Clarifying Distributions of Four Species of *Rana* in Southwestern British Columbia Using eDNA Methods

Final report, 2016 – 2018

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Cover photograph: Habitat in Skagit Valley where eDNA of Cascades frog was found in two of three years of study (photo by Lennart Sopuck)

Executive Summary

Environmental DNA (eDNA) methodologies are rapidly expanding our knowledge of distributions of species at risk, including amphibians in various geographical areas. This study investigated the distributions of four species of congeneric pond-breeding frogs of conservation concern in southwest British Columbia (BC) using eDNA methods. The target species were Oregon spotted frog (*Rana pretiosa*; endangered), Columbia spotted frog (*R. luteiventris*; COSEWIC candidate list), Northern red-legged frog (*R. aurora*; Special Concern), and Cascades frog (*R. cascadae*; globally Near Threatened, yet to be confirmed from BC). Objectives for the 3-year study were to (1) clarify occupancy and habitat use of the focal species within the study area using eDNA methods; (2) assess and validate eDNA sampling procedures for these species, and (3) improve management of these species and their habitats through communication of the project results.

During field seasons from 2016 to 2018, three one-litre water samples were collected from 176 sites in 86 water bodies (locations) for a total of 524 water samples (including replicates/site). The sites were within eight main watershed groups and distributed from Burns Bog in the southwest to Lillooet Lake in the north, and through the Lower Mainland to Skagit Valley and Manning Provincial Park in the east. Water samples were collected and filtered using the BC Ministry of Environment eDNA Collection Protocols. For validation purposes, skin swabs were collected from 48 frogs to obtain genetic material; the swab samples were from wild-caught frogs from nine field sites and from frogs in the Oregon spotted frog captive breeding programs. In addition, 16 water samples with concentrated frog DNA (1 L of water where a frog had been for 2 to 5 min) were collected at field sites. eDNA tests were developed *de novo* for this project, and isolated DNA was analysed for the focal species with quantitative polymerase chain reaction (qPCR) methods by our partners at the University of Victoria.

Oregon spotted frog eDNA was detected (biologist interpretation “yes” or “suspected”) at 14.6% of the sites, representing 15.1% of the water bodies tested. Columbia spotted frog eDNA was detected at 13.4% of the sites sampled, representing 22.9% of the water bodies tested. Northern red-legged frog eDNA was detected at 25% of the sites sampled, representing 26.3% of the water bodies tested. Cascades frog eDNA was detected at 7.9% of the sites sampled, representing 8.6% of the water bodies tested.

The main findings were as follows:

- Oregon spotted frog eDNA was detected at an extralimital site in the Skagit Valley (Skagit 3) at an elevation of 500 m, and the presence of the species there was confirmed by the analysis of a skin swab from a field-caught frog.
- Oregon spotted frog eDNA was detected at five previously unreported locations in the Harrison and Lower Fraser watersheds in the Lower Mainland.
- Oregon spotted frog eDNA was detected at two water bodies from where the species was known only from historical records, and a spotted frog was observed at one of the sites during sample collection in 2017.
- eDNA and swab sample analyses helped clarify potential contact zones between Columbia and Oregon spotted frogs. In the east, analysis of DNA from swab samples indicated that both species of spotted frogs were present in the same wetland in the Skagit

Valley. In the west, Columbia spotted frog's eDNA was detected in one wetland, 2.5 km northwest of a known Oregon spotted frog site in the Lower Mainland, but the validity of this result requires further corroborative evidence.

- eDNA of Northern red-legged frog was detected throughout the species' known distribution in the Lower Mainland and at one site north of Squamish, also within the known range. The species' eDNA was not detected east of the Lower Mainland in the Skagit Valley or Manning Provincial Park, corroborating previous reported understanding of the species' distribution in BC.
- eDNA of Cascades frog was detected at six locations in the Skagit and Similkameen watersheds. However, physical searches in 2017 and 2018 of five of these sites failed to detect the species, and further corroborative evidence is needed before the presence of the species in BC is confirmed.

This project provided an opportunity to investigate the effectiveness of eDNA methods for closely related species of frogs in lentic habitats, including often eutrophic, turbid pools. The eDNA method was effective for three of the target species, Oregon spotted frog, Northern red-legged frog, and Cascades frog, based on evidence from positive and negative field controls and relatively high congruence of results for sites sampled repeatedly across years. The eDNA method performed less well for Columbia spotted frog, and both false negative and false positive results were evident. Direct observations of Columbia spotted frog at the time of water sample collection indicated that eDNA analysis resulted in positive detections only 37% of the times when the species was known to be present. The potential reasons for false negatives include poor dispersion of eDNA in the water bodies and/or poor performance of the test at the low DNA concentrations encountered in the field. The false positive detections may be the result of interference by eDNA of Northwestern salamander (*Ambystoma gracile*), which may produce a positive signal with Columbia spotted frog test if present at very high concentrations, or by other untested organisms; it could also reflect genetic introgression between the two sister species of spotted frogs at some sites. This may be resolved by obtaining more extensive species DNA information than what is currently available through public databases and redesigning the eDNA test to target more discriminatory regions of DNA.

Accurate knowledge of species' distributions is essential for effective conservation, and eDNA methods developed for the four target species as part of this project provide an additional tool to obtain such information. The detection of Oregon spotted frog eDNA and Cascades frog eDNA at previously undocumented sites can be used to target physical surveys. Sites with only historical records of Oregon spotted frog where eDNA evidence suggests the species still occurs can be similarly prioritized for physical surveys and re-evaluation of habitat protection and restoration needs. The results of this study also raise the possibility of introgression among Oregon spotted frog and Columbia spotted frog where the distributions of the two species meet, an issue with implications for recovery planning that merits further study.

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1.0 Introduction

Southwestern British Columbia (BC) is a hotspot of biodiversity and contains unique ecosystems and numerous species at risk (SCCP 2019). This three-year project is intended to address knowledge gaps regarding the distribution and habitat use for four species of amphibians of conservation concern in this area through environmental DNA (eDNA) methods. The focal species are Oregon spotted frog (*Rana pretiosa*) (Endangered, SARA-listed), Northern red-legged frog (*R. aurora*) (Special Concern, SARA-listed), Columbia spotted frog (*R. luteiventris*) (COSEWIC candidate list), and Cascades frog (*R. cascadae*) (global status: vulnerable; yet to be confirmed to occur in BC). The recovery objectives for Oregon spotted frog call for survey effort "to prevent inadvertent loss of currently unidentified populations by conducting a comprehensive inventory of potentially suitable habitat" (Objective 4; Canadian Oregon spotted frog Recovery Team 2014).

Environmental DNA survey methods have been used with success in aquatic habitats for a variety of organisms, including amphibians (Ficetola *et al*. 2008; Pilliod *et al.* 2013; Veldhoen *et al*. 2016) and fish (Jerde *et al*. 2011; Thomsen *et al*. 2012) and are rapidly evolving with increased accuracy and applicability to a wide range of conservation issues (Goldberg *et al*. 2015, 2016; Hobbs *et al.* 2019). In BC, eDNA methods have been used to detect a variety of amphibians, including American bullfrog (*L. catesbiena*), Coastal Tailed frog (*Ascaphus truei*), Rocky Mountain Tailed frog (*A. montanus*) and Coastal Giant Salamander (*Dicamptodon tenebrosus*) in stream habitats and Western Toad (*Anaxyrus boreas*), Northern Red-legged Frog, Great Basin Spadefoot (*Spea intermontanus*), Northern Leopard Frog (*R. pipiens*), and Tiger Salamander (*Ambystoma mavortium*) in pond habitats (Hobbs *et al*. 2019; J. Hobbs, unpubl. reports).

Here we present the results of fieldwork carried out in May – July 2016, 2017, and 2018 and associated laboratory analyses as part of the 3-year study using eDNA methods to clarify distributions of the focal species.

The objectives were as follows:

- To clarify occupancy and habitat use of four species of amphibians of conservation concern within the study area using eDNA methods.
- To assess and validate eDNA sampling procedures for the focal species.
- To analyse field samples using eDNA sampling procedures and tests developed for the target species
- To improve management of the focal species and their habitats through communication of the project results.

2.0 Methods

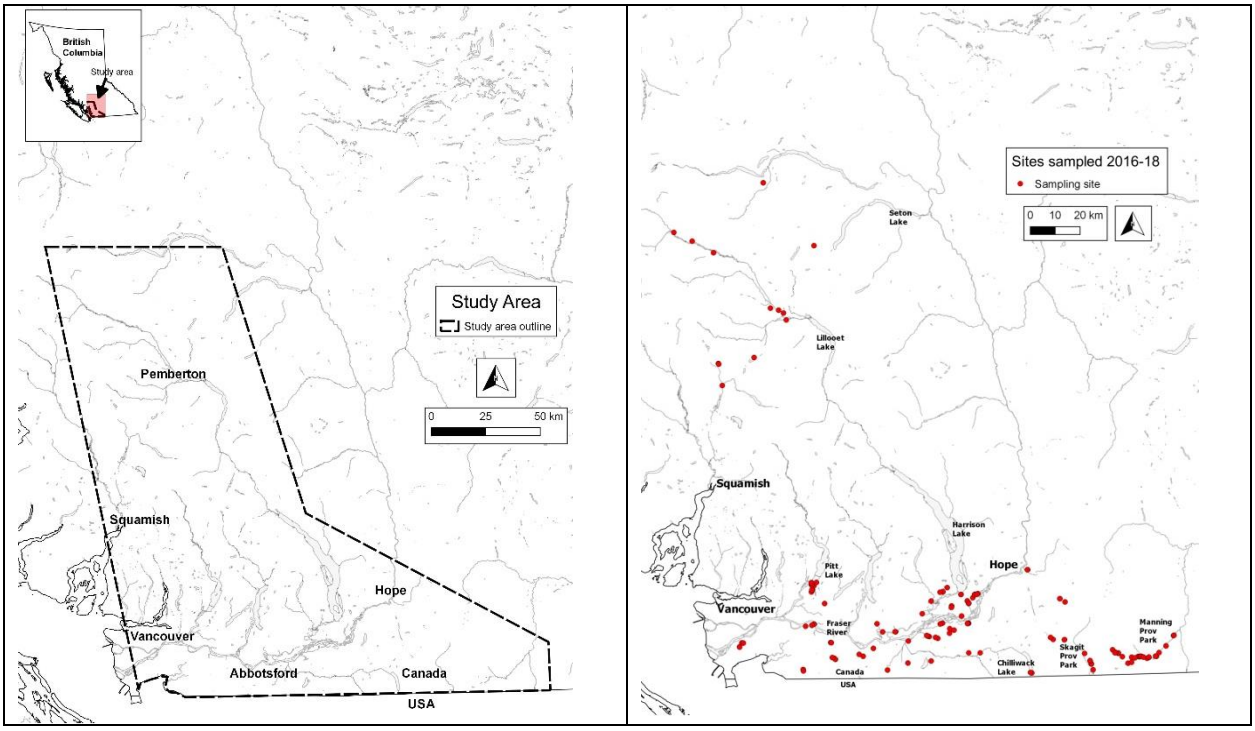
2.1 Site Selection

Wetlands within the target areas were mapped and prioritized for sampling based on predetermined criteria that included patch size, habitat connectivity, and habitat suitability for each of the focal species. In the Lower Mainland, we surveyed sites within Chilliwack, Harrison, and Lower Fraser river watersheds. The emphasis was on habitats of Oregon spotted frog, a species of high conservation concern. Priority was assigned to water bodies in areas with no or low previous survey efforts, as advised by the Oregon spotted frog Recovery Team. In addition to potential new sites, six known sites (in five water bodies) with recent records of Oregon spotted frog were included as positive controls; two sites with historical records only were also included.

Higher elevation (>500 m above sea level, asl) sites east of the Lower Fraser Valley in Skagit, and Sunshine valleys and in Manning Provincial Park in Skagit and Similkameen watersheds were sampled with emphasis on potential habitats for Cascades frog. In 2017 and 2018, the surveys in these areas focused on revisiting sites in Skagit Valley and Manning Park where Cascades frog DNA was detected and/or where Oregon spotted frog eDNA was detected outside the species' known range in the first year of the study. In 2018, sites to the north in Squamish, Lillooet, and Seton Lake watersheds were added because of observations of ranid frogs of unusual appearance reported to us from the Pemberton area (see **Figure 1** for overview of the study area).

The sampling sites were mapped using QGIS, version 2.18.2.

Figure 1. Overview of the study area and sampling sites in southwestern British Columbia.



2.2 Sample Collection, Handling, and Processing

Environmental DNA was collected and processed according to accepted BC Ministry of Environment provincial standards for surface water collection of eDNA (Hobbs and Goldberg. 2017). The number of sites sampled per water body ranged from one to five, depending on the size of the water body, availability of suitable habitat for the target species, and budgetary constraints. Three replicate one litre samples of water were collected per site. Survey conditions were recorded and included cloud cover, precipitation, wind speed, air temperature, water temperature, pH, and conductivity. Habitat attributes recorded included water body size class, water depth, water seasonality, and abundance of emergent vegetation.

After collection, each water sample was stored in a cooler in direct contact with ice during transport to a field laboratory for filtering. Site water was filtered through a 45 µm pore-size cellulose filter membrane to capture eDNA present in site water within each sample. Most samples were filtered on the day of collection; all samples were filtered within 24 h of collection. A de-ionized (distilled) water sample was also processed during each independent filtering session to provide a control to detect potential contamination during sample collection and filtration.

Upon completion of filtration, filter membranes were preserved by one of two methods:

1) filters were dried placed in individual coin-envelopes and stored in a zip-loc™ sealing storage bag with 15-30 mL of self-indicating silica desiccant; this method was used for all samples in 2018, or
2) filters were preserved via submersion in 95% molecular grade ethanol.

Preserved filters were transported to Dr. Caren Helbing`s laboratory at the University of Victoria for eDNA isolation and qPCR analysis.

2.3 Laboratory Analysis and Data Interpretation

2.3.1 eDNA TEST DEVELOPMENT AND VALIDATION

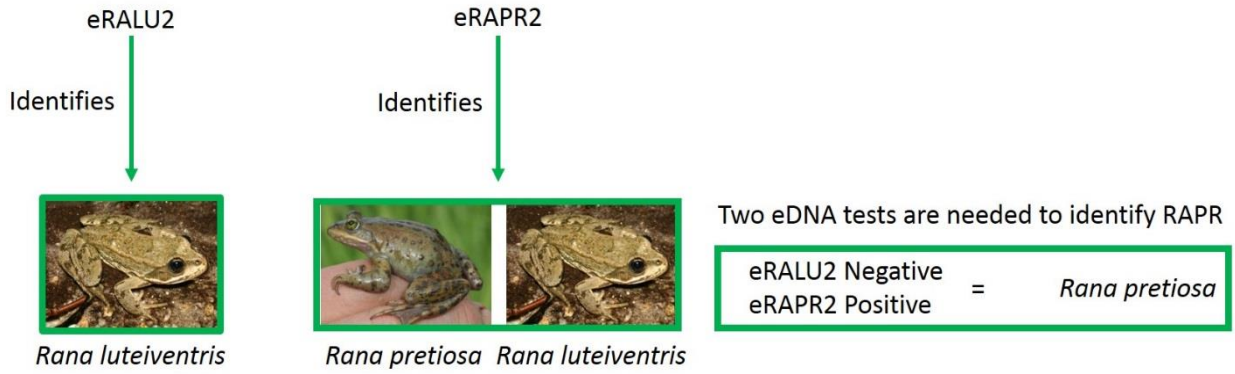
In Year 1, new eDNA tests (see **Appendix 1** for test fact sheets) were developed for four target species: Oregon spotted frog, Columbia spotted frog, Northern red-legged frog, and Cascades frog. An existing test for Northern red-legged frog from Dr. C. Goldberg's lab (Washington State University) was evaluated but was unable to distinguish between Cascades frog and Oregon spotted frog.

The four tests based upon quantitative real-time polymerase chain reaction (qPCR) were developed at Dr. C. Helbing's laboratory (University of Victoria) using a stringent, tiered validation system as described in Veldhoen *et al*. (2016) and Hobbs *et al.* (2019) sourcing publicly available DNA sequences. Specificity for each test to each respective target taxon was extensively queried and documented. Genetic material from voucher specimens of Cascades frog originated from Washington State (courtesy of Dr. C. Goldberg). Genetic material for the

remaining three focal taxa was obtained from species-verified tissue samples collected in BC. The four eDNA tests are referred to as: eRAAU1 (Northern red-legged frog), eRACA2 (Cascades frog), eRALU2 (Columbian spotted frog), and eRAPR2 (Oregon spotted frog).

There were no eDNA tests that could distinguish Oregon spotted frog from the closely-related Columbia spotted frog before commencement of this project. We were extremely limited by a paucity of genetic sequence information for these species and the similarity in DNA sequences available was considerable. Nevertheless, we were able to generate an eDNA test that distinguishes Columbia spotted frog from Oregon spotted frog (eRALU2; **Figure 2**). To identify the Oregon spotted frog, we had to use the eRALU2 test in tandem with another test (eRAPR2). The latter test hits both Columbia and Oregon spotted frog species (**Figure 2**). If the eRALU2 test result is negative but the eRAPR2 test is positive, then this provides supporting evidence for the presence of Oregon spotted frog (**Figure 2**). Unfortunately, this process cannot be used to detect Oregon spotted frog DNA where both species co-occur in the same site. Generation of more extensive mitochondrial DNA sequences for these two species would allow for the design and development of eDNA tests that could have greater ability to distinguish between these two closely-related species. During the course of the three-year study, we determined that an abundance of salamanders in an area may confound results for the Columbia spotted frog (eRALU2) test (see **Section 4.5**).

Figure 2. Interpretation of eDNA analysis using eDNA tests developed at the Helbing lab (University of Victoria) for distinguishing between Oregon spotted frog, *Rana pretiosa* (RAPR), and Columbia spotted frog, *R. luteiventris* (RALU).



2.3.2 Laboratory Analysis

The eDNA samples were evaluated using the process described in Veldhoen *et al* (2016) and refined as described in Hobbs *et al* (2019) that applies rigour in the design, validation, and execution steps of the qPCR tests to enhance confidence and interpretative power. eDNA test design and validation considerations included: explicitly designing and testing the eDNA tests against detection of human DNA that may be introduced at the sampling and/or analysis stages; additional aspects of primer and probe design considerations; qPCR run conditions to enhance specificity based on biochemical principles; and the ability to distinguish between true and false negatives through evaluation of endogenous DNA found in all field-collected eDNA samples (IntegritE-DNA test).

The process (**Figure 3**) includes the innovation of an IntegritE-DNA test that directly verifies the presence of amplifiable DNA in the sample based upon the presence of plant/algae chloroplast DNA that is ubiquitous and typically highly abundant in field samples (Veldhoen *et al* 2016; Hobbs *et al.* 2019). DNA can be isolated from a sample, but it can be degraded or modified by UV-radiation such that the qPCR detection method cannot function and may yield a false negative result. False negatives can also result from co-purification of DNA with impurities that inhibit the qPCR reaction or the assumption that DNA was collected on the membrane filter when it was not. The IntegritE-DNA test *directly* evaluates the eDNA sample and the test components and thus provides confidence of sample integrity for a negative species-specific result (**Figure 4)**. Four technical replicates of the IntegritE DNA test are run on every sample. The inclusion of the IntegritE-DNA test on every eDNA sample has greater power than the common laboratory-based practice of spiking samples with an external DNA template that will only detect the presence of inhibitors but fail to detect sample degradation.

Once sample integrity was confirmed, each eDNA sample was tested for the presence of eDNA from each respective target taxa using eight technical qPCR replicates. Laboratory procedures incorporated standard internal positive and negative plate controls to ensure the tests were performing properly and that samples were free from procedural contamination (for more details, see Hobbs *et al.* 2019).

In Year 3 of the project, an additional validation component was added to the eDNA tests where test sensitivity was further evaluated using synthetic DNA fragments as laboratory positive controls (as described in Hobbs *et al* 2019). These fragments correspond to the precise DNA sequences amplified by the targeted eDNA tests. The use of synthetic DNA fragments is notable for two major reasons: it eliminates the need for obtaining tissue from voucher specimens on a regular basis (which is not conducive to conservation) and it allows for inter-laboratory comparison of eDNA test performance (Hobbs *et al* 2019).

Figure 3. Schematic of the eDNA analysis process. An important innovation to eDNA analysis is the reduction of false negatives through the use of an IntegritE-DNA test prior to testing for specific frog species. Further details about the application of the IntegritE-DNA test can be found in Hobbs *et al* (2019).

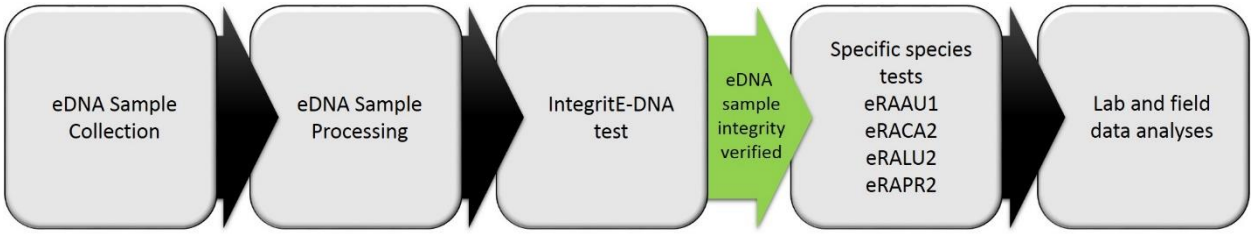
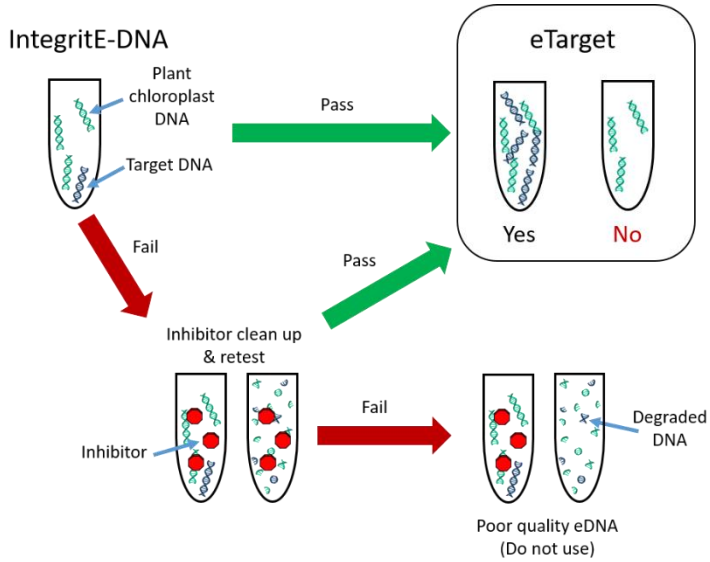


Figure 4. Use of the IntegritE-DNA test to increase confidence of taxa-specific DNA (eTarget) results.

The Integrit E-DNA test evaluates the ability of the isolated DNA in a sample to amplify plant chloroplast DNA prior to testing for the target species. If the sample passes the IntegritE-DNA test, then it is then tested for the target species (eTarget). If a sample fails the IntegritE-DNA test, it undergoes clean-up to remove inhibitors before being retested. If the cleaned-up sample fails the IntegritE-DNA test, it is deemed poor quality due to either degraded DNA or retention of inhibitors and the sample is not considered reliable. From Hobbs *et al.* (2019).



2.3.3 Interpretation of Laboratory Results

The laboratory determined that samples were positive for the presence of the target species' DNA if ≥3 of 8 runs/sample were positive (referred to as the "lab call"); uncontaminated de-ionized water reference samples have never had this result (Veldhoen *et al.* 2016; Hobbs *et al.* 2019). Following provincial eDNA standards (Hobbs and Goldberg 2017 and updates), we interpreted results at the site level with collective consideration of all three samples (referred to as the "biologist call") (see **Figure 5** for schematic of the scoring system).

Analytical rules were as follows:

1) If any of the three replicate samples/site resulted in ≥3/8 runs as positive, the entire site was considered positive ("yes"). Similarly, if any of the sites within a water body were positive ("yes"), the entire water body was considered positive.
2) If 2/8 runs for a sample were positive during qPCR testing for at least two of the three replicates collected at a site, then the site was assigned a status of "suspected". The site was also considered "suspected" if 2/8 runs for a sample were positive for one replicate and 1/8 runs were positive for the other two replicate samples. If any of the sites within a water body was categorized as "suspected" (yet there were no confirmed positive sites from the same waterbody), then the entire water body was classified as "suspected".
3) For all other combinations, the site was classified as negative.

We considered the results of the analytical rules in light of potential habitat suitability and connectivity, species ecology, and proximity to known occurrences. Consequently, we considered a result "unlikely" for positive detections ("yes") when outside known range and in atypical habitats. The rules for the "Biologist call" are summarized in **Table 1**.

Figure 5. Schematic of the scoring system used for assessing eDNA samples for target species' presence.

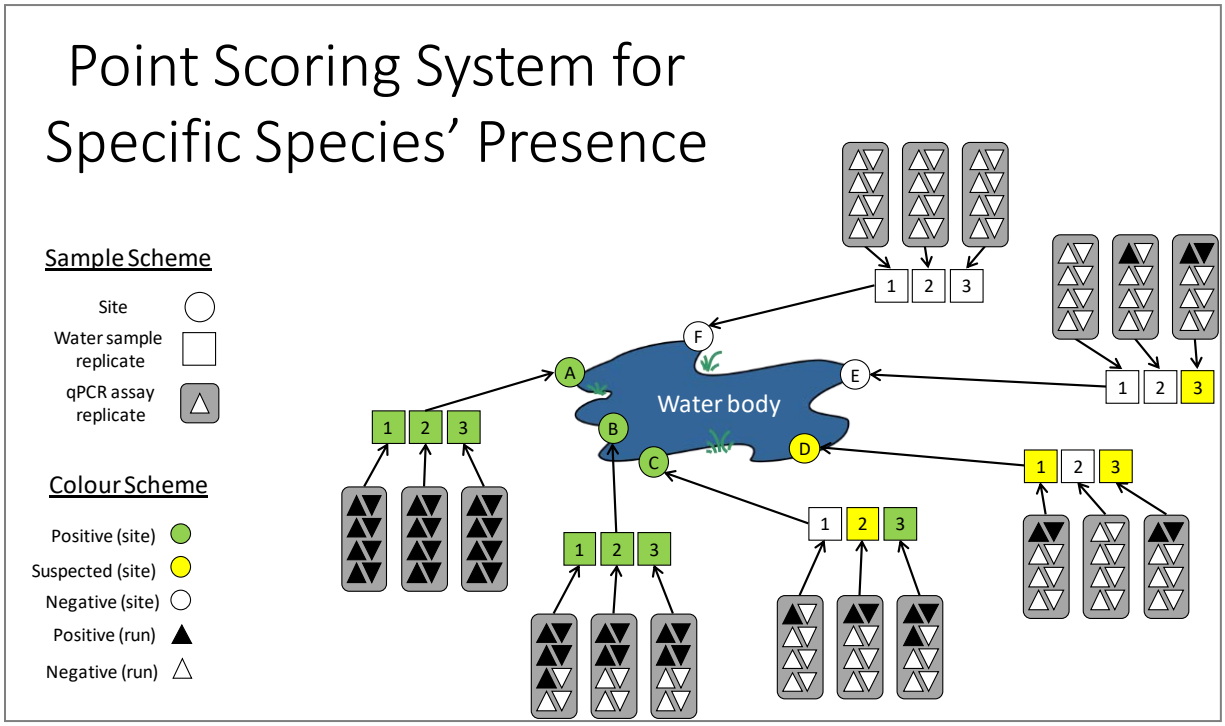


Table 1 . Interpretation rules of eDNA results for "Biologist call".

hits/runs	Biologist call for site	Biol call for water body	Code	Comments
3/8 or higher	positive	positive	Y	
2/8, 2/8, 2/8 or 2/8, 2/8, 1/8, or 2/8, 2/8, 0/8	suspected	suspected	S	
2/8, 1/8, 0/8 or 2/8, 0/8, 0/8 or 1/8, 0/8, 0/8, or all 0/8	negative	negative	N	
3/8 or higher but outside known range and habitat, unless other corroborative evidence is available (e.g., concentrated DNA or morphology of frog)	unlikely	ambiguous	U	Suspected category is not used if outside known range and in atypical habitats (i.e., higher criteria used for positive detections); this rule is extended for RALU/RAPR distinctions in the 2-tiered test, if outside known range

2.4 Visual Encounter Surveys and Collection of Genetic Material

In 2017 and 2018, visual encounter surveys as per methods described by Olson *et al*. (1997) were conducted at selected sites for eDNA validation and to facilitate collection of genetic material from focal taxa. Survey priority was afforded to sites where Cascades frog eDNA and Oregon spotted frog eDNA were detected in Year 1 outside the current known geographic distribution of either or both species. Observers located amphibians by slow visual searches of suitable habitats within the shoreline and shallow-water zone. Vigilance was applied to areas of abundant emergent vegetation and high incident solar exposure. Search time (effort) was recorded as person-hours per site. In addition to targeted surveys, we also recorded observations of amphibians encountered opportunistically while collecting water samples or walking between sites.

A sub-sample of spotted frogs detected at each site was swabbed for genetic material. Frogs were caught by hand or with a dip net. The swabbing for DNA analysis followed standard methods as described in Briggs NIH research group (2009). Briefly, the procedure consisted of swabbing the underside of the frog ~30 times with a sterile cotton swab to dislodge skin cells, air drying the swab, and storing it sealed in a dark, cool place until analysis. Immediately after collection in the field, each sample was placed in a small individual pre-sterilized vial or paper envelope that was sealed in a plastic bag with desiccant crystals. New disposable gloves were used when handling each individual frog. In addition to swabs, water samples with concentrated frog eDNA were collected by placing a frog in ~1 litre of site water (in 2017) or distilled water (in 2018) for 2 to 5 min and processing the sample as described for site water eDNA samples. Handling time was kept to a minimum, and frogs were released at their original capture locations immediately after processing.

British Columbia Ministry of Environment (2008) Standard Hygiene Protocols were followed to avoid spreading disease organisms among water bodies.

3.0 Results

3.1 Sampling Effort

Sampling took place during the following periods during three field seasons:

1) 2016: 13–18 May; 23–26 July
2) 2017: 4–6 July; 27–29 July
3) 2018: 16–20 June; 4–6 July

The surveys were timed for relatively dry periods to avoid dilution of eDNA due to recent rainfall, as per provincial standards (Hobbs and Goldberg 2017). The surveys were conducted before tadpoles had left breeding ponds. Low elevation sites were surveyed first, as breeding was expected to occur later at higher elevations. Furthermore, snow cover persisted well into summer in some years at higher elevations, hindering access.

3.1.1 SITE WATER SAMPLING EFFORT

In total, site water samples were collected from 176 sites in 86 water bodies for a total of 524 water samples (including replicates/site). **Table 2** provides a summary of the sites by major watershed group (see **Appendix 2** for list of sites and their coordinates and **Appendix 3** for overview of watershed groups). Sampling sites were distributed from Burns Bog in the southwest to Lillooet Lake in the north, and through the Lower Mainland to Skagit Valley and Manning Provincial Park in the east (**Figure 6**). Water samples from two additional sites in Washington State (Mt. Baker and Mt. Rainier; not shown in **Table 2** were analysed for positive controls for Cascades frog, which was not conclusively documented from BC.

Table 2. Distribution of sampling sites for amphibian eDNA collection within major watershed groups in southwestern British Columbia in 2016 - 2018.

Sample type	Chilliwack	Harrison	Lower Fraser	Fraser Canyon	Similka-meen	Skagit	Squamish	Lillooet & Seton Lake	Total collected	Total qPCR viable
Site water: # locations/water bodies	14	15	14	1	13	16	3	10	86	85
Site water: # sites	19	41	38	3	29	33	3	10	176	174
Site water: # samples	57	123	114	9	87	98	9	27	524	509*
Frog froth (# samples)	0	2	0	0	3	5	0	4	14*	13*
Skin swabs	2	11	0	0	22	12	0	2	48	48
Distilled water (control)	~	~	~	~	~	~	~	~	21	21

*excludes 2 reference samples from Washington State

3.1.2 CONCENTRATED DNA OF TARGET SPECIES

A total of 16 frog froth samples (i.e, a frog placed briefly into a bottle with ~1 litre of water) and 48 skin swabs were collected to confirm identity of ranid frogs and as additional positive controls for the eDNA tests (**Table 2**). Of the frog froth samples, two were from known Cascades frog sites in Washington State (Mt. Rainier, Mt. Baker). Of the swab samples, 35 were from field sites, and 13 were from captive breeding programs for Oregon spotted frog in the Lower Mainland of BC, provided to us by Kendra Morgan.

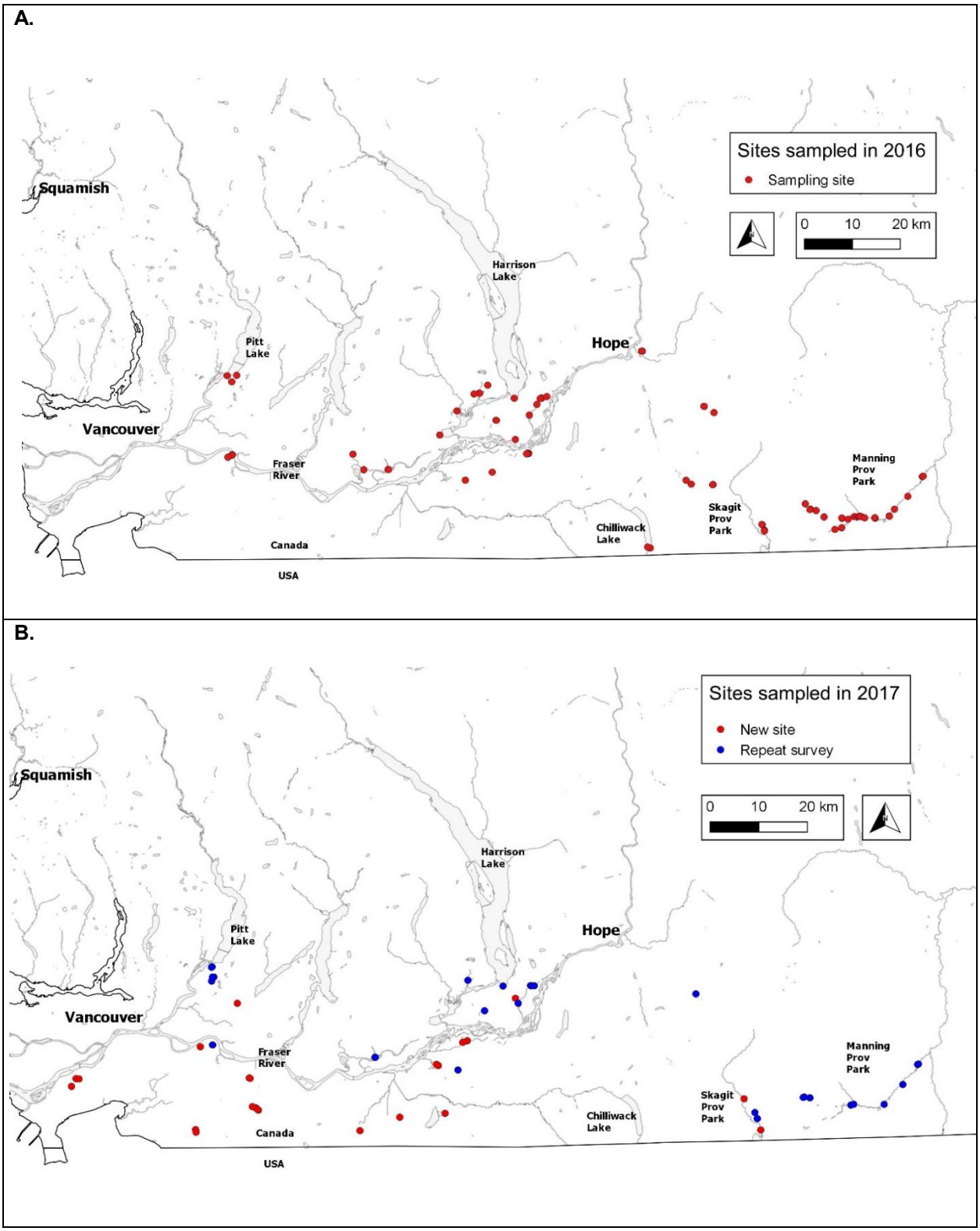
3.1.3 VISUAL ENCOUNTER SURVEYS FOR FROGS

During sampling sessions in 2017 and 2018, a total of 41.5 person-hours was spent searching for frogs at 36 sites (**Table 3**). Objectives included: (a) verification of extralimital positive qPCR test results for Cascades frog and Oregon spotted frog; and, (b) collection of genetic material (swabs and concentrated eDNA) from both species of spotted frogs to verify species identification, (c) verify identification of ranid frogs from north of Lower Mainland, especially from around Pemberton, as anecdotal information suggested they are morphologically unusual. In addition to above surveys, all amphibians observed opportunistically at the field sites during eDNA collection were recorded.

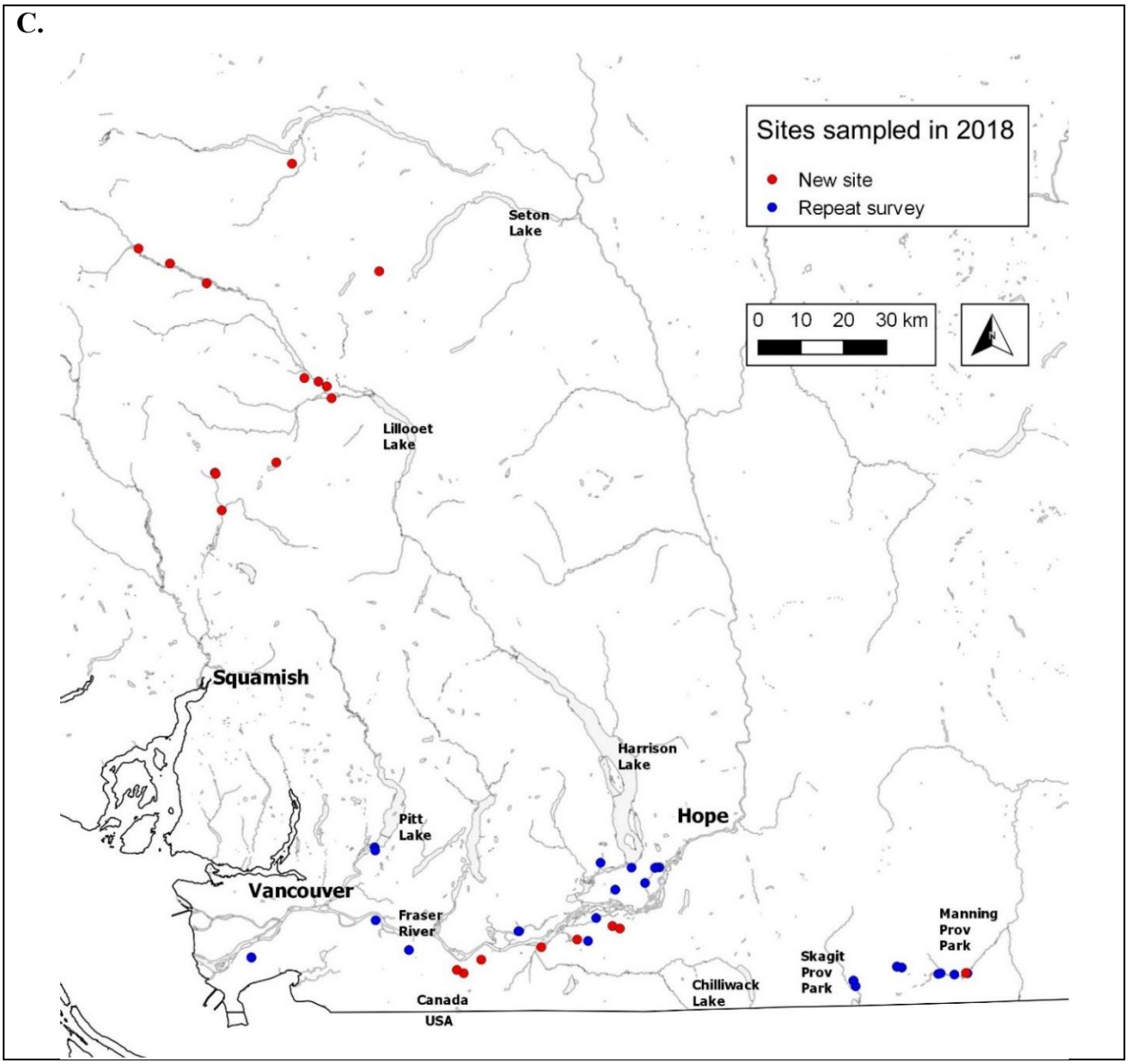
Table 3. Summary of visual encounter surveys conducted at selected water sample collection sites. The surveys focused on sites where eDNA results indicated that Cascades Frog might be present or where unusual ranids were suspected.

Watershed group	# water bodies	# sites	Total search time (person-h)
Harrison	2	2	0.5
Similkameen	9	10	14.9
Skagit	11	13	22.0
Lillooet, Seton Lake, Squamish	6	6	4.1
Total	28	31	41.5

Figure 6. Distribution of sampling sites in 2016 (A), 2017 (B), and 2018 (C).



C.


Sites sampled in 2018
New site
Repeat survey
0 10 20 30 km
Seton Lake
Lillooet Lake
Squamish
Harrison Lake
Hope
Pitt Lake
Vancouver
Fraser River
Canada
USA
Chilliwack Lake
Skagit Prov Park
Manning Prov Park

3.2 Sample Viability and Reference Samples

All samples collected during the 2016 – 2018 field seasons were processed in Dr. Helbing's laboratory (Biochemistry & Microbiology, University of Victoria). In total, 39 samples initially failed the IntegritE-DNA test (Hobbs *et al*. 2019). After clean up (**Figure 4**), 15 samples, including all three samples from one water body (Erock) and two sites (Cheam Lake 4, Nicomen Slough 2), still failed the IntegritE-DNA test and are therefore unreliable.

In 2017 and 2018, all the remaining site water samples that passed the IntegritE-DNA test were analysed for all four target species of *Rana*, while in 2016, samples from unlikely habitats (low elevation sites in Lower Mainland) were not analysed for Cascade frog, and only a randomized selection of unlikely sites in the Manning Park were analysed for Oregon spotted frog.

Of the frog froth samples, one sample failed the IntegritE-DNA test and was therefore unreliable. Of the 48 swab samples, four samples were negative for DNA of all four target species; two of these showed evidence of poor storage conditions and one represented a swab of a bag from which the frog had escaped. The fourth failed sample was from a frog at the captive breeding colony at Vancouver Zoo, where a second sample also showed a weak signal for Oregon spotted frog for unknown reasons.

A total of 21 reference samples of de-ionized water, filtered during the sample processing sessions, were analysed to test for sample contamination in the field. Six of the samples (3 from 2016, 3 from 2018) tested positive in the IntegritE-DNA test. All other de-ionized water samples returned 0/4 hits. Regardless, DNA from any of the target species was not detected in any of these samples, indicating that the samples were uncontaminated. This observation is consistent with previous studies where these negative controls occasionally have plant/algae material present in bottled water. One of the de-ionized water samples from 5 July 2017 tested positive for Columbia spotted frog DNA (3/8 runs), indicating contamination at some point during the field or lab processing. During the filtering process, it was noted that the sample contained specks of debris, which further indicated contamination of the distilled water control. However, the sampling during that survey session took place in the Lower Mainland, outside the known range of Columbia spotted frog, but DNA of this species was detected in some site water samples collected during the session (see "**eDNA Results from Site Water Samples**"). The reason for these positive detections remains enigmatic.

3.3 eDNA Results from Site Water Samples

3.2.1 Overview

Oregon spotted frog eDNA was detected (biologist call "yes" or "suspected") at 14.6% of the sites sampled, representing 15.1% of the water bodies tested. Columbia spotted frog eDNA was detected at 13.4% of the sites sampled, representing 22.9% of the water bodies tested. In addition, eDNA of Columbia spotted frog (13 sites in 2017) and Oregon spotted frog (one site in 2016) was detected in unexpected habitats and geographic areas; these are considered unreliable and are referred to as "unlikely" in the biologist call. The reasons for this are discussed in **Section 4.5**. For both species of spotted frogs, the detection rates were the highest in 2016 and lowest in 2018. Northern red-legged frog eDNA was detected at 25% of the sites sampled,

representing 26.3% of the water bodies tested. Cascades frog eDNA was detected at 7.9% of the sites sampled, representing 8.6% of the water bodies tested. The overall detections for the four target species are summarized in **Table 4** (all years combined) and **Table 5** (for each year).

Table 4. Percentage of sites (A) and water bodies (B) with eDNA detections of four species of Rana, based on water sample analysis, 2016 - 2018 results combined. Biologist call: YES - detected, SUSP - suspected, NO - not detected

A. Percentage of sites:

Species	YES	SUSP	NO	Unlikely	Sample size
Northern Red-legged Frog	22.2	2.8	75.0	0.0	144
Cascades Frog	7.9	0.0	92.1	0.0	139
Columbia Spotted Frog	10.5	2.9	79.1	7.6	172
Oregon Spotted Frog	10.8	3.8	84.8	0.6	158

B. Percentage of water bodies (locations):

Species	YES	SUSP	NO	Unlikely	Sample size
Northern Red-legged Frog	25.0	1.3	73.7	0.0	76
Cascades Frog	8.6	0.0	91.4	0.0	70
Columbia Spotted Frog	18.1	4.8	61.4	15.7	83
Oregon Spotted Frog	11.3	3.8	83.8	1.3	80

Table 5. Percentage of sites (A) and water bodies (B) with eDNA detections of four species of Rana, based on water sample analysis by year, 2016, 2017, and 2018 results.

	A. Percentage of sites			**B. Percentage of water bodies**		
eDNA detected	2016: % of total	2017: % of total	2018: % of total	2016: % of total	2017: % of total	2018: % of total
Northern Red-legged Frog:						
Yes	15.6	28.8	19.1	30.4	37.1	18.6
Suspected	4.4	3.8	0.0	4.3	0.0	0.0
No	80.0	67.3	80.9	65.2	62.9	81.4
Sample size	45	52	47	23	35	43
Cascades Frog:						
Yes	15.0	9.6	0.0	21.4	14.3	0.0
Suspected	0.0	0.0	0.0	0.0	0.0	0.0
No	85.0	90.4	100.0	78.6	85.7	100.0
Sample size	40	52	47	28	35	43
Columbia Spotted Frog:						
Yes	14.9	11.5	2.1	14.3	17.1	2.3
Suspected	4.1	1.9	0.0	4.1	2.9	0.0
No	81.1	61.5	97.9	81.6	42.9	97.7

A. Percentage of sites				B. Percentage of water bodies		
eDNA detected	2016: % of total	2017: % of total	2018: % of total	2016: % of total	2017: % of total	2018: % of total
Unlikely	0.0	25.0	0.0	0.0	37.1	0.0
Sample size	74	52	47	49	35	43
Oregon Spotted Frog:						
Yes	18.6	5.8	6.4	20.5	8.6	7.0
Suspected	8.5	1.9	0.0	10.3	2.9	0.0
No	71.2	92.3	93.6	66.7	88.6	93.0
Unlikely	1.7	0.0	0.0	2.6	0.0	0.0
Sample size	59	52	47	39	35	43

3.3.2 Oregon Spotted Frog

Oregon spotted frog eDNA was detected (biologist call "yes" or "suspected") in water samples from 23 sites, representing 12 water bodies (**Error! Reference source not found., Figure 7**). The sites included four known locations with recent records in the Lower Mainland within the Chilliwack and Harrison watersheds, two locations with historical records only in the Lower Fraser watershed, and five new locations (**Table 7, Table 8**).

Table 6. Summary of eDNA results for Oregon spotted frog from site water samples for sites (A) and water bodies (B), 2016 – 2018.

Numbers in cells refer to the number of sites or water bodies where eDNA of this species was detected (Yes) or Suspected, and where it was not detected (No) based on the Biologist call.

eDNA detected	Chilliwack	Harrison	Lower Fraser	Fraser Canyon	Simil-kameen	Skagit	Squamish	Lillooet & Seton Lake	Total
A. Sites									
Yes	3	10	2	0	0	2	0	0	17
Suspected	1	3	2	0	0	0	0	0	6
No	15	25	34	3	15	29	3	10	134
Unlikely	0	0	0	0	1	0	0	0	1
Total Sites:	19	38	38	3	16	31	3	10	158
B. Water bodies									
Yes	2	4	2	0	0	1	0	0	9
Suspected	0	2	1	0	0	0	0	0	3
No	12	8	11	1	8	14	3	10	67
Unlikely	0	0	0	0	1	0	0	0	1
Total Water Bodies	14	14	14	1	9	15	3	10	80

Of the six water bodies with recent records of Oregon spotted frog that were sampled for positive controls, eDNA of Oregon spotted frog was detected at four water bodies, but not in each year

(**Table 7**). Oregon spotted frog eDNA was also detected at two of five the historical locations sampled, but only in one year (**Table 7**). A spotted frog was seen at one of these sites (Nicomen Slough), verified by Kendra Morgan and Jared Hobbs, during sample collection on 6 July 2017.

Of the five new records, three were in the Lower Mainland and one from Pitt-Addington Marsh within the known range of the species. The remaining new record was from the Skagit Valley outside the previously known range of the species. The presence of Oregon spotted frog at this site (Skagit 3) was confirmed by a skin swab (see **Section 3.4**). Columbia spotted frog was also present at this site, based on swabbing results.

Table 7. Summary of water bodies by status (historical, recent, new) where Oregon Spotted eDNA was detected in water samples, 2016 - 2018.

Watershed group	Water body/location	Recent records	Historical records only	New site	Detected: # years	Years surveyed
Chilliwack	Semihault	yes	no	no	3	3
Harrison	Maria Slough Chaplin Rd	yes	no	no	0	3
Harrison	Maria Slough Dump	yes	no	no	0	3
Harrison	Maria-Kamp	yes	no	no	3	3
Harrison	Morris Valley	yes	no	no	2	3
Harrison	Mountain Slough CSC	yes	no	no	2	3
Harrison	Derby Reach 4	no	yes	no	1	3
Harrison	Nicomen Slough	no	yes	no	1	3
Lower Fraser	Aldergrove	no	yes	no	0	1
Lower Fraser	Campbell River Park	no	yes	no	0	1
Lower Fraser	West Creek Wetlands	no	yes	no	0	2
Chilliwack	Big Ditch	no	no	yes	1	1
Harrison	Maria Main Stem	no	no	yes	1	1
Lower Fraser	Dale Road	no	no	yes	1	1
Lower Fraser	Pitt-Addington	no	no	yes	1	3
Skagit	Skagit 3	no	no	yes	2	3

Figure 7. Map of eDNA survey results for Oregon spotted frog, 2016 - 2018. Concentrated DNA: from skin swabs and "frog froth" samples (i.e., frog in ~1 litre of water for 2-5 min)

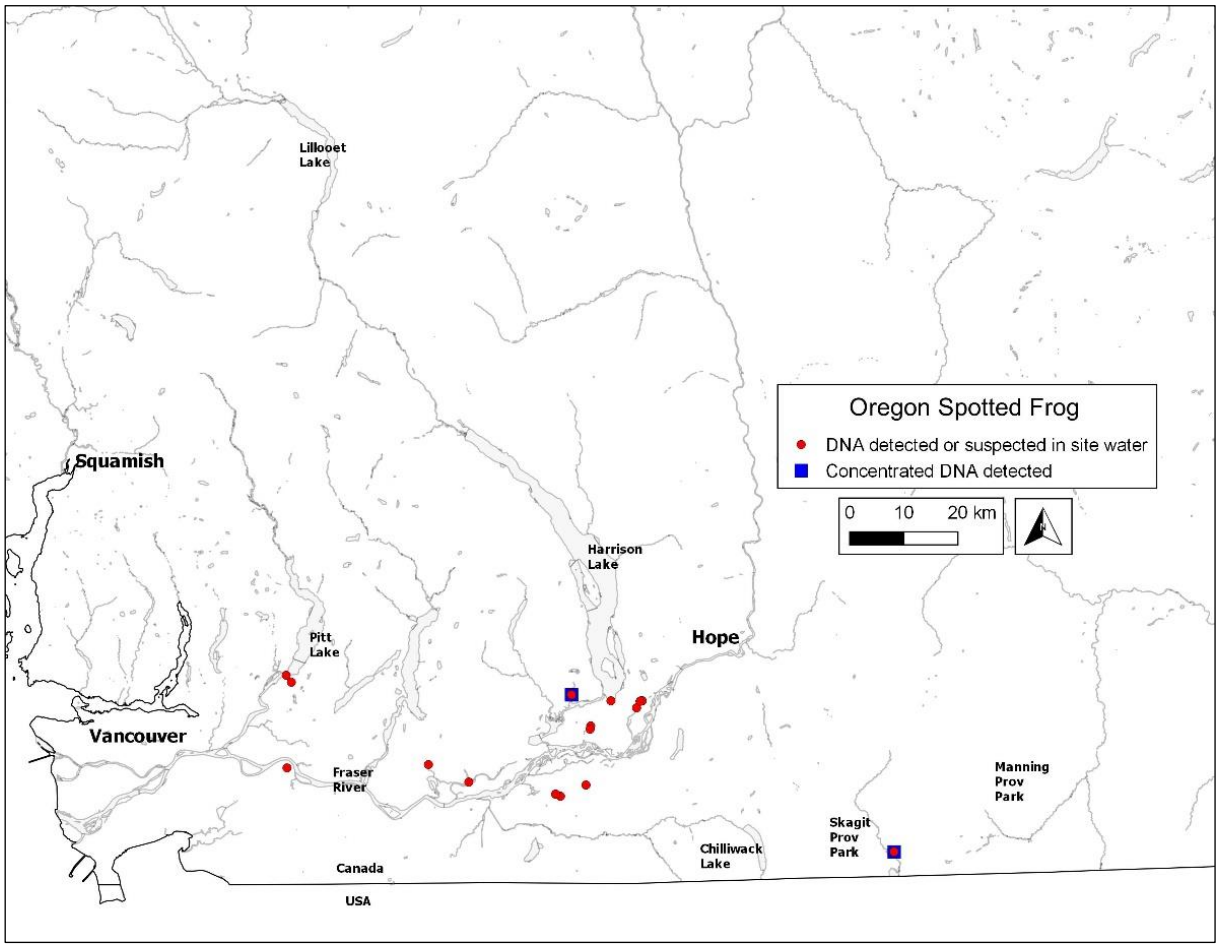


Table 8. Water bodies where eDNA of Oregon spotted was detected by survey year, 2016 - 2018.

eDNA detected (Biologist call)	Positive detections in 2016	Positive detections in 2017: NEW	Positive detections in 2017: REPEATS (from 2016)	Positive detections in 2018: NEW	Positive detections in 2018: REPEATS (from 2016 or 2017)
Yes	CHIL: Big Ditch HAR: Maria-Kamp*, Morris Valley*, Mountain Slough CSC*, Nicomen Slough^; LF: Derby Reach 4^, Pitt-Addington SK: Skagit 3	None	CHIL: Semihault* HAR: Morris Valley*, Mountain Slough CSC*	None	CHIL: Semihault* HAR: Maria-Kamp* SK: Skagit 3
Suspected	CHIL: Semihault* HAR: Maria Main Stem, Miami* LF: Dale Rd	None	HAR: Maria-Kamp*	None	None
Total	12	0	4	0	1

Watershed codes: CHIL-Chilliwack; HAR-Harrison; LF-Lower Fraser; SIM-Similkameen; SK-Skagit
*known site with recent records; ^site with historical records only

3.3.2 COLUMBIA SPOTTED FROG

Columbia spotted frog eDNA was detected (biologist call "yes" or "suspected") in water samples from 23 sites, representing 19 water bodies (**Table 9, Figure 8**). The majority of these were within in the Skagit Valley and Manning Provincial Park in the Skagit and Similkameen watersheds, where the species is known to occur (**Table 10**). One record was from Wolfe Lake in the Harrison watershed in the Lower Mainland and one in the northern portion of the study area in the Lillooet watershed. Swab samples confirmed the presence of the species at two additional sites, Gwyneth Pond, Lillooet, and Skagit 3, Skagit Valley (**Figure 8**; see **Section 3.4**), but eDNA results from the water samples were negative. In 2017, eDNA attributed to this species was detected from atypical habitats at several Lower Mainland sites (**Table 9**). The presence of Columbia spotted frog at these sites was deemed unlikely.

Table 9. Summary of eDNA results for Columbia spotted frog from site water samples for sites (A) and water bodies (B), 2016 - 2018.

Numbers in cells refer to the number of sites or water bodies where eDNA of this species was detected (Yes) or Suspected, and where it was not detected (No) based on the Biologist call.

eDNA detected	Chilliwack	Harrison	Lower Fraser	Fraser Canyon	Simil-kameen	Skagit	Squamish	Lillooet & Seton Lake	Total
A. Sites									
Yes	0	1	0	0	8	8	0	1	18
Suspected	0	0	0	0	2	3	0	0	5
No	17	30	33	3	19	22	3	9	136
Unlikely	2	6	5	0	0	0	0	0	13
Total Sites:	19	37	38	3	29	33	3	10	172
B. Water bodies									
Yes	0	1	0	0	7	6	0	1	15
Suspected	0	0	0	0	2	2	0	0	4
No	12	6	8	1	4	8	3	9	51
Unlikely	2	6	5	0	0	0	0	0	13
Total Water Bodies	14	13	13	1	13	16	3	10	83

Figure 8. Map of eDNA survey results for Columbia spotted frog, 2016 - 2018.

Concentrated DNA: from skin swabs and "frog froth" samples (i.e., frog in ~1 litre of water for 2-5 min)

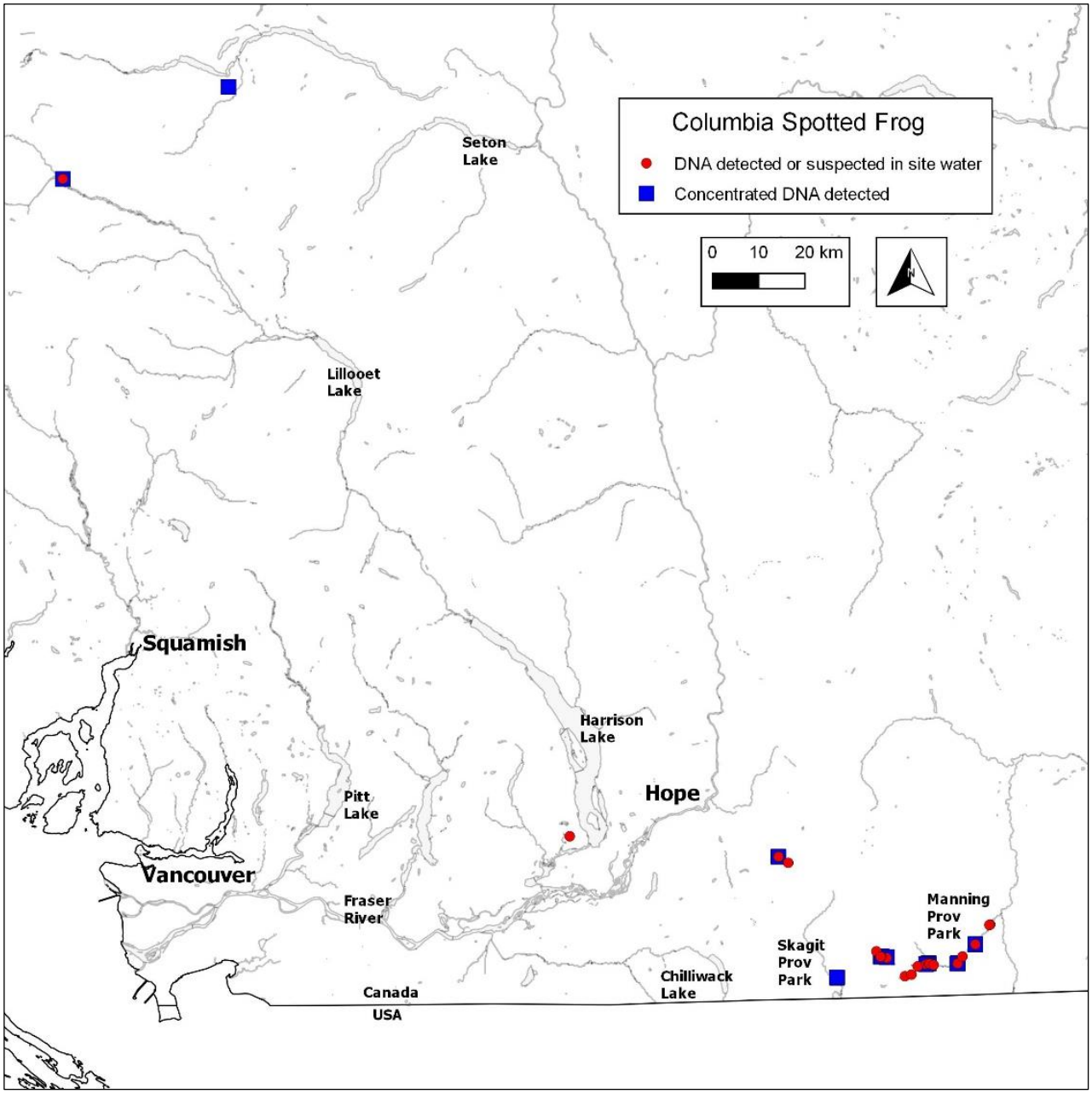


Table 10. Water bodies where eDNA of Columbia spotted frog was detected by survey year, 2016 - 2018.

eDNA detected (Biologist call)	Positive detections in 2016	Positive detections in 2017: NEW	Positive detections in 2017: REPEATS (from 2016)	Positive detections in 2018: NEW	Positive detections in 2018: REPEATS (from 2016 or 2017)
Yes	HAR: Wolfe L SK: Manning Park 2, 22, 3 (Poland L), 8.2, Sunshine Valley 5, 6	None	SIM: Manning Park 11, 13 SK: Manning Park 8.2, Sunshine Valley 5	LIL: Alena Cr	None
Suspected	SK: Manning Park 6, 8	SIM: Manning Park 104	None	None	None
Total	9	1	4	1	0

Watershed codes: CHIL-Chilliwack; HAR-Harrison; LF-Lower Fraser; SIM-Similkameen; SK-Skagit

3.3.4 Northern Red-legged Frog

Northern red-legged frog eDNA was detected (biologist call "yes" or "suspected") in water samples from 36 sites, representing 20 water bodies (**Table 11**, **Figure 9**). The positive sites were within Chilliwack, Harrison, and Lower Fraser watersheds in the Lower Mainland and to the north in the Squamish watershed (see **Table 12** for the locations**)**. The species was not detected in the Skagit or Similkameen watersheds or in the Lillooet/Seton Lake watersheds.

Table 11. Summary of eDNA results for Northern red-legged frog from site water samples for sites (A) and water bodies (B), 2016 - 2018.

Numbers in cells refer to the number of sites or water bodies where eDNA of this species was detected (Yes) or Suspected, and where it was not detected (No) based on the Biologist call.

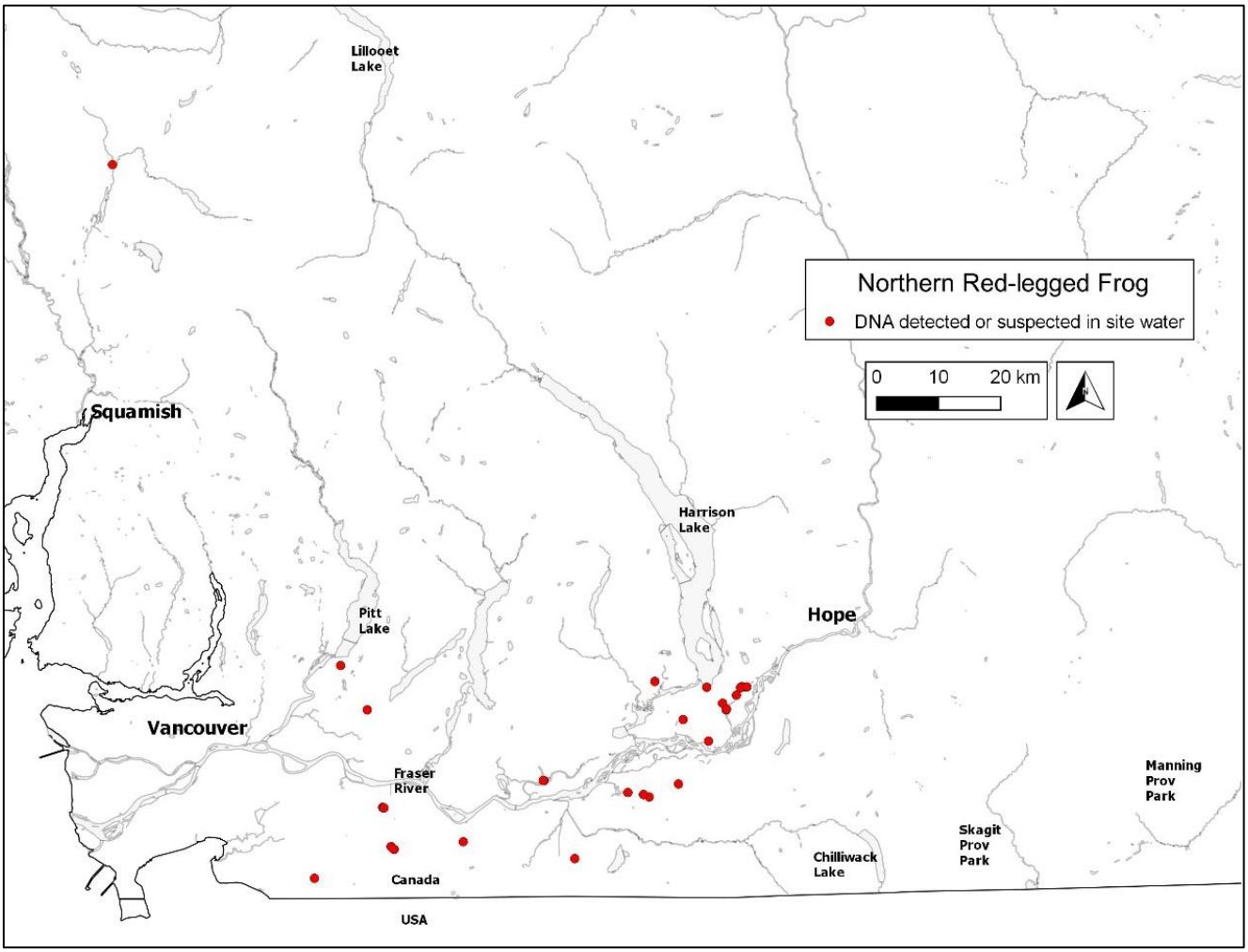
eDNA detected	Chilliwack	Harrison	Lower Fraser	Fraser Canyon	Simil-kameen	Skagit	Squamish	Lillooet & Seton Lake	Total	Washinton references
A. Sites										
Yes	6	16	9	0	0	0	1	0	32	0
Suspected	0	3	1	0	0	0	0	0	4	0
No	10	18	28	3	11	26	2	10	108	2
Total Sites:	16	37	38	3	11	26	3	10	144	
B. Water bodies										
Yes	4	10	4	0	0	0	1	0	19	0
Suspected	0	0	1	0	0	0	0	0	1	0
No	8	3	9	1	7	12	3	13	56	2
Total Water Bodies	12	13	14	1	7	12	3	13	76	2

Table 12. Water bodies where eDNA of Northern red-legged frog was detected by survey year, 2016 - 2018.

eDNA detected (Biologist call)	Positive detections in 2016	Positive detections in 2017: NEW	Positive detections in 2017: REPEATS (from 2016)	Positive detections in 2018: NEW	Positive detections in 2018: REPEATS (from 2016 or 2017)
Yes	CHIL: Semihault HAR: Cutler Rd, Maria Main Stem, Maria-Kamp, Morris Valley 3, Maria Slough, Chaplin Rd LF: Pitt-Addington	CHIL: Towne Cr HAR: Chaplin Rd UBC farm LF: Aldergrove, Campbell River Park, Mike L, West Creek Wetlands	CHIL: Semihault HAR: Maria Slough Chaplin Rd, Maria Slough Dump, Maria-Kamp, Miami, Mountain Slough CSC, Nicomen Slough	CHIL: Chilliwack Cr 2 LF: Glenmore Rd Ditch SQ: Callahan Pond 1	CHIL: Semihault HAR: Maria Slough Dump, Maria Slough Chaplin Rd, Maria-Kamp LF: West Creek Wetlands
Suspected	HAR: Nicomen Slough	~	~	~	~
Total	8	6	7	3	5

Watershed codes: CHIL-Chilliwack; HAR-Harrison; LF-Lower Fraser; SIM-Similkameen; SK-Skagit

Figure 9. Map of eDNA survey results for Northern red-legged frog, 2016 - 2018.



3.3.3 CASCADES FROG

Cascades frog eDNA was detected (biologist call "yes" or "suspected") in water samples from 11 sites, representing six water bodies in Manning Provincial Park and the Skagit Valley in the Similkameen and Skagit watersheds (**Table 13**, **Figure 10**). These locations were re-sampled in 2017 and 2018 (**Table 14**) following the detection of the species' eDNA there in 2016. One water body (Manning Park 3, Poland Lake) was not re-sampled due to logistical constraints. The results from 2017 were also positive for eDNA of this species (**Table 14**). However, visual encounter surveys conducted at all sites (except Poland Lake) in 2017 and 2018 failed to verify the presence of Cascades frog at any of these sites.

As a positive control, we sampled site water and concentrated DNA from two known Cascades frog sites in Washington State (with personal funding). Cascades frogs were found at one of the sites (Mt Rainier), and the species' eDNA was detected both in the water samples taken and in the frog froth sample.

Table 13. Summary of eDNA results for Cascades frog from site water samples for sites (A) and water bodies (B), 2016 - 2018.

Numbers in cells refer to the number of sites or water bodies where eDNA of this species was detected (Yes) or Suspected, and where it was not detected (No) based on the Biologist call.

eDNA detected	Chilliwack	Harrison	Lower Fraser	Fraser Canyon	Simil-kameen	Skagit	Squamish	Lillooet & Seton Lake	Total	Washington references
A. Sites										
Yes	0	0	0	~	6	5	0	0	11	1
Suspected	0	0	0	~	0	0	0	0	0	0
No	17	18	29	~	23	28	3	10	128	1
Total Sites:	17	18	29	0	29	33	3	10	139	2
B. Water bodies										
Yes	0	0	0	0	3	3	0	0	6	1
Suspected	0	0	0	0	0	0	0	0	0	0
No	13	8	11	0	9	10	3	10	64	1
Total Water Bodies	13	8	11	0	12	13	3	10	70	2

Figure 10. Map of eDNA survey results for Cascades frog, 2016 - 2018.

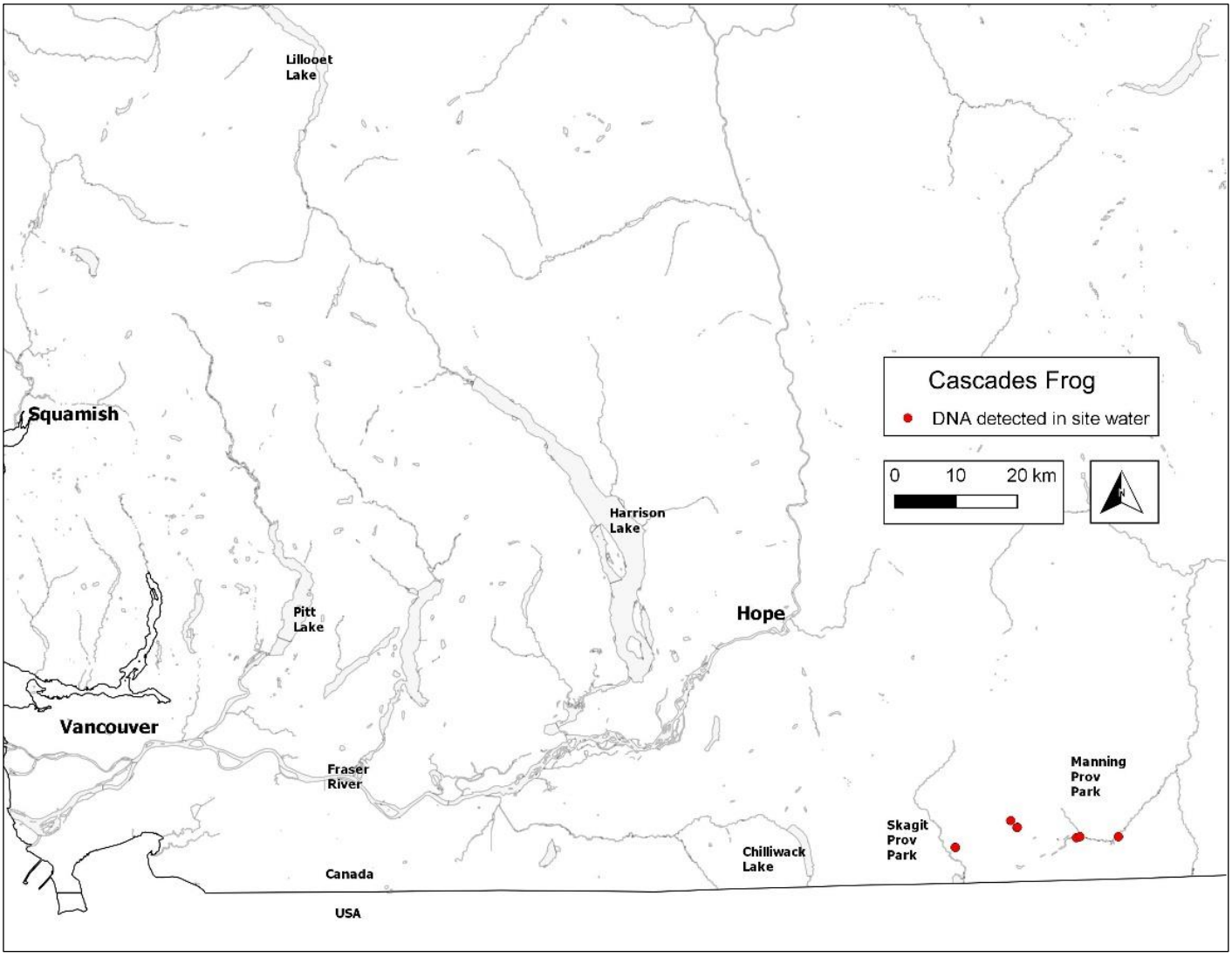


Table 14. Sampling of water bodies where Cascades frog eDNA was found, 2016 - 2018.

Watershed group	Water body	2016	2017	2018	# years sampled	# sites/water body sampled: 2016, 2017, 2018
Similkameen	Manning Park 101	Y	Y	N	3	2,1,1
Similkameen	Manning Park 11	Y	Y	N	3	1,1,1
Similkameen	Manning Park 13	Y	Y	N	3	1,1,1
Skagit	Manning Park 8	Y	Y	N	3	1,1,2
Skagit	Skagit14	Y	~	N	2	2,0,2
Skagit	Manning Park 3 (Poland L)	Y	~	~	1	1,0,0

3.4 Concentrated Frog DNA Samples

Concentrated DNA, consisting of skin swabs of frogs and eDNA from frog froth samples, were tested for all four species of *Rana* using the tests developed as part of this project. In 2017 – 2018, swabs were obtained from a total of 47 individuals, 13 of which were from captive breeding programs (**Table 15**). The swabs from the 36 wild-caught frogs were from nine field sites. The samples included swabs from two water bodies where Oregon spotted frog eDNA was detected in 2016 outside the known range (Skagit 3, Manning Park 6) and three water bodies where Cascades frog eDNA was detected in 2016 and 2017 (Manning Park 101, 11, 13). The swab samples from all wild-caught frogs tested positive for Columbia spotted frog, except one of four frogs from Skagit 3, which tested positive for Oregon spotted frog. The signal was strong (8/8 runs tested positive), and the same result was obtained when the sample was retested.

All samples of Oregon spotted frog from the captive breeding programs tested positive for that species, with the exception of one sample from Greater Vancouver Zoo that did not produce a positive result for any of the four species of *Rana* (**Table 15**). Another swab sample from the same facility showed only a weak signal; a strong signal is expected from swab samples.

Table 15. Species identification based on analysis of concentrated DND from skin swabs of frogs from field sites and from captive-breeding programs (blue shading).

Collection year	Watershed group	Location or source	# of frogs	Species ID from DNA*	Comments
2018	Seton Lake	Gwyneth Pond	2	~	Storage conditions suboptimal
2017	Similkameen	Manning Park 101	6	RALU	
2018	Similkameen	Manning Park 101	2	RALU	
2018	Similkameen	Manning Park 101	1	~	Weak signal maybe due to storage conditions
2017	Similkameen	Manning Park 104	3	RALU	
2018	Similkameen	Manning Park 11	6	RALU	
2017	Similkameen	Manning Park 13	2	RALU	
2018	Similkameen	Manning Park 13	1	RALU	
2017	Skagit	Manning Park 6	3	RALU	
2018	Skagit	Manning Park 8	3	RALU	
2017	Skagit	Manning Park 8.2	1	RALU	
2017	Skagit	Skagit 3	3	RALU	
2017	Skagit	Skagit 3	1	RAPR	Retested with same result
2018	Skagit	Skagit3	1	~	Weak signal; frog escaped & only bag was swabbed
2018	Harrison	Gr. Vancouver Zoo, Maria Slough	1	RAAU	Identified as RAAU and included for comparisons
2018	Harrison	Gr. Vancouver Zoo, Maria Slough	1	~	Possibly a poor swab or preservation
2019	Harrison	Gr. Vancouver Zoo, Maria Slough	1	RAPR	Weak signal; possibly a poor swab or preservation
2018	Harrison	Vancouver Aquarium, Maria Slough	2	RAPR	

Collection year	Watershed group	Location or source	# of frogs	Species ID from DNA*	Comments
2018	Harrison	Vancouver Aquarium, Mountain Slough	2	RAPR	
2018	Chilliwack	Vancouver Aquarium, Chilliwack (Elk)	2	RAPR	
2018	Harrison	Vancouver Aquarium, Morris Valley	2	RAPR	
2018	Harrison	Gr. Vancouver Zoo, Morris Valley	2	RAPR	

*RAAU-Northern red-legged Frog; RALU-Columbia spotted frog; RAPR-Oregon spotted frog

In total, 16 one litre water samples containing concentrated frog eDNA (frog froth) were collected for genetic testing purposes (**Table 16**). Oregon spotted frog eDNA was detected from both samples from a known site in the Lower Mainland (Morris Valley).

Two samples were from Washington State from known localities for Cascades frog. Cascades frog eDNA was detected in one of the samples, while Northern red-legged frog eDNA was detected in the other sample. These results concurred with the field identification of the frogs sampled. All remaining samples tested positive for Columbia spotted frog (**Table 16**).

Table 16. Species identification based on analysis of concentrated DND ("frog froth") from frogs kept in ca. 1 l of water for 2 - 5 min in the field.

Collection year	Watershed group	Water body/Location	Species ID from eDNA	Comments
2016	Harrison	Morris Valley	RAPR	
2016	Harrison	Morris Valley	RAPR	
2018	Lillooet	Alena Creek	~	Sample failed IntegritE-DNA test
2018	Lillooet	Alena Creek	RALU	
2018	Seton Lake	Gwyneth Pond	RALU	
2018	Seton Lake	Gwyneth Pond	RALU	
2017	Similkameen	Manning Park 101	RALU	
2017	Similkameen	Manning Park 101	RALU	
2017	Similkameen	Manning Park 104	RALU	
2017	Skagit	Manning Park 6	RALU	
2017	Skagit	Manning Park 6	RALU	
2016	Skagit	Skagit 3	RALU	
2017	Skagit	Skagit 3	~	Sample failed IntegritE-DAN test; skin swab from same frog was positive for RAPR
2017	Skagit	Sunshine Valley 5	RALU	

Collection year	Watershed group	Water body/Location	Species ID from eDNA	Comments
2016	Washington, Mt Baker	Nooksack R, North Fork	RAAU	Reference site with known RACA; field identification of frog was RAAU
2017	Washington, Mt Rainier	Mt Rainier, Frog Heaven	RACA	Reference site with known RACA

*RAAU-Northern red-legged Frog; RACA-Cascades frog; RALU-Columbia spotted frog; RAPR-Oregon spotted frog

3.5 eDNA Detection Effectiveness and Repeatability Across Years

During the course of this study, 25 water bodies were surveyed repeatedly in different years, nine in two years and 19 in three years. These water bodies were distributed among watersheds as follows: Chilliwack (n=2), Harrison (n=7), Lower Fraser (n=4), Similkameen (n=6), and Skagit (n=6). A congruence index was calculated for each of the four target species, based on whether a species' eDNA was detected in water samples from the same locations repeatedly in different years: score of 0 indicates a different result each year, 0.5 indicates that the result was the same in 2 of 3 years, and 1 indicates that the result was the same in each year. Note that many factors can affect congruence across years, including environmental conditions during sampling, as well as distribution and presence of the species, and the index does not directly address eDNA effectiveness.

The congruence index was relatively high (mean >0.8) for Oregon spotted frog, Cascades frog, and Northern red-legged frog, but lower for Columbia spotted frog (**Table 17;** see **Appendix 4** for details). Comparison of eDNA detection success for sites where Columbia spotted frogs were seen during sample collection also suggests that the effectiveness of eDNA sampling with the test used was lower for this species than for the other three target species under field conditions. Columbia spotted frog eDNA was detected only 37% of the times when the species was visually observed at the sampling locations (**Table 18;** see **Appendix 5** for details). The reasons for this are discussed in **Section 4.5**.

Table 17. Comparison of congruence of eDNA detection for water bodies sampled in two or three years, 2016 – 2018.

Congruence index	Northern red-legged frog	Cascades frog	Columbia spotted frog	Oregon spotted frog
Mean	0.88	0.88	0.54	0.83
SD	0.27	0.26	0.38	0.29
Median	1	1	0.5	1
n	21	25	25	23

Table 18. Detection of Columbia spotted frog eDNA from water collected from sites where spotted frogs were seen during sample collection.

eDNA detected*	2016	2017	2018	Total	% of total
Yes or Suspected	5	5	1	11	36.7
No	8	4	7	19	63.3
Total	13	9	8	30	100.0

*Biologist call for site

4.0 Discussion

This study applied eDNA sampling methods and investigated its effectiveness for a each of four species of congeneric, pond-breeding frogs (genus *Rana*) with varying degrees of sympatry based on three years of surveys. The main findings of the project for the target species are highlighted in the sections below.

4.1 Oregon Spotted Frog

- Oregon spotted frog eDNA was detected at an extralimital site in the Skagit Valley (Skagit 3), and the presence of the species there was confirmed by analysis of a concentrated DNA sample obtained by swabbing the skin of a field-caught frog.
- eDNA of this species was also detected at previously unreported locations in the Harrison and Lower Fraser watersheds in the Lower Mainland. Four were in the vicinity of known sites, while one was farther to the west in a large wetland complex at Pitt-Addington marsh. These results can be used to guide physical searches for this species as part of recovery efforts.
- Oregon spotted frog eDNA was detected at two water bodies sampled from where the species was known only from historical records (Nicomen Slough, Derby Reach 4); a spotted frog was observed at Nicomen Slough during sample collection in July 2017, supporting the validity of the positive eDNA result at that site.

In Canada, Oregon spotted frog was believed to be confined to the lowlands of the Lower Fraser Valley (<200 m asl; COSEWIC 2011). Both eDNA and genetic test results from swabbing collected during this study suggest that habitat use by this species in BC may be broader than previously documented. The elevation of the Skagit site is ~500 m, but the habitat consists of large marshland complex/sedge meadow, similar to Oregon spotted frog habitats in the Lower Fraser Valley. Both Oregon spotted frog and Columbia spotted frog occurred at this site, and there may be genetic introgression between the two species. The differences between the two species are subtle, and they can only be physically distinguished by consideration of a large number of morphometric measurements (Green *et al*. 1996). As such, this suggestion warrants further study. Genetic samples from individuals used to substantiate the original division of Oregon spotted frog from Columbia spotted frog in BC (by Green *et al*. 1997) are no longer available for genetic testing.

The known distribution of Oregon spotted frog occurs within low elevation lentic sites in Washington State (Bohannon *et al*. 2016), but sites up to 850 m have been described as potentially suitable for this species there (Germaine and Cosentino 2004). In Oregon, the species has been reported from elevations of 1,232–1,340 m (Parsnip Lakes: Parker 2009).

4.2 Columbia Spotted Frog

- eDNA and swab sample analyses helped clarify potential contact zones between Columbia and Oregon spotted frogs. In the east, analysis of swab samples indicated that both species of spotted frogs were present in the same wetland in the Skagit Valley (Skagit 3).
- In the west, Columbia spotted frog's eDNA was detected in one wetland (Wolfe Lake), 2.5 km northwest of a known Oregon spotted frog site in the Lower Mainland (Morris Valley). However, spurious results from other wetlands in the Lower Mainland in 2017 suggest caution in accepting this result until further corroborative evidence is obtaining through physical surveys and genetic data from swabs.
- eDNA sampling was less effective for Columbia spotted frog than for the other three target species as indicated by the congruence index for this species. In addition to producing apparently false positive detections as noted above, it also produced false negatives for sites where the species was observed during sample collection in the Skagit and Similkameen watersheds (see **Section 4.5** for further discussion of the limitations of the eDNA test for Columbia spotted frog).

4.3 Northern Red-Legged Frog

- eDNA of Northern red-legged frog was detected throughout the species' known distribution in the Lower Mainland and at one site north of Squamish, also within the known range.
- The species' eDNA was not detected, nor was the species found during physical searches, east of the Lower Mainland in the Skagit Valley or Manning Provincial Park, corroborating previous information on the species' distribution (COSEWIC 2015).

4.4 Cascades Frog

- eDNA of Cascades frog was detected at six locations including the Skagit Valley (1 location) and Manning Provincial Park (5 locations) in the Skagit and Similkameen watersheds.

The above findings are of particular interest as Cascades frog has not been recorded previously within Canada. Physical searches for this species at five of these localities in 2017 and 2018, totalling 15.5 person-hours, failed to visually locate the species. Without physical evidence, the presence of Cascades frog in British Columbia should be treated as unconfirmed, although it is strongly suspected based on eDNA results from this project. South of the USA - Canada border, the nearest confirmed record of Cascades frog is approximately 13 km south in Washington State. Occurrence in BC seems plausible.

4.5 Effectiveness of eDNA Methods for the Target Species

While the efficacy of the method as a conservation tool has been demonstrated for a number of species, including tailed frogs in stream habitats British Columbia (Hobbs *et al.* 2019), variability in efficacy can be expected among species and habitats. eDNA sampling poses particular challenges for frogs in lentic habitats, including the often eutrophic, turbid pools with little or no water flow inhabited by pond-breeding frogs. A further challenge for this project was to develop primers and probes that effectively discriminated among the closely related species, particularly between the sister species of Oregon and Columbia spotted frogs. The tests developed for the four target species were rigorously validated under laboratory conditions for both specificity and sensitivity by our collaborators in Dr. Helbing's lab in the University of Victoria, and they performed well. However, field conditions pose additional challenges. False negative results can result from low levels of eDNA at the sample location at the time of sample collection, including:

- The target taxa may use the site only seasonally outside the sampling period or only in some years.
- The target taxa may occur at a low abundance that is below the detection capabilities of qPCR methods.
- eDNA from the target taxa is distributed discontinuously even when species is present (Eichmiller *et al.* 2014), and the sample may have been collected from an unsuitable microsite within occupied habitat.
- Environmental factors, including water temperature and UVB exposure, are known to influence eDNA detection (Díaz-Ferguson and Moyer 2014). High water levels may also dilute the eDNA.

False positive eDNA detections may result from overlap of the DNA segment in the primers and probe with other taxa that were not anticipated or tested during the test development. The source of the genetic material used in test development is also important, especially for wide ranging species that may exhibit genetic variability across their geographic distribution within the DNA sequences used for test development.

Considering the above challenges, the congruence of eDNA results for the repeatedly sampled sites across the study years was remarkably high for three of the four focal taxa including: Oregon spotted frog, Northern red-legged frog, and Cascades frog. For Oregon spotted frog, six locations with recent records were included each year as positive controls. Positive results were obtained for two sites in three years (Maria-Kamp, Semihault), two sites in two years (Morris Valley, Mountain Slough CSC), but never for two sites (Maria Slough Dump, Maria Slough, Chaplin Road). False negative results may be best attributed to low levels of target taxa eDNA in the samples at the time of sample collection; water levels at most sites were relatively high in 2018. The analysis of concentrated DND (frog froth samples) obtained from wild-caught frogs (Morris Valley) and swabs from frogs from the captive breeding program tested positive for Oregon Spotted Frog, corroborating lab results of the effectiveness of the species-specific eDNA test.

For Cascades frog, five of six sites that tested positive for eDNA of this species in 2016 were resampled in the two following years. Positive results were obtained for these sites in two of three years. Site water samples and concentrated frog eDNA obtained from an occupied

Washington site tested positive for this species, providing support for the effectiveness of the test used to detect Cascades frog in this study.

For Northern red-legged frog, eDNA methods used in the study appeared to perform well, although positive controls were not actively included, and no concentrated DND samples were collected. The sites with positive detections were within the expected habitats and geographic range of the species, and no spurious or unexpected results were obtained.

For Columbia spotted frog, the eDNA method performed less well than for the other target species, and both false negative and false positive results were evident. The congruence of results among years was low when compared to the other species. In particular, there were false positive, extralimital detections in the Lower Mainland in 2017 and false negative detections in the Skagit and Similkameen watersheds in 2018. The differences among years are not readily explainable and remain enigmatic. Direct observations of Columbia spotted frog at the time of water sample collection indicated that eDNA analysis resulted in positive detections only 37% of the times when the species was known to be present. The potential reasons for false negatives include poor dispersion of eDNA in the water bodies and/or poor performance of the test at the low DNA concentrations encountered in the field. No issues with the test were noted in analysis of concentrated DNA samples (from swabs or frog froth) from the study sites. The source of genetic material from test development for Columbia spotted frog was from specimens sampled from the Kootenay region of BC. Incorporating genetic source material from specimens across a broader geographic area can further define the effectiveness of the test and its wider applicability.

We considered two possibilities to explain the reason for false positive results from the Columbia spotted frog eDNA tests. The first possibility could be the overlap of DNA sequences with Oregon spotted frog. However, this possibility is unlikely since the Columbia spotted frog test was rigorously validated in the lab and field and found not to detect Oregon spotted frog (see **Figure 2** and **Appendix 1**). Other notable sympatric species include the introduced green frog (*Lithobates clamitans*) that is ubiquitous in the Lower Mainland. The Columbia spotted frog eDNA test does not detect green frog DNA (**Appendix 1**).

The second possibility to explain false positive results from the Columbia spotted frog eDNA tests is that DNA sequences from another species might confound the results. Genetically, frogs and salamanders are generally divergent. However, it is possible that the limited DNA sequence available for test design has higher sequence identity than anticipated. When the primers and probe were designed, there was no available DNA sequence information available for relevant salamanders in the area.

In Year 2 of this project, the performance for Columbia spotted frog eDNA test was further scrutinized in light of the enigmatic results obtained that year. The investigations revealed that the Columbia spotted frog test can produce a positive result with very high concentrations of Northwestern salamander (*Ambystoma gracile*) DNA, and to a lesser extent, Ensatina salamander (*Ensatina eschscholtzii*) DNA (**Appendix 1**). Ensatina is a completely terrestrial salamander, and under field conditions it is extremely unlikely that its presence would generate a false positive result. However, the presence of Northwestern salamander in high abundance may have

confounded qPCR results when testing for Columbia spotted frog using the test designed for this study. The distribution of Northwestern salamander includes the Lower Mainland, and the species is known to occur at some of the localities where enigmatic test result were documented for Columbia spotted frog. At this time, sites containing aquatic habitats that support Northwestern salamander should be interpreted with caution when considering positive qPCR test results for Columbia spotted frog eDNA. Other potentially confounding species are Long-toed salamander (*Ambystoma macrodactylum*) and Rough-skinned newt (*Taricha granulosa*). These possibilities are currently being assessed.

The best solution for the Columbia spotted frog eDNA test, as well as for the development of a single eDNA test for Oregon spotted frog, is to secure more extensive mitochondrial DNA sequences from amphibia in the region. This would enable the informed identification of distinguishing DNA sequence regions from which robust eDNA tests can be designed.

4.6 Conservation Implications

Accurate knowledge of species' distributions is essential for effective conservation, and eDNA methods developed for the four target species of *Rana* as part of this project provide an additional tool to obtain such information. For Oregon spotted frog, this study resulted in the documentation of a new extralimital site in the Skagit Valley and identified of eDNA of Oregon spotted frog at historical and new sites in the Lower Mainland that merit further targeted searches for the species. The study also raises the possibility of introgression among Oregon spotted frog and Columbia spotted frog where the distributions of the two species meet, an issue with implications for sources for captive breeding programs and that merits further research.

Although Northern red-legged frog appears to occur relatively commonly within its Canadian range, it is listed federally as Special Concern in consideration of continued threats from multiple anthropogenic sources. It is important to continue monitoring the species' persistence and population health, especially within developed landscapes. eDNA methods provide an effective tool for this recovery objective.

The eDNA results obtained during this study suggest that the Cascades frog occurs in BC, although its presence could not be corroborated through visual searches. The results presented here help target further survey efforts to document physical evidence of the species' presence. Cascades frog is listed globally as "near threatened" by IUCN, and the documentation of its occurrence in BC would be of conservation significance both globally and in Canada.

5.0 Literature Cited

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6.0 Appendices

Appendix 1. Fact sheets for eDNA tests used in this study as provided by Dr. Helbing's lab, University of Victoria, including a new fact sheet for the Columbia spotted frog test.

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity.

General eDNA Assay Information

Target Species	:	Columbia Spotted Frog (*Rana luteiventris*)
Species Abbreviation	:	RALU
eDNA qPCR Tool	:	eRALU2
eDNA qPCR Format	:	TaqMan

eDNA Assay Specificity Tests

Multiple qPCR reactions (n=25) performed per target DNA. Detection within the standardized eDNA qPCR assay = Yes

ASMO	ANBO-VI	LICA	PSRE	RAAU	RAPR	RACA	RALU	LICL	AMGR	ENES	TAGR	HOSA	NTC
No	No	No	No	No	No	No	Yes	No	No*	No**	No	No	No

eDNA Assay Sensitivity Test

RALU

DNA (µg/L)	Detection Frequency (n=25)	Binomial Standard error (n=8)
5	100%	0%
1	92%	10%
0.2	36%	17%
0.04	12%	11%
0.008	0%	0%
0	0%	0%

AMGR

DNA (µg/L)	Detection Frequency (n=23)	Binomial Standard error (n=8)
5	36%	17%
1	4%	7%
0.2	9%	10%
0.04	4%	7%
0.008	0%	0%
0	0%	0%

ENES

DNA (µg/L)	Detection Frequency (n=23)	Binomial Standard error (n=8)
5	16%	13%
1	4%	7%
0.2	0%	0%
0.04	0%	0%
0.008	4%	7%
0	0%	0%

Appendix: Abbreviations

Rocky Mountain Tailed Frog (*Ascaphus montanus*)	ASMO	
Western Toad (*Anaxyrus (Bufo) boreas*)	ANBO-VI	Sourced from Vancouver Island (VI)
Bullfrog (*Lithobates (Rana) catesbeiana*)	LICA	
Pacific Chorus Frog (*Pseudacris (Hyla) regilla*)	PSRE	
Northern Red-legged Frog (*Rana aurora*)	RAAU	
Oregon Spotted Frog (*Rana pretiosa*)	RAPR	
Cascades Frog (*Rana cascadae*)	RACA	
Columbia Spotted Frog (*Rana luteiventris*)	RALU	
Rough-skinned Newt (*Taricha granulosa*)	TAGR	
Green Frog (*Lithobates(Rana) clamitans*)	LICL	
Northwestern Salamander(*Ambystoma gracile*)	AMGR	
Ensatina (*Ensatina eschscholtzii*)	ENES	
Human (*Homo sapiens*)	HOSA	
qPCR no template control	NTC	
quantitative real-time polymerase chain reaction	qPCR	
environmental DNA	eDNA	

Comments

*This tool weakly detects Northwestern Salamander (AMGR) at high DNA concentrations. It is possible that sites with very high AMGR abundance may produce false positive results.

**The tool weakly detects Ensatina (ENES). Under field conditions, false positive detection is highly unlikely.

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity.

General eDNA Assay Information

Target Species	:	Oregon Spotted Frog (*Rana pretiosa*)
Species Abbreviation	:	RAPR
eDNA qPCR Tool	:	eRAPR2
eDNA qPCR Format	:	TaqMan

eDNA Assay Specificity Tests

A. qPCR Activity : Multi-species analysis of eDNA tool efficacy

Multiple qPCR reactions (n=25) performed per target DNA. Detection within the standardized eDNA qPCR assay = Yes

ASMO	ANBO-VI	LICA	PSRE	RAAU	RAPR	RACA	RALU	TAGR	HOSA	NTC
No	No	No	No	No	Yes	No	Yes	No	No	No

B. Confirmation of gene-specificity in eDNA assay : Confirmed

eDNA Assay Sensitivity Test

DNA (ug/L)	Detection Frequency (n=25)	Binomial Standard error (n=8)
5	1	0.00
1	1	0.07
0.2	0.76	0.18
0.04	0.44	0.13
0.008	0.12	0.10
0	0	0.00

Appendix: Abbreviations

Rocky Mountain Tailed Frog (*Ascaphus montanus*)	ASMO	
Western Toad (*Anaxyrus (Bufo) boreas*)	ANBO-VI	Sourced from Vancouver Island (VI)
Bullfrog (*Lithobates (Rana) catesbeiana*)	LICA	
Pacific Chorus Frog (*Pseudacris (Hyla) regilla*)	PSRE	
Northern Red-legged Frog (*Rana aurora*)	RAAU	
Oregon Spotted Frog (*Rana pretiosa*)	RAPR	
Cascades Frog (*Rana cascadae*)	RACA	
Columbia Spotted Frog (*Rana luteiventris*)	RALU	
Rough-skinned Newt (*Taricha granulosa*)	TAGR	
Human (*Homo sapiens*)	HOSA	
qPCR no template control	NTC	
quantitative real-time polymerase chain reaction	qPCR	
environmental DNA	eDNA	

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity.

General eDNA Assay Information

Target Species	:	Northern Red-legged Frog (*Rana aurora*)
Species Abbreviation	:	RAAU
eDNA qPCR Tool	:	eRAAU1
eDNA qPCR Format	:	TaqMan

eDNA Assay Specificity Tests

A. qPCR Activity : Multi-species analysis of eDNA tool efficacy

Multiple qPCR reactions (n=25) performed per target DNA. Detection within the standardized eDNA qPCR assay = Yes

ASMO	ANBO-VI	LICA	PSRE	RAAU	RAPR	RACA	RALU	TAGR	HOSA	NTC
No	No	No	No	Yes	No	No	No	No	No	No

B. Confirmation of gene-specificity in eDNA assay : Confirmed

eDNA Assay Sensitivity Test

DNA (ug/L)	Detection Frequency (n=25)	Binomial Standard error (n=8)
5	1	0.00
1	0.88	0.11
0.2	0.4	0.17
0.04	0.2	0.14
0.008	0.28	0.16
0	0	0.00

Appendix: Abbreviations

Rocky Mountain Tailed Frog (*Ascaphus montanus*)	ASMO	
Western Toad (*Anaxyrus (Bufo) boreas*)	ANBO-VI	Sourced from Vancouver Island (VI)
Bullfrog (*Lithobates (Rana) catesbeiana*)	LICA	
Pacific Chorus Frog (*Pseudacris (Hyla) regilla*)	PSRE	
Northern Red-legged Frog (*Rana aurora*)	RAAU	
Oregon Spotted Frog (*Rana pretiosa*)	RAPR	
Cascades Frog (*Rana cascadae*)	RACA	
Columbia Spotted Frog (*Rana luteiventris*)	RALU	
Rough-skinned Newt (*Taricha granulosa*)	TAGR	
Human (*Homo sapiens*)	HOSA	
qPCR no template control	NTC	
quantitative real-time polymerase chain reaction	qPCR	
environmental DNA	eDNA	

Helbing Laboratory eDNA Technical Bulletin

All eDNA tools are validated through a rigorous multi-step evaluation protocol that includes tests of DNA target specificity and amplification sensitivity.

General eDNA Assay Information

Target Species	:	Cascades Frog (*Rana cascadae*)
Species Abbreviation	:	RACA
eDNA qPCR Tool	:	eRACA2
eDNA qPCR Format	:	TaqMan

eDNA Assay Specificity Tests

A. qPCR Activity : Multi-species analysis of eDNA tool efficacy

Multiple qPCR reactions (n=25) performed per target DNA. Detection within the standardized eDNA qPCR assay = Yes										
ASMO	ANBO-VI	LICA	PSRE	RAAU	RAPR	RACA	RALU	TAGR	HOSA	NTC
No	No	No	No	No	No	Yes	No	No	No	No

B. Confirmation of gene-specificity in eDNA assay : Confirmed

eDNA Assay Sensitivity Test

DNA (ug/L)	Detection Frequency (n=25)	Binomial Standard error (n=8)
5	0.96	0.07
1	1	0.00
0.2	0.76	0.15
0.04	0.36	0.17
0.008	0.08	0.10
0	0	0.00

Appendix: Abbreviations

Rocky Mountain Tailed Frog (*Ascaphus montanus*)	ASMO	
Western Toad (*Anaxyrus (Bufo) boreas*)	ANBO-VI	Sourced from Vancouver Island (VI)
Bullfrog (*Lithobates (Rana) catesbeiana*)	LICA	
Pacific Chorus Frog (*Pseudacris (Hyla) regilla*)	PSRE	
Northern Red-legged Frog (*Rana aurora*)	RAAU	
Oregon Spotted Frog (*Rana pretiosa*)	RAPR	
Cascades Frog (*Rana cascadae*)	RACA	
Columbia Spotted Frog (*Rana luteiventris*)	RALU	
Rough-skinned Newt (*Taricha granulosa*)	TAGR	
Human (*Homo sapiens*)	HOSA	
qPCR no template control	NTC	
quantitative real-time polymerase chain reaction	qPCR	
environmental DNA	eDNA	

Appendix 2. Summary of localities and eDNA analysis results as interpreted by biologists for sites surveyed in 2016 – 2018.
Y-detected; S-suspected; N-not detected; ~not tested; U-unlikely; red text-all 3 replicate samples failed the IntegritE-DNA test; RACA-*Rana cascadae*; RAAU-*Rana aurora*; RAPR-*Rana pretiosa*; RALU-*Rana luteiventris*

YEAR	New/ Repeat	Watershed group	Location name	Site ID	Collection date	Zone	Easting	Northing	RAAU	RACA	RALU	RAPR
2018	N	Chilliwack	Bert-Brink WMA	BERT	19-Jun-18	10	567153	5442929	N	N	N	N
2016	N	Chilliwack	Big Ditch	BD1	14-May-16	10	583699	5446077	Y	~	N	Y
2017	N	Chilliwack	Camp Slough1	CSL1	05-Jul-17	10	580892	5450115	N	N	N	N
2017	N	Chilliwack	Camp Slough2	CSL2	05-Jul-17	10	579969	5449777	N	N	U	N
2018	R	Chilliwack	Camp Slough2	CLS2_18	20-Jun-18	10	579985	5449759	N	N	N	N
2018	N	Chilliwack	Chilliwack Cr 2	CHC2	20-Jun-18	10	575552	5444712	Y	N	N	N
2016	N	Chilliwack	Chilliwack Purple	CHILP1	17-May-16	10	616697	5430154	~	N	N	N
2016	N	Chilliwack	Chilliwack Purple	CHIlP2	17-May-16	10	616747	5430154	~	N	N	N
2016	N	Chilliwack	Chilliwack Yellow	CHLY	17-May-16	10	616193	5430344	~	N	N	N
2018	N	Chilliwack	Hope Slough-McLeod Rd	HSMC	20-Jun-18	10	583782	5447890	N	N	N	N
2018	N	Chilliwack	Hope Slough-Yale Rd E	HSYA	20-Jun-18	10	585512	5447307	N	N	N	N
2017	N	Chilliwack	Parr Rd Slough	PARR1	06-Jul-17	10	574923	5444920	N	N	N	N
2017	N	Chilliwack	Parr Rd Slough	PARR2	06-Jul-17	10	574581	5445169	N	N	N	N
2017	N	Chilliwack	Saar Ditch	SD	06-Jul-17	10	558800	5431296	N	N	N	N
2016	N	Chilliwack	Semihault	SEM1	14-May-16	10	578082	5444389	Y	~	N	S
2017	R	Chilliwack	Semihault	SSD	06-Jul-17	10	578984	5444001	Y	N	N	Y
2018	R	Chilliwack	Semihault	SEM_18	20-Jun-18	10	578084	5444383	Y	N	N	Y
2017	N	Chilliwack	Smith Falls	SMF	06-Jul-17	10	576328	5434916	N	N	U	N
2017	N	Chilliwack	Towne Creek	TWN	06-Jul-17	10	566989	5434112	Y	N	N	N
2016	N	Fraser Canyon	Kawkawack	KW1	16-May-16	10	615077	5471514	N	~	N	N
2016	N	Fraser Canyon	Kawkawack	KW2	16-May-16	10	614925	5471501	N	~	N	N
2016	N	Fraser Canyon	Kawkawack	KW3	16-May-16	10	614952	5471506	N	~	N	N
2017	N	Harrison	Chaplin RD UBC farm	CUB	05-Jul-17	10	590812	5459017	Y	N	U	N
2016	N	Harrison	Cheam Wetlands	CW1	17-May-16	10	591348	5450030	N	~	N	N
2016	N	Harrison	Cheam Wetlands	CW2	17-May-16	10	591115	5450072	N	~	N	N
2016	N	Harrison	Cheam Wetlands	CW3	17-May-16	10	591102	5449955	~	~	~	~
2016	N	Harrison	Cheam Wetlands	CW4	17-May-16	10	590887	5449942	N	~	N	N
2016	N	Harrison	Cutler Road	CUT1	15-May-16	10	588542	5452953	Y	~	N	N
2016	N	Harrison	Erock	ER1	15-May-16	10	572740	5453877	~	~	~	~
2016	N	Harrison	Maria Main Stem	MMS1	14-May-16	10	593047	5460313	Y	~	N	S
2018	R	Harrison	Maria Slough Dump	DMP	20-Jun-18	10	594725	5461644	Y	N	N	N
2017	R	Harrison	Maria Slough, Chaplin Rd	CHP	05-Jul-17	10	591375	5457964	Y	N	N	N
2018	R	Harrison	Maria Slough, Chaplin Rd	CHP	20-Jun-18	10	591398	5457977	Y	N	N	N
2016	N	Harrison	Maria-Kamp	MK1	14-May-16	10	593967	5461665	S	~	N	Y
2016	N	Harrison	Maria-Kamp	MK2	14-May-16	10	594080	5461624	N	~	N	Y
2016	N	Harrison	Maria-Kamp	MK3	14-May-16	10	593716	5461541	Y	~	N	Y
2017	R	Harrison	Maria-Kamp	MK1	05-Jul-17	10	593935	5461673	Y	N	N	N
2017	R	Harrison	Maria-Kamp	MK2	05-Jul-17	10	594060	5461627	S	N	U	S
2018	R	Harrison	Maria-Kamp	KMP	20-Jun-18	10	594022	5461629	Y	N	N	N
2018	R	Harrison	Maria-Kamp	KW	20-Jun-18	10	593740	5461534	Y	N	N	Y
2016	N	Harrison	Miami	MIA1	15-May-16	10	588313	5461615	N	~	N	S
2017	R	Harrison	Miami	MR	05-Jul-17	10	588261	5461572	Y	N	U	N
2018	R	Harrison	Miami	MIA	20-Jun-18	10	588250	5461581	N	N	N	N
2016	N	Harrison	Morris Valley	MV1	13-May-16	10	581022	5462695	N	~	N	Y
2016	N	Harrison	Morris Valley	MV2	13-May-16	10	581072	5462662	N	~	N	Y
2017	R	Harrison	Morris Valley	MV	05-Jul-17	10	581009	5462773	N	N	U	Y

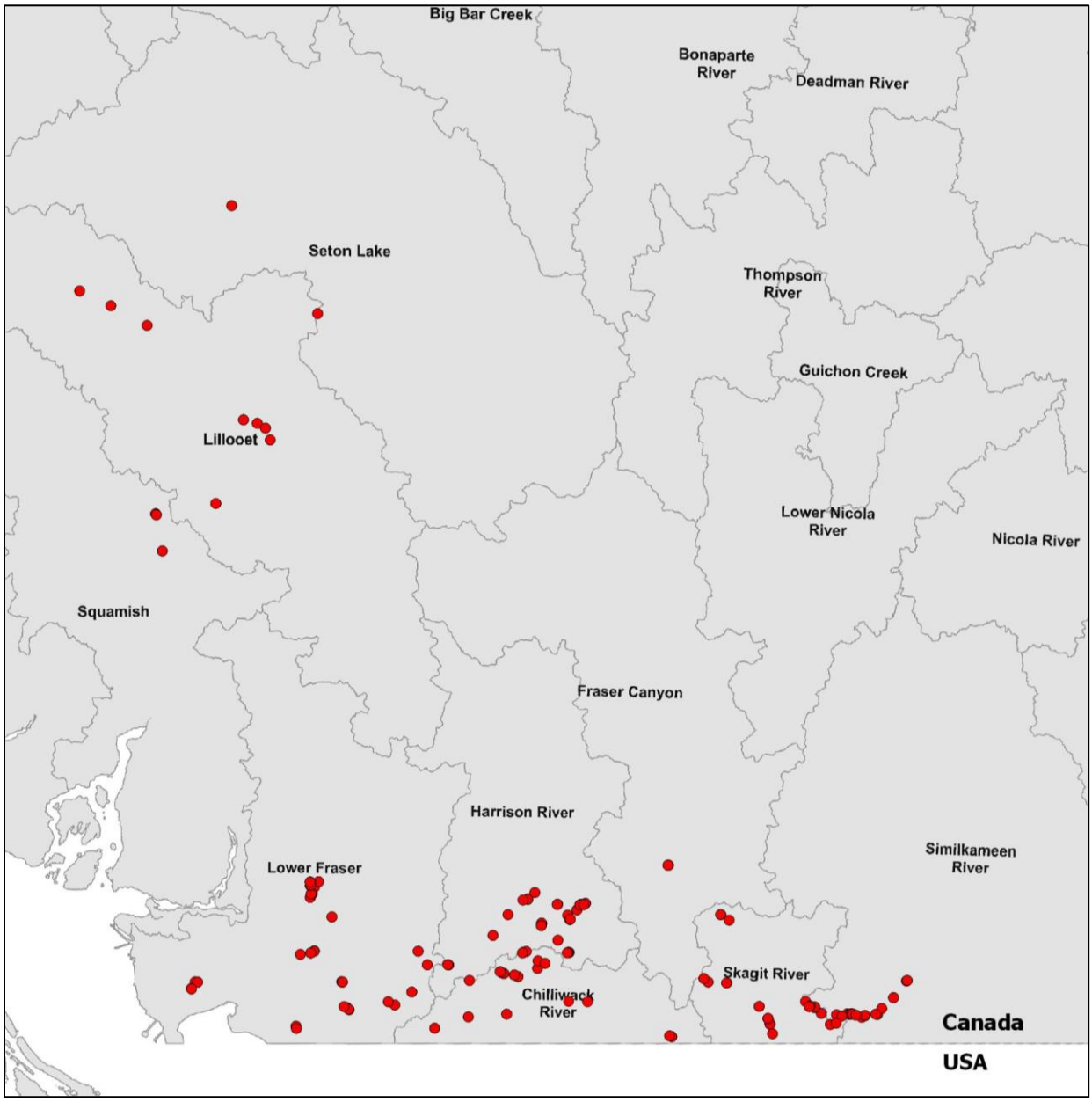
YEAR	New/ Repeat	Watershed group	Location name	Site ID	Collection date	Zone	Easting	Northing	RAAU	RACA	RALU	RAPR
2018	R	Harrison	Morris Valley	MS	20-Jun-18	10	581007	5462722	N	N	N	N
2016	N	Harrison	Morris Valley 3	MV3	14-May-16	10	579895	5462504	Y	~	N	N
2016	N	Harrison	Morris Valley 4	MV4	14-May-16	10	576316	5458951	N	~	N	N
2016	N	Harrison	Mountain Slough CSC	MSCSC1	13-May-16	10	584506	5456982	N	~	N	N
2016	N	Harrison	Mountain Slough CSC	MSCSC2	13-May-16	10	584565	5456973	N	~	N	Y
2017	R	Harrison	Mountain Slough CSC	CSC	05-Jul-17	10	584454	5456397	Y	N	N	Y
2018	R	Harrison	Mountain Slough CSC	CSC	20-Jun-18	10	584455	5456411	N	N	N	N
2016	N	Harrison	Nicomen Slough	NB1	15-May-16	10	561941	5446661	N	~	N	N
2016	N	Harrison	Nicomen Slough	NB2	15-May-16	10	562029	5446617	S	~	N	Y
2017	R	Harrison	Nicomen Slough	NS1	06-Jul-17	10	561893	5446653	Y	N	N	N
2017	R	Harrison	Nicomen Slough	NS2	06-Jul-17	10	561951	5446644	N	N	U	N
2018	R	Harrison	Nicomen Slough	Nic1	19-Jun-18	10	561949	5446657	N	N	N	N
2018	R	Harrison	Nicomen Slough	Nic2	19-Jun-18	10	561824	5446665	~	-	~	~
2016	N	Harrison	Wolfe Lake	WL1	14-May-16	10	582765	5464346	~	~	Y	N
2016	N	Harrison	Maria Slough Dump	MSDMP1	15-May-16	10	595108	5461955	N	~	N	N
2017	R	Harrison	Maria Slough Dump	DS	05-Jul-17	10	594695	5461640	Y	N	U	N
2016	N	Harrison	Maria Slough, Chaplin Rd	MSC1	13-May-16	10	591462	5458075	Y	~	N	N
2018	N	Lillooet	Alena Creek	ALC	17-Jun-18	10	473016	5606717	N	N	Y	N
2018	N	Lillooet	Arn drainage canal	ARN	19-Jun-18	10	511797	5576365	N	N	N	N
2018	N	Lillooet	Fulton Wetland	FUL	16-Jun-18	10	515077	5575550	N	N	N	N
2018	N	Lillooet	Green River Wetland	GRW	19-Jun-18	10	518155	5571641	N	N	N	N
2018	N	Lillooet	Lillooet Pond	LP	17-Jun-18	10	480375	5603212	N	N	N	N
2018	N	Lillooet	Pemberton Forest Road	PFR1	16-Jun-18	10	517033	5574416	N	N	N	N
2018	N	Lillooet	River of Humble Beginnings	RoHB	17-Jun-18	10	488939	5598579	N	N	N	N
2018	N	Lillooet	Whistler Wetland	WW	19-Jun-18	10	505240	5556581	N	N	N	N
2017	N	Lower Fraser	108 St Ditch, by Derby Reach	108ST	05-Jul-17	10	525919	5448865	N	N	N	N
2017	N	Lower Fraser	Aldergrove	ALD1	27-Jul-17	10	537881	5435599	S	N	N	N
2017	N	Lower Fraser	Aldergrove	ALD2	27-Jul-17	10	537378	5436057	Y	N	N	N
2017	N	Lower Fraser	Aldergrove	ALD3	27-Jul-17	10	537747	5435697	Y	N	U	N
2017	N	Lower Fraser	Aldergrove	ALD4	27-Jul-17	10	536647	5436319	N	N	N	N
2017	N	Lower Fraser	Burns Bog	BBOK1	04-Jul-17	10	499380	5440559	N	N	U	N
2017	N	Lower Fraser	Burns Bog	BBOK2	04-Jul-17	10	500318	5442187	N	N	N	N
2017	N	Lower Fraser	Burns Bog	BBOK3	04-Jul-17	10	500927	5442121	N	N	N	N
2018	R	Lower Fraser	Burns Bog	BBOG1_18	19-Jun-18	10	499389	5440557	N	N	N	N
2018	R	Lower Fraser	Burns Bog	BBOG5	19-Jun-18	10	499423	5440473	N	N	N	N
2017	N	Lower Fraser	Campbell River Park	CAM1	04-Jul-17	10	524936	5431516	N	N	N	N
2017	N	Lower Fraser	Campbell River Park	CAM2	04-Jul-17	10	525031	5430960	Y	N	N	N
2016	N	Lower Fraser	Dale Road	DR1	15-May-16	10	554571	5449855	N	~	N	S

YEAR	New/ Repeat	Watershed group	Location name	Site ID	Collection date	Zone	Easting	Northing	RAAU	RACA	RALU	RAPR
2016	N	Lower Fraser	Derby Reach 1-2	DERB1	18-May-16	10	529329	5449701	N	~	N	N
2016	N	Lower Fraser	Derby Reach 1-2	DERB2	18-May-16	10	529315	5449735	N	~	N	N
2016	N	Lower Fraser	Derby Reach 1-2	DERB3	18-May-16	10	529279	5449647	N	~	N	N
2016	N	Lower Fraser	Derby Reach 4	DERB4	18-May-16	10	528440	5449229	N	~	N	Y
2017	R	Lower Fraser	Derby Reach 4	DERNEW1	05-Jul-17	10	528430	5449246	N	N	N	N
2017	R	Lower Fraser	Derby Reach 4	DERNEW2	05-Jul-17	10	528460	5449237	N	N	U	N
2018	R	Lower Fraser	Derby Reach 4	DER4_18	18-Jun-18	10	528447	5449225	N	N	N	N
2018	N	Lower Fraser	Glenmore Rd Ditch	GLEN	18-Jun-18	10	549021	5436822	Y	N	N	N
2018	N	Lower Fraser	McLellan Creek	MCL	18-Jun-18	10	547425	5437587	N	N	N	N
2017	N	Lower Fraser	Mike Lake	ML1	04-Jul-17	10	533541	5457954	Y	N	N	N
2018	N	Lower Fraser	Page Creek	PAG	18-Jun-18	10	553130	5439993	N	N	N	N
2016	N	Lower Fraser	Pitt-Addington	PITF1	18-May-16	10	529244	5465065	Y	~	N	S
2016	N	Lower Fraser	Pitt-Addington	PITF2	18-May-16	10	530278	5466426	N	~	N	N
2016	N	Lower Fraser	Pitt-Addington	PITF3	18-May-16	10	528286	5466342	N	~	N	Y
2017	R	Lower Fraser	Pitt-Addington	UP1	04-Jul-17	10	528341	5465590	N	N	U	N
2017	R	Lower Fraser	Pitt-Addington	UP2	04-Jul-17	10	528222	5465497	N	N	N	N
2017	R	Lower Fraser	Pitt-Addington	PTNEW1	05-Jul-17	10	528246	5462599	N	N	N	N
2017	R	Lower Fraser	Pitt-Addington	PTNEW2	05-Jul-17	10	528708	5463458	N	N	N	N
2017	R	Lower Fraser	Pitt-Addington	PTNEW3	05-Jul-17	10	528437	5463465	N	N	N	N
2018	R	Lower Fraser	Pitt-Addington	PIT1_18	19-Jun-18	10	528213	5466347	N	N	N	N
2018	R	Lower Fraser	Pitt-Addington	PITKM	19-Jun-18	10	528338	5465605	N	N	N	N
2016	N	Lower Fraser	Sylvester Road	SR1	15-May-16	10	556852	5446593	N	~	N	N
2017	N	Lower Fraser	West Creek Wetlands	WCW1	04-Jul-17	10	536009	5442323	Y	N	N	N
2017	N	Lower Fraser	West Creek Wetlands	WCW2	04-Jul-17	10	536169	5442250	Y	N	U	N
2018	R	Lower Fraser	West Creek Wetlands	WCW2_18	18-Jun-18	10	536234	5442263	Y	N	N	N
2018	N	Seton Lake	Blackwater	BLK	18-Jun-18	10	529280	5601416	N	N	N	N
2018	N	Seton Lake	Gwyneth Pond	GP	17-Jun-18	10	508907	5626623	N	N	N	N
2016	N	Similkameen	Manning Park 10, 20 minute Lake	MAN10	25-Jul-16	10	659395	5436745	~	N	N	~
2016	N	Similkameen	Manning Park 100	MAN100.1	23-Jul-16	10	656819	5436386	~	N	N	N
2016	N	Similkameen	Manning Park 100	MAN100.2	23-Jul-16	10	656796	5436457	~	N	N	~
2016	N	Similkameen	Manning Park 101	MAN101.1	24-Jul-16	10	666719	5436867	~	N	S	~
2016	N	Similkameen	Manning Park 101	MAN101.2	24-Jul-16	10	666748	5436814	~	Y	N	~

YEAR	New/ Repeat	Watershed group	Location name	Site ID	Collection date	Zone	Easting	Northing	RAAU	RACA	RALU	RAPR
2017	R	Similkameen	Manning Park 101	MAN101.2ii	29-Jul-17	10	666752	5436805	N	Y	Y	N
2018	R	Similkameen	Manning Park 101	MAN101.1_18	05-Jul-18	10	666748	5436812	N	N	N	N
2016	N	Similkameen	Manning Park 102	MAN102.1	23-Jul-16	10	673640	5445148	~	N	N	U
2016	N	Similkameen	Manning Park 102	MAN102.2	23-Jul-16	10	673797	5445242	~	N	S	N
2017	R	Similkameen	Manning Park 102	MAN102.1	29-Jul-17	10	673640	5445148	N	N	Y	N
2017	R	Similkameen	Manning Park 102	MAN102.2	29-Jul-17	10	673797	5445242	N	N	N	N
2016	N	Similkameen	Manning Park 103	MAN103	24-Jul-16	10	667803	5438293	~	N	Y	~
2016	N	Similkameen	Manning Park 104	MAN104	23-Jul-16	10	670596	5441003	~	N	N	~
2017	R	Similkameen	Manning Park 104	MAN104.1	29-Jul-17	10	670596	5440958	N	N	S	N
2016	N	Similkameen	Manning Park 11	MAN11	25-Jul-16	10	659942	5436671	~	Y	N	~
2017	R	Similkameen	Manning Park 11	MAN11.2	29-Jul-17	10	659884	5436647	N	Y	Y	N
2018	R	Similkameen	Manning Park 11	MAN11_18	06-Jul-18	10	659922	5436655	N	N	N	N
2016	N	Similkameen	Manning Park 12	MAN12	25-Jul-16	10	660386	5436719	~	N	N	N
2016	N	Similkameen	Manning Park 13	MAN13	25-Jul-16	10	660470	5436825	~	Y	N	~
2017	R	Similkameen	Manning Park 13	MAN13	29-Jul-17	10	660470	5436825	N	Y	Y	N
2018	R	Similkameen	Manning Park 13	MAN13_18	05-Jul-18	10	660478	5436820	N	N	N	N
2016	N	Similkameen	Manning Park 15	MAN15.1	25-Jul-16	10	660799	5436757	~	N	Y	~
2016	N	Similkameen	Manning Park 15	MAN15.2	25-Jul-16	10	660729	5436721	~	N	Y	~
2016	N	Similkameen	Manning Park 19, Beaver Pond	MAN19.1	24-Jul-16	10	663760	5436407	~	N	N	N
2016	N	Similkameen	Manning Park 19, Beaver Pond	MAN19.2	24-Jul-16	10	663702	5436508	~	N	N	~
2018	R	Similkameen	Manning Park 19, Beaver Pond	MAN19_18	05-Jul-18	10	663704	5436515	N	N	N	N
2016	N	Similkameen	Manning Park 20	MAN20	25-Jul-16	10	658104	5436184	~	N	S	~
2016	N	Similkameen	Manning Park 4	MAN4	25-Jul-16	10	661568	5436492	~	N	Y	~
2018	N	Similkameen	Manning Park New	MANNEW1_18	05-Jul-18	10	666395	5436882	N	N	N	N
2016	N	Skagit	Manning Park 1, Shadow Lake	MAN1	23-Jul-16	10	653087	5436671	~	N	N	~
2016	N	Skagit	Manning Park 2	MAN2	25-Jul-16	10	655354	5434054	~	N	Y	N
2016	N	Skagit	Manning Park 22	MAN22	25-Jul-16	10	656757	5434401	~	N	Y	N
2016	N	Skagit	Manning Park 3, Poland Lake	MAN3	24-Jul-16	10	649169	5439437	~	Y	Y	N
2016	N	Skagit	Manning Park 6	MAN6	24-Jul-16	10	651422	5438020	~	N	S	N
2017	R	Skagit	Manning Park 6	MAN6	28-Jul-17	10	651462	5438168	N	N	N	N
2018	R	Skagit	Manning Park 6	MAN6_18	05-Jul-18	10	651417	5438147	N	N	N	N
2016	N	Skagit	Manning Park 8	MAN8	24-Jul-16	10	650201	5438307	~	Y	S	N
2017	R	Skagit	Manning Park 8	MAN8	28-Jul-17	10	650276	5438342	N	Y	N	N
2018	R	Skagit	Manning Park 8	MAN8.01_18	05-Jul-18	10	650276	5438342	N	N	N	N
2018	R	Skagit	Manning Park 8	MAN8.02_18	05-Jul-18	10	650303	5438404	N	N	N	N
2016	N	Skagit	Manning Park 8.2	MAN8.2	24-Jul-16	10	650102	5438231	~	N	Y	~
2017	R	Skagit	Manning Park 8.2	MAN8.2	28-Jul-17	10	650089	5438260	N	N	Y	N
2017	N	Skagit	Skagit 17_3	SK17-3	29-Jul-17	10	637903	5437961	N	N	N	N
2016	N	Skagit	Skagit 3	SK3.1	17-May-16	10	640612	5433874	N	N	N	N
2016	N	Skagit	Skagit 3	SK3.2	17-May-16	10	640625	5433821	N	N	N	N
2016	N	Skagit	Skagit 3	SK3.3	17-May-16	10	640608	5433722	N	N	N	Y
2017	R	Skagit	Skagit 3	SK3.4	28-Jul-17	10	640621	5433826	N	N	N	N
2018	R	Skagit	Skagit 3	SK3.1_18	04-Jul-18	10	640628	5433765	N	N	N	Y
2016	N	Skagit	Skagit12	SK12	16-May-16	10	625315	5443562	N	N	N	N
2016	N	Skagit	Skagit14	SK14.1	16-May-16	10	640159	5435047	N	Y	N	N
2016	N	Skagit	Skagit14	SK14.2	16-May-16	10	640090	5435096	N	N	N	N

YEAR	New/ Repeat	Watershed group	Location name	Site ID	Collection date	Zone	Easting	Northing	RAAU	RACA	RALU	RAPR
2017	R	Skagit	Skagit14	SK14.2ii	28-Jul-17	10	640130	5435090	N	Y	N	N
2018	R	Skagit	Skagit14	SK14.1_18	04-Jul-18	10	640124	5435024	N	N	N	N
2018	R	Skagit	Skagit14	SK14.2_18	04-Jul-18	10	640113	5435108	N	N	N	N
2017	N	Skagit	Skagit2017_2	SK17-2	29-Jul-17	10	641323	5431431	N	N	N	N
2016	N	Skagit	Skagit5	SK5	16-May-16	10	624235	5444377	N	N	N	N
2016	N	Skagit	Skagit8	SK8.1	16-May-16	10	629872	5443472	N	N	N	N
2016	N	Skagit	Skagit8	SK8.2	16-May-16	10	629789	5443441	N	N	N	N
2016	N	Skagit	Sunshine Valley 5	SV5.1	26-Jul-16	10	627968	5459919	N	N	Y	N
2016	N	Skagit	Sunshine Valley 5	SV5.2	26-Jul-16	10	628037	5459912	N	N	S	N
2017	R	Skagit	Sunshine Valley 5	SV5	27-Jul-17	10	627976	5459930	N	N	Y	N
2016	N	Skagit	Sunshine Valley 6	SV6	26-Jul-16	10	630072	5458604	N	N	Y	N
2018	N	Squamish	Callahan 1	CAL1	16-Jun-18	10	490889	5554167	N	N	N	N
2018	N	Squamish	Callahan 2	CAL2	16-Jun-18	10	491044	5553910	N	N	N	N
2018	N	Squamish	Callahan Pond 1	CP1	16-Jun-18	10	492466	5545349	Y	N	N	N
2016	NWA	Washington, Mt Baker	Nooksack R, North Fork	MtBAK	26-Jul-16	10	597726	5417167	N	N	~	~
2017	NWA	Washington, Mt Rainier	Mt Rainier, Frog Heaven	MTRAI	02-Sep-17	10	594727	5181182	N	Y	N	N

Appendix 3. eDNA sampling sites (red symbols) from 2016-2018 in relation to major watershed groups.



Appendix 4. Water bodies surveyed repeatedly over two or three years, showing congruence of eDNA detections (biologist call) for four species of *Rana*.

Biologist call: Yes -detected, S-suspected, N-not detected; Congruence Index: 0-different in all years, 0.5-same in 2 of 3 years; 1-same in all years; ~not tested

Watershed group	Water body	# years sampled	RAAU: 2016, 2017, 2018	RACA: 2016, 2017, 2018	RALU: 2016, 2017, 2018	RAPR: 2016, 2017, 2018	Congr. Index: RAAU	Congr. Index: RACA	Congr. Index: RALU	Congr. Index: RAPR
Chilliwack	Camp Slough 2	2	~,N,N	~,N,N	~,U,N	~,N,N	1	1	0	1
Chilliwack	Semihault	3	Y,Y,Y	~,N,N	N,N,N	S,Y,Y	1	1	1	1
Harrison	Maria Slough Chaplin Rd	3	Y,Y,Y	~,N,N	N,N,N	N,N,N	1	1	1	1
Harrison	Maria Slough Dump	3	N,N,N	~,N,N	N,U,N	N,N,N	1	1	0.5	1
Harrison	Maria-Kamp	3	Y,Y,Y	~,N,N	N,U,N	Y,S,Y	1	1	0.5	1
Harrison	Miami	3	N,Y,N	~,N,N	N,U,N	S,N,N	0.5	1	0.5	0.5
Harrison	Morris Valley	3	N,N,N	~,N,N	N,U,N	Y,Y,N	1	1	0.5	0.5
Harrison	Mountain Slough CSC	3	N,Y,N	~,N,N	N,N,N	Y,Y,N	0.5	1	1	0.5
Harrison	Nicomen Slough	3	S,Y,N	~,N,N	N,U,N	Y,N,N	0	1	0.5	0.5
Lower Fraser	Burns Bog	2	~,N,N	~,N,N	~,U,N	~,N,N	1	1	0	1
Lower Fraser	Derby Reach 4	3	N,N,N	~,N,N	~,U,N	~,N,N	1	1	0	1
Lower Fraser	Pitt-Addington	3	Y,N,N	~,N,N	N,U,N	Y,N,N	0.5	1	0.5	0.5
Lower Fraser	West Creek Wetlands	2	~,Y,Y	~,N,N	~U,N	~N,N	1	1	0	1
Similkameen	Manning Park 101	3	~N,N	Y,Y,N	S,Y,N	~N,N	1	0.5	0.5	1
Similkameen	Manning Park 102	2	~,N,~	N,N,~	S,Y,~	U,N,~	~	1	0	0
Similkameen	Manning Park 104	2	~,N,~	N,N,~	N,S,~	~,N,~	~	1	0	~
Similkameen	Manning Park 11	3	~N,N	Y,Y,N	N,Y,N	~,N,N	1	0.5	0.5	1
Similkameen	Manning Park 13	3	~N,N	Y,Y,N	N,Y,N	~,N,N	1	0.5	0.5	1
Similkameen	Manning Park 19, Beaver Pond	2	~,~,N	N,~,N	N,~,N	N,~,N	~	1	1	1
Skagit	Manning Park 6	3	~N,N	N,N,N	S,N,N	N,N,N	1	1	0.5	1
Skagit	Manning Park 8	3	~N,N	Y,Y,N	S,N,N	N,N,N	1	0.5	0.5	1
Skagit	Manning Park 8.2	2	~,N,~	N,N,~	Y,Y,~	~,N,~	~	1	1	~
Skagit	Skagit 3	3	N,N,N	N,N,N	N,N,N	Y,N,Y	1	1	1	0.5
Skagit	Skagit14	2	N,~,N	Y,~,N	N,~,N	N,~,N	1	0	1	1
Skagit	Sunshine Valley 5	2	N,N,~	N,N,~	Y,Y,~	N,N,~	1	1	1	1

Appendix 5. List of water bodies with where Columbia spotted frogs (RALU) were seen during sample collection in comparison with eDNA detection from same sites, 2016 - 2018.

Blue shading indicates where the species was seen and were eDNA was detected.

YEAR	Watershed group	Water body/ location name	Site ID	# Spotted frog seen (adult or juv.)	# Spotted frog tadpoles seen	RALU: eDNA detection	Comments
2017	Harrison	Nicomen Slough	NS1	1	0	No	
2018	Lillooet	Alena Creek	ALC	0	20	Yes	
2018	Lillooet	River of Humble Beginnings	RoHB	1	0	No	1 dead adult seen
2016	Similkameen	Manning Park 10, 20 minute Lake	MAN10	1	0	No	
2016	Similkameen	Manning Park 100	MAN100.1	1	0	No	
2016	Similkameen	Manning Park 101	MAN101.1	3	0	Susp	
2016	Similkameen	Manning Park 101	MAN101.2	6	0	No	
2018	Similkameen	Manning Park 101	MAN101.1_18	25	0	No	
2016	Similkameen	Manning Park 102	MAN102.2	1	0	Susp	
2017	Similkameen	Manning Park 102	MAN102.1	1	0	Yes	
2017	Similkameen	Manning Park 102	MAN102.2	1	5	No	
2016	Similkameen	Manning Park 104	MAN104	8	0	No	
2017	Similkameen	Manning Park 104	MAN104.1	7	1	Susp	
2016	Similkameen	Manning Park 11	MAN11	0	100	No	
2017	Similkameen	Manning Park 11	MAN11.2	0	20	Yes	
2018	Similkameen	Manning Park 11	MAN11_18	25	many	No	
2016	Similkameen	Manning Park 12	MAN12	2	20	No	
2016	Similkameen	Manning Park 13	MAN13	1	100	No	Only 1 of 3 replicate samples passed the IntegritE-DNA test
2017	Similkameen	Manning Park 13	MAN13	5	3	Yes	
2018	Similkameen	Manning Park 13	MAN13_18	0	2	No	

YEAR	Watershed group	Water body/ location name	Site ID	# Spotted frog seen (adult or juv.)	# Spotted frog tadpoles seen	RALU: eDNA detection	Comments
2016	Similkameen	Manning Park 15	MAN15.1	5	0	Yes	
2016	Similkameen	Manning Park 15	MAN15.2	Several	0	Yes	Frogs seen in sedges during walk around wetland between sites
2016	Similkameen	Manning Park 19, Beaver Pond	MAN19.2	0	10	No	
2016	Similkameen	Manning Park 4	MAN4	1	0	Yes	
2017	Skagit	Manning Park 6	MAN6	12	1	No	
2018	Skagit	Manning Park 6	MAN6_18	13	0	No	
2018	Skagit	Manning Park 8	MAN8.01_18	4	0	No	
2018	Skagit	Manning Park 8	MAN8.02_18	1	0	No	
2017	Skagit	Manning Park 8.2	MAN8.2	3	30	Yes	
2017	Skagit	Skagit 3	SK3.4	7	0	No	