Amphibian eDNA Pilot Report

A collaborative project between Innotech Alberta and ABMI

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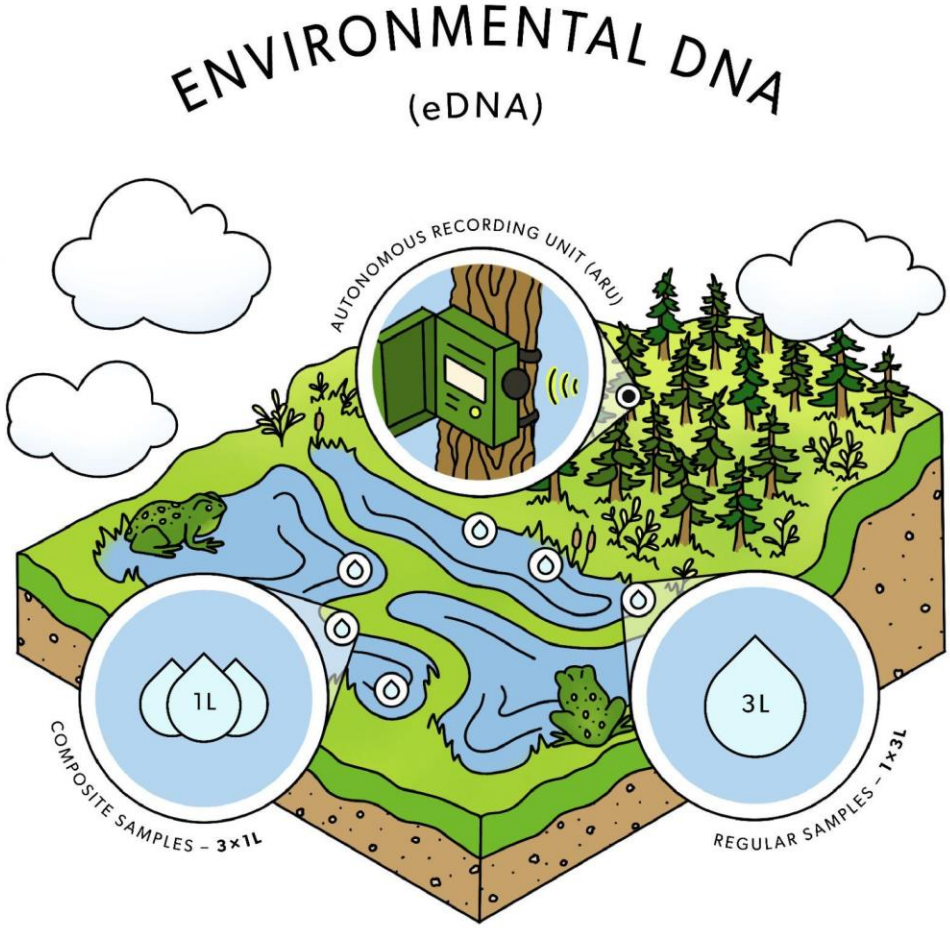

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Cover illustration: a conceptual drawing of the distribution of eDNA sampling points and an autonomous recording unit (ARU) used to collect amphibian calling data to validate the eDNA results.

Background/Introduction

Detecting rare and elusive species using traditional sampling methods can be challenging, often requiring significant time and cost investment for field work. The sampling effort needed to detect rare and elusive species can detrimentally impact both target and non-target organisms in the study area . Some species are difficult to detect using common sampling techniques because of specialized habitat use, or specific activity periods (e.g., overnight or triggered by rain events). To mitigate these challenges, approaches based on the use of environmental DNA (eDNA) to assess the presence of species of interest are increasingly being explored as a tool to augment other monitoring or sampling approaches.

eDNA refers to a collection of technologies and methodologies used to detect species based on DNA fragments in relatively small environmental samples. Because the technique can detect species without having to actually collect specimens, it is well-suited to detecting rare and elusive species, and all life-stages of a species with distinctly different developmental phases (e.g., egg, larval, adult).

By developing unique amplicons (DNA fragments) and corresponding species-specific molecular probes for multiple target species, we are able to detect a range of species from a common water sample. Testing these primer sets against tissue from the target species which has been collected as near to the study area as possible is a critical step in the validation process. Ideally, this should be followed by field validation to compare species detections using eDNA and other sampling techniques at the same sampling sites.

eDNA signals are expected to be strongest in areas where the target animals have recently been present, with the expectation that the presence of more individuals of a species is positively correlated with the abundance of eDNA for that species in the local environment (Bylemans et al. 2017). This is, however, impacted by the rate at which the eDNA fragments degrade under the ambient conditions for that area; factors such as temperature, microbial activity, water chemistry and UV-B radiation can influence eDNA degradation rates (Strickler et al. 2015; Foote et al. 2012; Pilliod et al. 2014). Current understanding estimates that eDNA in lotic aquatic environments can be detected for about two weeks after it is shed, depending on the type of tissue (i.e., skin versus toenail) and environmental conditions (Strickler et al. 2015; Harrison et al. 2019). For amphibians, the strongest eDNA signals are expected to be during breeding when adults congregate to mate and when aquatic larvae (i.e., tadpoles) are prevalent.

Autonomous Recording Units (ARUs) are audio recording devices that can be programmed to sample at scheduled times throughout the day. The ABMI currently uses ARUs to monitor amphibians across Alberta. The dominant audio signals from amphibians are breeding calls in early spring; these calls are generally considered an indication of the abundance of breeding adults (Nelson and Graves 2004, Corn et al. 2011) but not necessarily of successful breeding.

The overall goal of this project was to assess the use of eDNA methods to monitor amphibians in Alberta wetlands and potentially augment current ABMI sampling. During the project we completed several steps related to developing an eDNA approach to monitor amphibians. These included the following:

- Develop primers for target Alberta amphibian species,
- Test primer sets on tissue samples to ensure they are specific to target species,
- Collect samples from multiple wetlands and analyze, and
- Compare results to data collected using ARUs.

Methods

In this study, InnoTech Alberta and the Alberta Biodiversity Monitoring Institute (ABMI) partnered on a project examining the use of eDNA to detect six amphibian species (Table 1) in the Edmonton region at four study sites (1113-71-5, 1086-71-28, 1086-71-9, 1086-71-5; Figure 1).

Table 1. Target amphibian species for eDNA study

Common name	Scientific name
Wood Frog	*Lithobates sylvaticus*
Boreal Chorus Frog	*Pseudacris maculata*
Western (Boreal) Toad	*Anaxyrus boreas*
Canadian Toad	*Anaxyrus hemiophrys*
Northern Leopard Frog	*Lithobates pipiens*
Blotched Tiger Salamander*	*Ambystoma mavortium melanostictum*

*Blotched Tiger Salamanders do not broadcast breeding calls and therefore are not a target species for ARU detection.

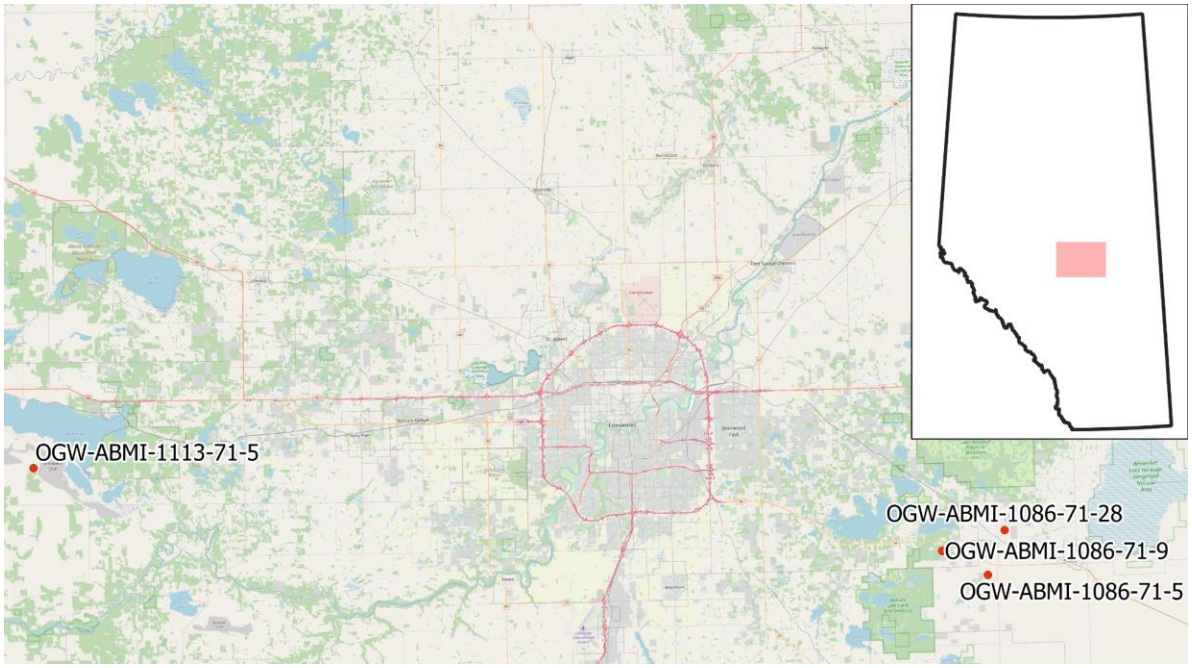

Figure 1. Sampling site locations.

Sample Collection

The study included nearly continuous ARU sampling and three eDNA sampling sessions (June 5-10, July 6-8 and August 5-10 of 2020; see Table A1 for detailed sampling dates) at each site.

The eDNA water sampling consisted of 3, 3 Liter (L) replicate water samples for each of three sites during each session (Figure 2). At site 1113-71-5, a set of 3 replicate eDNA samples were collected during each session (as for the other three sites), as well as 3 composite samples, which each consisted of 3 individual 1 L samples combined into a composite 3 L sample (Figure 2). The intent behind the composite sample was to filter the same total volume of water (9 L) at a site, but to distribute it over a greater spatial area (9 sampling locations instead of 3), to determine if sampling a larger area provides better detection of amphibian species.

The eDNA sampling locations were centred on the location of the ARU used to record amphibian calls. The 3 L samples were distributed 50 m apart and the 1 L composite samples were collected at least 25 m from other sampling locations (Figure 2).

Each eDNA sample was collected by filtering water through a set of filters (a 5 micron filter to remove larger debris, and a 0.45 micron filter which was used to capture the eDNA sample) in the field. The samples were filtered using a peristaltic pump, driven by a cordless drill. The filtering process was designed to minimize the chance of cross-contamination. Control samples (bottled water) were collected before and after sampling at each site to ensure proper handling procedures were being followed. Further details on the eDNA collection methods can be found in Open Water Wetland Environmental DNA (eDNA) Field Collection Protocols (ABMI 2021).

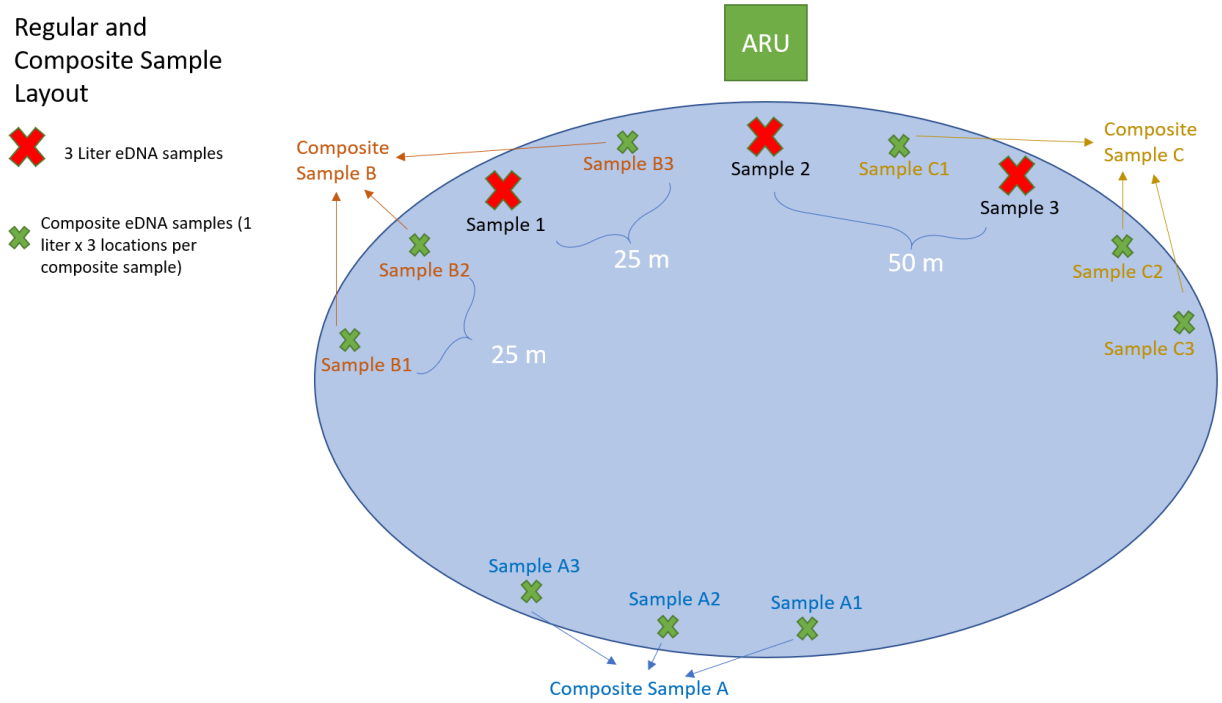

Figure 2. Distribution of eDNA water sample collection points at study sites. Regular samples were a series of three 3 L water samples. In addition, composite samples (see text for details) were collected at one study site.

DNA Methods

DNA Extractions

The filter papers were extracted using a Qiagen Powerwater DNA extraction kit (Qiagen 14900-100-NF) and stored at -80°C until thawed for use in the assay. Tissue samples used to validate the primer sets developed during this project were extracted using a Qiagen blood and tissue DNA extraction kit (Qiagen 69504) and stored at -80°C until thawed for testing each assay.

Amplification Check

For DNA extraction from each filter set, the samples were diluted to 50 pg/uL and triplicates of each extraction were run using a Sybr assay universal primer set (COI2) which indicated the ability of the template to be amplified by PCR. When a sample failed to produce a positive test on the universal Sybr assay, the DNA extracted was considered insufficient or non-amplifiable. Non-amplification can occur when various chemicals (e.g., 2+ ions, humic acids, phenolic compounds, and other complex chemicals) that can inhibit PCR reactions carry over from the environmental samples during the extraction process. When a sample failed to amplify during this step, it was removed from the dataset entirely. Samples that only amplified in one of three technical replicates were marked as questionable and further decisions on the sample set were made with a heavier weight on the biological replicates that did amplify. If only one

of the three technical replicates for a biological sample could be amplified, and no other biological replicates from a site could be amplified, the samples for that site were removed entirely from the data set and classified as non-amplifiable.

Primer Design and Validation

Taqman primers and probes to detect the target species were designed with a 6 FAM (6-Carboxyfluorescein) fluorophore reporter and a BHQ1 (Blackhole 1) quencher (Custom Sigma Primers). Figure 3 provides an explanation of how the primers work. Primers were designed using the CLC Genomics Workbench 20.0.4 (QIAGEN) and known sequence data for the COI and cytB genes for each target species available online as found on the NCBI and BOLD databases (Murphy et al. 2022, Ratnasingham et al . 2007, Sayers et al. 2022). Alignments of the species sequences and primer design for a taqman assay were done through CLC genomics and the resulting primers were exported and tested insilico for species specificity using NCBI-Primer Blast (Ye et al. 2012).

To test the primers against known positive tissue, 1.25 nmol of the forward and reverse primer, 0.5 nmol of probe, 5 µL of BioRad iTaq Universal Probes Supermix (Biorad #1725134), and 2 µL of a 1/10 dilution of 10 ng of genomic DNA extracted from tissue were mixed to produce a 10 µL Taqman reaction. The results of these tests were used to determine if the primers cross-react with DNA from the target and non-target species to assess the specificity of the primers.

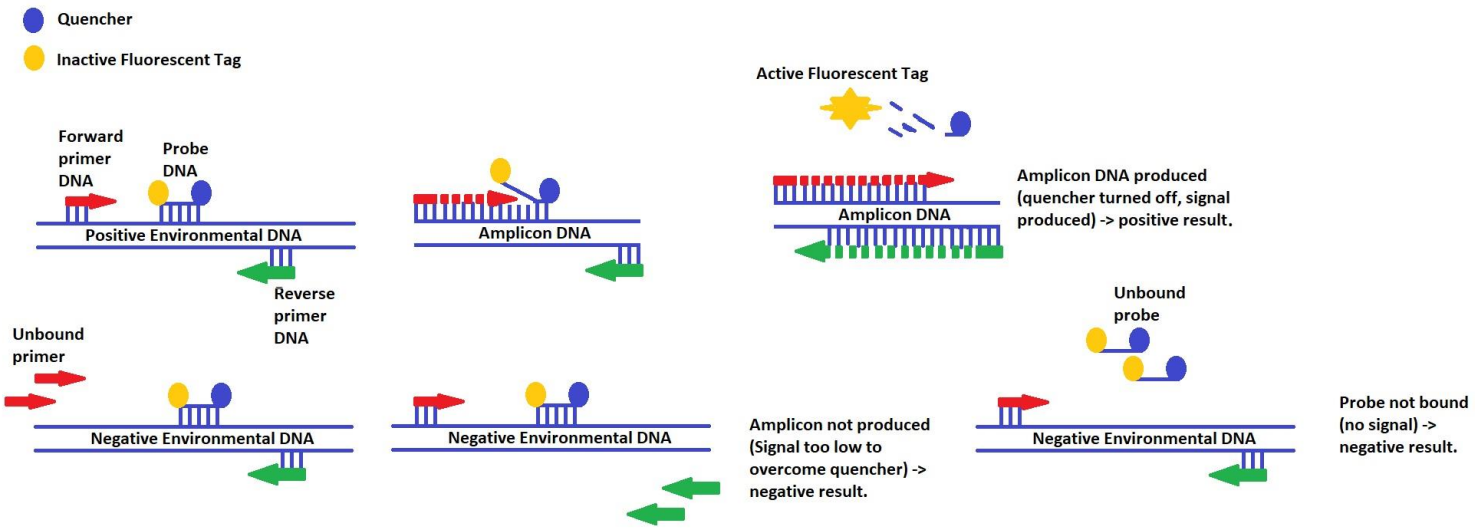

Figure 3. Explanation of how primers are used to identify target eDNA sequences.

To determine the specificity of each primer set, the primers were run against concentrated samples of DNA extracted from tissue samples from each target species, as well as some of the co-occurring non-target species. If non-target species were picked up below 30 cycles or within 10 cycles of the target species, the primer was considered as a failure, and was discarded. For example, if the target species was detected at 22 cycles and the non-target species was detected at 32 cycles, the primer set was considered a failure, despite 32 being above the 30 cycle threshold. If more than two primer sets passed this initial assessment, the two lowest sets that amplified target DNA at lower cycles were chosen for sensitivity testing. If only one set passed, then it was tested for sensitivity.

To estimate the sensitivity of each primer set, serial dilutions of the positive (e.g., target species) genomic DNA (ranging from concentrated to a 1/10,000,000 dilution) and a series of eight blanks were run in triplicate. A graph of the log of the dilution versus the cycle at which exponential change in the reaction was reached was examined to determine the range over which the reaction exhibited linear exponential increase. Primers with a broad linear range were favoured over those with a narrow linear range. For example, primers that increased amplification of their product linearly over 7 dilution levels were chosen over those that only worked over 5 dilution levels.

The lowest dilution level that still produced a read that was greater than the read for pure water was used to set the lower sensitivity level for the primer set. If a negative control, composed of mixed negative tissue (e.g., non-target species) samples (see Table 2), produced a read, this read level was used as a cut off for any other reads during the sensitivity trials; reads lower than this level were attributed to non-specific binding and not considered as positive reads. The species and number of independent samples used for specificity/sensitivity testing for all primer sets are provided in Table 2.

Table 2. Table of species used in cross-species testing (specificity testing) for the amphibian primer sets developed during this project.

Species	No. independent samples	Comments
Wood Frog	3	*Lithobates sylvaticus*
Northern Leopard Frog	2	*Lithobates pipiens (aka Rana pipens)*
Boreal Chorus Frog	1	*Pseudacris maculata*
Boreal Toad	11	*Anaxyrus boreas boreas*
Western Toad	4	Identified specifically as western toad (*Anaxyrus boreas*)
Canadian Toad	6	*Anaxyrus hemiophrys*
Blotched Tiger Salamander	7	*Ambystoma mavortium melanostictum*
Northern Bog Lemming	3	*Synaptomys borealis*
Water Shrew	3	*Sorex palustris*
Mink	2	*Neovison vison (aka Mustela vison)*
River Otter	1	*Lontra canadensis*
Human	1	
Leech	1	Species unidentified
Fish Mix	1	Mix of Northern Pike, Pearl Dace, White Sucker, Lake Chub, Yellow Perch, Longnose Sucker, Brook Stickleback, Fathead Minnow, Slimy Sculpin, Spottail Shiner, Arctic Grayling, Northern redbelly dace, and Finescale dace
Fungal Mix	1	Mix of Leptosphaeria maculans, Leptosphaeria biglobosa, genetically unidentified Leptosphaeria-like fungal cultures, and genetically unidentified Penicillium species
Plant Mix	1	Mix of Barley and Canola
Water	1	Blank

ARU Methods

The ARUs were set to record following ABMI's standard sampling schedule:

- 10 min at 00:00:00
- 3 min at 02:00:00
- 10 min at 30 min after sunrise ("Dawn")
- 3 min at 2 hrs. after sunrise ("Dawn + 1.5 hrs")
- 3 min at 12:00:00
- 3 min at 15:00:00
- 3 min at 1 hr. before sunset
- 3 min at 1 hr. after sunset

Only recordings from 00:00:00 and 02:00:00 were processed for analysis. The available recordings were subsampled based on ABMI's standard processing. A random 00:00:00 and 02:00:00 recording was selected from four blocks of time: March 31-May 20, May 21-June 9, June 10 - June 29, and June 30 - July 29, where available. In addition, a recording was processed for midnight the night before eDNA sampling occurred (once per month in June, July, and August).

During processing, the first minute of a recording was transcribed. Each unique species was tagged and assigned a calling intensity rank as a common measure of estimating amphibian abundance adapted from the North American Amphibian Monitoring Program (NAAMP) Amphibian Calling Index (ACI) (Mossman and Weir 2005).

Results

eDNA Primers

Descriptions of each primer set that was developed and used in this project, and commentary on their amplification quality, are provided in Table 3. Note that some of the primers require further development and testing to meet quality standards related to amplification; see the Discussion section for further details.

Table 3. Information on the primer sets used in this project.

Species	Sequences	Annealing temp	Sensitivity	Comments
Blotched Tiger Salamander	Forward - ATAGTAATACCTGTAATAATCG Reverse - CTAATAGAAGGAGGAATGA Probe - [6FAM] TGCACCAGATATAGCCTTCC [BHQ1]	60°C	1/10,000,000	No future adjustments needed.
Northern Leopard Frog	Forward – TTGGACTCACTTARGAAT Reverse – ATTGAATGGACTAAGWCTATG Probe – [6FAM] TACTTGATAGGACCTTCGCTT [BHQ1]	60°C	1/1,000,000	This primer set worked well. No future adjustments needed.
Wood Frog	Forward – AATAACGGCTGACTYCTA Reverse – AAGGCTGTWGCTATYACTA Probe – [6-FAM] TTTCAYATYGGACGAGGC [BHQ-1]	60°C	1/100,000	Sensitivity of this primer is a bit low, but it is still working. Decreasing annealing temperature should improve sensitivity.
Canadian Toad	Forward – GGGATTGGTGATGATATG Reverse – CAGACTTTCACACCTTTA Probe – [6FAM] TGCTCGATTATACATAGTATGTCCTTC [BHQ1]	60°C	1/100,000	This primer set may be affected by decreased sensitivity.
Boreal Chorus Frog	Forward – CCATGAGGACAGATATCC Reverse – CTCAGATTCATTGAACTAGG Probe – [6FAM] CCACTGTCATCACTAACCTCCTCTC [BHQ1]	59°C	1/10,000	This primer set is not as sensitive as required.
Western (Boreal Toad)	Forward – TCTGGCATCTCATAGTGG Reverse – CCTTCTTCTTATGCTAGACAA Probe – [6FAM] ATGGCACATTAACAAGGCTGTCC [BHQ1]	61°C	1/1,000,000	This primer set may also be picking up Boreal Chorus Frog at lower concentrations.

DNA Detections

The eDNA approach detected three amphibian species across the four sites sampled in this project (Table 4). Blotched Tiger Salamander was detected at two sites, Wood Frog was detected at every site, and Boreal Chorus Frog was detected at one site. Northern Leopard Frog, Canadian Toad, and Western Toad were not detected at any of the sampling sites. Table A1 (in the Appendix) displays the amphibian detection results from the eDNA analysis in more detail.

Table 4. Summary of the detection of amphibian species using eDNA at four Alberta wetlands.*

Site	Month	Blotched Tiger Salamander	Northern Leopard Frog	Wood Frog	Boreal Chorus Frog	Canadian Toad	Western Toad
1086-71-25	June						
	July						
	Aug						
1086-71-28	June						
	July						
	Aug						
1086-71-9	June						
	July						
	Aug						
1113-71-5	June						
	July						
	Aug						

***Note** that red indicates a species was not detected, green that it was detected with a strong signal during the molecular analysis, and yellow that it was detected, but the signal was weak.

ARU Detections

The amphibians that were detected by ARU at each site and identified by interpreters are shown in Figure 4. The ARUs did not record throughout the whole sample period as intended due to premature loss of battery power. The last recording date for each site was:

- Site 1113-71-5: August 12, 2020
- Site 1086-71-28: July 3, 2020
- Site 1086-71-25: June 11, 2020
- Site 1086-71-9: July 22, 2020

Three amphibian species were identified by ARU methods at the four sites. Boreal Chorus Frog and Wood Frog were detected at all sites and a single Western Toad was detected at one site.

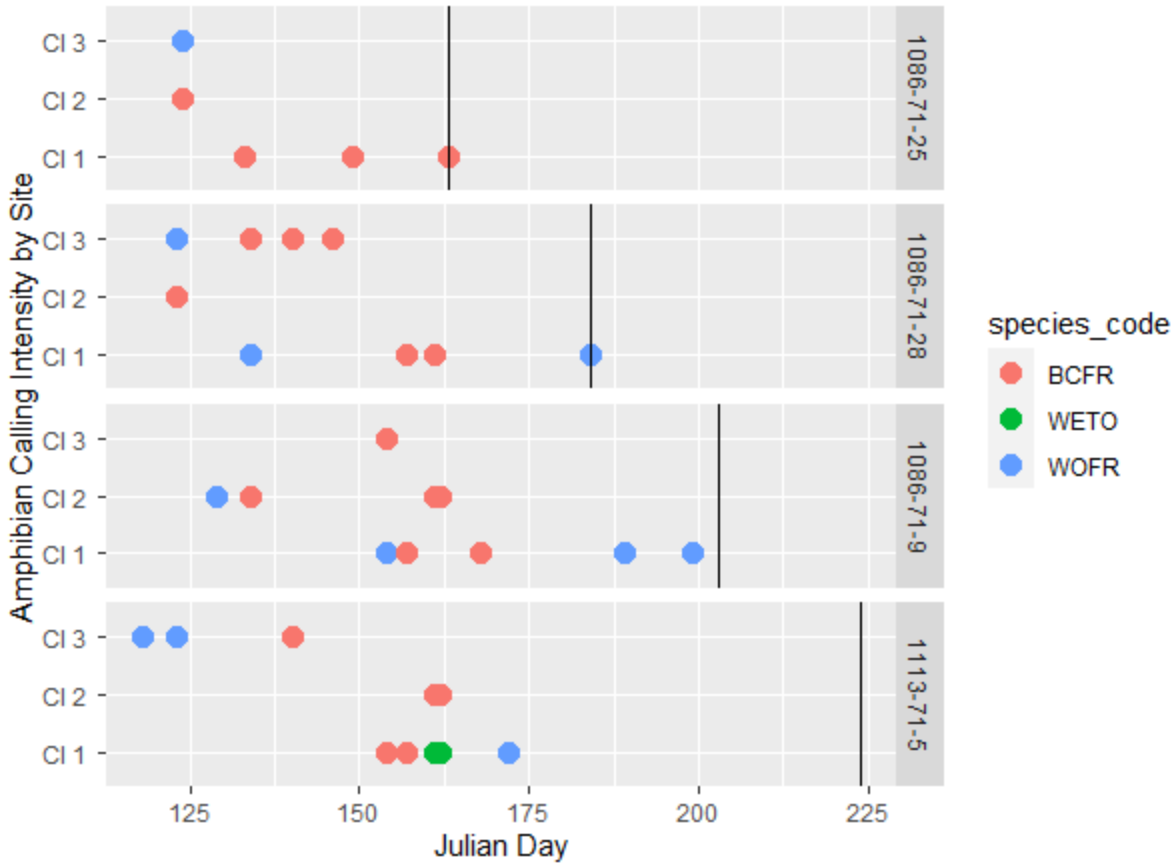

Figure 4. ARU detection of amphibians. Black vertical lines indicate the last ARU recording date for each site. **Note** Julian day 120 is April 30, day 160 is June 9 and day 200 is July 19; BCFR = Boreal Chorus Frog, WETO = Western Toad, WOFR = Wood Frog

Comparison of ARU and eDNA Detections

The audio and molecular methods were generally in agreement in terms of amphibian species detected. However, there were discrepancies between the two methods, namely, the absence of Blotched Tiger Salamander detections by the ARUs, the absence of Western Toad detections by eDNA at site 1113-71-5, and the absence of Boreal Chorus Frog detections by eDNA at sites 1086-71-25, 1086-71-28, and 1086-71-9 (Table 5).

Table 5. Comparison of amphibian detection using ARU and eDNA methods at study sites over the sampling period. *

Site	Month	Northern Leopard Frog	Wood Frog	Boreal Chorus Frog	Canadian Toad	Western Toad
1086-71-25	June	same	same	eDNA: no; ARU: yes	same	same
1086-71-28	June	same	same	eDNA: no; ARU: yes	same	same
	July	same	same	eDNA: no; ARU: yes	same	same
1086-71-9	June	same	same	eDNA: no; ARU - yes	same	same
	July	same	same	eDNA – no; ARU - yes	same	same
1113-71-5	June	same	same	same	same	eDNA – no; ARU - yes
	July	same	same	same	same	same
	Aug	same	same	same	same	same

***Note** that the rows for the months with no ARU data have been removed from the table. Red cells indicate a species was not detected by either method, green cells mean the species was detected using both methods, and grey cells indicate the species was detected by only one of the two methods. Blotched Tiger Salamander was not included in the table, as this species does not make breeding calls.

Discussion

Primer Sets Testing

Canadian Toad

Three different primer sets were developed to try to detect this species. The first set did not amplify, the second set had a low sensitivity of 1/1000, while the third set seemed to work reasonably during tissue validation, with a low but potentially usable sensitivity. No Canadian Toad detections were made during the field trial, and there was no evidence (from the ARU data or incidental observations during field work) that the species occurred at any of the four sites used in this study. It may be necessary to optimize the annealing temperatures for this primer set to make it more sensitive, though this must be balanced against a potential increase in the possibility of false positives. However, this step should not be taken until the primer set can be tested at sites with known Canadian Toad populations.

Boreal Chorus Frog

Six different primer sets were developed to elucidate this species, four based on the CytB gene and 2 based on the COI gene. The four primer sets based on the CytB gene did not react to the presence of chorus frog tissue (Primer sets 1, and 6), gave non-specific target reads (Primer set 2 - picked up Canadian Toad and Blotched Tiger Salamander), reacted at a very low level (e.g 31- 32 cycles, Primer sets 3 and 5), or exhibited low levels of sensitivity (Primer set 4) (e.g., were not able to detect the target species during serial dilution trials when the concentration dropped below 1/100,000). The two primer sets based on the COI gene picked up the presence of fish tissue during the validation step, and so were discarded. Therefore, the most sensitive of the CytB primer sets (Primer set 4) was used to examine the pond samples, with the annealing temperature dropped to 59°C with the hope that it would be sensitive enough to detect the target species without sacrificing specificity. This primer picked up a signal for Boreal Chorus Frog from four samples at site 1113-5, but did not detect the species from any of the other sites, even though they supported Boreal Chorus Frog in multiple months (often in high numbers) based on the acoustic sampling results (Figure 4). The sites with large choruses likely produced large numbers of tadpoles, which should have resulted in relatively high DNA inputs into the wetlands, which we should have picked up during collection of water samples for eDNA analysis. It seems likely that this primer set was less than ideal, and should be further optimized or redesigned to improve its sensitivity, and then tested in the field again.

Western (Boreal) Toad

Three different primer sets were developed for this species. In all cases Boreal Chorus Frog was detected by the Western Toad primer set during tissue validation tests. This may reflect reactivity of the primer sets to both Western Toad and Boreal Chorus Frog DNA, or perhaps the isolated Boreal Chorus Frog DNA was contaminated with a very small amount of Western Boreal Toad. Therefore, this primer set should be used with caution until more tests can be done with Boreal Chorus Frog tissue. The best of the three primer sets was Aborbor3, which showed a much lower reactivity to the Boreal Chorus Frog DNA during the tissue validation step when the annealing temperature was increased to 61°C to improve specificity. While increasing the annealing temperature improves specificity, it can also decrease sensitivity, which may cause false negatives in environmental samples.

There were two ARU detections of the Western (Boreal) Toad at site 1113-5 on June 9 and 10, shortly after eDNA samples were taken at the site on June 8. These acoustic detections were of only one individual on each night, and therefore a high concentration of Western Toad eDNA would not be expected in the pond. The failure of the primer set to detect this individual toad does not necessarily indicate a poor primer set, but is more likely a reflection of very low abundance of the target species at the site resulting in very localized distribution of the little eDNA signal that was potentially present. Further field tests should be conducted which include wetlands with significant numbers of Western Toads to facilitate better optimization of this primer set and a more realistic test of its ability to detect the target species.

Wood Frog

The wood frog primer worked acceptably, but had low sensitivity. The primer only amplified up to 1/100,000 dilutions, meaning that higher levels of wood frog DNA are needed to produce a positive test. Adjustments should be trialed with this primer set to optimize sensitivity. Conditions that could be modified to improve sensitivity include the annealing temperature and concentration of Mg2+ ions in the reaction.

Northern Leopard Frog

The Northern Leopard Frog primer set used in this project was based on a Sybr primer initially developed for a previous project. The addition of the Taqman probe to this primer has increased both the sensitivity and specificity of the assay, and this primer set is now considered a high quality primer. A slight adjustment to the annealing temperature may optimize the sensitivity further.Blotched Tiger Salamander

The primer set for the Blotched Tiger Salamander is working at full sensitivity. The specificity is good, but one should be cautious when testing in areas with other *Ambystoma mavortium* sub species or members of the Ambystoma genus. These primers were not tested against tissues from close taxonomic relatives and it is uncertain if they would cross react and give false detections. If use of this primer set is anticipated for samples from sites that may contain other abmystomid salamanders, the primer set should first be tested against tissue from these species.

Amplification Tests

The number of amplifiable universal COI reactions is provided in Table A1, *Amplification Check* column. All the environmental samples had a high level of reactive DNA. For one sample (August 10, 2020 at site 1113-5) only half of the extractions produced amplifiable DNA, which could explain why no target species at all were detected for this sample. Please see the discussion in the *Amphibian Detections* Section below for further explanation.

For the Taqman assay, the 50 pg dilutions of the pond water produced no positive reactions, so 2 uL of the concentrated unnormalized genomic DNA was used for subsequent analyses. The need to use the concentrated sample in the Taqman assay was not unexpected; the higher specificity of Taqman assays tends to exclude much of the nonspecific DNA binding observed in Sybr reactions, but provides a more definitive yes/no answer related to species detections. However, Taqman assays do require a higher concentration of DNA for a positive test until the primers are completely optimized, at which point sensitivity of the two approaches is similar, with the Taqman assay less likely to produce false positives than the corresponding Sybr assay.

Amphibian Detections

Unsurprisingly, Wood Frog and Boreal Chorus Frog were the most prevalent detections using both eDNA and ARU sampling. Using eDNA analysis, the Wood Frog was detected at every site and the Boreal

Chorus Frog was detected at a single site; in contrast, audio sampling detected both species at all four sites. There were two detections of a single Western Toad at site 1113-71-5 by the ARUs that were not detected by eDNA, and detections of Blotched Tiger Salamander at OGW-ABMI-1086-71-25 by eDNA that were not detected by the ARUs (Table 5).

At all sites, the August eDNA samples generally had lower positive counts for Boreal Chorus Frog and Wood Frog than other months (Table A1); the tadpoles of both these species generally undergo metamorphosis and leave the ponds in July. Although ARU detections of large numbers of Wood Frog and Boreal Chorus Frog (C3 level of abundance rating scale) occurred earlier in the season (from April 27th to June 2), there were no ARU detections in August. While some individuals may call outside of the breeding season, it would be expected that calling frequency and intensity would be very low. In addition, only one ARU was still recording in August. The lack of Wood Frog eDNA detections in the August samples may reflect the movement of individuals of this species out of the ponds and into the surrounding uplands to forage in preparation for overwintering. The same may be true for the Boreal Chorus Frog, but the challenges with the primer set for this species make it more difficult to draw conclusions related to temporal patterns of detection.

While the Wood Frog primers seemed to have a pond-wide reaction (i.e., if a filter tested positive in a pond, all filters for that pond tended to give at least one positive test), the Blotched Tiger Salamander samples did not exhibit this pattern. For this species, one filter could provide three very strong replicate positives, but the other filters taken at different points around the pond did not show the same positive test, suggesting that the DNA was not widely dispersed throughout the pond. This may reflect a more localized distribution and/or smaller numbers of individuals in the pond. This suggests that more samples will need to be collected at multiple points around the pond, or specific habitats within the pond will need to be targeted, when using eDNA methods for detecting species which occur in low abundance and/or in specific habitat types.

Composite Sampling

The eDNA results for the composite samples at site 1113-5 were similar to the results from the corresponding regular samples collected (Table A1). The results do not suggest that the composite sampling captured more DNA than the regular sampling procedure. However, this comparison was limited to one site and lacked replication and the ability to perform a statistical comparison. Furthermore, conducting the composite sampling entails using the same filter multiple times, with movement of personnel and the filter system between multiple sites, potentially increasing the chance for contamination. In order to be able to actually compare the efficacy of single versus composite samples, additional tests using multiple sites are needed.

Another potential approach to improve detection of species which are not well distributed around a pond would be to sample habitats where the species is most likely to be found during the sampling period. This would require some knowledge of differential habitat use by the target species, as well as

the ability to differentiate between different habitat types in the field so that sampling effort could be distributed appropriately.

Conclusions and Next Steps

The overall goal of this project was to assess the ability of using eDNA methods to monitor amphibians in Alberta wetlands. Achieving this goal required significant contributions to the development of eDNA amphibian monitoring methods in Alberta, including the development and testing of primers. The results of the project indicated that there is potential for eDNA methods to augment audio recording methods for amphibian detection, especially for non-vocal species like the Blotched Tiger Salamander. The results of this study also indicated that additional work is required to develop a fully functional eDNA amphibian monitoring program for use across Alberta. The following 'next steps' were identified as specific activities that would support development of amphibian eDNA monitoring methods for Alberta wetlands.

- Western (Boreal) and Canadian Toad primer sets need to be tested and optimized using samples from sites that are known to support populations of these species.
- The Northern Leopard Frog Primer set is a good candidate primer set for digital droplet PCR which would allow us to test when there are very low Northern Leopard Frog to total DNA ratios then typically examined using standard qPCR, giving us a much more sensitive assay then what a standard Taqman assay provides.
- Redesign the Boreal Chorus Frog primers to identify a primer set which has a 1/1,000,000 sensitivity or better. The ARUs confirmed the presence of Boreal Chorus Frog in large numbers at many sites, but the current Boreal Chorus Frog primer failed to detect them in most cases. Therefore, the following steps are suggested:
    - Continue with primer redesign instead of simply trying to optimize analysis conditions. Specifically, we suggest amplifying the whole gene sequence to help focus on a unique segment to develop a new primer. This is suggested specifically for Boreal Chorus Frog; however, all species of interest may benefit from this approach, which could be done in association with adopting a high throughput eDNA methodology.
    - Additionally, a number of new amphibian primer sets have been published recently. We suggest that we order these and test their performance against our known local frog genomic DNA and other controls. At the same time, it would be useful to test a few more newly designed primer sets, in case the published primers are seen to cross-react with other local species.

Resources

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Appendix

Table A1. Results from molecular analysis of wetland eDNA samples for target species.

							Blotched Tiger Salamander	Northern Leopard Frog	Wood Frog	Boreal Chorus Frog	Canadian Toad	Western (Boreal) Toad
Sample Date	**Type**	**Location**	**Avg ng/uL**	**Filters extracted**	**Total # technical replicates**	**Amplification Check - Universal COI**	**Atig1 Positive Test Count***	**Rpip1t Positive Test Count***	**Lsyl1 Positive Test Count***	**Pmac4 Positive Test Count***	**Ahem3 Positive Test Count***	**Abor3 Positive Test Count***
June 10/20	Sample	1086-9	20.85	3	9	9	0	0	8	0	0	0
July 8/20	Sample	1086-9	14.56	4	12	10	0	0	8	0	0	0
Aug 6/20	Sample	1086-9	16.28	5	15	10	7	0	1	0	0	0
June 5/20	Sample	1086-25	17.16	3	9	9	0	0	1	0	0	0
July 7/20	Sample	1086-25	30.72	6	18	16	3	0	6	0	0	0
Aug 5/20	Sample	1086-25	14.94	7	21	19	2	0	1	0	0	0
June 9/20	Sample	1086-28	18.80	3	9	6	0	0	5	0	0	0
July 7/20	Sample	1086-28	36.47	4	12	12	0	0	7	0	0	0
Aug 5/20	Sample	1086-28	49.79	3	9	9	0	0	1	0	0	0
June 8/20	Sample	1113-5	17.66	4	12	12	0	0	7	4	0	0
July 6/20	Sample	1113-5	35.10	4	12	12	0	0	7	3	0	0
Aug 10/20	Sample	1113-5	10.95	6	18	9	0	0	0	0	0	0
June 8/20	Composite	1113-5	14.06	3	9	6	0	0	6	6	0	0
July 6/20	Composite	1113-5	34.56	3	9	9	0	0	9	9	0	0
Aug 10/20	Composite	1113-5	18.32	7	21	15	0	0	1	0	0	0

Table A1 Key: Red highlighted cells indicate a negative result with no amplification, orange cells indicate a questionable result in which amplification is near the level of blank or negative controls, and Green cells reflect a positive result indicating a strong read; the numbers in the coloured cells indicate the number of positive tests. ***Note**: The number in the column for each species is the number of individual technical replicates for which that species was detected.

Table A2. Results of molecular analysis of the control samples collected during the project. There were no detections of any of the target species based on the control filters, suggesting there was no contamination of the samples.

							Blotched Tiger Salamander	Northern Leopard Frog	Wood Frog	Boreal Chorus Frog	Canadian Toad	Western (Boreal) Toad
Sample Date	**Type**	**Location**	**Month**	**Avg ng/uL**	**Filters extracted**	**Amplification Check - Universal COI**	**Atig1 Positive Test Count***	**Rpip1t Positive Test Count***	**Lsyl1 Positive Test Count***	**Pmac4 Positive Test Count***	**Ahem3 Positive Test Count***	**Abor3 Positive Test Count***
June 10, 2020	Control	1086-9	June	10.16	2	3	0	0	0	0	0	0
July 8, 2020	Control	1086-9	July	1.30	2	4	0	0	0	0	0	0
Aug 6, 2020	Control	1086-9	Aug	1.49	2	0	0	0	0	0	0	0
June 5, 2020	Control	1086-25	June	2.72	2	0	0	0	0	0	0	0
July 7, 2020	Control	1086-25	July	0.435	2	4	0	0	0	0	0	0
Aug 5, 2020	Control	1086-25	Aug	2.48	2	4	0	0	0	0	0	0
June 9, 2020	Control	1086-28	June	2.2	2	3	0	0	0	0	0	0
July 7, 2020	Control	1086-28	July	16.01	1	0	0	0	0	0	0	0
Aug 5, 2020	Control	1086-28	Aug	2.22	1	1	0	0	0	0	0	0
June 8/20	Control	1113-5	June	48.34	2	1	0	0	0	0	0	0
July 6/20	Control	1113-5	July	1.22	2	2	0	0	0	0	0	0
Aug 10/20	Control	1113-5	Aug	2.11	2	6	0	0	0	0	0	0

***Note**: The number in the column for each species is the number of individual control replicates for which that species was detected.

Table A3. Detailed data on the ARU detections of amphibians during the study.

Location	Date	Time	Species	Abundance*
OGW-ABMI-1086-71-25	2020-05-03	0:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-25	2020-05-03	0:00:00	Wood Frog	CI 3 (>100 Frogs)
OGW-ABMI-1086-71-25	2020-05-12	2:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-25	2020-05-28	0:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-25	2020-06-11	0:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-25	2020-06-11	2:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-28	2020-05-02	2:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-28	2020-05-02	2:00:00	Wood Frog	CI 3 (>100 Frogs)
OGW-ABMI-1086-71-28	2020-05-13	0:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1086-71-28	2020-05-13	0:00:00	Wood Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-28	2020-05-19	0:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1086-71-28	2020-05-25	2:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1086-71-28	2020-06-05	0:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-28	2020-06-09	2:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-28	2020-07-02	2:00:00	Wood Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-9	2020-05-08	2:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-9	2020-05-08	2:00:00	Wood Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-9	2020-05-13	0:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-9	2020-06-02	0:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1086-71-9	2020-06-02	0:00:00	Wood Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-9	2020-06-05	0:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-9	2020-06-09	0:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-9	2020-06-10	0:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1086-71-9	2020-06-16	0:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-9	2020-07-07	0:00:00	Wood Frog	CI 1 (1 Frog)
OGW-ABMI-1086-71-9	2020-07-17	0:00:00	Wood Frog	CI 1 (1 Frog)
OGW-ABMI-1113-71-5	2020-04-27	2:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1113-71-5	2020-04-27	2:00:00	Wood Frog	CI 3 (>100 Frogs)
OGW-ABMI-1113-71-5	2020-05-02	0:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1113-71-5	2020-05-02	0:00:00	Wood Frog	CI 3 (>100 Frogs)
OGW-ABMI-1113-71-5	2020-05-19	0:00:00	Boreal Chorus Frog	CI 3 (>100 Frogs)
OGW-ABMI-1113-71-5	2020-06-02	2:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1113-71-5	2020-06-05	0:00:00	Boreal Chorus Frog	CI 1 (1 Frog)
OGW-ABMI-1113-71-5	2020-06-09	0:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1113-71-5	2020-06-09	0:00:00	Western Toad	CI 1 (1 Frog)
OGW-ABMI-1113-71-5	2020-06-10	0:00:00	Boreal Chorus Frog	CI 2 (>10 Frogs)
OGW-ABMI-1113-71-5	2020-06-10	0:00:00	Western Toad	CI 1 (1 Frog)
OGW-ABMI-1113-71-5	2020-06-20	0:00:00	Wood Frog	CI 1 (1 Frog)

* CI = “Calling index”; CI 1: 1 frog heard; CI 2: >10 frogs heard; CI 3: > 100 frogs heard.