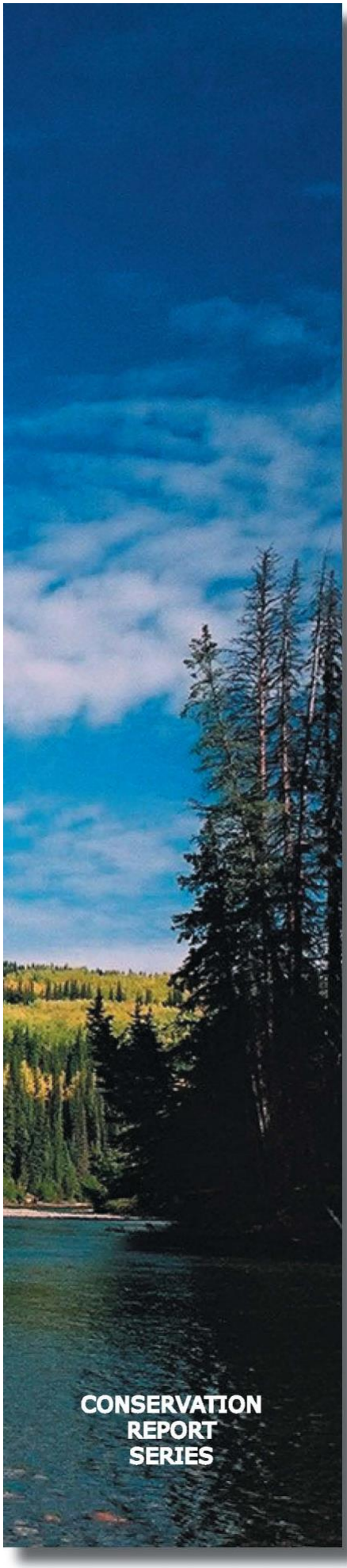

Comparison of Water and Sediment Sampling Techniques for the Evaluation of Amphibian eDNA in Lentic Waterbodies in Alberta

Alberta Conservation Association

Comparison of Water and Sediment Sampling Techniques for the Evaluation of Amphibian eDNA in Lentic Waterbodies in Alberta

Kris Kendell
Alberta Conservation Association
#101, 9 Chippewa Road
Sherwood Park, Alberta, Canada
T8A 6J7

Caren S. Goldberg
Washington State University
100 Dairy Rd./404 Heald Hall
Pullman, Washington, USA
99164-2812

Report Editors

DOUG MANZER
Alberta Conservation Association
P.O. Box 92, Provincial Bldg.
Blairmore, AB T0K 0E0

SUE PETERS
Alberta Conservation Association
#101, 9 Chippewa Rd.
Sherwood Park, AB T8A 6J7

Conservation Report Series Type: Data

ISBN: 978-1-989448-12-0

Suggested Citation:
Kendell, K. and C.S. Goldberg. 2020. Comparison of water and sediment sampling techniques for the evaluation of amphibian eDNA in lentic waterbodies in Alberta. Data Report, produced by Alberta Conservation Association, Sherwood Park, Alberta, Canada. 16 pp + App.

Cover photo credit: David Fairless

Digital copies of conservation reports can be obtained from:
Alberta Conservation Association
#101, 9 Chippewa Rd.
Sherwood Park, AB T8A 6J7
Toll Free: 1-877-969-9091
Tel: (780) 410-1998
Fax: (780) 464-0990
Email: info@ab-conservation.com
Website: www.ab-conservation.com

EXECUTIVE SUMMARY

Environmental DNA, or eDNA, refers to the DNA that organisms leave behind or shed as they pass through the environment. DNA technology has evolved to allow researchers to detect DNA signatures from material such as mucus, feces, urine, or sloughed skin that is naturally contained within water, soil and other mediums. Through this project, ACA worked towards a reliable method of detecting amphibians using eDNA in pond water and pond-bottom sediment. The first phase involved a collaboration on a M.Sc. project developing an approach for detecting amphibians with eDNA from water and sediment samples. The second phase, reported here, involved a partnership with Washington State University to further refine and compare techniques for collecting eDNA from water and sediment sampling methods. In 2018, we adjusted our sampling protocols to enable more complete coverage of study ponds to improve the likelihood that target species' eDNA were present in the samples of water and sediment collected. We believed this new strategy would improve our ability to detect species that occur in lower densities or have tadpoles with schooling behaviour (e.g., boreal toad) that may result in patchy distribution of their eDNA in a pond. Both sampling techniques were successful in detecting amphibians; the water filtration technique was as good as field surveys for detecting the presence of amphibian species at the ponds sampled, whereas detection was lower from samples assayed using pond-bottom sediment. When deciding on which method to use, the greater simplicity of sampling sediment but uncertainty from the potential of including relic DNA must be weighed against the increased detection ability of the water filtration technique. These results indicate eDNA sampling can be an effective alternative to more traditional amphibian monitoring methods in Alberta, when studying cryptic or non-vocalizing species, or species that occur at low abundance in aquatic systems.

Key words: Alberta, amphibian, eDNA, evaluation, filtration, lentic, sediment

ACKNOWLEDGEMENTS

First and foremost, we are indebted to the landowners that allowed us to sample ponds on their properties. This study would not have been possible without their support.

The authors acknowledge Shell Canada Limited and COSIA – Land Environmental Priority Area (EPA) for their financial contributions and assistance in delivering the project.

We are grateful to Bryce Phimester, Mark Sherrington, Rob Stewart and Ryan C. Smith of Shell Canada Ltd. for their help in laying the groundwork for our study, and their hospitality. We are also grateful to Brandon Booker, David Coltman, Corey Davis and Cynthia Paszkowski of the University of Alberta (UofA) for their helpful input and assistance through the early phase of this collaboration.

We also thank the many colleagues and volunteers who have helped us along the way during our research journey: Amanda Rezansoff, Brendon Ganton, Sue Peters, Britt Schmidt and Peter Aku of Alberta Conservation Association (ACA); Nils Anderson, Makena Kiguhda, Sean MacQueen, Mariah Pierini, Curtis Vieville, Arthur Whiting of the UofA; Brian Eaton of InnoTech Alberta; and, Gavin Berg, Lisa Matthias, Robin Gutsell, and Tanya Rushcall of Alberta Environment and Parks; and, citizen science volunteers Ian Kanda and Sarah Wylie.

Additional thanks go to ACA employees Lance Engley, Ken Kranrod and Doug Manzer for providing practical input on the study design.

For the collection of field samples in 2018, we thank Amanda Rezansoff and for laboratory analysis of the field samples, we thank Mary Sterling of Washington State University.

The quality of this report was improved by comments and suggestions provided by Doug Manzer and Sue Peters of ACA.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

1.0 INTRODUCTION

Amphibians are an integral component of Alberta's biodiversity, and knowledge of their distribution at a fine scale is essential to their conservation. Amphibian occurrence data can provide insights about their status and population trends in response to habitat availability (Ficetola et al. 2015), environmental change (Chen et al. 2011), and ongoing threats, such as emerging pathogens (Stuart et al. 2008, Collins and Crump 2009, YAP et al. 2017). Reliable species occurrence data are crucial to help guide management decisions (Gabrielle et al. 2013, Alberta Environment and Parks 2018, 2019), inform options for mitigating potential harm to amphibians from habitat loss or degradation, and facilitate human-mediated movements of amphibians for conservation and mitigation purposes (Randall et al. 2018).

The detection of amphibians using traditional survey methods (auditory techniques and visual encounter surveys) can be hampered by their complex behaviours and ecology, making them difficult to locate (Heyer et al. 1994, Russell and Bauer 2000). Although the distinct calls of most frogs and toads are relatively easy to detect during their breeding season, and can be used for species identification, some behaviours limit our ability to reliably detect them; for instance, chorusing males may be active or vocal at unpredictable times of the day or season, or only at night, or inactive under certain weather conditions, and some species may have weak calls or not produce a mating call. For example, breeding boreal toad (*Anaxyrus boreas boreas*) males in some populations do not produce a mating call (Olson et al.1986, Species at Risk Committee 2014). Salamanders are non-vocal at breeding, making call surveys futile. Some populations of amphibians may skip breeding in a year as a result severe drought, such as the Plains spadefoot (*Spea bombifrons*) and Great Plains toad (*Anaxyrus cognatus*) (Werner et al. 2004), or only become active in response to heavy rainfall events. While some species have a prolonged calling period, such as the boreal chorus frog (*Pseudacris maculata*), some species call over a very short period in early spring, such as the wood frog (*Lithobates sylvaticus*). Amphibian ecology may further hamper our ability to inventory or monitor populations; visual searches for amphibians are challenged by their changing life stages and seasonal habitat use. Many species of amphibians are well-camouflaged and elusive, and spend most of their lives in burrows, underground or under various types of cover. Taken together, multiple surveys are often needed at a given locality to ensure the full suite of potential species are detected; these challenges are intensified if amphibian occurrences are patchy and at low densities, or if nighttime work is required.

In comparison to traditional methods for surveying amphibians in aquatic habitats, the molecular analysis of genetic material recovered from environmental samples (e.g., water or sediments) has

the potential for greater sensitivity in detecting amphibians, and to be less time-consuming and costly (Rees et al., 2014). Environmental DNA (eDNA) may be used to detect cryptic, rare, nocturnal and transient species, as well as individuals hidden from view, without the need to isolate or sight a species (Jerde et al. 2011, Taberlet et al. 2018). However, we recognize that there are several sources of potential error that generate uncertainty in the results of eDNA survey methods. For example, the ability of DNA to persist in the soil or other substrates after an organism has left or died can produce a false positive in eDNA analysis; conversely, inhibition of the polymerase chain reaction (PCR) process at the molecular analysis stage, can result in failed or delayed amplification of the target species' DNA, decreasing the probability of detecting DNA when it is present, producing a false negative result. However, study design and procedural and quality control measures in the field and laboratory can limit these and other potential sources of error (Roussel et al. 2015, Goldberg et al. 2016, Harrison et al. 2019, Mathieu et al. 2020).

Through an initial phase of this work we collaborated with the University of Alberta. An eDNA sampling protocol was developed that enabled the identification of at least three species of amphibians by simply taking samples of water and aquatic sediment from ponds (Booker 2016). In 2016, we then embarked on a second phase to further refine the sampling methods to improve detection consistency. We assessed the reliability of eDNA collected from water filtration versus pond-bottom sediment in partnership with Washington State University (WSU). Laboratory results suggested that the field protocols had shortcomings for detecting amphibians that occur at low densities, or where eDNA from tadpoles with schooling behaviour is not well mixed into the aquatic system. In response, we adjusted our field sampling approach in 2018 so that eDNA from species with low density or tadpoles with schooling behaviour would not be missed as easily.

We modified the spatial distribution and number of sampling stations to improve detection and refined our field controls to reduce the potential of cross-contamination of eDNA samples. We compared the results of assays from samples collected with two different eDNA sampling techniques (i.e., water filtration vs. pond-bottom sediment) to determine if: a) our ability to detect amphibians from eDNA sampling differed from detections of amphibians using traditional surveys, and b) whether detections were more consistent with one eDNA sampling technique over the other.

The presence of eDNA provides evidence of a target species; it cannot verify whether the target species is alive or whether a viable population is present, only that it is, or has been, present in the recent past (Goldberg et al. 2016). DNA degrades in the environment at variable rates. Decay times in sediment can vary from days and weeks to thousands of years (Harrison et al. 2019),

whereas eDNA decays within a few days or weeks (or months) in the water column, reflecting present-day rather than past diversity (Pedersen et al. 2015, Strickler et al. 2015). As such, we anticipated the detection of amphibian-specific eDNA in the water and aquatic sediment samples collected from ponds in our study where amphibians were known to be present.

2.0 STUDY AREA

In cooperation with private landowners, five ponds in central Alberta were selected for eDNA sampling. Three ponds were situated approximately 13 km northwest of the town of Redwater, within Thorhild County, and two ponds were situated approximately 4 km west of Elk Island National Park, within Strathcona County (Figure 1).

Study ponds were standing (natural and human-made), fishless waterbodies containing varying amounts of native floating, aquatic (rooted) macrophytes, and aquatic emergent vegetation. The ponds were also characterized by variable water depths and shallow sloping shorelines, often with adjacent boggy areas (Appendix 1).

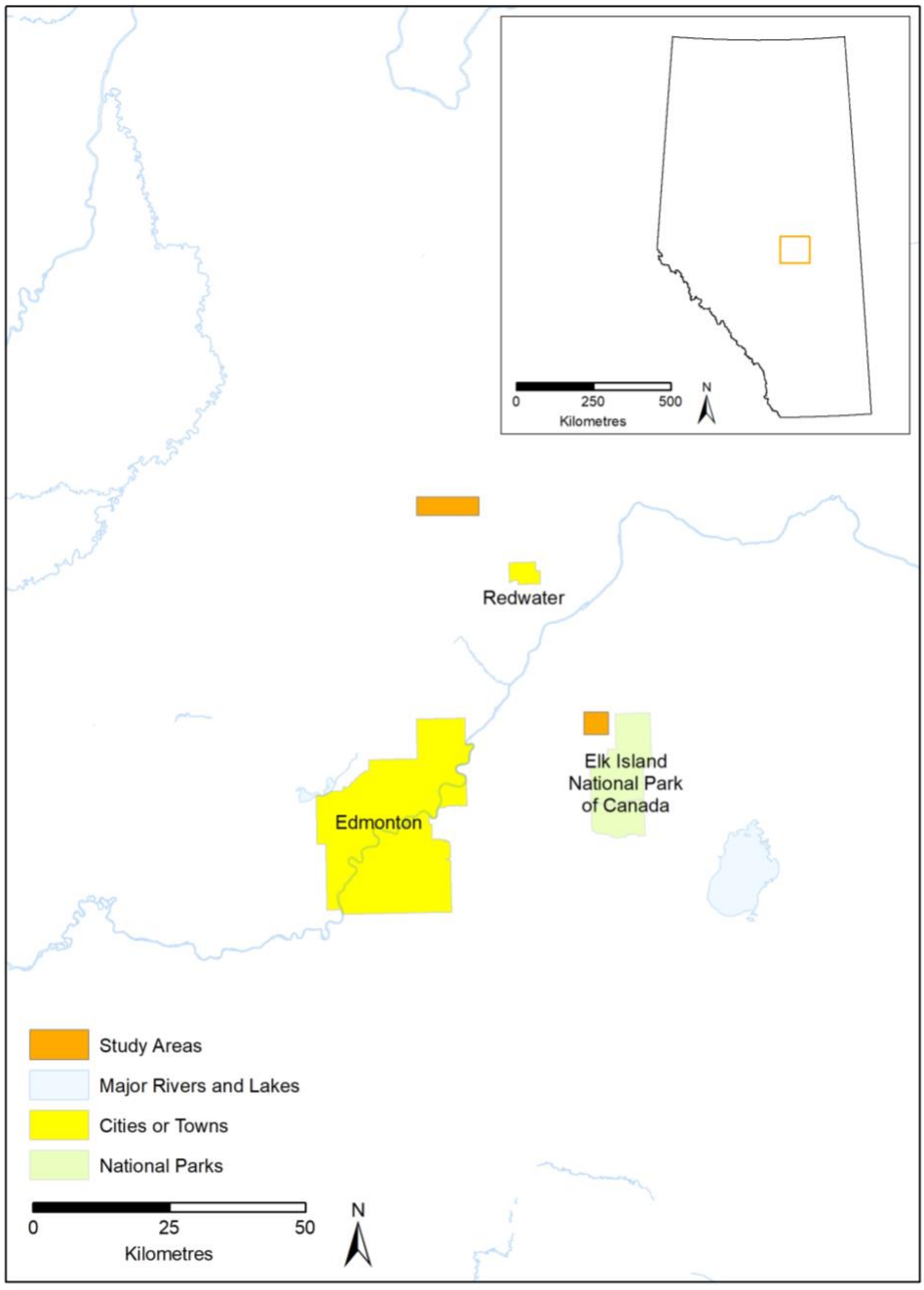

Figure 1. Map showing study areas (i.e., study ponds) within central Alberta.

3.0 MATERIALS AND METHODS

3.1 Visual amphibian surveys (traditional method)

We conducted traditional amphibian surveys (Alberta Conservation Association and Alberta Sustainable Resource Development 2010) at the five study ponds prior to eDNA sample collection (i.e., June 21, 2018) and during sample collection (i.e., June 27–29 and July 4, 2018). Visual surveys for amphibians involved slowly walking along the edges of the study ponds, watching carefully for the movement of all age classes of amphibians underfoot or in shallow water. Amphibian surveys were timed to coincide with the larval stage of development of the anticipated species at the ponds. Larval surveys were selected because that life stage can be found in high densities in some waterbodies allowing easy detection. Moreover, the larval stage of most amphibians is long-lasting, providing a protracted survey period for that stage of development. Importantly, the presence of larvae confirmed the use of habitat for breeding by any species detected. The survey periods also overlapped with the sudden and rapid emergence (metamorphosis) of larvae and when large numbers of young-of-the-year gather for a short time along the edges of their natal waterbody before dispersing into surrounding habitats, further improving potential detection. A dip-net was used when larvae were possibly hidden from view by dense aquatic vegetation, turbid water, and/or overhanging bank vegetation, or obscured by reflections or ripples on the water surface.

3.2 eDNA sample collection

We collected eDNA samples using two approaches, following the protocols used by Goldberg and Strickler (2015) and Booker (2016) with slight modification to improve spatial sampling at each pond. Sample collection occurred on June 27–29, 2018 and July 4, 2018. We focused on improving detection probability by increasing the number of sampling stations at each pond from a maximum of three equally spaced stations (depending on the size of the pond), to ten equally spaced stations. We also created a triplicate composite sampling scheme for both eDNA sampling techniques.

3.2.1 Water filter sampling

First, we stratified the perimeter of each of the five ponds into ten equi-spaced collection stations, to ensure sufficient spatial coverage for sampling. From each station, we collected three, 100-ml pond-water samples and placed them into three separate 1-L polypropylene containers. At each subsequent sampling station, a 100-ml sample was added into its respective 1-L container, creating three mixed 1-L samples (i.e., containers A–C in Table 3). Three separate

designated long-handled scoops were used to collect the 100 ml water samples for each respective 1-L polypropylene container. From each of the three mixed 1-L samples, we subsampled and filtered 250 ml of water, in triplicate. We processed water samples *in situ* using 250-ml disposable polypropylene filter funnels fitted with 0.45 μm nitrocellulose filter membranes. A 1-L polypropylene vacuum flask with tubulation and section of silicone tubing for connection to a hand-driven vacuum pump was used to filter the 250 ml water through the membranes. Each processed filter membrane was dried and stored individually at room temperature in a coin envelope containing a small amount of moisture-indicating silica desiccant beads to control humidity and prevent degradation of the samples until processed.

3.2.2 Pond sediment sampling

At each of the ten stations (after water sampling), we collected three small samples of pond-bottom sediment from the top 2 cm – 3 cm of the sediment profile and placed each sample into its own separate small plastic bag (i.e., bags A–C in Table 3). Each subsequent sediment grab sample was added into its own respective plastic bag, creating three bags of mixed pond sediment, each with a total of ten grab samples. From each of the three bags of mixed sediment and using 2 mL microcentrifuge tubes, we subsampled *in situ* 2 ml of the sediment, in triplicate. We wore a non-powdered disposable nitrile glove when collecting and handling pond-bottom sediment samples, being careful not to exceed the glove's cuff when reaching into the water. The microcentrifuge tubes containing the sediment samples were temporarily stored in a cooler with ice while in the field, and later moved to a household freezer until being shipped frozen to the laboratory to be processed.

We prepared field negative controls for each sampling technique, and for each pond. Deionized water was used for the water filter controls, and coir fiber, wetted with deionized water, was used for sediment controls. The controls were subjected to all aspects of sample collection, field processing, preservation, transportation and laboratory handling, and analyzed as environmental samples.

3.3 Laboratory analysis

Frozen sediment samples were sent to WSU and moved directly to a laboratory freezer until analyzed; the filter membranes were stored at room temperature inside a cabinet, until extraction. Prepared samples consisted of 50 filters preserved in ethanol and 50 sediment samples, nine of each from each of five field sites, plus a field negative control of each kind from each field site.

We extracted eDNA from the filters using a standard DNeasy/Qiashredder protocol (Goldberg et al. 2011) and from the sediment samples using a PowerSoil Kit (MoBio, now owned by Qiagen). We used the assays developed by Booker (2016) for wood frogs, boreal chorus frogs, and boreal toads. Reactions were run using Quantitect Multiplex PCR Mix (Qiagen, Inc, Hilden, Germany) with recommended multiplexing concentrations (1X QuantiTect Multiplex PCR mix, 0.2 µM of each primer, and 0.2 µM probe) on a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) at WSU. Reactions were 15 µL in volume and each included 3 µL of sample, as well as exo-IPC (Applied Biosystems) to test for inhibition. Cycling began with 15 min at 95°C followed by 50 cycles of 94°C for 60 s and 60°C for 60 s.

Data were analyzed with CFX Manager Software (BioRad). Each species was analyzed separately, in triplicate. If inconsistency was observed (1 or 2 out of 3 wells testing positive), the sample was re-tested and considered positive if at least 1 well tested positive in the second round. This accounts for low-probability false-positive events, estimated at 0.02 per well in a similar study (Smith and Goldberg 2020).

4.0 RESULTS

4.1 Visual amphibian surveys (traditional method)

Five species of amphibian have ranges that overlapped with the eDNA sampling pond locations. We detected amphibians at all five ponds using traditional survey methods (Table 1). Observations included the larval, young-of-the-year, sub- and/or adult age classes of wood frog, boreal chorus frog, and boreal toad (Figure 2).

Figure 2. Representative photos of amphibian species detected during traditional amphibian surveys at the five study ponds. **A.** wood frog (*Lithobates sylvaticus*); **B.** boreal chorus frog (*Pseudacris maculata*); **C.** boreal toad (*Anaxyrus b. boreas*). Photos: Kris Kendell.

We did not detect the Canadian toad (*Anaxyrus hemiophrys*) or tiger salamander (*Ambystoma mavortium*) at any of the study ponds in 2018. The wood frog was the most commonly encountered amphibian species at the ponds (Table 1).

Table 1. Amphibian species detected at study ponds during visual surveys in June and July of 2018.

Pond	Date surveyed (mm/dd/yy)	Field species detected				
		WOFR	BCFR	BOTO	CATO	TISA
1	06/21/18	●	-	-	-	-
	06/27/18	●	-	-	-	-
2	06/21/18	●	-	●	-	-
	06/28/18	●	-	●	-	-
3	06/21/18	●	●	-	-	-
	06/29/18	●	●	-	-	-
4	06/21/18	●	●	●	-	-
	06/29/18	●	●	-	-	-
5	06/21/18	●	-	-	-	-
	07/04/18	●	-	-	-	-

Codes: WOFR = wood frog, BCFR = boreal chorus frog, BOTO = boreal toad, CATO = Canadian toad; TISA = tiger salamander; (●) = Detected, (-) = Not detected

4.2 eDNA sample collection

Pond perimeters varied between approximately 57 m and 283 m, resulting in a 6 m to 28 m spacing between sample collection stations, depending on pond size (Table 2).

In total, we processed, *in situ*, 50 filters and 50 aquatic sediment samples from five ponds: nine of each from each pond, plus a negative field control of each sample type from each pond (Table 3).

Table 2. Approximate pond perimeter and distance between sampling stations around each pond.

Pond	Perimeter (m)	Station Spacing (m)
1	267	27
2	283	28
3	57	6
4	76	8
5	211	21

Table 3. Number of water filter, pond-bottom sediment and negative control samples taken for each eDNA sampling technique at ponds visited in June and July of 2018.

Pond	Date	Water filter†					Sediment‡				
		250 mL			Control	Total	2 mL			Control	Total
		A	B	C			A	B	C		
1	06/27/18	3	3	3	1	10	3	3	3	1	10
2	06/28/18	3	3	3	1	10	3	3	3	1	10
3	06/29/18	3	3	3	1	10	3	3	3	1	10
4	06/29/18	3	3	3	1	10	3	3	3	1	10
5	07/04/18	3	3	3	1	10	3	3	3	1	10
Total						50	**Total**				50

†10 stations, 100 mL water collected per station, homogenized into three 1L containers (A, B, C); 250 mL subsampled in triplicate

‡10 stations, grab sample of aquatic sediment collected per station, homogenized into three plastic bags (A, B, C); 2 mL subsampled in triplicate

4.3 Laboratory analysis

All field and laboratory controls tested negative, meaning there was no evidence of cross-contamination among samples within a pond or among ponds, or among extracts. All species

detected using traditional field surveys were also detected using the water filter technique, at all five ponds. In comparison, detection was lower using the sediment sampling technique, which failed to detect boreal chorus frogs and boreal toads at two of five ponds where they were known to be present based on field surveys (Table 4).

Table 4. Laboratory assay results of water filter and sediment samples from ponds where target field species were detected. The number of total positive samples (out of nine collected per site) is presented for each species.

Pond	Species	Detected in Field Survey	Number of Positive Triplicate Tests	
			Filter	Sediment
1	WOFR	●	9/9	9/9
	BCFR	-	0/9	0/9
	BOTO	-	1/9	0/9
2	WOFR	●	9/9	3/9
	BCFR	-	4/9	3/9
	BOTO	●	5/9	0/9
3	WOFR	●	9/9	9/9
	BCFR	●	9/9	0/9
	BOTO	-	0/9	0/9
4	WOFR	●	9/9	9/9
	BCFR	●	9/9	9/9
	BOTO	●	1/9	1/9
5	WOFR	●	9/9	9/9
	BCFR	-	0/9	0/9
	BOTO	-	0/9	0/9
Total number of positive tests (%)			74/135 55%	52/135 39%

Codes: BOTO = boreal toad, WOFR = wood frog, BCFR = boreal chorus frog; (●) = Detected, (-) = Not detected

At two ponds, eDNA sampling performed better than conventional amphibian monitoring methods. At Pond 1, boreal toads were detected in filters but not seen during field surveys. At Pond 2, boreal chorus frogs were detected in both filters and sediment, but they were not seen during field surveys. Overall, filters (55%) had higher proportions of samples testing positive for species detected in field surveys compared to sediment (39%). The wood frog had the highest number of total positive tests (100% filters, 87% sediment) for species confirmed present during field surveys (Table 4).

5.0 DISCUSSION

We have demonstrated that eDNA can be used to detect amphibian presence in ponds, using filtered pond water and pond-bottom sediment samples. Moreover, our results showed that eDNA analysis was a more sensitive method of species detection, and sometimes detected species that were not recorded during traditional amphibian surveys. Using eDNA sampling, we were able to improve our ability to detect the boreal chorus frog and the boreal toad. At two ponds, eDNA sampling performed better than conventional amphibian monitoring methods. At Pond 1, boreal toads were detected in filters but not seen during field surveys in 2018. It is worth noting that we did observe boreal toads at Pond 1 two years earlier, on June 30, 2016, so it is possible that their eDNA is still present even though there are no longer boreal toads in the pond, or that our traditional surveys failed to detect boreal toads in 2018. The boreal toad occurred in low densities in our study ponds and was expected to have a patchy distribution of eDNA because of tadpole behaviour; their tadpoles are known to form dense synchronized schools that seek out warm microhabitats in their natal ponds, often remaining near oviposition sites of the adults until metamorphosis is complete (Matsuda et al. 2006, Russell and Bauer 2000, Werner et al. 2004). At Pond 2, boreal chorus frogs were detected in both filters and sediment, where they were not seen during any field surveys, including two years earlier on June 28, 2016. Because of our strict laboratory protocols to minimize detection errors (false positive or false negatives), we are confident in these results.

It is worth noting that several ponds had only one sample out of nine detect the eDNA of the boreal toad. This seems to indicate that our field sampling intensity of ten stations per pond was barely adequate, and that increased sampling effort might detect more species, especially species with a patchy distribution.

The decay rate of DNA is one factor to consider when deciding to use pond-bottom sediment versus water filtration for collecting eDNA samples. It is well known that waterbody sediments adsorb eDNA (Thomsen and Willerslev 2015), providing a potential source for species detection.

For example, Turner et al. (2015) found that carp (*Hypophthalmichthys* spp.) eDNA could be extracted from aquatic sediment in experimental ponds and natural rivers. In water, eDNA breaks down within a few days to a few months, depending on environmental conditions that influence its decay (Strickler et al. 2015). In other environments, such as waterbody sediments, the persistence of eDNA can be much longer (Pedersen et al. 2015) because of anoxic conditions that reduce nuclease degradation, and the physical process that influence eDNA persistence (e.g., UV-B and high temperature) are lower at the bottom of a pond compared to within the water column (Goldberg et al. 2018). But not all research has shown eDNA decay to be longer in sediments versus water (e.g., Wei et al. 2018). So there are challenges to inferring current or historical site occupancy from water and sediment eDNA. Understanding eDNA decay under specific conditions is critical for robust data interpretation (Harrison et al. 2019).

Although our results show that water filtration had higher levels of species detection than sediment collected at the same time, we feel sediment collection for eDNA analysis may warrant further investigation. An advantage to sediment sampling is its simplicity when compared with that of the filter sampling method. For instance, filtration of water involves more elaborate steps than sediment collection and requires the use of nitrocellulose filter membranes, which are a hazardous material that requires special handling. In contrast, the sediment sampling method is relatively straightforward, and the samples have minimal post-collection handling requirements other than freezer storage. One constraint of the sediment sampling technique is the requirement to keep samples cool in the field until they can be stored in a freezer. Nonetheless, when deciding on which method to use, the greater simplicity of sampling pond-bottom sediment but uncertainty from the potential of including relic DNA must be weighed against the increased detection ability of the water filtration technique.

6.0 LITERATURE CITED

Alberta Conservation Association and Alberta Sustainable Resource Development. 2010. Alberta Volunteer Amphibian Monitoring Program – participants manual. Alberta Conservation Association, Edmonton, AB. 46 pp.

Alberta Environment and Parks (AEP). 2018. Master schedule of standards and conditions (MSSC). Alberta Environment and Parks, Government of Alberta, Edmonton, Alberta, Canada. 308 pp.

Alberta Environment and Parks (AEP). 2019. Pre-application for formal dispositions. Alberta Environment and Parks, Government of Alberta, Edmonton, Alberta, Canada. 71 pp.

Beja-Pereira, A., R. Oliveira, M.K. Schwartz, and G. Luikart. 2009. Advancing ecological understanding through technological transformation in noninvasive genetics. Mol. Ecol. Res. 9: 1279–1301.

Booker, B.K. 2016. Developing and assessing an environmental DNA protocol for detecting amphibian species in lentic systems in Alberta, Canada (M.A. Thesis), Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada. 106 pp.

Chen, I.C, J.K. Hill, R. Ohlemüller, D.B. Roy, and C.D. Thomas. 2011. Rapid range shifts of species associated with high levels of climate warming. Science Vol. 333 (6045):1024–1026

Collins, J.P. and M.L. Crump. 2009. Extinction in our times: global amphibian decline. New York: Oxford University Press USA. 304 pp.

Ficetola, G.F., C. Rondinini, A. Bonardi, D. Baisero, and E. Padoa-Schioppa. 2015. Habitat availability for amphibians and extinction threat: A global analysis. Diversity and Distributions 21: 302–311.

Gabrielle, G.J., K.A. Buhlmann, L.R. Wilkinson, and J.W. Gibbons (Eds.). 2013. Inventory and monitoring: Recommended techniques for reptiles and amphibians, with application to the United States and Canada. Partners in Amphibian and Reptile Conservation Technical Publication IM-1, Brimingham, Alabama, USA. 321 pp.

Goldberg, C.S., D.S. Pilliod, R.S. Arkle, and L.P. Waits. 2011. Molecular detection of vertebrates in stream water: A demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. PloS One 6: e22746.

Goldberg, C.S. and K.M. Strickler. 2015. eDNA Protocol sample collection. Unpublished manuscript, Washington State University (accessed online June 7, 2016).

Goldberg, C.S., K.M. Strickler, A.K. Fremier. 2018. Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: increasing efficacy of sampling designs. Sci. Total Environ. 633: 695–703.

Goldberg, C.S., C.R. Turner, K. Deiner, K.E. Klymus, P.F. Thomsen, M.A. Murphy, S.F. Spear, A. McKee, S.J. Oyler-McCance, R.S. Cornman, M.B. Laramie, A.R. Mahon, R.F. Lance, D.S. Pilliod, K.M. Strickler, L.P. Waits, A.K. Fremier, T. Takahara, J.E. Herder, and P. Taberlet. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7(11): 1299–1307. Available online at https://doi.org/10.1111/2041-210X.12595.

Harrison, J.B., J.M. Sunday, and S.M. Rogers. 2019. Predicting the fate of eDNA in the environment and implications for studying biodiversity. Proc. R. Soc. B 286: 20191409.

Heyer, W.R, M.A. Donnely, R.W. McDiarmid, L.-A. C. Hayek, and M. Foster. 1994. Measuring and monitoring biological diversity: standard methods for amphibian diversity. Smithsonian Institution Press, Washington, D.C., USA. 364 pp.

Jerde, C.L., A.R. Mahon, W.L. Chadderton, and D.M. Lodge. 2011. “Sight unseen” detection of rare aquatic species using environmental DNA. Conservation Letters 4: 150–157.

Mathieu, C., S.M. Hermans, G. Lear, T.R. Buckley, K.C. Lee, and H.L. Buckley. 2020. A systematic review of sources of variability and uncertainty in eDNA data for environmental Monitoring. Front. Ecol. Evol. 8:135.

Matsuda, B.M., D.M. Green, and P.T. Gregory. 2006. Amphibians and reptiles of British Columbia. Victoria: Royal British Columbia Museum. 266 pp.

Olson, D.H., A.R. Blaustein, and R.K. O'Hara. 1986. Mating pattern variability among western toad (*Bufo boreas*) populations. Oecologia (Berlin) Vol. 70, No. 3: 351–356.

Pedersen, M.W., S. Overballe-Petersen, L. Ermini, C.D. Sarkissian, J. Haile, M. Hellstrom, J. Spens, P.F. Thomsen, K. Bohmann, E. Cappellini, I.B. Schnell, N.A. Wales, C. Carøe, P. F. Campos, A.M.Z. Schmidt, M.T.P.Gilbert, A.J. Hansen, L. Orlando, and E. Willersley. 2015. Ancient and modern environmental DNA. Philos Trans R Soc Lond B Biol Sci. 370 (1660): 20130383.

Randall, L., N. Lloyd, and A. Moehrenschlager. 2018. Guidelines for mitigation translocations of amphibians: Applications for Canada's prairie provinces. Version 1.0. Centre for Conservation Research, Calgary Zoological Society. Calgary, Alberta, Canada. 94 pp.

Rees, H.C., K. Bishop, D.J. Middleditch, J.R.M. Patmore, B.C. Maddison and K.C. Gough. 2014. The application of eDNA for monitoring of the great crested newt in the UK. Ecol. Evol. 4: 4023–4032.

Roussel, J.M., J.M. Paillisson, A. Tréguier, and E. Petit. 2015. The downside of eDNA as a survey tool in water bodies. Journal of Applied Ecology 52(4): 823–826.

Russell, A.P., and A.M. Bauer. 2000. The amphibians and reptiles of Alberta: A field guide and primer of boreal herpetology. Calgary, 2nd Edition: University of Calgary Press. 279 pp.

Smith, M.M., and C.S. Goldberg. 2020. Occupancy in dynamic systems: accounting for multiple scales and false positives using environmental DNA to inform monitoring. Ecography. 42: 1–11.

Species at Risk Committee. 2014. Species status report for western toad (*Anaxyrus boreas*) in the Northwest Territories. Species at Risk Committee, Yellowknife, NT. 54 pp.

Strickler, K., A.K. Fremier, and C. Goldberg. 2015. Quantifying the effects of UV, temperature, and pH on degradation rates of eDNA in aquatic microcosms. Biological Conservation 183: 85–92.

Stuart, S.N., M. Hoffmann, J.S. Chanson, N.A. Cox, R.J. Berridge, P. Ramani, and B.E. Young. (eds.) 2008. Threatened amphibians of the world. Lynx Edicions, Barcelona, Spain; IUCN, Gland, Switzerland; and Conservation International, Arlington, Virginia, USA. 151 pp.

Taberlet, P., A. Bonin, L. Zinger and E. Coissac. 2018. Environmental DNA: for biodiversity research and monitoring. Oxford University Press, Oxford, UK.

Thomsen, P.F., and E. Willerslev. 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation1 83: 4–18.

Turner, C.R., K.L. Uy, and R.C. Everhart. 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biological Conservation 183: 93–102.

Wei, N, F. Nakajima, and T. Tobino. 2018. A microcosm study of surface sediment environmental DNA: decay observation, abundance estimation, and fragment length comparison. Environ. Sci. Technol. 52: 12,428–12,435.

Werner, J.K., B.A. Maxell, P. Hendricks, and D.L. Flath. 2004. Amphibians and reptiles of Montana. Missoula, Montana: Mountain Press Publishing. 262 pp.

Yap, T.A., N.T. Nguyen, M. Serr, A. Shepack and V.T. Vredenburg. 2017. *Batrachochytrium salamandrivorans* and the risk of a second amphibian pandemic. Ecohealth 14(4): 851–864.

7.0 APPENDICES

Appendix 1. Five ponds in central Alberta that were selected for eDNA sampling in June and July of 2018: **A.** = Pond 1 (315420-B); **B.** = Pond 2 (045923-S); **C.** = Pond 3 (315822); **D.** = Pond 4 (025923); **E.** = Pond 5 (315420-A). Photos: Kris Kendell.


Alberta Conservation
Association