA amplification via PCR	42
2.2.5.1.4	Gel electrophoresis	42
2.2.5.1.5	DNA extraction from agarose gel	43
2.2.5.1.6	DNA sequencing and nucleic acid quantification	43
2.2.5.2	Developing synthetic oligonucleotide standards and synthetic insert	44
2.2.6	eDNA barcoding assay optimization	45
2.2.6.1	Primers and probe test	45
2.2.6.2	Primer concentration optimization	49
2.2.6.3	Probe concentration optimization	49
2.2.6.4	Optimization of annealing temperature	50
2.2.6.6	Multiplexing	52
2.2.6.7	Sensitivity of eDNA barcoding assays	53
2.2.7	eDNA sample processing	53
2.2.7.1	eDNA integrity	53

2.2.7.2	eDNA barcoding assay application.....	54
2.2.8	Analysis.....	55
2.2.8.1	Audio data analysis using Kaleidoscope Pro analysis software.....	55
2.2.8.2	Training Kaleidoscope Pro and advanced classifiers.....	55
2.2.8.3	eDNA statistical analysis	57
2.3	Results	59
2.3.1	Conventional Data	59
2.3.1.1	Acoustic surveys	59
2.3.1.1.1	Active acoustic monitoring – Marsh monitoring protocol	59
2.3.1.1.2	Passive acoustic surveys – Mounted song meters	62
2.3.1.2	Visual encounter surveys	64
2.3.2	qPCR.....	66
2.3.2.1	Assay optimization.....	66
2.3.2.1.1	Optimal assay conditions for eDNA barcoding assays	66
2.3.2.1.2	Limit of detection and quantification	68
2.3.2.2	Detection of eDNA shed from target amphibians in vernal pools	70
2.3.2.2.1	Quality control of eDNA samples	70
2.3.2.2.2	Amphibian eDNA detections	72
2.3.2.2.3	Ancient Woods Swamp eDNA detections	74
2.3.2.2.4	Ancient Woods Pond eDNA detections	74
2.3.2.2.5	Preston Flats Pond eDNA detections	75
2.3.3	Amphibian eDNA concentrations detected	83
2.3.4	Species detection method comparison	86
2.4	Discussion.....	94
2.4.1	Method comparison	94
2.4.2	Spatial and temporal sampling.....	95
2.4.3	False positives and negatives	99
2.4.3.1.1	Conventional methods.....	99
2.4.3.1.2	eDNA barcoding.....	100
2.4.4	Data analysis methods.....	102
2.4.5	Conclusion	103
3	General Conclusions	104

3.1	Objectives and overall findings	104
3.2	Research considerations	104
3.3	Future Directions.....	109
	<i>References</i>	<i>111</i>
	<i>Appendix A Supplementary Data</i>	<i>127</i>

List of Figures

Figure 2.1. Map of vernal pools surveyed at <i>rare</i> Charitable Research Reserve.	30
Figure 2.2. Sampling locations selected at three vernal pools to survey for amphibians using conventional and eDNA surveys.	33
Figure 2.3. Temporal anuran amphibian call detections with passive acoustic monitoring using song meters.	63
Figure 2.4. Amphibian species detections across three vernal pools using visual encounter surveys.	65
Figure 2.5. eDNA integrity assessed with ePlant5 assay.	71
Figure 2.6. Spatial and temporal detection of six target amphibian species using species-specific environmental DNA barcoding assays.	76
Figure 2.7. eDNA copy number across a temporal scale for five target amphibian species at each vernal pool.	84
Figure 2.8 Comparison of mean cumulative species richness for eDNA barcoding and conventional methods of species detection.	87
Figure 2.9. Comparison of detection methods for target amphibians using eDNA and conventional monitoring.	89

List of Tables

Table 1.1. Target species' physical descriptors and information on life history through breeding, post-breeding, and overwintering.	21
Table 2.1 Dates of conventional and eDNA surveys conducted for all three vernal pools, Ancient Woods Swamp (AWS), Ancient Woods Pond (AWP), and Preston Flats Ponds.	36
Table 2.2. Summary of eDNA barcoding qPCR primer and probe sequences used in this study.	47
Table 2.3. Conditions tested for eDNA barcoding assay optimization.	51
Table 2.4 Amphibian call detections at three vernal pools with active acoustic surveys using the Marsh Monitoring Protocol.	61
Table 2.5. Optimized assay conditions for eDNA barcoding assays for six target amphibian species.	67
Table 2.6. Limit of detection (LOD) and quantification (LOQ) for amphibian eDNA barcoding assays.	69
Table 2.7. Confidence matrix of species detection based on eDNA barcoding amplification.	73
Table 2.8. Binomial logistic regression models for the effect of date and sampling location on eDNA amplification for six target amphibians at three vernal pools.	79
Table 2.9. Comparison of eDNA concentration from amplified eDNA samples from duplicate biological field replicate water samples.	82
Table 2.10 Binomial generalized mixed effects regression models determining the effect of survey methods on species detection with mixed effects.	93
Supplementary Table 1. Synthetic oligonucleotide standard sequences for target amphibians.	127

Supplementary Table 2. Summary of water quality metrics from three vernal pools.	128
Supplementary Table 3. Standard curves from singleplex and multiplex of species-specific assays (FAM) and synthetic insert assay (VIC).....	129

List of Abbreviations

AMTO – American toad

AWS – Ancient Woods Swamp

AWP – Ancient Woods Pond

Bd – *Batrachochytrium dendrobatidis*

BF – Blair Flats Wetland

Bsal – *Batrachochytrium salamandrivorans*

BSC – Biosafety cabinet

COI – cytochrome oxidase c subunit 1

Cytb – cytochrome b

DNA – deoxyribonucleic acid

eDNA – environmental DNA

FV3 – Frog virus 3

GPS – Global Positioning System

GRFR– Green frog

mtDNA – mitochondria DNA

NLFR – Northern leopard frog

NTC – No template control

PCR – polymerase chain reaction

PF – Preston Flats Pond

qPCR – quantitative polymerase chain reaction

RNA – ribonucleic acid

rRNA – ribosomal ribonucleic acid

SPPE – Spring peeper

SPSA – Spotted salamander

SYNTH – synthetic probe

WOFR – Wood frog

1 General Introduction

1.1 Global amphibian decline

The amphibian taxa is facing a global decline in populations at a faster rate than any other taxa (Collins and Storfer 2003; Hussain and Pandit 2012). In a 2023 study that explored the defaunation of the Anthropocene, results indicated that not only do amphibians represent the highest proportion of decreasing populations compared to mammals, birds, reptiles, and invertebrates, but additionally, they had the fewest stable or actively increasing populations (reviewed in Finn et al. 2023). This taxa contains 8,615 described amphibian species; 8,011 species being assessed for global status and trends in extinction risk (IUCN 2023). As of 2023, approximately 41% of assessed amphibian species are considered threatened, which is more than any other vertebrate taxa (IUCN 2023). Currently 2,364 amphibian species are currently considered threatened, however, there are an additional 1,145 species that are “data-deficient” indicating not enough information to assess the level of extinction threat they face. The number of data-deficient species indicates the need for effective and ongoing monitoring of amphibians to better understand all species in this at-risk taxa. This data deficient category may include species that, with proper assessment, would also be classified as threatened and therefore the current number of species likely underestimates the true level of the threat facing the amphibian taxa. (Howard and Bickford 2014). Species assessments and the lack of effective monitoring, especially for data-deficient species, indicate the need to better understand the rapid species decline and the contributing threats they face.

The declines exhibited by the amphibian taxa are related to the threats faced by the species throughout their life history. Common threats that amphibians are facing include overexploitation, introduced species, pollutants, disease, habitat reduction, and global climate change (Baille et al. 2004; Stuart et al. 2005; Oshiro 2021). Overexploitation is the purposeful removal of amphibians from their natural habitat for use in the pet trade, medicine, and the food industry (reviewed in Luedtke et al. 2023). This overexploitation is an added stressor leading to amphibian declines and resulted directly in the deterioration of at

least 35 amphibian species' status since 1980 (reviewed in Luedtke et al. 2023). Introduced species can have a direct effect on native amphibian populations through predation and indirectly through an increased level of competition for resources and space (Falaschi et al. 2020). A review paper in 2023 concluded that invasive species have also acted as a vector for introduced pathogens and in a majority of the cases, results in invasion-facilitated disease amplification (reviewed in Atkinson and Savage 2023). Pollutants pose a threat via chemical pollutants but also physical contaminants such as microplastics. Chemical contaminants, such as deicing solutions, nitrogenous compounds, heavy metals, and wastewater, are known to decrease amphibian survival, and body mass, and increase the rate of abnormalities in offspring (Egea-Serrano et al. 2012). Physical contaminants, such as microplastics, pose a threat and some studies suggest they cause a decrease in survival and impairment of development in tadpoles (Calderon et al. 2019; Boyero et al. 2020). Another threat contributing to amphibian declines are pathogens and diseases (Venter et al. 2006). An example of pathogens that many amphibian species are susceptible to include frog virus 3 (FV3), *Batrachochytrium dendrobatidis* (*Bd*), and *Batrachochytrium salamandrivorans* (*Bsal*) (Standish et al. 2018; Fisher and Garner 2020; Spitzen - van der Sluijs et al. 2020; Vilaça et al. 2020). These pathogens cause an increased rate of mortality in tadpoles compared to adults; however, the pathogens can have both lethal and sublethal effects for all life stages. Habitat reduction is a major threat experienced by amphibians and ranges from total habitat destruction to fragmentation of vital habitat. Habitat destruction causes a total loss of the required habitat whereas habitat fragmentation is the selective destruction of habitat creating isolated regions still capable of maintaining a small population of species. In some cases, the habitat is altered to a point where the habitat for all life history strategies or life stages is no longer accessible. Habitat fragmentation causes any remaining populations not eliminated to be forcibly isolated from the surrounding populations, subsequently disrupting gene flow and reducing genetic diversity (Cushman 2006). Habitat fragmentation disrupts natural migration routes causing amphibians to traverse more dangerous terrain such as roadways leading to increased road mortality (Cushman 2006; Beebee 2013). Additionally, global climate change poses a vast and complex threat to amphibian

populations. As the global climate changes, amphibian survival, growth, reproduction, and dispersion could all be affected (Blaustein et al. 2010). Not only could global climate change affect individual amphibian populations, but there could be increased effects on habitat, pathogen-host interactions, predator-prey relationships, and overall level of resources. The effects of these threats differ based on species and geography, but together, these have led to devastating global amphibian population declines.

Due to the biphasic life history strategy shared by many amphibian species, they face threats on two fronts and depend on both aquatic and terrestrial habitats throughout their development and seasonality. This means they are susceptible to threats facing the aquatic breeding ground, hibernation sites, terrestrial foraging areas, and throughout seasonal migrations or distributions (reviewed in Nolan et al. 2023). The requirement for multiple habitat types causes a potentially increased effect of the threats facing both habitat types (reviewed in Nolan et al. 2023). According to a 2022 review paper, approximately 65% of anuran species and 36.3% of salamanders require aquatic habitat for egg-laying or larval development (reviewed in Liedtke et al. 2022). The aquatic larval stages may be more highly susceptible to pathogens compared to adult amphibians (reviewed in Luedtke et al. 2023). Chemical threats such as pesticides, are often considered to play a role in the aquatic environment, however, a study of American toads has shown how rapid uptake of these substances can occur from treated soil as well, affecting adult amphibians (Brühl et al. 2011). Studies targeting threats across all life stages and their required habitat are scarce for amphibians but are much needed to inform conservation efforts since amphibians play a vital role in the ecosystem.

Amphibians play a critical role in the ecosystems, with different species branching across a variety of trophic niches. Amphibians are known to be carnivores, planktivores, and omnivores, as well as important prey within the system (Hopkins 2007). Not only are different species capable of filling multiple niches, but amphibians can play different roles in the ecosystem at different life stages (Morin 1983). In a previous study, amphibians including a salamander and frog species are known to have both top-down and bottom-up

control on the phytoplankton and zooplankton communities in wetlands (Rowland et al. 2017). This study also showed the role of both salamander and frog larvae as both predator and prey for wetland interactions. Studies suggest that the decline of amphibians at any life stage, but especially juvenile recruitment, could interrupt the energy flow between the aquatic and terrestrial habitats (Gibbons et al. 2006). This loss of energy transfer can have lasting effects on species diversity, primary producer growth and communities, organic matter composition, and consumer species such as invertebrates and other predator species (Gibbons et al. 2006; Whiles et al. 2006; Petranka et al. 2007; Rowland et al. 2017). The important trophic role and sensitivity of amphibians to threats has often labelled them as indicator species, similar to a canary in a coal mine, suggesting the health of the local amphibian populations is indicative of the overall health of an ecosystem (Welsh and Ollivier 1998; Venturino et al. 2003; Hussain and Pandit 2012; Miaud et al. 2019). Understanding how amphibian populations react to threats could therefore serve two purposes: to address the growth or decline of amphibian populations and to provide a predictor of threats that could soon affect other taxa. To discern these patterns, there is a need for consistent and effective monitoring of amphibian populations across the globe. This monitoring must provide accurate information about amphibian populations and must be conducted through feasible methodologies to detect a wide range of amphibian species.

There is evidence of some amphibian declines across Canada and Ontario but overall, the quantitative information on species abundance, range, and life history is deficient (Bishop and Pettit 1992; Badzinski and Archer 2008). COSEWIC, Committee on the Status of Endangered Wildlife in Canada, has found that as of 2016, 44% of Canadian amphibians are listed as endangered, threatened, or special concern (CHS 2023). Habitat loss in Ontario has been a major contributing factor to declines in local amphibian populations and species diversity. In southern Ontario, it is believed that 90% of wetlands in the Great Lake region have been lost (Hecnar 2004). There is a need for accessible and long-term monitoring of amphibians despite differing life histories or current risk status since shifts can occur during periods of little to no monitoring.

1.2 Conventional monitoring techniques for amphibians

Monitoring of amphibian species is conducted through various conventional methods to obtain a variety of information on trends of species composition, number of individuals, population demographics, and spatial-temporal habitat use (Walls 2014; Walls and Gabor 2019). Conventional methods of monitoring amphibians include trapping, acoustic monitoring, and visual encounter surveys (Gunzburger 2007). These methods aim to capture robust data about populations. However, these methods often overlook larval stages due to non-calling at this stage, inability to visually discriminate between species, and the need for genetic testing to determine species. Therefore, conventional methods have primarily targeted juvenile and adult life stages for species detection. Historically, conventional methods of monitoring have been used to monitor amphibians, however, potential inaccuracies in these methods could have contributed to poorly informed conservation efforts.

1.2.1 Trapping

A conventional method of species detection includes trapping or netting which is a highly invasive but informative method (Gunzburger 2007; De Souza et al. 2016; Swartz and Miller 2018). Trapping provides a passive method for capturing amphibian species while netting requires an active search and capture using adapted fishing nets (Ernst and Rödel 2004; Gunzburger 2007). Trapping can be conducted by using baited traps to lure amphibians into modified structures, such as crayfish traps or pitfall traps. Netting requires active searching for amphibians followed by rapid capture. After capture, observers gain information on the size, sex, life stage, and overall health of the individual including evidence of pathogens (Brown 1997). An advantage of this method includes the robust data collected, and the possibility to run mark-recapture studies. The disadvantages of trapping and netting relate to the invasive nature of the methodologies. This method requires direct handling of the organisms which increases the stress they experience (Smart et al. 2015; Swartz and Miller 2018). Additionally, within the traps, organisms may experience levels of predation from other amphibians or by-catch organisms. Another disadvantage to this method

is the time-intensive methods, either through extensive netting, data collection, or due to the required trap checking to ensure no species deaths. The disadvantages related to trapping and netting do not make it optimal for the detection of threatened species (Pilliod et al. 2013). Trapping and netting can be highly beneficial in the qualitative and quantitative data recorded. However, the invasive nature of this method to capture the target amphibian must be considered, due to increased stress on the organism and risk of fatality, before implementation of trapping.

1.2.2 Acoustic surveys

Acoustic surveys are a conventional way of detecting amphibians targeting the breeding seasons of calling anuran species. Acoustic surveys can be conducted either actively or passively (Melo et al. 2021). Active acoustic surveys are conducted in person using a protocol with a set of guidelines for weather conditions, observation times, and level of calling, such as the marsh monitoring protocol (Bird Studies Canada 2009). The passive acoustic survey is an alternate acoustic method that consists of mounting an acoustic recording device to record amphibian calls in audio file format (Brauer et al. 2016; Melo et al. 2021). This method allows for remote devices to record on a set schedule and be later processed for species presence. Both active and passive acoustic surveys are conducted near wetlands to capture the breeding activity of anuran species. Detections with this method capture species-specific calls of breeding male amphibians. Acoustic methods are beneficial for the non-invasive nature of data collection and the ability to locate active breeding grounds. This method also has the ability for easy multi-species detection and approximate counts for each species. However, this method has a sex bias and only detects male amphibians, as they will call to attract female mates (Gunzburger 2007; Brauer et al. 2016; Melo et al. 2021; Postaire et al. 2023). Additionally, this method completely excludes the order Caudata and Gymnophiona which contain non-calling amphibian species such as salamanders, newts, and caecilians. Acoustic surveys are also time-intensive and depend on the observer's expertise in knowing amphibian calls. Acoustic surveys are advantageous since they can detect active amphibian breeding grounds however, due to the inability to

detect non-calling species and the time-intensive methods, it is vital to consider other methods of species detection.

1.2.3 Visual encounter surveys

Visual encounter surveys (VES) offer another conventional method of amphibian species detection. This method is based on incidental observations of amphibians or conducted using a defined space or time to moderate search effort (Flint and Harris 2005; Gunzburger 2007). Visual encounter surveys consist of searching wetland habitats and the surrounding area for amphibian species. The success of VES depends on the observer's expertise and ability to accurately identify and count each species (Dejean et al. 2012; Ruso et al. 2019). Another indicator of the success of this method for species detection is the intensity of searching. Intensive VES can include disturbing the habitat and moving cover while searching for amphibians, which can increase the number of species detected. However, to avoid disturbing vital habitats, lower intensity VES are performed but come at a cost of lower species detections. Visual encounter surveys can offer a non-invasive method of species detection that does not discriminate based on the sex or calling ability of the species. Additionally, amphibians can be identified using VES throughout various life stages, such as adults, eggs, and larvae. A downside of visual encounter surveys is the time-intensive field methods required to detect species (Boullhesen et al. 2021). Additionally, visual encounter surveys risk the possibility of inaccurate identifications due to similar amphibian appearances or due to fleeing behaviours of amphibians obscuring them from view from observers. Depending on the search effort, visual encounter surveys can fail to detect cryptic species or those at low population densities (Chen and Watkins 2005; Barata et al. 2017). Visual encounter surveys are a widely used method of amphibian detection, however, the associated challenges indicate the need for a more effective method.

1.2.4 The current state of conventional amphibian monitoring

Monitoring of amphibians has historically focused on observing fluctuations in numbers of amphibian populations and short-term, local observations of amphibian

populations (Petranka et al. 2004). Ongoing monitoring efforts can aid in establishing baseline population numbers, understanding species ranges, taxonomic information, life history strategies, and habitat use (Baillie et al. 2004), all of which indicate not only the health of a species but the habitats they depend on. For instance, a required habitat that has faced degradation or urbanization will see a decrease in overall amphibian diversity, especially of specialist species, resulting in an altered amphibian community (Hamer and McDonnell 2008). Amphibian monitoring can also provide insight into how the interactions of their ecology or distribution play a role in their decline, which threats pose the greatest risk, and inform best conservation measures to pursue species recovery (Grant et al. 2013; Howell et al. 2020; Lee et al. 2022). There are multiple ways that amphibians have been previously monitored which target both their aquatic and terrestrial habitat (Gunzburger 2007). Some of the terrestrial methods of monitoring include cover board surveys, pitfall traps, or PVC pipe surveys, which target amphibians that seek out damp cover in their terrestrial habitat. Methods of detection including trapping, acoustic surveys, and visual encounter surveys tend to target amphibians in their aquatic habitats. Most species detection methods for amphibians target the aquatic environment due to the requirements of aquatic habitats and the predictability of use of the habitats for breeding (reviewed in Liedtke et al. 2022). Aquatic habitats also provides a location where many species of amphibians will converge in larger numbers rather than scattered in the terrestrial habitats.

1.3 Environmental DNA

1.3.1 Introduction to environmental DNA

Environmental DNA (eDNA) is genetic material, originating from the nuclear or mitochondrial genomes, of an organism that persists in substrates of the environment, such as air, water, or soil (Taberlet et al. 2012; Rees et al. 2014). eDNA can be left behind by micro-organisms and macro-organisms through processes such as the sloughing of tissue cells, fecal matter or excrement, natural secretions (mucus), decomposing organisms, release of gametes, or other bodily functions (Barnes and Turner 2016). eDNA is shed into the environment at

various rates and concentrations. Overall biomass of organisms can play a role in quantity of eDNA shed, and in many cases, a high biomass often results in a higher concentration of eDNA in the environment (Klymus et al. 2015). High metabolic activity, such as increased movement by organisms and processes related to growth or development can increase the rate at which eDNA is shed (Maruyama et al. 2014; A.A. Breton et al. 2022). High environmental temperatures or stressors can also increase the shed of eDNA (Strickler et al. 2015; De Souza et al. 2016). After eDNA is shed into the environment, it is capable of persisting in the environment over time.

eDNA is subject to degradation and dispersal after being shed into the environment in freshwater, marine, and terrestrial environments (Goldberg et al. 2016; Goldberg et al. 2018; Murakami et al. 2019; Bairoliya et al. 2022). Degradation is a universal problem when trying to detect eDNA as many factors cause fragmentation and breakdown of eDNA such as UV-B exposure, high temperatures, and pH variations (Strickler et al. 2015). eDNA can be either free-floating or cell-bound, where cell-bound eDNA is able to persist longer in the environment due to the protection from the cell membrane (Moushomi et al. 2019). Mitochondrial DNA (mtDNA) is the target of many eDNA-based studies because the double membrane of the mitochondria contributes to the protection of the mtDNA from degradation allowing for it to persist longer in the environment (Rickwood and Chambers 1981; Moushomi et al. 2019). Mitochondrial DNA is also more abundant, with many copies of the genome from the numerous mitochondria in a single cell compared to the one copy of nuclear DNA per cell (Ficetola et al. 2008; Takahara et al. 2012; Jo et al. 2020; Jo et al. 2022). Dispersal of eDNA is common in the air and aquatic environments, but is also possible with soil substrate when disrupted, releasing trapped eDNA molecules. Aquatic environments can be divided into two larger subtypes, being standing wetlands known as lentic systems, and flowing water such as rivers and streams known as lotic systems (Bedwell and Goldberg 2020). In an aquatic environment, the system, lentic or lotic, is a contributor to eDNA dispersal (Bedwell and Goldberg 2020). In lentic systems, sampling design plays a greater role in species detection. Proximity of sample collection to the

organism(s) shedding eDNA plays a role in detection probability. Therefore, a well-planned sampling technique using both spatial and temporal replicates can influence detections in lentic wetlands (Deiner and Altermatt 2014; Barnes and Turner 2016). It is important to consider flow directions and speed in lotic systems, and factors such as sample volume, sampling date, and spatial design play a large role in detection probability. To use eDNA for species detection, all the factors above must be considered to sample at an appropriate frequency and target appropriate spatial locations for that system to capture eDNA during sampling. Collected samples containing eDNA measure the steady-state level of eDNA in the environment which is a culmination of eDNA being produced, persisting, degrading, and dispersing through the system.

Many Canadian amphibians prefer to inhabit lentic wetlands such as ponds, lakes, bogs, marshes, swamps, and vernal pools (Table 1.1). Sampling design for amphibian eDNA in lentic systems should be informed by the system itself and the biology of the organisms. eDNA distribution through lentic systems can be stratified with irregular distributions which requires an exhaustive sampling design to best capture eDNA present (Takahara et al. 2012; De Souza et al. 2016; Harper et al. 2019; Bedwell and Goldberg 2020). This patchy distribution of eDNA through lentic systems, such as vernal pools, can be related to heterogeneity in the systems such as differing habitats, seasonal water levels, hydrology effect on water dispersal, and seasonality of species presence. Vernal pools are an important wetland type as many amphibian species are obligate vernal pool breeders which directly require these ephemeral wetlands for reproduction. Amphibians select for the desired habitat in the aquatic environment, often linked to available vegetation (Strijbosch 1979). Lentic systems can be diverse in vegetation types and therefore different wetlands or parts of wetlands may house certain amphibians. Additionally, lentic systems are subject to seasonal evaporation and nutrient mixing depending on the climate and weather. These lead to possible concentration or dispersal of eDNA. Many amphibian species follow a biphasic life history or utilize the aquatic environment for foraging. Therefore, the use of the wetlands and the time when eDNA is shed is dependent on the seasonal use by each species. With these

considerations, there is a need for spatial and temporal sampling to optimize the capture of amphibian eDNA in lentic systems.

1.3.2 Species detection using eDNA

The detection of eDNA as a methods of species detection is carried out through either metabarcoding or species-specific eDNA barcoding (Harper et al. 2018; Yu et al. 2022). Both of these methods begin with the same general procedure; sample collection, eDNA filtration, and eDNA extraction, but are followed by different methods of species detection (Langlois et al. 2020). eDNA metabarcoding offers a broad, community-based approach to species detection while eDNA barcoding offers a more targeted and specific approach to species detection. Both eDNA metabarcoding and eDNA barcoding have the ability to detect low concentrations of persisting eDNA in the environment and then identify sequences to a species-level (Ficetola et al. 2008). Selecting a molecular eDNA detection method for species detection depends on the overall goal of the study. Both eDNA metabarcoding and barcoding methods offer a method of detecting species while negating the need for a direct species observation (Ficetola et al. 2008; Taberlet et al. 2012; Moss et al. 2022). This can be beneficial for cryptic species, low-density populations such as endangered or threatened species, or species intolerant to high levels of disturbance associated with other conventional methods of detection (Ficetola et al. 2008; Taberlet et al. 2012; Moss et al. 2022). However, there is a level of error associated with eDNA detections, the same as any method of detection, that can lead to levels of false positives or negatives. eDNA also does not provide biological or observational information such as demographics, health of organisms, and number of individuals (Abbott et al. 2021; Wikston et al. 2023). Detections using eDNA require high levels of *in vitro* and *in situ* validations or optimizations to ensure high confidence in the molecular results (Thalinger et al. 2021; Abbott et al. 2021). eDNA barcoding and metabarcoding as methods of species detection are highly sensitive and offer promising methods for amphibian species detection.

1.3.2.1 Metabarcoding

The metabarcoding approach focuses on identifying a broad community of species detections across many taxa (Harper et al. 2018; Yu et al. 2022). This method consists of targeting universal DNA sequences with universal primers on either side of a region with species-level variability. This region undergoes amplification and sequencing via high-throughput sequencing (Ruppert et al. 2019). The resulting reads can be matched using a sequence database to build a community of species detected (Liu et al. 2020). This method also provides the composition of the community with associated levels of amplification for each organism in the form of sequencing read counts (Di Muri et al. 2020; Skelton et al. 2023). This method is emerging as a popular choice for eDNA detection due to the ability to detect a wide range of species and get a snapshot of the community within a sample without the need for prior knowledge of the species present (Taberlet et al. 2012; Valentini et al. 2016). However, a potential downside includes primer bias, which is preferential primer binding to sequences of some species over others (Gargan et al. 2022; Yu et al. 2022). The primer bias leads to false negatives when primers do not bind to eDNA sequences at a low concentration due to competition for the available primer. Additionally, eDNA metabarcoding assays include universal primers which may contain mismatches on the primer binding site for some taxa or species (Deiner and Altermatt 2014; Valentini et al. 2016; Liu et al. 2020). These mismatches can lead to primers having a higher affinity for binding to some sequences over others, and therefore, the resulting proportion of reads is biased. Additionally, there is the issue of competition for primers in the reactions which warrants the need for optimization of metabarcoding assays to ensure optimal conditions. This method was not used for species detection in this study, therefore eDNA barcoding will be the method of focus.

1.3.2.2 eDNA barcoding

Another method of species detection relies on the detection and amplification of species-specific fragments of eDNA known as eDNA barcoding. eDNA barcoding uses quantitative polymerase chain reaction (qPCR) to detect and amplify unique, species-specific

nucleic acid sequences even at low concentrations (Bustin et al. 2009). eDNA barcoding consists of species-specific primers binding to nucleic acids and amplifying the sequence and measuring the associated fluorescence, allowing for quantitative data to be collected. Fluorescence is detected via SYBR green assays where dye binds to any double-stranded DNA and fluorescence increases throughout the amplification process (Tajadini et al. 2014). An alternate method, primarily used in this study, is the use of TaqMan probes which are made up of a unique species-specific nucleic acid sequence surrounded by a quencher on the 3' end and a reporter dye on the 5' end (Tajadini et al. 2014). During amplification, the probe binds to the species-specific sequence and then during the extension phase, the reporter dye is cleaved off and fluoresces indicating the species "barcode" was present (Tajadini et al. 2014). eDNA detection via qPCR through amplification indicates species detection using these nucleic acid "barcodes". An advantage of this method is the targeted, sensitive approach to species detection for a single species. This can be optimal when looking for endangered or threatened species as well as invasive species (Dejean et al. 2012; Veldhoen et al. 2016; Hobbs et al. 2020) to track the distribution or habitat use. A disadvantage is that the eDNA barcoding approach does not offer the full community composition that metabarcoding offers but does allow for more customized and increased primer and probe options since metabarcoding depends on locating a highly variable sequence region between two very conserved regions for primer binding (Harper et al. 2018; Gargan et al. 2022). The use of species-specific sequences is more flexible in target regions and results in an increased level of sensitivity for low copy number collections of eDNA when using eDNA barcoding (Lesperance et al. 2021). Additionally, the results from eDNA barcoding via qPCR are easily interpreted compared to the sequencing required for the metabarcoding (Yu et al. 2022). A disadvantage to eDNA barcoding is the need to develop and fully optimize an assay for each target species in a study. This takes time and effort, however, following optimization, processing is efficient and easily interpretable compared to eDNA metabarcoding since there is no need for bioinformatic analysis (Yu et al. 2022). eDNA barcoding could potentially offer an additional method of amphibian species detection to aid in ongoing monitoring.

1.3.3 eDNA processing standards for eDNA barcoding

With the increased use of eDNA barcoding, users outline the need for increased use of controls and overall standardization of methodologies to encourage the adoption of eDNA barcoding as a method of species detection (Helbing and Hobbs 2019). Use of molecular methods for species detection is possible without direct species observation, however, it is possible to introduce errors or bias when inferring the presence of a species. The increased use of controls during *in silico* design testing, field methods, and eDNA processing aid in determining the limitations of the study. Standardizations are meant to be implemented throughout the main stages of eDNA barcoding methodology; sample collection, development of eDNA barcoding assay, and sample processing (Veldhoen et al. 2016). These standardizations include; the importance of validations of assays *in silico* with target DNA, proper eDNA extraction techniques, comprehensive reporting of assay conditions for PCR, testing for cross-species amplification of non-target DNA, testing environmental samples, assay sensitivity and specificity, design of field testing, and accounting for patterns in ecology and abiotic factors when analyzing results (Thalinger et al. 2021; Abbott et al. 2021). Controls and standardizations are implemented through three main stages: laboratory validation of eDNA barcoding assays, field methodologies, and sample processing.

A vital stage of eDNA barcoding is the qPCR assay design *in silico* and testing for optimal conditions *in vitro*, followed by application to eDNA samples collected in the field (Helbing and Hobbs 2019). Selection of the target region of the mitochondrial genome should be optimized to meet probe-based qPCR requirements, such as selection of a target gene, target sequence length, GC content, melting temperature (Bustin et al. 2009; Langlois et al. 2020), and ensuring no possibility for cross-species amplification. Efforts should be made to test the qPCR assay for cross-species amplification of closely related species and species with overlapping ranges *in vitro* (Helbing and Hobbs 2019; Thalinger et al. 2021; Abbott et al. 2021). Following assay design and initial cross-species amplification testing, each eDNA barcoding assay should be optimized for primer concentration, probe concentration, and annealing temperature *in vitro*. These optimizations should allow for

efficient assays even when detecting low copy numbers of eDNA. The sensitivity of the assays at low concentrations of target eDNA is assessed by determining the limit of detection (LOD) and limit of quantification (LOQ) (Klymus et al. 2019; Lesperance et al. 2021). The limit of detection is defined as the lowest concentration of DNA that can be detected with 95% confidence. The limit of quantification has a more debated definition. In some cases, LOQ is the same as the limit of detection while other studies have an LOQ of a much higher concentration due to use of a preset precision metric (reviewed in Armbruster and Pry 2008). The precision metric often refers to a predefined coefficient of variation deemed acceptable for the study (reviewed in Armbruster and Pry 2008). For this thesis, the limit of quantification refers to the lowest DNA standard concentration that amplifies with a coefficient of variation below 35%. The final step is to apply the assays to eDNA samples and interpret results using the controls to understand the limitations of the study.

Methods for eDNA sampling requires the implementation of stringent controls and standardizations. The controls used in studies must all undergo the same processing that samples undergo to ensure the controls are effective (Goldberg et al. 2016). The use of controls in the field, such as sterile water field blanks, is a necessary control for tracing contamination of introduced DNA during the eDNA methodologies. Decontamination measures, such as using bleach on surfaces, can be used to break down DNA before it could potentially contaminate samples or field blanks. Maintaining DNA-free surfaces as much as possible during fieldwork is important to ensure all eDNA collected originates from the sample avoiding false positives from foreign DNA contamination. The methods of sample processing includes filtration and concentration of eDNA onto a filter and the subsequent extraction of eDNA from that filter. For standardization of sample processing, vital information such as all products used is necessary to provide transparency and reproducibility. The use of controls, such as sterile filters extracted alongside eDNA sample filters, ensures the detection of foreign DNA contamination through the stages of eDNA processing. Through the use of controls and standardizing field methods, studies will better inform future work and allow for increased comparability.

An additional control in the field of eDNA processing is to screen all eDNA samples for quality metrics such as inhibitors within the sample and integrity of the present eDNA (Goldberg et al. 2016; Veldhoen et al. 2016; Hobbs et al. 2019; Doi et al. 2021; Thalinger et al. 2021). Inhibitors, which are compounds originating from organic materials and soil, such as tannins or humic acids, can inhibit the PCR reactions used to amplify and detect eDNA (Hunter et al. 2019). To decrease the effect of inhibitors on eDNA barcoding reactions, eDNA samples can be diluted or cleaned using an inhibitor removal kit (McKee et al. 2015). These options can lead to diluted genetic material or loss of DNA but can increase the number of successful detections due to no compounds significantly inhibiting reaction interactions during amplification. This tradeoff is an important consideration and the increased amount of inhibitors in lentic wetlands due to lack of flow and increased organic material can make this a crucial step for testing in these wetlands (Harper et al. 2019). Inhibitors can lead to false negatives during detection, but another possible reason for non-detection is the degradation of the collected eDNA. To ensure degradation has not occurred above an accepted level, the integrity of the target eDNA can be evaluated by proxy. Chloroplasts are ubiquitous in wetlands systems and provide abundant eDNA. Therefore, to determine if eDNA is intact, an assay targeting the chloroplast 23S rRNA gene can be implemented (Veldhoen et al. 2016; Hobbs et al. 2019; Randall et al. 2023). Amplification of the gene below a set threshold, 30 cycles, indicates that eDNA did not experience high levels of degradation. Ensuring uninhibited samples and testing for intact eDNA aid in preventing false negatives and lends confidence when interpreting negative results in the data.

1.3.4 Sampling design

During sample collection, it is important to consider the sampling effort such as the number of replicates, number of sampling locations, and repeated sampling over time. The frequency of spatial and temporal sampling is dependent on wetland type and size. For instance, the sampling in lentic and lotic systems should be approached with different sampling designs due to dispersal patterns either from the flowing of water or the horizontal and vertical mixing of a lentic system (Moyer et al. 2014; Bedwell and Goldberg 2020). The

area of a wetland should be accounted for in the sampling effort, with increased sampling locations for larger areas. Additionally, in lentic systems, such as lakes and ponds, it is suggested to sample around the circumference of the wetland (Bedwell and Goldberg 2020). For lotic systems, sampling across the flowing water and at different points upstream and downstream is an example of optimal design (Bedwell and Goldberg 2020). During sample collection, it is important to ensure eDNA samples are handled in a way that decreases the degradation of eDNA. This results in ensuring the eDNA samples remain cold during transport to a laboratory for filtration or conducting in-field filtration with the use of self-preserving filters (Yamanaka et al. 2017).

1.3.5 Current use of eDNA barcoding for amphibian species detection

Conserving amphibians and their habitat is more important than ever with the increased threats and global declines, therefore it is important to determine amphibians' preferred habitat. Canadian amphibian species can inhabit lentic wetlands, lotic wetlands or terrestrial habitats and can be either generalists or specialists for the habitat (CHS 2024). For example, generalist green frogs will utilize many types of lentic wetlands including ponds, and lakes (Stefan 2023) while some species are specialists such as the coastal tailed frog inhabiting running streams (Hobbs et al. 2019). To conserve target species, all required habitat must be preserved. For example, a tree frog species, such as the spring peeper, requires protection of their terrestrial forest habitat but also of the aquatic breeding grounds (Ontario Nature 2023; CHS 2024). In Canada, specifically Ontario, loss of habitat has been a major factor in species declines (Bishop and Pettit 1992; Hecnar 2004; Badzinski and Archer 2008). An often overlooked wetland feature are vernal pools which are ephemeral wetlands. These wetlands are fed only by snow melt, and rainfall, and are hydrologically separated from other water sources. Fluctuations from year to year, and the nature of these small but vital wetlands, have led them to remain an unprotected wetland type in Canada. These vernal pools are a refuge for multiple species including multiple amphibians such as the spotted salamander and the wood frog. Conserving amphibians within these vernal pool habitats could increase juvenile recruitment of amphibians, boosting populations due to the lack of fish as predators in these

wetlands. These wetlands may also act as connectors to other larger wetlands and are vital to gene flow and the flow of energy between systems. The role of amphibians in the ecosystem is vital as they play a role as prey species and as predators of insects which can control their populations and aid in human health. This emphasizes the need for monitoring of amphibian species and vernal pool habitats.

Current use of eDNA barcoding for amphibians has been used to determine species range, detection of threatened or invasive species, and estimation of species abundance. Evaluating the species distribution and occupancy is a common use for eDNA species detection (Pilliod et al. 2013; Ruso et al. 2019; Hobbs et al. 2020; Postaire et al. 2023; Quilumbaquin et al. 2023). In the case of the mudpuppy (*Necturus maculosus*), the known range of the species in Ohio, United States, was expanded to several new river systems based on eDNA surveys (Collins et al. 2019). Amphibian detections using eDNA barcoding have been used to determine occupancy of species in certain wetlands types, such as agricultural wetlands (Ruso et al. 2019). eDNA barcoding has also been used to detect endangered species, such as the pool frog (*Pelophylax lessonae*) in Sweden (Eiler et al. 2018). As well as detection of rare species, eDNA barcoding has been used to detect invasive species, a method which could allow for early detection of the introduced species (Smart et al. 2015). Attempts at estimating species abundance of amphibian tadpoles has been attempted using eDNA barcoding, however, there is much debate if these models are accurate due to the high level of confounding variables (Breton et al. 2022; Rourke et al. 2023). Overall, eDNA barcoding has been used to gather more information about amphibians globally, however, it is still an under-used tool for this taxon.

The use of eDNA as a method of detection has now been used across the globe and on multiple taxa, including here in Canada. Multiple Canadian government bodies have concluded that the use of eDNA as a method of species monitoring can be beneficial and therefore should be optimized and standardized (Abbott et al. 2021). Amphibians have become a target of eDNA studies such as the detection of the invasive bullfrog in Western Canada (Ficetola et al. 2008). Until 2019, there were no eDNA assays developed for most of

Ontario's amphibian species (Beauchlerc et al. 2019). After the development of these assays, they have been used for mesocosm eDNA experiments to look at amphibian eDNA biomass and to conduct a comparative analysis of eDNA barcoding versus conventional methods of detection of anuran species in Ontario (Breton et al. 2022; Wikston et al. 2023). Optimal sampling design for targeting Canadian amphibians is still an area of research. The current use of amphibian eDNA assays has not adequately targeted the spatial-temporal variations possible in eDNA detection with few studies sampling multiple locations per wetlands or multiple times per season. A study with increased spatiotemporal sampling design will aid in informing future amphibian sampling design regarding when to sample and how many spatial replicates are required. Spatial temporal studies will also aid in determining if there should be species-specific considerations when conducting eDNA sampling. Another knowledge gap includes the targeting of vernal pool wetlands (Brooks 2004; Pyke 2005; Dean et al. 2009). These ephemeral wetlands, fed only by snow melt and rain, can offer vital habitats to amphibians such as obligate vernal pool breeders like wood frogs and spotted salamanders. Determining if eDNA barcoding offers a reliable method of species detection is important since it could inform government bodies on species range and habitat use, and as a result, benefit amphibian conservation efforts.

1.4 Target species

In this study, I will focus on species detection of six amphibian species, whose ranges include southern Ontario, with differences in habitat use and life history strategies. The species selected are the American toad (*Anaxyrus americanus*), Green frog (*Lithobates clamitans*), Northern leopard frog (*Lithobates pipiens*), Spotted salamander (*Ambystoma maculatum*), Spring peeper (*Pseudacris crucifer*), and Wood frog (*Rana sylvatica*) (Table 1.1). The rationale for selecting this subset of species was related to the diversity in breeding behaviours, threatened status, and habitat use (Table 1.1). Since sampling was completed in vernal pools, which are ephemeral wetlands (Dean et al. 2009), both the spotted salamander and wood frog were selected as they are obligate vernal pool breeders and require ephemeral wetlands for breeding. The green frog and northern leopard frog were selected as both

common, widespread species in southern Ontario and, as generalists for breeding location, they are present in many wetland types. Despite green frogs not targeting ephemeral wetlands as vital breeding grounds, juvenile frogs use these wetlands during the spring and summer seasons. Additionally, the northern leopard frog is a threatened species in western Canadian populations indicating a need for effective monitoring of this species which eDNA barcoding could offer (Venter et al. 2006; COSEWIC 2009). Multiple species are also good examples of explosive breeders with a short, defined breeding season such as American toad, spotted salamander, and the wood frog (Table 1.1). All these explosive breeding species breed in early spring, often coinciding with specific conditions, and then seek out cover in terrestrial habitats for the remainder of the season. Detection of eDNA from these species would indicate adult presence in early spring but later in the season would indicate detection of larval amphibians or persistence of adult amphibian eDNA in the environment. The spotted salamander also represented a species from the order Caudata, which are all non-calling salamander species (Bishop and Pettit 1992; Baillie et al. 2004; Streicher et al. 2020). The spring peeper was selected since it is a widespread species in eastern Canada and represents a member of the family Hylidae, which contains all species of treefrogs indicating that they spend most of the year in an arboreal environment after the breeding season. (Faivovich et al. 2005). This species is small in biomass and often persists at low density within wetlands. These target species provide a subset of different amphibian life history strategies and therefore will provide some insight into comparative species detection via conventional methods or eDNA barcoding.

Table 1.1. Target species’ physical descriptors and information on life history through breeding, post-breeding, and overwintering.

	American toad	Green frog	Northern leopard frog	Spotted salamander	Spring peeper	Wood frog
	<i>Anaxyrus americanus</i>	<i>Lithobates clamitans</i>	<i>Lithobates pipiens</i>	<i>Ambystoma maculatum</i>	<i>Pseudacris crucifer</i>	<i>Rana sylvatica</i>
						
Physical characteristics	Maximum 11 cm in length, brown to olive in colour, brown wart-like bumps. Desiccation tolerant.	Up to 11 cm. Dark green to brown dorsal colour with patchy banding on hind legs. White or yellow belly. Defined dorsal lateral ridge.	Ranging from 6–11 cm in length, green to brown dorsal colouration with black white lined spots, light coloured belly.	Thick bodied salamander up to 25 cm in length. Dark black dorsal colouration with bright yellow spots on head, body and tail.	Below 4 cm in length, light brown colour with darker “x” shape on back. Lack of dorsal lateral ridges. White-yellow inner legs.	UP to 8 cm length. Body colour brown to reddish brown with dark mask around eyes. White coloured belly and upper lip.
Breeding season	1-2 weeks in April-June.	Prolonged breeding, June to August.	May-June	Early spring, ice off	April-early June	Explosive breeders, 1-2 weeks in April-May.
Breeding habitat	Permanent and temporary wetlands including bogs, fens, marshes, ponds, lakes.	Permanent wetland	Shallow wetlands, both permanent and ephemeral wetlands.	Fish free wetlands, obligate vernal pool breeder.	Fishless wetlands with woody brush such as vernal pools, beaver ponds, bogs, and marshes.	Obligate vernal pool breeders, ephemeral wetlands.
Post-breeding habitat	Terrestrial habitats ranging from woodlands, grasslands, meadows, and urban areas	Wetland generalist	Shallow marshes, meadows, grasslands.	Deciduous and mixed forest, under cover such as logs, leaf litter, and rocks.	Forest regions within hundreds of meters from breeding location.	Terrestrial, deciduous and boreal forests.
Overwintering habitat	Terrestrially, below frost line in dug burrows and rock crevices.	Aquatic, burrowed in mud below the frost line.	Deep aquatic wetlands, below frost line.	Terrestrial below frost line in burrows and root crevices.	Root crevices, mammal burrows, beneath leaf litter below frost line.	Under leaf litter in forest habitats.
Number of eggs	2000-15000 in strings	1000-5000	3000-70000	1-250	200-1500	300-2000
Time to metamorphosis	42-63 days	Overwinter as tadpoles	30-90 days	Up to 90 days	60-90 days	43-84 days
Sources	(Canadian Herpetological Society 2023a; Ontario Nature 2023a)	(Canadian Herpetological Society 2023b; Ontario Nature 2023b; Stefan 2023)	(COSEWIC 2009; Canadian Herpetological Society 2023c; Ontario Nature 2023c)	(AmphibiaWeb 2023a; Canadian Herpetological Society 2023d; Ontario Nature 2023d)	(Dean et al. 2009; Canadian Herpetological Society 2023e; Ontario Nature 2023e; Postaire et al. 2023)	(Dean et al. 2009; AmphibiaWeb 2023b; Canadian Herpetological Society 2023f; Ontario Nature 2023f)

1.5 Objectives and Hypothesis

The key question remains of how to increase effective amphibian monitoring to allow for conservation of the taxa. This question has not been addressed enough globally or here in Canada. By increasing the ability to detect amphibians, this will aid in targeting conservation efforts towards identified populations and their associated habitat. To achieve increased amphibian detection ability, it is vital to explore the possible methods of detection and the optimal sampling design for each method. In a recent study by Wikston et al. (2023), they strove to compare conventional amphibian monitoring techniques to eDNA barcoding for anuran species. They concluded that eDNA barcoding offered a reliable method of species detection but needed to be used alongside a conventional method, and identified visual encounter surveys as the best conventional method to use. This thesis will potentially provide further evidence supporting the use of eDNA barcoding as a viable and effective method of monitoring compared to the widespread conventional methods. This research is needed as it could aid in the uptake of the eDNA barcoding detection method for conservation and species monitoring by end users such as government bodies and industry users. Few studies have focused on eDNA sampling with both spatial sampling around wetlands and temporal sampling by repeated sampling across a season. Spatial and temporal sampling are important to help inform future studies on the number of replicates, number of sampling events, and sampling effort required to obtain species detections. There is also a need for studies targeting various amphibian habitats due to the use of a diverse number of wetlands by various amphibian species. A habitat of interest, which are often overlooked, are vernal pools which are temporary and seasonal wetlands. It is crucial to explore the use of vernal pools by various amphibian species including specialist species to support the conservation of these wetlands in the future. Overall, it is vital to conduct amphibian studies dedicated to comparing monitoring methods, including molecular methods such as eDNA barcoding to increase the ability to detect many different amphibian species despite life history and be able to apply these methods to different wetland types.

The goal of this research is to compare the detection capability of non-invasive methods of amphibian surveying to improve detection ability. This study focused on detecting amphibians in three vernal pools in southern Ontario. Conventional methods often require high levels of effort and are fraught with challenges that overlook the larval stages of amphibians, low-density species, and cryptic species. Conventional methods of monitoring amphibians were compared to the molecular method of eDNA barcoding which negates the need for direct species observation. For this study, eDNA barcoding may either outperform conventional methods, match the level of detection, or be insufficient depending on the species of interest. The **objective** of the study is to conduct non-invasive conventional methods of detection and then directly compare the sensitivity and detection ability of each method to the non-invasive molecular eDNA barcoding method. I hypothesized that eDNA barcoding would result in an equal number of binary presence-absence detections on each sampling date when compared to non-invasive conventional methods of detection (audio/visual). I predicted that eDNA barcoding would detect a greater species richness and accumulation per sampling event compared to conventional methods when observing species detection.

2 Detection of amphibians using environmental DNA barcoding compared to conventional methodologies in southern Ontario vernal pools

2.1 Introduction

Amphibian populations have been facing global declines with nearly 41% of all species being classified as threatened or endangered (reviewed in Luedtke et al. 2023; IUCN 2023). The threats facing amphibians are numerous and include global climate change, habitat loss and fragmentation, pathogens, pollutants, over-exploitation, and invasive species (Baillie et al. 2004; Stuart et al. 2005; Oshiro 2021; reviewed in Luedtke et al. 2023). The amphibian taxa has the fastest number of declining populations and the fewest stable populations when compared to mammals, birds, reptiles, and invertebrates (reviewed in Finn et al. 2023). Amidst these current declines, these are likely underestimates of the amphibian populations and the true risk to the taxa due to the lack of effective monitoring and the data-deficient status of many amphibian species (Howard and Bickford 2014). The combination of the declining populations and lack of long-term monitoring, results in a dire need for effective and ongoing monitoring.

Effective monitoring of amphibian populations is challenging due to their varied life histories and conservation status. Many amphibians, including Canadian species, utilize a biphasic life cycle and depend on both aquatic habitats, for reproduction and rearing, and terrestrial habitats, for foraging or overwintering (reviewed in Liedtke et al. 2022; reviewed in Nolan et al. 2023). Due to the required use of two distinct habitats, amphibians face threats from both environments. This is one of the reasons amphibians have been referred to as an indicator species of the ecosystem health (Welsh and Ollivier 1998; Venturino et al. 2003; Hecnar 2004; Rice et al. 2007; Hussain and Pandit 2012; Miaud et al. 2019). Amphibian monitoring is often biased towards generalist species and excludes species that have explosive breeding strategies, cryptic life histories, nocturnal behaviours, or persist at low densities in the ecosystem, such as threatened species (Scheffers and Paszkowski 2012). The

biases in sampling arise from the mistiming of surveys, insufficient search effort, and/or insufficient spatial sampling. These behavioral characteristics, life history strategies, and conservation status must be considered when attempting amphibian monitoring to properly capture species richness in a given habitat. Amphibian monitoring often overlooks or still contains biases excluding species from detection.

Current conventional methods for monitoring amphibian populations include acoustic surveys, visual surveys, and/or trapping and vary in their ability to detect entire amphibian communities, and in the level of invasiveness posed to the species or habitat (Ernst and Rödel 2004; Gunzburger 2007; Moss et al. 2022). Acoustic surveys, through active or passive survey methods, allow for the detection of calling male amphibians in the order Anura and can locate active breeding sites (Melo et al. 2021). Both acoustic methods are considered non-invasive and can allow for multi-species detection relatively quickly but have a sex bias for only male individuals, are dependent on specific environmental cues, and completely neglect non-calling amphibians such as the orders Caudata and Gymnophiona. Visual encounter surveys detect amphibians by either coincidental observation or active searches of a set area or for a set time (Gunzburger 2007). This method offers an additional non-invasive technique to detect species though it can be disruptive to the habitat, particularly when conducted extensively, and relies on the accurate identification skills of the field technicians. Trapping is a highly invasive method of species detection that puts the captured animals at risk of predation in the traps as well as high levels of stress during the collection of data (Gunzburger 2007; Todd et al. 2007; Farmer et al. 2009). Though this method collects extensive data on health, size, sex, and other quantitative and qualitative data, this method is highly invasive and use of this method is unfavourable for use in areas containing threatened amphibians as well as requiring special permits to handle threatened species (Rees et al. 2014). The decline in amphibians not only requires ongoing monitoring but requires sensitive, non-invasive techniques to maximize detection while minimizing the impact of the monitoring on this imperiled taxon. These conventional methods have advantages and disadvantages that cater to some species more than others, which supports that there is no

“one size fits all” method for amphibian monitoring (Fediajevaite et al. 2021). This has led to the development of novel methods of detection to replace or to be used in combination with conventional methods. There is a need for a detection method that has an increased sensitivity to detect across life stages, at all species densities, to account for species behaviour, and various life history strategies of all amphibians.

A non-invasive, sensitive molecular method of species detection has gained popularity for species detection and uses the collection and detection of environmental DNA (eDNA) to identify species presence (Ficetola et al. 2008). eDNA consists of genetic material that persists in the environment (air, water, and soil) that originated from macro and microbiota through the sloughing of cells, from bodily fluids, the release of gametes, and the decomposition of dead organisms (Barnes and Turner 2016). eDNA from these organisms can then be collected in water samples to undergo species detection. eDNA detection within the collected samples is used as a method of species detection through a method known as eDNA barcoding (Ficetola et al. 2008; Dejean et al. 2012; Mauvisseau et al. 2019). This uses qPCR to amplify species-specific sequences of nucleic acids which act as a “barcode” for the target species, negating a need for direct species observation. This method has been previously used within Canada to detect a wide variety of species including invertebrates, fish, mammals, and amphibians (Hernandez et al. 2020; Abbott et al. 2021; Suarez-Bregua et al. 2022; Bernos et al. 2023; Wikston et al. 2023). In a recent study, Ontario amphibians were targeted for species detection using eDNA barcoding (Wikston et al. 2023). This study showed that eDNA barcoding alongside visual encounter surveys was optimal for amphibian species detection in many wetland systems. This supports that studying the ability of eDNA barcoding for species detection could aid in increased amphibian species detection. This emphasizes the importance of determining the effectiveness of eDNA barcoding for detection of cryptic species, low-density species, and detection across life stages for amphibians, such as tadpoles. There have been few overall studies of eDNA detection of amphibians and even fewer studies that directly compare conventional methods of detection to eDNA barcoding. This study aims to further previous studies by supporting the use of eDNA barcoding as an

optimal method of species detection compared to other non-invasive methods through implementing an in-depth spatial temporal design in ephemeral vernal pools. The uptake of eDNA barcoding as a method for conservation, by government bodies and industry, has been slow but efforts focusing on standardizations in the field strive for the implementation of this method. Some of these standardizations include careful sample design, use of controls in the field and laboratory, reporting assay sensitivity and quality metrics, extensive qPCR validations, and reporting qPCR conditions (Thalinger et al. 2021). In this study, sensitivity was determined for each assay through the calculation of a limit of detection (LOD) and quantification (LOQ) (Armbruster and Pry 2008; Klymus et al. 2019; Lesperance et al. 2021). LOD and LOQ are defined as the concentration of DNA at which detection occurred at a rate of 95% confidence and when detections occurred with a coefficient of variation of 35%, respectively (Klymus et al. 2019; Lesperance et al. 2021). With increased guidelines, routine species monitoring using eDNA barcoding may become more widespread and accepted as a method of species detection. For eDNA barcoding to be justified as a valid option for species detection, increased evidence is needed that it is a viable and sensitive option when compared to conventional methods of species detection.

This study aims to compare non-invasive methods of amphibian species detection by contrasting non-invasive conventional detection methods (audio surveys and visual surveys) with eDNA barcoding. This comparison was conducted in three vernal pool wetland habitats in southern Ontario for six target species; American toad (*Anaxyrus americanus*), Green frog (*Lithobates clamitans*), Northern leopard frog (*Lithobates pipiens*), Spotted salamander (*Ambystoma maculatum*), Spring peeper (*Pseudacris crucifer*), and Wood frog (*Rana sylvatica*). I hypothesized that eDNA barcoding will provide an equal or greater number of binary presence-absence detection of the target species on each sampling date compared to conventional methods of detection. This study aims to determine if eDNA barcoding is an effective method of species detection compared to the non-invasive conventional methods used when targeting amphibians with varying life history strategies. Additionally, this study

will explore the use of spatial and temporal sampling for detection of amphibian eDNA in vernal pool wetlands.

2.2 Methods

2.2.1 Field Methods

2.2.1.1 Site Selection

Conventional and eDNA surveys were conducted at four vernal pools in collaboration with *rare* Charitable Research Reserve (Cambridge, Ontario, Canada) from April 4 – July 7, 2019 (Figure 2.1). Vernal pools were selected based on size and proximity to anthropogenic influence. The larger Blair Flats Wetland (BF, 17T 551074 4803618) and the smaller Preston Flats Pond (PF, 17T 550659 4804384) were selected based on proximity to a roadway, Fountain Street S and Blair Road, respectively. A second larger vernal pool, Ancient Woods Swamp (AWS, 17T 551053 4802838), and a second smaller vernal pool, Ancient Woods Pond (AWP, 17T 551404 4802660), were selected for their distance from a roadway and were located at least 750 m from a roadway within an old growth forest that merges with a deciduous-mixed swamp. Despite the relatively large area of Blair Flats Wetland, this wetland was relatively shallow and dried up in May 2019, preventing further survey and was removed from this study. Vernal pools were surveyed using conventional survey methods (Section 2.2.2) and eDNA survey methods (Section 2.2.3).



Figure 2.1. Map of vernal pools surveyed at *rare* Charitable Research Reserve.

Locations of vernal pools selected for conventional (audio and visual) and eDNA surveys are outlined in blue and numbered in orange circles. Green outlines depict the lands that are part of the *rare* Charitable Research Reserve. Two vernal pools were selected for their proximity to roadways: (1) Preston Flats Pond adjacent to Fountain Street S, and (2) Blair Flats Wetland adjacent to Blair Road. Similarly, two vernal pools were selected based on their distance from roadways and are nested in mixed woods: (3) Ancient Woods Swamp and (4) Ancient Woods Pond. Global Positioning System (GPS) location and approximate size of each selected vernal pool are noted below each sampling location listed in the legend on the image. [Figure credit: *rare* Charitable Research Reserve].

2.2.2 Conventional amphibian monitoring

2.2.2.1 Acoustic surveys

Two audio surveys were performed to record amphibian calls at all vernal pools surveyed: an active acoustic survey using the marsh monitoring protocol (MMP) and a passive acoustic survey using mounted song meters (Figure 2.2). Active MMP audio surveys were conducted once a month at each vernal pool from April to June (Table 2.1). The MMP survey was performed by visiting each vernal pool after sunset between 20:45 and 23:00 h, waiting one minute in silence to allow amphibians to settle after arrival, and listening for amphibian calls for 3 min (Environment Canada, Participants guide). The wind speed (Beaufort Wind scale), background noise (scale from MMP), and GPS location (Garmin eTrex® 30X) were recorded on each sampling date and vernal pool (Bird Studies Canada 2009). MMP surveys were only conducted if night temperatures exceeded 5 °C and wind speed did not exceed approximately 19 km/h. The observer for the active acoustic surveys was PhD student Nathanael Harper along with a field technician who both have experience identifying amphibian mating calls to the species level for Ontario frog species. At Preston Flats Pond and Ancient Woods Swamp, the observers stood on the North side of the vernal pool facing the south side of the vernal pool (Figure 2.2). For Ancient Woods Pond, the observers stood on the southwest shore facing the northeast side of the vernal pool. All amphibian species calling were recorded at each field site with approximate location and codes related to the level of calling and relative species abundance (Bird Studies Canada 2009). Level of calling was recorded as code 1 indicating that the calls of the same species are not overlapping, and individuals are countable, code 2 indicating that there are overlapping calls of the same species and individuals can be isolated spatially though true abundance is difficult to determine, or code 3 indicating a full chorus of calls from the same species meaning calls are overlapping, continuous, and creates a constant wall of sound. For passive acoustic song meter surveys, one SM4 acoustic song meter (Wildlife Acoustics, Maynard, MA, USA) was placed at each vernal pool and was mounted to a tree approximately 1.2 m from the ground and within 5 m of the vernal pool edge (Figure 2.2).

The song meters were programmed to record one hour of acoustic data at dawn and dusk using acoustic song meter preset settings based on the location's associated day-night cycle from May 2 to July 7, 2019. Due to a power failure of the SM4 song meters, recordings were not obtained from May 25 to June 7, 2019.

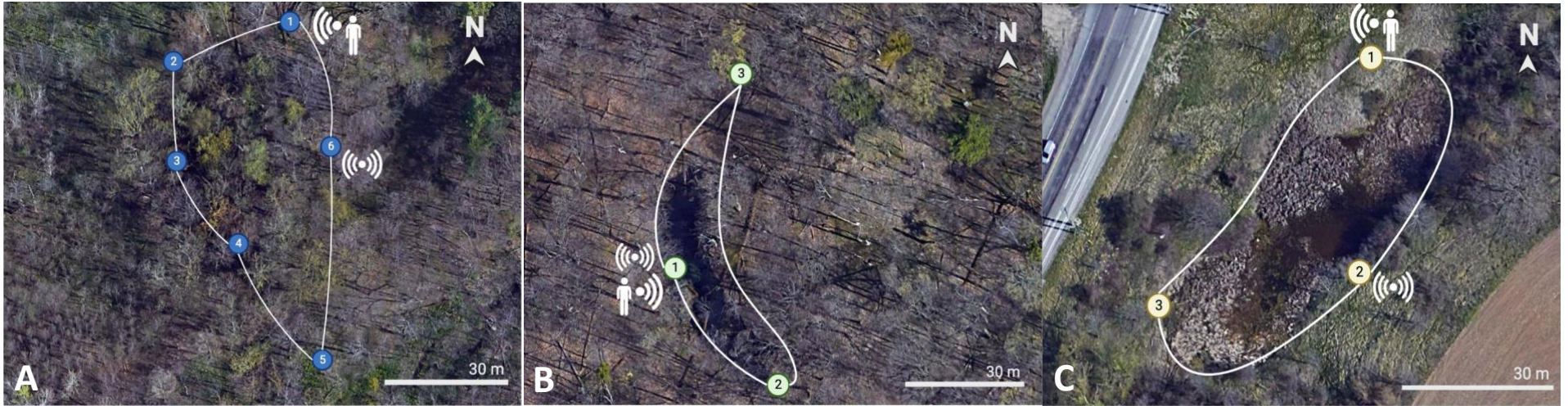


Figure 2.2. Sampling locations selected at three vernal pools to survey for amphibians using conventional and eDNA surveys.

Conventional and eDNA surveys were conducted at (A) Ancient Woods Swamp, (B) Ancient Woods Pond, and (C) Preston Flats Pond. The signal symbol in conjunction with a person represents the location at which the active acoustic surveys using the marsh monitoring protocol were conducted. The signal symbol represents the location of the Wildlife Acoustics SM4 acoustic song meter placement used for passive acoustic surveys. The white line approximates the path of visual encounter surveys around the periphery of each vernal pool. Numbering represents the number of sampling locations where two replicate bottles of water were collected for downstream eDNA barcoding. Each map is scaled and contains an arrow representing North. Figure created using Google Maps 2023 and BioRender. Note: Images are from Fall 2015 and water levels vary based on season, snowmelt, and rainfall.

2.2.2.2 Visual Surveys

Vernal pools were visited once every 6 - 10 days from April to mid-May and then every 14 - 20 days until mid-July in 2019 (Table 2.1). Ancient Woods Swamp was surveyed five times throughout the season. Both Ancient Woods Pond and Preston Flats Pond were surveyed a total of seven times. During each visit, visual encounter surveys were conducted by traversing around the entire periphery of each vernal pool while being careful not to disturb the water or startle amphibians. Visual encounter surveys were conducted by PhD student Nathanael Harper and a field technician, both with knowledge of Ontario amphibian ID based on visual appearance. All detections were recorded including species and number of individuals detected.

2.2.3 eDNA sampling

Each vernal pool was sampled at multiple sampling locations: three sampling locations for Ancient Woods Pond and Preston Flats Pond, or six sampling locations for Ancient Woods Swamp (Figure 2.2). At each sampling location around the periphery of the vernal pool, two replicate 1 L amber-stained glass bottles (Systems Plus, Baden, ON, Canada; Cat. # GL 1000 A/PC) were used to collect 1 L of water in each replicate bottle approximately 0.5 m from the vernal pool's edge. The field technician wore gloves disinfected with 70 % ethanol and collected water by reaching as far into the pool without wading so as not to disturb the sediment of the vernal pool. The bottle was submerged just below the surface of the vernal pool to collect 1 L of water. Gloves were changed when beginning sampling at a new sampling location at the same vernal pool and when changing vernal pool locations. Additionally, if the gloves touched the water they were also changed. In any instance when gloves were changed, the wearer sprayed their gloved hands with 70 % ethanol and let them dry before proceeding with additional sampling. At one sampling location at each vernal pool, a 1 L bottle containing molecular-grade water (Fisher Scientific, Ottawa, ON, Cat. #BP28251) was opened at the beginning of sampling and closed after sampling was complete for that sampling location. This acted as a field blank to track the possible introduction of contamination from methodologies in the field and better understand baseline levels of contamination from this field process. All bottles of water were kept on ice

in an insulated cooler to minimize the degradation of DNA within samples during transport back to the University of Waterloo. Samples were stored at 4 °C and filtered (Section 2.2.4.2) within 24 h of collection. Alongside the collection of eDNA water samples, at one sampling location for each vernal pool, the Professional Plus (Pro Plus) Multiparameter Instrument (YSI, Yellow Springs, Ohio, SKU: 6050000) was used to test water for temperature (°C), dissolved oxygen (%), pH, conductivity (mS/cm), TDS (g/L), ammonium levels (mg/L - N), and nitrate levels (mg/L - N).

Table 2.1 Dates of conventional and eDNA surveys conducted for all three vernal pools, Ancient Woods Swamp (AWS), Ancient Woods Pond (AWP), and Preston Flats Ponds.

Dates	Active acoustic survey			Visual encounter survey			eDNA sample collection		
	AWS	AWP	PF	AWS	AWP	PF	AWS	AWP	PF
2019-04-08				✓	✓	✓	✓	✓	✓
2019-04-18					✓	✓		✓	✓
2019-04-19	✓	✓	✓						
2019-04-24				✓	✓	✓	✓	✓	✓
2019-05-02					✓	✓		✓	✓
2019-05-10				✓	✓	✓	✓	✓	✓
2019-05-19	✓	✓	✓						
2019-05-24				✓	✓	✓	✓	✓	✓
2019-06-07				✓	✓	✓	✓	✓	✓
2019-06-19	✓	✓	✓						
2019-06-27				✓	✓	✓	✓	✓	✓

Amphibian detection surveys were conducted at Ancient Woods Swamp (AWS), Ancient Woods Pond (AWP), and Preston flat pond (PF) between April 08, 2019, and June 27, 2019. Surveys included both conventional methods and eDNA collection. Conventional methods included active acoustic surveys using the marsh monitoring protocol, passive acoustic surveys using song meters, and visual encounter surveys. Checks indicate the dates and locations surveys were conducted. Note: Passive acoustic surveys using mounted acoustic song meters recorded twice daily (dawn and dusk) from May 02 to May 24, 2019, and June 07 to July 02, 2019. All recordings were analyzed for species detection.

2.2.4 eDNA sample filtration, extraction, and quality control

2.2.4.1 Decontamination for eDNA molecular processes

After field methods, eDNA filtration, extraction, quality control, and all qPCR preparation were completed. Throughout these processes, methodologies to mitigate potential contamination were conducted by using multiple decontamination procedures. These processes are completed in biosafety cabinets (BSC), or PCR hood lab stations depending on the stage of sample processing. This lab equipment was all decontaminated in similar ways. BSCs were decontaminated by spraying surfaces down with 10% bleach and left to sit for under 1 minute before removing liquid with clean lint-free wipes (Kimwipes™, Kimtech™, Fisher Scientific, Mississauga, ON, Cat. #06-666). Following bleaching, all surfaces were sprayed generously with 70% ethanol and let stand for under 1 minute before removing the liquid with clean lint-free wipes. The same process was completed for all PCR hoods with the substitution of 10% bleach for RNase AWAY surface decontaminant (ThermoFisher Scientific, Mississauga, ON, Cat. #7000TS1). Users of these lab stations wore lab coats used solely for that room or piece of equipment. Users additionally equipped gloves that were decontaminated in the same method as above, 10% bleach or RNase AWAY for under 1 minute, followed by 70% ethanol, with liquid removed via lint-free wipes. Any object or equipment entering the hood was also treated with the same decontamination solutions as used for the lab station. Following chemical decontamination, the hood and equipment inside were exposed to germicidal ultraviolet light (UV) for 30 minutes.

2.2.4.2 eDNA sample filtration

After the collection of water samples containing eDNA, the next step was to concentrate the eDNA through a filtration process. Before filtration, a laminar flow BSC and all required equipment underwent the decontamination processes (Section 2.2.4.1). After the chemical decontamination, the hood was also sterilized with UV for 30 minutes. Water samples were filtered by passing the entire sample through 0.45 µm cellulose nitrate filters

(Thermo Scientific, Mississauga, ON, Cat. #09-740-30K). Each water sample was poured in 250 mL increments into the filter cup of the single-use analytical filter funnels (Thermo Scientific, Mississauga, ON, Cat. #1452045) and a peristaltic pump was set to 70% power, which forced the sample through the filter. Generally, the entire 1 L was filtered through a single filter. In the case of a clog, where no additional volume had passed through the filter for 10 minutes, a second filter was used. All remaining water was passed through the second filter and then eDNA was pooled from both filters during the extraction process (Section 2.2.4.3). All filters were removed from the apparatus using forceps that underwent decontamination but were additionally dipped in 70% ethanol and flamed using a Bunsen burner. Filters were placed in a sterile petri dish so as not to touch the hood directly and filters were halved with scissors that underwent the decontamination process, were dipped in 70% ethanol and flamed in a Bunsen burner for 10 seconds. Filter halves were placed individually in labelled Axygen 5 mL RNase/DNase free screw cap tubes (Corning, NY, USA, Cat. #SCT-5ML) and stored frozen at -80 °C until eDNA extraction (Section 2.2.4.3).

2.2.4.3 eDNA extraction

eDNA was extracted from filters used to collect eDNA from water samples, field blank samples, and an extraction blank and was conducted in a decontaminated biosafety cabinet (BSC) (Section 2.2.4.1). An extraction blank, consisting of a new and sterile filter was extracted alongside samples from each vernal pool on a set sampling date. This extraction blank was used to detect potential contamination during the extraction process. DNA was extracted from filters using the SOX soil DNA extraction kit (MetagenomBio, Waterloo, ON, Cat. #18011_S0) according to the kit manual with minor modifications. This kit was used based on the ability to remove possible qPCR inhibitors such as humic acids. Each filter was rolled and cut into approximately 8–12 thin pieces using scissors and a scalpel dipped in 70% ethanol and then flamed for 10 seconds and sterile forceps. Filter fragments were placed in a SOX-bead beating tube with 850 µL of SOX1 solution and tubes were agitated using the Fisher brand Bead Mill 24 homogenizer (FisherScientific, Cat. #15-340-163) for 10 minutes. The remaining steps were carried out according to the SOX DNA

isolation kit user manual including optional centrifugation steps to account for high levels of inhibitory substances such as humic acids. Steps were also taken during the handling of the solution SOX3 to keep all working tubes and the reagent in dim light until the steps no longer contained solution SOX3. eDNA was eluted in 100 µL of the provided elution buffer into a 1.5 mL microcentrifuge tube (Fisherbrand™, Fisher Scientific, Ottawa, ON, Cat. #05-408-129) and stored at -80 °C until quality control of eDNA samples (Section 2.2.4.4) and eDNA amplification (Section 2.2.7.2).

2.2.4.4 eDNA quality control

eDNA samples commonly contain compounds and materials that can inhibit PCR reactions and, therefore, lead to false negatives. The DR-1 assay, designed in-house by Nathanael Harper and Dr. Michael Lynch, was used to determine if the extracted eDNA from vernal pool samples contained inhibitors by spiking an aliquot of the extracted eDNA sample with a known quantity of zebrafish (*Danio rerio*) DNA and comparing amplification against a control amplification with the same concentration of zebrafish DNA spiked into molecular-grade water. An aliquot of each eDNA sample based on vernal pool, date, and sampling location was spiked with 0.2 ng of zebrafish DNA containing a 298 bp section of the cytochrome b (*cytb*) gene (Supplementary Table 1). Amplification occurred under the following reaction conditions in a total 15 µL reaction volume; 7.5 µL TaqMan™ Environmental Master Mix 2.0 (ThermoFisher, Cat. # 4396838), 60 nM forward primer, 60 nM reverse primer, 16.6 nM DR-1 probe, 2 µL of 0.2 ng of internal positive control, 2 µL aliquot eDNA sample, and adjusted to final volume using molecular-grade water (Fisher Scientific). All reactions were run with control IPC reactions which have the same reaction conditions as the samples but substitute the eDNA aliquot with a matching volume of ultrapure water. Finally, for each vernal pool, 1 µL of eDNA from all sampling locations from the same sampling date was pooled. This pooled sample was used to determine background levels of amplification using the DR-1 assay. Triplicate 15 µL reactions were run with 7.5 µL TaqMan™ Environmental Master Mix 2.0 (ThermoFisher, Cat. # 4396838), 60

nM forward primer, 60 nM reverse primer, 16.6 nM DR-1 probe, 2 µL pooled eDNA aliquot sample, and adjusted to final volume using ultrapure water. All reactions underwent the same thermal cycling conditions; 50 °C hold for 2 minutes, 95 °C hold for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. These assays were amplified using QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON). Due to detection of some inhibited samples all samples were treated with ZYMO one step inhibition removal kit, and then the inhibited samples were retested.

The next step for quality assurance of eDNA samples is to ensure there is intact DNA present and that field methods, extraction, and storage did not lead to nucleic acid degradation. To test for eDNA integrity, samples were amplified using the ePlant5 assay which targeted a region (147 bp) in the chloroplast 23S rRNA gene (Veldhoen et al. 2016; Hobbs et al. 2019). Reactions with a final volume of 10 µL contained 5 µL TaqManTM Environmental Master Mix 2.0, 900 nM forward primer, 900 nM reverse primer, 250 nM ePlant5 probe, 0.95 µL ultrapure water, and 2 µL aliquot of eDNA sample. eDNA samples were run in duplicate and run alongside a standard curve of ePlant5 synthetic oligonucleotide standards. Thermal cycling conditions consisted of a hold at 50 °C for 2 minutes, a hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. These assays were amplified using QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON).

2.2.5 eDNA barcoding assay optimization

2.2.5.1 Generation of target sequence amplicons for use as target species qPCR standards

2.2.5.1.1 Tissue procurement

To obtain DNA for target amphibian species, tissues from roadkill or voucher specimens were obtained. Some tissue was collected in the form of roadkill near the field site at *rare* Charitable Research Reserve, roads near the University of Waterloo, and the

Kitchener-Waterloo region. Tissues from roadkill was obtained for the American toad, and spotted salamander. The American toad was found in April 2022 on the University of Waterloo campus, on Hagey Blvd, Waterloo, ON and was stored at -80 °C as a whole specimen, with muscle tissue cut from the leg used for DNA extraction (Section 2.2.5.1.2). The spotted salamander was found in the Spring of 2019 in Waterloo and was stored at -80 °C as a whole specimen, with a small section of muscle and skin from the tail used for genomic DNA extraction (Section 2.2.5.1.2). The wood frog tissue was collected in the summer of 2018 and provided as frozen dissected muscle tissue from the Katzenback lab (animal care protocol # 30008). The remaining tissue for three target species, the green frog, northern leopard frog, and spring peeper, was obtained from the Royal Ontario Museum's Natural History Department and provided as muscle tissue preserved in 95 % ethanol. All tissue used in this study originated from Ontario-caught amphibians. The northern leopard frog was collected in Minden, ON, the green frog was collected in Harburn, ON, and the spring peeper was collected in Ancaster, ON. Tissues stored in ethanol were rinsed with molecular-grade water before beginning genomic DNA extraction (Section 2.2.5.1.2). All tissues acted as a source of DNA for downstream use in standard curves during qPCR (Sections 2.2.5.1.2 2.2.5.1.6).

2.2.5.1.2 Genomic DNA extraction

Tissues, in this case, muscle and/or skin tissues, from target amphibian species were used to isolate DNA for downstream use in standard curves in qPCR. Between 10 to 25 mg of tissue was weighed, macerated with decontaminated scissors, and prepared according to Qiagen's DNEasy Blood and tissue kit (QIA-GEN Inc., Mississauga, ON, Canada; Cat. # 69506). Extraction was completed according to the DNEasy Blood and Tissue kit user manual but modified to contain a reduced volume of ATL buffer solution (160 µL) and an increased volume of proteinase K (40 µL) at the digestion stage of DNA extraction. Washes were completed according to the kit instructions with an additional centrifugation at 20,000 × *g* after washing with Buffer AW2. Eluted DNA was tested for quantity of double stranded nucleic acids using the absorption of 260 nm wavelength by the Take3 Trio on the Cytation 5

spectrophotometer (Agilent Technologies Canada Inc., Mississauga, ON L5N 5M4). Samples were measured and the absorbances at 280 nm, 260 nm, and 230 nm were used to determine the 260/280 ratio to indicate the presence of phenols or proteins, and the 260/230 ratio to determine the presence of organic contaminants. All DNA was stored at -20 °C until further use.

2.2.5.1.3 DNA amplification via PCR

A region of the cytochrome c oxidase subunit (COI) was amplified from the extracted amphibian DNA (American toad, green frog, spotted salamander, and wood frog) using universal primers (LC01490 and HC02198) through PCR (Folmer et al. 1994). This was completed using the New England Biolabs Standard Taq DNA Polymerase with Standard Taq Buffer Kit and protocol (New England Biolabs Inc. Whitby, ON, Canada; Cat. # M0273) in 50 µL reactions with final concentrations and volumes of 1× Standard Taq reaction buffer, 200 µM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 0.25 µl Taq polymerase, ~10 ng of DNA, and molecular grade water to adjust to the final volume. All amplifications were run alongside two no template control reactions which acted as negative controls. PCR thermal cycling was completed using a BioRad T100™ Thermal Cycler (BioRad, Mississauga, ON, Canada) with thermal cycling conditions as follows: initial denaturation of 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. A final extension at 72°C for 10 minutes was completed following the 40 cycles. PCR reactions were held at 4°C until gel electrophoresis (Section 2.2.5.1.4).

2.2.5.1.4 Gel electrophoresis

PCR product was verified to have only a single amplicon and expected size of the target amplicon through gel electrophoresis. Loading dye (6 ×) (FroggaBio, Concord, ON, Cat. #LD010) was added in 4 µL volume for every 20 µL of PCR product and was loaded into 1.5% agarose gel prepared with 1× TAE buffer and RedSafe Nucleic Acid Staining Solution (FroggaBio, Concord, ON, Cat. #21141). Samples were run alongside no template control reactions and 5 µl Genedirex 100 bp Ladder H3 RTU (Thermo Fisher Scientific,

Mississauga, ON, Canada; Cat. #SM0243). PCR products were electrophoresed for 60 minutes at 100 V. The resulting bands, if they correlated with the desired amplicon and there was no evidence of secondary bands, were excised from the gel with a clean scalpel and used directly for DNA extraction (Section 2.2.5.1.5).

2.2.5.1.5 DNA extraction from agarose gel

DNA was extracted from agarose gel slices using the EZ-10 Spin Column DNA Gel Extraction Kit (BioBasic Inc, Markham, ON, Canada; Cat. # BS354) according to manufacturer's instructions. DNA was eluted off the spin column with 50 µl of elution buffer and the nucleic acid concentration quantified using Qubit 4 Fluorometer (ThermoFisher Scientific, Mississauga, ON, Canada; Cat. #Q33226). Working solution was prepared for the fluorometer by adding 1 µL of Qubit HS reagent for every 199 µL Qubit buffer solution. For each sample, duplicate DNA reactions were prepared with 2 µL of DNA and 5 µL of DNA and adjusted to a final volume of 200 µL with Qubit working solution. The Qubit 4 fluorometer was first calibrated using the standard dilutions prepared as outlined in the machine's user manual. Nucleic acid quantification was completed using the dsDNA high sensitivity protocol. Remaining extracted DNA was stored at -20 °C for sequencing and standard preparation (Section 2.2.5.1.6 and Section 2.2.5.2).

2.2.5.1.6 DNA sequencing and nucleic acid quantification

The isolated PCR product was sent for Sanger sequencing at The Centre for Applied Genomics, The Hospital for Sick Kids, Toronto, Ontario. Sequences provided by TCAG, along with the nucleic acid concentration of the respective extracted DNA sample (Section 2.1.5.1.5) were used to calculate the molecular weight of each species-specific COI amplicon using Equation 1.

$$\text{Number of copies} = \frac{x \text{ (ng)} * 6.0221 * 10^{23} \left(\frac{\text{molecules}}{\text{mole}} \right)}{(N * 660 \left(\frac{\text{g}}{\text{mole}} \right) * 1 * 10^9 \left(\frac{\text{ng}}{\text{g}} \right))}$$

Equation 1

The concentration of isolated COI amplicons for each species was determined and then diluted to 1×10^8 copies/ μ L. These dilutions acted as tissue derived DNA standards for qPCR as the larger amplicon contained a nested region with the associated species-specific eDNA barcoding qPCR binding regions for primer and probes. The tissue derived standards were prepared for the American toad, green frog, spotted salamander and wood frog and used for optimization of eDNA barcoding assays via qPCR (Section 2.2.5). Issues with COI primer binding resulted in no preparation of tissue derived standards for northern leopard frog and spring peeper resulting in the design of synthetic standards for all species (Section 2.2.5.1).

2.2.5.2 Developing synthetic oligonucleotide standards and synthetic insert

Tissue derived standards offered the ability to use nested species-specific primers for qPCR, however, amplification of the correct region using universal COI primers was challenging for the northern leopard frog and spring peeper due to lack of available sequence data for regions where universal primers would bind. As a result, there was no way to ensure this region did not contain mismatches which lead to amplification issues. As a result, synthetic oligonucleotides (Integrated DNA Technologies, Coralville, Iowa, USA) were designed to match the target sequence of each species-specific qPCR region using sequences from Sanger sequencing (Section 2.2.5.1.6) or from Canadian sequences from BOLD (Supplementary Table 1). Sequences were aligned with top NCBI Blast (National Center for Biotechnology Information (NCBI)) matches as well as the previously designed species-specific amphibian primers and probes (Beauchlerc et al. 2019). The synthetic oligonucleotides, which act as known standards during eDNA processing, were designed to include a synthetic insert sequence (Wilson et al. 2016). The synthetic insert included three partial sequences derived from three restriction enzymes: *Bam*HI, *Hind*III, and *Stu*I. The synthetic insert sequence was queried against other sequences in the BLAST database to ensure no overlap with sequences from other amphibian species or geographically relevant flora and fauna. Additionally, each amphibian sequence with the synthetic insert was searched against the BLAST database for matches. Addition of the insert sequence did not

align with any species and was therefore used in the synthetic standards. The synthetic insert was used as a secondary probe binding region to allow for multiplexing and, therefore, detection of potential contamination from synthetic standards during qPCR in eDNA samples being evaluated. The synthetic oligonucleotides were used as a known standard for optimization (Section 2.2.6) of northern leopard frog and spring peeper eDNA barcoding assays. Optimization (Section 2.2.6) of the other four assays (American toad, green frog, spotted salamander, and wood frog) were completed with tissue derived standards. For assay optimized with tissue derived standards, standard curves of both tissue derived, and synthetic standards were compared to determine if results varied in respect to efficiency, fit, slope, and intercept. Synthetic standards were used as standard curves during the eDNA processing (Section 2.2.7.2) for all target amphibian species.

2.2.6 eDNA barcoding assay optimization

Each eDNA barcoding assay was optimized for optimal reaction conditions. Optimization was completed for primer (Section 2.2.6.2) and probe concentrations (Section 2.2.6.3) and annealing temperature (Section 2.2.6.4) of the reaction by using standard curves of known DNA copy number. Each optimization was carried out independently and the optimal condition carried forward to the next optimization step. Standards were prepared for each target species by first serially diluting either a tissue derived standard or a synthetic oligonucleotide standard from a known starting concentration of 1×10^8 copies/ μ L to 1×10^5 copies per reaction and then using this dilution to generate six standards using a 10 -fold serial dilution (10^5 to 10^0 copies per reaction). Standards were always prepared in the same manner but occasionally only a subset of standards were used for amplification of standard curves for certain optimization steps.

2.2.6.1 Primers and probe test

Custom primers and TaqMan MGB probes were used for all amphibian eDNA barcoding assays (Table 2.2). Custom primers used for all qPCR eDNA barcoding assays were delivered lyophilized and were resuspended in TE buffer (10 mM Tris, 1 mM EDTA,

pH 8.0). Resuspensions were performed according to manufacturer's guidelines regarding volume to create stock solutions of 100 μ M (Millipore Sigma, Oakville, ON, Pure and Simple Primers). All custom MGB TaqMan (Thermo Fisher) probes were resuspended by the manufacturer to a stock solution of 100 μ M.

Upon first use of primers and probes, an initial standard curve was run using PowerUp SYBR Green Master mix (Thermo Fisher) and another using TaqMan Environmental Master Mix 2.0 (Applied Biosystem, Mississauga, ON; Cat. # 43-968-38) to ensure initial reaction conditions resulted in amplification and for the later, that the probe was being detected throughout amplification. Reactions were prepared to meet the preliminary reaction concentrations seen in Beauclerc et al. 2019. Initial qPCR reactions for standard curves were completed at a volume of 15 μ L containing 7.5 μ L TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 200 nM of forward primer, 200 nM of reverse primer, 200 nM fluorescent TaqMan species-specific probe, 4.6 μ L molecular grade water, and 2 μ L of template DNA. Standards ranging from 1×10^5 copies per reaction diluted 10-fold to a final standard of 1 copy per reaction were amplified in triplicate technical replicates. The standard curve was run alongside three technical replicates of a positive control containing total extracted species DNA and three technical replicates of no template controls where the standard was substituted for ultrapure water. Amplification and quantification were carried out using a QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON) with the following thermal cycling conditions; an initial hold at 50 °C for 2 minutes, hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Table 2.2. Summary of eDNA barcoding qPCR primer and probe sequences used in this study.

Name	Latin name	Sense Primer	Anti-sense Primer	Probe	Target gene	Amplicon (bp)	Source
American toad	<i>Anaxyrus americanus</i>	GCAGGACCAT CAGTT GACTTAACC	GTGGTAATAA AATTA ATTGCCCCAAG	ACACCTGCT AGATGGAGG	COI subunit	86	Beauclerc et al. 2019
ePlant5	-	TCTAGGGATAA CAGGCTGAT	TGAACCCAGC TCACGTAC	TTTGGCACCT CGATGTCGG	chloroplast 23S rRNA	147	Veldhoen et al. 2016
Green frog	<i>Lithobates clamitans</i>	CATCCTCAACT ACACAATACCAA ACA	GGAGTATAGT AATTCCGGCAGC TARTA	TCTATTTGTC TGATCAGTCTT	COI subunit	111	Beauclerc et al. 2019
Internal positive control (zebrafish)	<i>Danio rerio</i>	TGCGAAAAAC ACACCCAG	GGCAGATGAA GAAGAAGGAAG	CAATACACT ACACCTCAGAC ATCT CAACAGCA	Cytochrome b (<i>cytb</i>)	298	Nathanael Harper, Michael Lynch (UWaterloo)
Northern leopard frog	<i>Lithobates pipiens</i>	CACACAGTACC AAAC ACCCCTATTT	GAGTATAGTA ATCCC TGCCGCTAGAA	TGTTTTGATC ACCGCAGTT	COI subunit	100	Beauclerc et al. 2019

Spotted salamander	<i>Ambystoma maculatum</i>	CGCATTCGTAA TGAT TTTCTTCAT	AAAGCTATAT CTGGT GCCCCAAT	ATGCCTGTTA TAATTGGCGGA	COI subunit	99	Beauclerc et al. 2019
Spring peeper	<i>Pseudacris crucifer</i>	CCATCTTTCCT TCTT CTCCTCG	TGCATGTGCT AAGTT CCCAGC	GTGTAGAGG CAGGTGC	COI subunit	99	Beauclerc et al. 2019
Synthetic insert	-	-	-	GGATCCAAG CTTAGGCCT	<i>Bam</i> HI, <i>Hind</i> III, <i>Stu</i> I	Assay + 18	Wilson et al. 2016
Wood frog	<i>Rana sylvatica</i>	GCCCCTCAGTA GATTTAGCTATTT TC	AGRGGTGTTT GATATTGTGTAG TTGATGA	CACCTAGCT GGTGTTTC	COI subunit	130	Beauclerc et al. 2019

Primers and probes for qPCR assays to detect eDNA through eDNA barcoding for six target amphibian species. Additionally, quality control assays, internal positive control, and ePlant5 primer and probe sequences are included. Note: For all amphibian assays, amplicons used as standards include an additional 18 bp from the synthetic probe insert. The synthetic probe does not have associated primers since it uses the species-specific primers for each amphibian assay.

2.2.6.2 Primer concentration optimization

To optimize the concentration of primer used for future assays, the standards were prepared as above (Section 2.2.6). A modified standard curve was run in triplicate with standards of 1×10^3 , 1×10^2 , 1×10^1 copies per reaction along with triplicate NTCs for each curve. Each qPCR reaction for the standard curves was completed at 15 μ L containing 7.5 μ L TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 200 nM fluorescent TaqMan probe, 2 μ L of sample, both forward and reverse primers at one of the following: 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 600 nM, 700 nM, or 800 nM, and volume adjusted with molecular water (Table 2.3). All qPCR reactions were amplified using a QuantStudio 5 real-time PCR machine with the following thermal cycling conditions; an initial hold at 50 °C for 2 minutes, hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Standard curves were compared, and optimal conditions were selected for based on efficiency, R^2 , slope, error, y-intercept, and detection at low copy number of standard. In cases of multiple good contenders in order to select the optimal conditions, the methods above were repeated with an extended standard curve with template DNA starting at 1×10^5 copies per reaction followed by a 10-fold dilution series to a final standard of 1 copy per reaction.

2.2.6.3 Probe concentration optimization

To optimize the concentration of probe used for future assays, the standard curves were prepared by diluting target template synthetic oligonucleotides from a starting concentration of 1×10^8 to 1×10^4 copies per reaction followed by a 10-fold serial dilution to a final quantity of 1×10^0 copy per reaction. Each qPCR reaction for the standard curves was completed at a volume of 15 μ L containing 7.5 μ L TaqMan Environmental Master Mix 2.0 (Applied Biosystems), species-specific forward and reverse primers at the concentration deemed optimal (Section 2.2.6.2) (Table 2.5), 2 μ L of sample, a fluorescent TaqMan probe concentration of one of the following: 75 nM, 125 nM, 200 nM, 275 nM, 350 nM, and final reaction volume adjusted using molecular water (Table 2.3). Standard curves were amplified

using QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON) with the following thermal cycling conditions; an initial hold at 50 °C for 2 minutes, hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Standard curves were used to determine optimal for efficiency, R^2 , slope, error, y-intercept, and detection at low copy number of standard.

2.2.6.4 Optimization of annealing temperature

Standard curves were prepared as seen in Section 2.2.6, with standard ranging from 1×10^5 to 1×10^0 copies per reaction with a 10-fold dilution between each standard concentration. The optimal annealing temperature was determined by running five separate standard curves with seven concentrations of standards. Standards were amplified in triplicate technical replicates alongside three technical NTC reactions and three technical positive control reactions containing total DNA from the target species. All amplifications were carried out with the optimized assay concentrations for each species-specific assay (Table 2.5). Each of these five standard curves was amplified using QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON) with the thermal cycling condition of 50 °C hold for 2 minutes, 95 °C hold for 10 minutes, followed by 45 cycles at 95 °C for 15 seconds and one of the following annealing temperatures for 1 minute; 55 °C, 57.5 °C, 60 °C, 62.5 °C, or 64 °C. Standard curves optimized for efficiency, R^2 , slope, error, y-intercept, and detection at low copy number of standard. Following the determination of optimal annealing temperature, eDNA barcoding assays were applied under the optimal concentrations and conditions for multiplex testing, sensitivity testing, and eDNA sample processing (Table 2.5) (Sections 2.2.6.6-2.2.6.7).

Table 2.3. Conditions tested for eDNA barcoding assay optimization.

Forward primer concentration (nM)	Reverse primer concentration (nM)	Probe concentration (nM)	Annealing temperature (°C)
100	100	200	60
200	200		
300	300		
400	400		
500	500		
600	600		
700	700		
800	800		
*Previously determined primer concentrations		75	60
		125	
		200	
		275	
		350	
*Previously selected primer and probe concentrations			54
			57.5
			60
			62.5
			64

All conditions tested for assay optimization including all combinations of forward and reverse primer concentration (nM), probe concentration (nM), and annealing temperature (°C). Condition being tested was altered while all others were held steady to allow for direct comparison and selection of condition.

2.2.6.6 Multiplexing

After assays were fully optimized, the designed synthetic oligonucleotide standards (Integrated DNA Technologies, USA), which were designed to contain a unique sequence for the binding of a synthetic probe (Wilson et al. 2016), were tested for multiplexing compatibility. The goal of multiplexing with a synthetic insert was to detect false positives from contamination originating from standard curves run on each qPCR plate. These samples would amplify with the second probe if detected while processing field samples for eDNA. Using synthetic oligonucleotide standards, a 10-fold dilution series was prepared from a stock of 1×10^8 copies/ μL to make four standards ranging from 1×10^4 to 1×10^1 copies. Three standards curves were prepared, each with triplicate technical replicates of four standards at concentrations ranging from 1×10^4 to 1×10^1 copies per reaction alongside three reactions containing total amphibian DNA and three no template controls. Reactions containing total amphibian DNA acted as a positive control for the species-specific eDNA barcoding assays and as a negative control for the synthetic probe assay since the DNA does not contain the synthetic insert. The three standard curves prepared were a singleplex targeting the amphibian species-specific probe, a singleplex targeting the synthetic probe, and a multiplexed standard curve containing both the amphibian species-specific and synthetic insert probe. Each qPCR reaction had a final volume of 15 μL containing 7.5 μL of TaqMan Environmental Mastermix 2.0 (Applied Biosystem, Mississauga, ON; Cat. # 43-968-38), and the optimized concentration for forward primer, reverse primer, species specific probe and 2 μL of standard (Table 2.5). All qPCR reactions were amplified using QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON) with the following thermal cycling conditions; an initial hold at 50 °C for 2 minutes, hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and the optimized annealing temperature of 57.5 °C or 60 °C for 1 minute. Results were analyzed using Design and Analysis Software 2.6.0 (Applied Biosystems, Mississauga, ON) and singleplex standard curves were compared to the multiplex standard curves based on efficiency, R^2 , slope, error, y-intercept, and detection at low copy number of standard (Supplementary Table 1).

2.2.6.7 Sensitivity of eDNA barcoding assays

Assays with completed optimizations for primer concentration, probe concentration, and annealing temperature were tested for assay sensitivity. This was completed by determining the limit of detection (LOD) and limit of quantification (LOQ) for each amphibian eDNA barcoding assay. Dilution series of a known quantity of target COI amplicons were prepared using previously extracted, amplified, and isolated DNA of spotted salamander and wood frog. For the remaining target species, synthetic oligonucleotide standards were prepared to determine the LOD and LOQ of the assays. A standard curve was prepared in triplicate starting at 1×10^5 copies per reaction and continuing with a 10-fold dilution series to a final concentration of 1×10^0 copies per reaction. To calculate the LOD and LOQ, six 4-fold dilutions were prepared starting at a quantity of 1024 copies per reaction to a single copy per reaction (1024, 256, 64, 16, 4, 1). Each of these LOD and LOQ standards were amplified in replicates of 24 (Klymus et al. 2019) by adding 2 μ L of standard to each 15 μ L reactions containing the optimized assay conditions for the target species as previously specified (Table 2.5). LOD and LOQ determinations for a single target species were prepared on the same day, with the same standards, and used shared master mix of qPCR reagents to ensure accuracy between plates. Thermal cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes followed by 45 cycles of 95 °C for 15 seconds, and the optimized annealing temperature for 1 minute (Table 2.5). All qPCR reactions were amplified using QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON). Amplification results were exported and used to determine the LOD and LOQ using the R script “eLowQuant” adapted from Lesperance et al. 2021. All calculations were completed using R studio 2022.07.1.

2.2.7 eDNA sample processing

2.2.7.1 eDNA integrity

eDNA underwent long-term storage at -80 °C from April-June 2019 until April 2023, so prior to applying developed eDNA barcoding assays, the eDNA was re-tested for integrity

using the ePlant5 qPCR assay (Veldhoen et al. 2016; Hobbs et al. 2019) targeting chloroplast 23S rRNA gene of mtDNA (Table 2.2). In a PCR hood, 10 μ L qPCR reactions were prepared to contain 5 μ L of TaqMan Environmental Mastermix 2.0 (Applied Biosystem, Mississauga, ON; Cat. # 43-968-38), 900 nM of forward primer, 900 nM of reverse primer, 250 nM of ePlant5 probe, 2 μ L of sample, and adjusted to final volume with molecular-grade water. Each plate included a standard curve of ePlant5 target synthetic oligonucleotide standards with 10 $\times$ serial dilutions from 1×10^6 copies per reaction to 1 copy per reaction. Alongside the standard curve, three NTC reactions were prepared to control for level of contamination during qPCR set up. All eDNA samples were tested for chloroplast DNA and run only in duplicate to conserve eDNA sample for amphibian barcoding. The technical replicate reactions, run in duplicate, for each sample were randomly assigned a well position side by side on the plate. Thermal cycling conditions for the ePlant5 reactions were completed using the QuantStudio 5 real-time PCR machine (Applied Biosystems, Mississauga, ON) with conditions as follows; an initial hold at 50 °C for 2 minutes, hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Reactions with Ct values below 30 cycles were considered to contain intact eDNA.

2.2.7.2 eDNA barcoding assay application

A total of 142 eDNA samples were processed, 60 from Ancient Woods Swamp, 42 samples from Ancient Woods Pond, and 40 samples from Preston flats. On each processing day, a subset of 22 vernal pool eDNA samples were thawed, re-tested for integrity (Section 2.2.7.1), and amplified with each species-specific eDNA barcoding assay. Each plate was set to contain a standard curve, containing 1×10^5 copies per reaction to 1 copy per reaction in triplicate, 12 NTCs, and 22 randomly selected samples (including vernal pool eDNA samples and field/extraction blanks). Each of the samples were run in triplicate and randomly assigned to well positions, though sample replicates remained adjacent. During pipetting of eDNA samples and synthetic oligonucleotide standards, sections of the qPCR plate not receiving sample was covered to reduce cross contamination. Each reaction was set up

according to the assay's optimized primer and probe concentrations (Table 2.5). Final reactions contained 7.5 μ L of TaqMan Environmental Mastermix 2.0 (Applied Biosystem, Mississauga, ON; Cat. # 43-968-38), the optimized concentration of forward primer, the optimized concentration of reverse primer, optimized concentration of species-specific probe, equal concentration of synthetic probe as species-specific probe, 2 μ L of sample, volume adjusted to a final 15 μ L with ultrapure water. All qPCR plates were run on a QuantStudio 5 real-time PCR machine with conditions as follows; an initial hold at 50 °C for 2 minutes, hold at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and optimized temperature for 1 minute.

2.2.8 Analysis

2.2.8.1 Audio data analysis using Kaleidoscope Pro analysis software

Audio data from the SM4 acoustic song meters, which consisted of one hour of audio recordings at dusk and dawn from May 02 to July 02, 2019, were collected and analyzed with Kaleidoscope Pro analysis software (Wildlife Acoustics). To conduct presence-absence determination from all audio files, detections were clustered using advanced classifiers to extract and group similar vocalizations.

2.2.8.2 Training Kaleidoscope Pro and advanced classifiers

To create advanced classifiers, initial cluster analysis was conducted with default settings and files were scanned to form clusters between the frequency range of 100 Hz to 4000 Hz. From initial cluster analysis from each location, species detections were identified and the corresponding audio files with high-quality examples of each species calls were set aside for training the classifiers. After multiple training files for each species were isolated, from multiple vernal pool locations where possible, kaleidoscope advanced classification was conducted.

Training files were loaded into Kaleidoscope Pro Analysis software (Wildlife Acoustics), then they were scanned for all detections, and clustered into “like” detections.

For this process, Kaleidoscope Pro was set to non-bat analysis mode to extract sounds at the proper frequency settings. The batch tab was used to select the input directory, containing training files with good examples of the vocalizations of the species of interest, and set the output directory where kaleidoscope will deposit the resulting cluster data. Signal parameters were set to scan recording frequencies between 100 and 4000 Hz. The possible call length was set from 0.1 seconds to 20 seconds. Finally, the program determines where a new call starts based on the inter-syllable gap which was set as 0.35 to 0.5 seconds. Cluster settings remained on default except cluster numbers which were modified to 300 possible clusters.

Clusters were manually identified as either the target species, a blank for unclear or overlapping calls of target species, or “not frog” when clusters misidentified a species. All clusters with non-target species remained unlabeled and the program excluded them from further training. The first cluster .csv file was saved and used to rescan training files using a hidden Markov model which considers only labelled detection and omits poor/blank IDs from the model to produce new clusters. This rescan process is repeated until the target cluster contains high-quality examples of species detections with minimal to no false positives. The final pass of the cluster analysis was used as an advanced classifier and applied to all audio data.

For each amphibian detected via audio files, two advanced classifiers were made, one where overlapping calls were included in the classifier to decrease false negatives and a strict advanced classifier with only high-quality calls included to decrease false positives. The strict advanced classifier was trained with the same signal parameters as the general advanced classifier, however, when analyzing the clusters containing target species calls, the best detections within a distance of 0.50 to the cluster center were selected and all other calls were excluded from the training model. The two classifiers were both run and compared to determine the results used for upstream analysis. The selected classifier for each species was applied to all audio files and species detections were all manually reviewed and reassigned as target or non-target to a distance of 1.0 from the cluster center. Each detection, which

consisted of an instance when Kaleidoscope identified a separate instance of calling, was totalled to determine frequencies of calls on each sampling date and used for data analysis.

2.2.8.3 eDNA statistical analysis

The coefficient of variation for eDNA samples was calculated to determine the level of variation between detections of eDNA samples that were amplified in all three triplicates. This was calculated by quantifying the eDNA concentration of all eDNA samples that were amplified in triplicate above the limit of quantification and averaged. Calculations were completed by dividing the mean standard deviation by the mean quantity of eDNA from each sample. These calculations were completed using R studio 2022.07.1. For binary presence-absence data, eDNA amplifications at all confidence levels were considered as detection of presence. Additionally, amplifications below the LOD were considered for binary presence/absence data due to the use of negative controls and synthetic insert during the qPCR amplification (Thalinger et al. 2021; Abbott et al. 2021). No amplifications below the LOQ were used for any quantitative analysis and only represented presence/absence in a sample.

Species richness was calculated for each survey type by determining the mean number of species detected on each sampling date and averaged for detection from the three vernal pools. The associated standard of error was calculated for each species detection method as well. Calculations were completed using Microsoft Excel.

Detection of eDNA as presence-absence data was analyzed to determine if the date, vernal pool, sampling location or water quality metrics (Supplementary Table 2) significantly affected species detection. Each species assay was assessed using generalized linear regression models (GLMs) with binary detection as the response and using the “bestglm” function in R studio 2022.07.1 to determine the relevant predictor variables for each species detection. The significance of these functions was determined using a type III likelihood-ratio test with the function anova in in R studio 2022.07.1. eDNA detection data was also

analyzed to determine if spatiotemporal patterns played a role in eDNA detection. This was determined by using generalized linear regression models with detection as the response variable and date, vernal pool, and sampling location around the vernal pool as predictor variables. These GLMs were analyzed for significance using the *anova* function of the *car* package in R studio 2022.07.1 specifying type III likelihood ratio testing. This was not completed for the spring peeper as there was insufficient eDNA detection. Additional analysis was completed to determine if there were significant differences in eDNA quantity detected between the duplicate biological field replicates collected during eDNA sampling using ANOVA. Analysis was completed in R studio 2022.07.1 to determine if eDNA quantity was significantly different between biological field replicates using an ANOVA followed by a post-hoc Tukey test.

eDNA barcoding as a method of species detection was compared to the three conventional monitoring techniques by using generalized logistic mixed effects models (GLMERs). This method allowed for binary detections to act as the response variable with each method as the predictor with the addition of vernal pool location and date as a random effect. The use of the vernal pool and date as random effects controls for the non-independence of sampling due to sampling the same vernal pools over time. The significance of these models was determined using a Wald's type III likelihood-ratio test with the function *anova* using *car* package in R studio 2022.07.1. These analyses (p-values) alongside observed patterns in the data were used to examine the effect of eDNA barcoding compared to the conventional monitoring techniques.

2.3 Results

2.3.1 Conventional Data

Current amphibian monitoring relies on the use of conventional methods of species detection such as trapping, acoustic surveys, and visual encounter surveys. This study focuses on the use of non-invasive methods of species detection. Through non-invasive acoustic surveys and visual encounter surveys, conventional methods successfully detected five of the six target species using three different methods of detection. Depending on the method of detection, different species were better targeted than others. Each survey method had benefits and deficits in the associated detection ability.

2.3.1.1 Acoustic surveys

2.3.1.1.1 Active acoustic monitoring – Marsh monitoring protocol

Active acoustic monitoring surveys were conducted at each of the three vernal pools once a month, for three months (April, May, June), using the marsh monitoring protocol (Bird Studies Canada 2009), which detects calling males during the species' breeding season. This method is field intensive, potentially disturbing habitat and breeding activity, but gains quick and easily interpretable species detections. Overall, six anuran species were detected across the vernal pools on three dates ranging from April 19, 2019, and June 19, 2019. Of these six species, the five target calling anurans (American toad, green frog, northern leopard frog, spring peeper, wood frog) and one non-target anuran species (grey treefrog, *Hyla versicolor*) were detected. At Preston Flats Pond, northern leopard frog, American toad, and green frog were detected. Northern leopard frog calls were detected at a call level code 1 on April 19, and was the only calling amphibian species detected in April at this vernal pool. American toads and green frogs were detected in May at a call level 1. The green frog was the only calling amphibian species detected in June at Preston Flats Pond (Table 2.4). At Ancient Woods Pond, only two calling amphibian species were detected. Wood frogs were detected during the April survey date at a calling code 1. No amphibian calls were detected

on the second survey date in May. However, a single non-target amphibian species, the grey tree frog (*Hyla versicolor*), was detected in June. At Ancient Woods Swamp, three amphibian species were detected through observation of audio calls through active acoustic surveys. Spring peeper calls were detected in April at a calling code 2. Northern leopard frogs were detected in May, calling at a code level 1. In June, the non-target gray treefrog was detected at a calling code 1. Active acoustic surveys were sporadic in species detection between survey date and vernal pool sampling location potentially related to breeding period and environmental conditions.

Table 2.4 Amphibian call detections at three vernal pools with active acoustic surveys using the Marsh Monitoring Protocol.

Sampling date	Vernal pool	Target species					Non-target species
		American toad	Green frog	Northern leopard frog	Spring peeper	Wood frog	Gray treefrog
2019-04-19	Ancient Woods Swamp	0	0	0	Code 2	0	0
2019-04-19	Ancient Woods Pond	0	0	0	0	Code 1	0
2019-04-19	Preston Flats Pond	0	0	Code 1 (4)	0	0	0
2019-05-19	Ancient Woods Swamp	0	0	0	0	0	0
2019-05-19	Ancient Woods Pond	0	0	0	0	0	0
2019-05-19	Preston Flats Pond	Code 1 (4+)	Code 1 (1)	0	0	0	0
2019-06-19	Ancient Woods Swamp	0	0	0	0	0	Code 1 (1)
2019-06-19	Ancient Woods Pond	0	0	0	0	0	Code 1 (7)
2019-06-19	Preston Flats Pond	0	Code 1	0	0	0	0

Codes indicate level of calling, code 1 indicates no overlapping calls, code 2 indicates some overlapping calls but still distinct individuals and code 3 indicates a full chorus as defined in the Marsh Monitoring Protocol (MMP) (Bird Studies Canada 2009). Numerical values in brackets beside the calling codes indicate the number of individuals of that species counted on that date if possible. A 0 indicated no species detection at that location and date.

2.3.1.1.2 Passive acoustic surveys – Mounted song meters

Passive acoustic monitoring can be used to detect amphibian calls over a long time frame to record data more frequently (Melo et al. 2021), though the required data analysis is more extensive. This method is also non-invasive as it limits the number of times field technicians must go to the field and disturb breeding habitats and behaviours while still capturing the calls of species that call asynchronously through the season. In this study, acoustic song meters were set to record twice daily from May 02 to July 04, 2019. Collectively, this method detected four target species, the American toad, green frog, northern leopard frog, spring peeper, and an additional non-target amphibian species, the Grey treefrog (*Hyla versicolor*) across the vernal pools and timeframe studied. The number of detections and seasonal time of detection often mimics the species' associated life history with calls generally matching the expected breeding period for the American toad, green frog, and spring peeper (Figure 2.3). Spring peeper detections were also more prevalent at both Ancient Woods Swamp and Ancient Woods Pond with few detections at Preston Flats Pond. The calls of the northern leopard frog were sporadic and detection with acoustic song meters showed a few peak days of calling but overall, no distinct patterns. The American toad called for two one-week periods at Preston flats wetland (Figure 2.3). This is similar to the expected breeding habits of this species, with approximately two weeks of calling with a few days of non-calling, likely related to poor conditions such as temperature, wind speed, or background noise. This method of species detection allowed for more extensive temporal data collection and controlled for the asynchronous calling behaviour of some amphibian species.

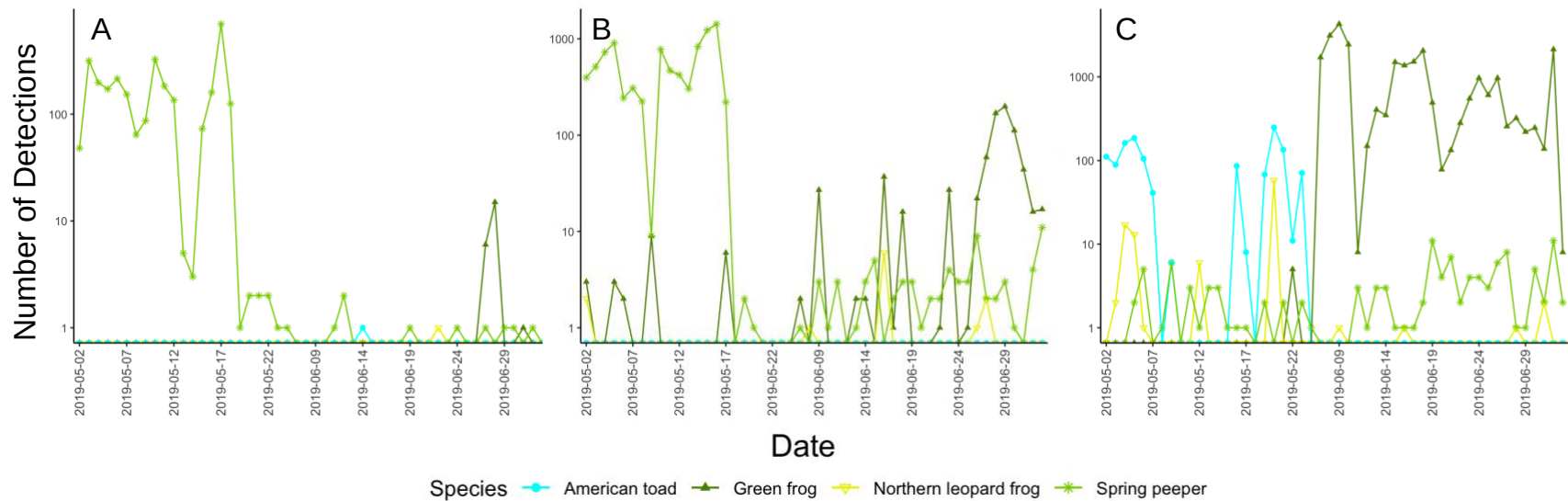


Figure 2.3. Temporal anuran amphibian call detections with passive acoustic monitoring using song meters.

Amphibian call recordings were captured at dawn and dusk at three vernal pools from May 02 to July 4, 2019, using SM4 acoustic song meters. The number of amphibian call detections at (A) Ancient Woods Swamp, (B) Ancient Woods Pond, and (C) Preston Flats Pond from recordings at dawn and dusk from May 02 to July 4 using SM4 acoustic song meters (Wildlife Acoustics, Maynard, MA, USA). Detections were determined using Kaleidoscope Pro advance classifier cluster analysis and plotted on a log₁₀ scale. Call detections are reported for American toad, green frog, northern leopard frog, and spring peeper. Note: Song meters experienced power failure from May 24, 2019, to June 07, 2019, and thus there is no call data from this time period.

2.3.1.2 Visual encounter surveys

Visual encounter surveys were used to detect amphibian species through an alternative non-invasive conventional method. Non-invasive visual encounter surveys were conducted five times at Ancient Woods Swamp, and seven times at both Ancient Woods Pond and Preston Flats Pond between April 08 and June 27, 2019. Visual encounter surveys were conducted by walking around the periphery of each vernal pool and observations for amphibian species and numbers were recorded. Using this method, three target amphibian species were detected (green frog, northern leopard frog, wood frog) across all vernal pools and survey dates. In some cases, during visual encounter surveys, other amphibians were detected but not accurately identified before fleeing, resulting in the recording of an “unknown” amphibian detection. Overall, 54.6 % of the detections were green frog, 6.8% of detections were northern leopard frog, and 1.2% were wood frog detections (Figure 2.4). The remaining 36.7% of detections were classified as unknown, due to amphibian being spotted but not identified before the organism was obscured (Figure 2.4). At Ancient Woods Swamp, two species, green frogs and northern leopard frogs, were detected using the VES method. Green frogs, northern leopard frogs, and wood frogs were detected at Ancient Woods Pond. At Preston Flats Pond, green frog, northern leopard frog, and wood frog were all detected. Although there was a detection of one wood frog at Preston flats there was no evidence of calling or other detections at this site so this could have been a migrating individual. At all three vernal pools, a large proportion of amphibian detections were “unknown” indicating that the amphibian spotted was not identifiable before becoming obscured from view.

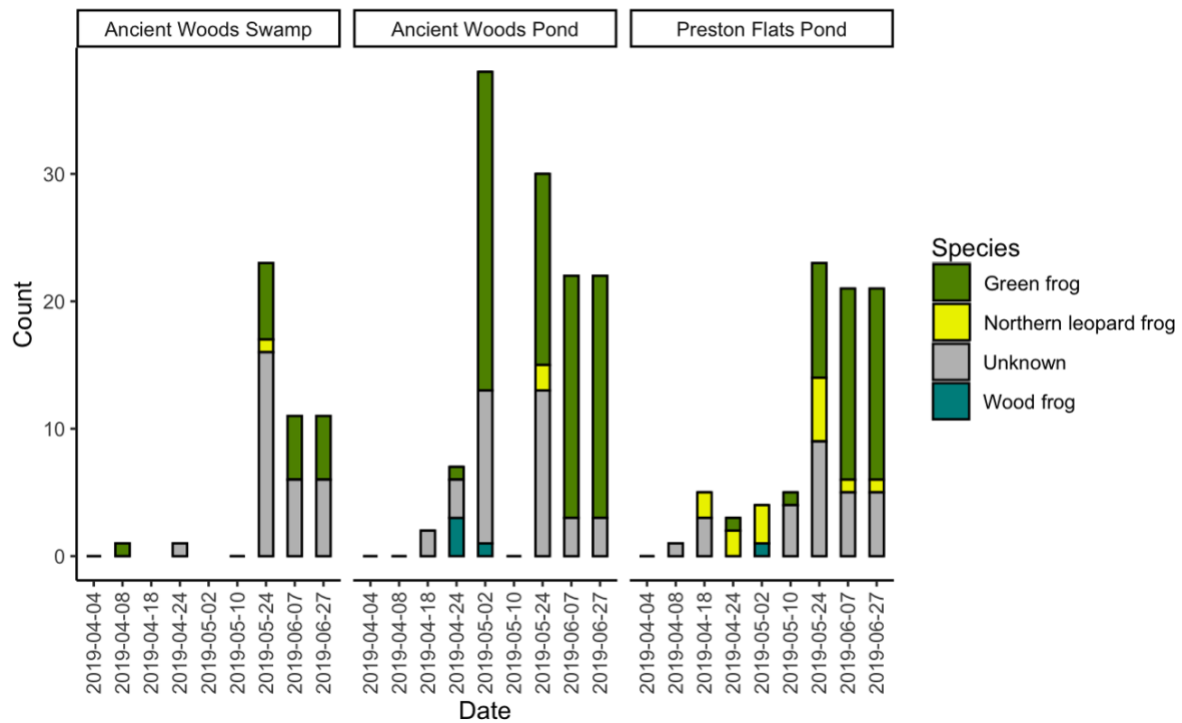


Figure 2.4. Amphibian species detections across three vernal pools using visual encounter surveys.

Detections of amphibian target species using conventional visual encounter surveys for three vernal pools; Ancient Woods Swamp, Ancient Woods Pond, and Preston Flats Pond. Counts for all species detected on each survey date from May 4, 2019, to June 27, 2019. In addition to target species, any amphibian spotted but not positively identified was classified as “unknown”.

2.3.2 qPCR

2.3.2.1 Assay optimization

2.3.2.1.1 Optimal assay conditions for eDNA barcoding assays

Each eDNA barcoding assay was optimized for optimal primer (forward and reverse) concentration, probe concentration, and annealing temperature (Table 2.5). Optimized assays achieved efficiencies of 88.7 % - 101.7 % and fall within the acceptable qPCR assay efficiency range of 80-110 % (Bustin et al. 2009; Nybo 2011). Using tissue derived or synthetic oligonucleotide standards, assays were tested for multiple primer concentrations, probe concentrations, and annealing temperatures to determine the optimal conditions. An equal concentration of synthetic insert probe was multiplexed with the species-specific probes to detect possible contamination from standards. The resulting standard curves showed no change to standard curve R^2 or efficiency between singleplex or multiplexed assays for species-specific assays or the synthetic insert assay for each species (Supplementary Table 1). These optimized assays were subsequently used during eDNA sample processing (Section 2.2.7.2) to detect target eDNA. Standards were multiplexed with a synthetic insert (Wilson et al. 2016) to detect an insertion sequence in the synthetic oligonucleotide used in preparing standard curves and thus differentiate potential contamination from these synthetic oligonucleotides.

Table 2.5. Optimized assay conditions for eDNA barcoding assays for six target amphibian species.

Species	Common name	Optimal sense primer concentration (nM)	Optimal anti-sense primer concentration (nM)	Optimal probe concentration (nM)	Optimal annealing temperature (°C)	R ²	Efficiency
<i>Anaxyrus americanus</i>	American toad	200	200	200	60.0	0.99	98.6%
<i>Lithobates clamitans</i>	Green frog	200	200	275	57.5	0.99	88.7%
<i>Lithobates pipiens</i>	Northern leopard frog	100	100	125	57.5	0.99	94.3%
<i>Ambystoma maculatum</i>	Spotted salamander	200	200	125	57.5	0.98	101.7%
<i>Pseudacris crucifer</i>	Spring Peeper	200	200	275	57.5	0.98	95.0%
<i>Rana sylvatica</i>	Wood frog	300	300	275	60.0	0.99	92.0%

2.3.2.1.2 Limit of detection and quantification

Assay sensitivity was determined using the “eLowQuant” R script adapted from Lesperance et al. 2020 and the experimental set up from Klymus et al. 2019 (Table 2.6) (Klymus et al. 2019; Lesperance et al. 2021). All species-specific assays were determined to best fit a non-intercept model. No assay had a level of blank (LOB) indicating no amplification in no template control reactions which indicates no background noise from the assay. A serial dilution of species-specific DNA standards was used to determine the standard at which amplification no longer occurred at a rate of 95% to determine the DNA copy number that represents the limit of detection. The limit of detection was determined for each species-specific assay and the total range of the limit of detection when modelling for 3 technical replicates was 3.4 copies per reaction for the spotted salamander assay to 11.9 copies per reaction for the wood frog assay. All limits of detection had a calculated confidence of a lower and upper limit to the limits (Table 2.6).

The limit of quantification, which is the DNA concentration at which the coefficient of variation below 35% (Klymus et al. 2019) but was fitted with models using standard curves, standard error and a Binomial-Poisson statistical distribution to avoid the previously used overfitting of higher order polynomials. Data was obtained using the experimental design from Klymus et al. 2019, and the eLowQuant script adapted from Lesperance et al. 2020 determined the limit of quantification for each assay modelled for 3 technical replicates with an associated upper and lower confidence limit (Table 2.6). The limit of quantification ranged from 7.7 copies per reaction to 26.7 copies per reaction for the spotted salamander assay and wood frog assay respectively. The limit of quantification represents that the cut off and eDNA quantities below the limit of quantification cannot accurately be determined but binary presence-absence can be inferred.

Table 2.6. Limit of detection (LOD) and quantification (LOQ) for amphibian eDNA barcoding assays.

Species	Abbreviation	alpha	aSE	beta	bSE	LOB	LODL	LOD	LODU	LOQL	LOQ	LOQU
<i>Anaxyrus americanus</i>	AMTO	0.0	0.0	0.1	0.0	0	6.9	9.4	14.5	15.5	21.0	32.5
<i>Lithobates clamitans</i>	GRFR	0.0	0.0	0.2	0.0	0	4.7	5.9	7.8	10.5	13.1	17.5
<i>Lithobates pipiens</i>	NLFR	0.0	0.0	0.2	0.0	0	3.9	5.3	7.9	8.8	11.8	17.7
<i>Pseudacris crucifer</i>	SPPE	0.0	0.0	0.1	0.0	0	8.5	11.6	18.4	19.0	26.0	41.2
<i>Ambystoma maculatum</i>	SPSA	0.0	0.0	0.3	0.1	0	2.7	3.4	4.7	6.1	7.7	10.5
<i>Rana sylvatica</i>	WOFR	0.0	0.0	0.1	0.0	0	10.0	11.9	14.9	22.3	26.7	33.3

Amplification data from DNA standards from six target species assays were analyzed for LOD and LOQ and fit with the non-intercept model due to no amplification of the no template control reactions and no detection of a limit of blank (LOB). Limit of detection (LOD) is reported with a lower confidence limit (LODL) and an upper confidence limit (LODU). The limit of quantification (LOQ) is reported with a lower confidence limit (LOQL) and an upper confidence limit (LOQU). Both LOD and LOQ along with associated confidence limits were reported in copies per reaction. Alpha represents the occurrence rate for the number of no template controls amplified along with the associated standard error (aSE). Beta represents the number of failures of amplification in a standard reaction with the associated standard error (bSE).

2.3.2.2 Detection of eDNA shed from target amphibians in vernal pools

2.3.2.2.1 Quality control of eDNA samples

All eDNA samples were screened for qPCR inhibitors by spiking in aliquots of a known quantity of zebra fish DNA. Amplification of the samples saw no inhibition of reactions at any vernal pool in April. Samples in April amplified within three cycles of the control, indicating an acceptable level of inhibitors. However, May and June, amplification was not achieved in Ancient Woods Swamp (AWS) due to inhibition. All samples were treated with ZYMO one step inhibition removal kit, and then the inhibited samples were retested. This resulted in reduction of PCR inhibitors so that all amplifications were no later than three cycles compared to the control IPC reactions.

To test for integrity of DNA, amplification of chloroplast DNA was conducted via qPCR due to the widespread and ubiquitous nature of chloroplasts in waterbodies. eDNA was deemed intact when amplification was detected below 30 cycles. The average number of cycles is significantly lower than 30 cycles for all sampling dates and for each vernal pool (Figure 2.5) indicating no significant level of eDNA degradation.

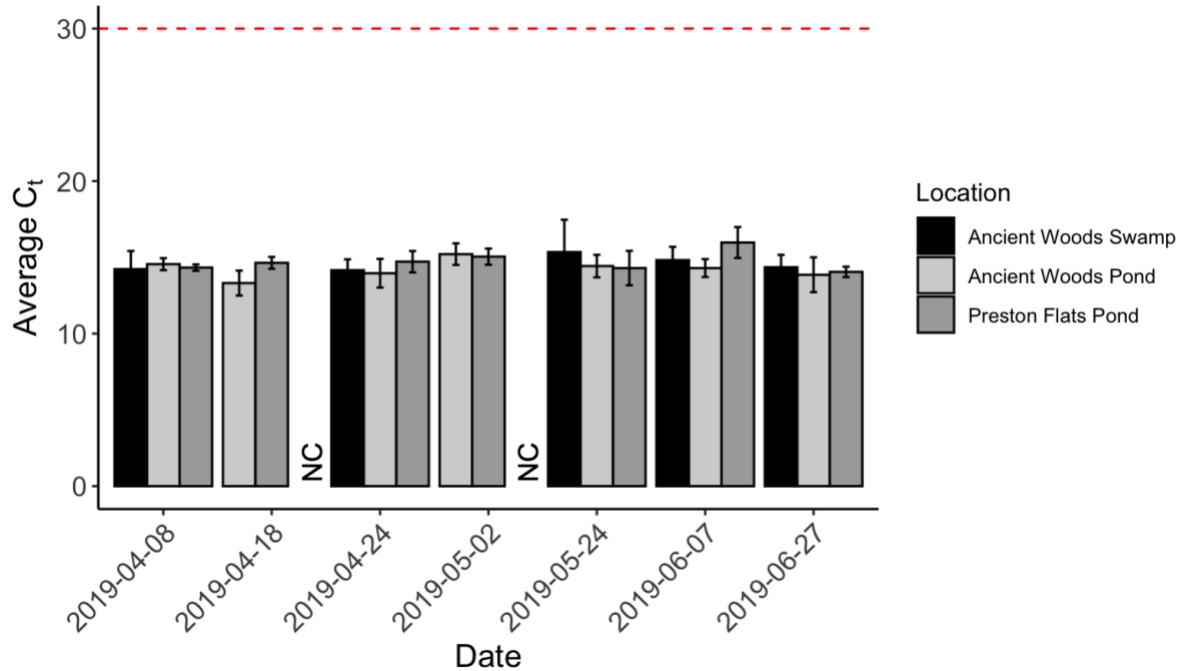


Figure 2.5. eDNA integrity assessed with ePlant5 assay.

Average amplification of chloroplast 23S rRNA gene via qPCR using the ePlant5 assay for vernal pool eDNA samples. Samples from Ancient Woods Swamp (n=5), Ancient Woods Pond (n=7), and Preston Flats Pond (n=7) were amplified for the chloroplast DNA to determine eDNA degradation. eDNA was considered to have minimal degradation if amplified below the dotted red threshold of 30 cycles (C_t). Each bar represents the average C_t for each vernal pool's samples $\pm$ standard deviation error bars from sampling dates ranging from April 8, 2019, to June 27, 2019. NC indicates dates when Ancient Woods Swamp was not sampled and therefore no integrity results were obtained.

2.3.2.2.2 Amphibian eDNA detections

To determine presence or absence of amphibians using eDNA barcoding, DNA extracted from water samples were amplified with species-specific qPCR assay. All six of the target amphibians were detected using eDNA barcoding at a minimum of one timepoint, in one vernal pool, and at one sampling location.

The coefficient of variation for eDNA samples determines the level of variability between the eDNA concentration of successfully amplified qPCR triplicate replicates for each species (Mauvisseau et al. 2019). To determine the coefficient of variation the mean standard deviation of eDNA quantity is divided by mean eDNA concentration. The percent coefficient of variation (% CV) for each amphibian's eDNA detections was 51.1%, 19.17%, 7.46%, 12.76%, 17.78%, and 13.09% for American toad, green frog, northern leopard frog, spotted salamander, spring peeper, and wood frog respectively. Due to variation in detections in both biological field replicates and qPCR technical replicates, I am proposing a confidence matrix to aid in interpretation of eDNA amplification results (Table 2.7). Amplifications with a low confidence were still considered as species presence, due to the added measures taken to control for contamination (Thalinger et al. 2021; Abbott et al. 2021). Reactions with an eDNA quantity below the limit of detection were considered as species presence however, it was important to note that non-detections could indicate that the species was absent or that species was present but the eDNA concentration persists below the limit of detection (Thalinger et al. 2021; Abbott et al. 2021).

Table 2.7. Confidence matrix of species detection based on eDNA barcoding amplification.

Confidence	Amplification in technical qPCR replicates	Amplification in duplicates biological field replicates
High	3/3	1 or 2
	2/3	2
Medium	2/3	1
	1/3	2
Low	1/3	1

Confidence in species detections determined from the amplification based on both technical qPCR replicates and biological field replicates. Technical qPCR replicates indicated by the number of replicate wells that amplified. The biological field replicates are represented by the amplification from the duplicate bottles of water collected in the field. Together, success of amplification across technical and biological replicates are given a confidence level of high, medium, or low.

2.3.2.2.3 Ancient Woods Swamp eDNA detections

The larger vernal pool, Ancient Woods Swamp, was sampled for eDNA five times across the sampling season with six sampling locations and duplicate water samples collected at each sampling location. This allowed for spatial and temporal patterns of eDNA to be detected. At this site, five target amphibian species were detected using eDNA barcoding (Figure 2.6). American toad was detected on three sampling dates and at two different sampling locations with either low or medium detection confidences (Figure 2.6). The green frog was detected on every sampling date but not consistently at all sampling locations (Figure 2.6). On each sampling date, there was detection of green frog with either medium or high confidence, however, the confidence of detection varied between each sampling location. The spring peeper was detected with high confidence on one occasion and at one sampling location (Figure 2.6). Finally, the wood frog was detected with high confidence starting on the third sampling date and then consistently throughout the season. No amphibian eDNA was detected in field blanks or extraction blanks.

2.3.2.2.4 Ancient Woods Pond eDNA detections

Ancient Woods Pond, representing a smaller vernal pool, was located in a mixed deciduous forest and was sampled for eDNA seven times throughout the sampling season. Due to the smaller size of the vernal pool, it was sampled at only three sampling locations where duplicate biological field replicates of water were collected. Across all eDNA sampling at this vernal pool, five amphibian species, American toad, green frog, northern leopard frog, spotted salamander, and wood frog were all detected (Figure 2.6). American toad was detected at low confidence on two sampling dates. Green frog was detected with consistent high confidence starting on the third sampling date and then continued throughout the season. Northern leopard frog was detected with medium confidence on only one sampling date at this vernal pool. This sampling location exhibited the most consistent detections of the obligate vernal pool breeding species, the spotted salamander and the wood frog. Ancient Woods Pond was the only location to detect spotted salamanders. Detection of this species had initial high confidence detections, a decrease in confidence for two sampling

dates but increased detection once again at the end of the sampling season. Wood frog was detected with high confidence starting on the second sampling date and was consistent throughout the season (Figure 2.6). No detections occurred in field blanks or extraction blanks.

2.3.2.2.5 Preston Flats Pond eDNA detections

Preston Flats Pond, a smaller vernal pool, located near a roadway was sampled seven times at three sampling locations throughout the season. Three amphibian species, American toad, green frog, and northern leopard frog were detected at this vernal pool (Figure 2.6). The American toad was not detected on the initial sampling dates, however, an increase in detections occurred in June with high confidence detections later in the month (Figure 2.6). Both the green frog and northern leopard frog were detected at high confidence across all sampling dates for at least one sampling location. No detections occurred in field blanks or extraction blanks.

		ANCIENT WOODS SWAMP														ANCIENT WOODS POND								PRESTON FLATS POND								
		1		2		3		4		5		6		BLANKS		1		2		3		BLANKS		1		2		3		BLANKS		
		A	B	C	D	E	F	G	H	I	J	K	L	FB	EB	A	B	C	D	E	F	FB	EB	A	B	C	D	E	F	FB	EB	
APRIL	2019-04-08								1/3																						AMERICAN TOAD	
	2019-04-18																															
	2019-04-24																															
	2019-05-02																															
MAY	2019-05-24																		1/3													
	2019-06-07							1/3	1/3																							
JUNE	2019-06-27											1/3																				

Figure 2.6. Spatial and temporal detection of six target amphibian species using species-specific environmental DNA barcoding assays.

eDNA was sampled from three vernal pools, Ancient Woods Swamp, Ancient Woods Pond, and Preston Flats Pond from April 08, 2019, to June 27, 2019. On each sampling date, duplicate water samples (A-L) containing eDNA were collected from either three sampling sites (Ancient Woods Pond and Preston flats), or six sampling sites (Ancient Woods Swamp). All eDNA samples, including a field blank (FB) and extraction blank (EB), were tested to detect species by running qPCR eDNA barcoding assays for the six target amphibian species, the American toad (light blue shading), Green frog (dark green shading), northern leopard frog (yellow shading), spring peeper (light green shading), spotted salamander (orange shading), and wood frog (dark blue shading). Within each species section, each individual row shows detections on a specific date. Black squares indicate 0/3 detections, grey indicates dates where no eDNA was collected or processed via qPCR. Each coloured cell indicating a detection contains a ratio of how many of the 3 triplicate reactions resulted in amplified eDNA.

Generalized linear regression models were used to determine if sampling location around the vernal pool and date significantly explained the variance in amphibian eDNA detections. These were compared for each target species and for each vernal pool using Wald's Chi-Squared test. Both sampling location and sampling date were significant variables in the models for American toad detections at all three vernal pools (Table 2.8). Logistic regression models for green frog detections were significantly affected by sampling date at all three vernal pools, but sampling location was only significant at Preston Flats Pond and Ancient Woods Swamp (Table 2.8). Sampling location and date significantly contributed to the models of northern leopard frog eDNA detections at only Preston Flats Pond (Table 2.8). The spotted salamander was only detected at one vernal pool, Ancient Woods Pond, and sampling date and location were significant in fitting the model (Table 2.8). No model was possible for spring peeper due to low level of eDNA detections. Finally, the wood frog generalized linear regression model for Ancient Woods Pond and Ancient Woods Swamp were both significantly affected by sampling date and sampling location (Table 2.8).

Table 2.8. Binomial logistic regression models for the effect of date and sampling location on eDNA amplification for six target amphibians at three vernal pools.

Species	Site	Variable	LR Chisq	Df	Pr(>Chisq)
American toad	AWS	date	20.968	4	0.0003214 ***
American toad	AWS	sampling location	52.623	5	4.018e-10 ***
American toad	AWP	date	29.907	6	4.094e-05 ***
American toad	AWP	sampling location	10.081	2	0.006471 **
American toad	PF	date	195.315	6	< 2.2e-16 ***
American toad	PF	sampling location	48.845	2	2.475e-11 ***
Green frog	AWS	date	158.119	4	< 2.2e-16 ***
Green frog	AWS	sampling location	29.216	5	2.103e-05 ***
Green frog	AWP	date	286.648	6	< 2e-16 ***
Green frog	AWP	sampling location	2.843	2	0.2414
Green frog	PF	date	146.83	6	< 2.2e-16 ***
Green frog	PF	sampling location	185.21	2	< 2.2e-16 ***
Northern leopard frog	AWS	date	29.656	4	5.750e-06 ***
Northern leopard frog	AWS	sampling location	36.393	5	7.925e-07 ***
Northern leopard frog	AWP	date	48.835	6	8.046e-09 ***
Northern leopard frog	AWP	sampling location	29.519	2	3.891e-07 ***
Northern leopard frog	PF	date	421.66	6	< 2.2e-16 ***
Northern leopard frog	PF	sampling location	203.82	2	< 2.2e-16 ***
Spotted salamander	AWS		NA		
Spotted salamander	AWS		NA		
Spotted salamander	AWP	date	121.685	6	< 2.2e-16 ***
Spotted salamander	AWP	sampling location	17.212	2	0.000183 ***
Spotted salamander	PF		NA		
Spotted salamander	PF		NA		
Wood frog	AWS	date	722.68	4	< 2.2e-16 ***
Wood frog	AWS	sampling location	277.46	5	< 2.2e-16 ***
Wood frog	AWP	date	460.61	6	< 2.2e-16 ***
Wood frog	AWP	sampling location	62.89	2	2.202e-14 ***
Wood frog	PF		NA		
Wood frog	PF		NA		

Binary logistic regression models using formula: glm (formula = amplification ~ date + sampling location, family = binomial()). Models were made for six target amphibians at three vernal pools, Ancient Woods Swamp (AWS), Ancient Woods Pond (AWP), and Preston

Flats Ponds (PF). NA indicates no detections at vernal pool site and therefore no model produced.

Additional analysis was completed to determine if there were significant differences in eDNA quantity detected between the duplicate biological field replicates collected during eDNA sampling (Table 2.9). An ANOVA was used to determine if there was a difference in quantity of eDNA amplified when considering vernal pool and sampling location. There was no significant difference in eDNA quantity when compared using an ANOVA followed by a Tukey test in R (Table 2.9). This determines there is no significant difference between biological field replicates when collected from the same sampling location.

Table 2.9. Comparison of eDNA concentration from amplified eDNA samples from duplicate biological field replicate water samples.

Sites compared	difference	lower	upper	p-adjusted
AWS_B vs AWS_A	12.5937897	2716.06043	2741.248	1
AWS_D vs AWS_C	19.7437249	-2064.3004	2103.7878	1
AWS_F vs AWS_E	-46.466047	-2104.073	2011.1409	1
AWS_H vs AWS_G	-86.263105	-2050.5504	1878.0242	1
AWS_J vs AWS_I	7.28924	-3851.6106	3866.189	1
AWS_L vs AWS_K	3.0294496	-2195.7828	2201.8417	1
AWP_B vs AWP_A	-67.997056	-1658.7563	1522.7622	1
AWP_D vs AWP_C	72.2882582	-1360.8712	1505.4477	1
AWP_F vs AWP_E	30.3679002	-1293.2238	1353.9596	1
PF_B vs PF_A	735.408782	-819.71355	2290.5311	0.9894357
PF_D vs PF_C	1074.69054	-290.30323	2439.6843	0.3798066
PF_F vs PF_E	-1119.2769	-2463.3906	224.8368	0.2697502

Vernal pools Ancient Woods Swamp (AWS), Ancient Woods Pond (AWP), and Preston Flats Pond (PF) compared by sampling locations and the associated duplicates. (1-A&B, 2-C&D, 3-E&F, 4-G&H, 5-I&J, 6-K&L).

2.3.3 Amphibian eDNA concentrations detected

The quantity of eDNA detected varied based on both species and vernal pool location. For the American toad, no eDNA was detectable above the limit of quantification at both Ancient woods vernal pools. However, at Preston Flats Pond, eDNA was detected above this threshold on the last day of sampling on June 27, 2019 (Figure 2.7.A). Green frog copy number remained more uniform throughout the season starting on April 24, 2019, especially at Ancient Woods Swamp and Ancient Woods Pond (Figure 2.7.B). All green frog detections at Ancient Woods Pond on May 05, 2019, were above the limit of quantification. Quantity of eDNA for green frog reached the highest at Preston Flats Pond and was detected above the limit of quantification on all dates at this site. eDNA quantity of northern leopard frog was not detected within all three technical qPCR replicates and therefore was not quantifiable at either Ancient Woods Swamp or Ancient Woods Pond. Northern leopard frog eDNA quantities at Preston flats pool exhibited a general trend of increasing in quantity throughout the season with most detections above the limit of quantification past April 24, 2019 (Figure 2.7.C). Spotted salamander eDNA quantities at Ancient Woods Pond were only detected in all three technical qPCR replicates and above the LOD on the first eDNA sampling date, April 08, 2019 (Figure 2.7.D). After this date there is a decrease in spotted salamander eDNA amplification to quantities likely closer to the limit of detection. Finally, the wood frog eDNA was detected in the highest quantity at Ancient Woods Pond on June 07, 2019 (Figure 2.7.E). Both Ancient woods vernal pools had wood frog detections above the LOQ, and the majority of detections occurred in all three technical replicates. eDNA quantification varies for each species showing peaks in quantity not only at different sites but exhibited different patterns throughout the sampling season.

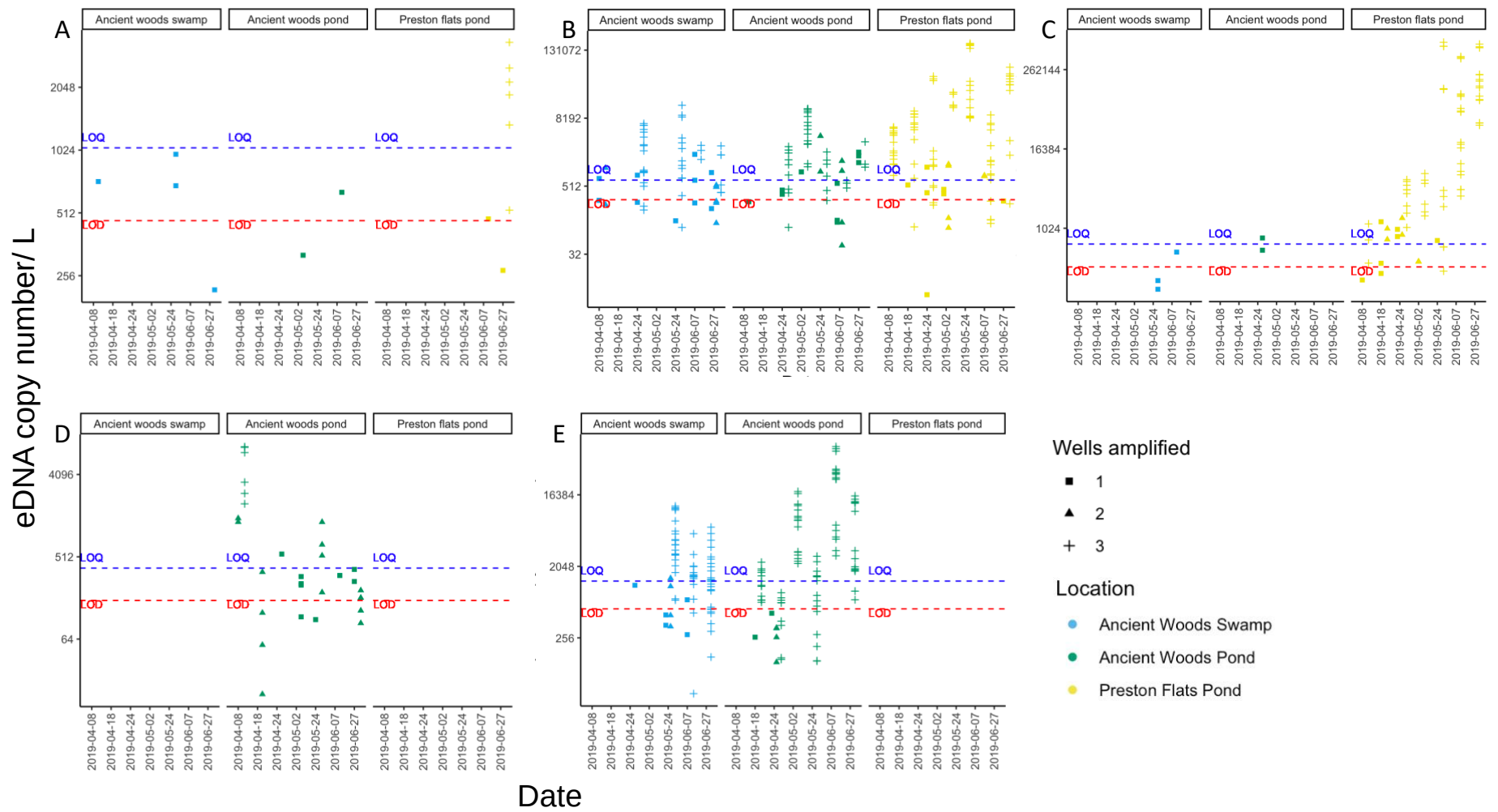


Figure 2.7. eDNA copy number across a temporal scale for five target amphibian species at each vernal pool.

Quantity of eDNA in copies per litre for all sampling dates from April 8, 2019, to June 27 2019 for all successful amplifications for the target six amphibian species at Ancient Woods Swamp, Ancient Woods Pond, and Preston Flats Pond. This figure depicts separate plots per target species (A) American toad, (B) green frog, (C) northern leopard frog, (D) spotted salamander, (E) wood frog, and the corresponding limit of detection (LOD) threshold (dashed red line) and limit of quantification (LOQ) threshold (dashed blue line).

2.3.4 Species detection method comparison

Amphibian monitoring and determining the optimal method to detect species has been a continuous challenge. In this study, we compare eDNA barcoding as a method of species detection to conventional methods of detection including active acoustic monitoring, passive acoustic monitoring, and visual encounter surveys. The method of detecting amphibians in vernal pools had different levels of effectiveness. Overall, eDNA barcoding detected the most species on a single sampling date with up to four species detected with this single method. Conventional methods, on a single sampling date, detected two species with active acoustic surveys, three species with passive acoustic surveys, and two species via visual encounter surveys. I calculated the mean species richness based on each sampling event for each method of detection in order to determine the cumulative species richness (Figure 2.8). eDNA barcoding exhibited the highest overall cumulative species richness (4.00 ± 0.82) and the greatest increase in species accumulation as number of sampling events increased (Figure 2.8). Passive acoustic surveys had a similar starting species richness as eDNA barcoding, however, species accumulation did not initially increase with additional sampling events and only increased to a mean of 3.67 ± 0.33 (Figure 2.8). Visual encounter surveys displayed the greatest increase in species accumulation over the total sampling events, however, the maximum mean species richness (3.00 ± 0.00) still did not exceed that of eDNA barcoding or passive acoustic surveys (Figure 2.8).

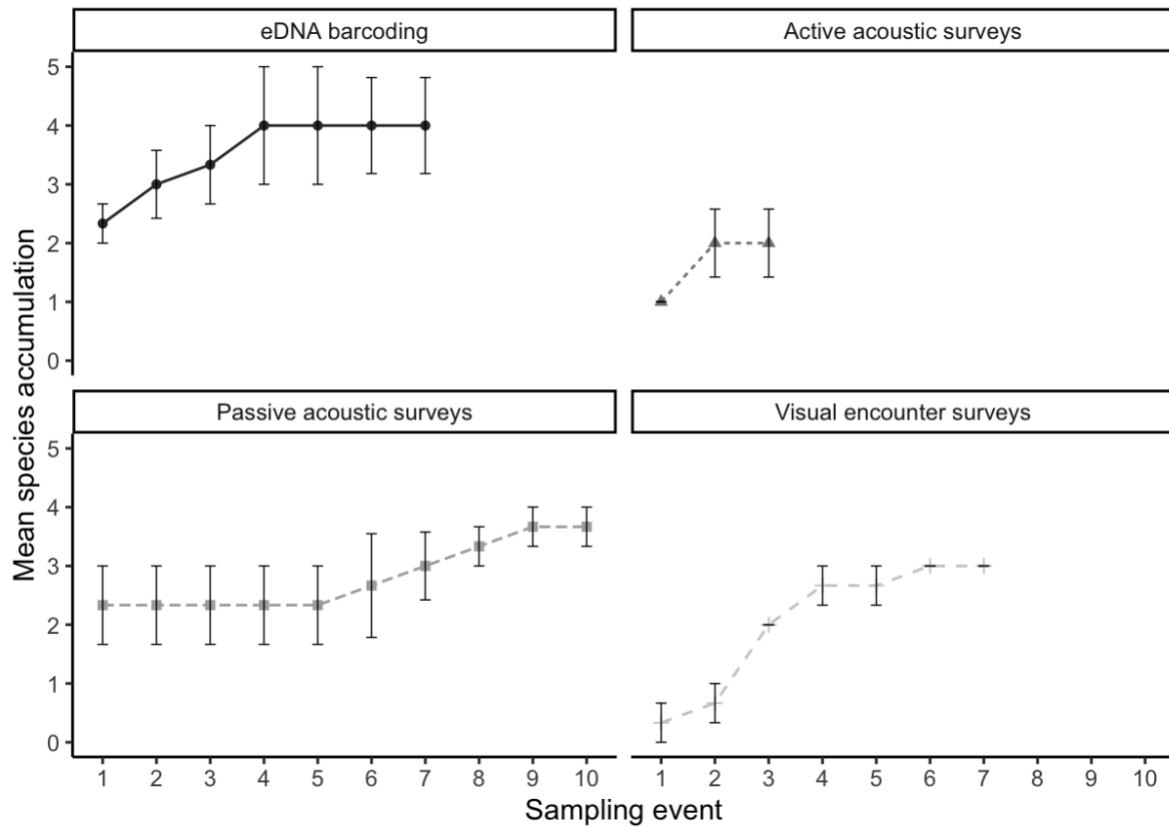


Figure 2.8 Comparison of mean cumulative species richness for eDNA barcoding and conventional methods of species detection.

Species accumulation plots were averaged for species detections ($\pm$ standard error) from three vernal pools and over multiple sampling events for each method. Sampling events varies between method consisting of seven sampling events for eDNA barcoding, three sampling events for active acoustic surveys, ten sampling events for passive acoustic surveys, and seven sampling events for visual encounter surveys. Since recordings for the passive acoustic monitoring were continuous from May 02, 2019, to July 04, 2019, a sampling event for this method was defined as each five-day period of recording.

To aid in ongoing amphibian monitoring, determining the optimal method or combination of methods of species detection is an important step for amphibian conservation. Conventional methods of amphibian detection have advantages and disadvantages leading to biases in species detection. eDNA barcoding is an emerging method of species detection that may offer an increased detection ability. In this study, there were cases of eDNA barcoding detecting species with greater, lesser, and equal ability (Figure 2.9). At Preston Flats Pond, American toad was detected through conventional methods prior to detection with eDNA. At all vernal pools, spring peeper was detected more consistently with passive acoustic surveys (Figure 2.9). Species detection using eDNA barcoding performed similar to conventional methods for both the green frog and northern leopard frog. eDNA barcoding was optimal for detection of the obligate vernal pool species, spotted salamander and wood frog (Figure 2.9). Spotted salamander was only detected using eDNA barcoding. Wood frog at Ancient Woods Pond was detected through eDNA barcoding and conventional methods early in the season. However, eDNA barcoding outperformed conventional methods by detecting consistently later in the season. Additionally, at Ancient Woods Swamp, wood frog was consistently detected with eDNA barcoding where there was no conventional detection of the species. Based on observations, eDNA barcoding is a beneficial method of species detection, however, it cannot completely replace conventional methods of species detection.

METHOD OF DETECTION	ANCIENT WOODS SWAMP							ANCIENT WOODS POND							PRESTON FLATS POND							SPECIES
	APRIL			MAY		JUNE		APRIL			MAY		JUNE		APRIL			MAY		JUNE		
	2019-04-08	2019-04-18	2019-04-24	2019-05-02	2019-05-24	2019-06-07	2019-06-27	2019-04-08	2019-04-18	2019-04-24	2019-05-02	2019-05-24	2019-06-07	2019-06-27	2019-04-08	2019-04-18	2019-04-24	2019-05-02	2019-05-24	2019-06-07	2019-06-27	
Active acoustic (MMP)	NC	NC		NC		NC		NC	NC		NC		NC		NC	NC		NC	Code 1	NC		AMERICAN TOAD
Passive acoustic (SM4)	NC	NC	NC					NC	NC	NC					NC	NC	NC	1	1			
VES																						
eDNA	L	NC		NC	M		L				L		L							L	H	
Active acoustic (MMP)	NC	NC		NC		NC		NC	NC		NC		NC		NC	NC		NC	Code 1	NC	Code 1	GREEN FROG
Passive acoustic (SM4)	NC	NC	NC				1	NC	NC	NC	1		1	1	NC	NC	NC			1	1	
VES	1						1			1	1	1	1	1			1		1	1	1	
eDNA	M	NC	H	NC	H	H	H			H	H	H	H	H	H	H	H	H	H	H	H	
Active acoustic (MMP)	NC	NC		NC	Code 1	NC		NC	NC		NC		NC		NC	NC	Code 1	NC		NC		NORTHERN LEOPARD FROG
Passive acoustic (SM4)	NC	NC	NC					NC	NC	NC			1	1	NC	NC	NC	1	1	1	1	
VES					1							1					1	1	1	1	1	
eDNA		NC		NC	L	L									H	M	H	H	H	H	H	
Active acoustic (MMP)	NA	NA		NA		NA		NA	NA		NA		NA		NA	NA		NA		NA		SPOTTED SALAMANDER
Passive acoustic (SM4)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
VES																						
eDNA		NC		NC				H	H	L	M	H	L	H								
Active acoustic (MMP)	NC	NC	Code 2	NC		NC		NC	NC		NC		NC		NC	NC		NC		NC		SPRING PEEPER
Passive acoustic (SM4)	NC	NC	NC	1	1			NC	NC	NC	1		1	1	NC	NC	NC			1	1	
VES																						
eDNA	H	NC		NC																		
Active acoustic (MMP)	NC	NC		NC		NC		NC	NC		NC		NC		NC	NC		NC		NC		WOOD FROG
Passive acoustic (SM4)	NC	NC	NC					NC	NC	NC					NC	NC	NC					
VES																						
eDNA		NC	L	NC	H	H	H											1				

Figure 2.9. Comparison of detection methods for target amphibians using eDNA and conventional monitoring.

eDNA barcoding as a method of detection was compared to conventional methods of detection which include active acoustic (MMP), passive acoustic (SM4), and visual encounter surveys. Detections were collected from three vernal pools (Ancient Woods Swamp, Ancient Woods Pond, and Preston Flats Pond) through April-June (columns) for each target species. Each individual row indicates the method of detection nested in the larger rows representing the six target species; American toad (light blue shading), green frog (dark green shading), northern leopard frog (yellow shading), spring peeper (light green shading), spotted salamander (orange shading), and wood frog (dark blue shading). Cells under the active acoustic surveys containing a code level, indicate detections based on anuran calling levels from the marsh monitoring protocol ranging from (code 1) no overlapping calls, (code 2) some overlapping calls, (code

3) full chorus. Cells filled with shaded colour and containing “1” indicate a detection for passive acoustic surveys and visual encounter surveys. For eDNA barcoding, shaded cells indicate species detection with low (L), medium (M), and high (H) confidence of detection. Black shaded cells indicate non-detections, grey shaded cells indicate either “NC” for not conducted, or “NA” for not applicable for the non-calling spotted salamander.

Amphibian species detection can vary based on method and species of interest, so it is important to compare and contrast the new eDNA barcoding method to conventional methods of amphibian species detection. Comparing methods of detections using binomial generalized mixed effects regression (GLMER) models showed that species detection could be both species and method specific. GLMER models were run for four target species with the exceptions of spotted salamander which was only detected via one method and the spring peeper which did not meet the model assumptions due to low number of detections. Models were built to determine the effect of method on species detection using both vernal pool location and date as a random effect to account for non-independent sampling (Equation 2).

$$\text{Detection} \sim \text{Method} + (1|\text{Date}) + (1|\text{Location}) \quad \text{Equation 2}$$

Method of detection was a significant factor affecting the detection of three amphibian species with the exception of the northern leopard frog (Table 2.10). With date as a random effect for all models, no method of detection significantly outperformed another for the American toad. Both conventional acoustic methods of detection for the green frog were significantly different in predicting detection compared to eDNA barcoding as a method of detection. For the northern leopard frog, conventional methods of detection were not significantly different than eDNA barcoding at predicting the variance in species detections. Finally, for the wood frog, eDNA as a method of detection was significantly better at species detection compared to the visual encounter surveys. The passive acoustic receivers were not considered in this model since they contributed no detection of wood frog throughout the season because they were mounted after the wood frog breeding season. Although these models indicate a level of significance, it is important to also account for observational data and patterns despite the p-value (Nahm 2017; Halsey 2019; Wikston et al. 2023). Similar to observational conclusions, statistical analysis did not find eDNA barcoding significantly different than conventional methods of detection for the American toad. The statistical analysis determined that eDNA barcoding outperformed acoustic surveys for the green frog. In the case of the northern leopard frog, there appeared to be site specific observations concluding that eDNA barcoding outperformed conventional methods, however, across all

locations, method of detection was not significant. Statistical significance determined for the wood frog models matched the conclusions for the species observations. Discrepancies between observational patterns and statistical analysis could be related to sampling size, number of sampling events, and sampling designs. Potentially, with follow up studies or multi-year studies, patterns could become clearer and strengthen statistical outputs.

Table 2.10 Binomial generalized mixed effects regression models determining the effect of survey methods on species detection with mixed effects.

	Coefficient	SE	Z	P
American toad				
X²=11.306, df=3, P=0.01018*				
eDNA:MMP	-1.36	1.157	1.198	0.231
eDNA:SM4	-91.63	90.24	-1.015	0.31
eDNA:VES	-32.52	3.56E+06	0	1
Green frog				
X²=20.04, df=3, P=0.0001665*				
Intercept	2.786	1.225	2.275	0.02290*
eDNA:MMP	-5.419	1.693	-3.201	0.00137**
eDNA:SM4	-3.675	1.429	-2.571	0.01013*
eDNA:VES	-2.045	1.053	-1.941	0.05223
Northern leopard frog				
X²=3.5254, df=3, P=0.3175				
Intercept	-0.03709	1.10591	-0.034	0.9732
eDNA:MMP	-2.23639	1.28616	-1.739	0.0821
eDNA:SM4	-0.53315	0.94433	-0.565	0.5724
eDNA:VES	-0.63846	0.78439	-0.814	0.4157
Wood frog				
X²=16.796, df=3, P=0.0007783*				
Intercept	-0.1516	1.0287	-0.147	0.8828
eDNA:MMP	-2.9409	1.5418	-1.907	0.0565
eDNA:VES	-2.4017	1.0495	-2.288	0.0221 *

Use of binomial mixed effect regression models with formula: Detection ~ Method +(1|Date) + (1|Location). Model test statistic (X²), degrees of freedom (df), and p-value (P) are outlined beneath each species subheading. Wald's Chi Square test used to determine the associated coefficient, standard error (SE), test statistic (Z), and p-value (P) for interactions of each model.

2.4 Discussion

2.4.1 Method comparison

Conducting a comparative study for eDNA barcoding and conventional methods of amphibian detection provides insight into the strengths and weaknesses of amphibian monitoring as well as aims to inform future monitoring efforts. Together, eDNA barcoding and conventional methods of species detection successfully detected all 6 target amphibians across the three vernal pools surveyed. However, the ability to detect a target amphibian species varied depending on the selected method of detection and each detection method had false negatives concerning target species detection events with the comparator detection methods. Generally, eDNA barcoding detected the most species overall and the most species on a single sampling date compared to the visual encounter surveys and acoustic surveys performed. The one exception to this general observation was for spring peepers where eDNA survey and barcoding detection methods, even performed temporally across the spring and early summer, were unable to detect eDNA from this target species.

The relative success of eDNA sampling and detection methods in comparison to conventional survey methods has been reported in previous studies, including a recent study performed in southern Ontario to detect an overlapping set of amphibian target species using eDNA barcoding (Wikston et al. 2023). Wikston et al. (2023) concluded that eDNA barcoding or visual encounter surveys were the best methods for the detection of the selected target anuran species but that eDNA barcoding offered a more efficient method (Wikston et al. 2023). Similarly, other studies have proposed the use of eDNA surveys alongside visual encounter surveys to best capture the aquatic and terrestrial stages of amphibians (Moss et al. 2022). Although the findings presented in this thesis also support the combined use of eDNA surveys with a conventional survey method, we did not find visual encounter surveys to be the only optimal conventional survey method for the detection of target species studied in this thesis and caution a one-size-fits-all approach. The selection of a complementary conventional method to pair alongside eDNA surveys is likely dependent on the species

being surveyed, the effort expended on the survey method, and the timing of when surveys are conducted, given the diversity of amphibian life histories.

Based on the cumulative species richness and species detections, I propose that eDNA barcoding be used as a method of species detection alongside passive acoustic surveys. This suggests that combining methods of detection can lead to the most effective species detection. Incorporating methods that target breeding season, such as passive acoustic surveys, alongside a method that is less specific to life history, such as eDNA barcoding, could optimize overall species detection. This would target the active breeding grounds of anuran species, indicating vital habitat for protection. Conducting these surveys alongside eDNA barcoding would allow for the detection of species despite life stages, cryptic life history, or breeding behaviours. I am proposing the use of passive acoustic monitoring alongside eDNA over active acoustic monitoring due to the time-intensive field sampling required for this method and the possibility that the observers could miss an explosive breeding event or optimal calling periods due to confounding environmental variables not indicating the start of breeding season yet. Passive acoustic monitoring allows for asynchronous recording and accounts for multiple species circadian rhythms. Passive acoustic monitoring was also selected over visual encounter surveys due to successful species detections and the bias of visual encounter surveys towards generalist or high-density species, such as the green frog. Additionally, species detections via visual encounter surveys are dependent on the search effort and level of invasiveness to the habitat, such as lifting cover. Amphibian species detection, despite the method, is dependent on both spatial and temporal sampling efforts.

2.4.2 Spatial and temporal sampling

In this study, there were distinct variations in detections based on spatial and temporal sampling. Community assemblages of amphibians vary based on their breeding habitat, foraging habitat, and their seasonal use of different habitats based on behaviour and life stages such as egg, juvenile, or adult (Bulger et al. 2003; Cushman 2006; Simon et al. 2009). When considering spatial sampling, this must also include geographical considerations. For

instance, these vernal pools are wetlands based in a more urban location, near city activity, which could explain the lower populations and lower calling levels throughout the whole season (Hamer and McDonnell 2008; Scheffers and Paszkowski 2012). The seasonal use of habitats is varied and distinct for each amphibian species. In this study, we addressed spatial patterns by sampling the entire periphery of the vernal pools with conventional VES methods, recording calls from vernal pools, and using multiple sampling locations for eDNA sampling. We found that duplicate eDNA sampled from the same sampling location were similar in species detections, but the use of multiple sampling locations resulted in increased species detections. On certain sampling dates, if spatial sampling was not conducted, species would have been missed using eDNA barcoding. For instance, the spring peeper was only detected on a single sampling date and at one sampling site (Figure 2.6) and without spatial sampling effort, detection would have been missed. This spatial sampling avoided non-detections for all other target amphibians as well, on at least one sampling date.

In this study, we have attempted to survey over a period of three months to best capture seasonal amphibian movement throughout the study period. Temporal sampling increased the number of sampling events which increased the cumulative species detected (Figure 2.8). Despite the temporal aspect of surveying in this study, the conventional survey methods targeted only adult or late-stage juveniles. This could lead to a bias that was addressed in other studies through use of larval bait traps and tadpole identifications (Wikston et al. 2023). With eDNA barcoding, we found that we could detect species such as the wood frog with eDNA detections when adults were no longer detected with conventional methods. This suggests that eDNA barcoding detects across life stages, including tadpoles, when non-invasive conventional methods fail. Temporal sampling using a multi-method approach with a conventional and eDNA barcoding method of species detection can be beneficial to aid in targeted sampling. For instance, some species, such as the American toad, were detected through a conventional method of detection and followed by a delayed detection of eDNA (Figure 2.6). American toad detections also went from low confidence to high confidence at many sampling locations, which could indicate that if sampling had

continued into late summer months, more detections via eDNA barcoding could have been possible. A similar pattern was seen with the northern leopard frog (Figure 2.6), where a delayed eDNA detection occurred supporting that eDNA persists in the environment allowing for extended species detection time or the detection of a larval life stage. In a study comparing species detection methods for an endangered pool frog, they found that traditional methods of species detection were better in June but that eDNA barcoding had a higher probability of detecting the species at other times of the year (Eiler et al. 2018). In our findings, there were cases when eDNA barcoding performed similarly, with eDNA barcoding detecting species past when conventional method were no longer detecting species presence. Many amphibian species have short breeding seasons, or begin the breeding season based on a set of specific environmental and physiological cues (Bosch and De La Riva 2004). The combination of these factors could also account for the discrepancy between the methods, even between audio methods. In some cases, on the same date as non-detections using active acoustic surveys, the passive acoustic surveys would successfully detect calls within the same 24 hours. This was seen with the green frog audio calls at Ancient Woods Pond around May 19, 2019. In a previous study, an increased wait time for active acoustic monitoring allowed amphibians to return to their normal behaviours of calling for mates and acclimate after the disturbance by field technicians (Bosch and De La Riva 2004). Temporal sampling for amphibians is not only vital due to the increased number of sampling events and therefore greater chance to detect a high number of species, but additionally, the life history of amphibians includes seasonal shifts in habitat use, changing their detectability.

The ecology of amphibian species is a driving factor of species detection and must be incorporated into the sampling design. One of the many ways to account for the ecology and life history strategies of amphibians is to implement spatial and temporal sampling with special consideration for the target species. The target species in this study all follow a biphasic life history, meaning they require aquatic habitat for reproduction and development, and then utilize terrestrial habitat for foraging and hibernation (reviewed in Liedtke et al. 2022). Additionally, behavioral aspects play a role in species detection. Amphibians may

have preferred habitat types, exhibit cryptic or nocturnal behaviours, or seek out cover when startled leading to non-detections (reviewed in Nolan et al. 2023). Amphibians are adept at camouflage and can be well hidden in both aquatic and terrestrial environments. They may also seek out cover, requiring flipping cover to see adults, however, this disturbs these vital microhabitats which could jeopardize the use of the habitat in the future. Amphibian breeding is an important ecological process which is often the reason aquatic habitat is targeted for amphibian detection. Acoustic surveys target specifically breeding individuals within these habitats. However, even with the stipulations of the Marsh monitoring protocol, some species detections were missed through active acoustic surveys. Timing surveys to the appropriate breeding season and to the proper environmental conditions that act as cues for breeding activity can be difficult and differ between sampling years or locations (Green 2017). For example, mounted acoustic song meters were likely placed too late to detect wood frogs, and therefore, without other methods, would have failed to detect the species. However, acoustic methods of detection were not viable as the sole method of detection since members of the order Caudata, such as the spotted salamander, are non-calling amphibian species and utilize other strategies for mating. Targeting this species outlines that one of the common non-invasive conventional methods completely overlooks salamanders, newts, and caecilian amphibian species. Without targeted cover board surveys or flipping cover, this species and others with this cryptic life history could be missed with conventional methods of detection. eDNA barcoding offers a more reliable method of species detection in the case of non-calling and cryptic species. Many amphibians, although they use aquatic habitats for breeding, the adults will leave to seek out terrestrial habitats for the majority of the spring and summer (Liedtke et al. 2022; Nolan et al. 2023). This is the case for both obligate vernal pool breeders, the spotted salamander and the wood frog. These species follow explosive breeding events and then adults return to terrestrial habitats which are trickier to survey. This means that conventional methods will only detect adults for a very short window. eDNA barcoding, however, can detect eDNA from all life stages including the developing larval stages which makes this method beneficial across life history and ecology strategies. Life histories can change where amphibians spend their time and the length of time spent due to a natural cycle

and false negatives can occur when targeting the improper habitat at an improper time or with a less effective method.

2.4.3 False positives and negatives

2.4.3.1.1 Conventional methods

Accurate identification of amphibians in their wetland habitat is a crucial step towards conservation through ongoing amphibian monitoring. These detections can be obtained through conventional methods or eDNA surveys. All methods of detection are fraught with challenges and can exhibit rates of false negatives and false positives. In conventional methods, these errors often occur related to life histories, cryptic species or due to low density populations leading to non-detections when species are present in a habitat (Tanadini and Schmidt 2011). Observer error is an important consideration that can lead to both false positives and negatives. Observers must be accurate in ID skills for visual appearance and breeding calls, but human error cannot be completely avoided. In this study we attempted to control for some of these sources of error by indicating “unknown” during VES to not introduce bias. We also used two audio methods, though both can have their own associated level of error. In a study comparing human-screened amphibian call files and the use of an automated program, less error was detected in human-screened call detections (Brauer et al. 2016). In our study, advanced classifiers that were trained were used to cluster detections, but all detections were also manually screened for a true positive detection to control for these introduced errors. Both acoustic survey methods were time-intensive in different ways. Active acoustic surveys were field intensive while passive acoustic surveys required the training of a program and quality controlling which is time intensive. However, once advanced classifiers are developed, they would be much simpler to apply to a multi-year study. Overall, extensive effort must be applied to ensure control for these levels of error in conventional detection methods.

Non-invasive conventional methods were able to detect some of the target amphibians however, each was selective for the species it accurately detected. Non-invasive methods were the only conventional methods explored in this study because of the increased stress and risk of fatality towards the species during invasive sampling methods, such as trapping. As seen in a study in 2018, using trapping as a method of long-term amphibian monitoring did not accurately or consistently predict amphibian population trends in wetlands (Greenberg et al. 2018). Knowing that the increased level of invasiveness still does not provide accurate monitoring further validates the focus towards non-invasive methods of species detection. The current methods for ongoing monitoring of amphibians is subject to levels of variability and there is a need for re-evaluating how we monitor for amphibians.

2.4.3.1.2 eDNA barcoding

eDNA barcoding has the benefit of negating many of the sources of error seen in conventional methods, however, this method comes with its associated chance of false positives or negatives. eDNA barcoding can negate the need for direct species observation and therefore some of the behaviour introduced levels of error such as habitat use, detection of life stage, cryptic behaviours, and low species density could possibly be avoided (Dejean et al. 2012; Biggs et al. 2015; Moss et al. 2022). However, this method faces challenges related to physical and environmental processes that can lead to inaccurate detections. eDNA is subject to levels of degradation and dispersion that can affect detection despite intensive field efforts (Strickler et al. 2015; Goldberg et al. 2018; Murakami et al. 2019; Ruso et al. 2019; Bedwell and Goldberg 2020).

A negative result is a difficult data point to interpret due to the possibility of false negatives in the dataset. This study attempts to control for many of the possible methods of false negatives, however not all variables could be controlled for. Controls began at sampling design of the field methods. All sampling locations were sampled in duplicate to increase the total volume of water collected and processed from each sampling location. Previous studies

have shown that increasing the volume of water filtered can increase the DNA yield (Hunter et al. 2019; Bedwell and Goldberg 2020). Water was also collected from multiple sampling locations at each vernal pool to obtain a robust number of spatial samples on each sampling date. Samples were collected from surface water due to the request of the collaborator *rare* Charitable Research Reserve, to not disturb vernal pool sediments for future sediment core research. This was beneficial as it ensured that eDNA trapped in sediment layers were less likely collected in the water samples. However, this did limit the eDNA collection as sampling could not take place towards the center of the vernal pool or deeper than the surface water. In previous studies on fish in lentic systems, eDNA detection varied based on collection depths due to species preferentially using different vertical distributions in the water column (Shu et al. 2020; Littlefair et al. 2021). Though vernal pools are shallow lentic wetland systems, if amphibians preferentially use certain habitats or depths, this could account for false negatives during sampling. Determining a limit of detection (LOD) *in vitro* prior to use of the assay is vital to interpreting negative eDNA results. Knowing that eDNA below a certain concentration is not accurately detected and attempting to optimize assays to have a low LOD can help avoid false negatives but cannot negate them. The interpretation of eDNA false negatives is an area of much discussion (Veldhoen et al. 2016; Helbing and Hobbs 2019; Thalinger et al. 2021) and leads to the slow uptake of eDNA barcoding as a method of monitoring.

Another common cause of false negatives is the presence of inhibitors in the samples or degraded eDNA. Inhibitors were a problem in this study due to use of vernal pools where the stagnant water is rich in contaminants such as humic acids, flavic acids, and tannins (Crecchio and Stotzky 1998; Faccio et al. 2019; Valley and Pools 2021). This study aimed to address inhibition possibilities by using a DNA extraction kit designed for soil and through the use of a spin column inhibitor removal kit. Both the extraction technique and inhibitor removal kit can result in a lower DNA yield, though it is necessary to remove the inhibitory compounds. Although two inhibitor removal methods were used it cannot be definitively proven that no inhibitors remain due to lack of sample for rerunning all reactions and only

those considered previously inhibited were rerun. Both the loss of potential eDNA from the methods used to remove inhibiting compounds and the risk of some remaining inhibitors could account for some false negatives in eDNA detection. Some species were detected conventionally at time points when eDNA barcoding did not result in detections. This could be a result of delayed eDNA detection, reaction inhibition, or low copy numbers remaining after inhibitor removal steps. Overall, inhibition of eDNA barcoding reactions is an area of eDNA studies that must be considered to account for the variation in detections.

False positives offer an additional area for consideration when interpreting eDNA results. Many methods have been adopted as standard in the field of eDNA research to control for false positives. One of the controls used in this study to control for false positives was the introduction of a field blank during sampling. This study did not amplify any eDNA in the field blanks indicating that contamination was minimal and if it took place, it did so at levels below the limit of detection. Additional blanks during eDNA extraction also offered another measure to trace introduced contaminants which yet again, did not amplify indicating no contamination or levels below the limit of detection. Increasing the blanks at other stages such as during filtration should be utilized to increase detection of contamination and ensure low levels of false positives. During eDNA barcoding via qPCR, controls for detecting false positives were added through no template control reactions on every plate. A common source of contamination during qPCR is from the species standards used in the standard curve. This was controlled for by using the synthetic oligonucleotides with the added synthetic insert. The lack of synthetic probe amplification in the eDNA samples further supports the low levels of cross-contamination throughout these methodologies.

2.4.4 Data analysis methods

Although there has been a shift in the eDNA field towards using occupancy modelling when comparing conventional methods of species detection and eDNA methods, this study has used logistic linear regression models and generalized logistic mixed effects models. These were used instead of occupancy modelling due to the data set and the constant fluctuation of amphibians in and out of wetland systems. The biphasic life history strategy

shared by the six target amphibians does not fit the assumptions of occupancy modelling that the system is closed (Barata et al. 2017; Randall et al. 2023). Therefore, modeling was used to aid in explaining variation in the data to better understand the role of water quality, date, time, and ultimately, method of detection on species detection. This study found that the p-values generated from these models, though helpful, occasionally overlooked trends in the data supporting the need to consider the p-values but also give merit to exhibited trends (Halsey 2019).

2.4.5 Conclusion

Optimal amphibian species detection should be achieved by using multiple methods of detection including conventional methods alongside eDNA barcoding. The optimal conventional method selected to run in conjunction with eDNA barcoding is dependent on the species being targeted and their associated physiological and behavioral strategies. For this study, I am proposing that using passive acoustic monitoring alongside eDNA barcoding offers the most species detection and accounts for multiple life history strategies of these target species. When monitoring for other species, eDNA barcoding is an ideal method of detection alongside a conventional detection method which can be catered the species of interest. The false positives and negatives associated with conventional methods and eDNA barcoding can be optimized by adapting field methods to include controls, effective training, stringent decontamination procedures, and optimization of laboratory eDNA assay performance and sensitivity. Additionally, sampling over extended time is optimal for amphibian detections and attempting to capture spatial variation of wetlands can increase species detections on a single sampling date. This study focused sampling on vernal pools to show the use of eDNA barcoding as a viable detection method in stagnant, seasonal pools and provide evidence that these wetlands must be protected in the future for ongoing amphibian conservation. Amphibian monitoring is a vital practice for amphibian conservation and the use of eDNA barcoding can offer a new method for academics, government bodies, and industry end users.

3 General Conclusions

3.1 Objectives and overall findings

The overall objective of my thesis was to compare non-invasive conventional methods of amphibian species detection to eDNA barcoding, which negates the need for direct species observations. The findings presented in this thesis demonstrate that eDNA barcoding offers a reliable method of species detection but further supports the importance of a multi-method approach to amphibian monitoring. The use of molecular methods of species detection, such as eDNA barcoding, must be fully standardized to allow for uptake by end-users in academia, government, and private industry. In both field and laboratory settings, standardizations will allow for the comparison of eDNA studies and the evaluation of the molecular method in all settings. These standardizations must include the use of controls and transparent reporting of methodology, assay conditions, and assay sensitivity. Furthermore, I propose the use of eDNA surveys coupled with a conventional method of amphibian detection that is selected with careful consideration for the life history of the target amphibian species for which monitoring is being performed to define the spatiotemporal sampling that might be required to optimize detection ability. For example, obligate vernal pool breeders, the spotted salamander and wood frog, were optimally detected using eDNA barcoding. All other species, with the exception of the spring peeper, were best detected with eDNA barcoding and an additional conventional method, in most cases, passive acoustic surveys. The spring peeper was best detected with conventional passive acoustic surveys. Together, the use of rigorously implemented eDNA surveys alongside a conventional detection method will improve the monitoring of this threatened taxa. Increased monitoring will allow for well-informed conservation efforts to maintain vital habitat and support healthy amphibian populations which benefit the overall ecosystem.

3.2 Research considerations

Extensive temporal sampling for amphibians is vital when surveying new wetland systems and attempting to capture broad amphibian communities. In this study, field

sampling in 2019 began in early April, however, not all methods were able to be implemented at one time due to the SM4 acoustic song meters still being in transit. This resulted in the delayed installation of the passive acoustic survey method. These devices likely missed the breeding window of the wood frog due to the explosive nature of their breeding behaviour early in the breeding season. In future works, I suggest mounted acoustic song meters be placed before other sampling methods so that all breeding activity can be captured. This could also help aid in targeting the beginning of sampling when the first calls of the season are heard indicating amphibian presence. The overall sampling period ranged from April 08, 2019, until July 02, 2019, however, this excludes the detection of species using vernal pools later in the summer in the larval, juvenile, or adult stage of life. I would suggest increasing the sampling period until the end of August to ensure robust species detection. The eDNA detection of the American toad at Preston Flats Pond suggests that this species could have begun using that vernal pool on the last eDNA sampling date but due to no additional sampling, this pattern could not be verified (Figure 2.6).

Another important consideration when analyzing the comparison of methods is to account for sampling effort. Visual encounter survey methods for this study were conservative, ensuring that sampling was conducted in a non-invasive manner. This differs from the methods of other studies conducting visual encounter surveys (Gunzburger 2007; Wikston et al. 2023). Increasing the scope of visual encounter surveys and including movement of cover could increase species detection but at the cost of a level of invasiveness to the surrounding and potentially vital habitat. Additionally, active acoustic surveys were conducted a total of three times which was fewer sampling events than any other method. Species detection using this method may have been greater if a similar number of sampling events had occurred. Considering sampling effort during data interpretation can aid in understanding the study's biases going forward.

Implementation of spatial sampling is vital to the success of amphibian monitoring and can be a limitation during field sampling. Due to the requirements of the collaborator, *rare* Charitable Research Reserve, researchers could not disturb the sediment of the vernal

pools due to a future study on sediment cores. This resulted in all eDNA samples being collected from the pools' edge which could affect the eDNA collected. Sampling at the pool edge without disturbing sediment could result in sampling in locations where possible barriers of eDNA dispersion exist, such as vegetation, resulting in low eDNA collected (Harper et al. 2019). However, a study conducted in similar lentic wetlands found that sampling at the surface was the optimal location for eDNA detection (Moyer et al. 2014). In future sampling, spatial sampling could include an alternate depth and location towards the middle of the vernal pool to determine if similar patterns exist as seen in other studies.

To allow for the uptake of eDNA barcoding as a viable method for species detection and monitoring, it must be tested and levels of error minimized. A step to ensuring accuracy of results is to optimize assay conditions *in vitro* prior to testing on environmental samples. This process is time consuming but leads to increased detection accuracy. Optimization conditions have been known to be lab specific and can lead to different performance of the same assays (Klymus et al. 2020; Lesperance et al. 2021). The assay conditions and associated efficiency, fit, and level of error should be reported for transparency and reproducibility (Bustin et al. 2009; Mauvisseau et al. 2019; Abbott et al. 2021).

A challenge to using eDNA methods for species detection is the lack of complete sequence data for amphibian species. Lots of time was spent on creating tissue derived standards to best represent local amphibian haplotypes. However, due to lack of sequence data for some species, there was potential mismatches in primer binding region of the PCR universal COI primers resulting in no amplification of target regions. This led to optimization of some assays using the tissue derived standards while others, such as spring peeper and northern leopard frog, were optimized with synthetic oligonucleotides. To save time in future optimizations, synthetic oligonucleotides would be used for all optimizations.

Synthetic oligonucleotide standards used for eDNA barcoding standard curves should include a synthetic insert that could be used for multiplexing and tracking contamination during qPCR set up from the standards (Wilson et al. 2016). Since standards used for standard curves can exist at high concentrations alongside low concentration eDNA samples,

it is important to ensure detections in the sample are not false positives from standards. The addition of the synthetic insert allowed for the multiplexing of reactions to track the introduction of contamination. Despite no contamination detected in the eDNA samples of this study, this is an important addition to lend confidence in the amplified results.

Additionally, eDNA is often present in low concentrations making it vital to understand the limitations of an assay for such concentrations. For this reason, indicators of sensitivity, such as LOD and LOQ, should be reported when discussing eDNA studies. In the context of this study, these sensitivity metrics aid in better interpreting negative results. These negative results are often difficult to interpret but it is important to recognize that non-detections can indicate species absence or species eDNA concentrations below the limit of detection. The definitions of these values have been slightly debated in the field and the method for determining the LOD and LOQ varies between studies. In this study, I used the experimental design of Klymus et al. 2019, however, the analysis used for determining the LOQ often resulted in overfitting high order polynomial models which leads to inaccurate values for LOQ. Therefore, analysis of the LOD and LOQ was completed with the eLowQuant script developed by Lesperance et al. 2021. Many other studies suggest alternate methods of calculating the same values leading to difficult comparisons of values between studies (Hunter et al. 2017; Leung et al. 2017; Klymus et al. 2019; Lesperance et al. 2021). Standardization of these values will increase transparency and understanding of assay sensitivity between studies.

Considerations during use of eDNA samples for quality control and amphibian detections are vital for optimizing future studies. Following the eDNA extraction process, the preparation of eDNA aliquots would have helped to ensuring ample sample for quality control and species detection assays. Additionally, the DR-1 assay used to determine inhibition of samples was designed for a large amplicon (298 bp), which is above the normal acceptable cut off of 200 bp for highly efficient assays (Holm et al. 2021). The design of an internal positive control assay that amplifies a DNA fragment below the 200 bp cut off would be preferable. A DNA fragment from a fish species could still be used as the target due to

the lack of fish in vernal pools while ensuring no overlap in sequences with other flora, fauna, or microbes present in the vernal pool. Lastly, adequate eDNA sample volume is needed for quality control assays and amphibian detection assays. Lack of sufficient sample volume posed a challenge in this study due to multiple factors. Some samples were used for pilot studies for quality control assays and amphibian assays prior to optimization in 2020. This led to low levels of sample for certain sampling dates in May and June. For future studies, I suggest a pilot study with a lessened sampling effort to conduct optimization of quality control assays and complete initial test of eDNA barcoding assays. Additionally, due to the COVID-19 global pandemic, there was prolonged storage of samples at -80 °C. Although this storage did not affect the eDNA integrity, the samples experienced potential sample loss due to sublimation and volume loss. Ideally, optimization of eDNA barcoding assays should be complete prior to sample collection to decrease the storage period. Overall, this study met many of the standards for eDNA barcoding studies, but improvements are always possible with further considerations.

Many studies have focused not only on the ability for species detection using eDNA barcoding, but a look at cost benefit analysis and time investment (Rees et al. 2014; Smart et al. 2016; Barata et al. 2017; Thalinger et al. 2021; Bernos et al. 2023). There are differing opinions on whether this method is truly more cost-effective as there are trade-offs when increasing sampling to increase detection ability, but it comes at an increased cost. The goal is to find a balance for an effective method of species monitoring but with a sampling effort that is cost-effective. Studies must aim to determine the point when increased sampling, optimization, and controls, no longer drastically affects the detection of species. As seen in this study, there is a large time commitment and associated cost to optimizing eDNA barcoding assays to improve performance and increase sensitivity for species detection. The upfront cost to allow for eDNA studies to withstand scrutiny could be worth the cost if future long-term monitoring is conducted. Long-term monitoring with eDNA barcoding can be less costly than other conventional methods which can be costly on this time scale (Smart et al. 2016). Using eDNA barcoding in an established lab with an established and optimized

protocol for long term studies neutralizes the initial cost of optimization. eDNA barcoding could offer a more cost-effective method for long-term studies of Canadian amphibians, though this requires an official review of these methodologies and the associated costs.

3.3 Future Directions

Amphibian monitoring is an area of active research and there are many ways in which the research could be further explored. I suggest that some possible follow-up studies could include the use of multi-year testing to better understand the effectiveness of species detection. This would allow for methods to be compared over multiple seasons which is more representative of the desired long-term monitoring required for amphibian conservation. Determining the species detection of eDNA barcoding compared to conventional methods over a longer timespan can help support the uptake of this method as a monitoring technique. Vernal pools are vital habitat but due to their natural fluctuations, chances of underestimating their use is highly possible. A future study could also include increasing the number of species targeted including adding additional species from the order Caudata, such as the blue spotted salamander (*Ambystoma laterale*). I also suggest adding a conventional method better suited to salamander detection such as coverboard surveys to be set up and monitored nearby to the vernal pool wetlands. Species detection in vernal pools using eDNA may also be benefitted by utilizing an additional method such as eDNA metabarcoding. eDNA barcoding is often more specific however, metabarcoding can capture a broader amphibian community outside of the six target species from this study. This could indicate a more expansive use of vernal pools by a larger number of amphibian species further proving how vital these ephemeral wetlands are. A similar study could be conducted with stricter parameters for survey effort and cost effectiveness to determine the optimal surveys for amphibian species detection in wetlands. All these studies would allow for a broader picture to aid in ongoing amphibian monitoring and conservation efforts. An additional follow up study to aid in conservation efforts is to not only determine species presence in vernal pools but investigate

other threats facing these amphibians such as pathogens. eDNA barcoding for pathogens such as the ranavirus frog virus 3 (FV3) or *Batrachochytrium dendrobatidis* (Bd) could be used alongside amphibian eDNA detection to determine patterns in pathogen introduction (Teacher et al. 2010; Kik et al. 2011; Kamoroff and Goldberg 2017; Leung et al. 2017; Standish et al. 2018; Miaud et al. 2019). A study like this would help determine possible vector species of pathogens into vernal pool wetlands. These pathogens are responsible for mass mortality events and a better understanding of the transmission can aid in effective monitoring and prevention (Kik et al. 2011; Kamoroff and Goldberg 2017; Burgos et al. 2019). The use of molecular methods to detect pathogens could detect pathogens before the pathogen load is high enough to cause a mass mortality event.

References

- Abbott C, Coulson M, Gagné N, Lacoursière-Roussel A, Parent GJ, Bajno R, Dietrich C, May-McNally S. 2021. Canadian Science Advisory Secretariat (CSAS) Guidance on the Use of Targeted Environmental DNA (eDNA) Analysis for the Management of Aquatic Invasive Species and Species at Risk. Sci Advis Sec Res Doc.:42. <http://www.dfo-mpo.gc.ca/csas-sccs/>.
- Armbruster DA, Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 29:49–52.
- Atkinson MS, Savage AE. 2023. Invasive amphibians alter host-pathogen interactions with primarily negative outcomes for native species. Biol Conserv. 286:110310. doi:10.1016/j.biocon.2023.110310.
- Badzinski D, Archer R. 2008. Assessment of trends in frog and toad populations in Ontario using citizen science monitoring data. <http://www.citeulike.org/group/13602/article/7303694>.
- Baillie JEM, Bennun LA, Brooks TM, Butchart SHM, Janice S, Chanson JS, Cokeliss Z, Hilton-Taylor C, Hoffmann M, Mace GM, Mainka SA, et al. 2004. IUCN Red List of Threatened Species. A Global Species Assessment. IUCN, Gland, Switzerland and Cambridge.
- Bairoliya S, Zhi Xiang JK, Cao B. 2022. Extracellular DNA in Environmental Samples: Occurrence, Extraction, Quantification, and Impact on Microbial Biodiversity Assessment. Appl Environ Microbiol. 88(3):1–13. doi:10.1128/aem.01845-21.
- Barata IM, Griffiths RA, Ridout MS. 2017. The power of monitoring: Optimizing survey designs to detect occupancy changes in a rare amphibian population. Sci Rep. 7(1):1–9. doi:10.1038/s41598-017-16534-8. <http://dx.doi.org/10.1038/s41598-017-16534-8>.
- Barnes MA, Turner CR. 2016. The ecology of environmental DNA and implications for conservation genetics. Conserv Genet. 17(1):1–17. doi:10.1007/s10592-015-0775-4.
- Beauclerc K, Wozney K, Smith C, Wilson C. 2019. Development of quantitative PCR primers and probes for environmental DNA detection of amphibians in Ontario. Conserv Genet Resour. 11(1):43–46. doi:10.1007/s12686-017-0962-3. <http://dx.doi.org/10.1007/s12686-017-0962-3>.
- Bedwell ME, Goldberg CS. 2020. Spatial and temporal patterns of environmental DNA detection to inform sampling protocols in lentic and lotic systems. Ecol Evol. 10(3):1602–1612.

doi:10.1002/ece3.6014.

Beebee TJC. 2013. Effects of road mortality and mitigation measures on amphibian populations. *Conserv Biol.* 27(4):657–668. doi:10.1111/cobi.12063.

Bernos TA, Yates MC, Docker MF, Fitzgerald A, Hanner R, Heath D, Imrit A, Livernois J, Myler E, Patel K, et al. 2023. Environmental DNA (eDNA) applications in freshwater fisheries management and conservation in Canada: overview of current challenges and opportunities. *Can J Fish Aquat Sci.* 80(7):1170–1186. doi:10.1139/cjfas-2022-0162.

Biggs J, Ewald N, Valentini A, Gaboriaud C, Dejean T, Griffiths RA, Foster J, Wilkinson JW, Arnell A, Brotherton P, et al. 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biol Conserv.* 183:19–28. doi:10.1016/j.biocon.2014.11.029.

Bird Studies Canada. 2009. Marsh Monitoring Program Participant's Handbook for Surveying Amphibians.

Bishop CA, Pettit KE. 1992. Declines in Canadian amphibian populations: designing a national monitoring strategy. Ottawa.

Blaustein AR, Walls SC, Bancroft BA, Lawler JJ, Searle CL, Gervasi SS. 2010. Direct and indirect effects of climate change on amphibian populations. *Diversity.* 2(2):281–313. doi:10.3390/d2020281.

Bosch J, De La Riva I. 2004. Are frog calls modulated by the environment? An analysis with anuran species from Bolivia. *Can J Zool.* 82(6):880–888. doi:10.1139/Z04-060.

Boullhesen M, Vaira M, Barquez RM, Akmentins MS. 2021. Evaluating the efficacy of visual encounter and automated acoustic survey methods in anuran assemblages of the Yungas Andean forests of Argentina. *Ecol Indic.* 127:107750. doi:10.1016/j.ecolind.2021.107750. <https://doi.org/10.1016/j.ecolind.2021.107750>.

Boyero L, López-Rojo N, Bosch J, Alonso A, Correa-Araneda F, Pérez J. 2020. Microplastics impair amphibian survival, body condition and function. *Chemosphere.* 244(125500):1–7. doi:10.1016/j.chemosphere.2019.125500.

Brauer CL, Donovan TM, Mickey RM, Katz J, Mitchell BR. 2016. A comparison of acoustic monitoring methods for common anurans of the northeastern United States. *Wildl Soc Bull.*

40(1):140–149. doi:10.1002/wsb.619.

Breton AA, Beaty L, Bennett AM, Kyle CJ, Lesbarrères D, Torres S, Madison V, Wilson CC, Murray DL. 2022. Testing the effectiveness of environmental DNA (eDNA) to quantify larval amphibian abundance. *Environ DNA*.(August 2021):1–12. doi:10.1002/edn3.332.

Breton BAA, Beaty L, Bennett AM, Kyle CJ, Lesbarrères D, Vilaça ST, Wikston MJH, Wilson CC, Murray DL. 2022. Testing the effectiveness of environmental DNA (eDNA) to quantify larval amphibian abundance. *Environ DNA*. 00:1–12. doi:10.1002/edn3.332.

Brooks RT. 2004. Weather-related effects on woodland vernal pool hydrology and hydroperiod. *Wetlands*. 24(1):104–114. doi:10.1672/0277-5212(2004)024[0104:WEOWVP]2.0.CO;2.

Brown LJ. 1997. An Evaluation of Some Marking and Trapping Techniques Currently Used in the Study of Anuran Population Dynamics. *J Herpetol*. 31(3):410–419.

Brühl CA, Pieper S, Weber B. 2011. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environ Toxicol Chem*. 30(11):2465–2472. doi:10.1002/etc.650.

Bulger JB, Scott NJ, Seymour RB. 2003. Terrestrial activity and conservation of adult California red-legged frogs *Rana aurora draytonii* in coastal forests and grasslands. *Biol Conserv*. 110(1):85–95. doi:10.1016/S0006-3207(02)00179-9.

Burgos G, Narváez-Narváez DA, Freire-Paspuel B, Merino-Viteri A, Muslin C, Genoy-Puerto A. 2019. Development of a multiplex real-time PCR surveillance assay for monitoring the health status of Ecuadorian amphibians at risk of extinction. *Forensic Sci Int Genet Suppl Ser*. 7(1):674–676. doi:10.1016/j.fsigss.2019.10.134.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 55(4):611–622. doi:10.1373/clinchem.2008.112797.

Calderon MR, Almeida CA, González P, Jofré MB. 2019. Influence of water quality and habitat conditions on amphibian community metrics in rivers affected by urban activity. *Urban Ecosyst*. 22(4):743–755. doi:10.1007/s11252-019-00862-w.

Chen MH, Watkins GG. 2005. Sampling Amphibians and Reptiles in the Iwokrama Forest Ecosystem. *Acad Nat Sci*. 154:55–69. doi:129.97.193.44.

CHS. 2023. Status of Amphibians and Reptiles in Canada. Can Herpetol Soc. [accessed 2023 Dec 19]. <https://canadianherpetology.ca/species/decline.html>.

CHS. 2024. About Amphibians. Can Herpetol Soc. [accessed 2024 Jan 12]. <https://canadianherpetology.ca/species/biology/amphibians.html>.

Collins JP, Storfer A. 2003. Global amphibian declines: Sorting the hypotheses. *Divers Distrib.* 9(2):89–98. doi:10.1046/j.1472-4642.2003.00012.x.

Collins MK, Spear SF, Groves JD, Williams LA, Kuchta SR. 2019. Searching for a Salamander: Distribution and Habitat of the Mudpuppy (*Necturus maculosus*) in Southeast Ohio Using eDNA as a Rapid Assessment Technique. *Am Midl Nat.* 182(2):191–202. doi:10.1674/0003-0031-182.2.191.

COSEWIC. 2009. COSEWIC assessment and update status report on the Northern Leopard Frog *Lithobates pipiens*, Rocky Mountain population, Western Boreal/Prairie populations and Eastern populations, in Canada. vii. Ottawa: Committee on the Status of Endangered Wildlife in Canada.

Crecchio C, Stotzky G. 1998. Binding of DNA on humic acids: Effect on transformation of *Bacillus subtilis* and resistance to DNase. *Soil Biol Biochem.* 30(8–9):1061–1067. doi:10.1016/S0038-0717(97)00248-4.

Cushman SA. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. *Biol Conserv.* 128(2):231–240. doi:10.1016/j.biocon.2005.09.031.

Dean A, Hunter A, Morse D. 2009. Ephemeral Wetland Restoration of an Agricultural Floodplain in Walsingham Township, Ontario.

Deiner K, Altermatt F. 2014. Transport distance of invertebrate environmental DNA in a natural river. *PLoS One.* 9(2). doi:10.1371/journal.pone.0088786.

Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bullfrog *Lithobates catesbeianus*. *J Appl Ecol.* 49(4):953–959. doi:10.1111/j.1365-2664.2012.02171.x.

Doi H, Minamoto T, Takahara T, Tsuji S, Uchii K, Yamamoto S, Katano I, Yamanaka H. 2021. Compilation of real-time PCR conditions toward the standardization of environmental DNA methods. *Ecol Res.* 36(3):379–388. doi:10.1111/1440-1703.12217.

Egea-Serrano A, Relyea RA, Tejedo M, Torralva M. 2012. Understanding of the impact of chemicals

- on amphibians: A meta-analytic review. *Ecol Evol.* 2(7):1382–1397. doi:10.1002/ece3.249.
- Eiler A, Löfgren A, Hjerne O, Nördén S, Saetre P. 2018. Environmental DNA (eDNA) detects the pool frog (*Pelophylax lessonae*) at times when traditional monitoring methods are insensitive. *Sci Rep.* 8(1):1–9. doi:10.1038/s41598-018-23740-5.
- Ernst R, Rödel M. 2004. Measuring and monitoring amphibian diversity in tropical forests . I . An evaluation of methods with recommendations for standardization. *Ecotropica.* 10:1–14.
- Faccio SD, Buckman KL, Lloyd JD, Curtis AN, Taylor VF. 2019. Bioaccumulation of methylmercury in wood frogs and spotted salamanders in Vermont vernal pools. *Ecotoxicology.* 28(7):717–731. doi:10.1007/s10646-019-02068-4.
- Faivovich J, Haddad CFB, Garcia PCA, Frost DR, Campbell JA, Wheeler WC. 2005. Systematic review of the frog family Hylidae, with special reference to Hyalinae: Phylogenetic analysis and taxonomic revision. *Bull Am Museum Nat Hist.*(294):1–227. doi:10.1206/0003-0090(2005)294[0001:srotff]2.0.co;2.
- Falaschi M, Melotto A, Manenti R, Ficetola GF. 2020. Invasive species and amphibian conservation. *Herpetologica.* 76(2):216–227. doi:10.1655/0018-0831-76.2.216.
- Farmer AL, Smith LL, Castleberry SB, Gibbons JW. 2009. A comparison of techniques for sampling amphibians in isolated wetlands in Georgia, USA. *Appl Herpetol.* 6(4):327–341. doi:10.1163/157075309X12470350858433.
- Fediajevaite J, Priestley V, Arnold R, Savolainen V. 2021. Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecol Evol.* 11(9):4803–4815. doi:10.1002/ece3.7382.
- Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. *Biol Lett.* 4(4):423–425. doi:10.1098/rsbl.2008.0118.
- Finn C, Grattarola F, Pincheira-Donoso D. 2023. More losers than winners: investigating Anthropocene defaunation through the diversity of population trends. *Biol Rev.* 4:000–000. doi:10.1111/brv.12974.
- Fisher MC, Garner TWJ. 2020. Chytrid fungi and global amphibian declines. *Nat Rev Microbiol.* 18(6):332–343. doi:10.1038/s41579-020-0335-x.

- Flint WD, Harris RN. 2005. The efficacy of visual encounter surveys for population monitoring of *Plethodon punctatus* (Caudata: Plethodontidae). *J Herpetol.* 39(4):578–584. doi:10.1670/255-04A.1.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. On the pseudoscorpionidea of australia. *Mol Mar Biol Biotechnol.* 14(2):275–303. doi:10.1071/ZO9660275.
- Gargan LM, Brooks PR, Vye SR, Ironside JE, Jenkins SR, Crowe TP, Carlsson J. 2022. The use of environmental DNA metabarcoding and quantitative PCR for molecular detection of marine invasive non-native species associated with artificial structures. *Biol Invasions.* 24(3):635–648. doi:10.1007/s10530-021-02672-8.
- Gibbons JW, Winne CT, Scott DE, Willson JD, Glaudas X, Andrews KM, Todd BD, Fedewa LA, Wilkinson L, Tsaliagos RN, et al. 2006. Remarkable amphibian biomass and abundance in an isolated wetland: Implications for wetland conservation. *Conserv Biol.* 20(5):1457–1465. doi:10.1111/j.1523-1739.2006.00443.x.
- Goldberg CS, Strickler KM, Fremier AK. 2018. Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of sampling designs. *Sci Total Environ.* 633:695–703. doi:10.1016/j.scitotenv.2018.02.295.
- Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS, et al. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods Ecol Evol.* 7(11):1299–1307. doi:10.1111/2041-210X.12595.
- Grant EHC, Zipkin EF, Nichols JD, Campbell JP. 2013. A Strategy for Monitoring and Managing Declines in an Amphibian Community. *Conserv Biol.* 27(6):1245–1253. doi:10.1111/cobi.12137.
- Green DM. 2017. Amphibian breeding phenology trends under climate change: predicting the past to forecast the future. *Glob Chang Biol.* 23(2):646–656. doi:10.1111/gcb.13390.
- Greenberg CH, Zarnoch SJ, Austin JD. 2018. Long term amphibian monitoring at wetlands lacks power to detect population trends. *Biol Conserv.* 228:120–131. doi:10.1016/j.biocon.2018.10.015.
- Gunzburger MS. 2007. Evaluation of seven aquatic sampling methods for amphibians and other aquatic fauna. *Appl Herpetol.* 4(1):47–63. doi:10.1163/157075407779766750.
- Halsey LG. 2019. The reign of the p-value is over: What alternative analyses could we employ to fill

the power vacuum? Biol Lett. 15(5). doi:10.1098/rsbl.2019.0174.

Hamer AJ, McDonnell MJ. 2008. Amphibian ecology and conservation in the urbanising world: A review. Biol Conserv. 141(10):2432–2449. doi:10.1016/j.biocon.2008.07.020.

Harper LR, Buxton AS, Rees HC, Bruce K, Brys R, Halfmaerten D, Read DS, Watson H V., Sayer CD, Jones EP, et al. 2019. Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. Hydrobiologia. 826(1):25–41. doi:10.1007/s10750-018-3750-5.

Harper LR, Lawson L, Christoph H, Boonham N, Rees HC, Gough KC, Lewis E, Adams IP, Brotherton P, Phillips S, et al. 2018. Needle in a haystack ? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). Ecol Evol.:6330–6341. doi:10.1002/ece3.4013.

Hecnar SJ. 2004. Great Lakes wetlands as amphibian habitats: A review. Aquat Ecosyst Heal Manag. 7(2):289–303. doi:10.1080/14634980490461542.

Helbing CC, Hobbs J. 2019. Environmental DNA standardization needs for fish and wildlife population assessments and monitoring. Stand Res.(2019):41.

Hernandez C, Bougas B, Perreault-Payette A, Simard A, Côté G, Bernatchez L. 2020. 60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. Environ DNA. 2(3):373–386. doi:10.1002/edn3.89.

Hobbs J, Adams IT, Round JM, Goldberg CS, Allison MJ, Bergman LC, Mirabzadeh A, Allen H, Helbing CC. 2020. Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus*, in British Columbia, Canada, using environmental DNA methods. Environ DNA. 2(3):350–361. doi:10.1002/edn3.82.

Hobbs J, Round JM, Allison MJ, Helbing CC. 2019. Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei*, in British Columbia, Canada, using robust eDNA detection methods. PLoS One. 14(3):1–16. doi:10.1371/journal.pone.0213849.

Holm W Van, Ghesquière J, Boon N, Verspecht T, Bernaerts K, Zayed N, Chatzigiannidou I, Teughels W. 2021. A Viability Quantitative PCR Dilemma: Are Longer Amplicons Better? Appl Environ Microbiol. 87(5):1–11. doi:10.1128/AEM.02653-20.

Hopkins WA. 2007. Amphibians as models for studying environmental change. ILAR J. 48(3):270–

277. doi:10.1093/ilar.48.3.270.

Howard SD, Bickford DP. 2014. Amphibians over the edge: Silent extinction risk of Data Deficient species. *Divers Distrib.* 20(7):837–846. doi:10.1111/ddi.12218.

Howell PE, Hossack BR, Muths E, Sigafus BH, Chandler RB. 2020. Informing amphibian conservation efforts with abundance-based metapopulation models. *Herpetologica.* 76(2):240–250. doi:10.1655/0018-0831-76.2.240.

Hunter ME, Dorazio RM, Butterfield JSS, Meigs-Friend G, Nico LG, Ferrante JA. 2017. Detection limits of quantitative and digital PCR assays and their influence in presence–absence surveys of environmental DNA. *Mol Ecol Resour.* 17(2):221–229. doi:10.1111/1755-0998.12619.

Hunter ME, Ferrante JA, Meigs-Friend G, Ulmer A. 2019. Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. *Sci Rep.* 9(1):1–9. doi:10.1038/s41598-019-40977-w.

Hussain QA, Pandit AK. 2012. Global amphibian declines: A review. *Int J Biodivers Conserv.* 4(10):348–357. doi:10.5897/ijbc12.008.

IUCN. 2023. The IUCN Red List of Threatened Species. Version 2022-2. IUCN Red List. [accessed 2023 Sep 11]. <https://www.iucnredlist.org>.

Jo T, Arimoto M, Murakami H, Masuda R, Minamoto T. 2020. Estimating shedding and decay rates of environmental nuclear DNA with relation to water temperature and biomass. *Environ DNA.* 2(2):140–151. doi:10.1002/edn3.51.

Jo T, Takao K, Minamoto T. 2022. Linking the state of environmental DNA to its application for biomonitoring and stock assessment: Targeting mitochondrial/nuclear genes, and different DNA fragment lengths and particle sizes. *Environ DNA.* 4(2):271–283. doi:10.1002/edn3.253.

Kamoroff C, Goldberg CS. 2017. Using environmental DNA for early detection of amphibian chytrid fungus *Batrachochytrium dendrobatidis* prior to a ranid die-off. *Dis Aquat Organ.* 127(1):75–79. doi:10.3354/dao03183.

Kik M, Martel A, Sluijs AS van der, Pasmans F, Wohlsein P, Gröne A, Rijks JM. 2011. Ranavirus-associated mass mortality in wild amphibians, The Netherlands, 2010: A first report. *Vet J.* 190(2):284–286. doi:10.1016/j.tvjl.2011.08.031.

- Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, et al. 2019. Reporting the limits of detection and quantification for environmental DNA assays. *Environ DNA*. 2(3):271–282. doi:10.1002/edn3.29.
- Klymus KE, Richter CA, Chapman DC, Paukert C. 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biol Conserv*. 183:77–84. doi:10.1016/j.biocon.2014.11.020.
- Klymus KE, Ruiz Ramos D V., Thompson NL, Richter CA. 2020. Development and Testing of Species-specific Quantitative PCR Assays for Environmental DNA Applications. *J Vis Exp*.(165):1–25. doi:10.3791/61825.
- Langlois VS, Allison MJ, Bergman LC, To TA, Helbing CC. 2020. The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. *Environ DNA*. 3(3):519–527. doi:10.1002/edn3.164.
- Lee TS, Randall LA, Kahal NL, Kinas HL, Carney VA, Rudd H, Baker TM, Sanderson K, Creed IF, Moehrensclager A, et al. 2022. A framework to identify priority wetland habitats and movement corridors for urban amphibian conservation. *Ecol Solut Evid*. 3(2):1–15. doi:10.1002/2688-8319.12139.
- Lesperance ML, Allison MJ, Bergman LC, Hocking MD, Helbing CC. 2021. A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. *Environ DNA*. 3(5):970–981. doi:10.1002/edn3.220.
- Leung WTM, Thomas-Walters L, Garner TWJ, Balloux F, Durrant C, Price SJ. 2017. A quantitative-PCR based method to estimate ranavirus viral load following normalisation by reference to an ultraconserved vertebrate target. *J Virol Methods*. 249:147–155. doi:10.1016/j.jviromet.2017.08.016.
- Liedtke HC, Wiens JJ, Gomez-Mestre I. 2022. The evolution of reproductive modes and life cycles in amphibians. *Nat Commun*. 13(1):1–15. doi:10.1038/s41467-022-34474-4.
- Littlefair JE, Hrenchuk LE, Blanchfield PJ, Rennie MD, Cristescu ME. 2021. Thermal stratification and fish thermal preference explain vertical eDNA distributions in lakes. *Mol Ecol*. 30(13):3083–3096. doi:10.1111/mec.15623.
- Liu M, Clarke LJ, Baker SC, Jordan GJ, Burrridge CP. 2020. A practical guide to DNA metabarcoding

for entomological ecologists. *Ecol Entomol*. 45(3):373–385. doi:10.1111/een.12831.

Luedtke JA, Chanson J, Neam K, Hobin L, Maciel AO, Catenazzi A, Borzée A, Hamidy A, Aowphol A, Jean A, et al. 2023. Ongoing declines for the world's amphibians in the face of emerging threats. *Nature*. 622(7982):308–314. doi:10.1038/s41586-023-06578-4.

Maruyama A, Nakamura K, Yamanaka H, Kondoh M, Minamoto T. 2014. The release rate of environmental DNA from juvenile and adult fish. *PLoS One*. 9(12):1–13. doi:10.1371/journal.pone.0114639.

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M. 2019. Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. *Sci Rep*. 9(1):1–10. doi:10.1038/s41598-018-37001-y. <http://dx.doi.org/10.1038/s41598-018-37001-y>.

McKee AM, Spear SF, Pierson TW. 2015. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biol Conserv*. 183:70–76. doi:10.1016/j.biocon.2014.11.031.

Melo I, Llusia D, Bastos RP, Signorelli L. 2021. Active or passive acoustic monitoring? Assessing methods to track anuran communities in tropical savanna wetlands. *Ecol Indic*. 132. doi:10.1016/j.ecolind.2021.108305.

Miaud C, Arnal V, Poulain M, Valentini A, Dejean T. 2019. eDNA increases the detectability of ranavirus infection in an alpine amphibian population. *Viruses*. 11(6):1–15. doi:10.3390/v11060526.

Morin PJ. 1983. Predation , Competition , and the Composition of Larval Anuran Guilds. *America (NY)*. 53(2):119–138.

Moss WE, Harper LR, Davis MA, Goldberg CS, Smith MM, Johnson PTJ. 2022. Navigating the trade-offs between environmental DNA and conventional field surveys for improved amphibian monitoring. *Ecosphere*. 13(2). doi:10.1002/ecs2.3941.

Moushomi R, Wilgar G, Carvalho G, Creer S, Seymour M. 2019. Environmental DNA size sorting and degradation experiment indicates the state of *Daphnia magna* mitochondrial and nuclear eDNA is subcellular. *Sci Rep*. 9(1):1–9. doi:10.1038/s41598-019-48984-7.

Moyer GR, Díaz-Ferguson E, Hill JE, Shea C. 2014. Assessing environmental DNA detection in

controlled lentic systems. PLoS One. 9(7). doi:10.1371/journal.pone.0103767.

Murakami H, Yoon S, Kasai A, Minamoto T, Yamamoto S, Sakata MK, Horiuchi T, Sawada H, Kondoh M, Yamashita Y, et al. 2019. Dispersion and degradation of environmental DNA from caged fish in a marine environment. Fish Sci. 85(2):327–337. doi:10.1007/s12562-018-1282-6.

Di Muri C, Handley LL, Bean CW, Li J, Peirson G, Sellers GS, Walsh K, Watson H V., Winfield IJ, Hänfling B. 2020. Read counts from environmental DNA (eDNA) metabarcoding reflect fish abundance and biomass in drained ponds. Metabarcoding and Metagenomics. 4:97–112. doi:10.3897/MBMG.4.56959.

Nahm FS. 2017. What the P values really tell us. Korean J Pain. 30(4):241–242.

National Center for Biotechnology Information (NCBI). No Title. Bethesda Natl Libr Med. <https://www.ncbi.nlm.nih.gov/>.

Nolan N, Hayward MW, Klop-Toker K, Mahony M, Lemckert F, Callen A. 2023. Complex Organisms Must Deal with Complex Threats: How Does Amphibian Conservation Deal with Biphasic Life Cycles? Animals. 13(10):1–22. doi:10.3390/ani13101634.

Nybo K. 2011. qPCR efficiency calculations. Biotechniques. 51(6):401–402. doi:10.2144/000113776.

Ontario Nature. 2023. Spring peeper. Ontario Nat Cent Soc Innov. [accessed 2023 Oct 12]. <https://ontarionature.org/programs/community-science/reptile-amphibian-atlas/spring-peeper/>.

Oshiro J. 2021. Why save amphibians? AmphibiaWeb. [accessed 2022 Nov 4]. <https://amphibiaweb.org/declines/conservation.html>.

Petranka JW, Harp EM, Holbrook CT, Hamel JA. 2007. Long-term persistence of amphibian populations in a restored wetland complex. Biol Conserv. 138(3–4):371–380. doi:10.1016/j.biocon.2007.05.002.

Petranka JWP, Smith CKS, Scott FA. 2004. Pond-breeding amphibians. 14(4):1065–1078.

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Can J Fish Aquat Sci. 70(8):1123–1130. doi:10.1139/cjfas-2013-0047.

Postaire B, Dupuch A, Ladent E, Surget-Groba Y. 2023. Vernal Pool Amphibian Inventories in

- Temperate Forests of Eastern North America: Can Environmental DNA Replace Traditional Methods? *Forests*. 14(1930). doi:[https:// doi.org/10.3390/f14101930](https://doi.org/10.3390/f14101930).
- Pyke CR. 2005. Assessing climate change impacts on vernal pool ecosystems and endemic branchiopods. *Ecosystems*. 8(1):95–105. doi:10.1007/s10021-004-0086-y.
- Quilumbaquin W, Carrera-Gonzalez A, Van der Heyden C, Ortega-Andrade HM. 2023. Environmental DNA and visual encounter surveys for amphibian biomonitoring in aquatic environments of the Ecuadorian Amazon. *PeerJ*. 11:1–25. doi:10.7717/peerj.15455.
- Randall LA, Goldberg CS, Moehenschlager A. 2023. Environmental DNA surveys can underestimate amphibian occupancy and overestimate detection probability: implications for practice. *J Wildl Manage*. 87(7):1–18. doi:10.1002/jwmg.22463.
- Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC. 2014. The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *J Appl Ecol*. 51(5):1450–1459. doi:10.1111/1365-2664.12306.
- Rice KG, Mazzotti FJ, Waddle JH. 2007. Use of Amphibians as Indicators of Ecosystem Restoration Success. *Edis*. 2007(14):1–5. doi:10.32473/edis-uw233-2007.
- Rickwood D, Chambers JAA. 1981. Evidence for protected regions of DNA in the mitochondrial nucleoid of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett*. 12(2):187–190. doi:10.1111/j.1574-6968.1981.tb07639.x.
- Rourke ML, Walburn JW, Broadhurst MK, Fowler AM, Hughes JM, Fielder DS, DiBattista JD, Furlan EM. 2023. Poor utility of environmental DNA for estimating the biomass of a threatened freshwater teleost; but clear direction for future candidate assessments. *Fish Res*. 258. doi:10.1016/j.fishres.2022.106545.
- Rowland FE, Rawlings MB, Semlitsch RD. 2017. Joint effects of resources and amphibians on pond ecosystems. *Oecologia*. 183(1):237–247. doi:10.1007/s00442-016-3748-5.
- Ruppert KM, Kline RJ, Rahman MS. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Glob Ecol Conserv*. 17:e00547. doi:10.1016/j.gecco.2019.e00547.
- Ruso GE, Morrissey CA, Hogan NS, Sheedy C, Gallant MJ, Jardine TD. 2019. Detecting Amphibians

in Agricultural Landscapes Using Environmental DNA Reveals the Importance of Wetland Condition. *Environ Toxicol Chem.* 38(12):2750–2763. doi:10.1002/etc.4598.

Scheffers BR, Paszkowski CA. 2012. The effects of urbanization on North American amphibian species: Identifying new directions for urban conservation. *Urban Ecosyst.* 15(1):133–147. doi:10.1007/s11252-011-0199-y.

Shu L, Ludwig A, Peng Z. 2020. Standards for methods utilizing environmental dna for detection of fish species. *Genes (Basel).* 11(3). doi:10.3390/genes11030296.

Simon JA, Snodgrass JW, Casey RE, Sparling DW. 2009. Spatial correlates of amphibian use of constructed wetlands in an urban landscape. *Landsc Ecol.* 24(3):361–373. doi:10.1007/s10980-008-9311-y.

Skelton J, Cauvin A, Hunter ME. 2023. Environmental DNA metabarcoding read numbers and their variability predict species abundance, but weakly in non-dominant species. *Environ DNA.* 5(5):1092–1104. doi:10.1002/edn3.355.

Smart AS, Tingley R, Weeks AR, Van Rooyen AR, McCarthy MA. 2015. Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecol Appl.* 25(7):1944–1952. doi:10.1890/14-1751.1.

Smart AS, Weeks AR, van Rooyen AR, Moore A, McCarthy MA, Tingley R. 2016. Assessing the cost-efficiency of environmental DNA sampling. *Methods Ecol Evol.* 7(11):1291–1298. doi:10.1111/2041-210X.12598.

De Souza LS, Godwin JC, Renshaw MA, Larson E. 2016. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One.* 11(10):1–15. doi:10.1371/journal.pone.0165273.

Spitzen - van der Sluijs A, Stark T, DeJean T, Verbrugghe E, Herder J, Gilbert M, Janse J, Martel A, Pasmans F, Valentini A. 2020. Using environmental DNA for detection of *Batrachochytrium salamandrivorans* in natural water. *Environ DNA.* 2(4):565–571. doi:10.1002/edn3.86.

Standish I, Leis E, Schmitz N, Credico J, Erickson S, Bailey J, Kerby J, Phillips K, Lewis T. 2018. Optimizing, validating, and field testing a multiplex qPCR for the detection of amphibian pathogens. *Dis Aquat Organ.* 129(1):1–13. doi:10.3354/dao03230.

- Stefan A. 2023. Green Frog. *Thecanadianencyclopedia.ca*. [accessed 2023 Oct 11].
<https://www.thecanadianencyclopedia.ca/en/article/green-frog>.
- Streicher JW, Sadler R, Loader SP. 2020. Amphibian taxonomy: early 21st century case studies. *J Nat Hist*. 54(1–4):1–13. doi:10.1080/00222933.2020.1777339.
- Strickler KM, Fremier AK, Goldberg CS. 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biol Conserv*. 183:85–92.
doi:10.1016/j.biocon.2014.11.038.
- Strijbosch H. 1979. Habitat Selection of Amphibians during Their Aquatic Phase. 33(3):363–372.
- Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Comment on “Status and trends of amphibian declines and extinctions worldwide”. *Science* (80-). 306(5743):1783–1787. doi:10.1126/science.1112996.
- Suarez-Bregua P, Álvarez-González M, Parsons KM, Rotllant J, Pierce GJ, Saavedra C. 2022. Environmental DNA (eDNA) for monitoring marine mammals: Challenges and opportunities. *Front Mar Sci*. 9(September):1–9. doi:10.3389/fmars.2022.987774.
- Swartz TM, Miller JR. 2018. Trapping Amphibians and Their Predators: Tradeoffs in Trap Design and Performance. *Herpetol J*. 49(2):238–243.
- Taberlet P, Coissac E, Hajibabaei M, Rieseber LH. 2012. Environmental DNA. *Mol Ecol*. 32(21):1789–1793. doi:10.1016/j.cub.2022.09.052.
- Tajadini M, Panjehpour M, Javanmard S. 2014. Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Adv Biomed Res*. 3(1):85. doi:10.4103/2277-9175.127998.
- Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z. 2012. Estimation of fish biomass using environmental DNA. *PLoS One*. 7(4):3–10. doi:10.1371/journal.pone.0035868.
- Tanadini LG, Schmidt BR. 2011. Population size influences amphibian detection probability: Implications for biodiversity monitoring programs. *PLoS One*. 6(12).
doi:10.1371/journal.pone.0028244.
- Teacher AGF, Cunningham AA, Garner TWJ. 2010. Assessing the long-term impact of Ranavirus infection in wild common frog populations. *Anim Conserv*. 13(5):514–522. doi:10.1111/j.1469-

1795.2010.00373.x.

Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS, Bruce K. 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environ DNA*. 3(4):823–836. doi:10.1002/edn3.189.

Todd BD, Winne CT, Willson JD, Gibbons JW. 2007. Getting the drift: Examining the effects of timing, trap type and taxon on herpetofaunal drift fence surveys. *Am Midl Nat*. 158(2):292–305. doi:10.1674/0003-0031(2007)158[292:GTDETE]2.0.CO;2.

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F, et al. 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol Ecol*. 25(4):929–942. doi:10.1111/mec.13428.

Valley C, Pools V. 2021. EPA Public Access. doi:10.1016/j.envpol.2019.113486.A.

Veldhoen N, Hobbs J, Ikonomou G, Hii M, Lesperance M, Helbing CC. 2016. Implementation of novel design features for qPCR-based eDNA assessment. *PLoS One*. 11(11):1–23. doi:10.1371/journal.pone.0164907.

Venter O, Brodeur NN, Nemiroff L, Belland B, Dolinsek IJ, Grant JWA. 2006. Threats to endangered species in Canada. *Bioscience*. 56(11):903–910. doi:10.1641/0006-3568(2006)56[903:TTESIC]2.0.CO;2.

Venturino A, Rosenbaum E, Caballero De Castro A, Anguiano OL, Gauna L, Fonovich de Schroeder T, Pechen de D'Angelo AM. 2003. Biomarkers of effect in toads and frogs. *Biomarkers*. 8(4/3):167–186. doi:10.1080/1354700031000120116.

Vilaça ST, Grant SA, Beaty L, Brunetti CR, Congram M, Murray DL, Wilson CC, Kyle CJ. 2020. Detection of spatiotemporal variation in ranavirus distribution using eDNA. *Environ DNA*. 2(2):210–220. doi:10.1002/edn3.59.

Walls SC. 2014. Identifying monitoring gaps for amphibian populations in a North American biodiversity hotspot, the southeastern USA. *Biodivers Conserv*. 23(13):3341–3357. doi:10.1007/s10531-014-0782-7.

Walls SC, Gabor CR. 2019. Integrating behavior and physiology into strategies for amphibian conservation. *Front Ecol Evol*. 7(234):1–13. doi:10.3389/fevo.2019.00234.

Welsh HH, Ollivier LM. 1998. Stream amphibians as indicators of ecosystem stress: A case study from California's redwoods. *Ecol Appl.* 8(4):1118–1132. doi:10.1890/1051-0761(1998)008[1118:SAAIOE]2.0.CO;2.

Whiles MR, Lips KR, Pringle CM, Kilham SS, Bixby RJ, Brenes R, Connelly S, Colon-Gaud JC, Hunte-Brown M, Huryn AD, et al. 2006. The effects of amphibian population declines on the structure and function of neotropical stream ecosystems. *Front Ecol Environ.* 4(1):27–34. doi:10.1890/1540-9295(2006)004[0027:TEOAPD]2.0.CO;2.

Wikston M, Breton B-AA, Vilaça ST, Bennett AM, Kyle CJ, Beresford D V., Lesbarrères D, Wilson CC, Green DM, Fortin M-J, et al. 2023. Comparative efficacy of eDNA and conventional methods for monitoring wetland anuran communities. *Front Ecol Evol.* 11:1–14. doi:10.3389/fevo.2023.1179158.

Wilson CC, Wozney KM, Smith CM. 2016. Recognizing false positives: Synthetic oligonucleotide controls for environmental DNA surveillance. *Methods Ecol Evol.* 7(1):23–29. doi:10.1111/2041-210X.12452.

Yamanaka H, Minamoto T, Matsuura J, Sakurai S, Tsuji S, Motozawa H, Hongo M, Sogo Y, Kakimi N, Teramura I, et al. 2017. A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. *Limnology.* 18(2):233–241. doi:10.1007/s10201-016-0508-5.

Yu Z, Ito SI, Wong MKS, Yoshizawa S, Inoue J, Itoh S, Yukami R, Ishikawa K, Guo C, Ijichi M, et al. 2022. Comparison of species-specific qPCR and metabarcoding methods to detect small pelagic fish distribution from open ocean environmental DNA. *PLoS One.* 17(9):1–23. doi:10.1371/journal.pone.0273670.

Appendix A

Supplementary Data

Supplementary Table 1. Synthetic oligonucleotide standard sequences for target amphibians.

Species common name	Species Latin name	Sequence with synthetic probe	Length	Source of sequence
American toad	<i>Anaxyrus americanus</i>	CTTGCCACGCAGGACCATCAGTTGACTTAACCATGGAT CCAAGCTTAGGCCTTTTTCCTCCATCTAGCAGGTGAT CATCTATTCTGGGGCAATTAATTAATACCACAACCCCT AAACATGAAACC	131	>AMT02_COI-user_added_CHROMAT_ID=1849847_358-443
Green frog	<i>Lithobates clamitans</i>	ACCATCCTCAACTACACAATACCAAACA CTCTATTTGTCTGATCAGTCTTAATCACCAGTTCTACTACTTTTATCTC TCCCGGATCCAAGCTTAGGCCTAGTACTAGCTGGCCGAA TTACTATACTCTC	133	>GRFR_COIuniversal_F_2-user_added/460-570 CHROMAT_ID=1955471
Northern leopard frog	<i>Lithobates pipiens</i>	CTTCATCTACACACAGTACCAAACACCCCTATTGTCTG ATCTGTTTTGATCACCAGCTTTACTTCTCTTATCTCTC GGATCCAAGCTTAGGCCTCCAGTCTAGCGGCAGGGAT ACTATACTCTTAACAGACCG	139	>MG423306.1/481-580 Lithobates pipiens voucher BIOUG31184-H02 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
Spotted salamander	<i>Ambystoma maculatum</i>	AGCACACGCATTTCGTAATGATTTTCTTCATAGTAATGCCT GTTATAATTGGCGGATTCGGAACCTGACTAGTGCCACTG GATCCAAGCTTAGGCCTAATAATGGGGCGCCAGATATA GCTTCCCCCGT	130	>SPSA1_COI-user_added_Sample_Name=183022 2_Chromat_id=1849907_Read_id=1 829798_Version=1_Length=683/134-232
Spring peeper	<i>Pseudacris crucifer</i>	TACTCCCCCATCTTTCTTCTCTCTCTCGCATCAGCAGC TGTAGAGGCAGGTGCTGGCACCAGGGTGAACCTGTTACC CCCCGGATCCAAGCTTAGGCCTTCTACTGGGAACCTTA CACATGCAATGGCACC	132	>EF525842.1/33-131 Pseudacris crucifer voucher MAS074-05 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
Wood frog	<i>Rana sylvatica</i>	GCCCCCTCAGTAGATTAGCTATTTCTCACTACACCTAGC TGGTGTTCATCTATCTTGGGGGCTATTAATTTATTACCA CAATCATTAATATAAAAAGATCCAAGCTTAGGCCTCCC ATCAACTACACAATATCAAAACACCCCT	148	>WOFR1_COI-user_added/371-500 Sample_Name=1830214 Chromat_id=1849906 Read_id=1829834 Version=1 Length=689
Zebra fish	<i>Danio rerio</i>	CAAGCCTGCGAAAAACACACCCAGTTTAAAAATCGCT AATGACGCATTAGTTGATTTGCCAACGCCACTAA ATATTTACGCGTGATGAAATTTTGATCTCTCCTTGGATT ATGTCTTATTACACAAATTTAACAGGACTAT TTTAGCAATACACTACACCTCAGACATCTCAACAGCAT TTTCATCTGTTGTGCATATTTGCCGAGATGTAA ATTCGGCTGACTTATTCGGAGCATCCATGCCAATGGGG CTTCCTTCTCTTCATCTGCCGTGATAT	298	>In house (Nathanael Harper and Michael Lynch)

Sequences for synthetic oligonucleotide standards with the associated forward primer region (yellow shading), reverse primer region (dark blue shading), species specific probe region (green shading), and synthetic insert probe (light blue shading).

Supplementary Table 2. Summary of water quality metrics from three vernal pools.

Date	Site	Wind (Beaufort Scale)	Canopy Cover	Water Temperature (°C)	Dissolved Oxygen (%)	Dissolved Oxygen (mg/L)	pH	Specific Conductance (SPC)	Conductivity	Total Dissolved Solids (TDS)	NH ₄	NH ₃
2019-04-04	Ancient Woods Pool	2	10	6.2	34.5	4.26	7.9	165.9	106.9	107.9	0.37	0.01
2019-04-08	Ancient Woods Pool	1	10	8.9	16.2	1.89	6.91	207.1	143.8	139.55	0.92	0
2019-04-18	Ancient Woods Pool	1	10	9.1	50.6	5.95	7.47	299.4	208.2	195	0.057	0
2019-04-24	Ancient Woods Pool	0	10	12.2	20.5	2.14	7.19	330.6	250.8	215.15	1.05	0
2019-05-02	Ancient Woods Pool	0	10	14	49.7	5.12	7.39	364.2	287.6	236.6	1.92	0.01
2019-05-24	Ancient Woods Pool	1	10	14.4	11.3	1.11	6.643	451.5	360	293.8	NA	NA
2019-06-07	Ancient Woods Pool	0	10	16.3	10.9	1.06	6.493	402	355.6	261.3	NA	NA
2019-06-27	Ancient Woods Pool	0	10	20	NA	NA	7.26	459.6	415	299	NA	NA
2019-04-04	Ancient Woods Swamp	2	10	5.1	15.4	1.94	8.95	127.5	79.2	83.2	0.5	0.03
2019-04-08	Ancient Woods Swamp	1	10	10.3	39.5	4.43	6.9	204.2	146.9	132	1.22	0
2019-04-24	Ancient Woods Swamp	1	10	14	30.1	3.14	7.17	289.9	230.6	188.5	0.99	0
2019-05-24	Ancient Woods Swamp	1	10	15.6	6	0.59	6.281	343.3	282.2	223.6	NA	NA
2019-06-07	Ancient Woods Swamp	0	10	16.1	12.4	1.18	6.306	321.3	266.9	208.65	NA	NA
2019-06-27	Ancient Woods Swamp	0	10	20.1	NA	NA	7.49	310.2	279.2	200.2	NA	NA
2019-04-04	Preston Flats Pool	2	0	8.7	67.8	7.91	7.98	372.7	261.2	242.45	0.54	0.01
2019-04-08	Preston Flats Pool	2	0	12.3	70.1	7.49	7.43	444	335.7	288.6	0.042	0
2019-04-18	Preston Flats Pool	1	0	11.3	50.5	5.56	7.53	605.1	445.2	393.9	0.34	0
2019-04-24	Preston Flats Pool	2	0	14.3	65.1	6.67	7.39	704	557	455	NA	NA
2019-05-02	Preston Flats Pool	0	0	15.5	71	7.11	7.4	NA	668	533	0.88	0.01
2019-05-24	Preston Flats Pool	1	0	16.5	41.4	4.05	6.614	887	743	758.8	NA	NA
2019-06-07	Preston Flats Pool	0	0	19.3	18	1.63	6.51	753	670	500.5	NA	NA
2019-06-27	Preston Flats Pool	0	0	22	NA	NA	7.77	399.6	374.8	259.35	NA	NA

NA represents dates when YSI malfunctions for certain water quality metrics.

Supplementary Table 3. Standard curves from singleplex and multiplex of species-specific assays (FAM) and synthetic insert assay (VIC).

Target species	Assay	Multiplex?	Slope	R ²	Y-intercept	Efficiency (%)	Error
American toad	Species-specific	singleplex	-3.673	0.976	42.734	87.169	0.192
American toad	Species-specific	multiplex	-3.368	0.989	41.691	98.123	0.119
Green frog	Species-specific	singleplex	-2.915	0.943	40.721	120.304	0.238
Green frog	Species-specific	multiplex	-3.002	0.944	40.799	115.333	0.244
Spotted salamander	Species-specific	singleplex	-3.569	0.974	41.802	90.627	0.204
Spotted salamander	Species-specific	multiplex	-3.772	0.995	42.55	84.113	0.086
Wood frog	Species-specific	singleplex	-3.518	0.995	41.516	92.411	0.092
Wood frog	Species-specific	multiplex	-3.821	0.991	42.264	93.69	0.12
American toad	Synthetic probe	singleplex	-3.599	0.997	42.389	89.608	0.075
American toad	Synthetic probe	multiplex	-3.319	0.99	41.743	100.135	0.113
Green frog	Synthetic probe	singleplex	-2.834	0.947	40.508	125.359	0.223
Green frog	Synthetic probe	multiplex	-2.987	0.95	40.826	116.163	0.229
Spotted salamander	Synthetic probe	singleplex	-3.674	0.994	42.029	87.149	0.092
Spotted salamander	Synthetic probe	multiplex	-3.792	0.995	42.561	83.531	0.088
Wood frog	Synthetic probe	singleplex	-3.683	0.995	41.916	86.86	0.087
Wood frog	Synthetic probe	multiplex	-3.778	0.99	41.837	83.962	0.124