Environmental DNA Protocol for Freshwater Aquatic Ecosystems Version 2.2

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PREFACE

This protocol presents instruction and recommended methods for conducting environmental DNA (eDNA) surveys for freshwater aquatic species, including species with aquatic life history phases, in British Columbia. The objective of the protocol is to ensure consistency amongst practitioners applying eDNA as a survey method for freshwater aquatic species. A standardized approach ensures reliability in results and increases confidence of interpretation of results amongst practitioners and resource managers.

The protocol provides important background information and should be reviewed before implementing surveys using eDNA methods. The evolution of standards and guidelines for collection, analysis and interpretation is an ongoing process and is the result of collaboration amongst many practitioners. New learnings and advances were incorporated from an extensive review of available literature and from discussion and input provided by experts in the method. Standard data collection and sample management procedures are suggested and provided for eDNA studies.

The following methods have not been formalized by the Resources Information Standards Committee (RISC), but we would like surveyors to test these methods and provide feedback to the Wildlife Species Inventory team at RISCWeb@gov.bc.ca. Of particular interest is what worked well, what didn't and any recommended changes to either the sampling techniques or equipment.

As these are interim methods, there currently is no standardized data template, so the preferred option for data collection and submission is the ***Biological Sample Collection*** template (http://www.env.gov.bc.ca/wildlife/wsi/data_templates/biological_sample_collection.htm). Any additional information listed in these methods can be added to the spreadsheet. Data and reports should be submitted to the Wildlife Species Inventory data submission site as outlined in the ***Inventory Projects and Surveys*** section on the following website: http://www.env.gov.bc.ca/wildlife/wsi/index.htm

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Many previous publications summarizing eDNA studies and learnings were reviewed and incorporated into the eDNA protocol. The effort and contribution from all authors, as noted in the References section, is greatly appreciated. In addition, review and comment was provided by several individuals including Kathy Paige and Dr. Purnima Govindarajulu (MOE).

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1.0 INTRODUCTION

As living organisms complete their life processes their genetic material, or DNA, is shed exogenously into the surrounding environment. For aquatic and semi-aquatic species environmental DNA (eDNA) can be collected in water samples, filtered to capture eDNA, and effectively assayed to detect the presence of aquatic and semi-aquatic species without direct observation. This method is referred to as eDNA (analysis) and provides a promising and emerging method for more cost-effective, less invasive and more efficient survey of aquatic species living in natural lotic and lentic systems (Biggs et al. 2014; Herder et al. 2015). Environmental DNA methods are currently being used to survey a diverse suite of freshwater environments including lotic (e.g., stream) and lentic (e.g., wetland) systems for a variety of aquatic and semi-aquatic species including benthic invertebrates, fishes, amphibians and semi-aquatic mammals (Goldberg et al. 2011, Thomsen et al. 2012, Farrington and Lance 2014). The reliable detection of aquatic vertebrate species using eDNA, from a variety of freshwater systems, has been confirmed as an effective survey method for many species (Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012).

Water and/or sediment samples from environments potentially inhabited by the species in question are collected for subsequent analysis to detect the presence of DNA from the target taxa. Testing for the presence of a species' DNA is completed *ex situ* (in a laboratory), generally using quantitative polymerase chain reaction (qPCR) methods. This method enzymatically increases the abundance of a specific sequence of DNA from target taxa in a solution using polymerase chain reaction (PCR). The PCR process repeatedly subjects the sample to alternating heat (to denature, or separate, the double helix) followed by cooling to copy the original DNA in the sample. This process is repeated many times (e.g. 50 cycles) resulting in an exponential increase in copy number of the DNA from the target taxa, and amplicons are detected using a species specific probe. Each process, including all cycles, is referred to as a run; samples are typically run between three to eight times during laboratory analysis, for each sample collected, to arrive at a binary conclusion regarding presence or absence of DNA from the target taxa. Samples from each site are then considered collectively to derive support for a positive or negative assignation (for the presence of DNA from the target taxa) at the site level. The use of a qPCR assay requires development of species-specific assays (primers and probe) that target a small section of the genome; primer specificity is key to this process (Farrington and Lance 2014).

As eDNA methods are refined, the approach is expected to become an increasingly widespread tool for biological monitoring in aquatic systems, particularly for inventory of at-risk and invasive species (Jerde et al. 2011, Goldberg et al. 2015). This document provides a high-level overview of the advantages and limitations of the eDNA method based on the best available information from the literature. It discusses recommended approaches for study design, outlines the steps required for tissue and surface water sample collection, and provides guidance for reporting and discussion of results. The objective of this document is to provide a standardized protocol for eDNA studies in British Columbia (BC) for both lotic and lentic freshwater ecosystems.

2.0 METHODOLOGICAL BACKGROUND

For freshwater aquatic systems, eDNA inventory is facilitated by the collection of water samples from the study area. The location, volume, spacing, replication, and timing of samples vary across systems and species, but generally follow a linear process (**Figure 1**).

Figure 1 Sequence of Steps in the eDNA Method

Each step of the method has advantages and limitations that must be considered to ensure accurate, reliable results that create confidence in the ability of the method to answer the study objective. These are summarized in Table 1.

Table 1 Summary of Comparison of Attributes between Conventional Methods and eDNA

Attribute	Conventional Methods	eDNA
Efficacy	Low-High	High
Multi-species	Sometimes	Yes
Retro-active addition of taxa	No	Yes
Adaptive design/testing	No	Yes
Observer bias	High	Low
Permitting required	Yes	No
Invasiveness	High	Low
Pathogen transfer risk	Moderate with protocol disinfection	Low
Timing	Restrictive	Less Restrictive
Special equipment/training	Medium-high	Low-medium

2.1 ADVANTAGES

Depending on the objective of the field program (e.g., presence/absence vs. abundance), eDNA methods may prove more efficient than traditional baited trapping and/or physical search methods. This is especially true for inventory of rare species (Jerde et al. 2011, Pilliod et al. 2013) or for cryptic species that persist at low density or with discontinuous distributions. Relative to traditional sampling methods, the following is true for eDNA methods[1]:

- Non-invasive (damaging) to the species and less invasive to the habitat.

[1] (Goldberg et al. 2011, 2015, Jerde et al. 2011, Thomsen et al. 2012, Pilliod et al. 2013, Deiner and Florian 2014, Thomsen and Willerslev 2015)

- Highly accurate for detection of species (i.e., high detection probability).
- Cost-effective.
- Able to detect the presence of pathogens that cause infectious disease (e.g., *Batrachochytrium dendrobatidis* (chytrid) and iridovirus in amphibians).
- Minimize the risk of pathogen transfer between sites.
- Can be completed by staff without extensive experience in species-specific survey techniques.
- Can be completed within less restrictive phenological stages for many target taxa.
- Not as dependent on or sensitive to environmental conditions (e.g., time of day, wind speed).

The eDNA method also offers improved efficiency in its ability to test for more than one species in one sample (Thomsen and Willerslev 2015). Another advantage is the ability to hold preserved samples in storage at a laboratory and perform testing months to years after they were collected and preserved. Preserved samples can also be later subsequently tested for additional species that were not the intended target taxa at the time of collection.

2.2 LIMITATIONS

The use of eDNA methods is not a panacea for species inventory. Current eDNA methods do not facilitate accurate quantification of species abundance (Jerde et al. 2011), as the relationship between the concentration of eDNA detected in a sample and the species' biomass in the system remains undefined (Thomsen et al. 2012, Kelly et al. 2014). Understanding the limitations of the eDNA method is critical to the accurate interpretation of presence/absence data (Maruyama et al. 2014). There are several processes and factors that must be considered when using eDNA methods: contamination, inhibition and error (Thomsen and Willerslev 2015).

2.2.1 Contamination

Contamination occurs when the sample comes in contact with a source of DNA and results in a false positive result. Sample contamination can occur at the stage of sample collection, filtration, preservation, extraction, or analysis. In the field, cross-contamination of samples can occur from not wearing or changing gloves between sites at the collection stage, or from failing to sufficiently sterilize filtration equipment between samples. This manual provides guidance on how to reduce the potential for contamination during the collection, filtration and preservation stages.

In the laboratory (extraction and qPCR preparation), the risk of contamination of samples is very high unless the activities are carried out in a lab dedicated to the extraction of DNA and decontamination procedures are strictly adhered to (Champlot et al. 2010, Goldberg et al., in press). Submitting field blanks (i.e. negative control samples) to the lab to monitor for contamination is essential to create confidence in the results and to quantify error if contamination is detected.

2.2.2 Type 1 and Type 2 Errors

The value of a species inventory program ultimately lies in the ability to understand and quantify false positive (type 1) and false negative (type 2) error rates (Moyer et al. 2014). Traditional methods used to sample species in aquatic habitats are prone to type 1 errors (i.e., recording a species when it is absent; generally through misidentification of an observation) or type 2 errors (i.e., failing to detect a species when it is actually present). Environmental DNA methods are also subject to these potential errors; however, eDNA methods have a reduced likelihood for type 2 errors as PCR analysis methods are highly sensitive (Thomsen et al. 2012, Pilliod et al. 2013).

2.2.3 Inhibition

Inhibition of the PCR reaction is detected at the analysis stage of the method (**Figure 1**). When an eDNA sample is collected, non-target substances from the system (e.g., total suspended solids) are also present in that sample. Many of the substances are removed from the sample during filtration and extraction processes, but some substances may be co-extracted with the DNA and inhibit the PCR reaction (Goldberg et al. 2015). If inhibition goes undetected it may lead to a false negative result (Goldberg et al. 2015, Thomsen and Willerslev 2015).

2.3 INTERPRETATION OF RESULTS

The outcome of the eDNA method is either a positive detection of the target species' DNA in the sample or a negative result (no DNA was detected). The key to interpreting the outcome is a thorough understanding of the three primary processes that influence the detection of eDNA: production, transport and degradation (Goldberg et al. 2015). More detail regarding appropriate interpretation of results from qPCR analysis are provided in **Appendix C**.

The rate of eDNA production and subsequent rate of release into the habitat varies across species (e.g., Thomsen et al. 2012). Within species, the rate varies across life stages and even across individuals (Maruyama et al. 2014, Klymus et al. 2015) but eDNA methods cannot distinguish between different life stages as the source of the DNA. The density of individuals in the habitat also influences the rate of DNA production and ultimately the rate of detection (Goldberg et al. 2015). In general, the higher the density of individuals, the more DNA is present in the habitat to be sampled, and the higher the likelihood of positive detection of DNA (Takahara et al. 2012, Thomsen et al. 2012) however eDNA methods cannot currently be used to draw correlative inference regarding species abundance as concentration of eDNA in an aquatic system is influenced by many confounding variables (Herder et al 2014, Goldberg et al 2016) including transport, degradation, distance to source and phenological variation in eDNA production rates.

The rate of transport (diffusion) of eDNA away from its source differs in lotic and lentic systems (Goldberg et al. 2011, Takahara et al. 2012, Pilliod et al. 2013). Study design must take into account these differences to yield useful inference about species' presence in the system. Quantification of the rate of

transport of eDNA in lotic systems and the processes that influence this distance has not been done for most species (Deiner and Florian 2014, Goldberg et al. 2015). Field studies in natural systems indicate that for some aquatic species (e.g., invertebrates) eDNA can persist and be detected after being transported over large distances nearly 12 km) from the source (Deiner and Altermatt 2014). The residence time of the organism in the system also influences the amount of eDNA that may be detected in a lotic system (Pilliod et al. 2014), and it is possible that the longer the organism is present in the system the further the eDNA will be transported from the source.

The rate of eDNA degradation also strongly influences the amount of eDNA present in a sample, and therefore its detectability (Barnes et al. 2014, Strickler et al. 2015). The timeframe for which DNA persists in the environment depends on several factors, including the type of flows in the system (e.g., lotic or lentic), ultraviolet rays, water temperature, pH, salinity, substrate type and microbial community activity (Strickler et al. 2015). Understanding the rate of eDNA degradation of the target organism in the aquatic system will help when determining whether a positive detection represents genetic material from an organism currently occupying the habitat or genetic material that persisted from recent use by the target species but the species is no longer present (Barnes et al. 2014). The published rates of eDNA degradation from controlled (i.e., lab) experiments vary widely across species and across local conditions (e.g. water temperature); however, a consistent pattern that emerges is an exponential decay of detectable eDNA over time (Thomsen et al. 2012, Barnes et al. 2014, Strickler et al. 2015). Experiments have shown that eDNA persistence in water after the removal of one or several individuals can range from less than one hour in a lotic system (Pilliod et al. 2014) up to approximately 58 days in a mesocosm (Strickler et al. 2015), with 7 to 25 days being the mid-range (Dejean et al. 2011, Thomsen et al. 2012). The length of persistence of eDNA in a system will depend on local environmental conditions at the time of sampling (Strickler et al. 2015). While no general conclusions for eDNA persistence rates can yet be drawn across taxa or systems, a conservative guideline for persistence of eDNA in an aquatic system is 7 to 21 days after the removal of the organism from the system.

Finally, positive detection of the target species' DNA in a sample indicates that the DNA was present in the water feature, but a single positive detection does not rule out the possibility that the DNA came from an external source such as contaminated gear, stormwater runoff, or feces of a predator (Merkes et al. 2014). External sources of DNA could lead to the conclusion that a species was present in an aquatic feature when in fact it was not using the habitat at or shortly preceding sampling, so care is required when interpreting a single positive result. Sample replication can help increase confidence during interpretation of qPCR lab results.

2.4 SUMMARY

Environmental DNA methods have proven advantages for detecting aquatic species and are a promising alternative to traditional inventory methods for determining species presence (Goldberg et al. 2015). While challenges remain, field and laboratory methods for eDNA are currently undergoing rapid development and refinement to improve accuracy and to quantify error (Goldberg et al. 2015). In addition, primers will be refined and developed as research into eDNA methods evolve. As such, eDNA practitioners should stay informed with current developments from the eDNA literature to allow for constant refinement of field design and laboratory methods to ensure the most robust study design and the most accurate interpretation of the data.

3.0 STUDY DESIGN

Environmental DNA methods are quickly proving to be a very powerful for aquatic applications when the objective of a project is to understand presence of the target species in the study area; however, the inability of eDNA methods to accurately and precisely link the concentration of eDNA in a habitat to species abundance is a notable limitation (Thomsen et al. 2012). Careful consideration of study objectives are required before deciding on the most cost-effective and efficient method. Depending on the objective the most appropriate approach may require application of more traditional methods (currently used to measure relative or absolute abundance in natural systems) and / or a combination of eDNA and conventional methods.

3.1 SAMPLE SITE SELECTION AND SURVEY TIMING

The study objective will influence appropriate survey design and sample site selection. Samples may be collected from water features where the target species is known to occur (e.g., previous sampling effort), is suspected to occur, has historically occurred (e.g., BC Conservation Data Centre occurrences), or where presence is unknown (e.g., un-sampled areas on the periphery of a species known range). The sampling effort (i.e., spatial and temporal replication) will vary across program objectives and may need to be increased to provide confidence to clients and regulators in results obtained using a relatively new method, particularly in areas of unknown or historic species occurrence.

Similar to traditional methods (i.e. RISC standards), survey timing should coincide with the breeding season of the target species or other appropriate biological timing windows when the concentration of the species eDNA is suspected to be the most abundant in the ecosystem (e.g., late summer breeding season and low flow periods for tailed frogs). Failure to sample during an appropriate biological timing window may decrease detection probabilities and reduce confidence in the ability of eDNA methods to accurately determine species presence/non-detection.

When interpreting results, survey timing must be considered to ameliorate the risk of concluding that a negative result means the species does not use the habitat for *any* life history requirements when in fact it does. For example, not detecting the species in the study area in the breeding season doesn’t preclude the value of the habitat for over-wintering.

3.2 SAMPLING EFFORT

For field programs designed to detect at-risk species that often occur in low densities or with discontinuous distributions, increased sampling effort generally improves detection probabilities (Green and Young 1993). For traditional inventory methods like baited trapping (e.g., Pacific water shrew) or time-constrained searches (e.g., amphibians), increasing sampling effort to a level that yields high confidence in results often increases project labour and material costs to a level that becomes prohibitive

(Jerde et al. 2011). For some species, eDNA can yield higher confidence in results for presence/non-detection objectives at a fraction of the cost of traditional methods (Biggs et al. 2014). Herder et al. (2014) recognize that for some species that are relatively easy to detect (e.g., spadefoot [*Spea intermontana]*), traditional methods may ultimately be more efficient but eDNA methods may still confer some advantage as samples can be collected under less restrictive sampling conditions; tadpoles can be detected even after they have metamorphosed into an adult terrestrial phase and abandoned the aquatic feature (within 7 to 25 days). Several studies report shorter handling time and lower cost using eDNA compared to traditional monitoring techniques (Biggs et al. 2014, Sigsgaard et al. 2015).

The sampling effort (i.e., sample size and temporal and spatial replication) of a study will depend on the objective (e.g., documenting species presence in a small restoration site vs. understanding a species distribution across the landscape or in large aquatic features). Generally, the greater the sampling effort, the greater the strength of evidence for species presence/absence at a site (Jerde et al. 2011). For example, detecting the presence of a species through a single positive sample indicates only that the target DNA was present in the sample (Jerde et al. 2011, Pilliod et al. 2013), but repeated sampling and positive detections over multiple years yields stronger evidence and higher confidence that the species uses the habitat to carry out life history requirements (Jerde et al. 2011). At the landscape scale, eDNA may provide a measure of a species' relative abundance, over time, when collected over longer time periods.

3.2.1 Water Sample Replication

The number of water sample replicates collected at each site will depend on several factors: the study objective (desired confidence), the budget, the system (lotic or lentic) and the target species. For species where detection probabilities are well-studied and protocols reliably produce detection probabilities consistent with an acceptable probability of false negatives, a single replicate may be sufficient for high confidence in sample results. Triplicate samples are recommended when detection probabilities are unknown. If temporal replication (e.g., multiple years) is anticipated, the results of the first set of samples can inform the sampling effort required for the next collection period.

Quality control to monitor for contamination of field and lab procedures is also part of appropriate sample replication. For new species and new practitioners, one negative control sample (distilled or deionized water) per processing session, or event, is recommended.

3.2.2 Spatial Replication

To ensure sufficient spatial coverage or to improve detection probability of the target species, sample spacing needs to be carefully considered. Preliminary data indicates that for amphibian species in lentic systems, samples spaced up to 50 m apart will yield full spatial coverage. Water samples should be collected at locations within the site where the target species is most likely to occur (i.e., suitable

microhabitats). If a lentic system is >1 ha, more sites should be sampled to ensure approximately 50 m spacing of sample collection locations especially where suitable micro-habitats are continuous. Appropriate micro-habitats for target taxa should be sampled to increase detection probability. Discretion is often required during sample collection to balance survey objectives with available funding (i.e., project costs) with full consideration of the implication of choices made during survey design and/or sample collection. For lotic systems, sampling in the mainstem will only allow inference that the target species is present somewhere in the system[2]; sampling within headwater streams and tributaries allows for finer scale understanding of species presence and distribution in the watershed.

While the number of samples required from each site will vary depending on total area (lentic) or linear distance (lotic) of the habitat being sampled, the sample volume should be consistent across all sites so that comparable detection probabilities can be estimated. Sample volume requirements vary across species and systems (e.g., lentic vs lotic), but should be consistent within the study design for a single study. Generally sample volume of 1L per sample, with three samples per site, is recommended. Recommended sample volume may be restricted during the filtration phase due to substances (tanins or suspended particulate matter) present in the system that may clog filter membranes. This is particularly problematic for samples collected from lentic systems and can ultimately lead to a reduction in detection probabilities. It is important to record the initial sample volume collected as well as the final volume filtered to inform interpretation of results.

Sample location in the water column should also be consistent across sites. For example, samples should be collected from surface water to prevent capture of sediment in the sample which can bind DNA for long periods of time[3] and can lead to a false positive from historical DNA (Turner et al. 2015), Goldberg et al. 2015). Lotic samples should be collected from the surface water in the thalweg (if possible), and if multiple samples from the same feature are required, sampling from subsequent sites should occur in an upstream direction to prevent contamination. In addition, if research is being conducted in the same aquatic feature (i.e., for unrelated studies) eDNA sampling should occur prior to other studies, or at least one month after other studies, to avoid contamination from in-stream activities by other researchers.

3.2.3 Temporal Replication

Study objectives and budget constraints will ultimately dictate the level of sampling effort, but increased temporal replication will always increase strength of evidence for species presence (Jerde et al. 2011). Replication can be increased by sampling across seasons within one year (strong), or in one season across multiple years (stronger), or across multiple seasons in multiple years (strongest). Temporal replication combined with spatial replication will yield the highest strength of evidence for determining

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[2] Deiner and Altermatt (2014) detected DNA of macro-organisms up to 12 km from established populations

[3] Turner et al (2015) reported eDNA was 8-1800 times more concentrated per gram of sediment than per milliliter of water and that DNA was detected in sediment up to 132 days after removal of the target taxa from the water.

species presence in the study area, but this level of effort may not always be required if study objectives do not require this degree of rigour. Another advantage of temporal and spatial replication is the ability to use the data set to calculate detection probabilities.

3.2.4 Detection Probability

All eDNA studies should calculate an estimate of detection probability in order to quantify the efficiency of the method at detecting a species when it is present, and the likelihood that a species is actually absent from a site (Green and Young 1993). This is especially true if there is temporal (seasonal) or spatial variability in the detection of a species (Goldberg et al. 2011, Kotze et al. 2012). Increasing detection probabilities of target species can be accomplished by ensuring survey timing coincides with anticipated periods of highest eDNA concentration. Detection probabilities can be calculated using the qPCR results by examining the proportion of eDNA replicates testing positive at sites where the species is known to occur (through field observations or some replicates testing positive). Because detection probabilities can vary with sampling and environmental variables (e.g., sample volume if it varied among sites, temperature), a modeling approach should be taken to understanding these influences before inference is drawn (MacKenzie and Royle 2005).

4.0 BIOLOGICAL ASSAY DEVELOPMENT

The premise of eDNA methods relies on the ability to test a sample for the presence of a target species DNA. Methods exist that can sequence all or specific DNA in a sample (meta-barcoding), but are currently not cost-effective for the single-species monitoring that this protocol focuses on. For single-species testing, eDNA detection requires the existence of a species-specific DNA assay[4] for testing. For some species, this assay will already exist but for others it will require development or additional validation. Ideally, an eDNA study should not be initiated until assay development is completed or underway; however, sample collection can precede assay development as filtered samples can be preserved. **Appendix D** details several species in BC for which assays have been developed, tested and applied.

The cost to develop an assay can vary depending on lab rates and genetic divergence within target taxa relative to other sympatric species and availability of sequence data in GenBank®. The labour associated with collection of tissue samples and costs associated with permitting to facilitate shipment to the lab, must also be considered in the budget in addition to the lab assay development cost. The amount of time and effort required to collect, prepare and ship the tissues can be a constraint to project timing; these steps should be completed well in advance of the need for project results.

4.1 TISSUE COLLECTION PROTOCOL

The eDNA samples cannot be analyzed using qPCR until the species-specific assay has been developed. It is recommended that eDNA sample collection be completed after the assay has been developed and verified. However, if timing constraints require sample collection prior to assay development, the water samples can be filtered, preserved and stored in molecular grade ethanol. Once preserved, the filters should remain viable for at least six months in storage. RNAlater can be used in place of molecular grade ethanol for tissue preservation to obviate the need for the requirement of a Transportation of Dangerous Goods permit.

Biological tissue samples in molecular grade ethanol[5],[6] or RNAlater™ can be stored in the dark in a non-self-defrosting -20°C freezer for 5-10 years prior to assay development, or longer after DNA extraction, so tissue collection can be initiated well in advance of eDNA sampling.

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4 An assay consists of a set of species-specific molecular markers (i.e., primers and probe) to be designed and implemented under controlled conditions in a laboratory following carefully designed, validated and executed qPCR-based procedures (Veldhoen et al 2016)

5 Mouth swabs must be stored in a lysis buffer and can be stored and remain viable for up to five years (Goldberg et al. 2003).

6 Only 95 to 100% molecular grade ethyl alcohol (i.e. ethanol) can be used for sample preservation. Denatured ethanol cannot be used to preserve tissues or filters as it contains acetone and will immediately degrade DNA and destroy the sample. The purchase of molecular grade ethyl alcohol is regulated by the BC Liquor Board and is restricted to Ethyl Alcohol permit holders. A permit can be obtained through the BC Liquor Control and Licensing Branch.

To develop a species-specific assay, the lab will typically require ten tissue samples from ten different individuals of the target species and five tissue samples of each closely related and sympatric species present in the study area. Ideally, tissue samples will be obtained from individuals present in the study area or from the same population as those in the study area to reduce differences in genetic variation across the species range. Ideally, tissue samples should be collected from individuals from several different sites (e.g., not all samples from the same pond) to capture genetic variation across individuals.

An entire individual or part of an individual may be used for assay development. Acceptable parts of an individual include fresh scales, toes, tail tips, eggs and buccal material (mouth swabs). There may be a substantial lead time (months) to obtain these samples either through field collection or through contacting government staff or other conservation professionals who may have frozen or dried samples of the target species. The collection of live parts of animals or dead specimens, including roadkill, requires a General Wildlife permit under the provincial *Wildlife Act*. The export of these tissue samples to laboratories outside of Canada also requires a permit (see **Section 4.1.3**).

The following protocol and list of materials has been adapted from the *Tissue Sampling Protocol for eDNA Test Validation* developed by Goldberg (2014).

4.1.1 Required Equipment

The following materials are required for tissue sample collection:

- 50 mL container of 50% household bleach and 50% distilled, deionized or municipally treated tap water
- 50 mL container of distilled or deionized water
- sterile gauze bandages or similar clean material
- non-powder latex gloves (if multiple species handled)
- small scissors or scalpel
- 95% (or undiluted) molecular grade ethanol OR RNAlater™ (from ThermoFisher or Sigma-Aldrich)
- polypropylene[7], cryogenic collection vials (2 mL size is recommended for sample storage in ethanol)
- lab (alcohol resistant) marker for labelling vials (note that industrial Sharpies are not suitable)
- whirl-paks for individual storage of samples in vials

[7] Polypropylene is a hydrophobic material resistant to the adsorption of highly hydrophilic DNA. Adsorption of DNA to the sample container could decrease the concentration of DNA in the sample and reduce detection probabilities (Marwood et al. no date)

4.1.2 Tissue Sample Collection Procedures

During tissue sample processing, it is imperative that no cross-contamination occurs between specimens, particularly between specimens of different species. Clean gloves must be worn at all times and different scissors or scalpel blades must be used on different species. Scalpel blades can be re-used between specimens of the same species; however, they should be cleaned in a 50% bleach solution and then rinsed in deionized or distilled water between individual specimens.

The following protocol should be followed when collecting biological tissues:

- Check to make sure there is an o-ring (gasket) in the cap of the vial to prevent leakage during shipping (Sarstedt brand tubes are strongly recommended).
- Use the ethanol-resistant marker to label the vial with the species name and collection date, location and collector.
- Handle the organism or tissue only with clean hands (no other species handled) or latex non-powder gloves.
- Wipe the open scissors or scalpel on the gauze to remove any excess tissue from previous sampling.
- Swirl the open scissors or scalpel in the 50% bleach solution for at least 30 seconds.
- Rinse the open scissors or scalpel in distilled/deionized water for at least 30 seconds.
- Fill the vial with molecular grade ethanol (**use lysis buffer for mouth swab**). If you are using RNAlater™, fill the vial to 80% of the maximum volume to allow for expansion of the liquid when frozen.
- Clip a small piece (~0.5 cm cube) of tissue and place it into the vial ensuring complete coverage of the tissue sample with the preservative.
- Securely tighten the lid on the vial.
- Place the vial in a whirl-pak and label with the species name and collection date, location and collector name. This will ensure proper sample identification in the event that the vial leaks and obscures the labelling on the vial.
- Store collection vials upright to ensure complete tissue immersion in a dark, cool location (e.g. refrigerator) for at least 24 hours to allow the preservative to penetrate the tissue sample. Following this important step, samples can be transferred to a non-self-defrosting -20°C freezer.
- When shipping samples to the receiving lab, ship them on blue ice (ice packs).

4.1.3 Tissue Sample Shipping

Under the provincial *Wildlife Act Permit Regulation*, the export of wildlife and wildlife parts (including eggs) out of BC requires individuals and organizations to possess either a valid general wildlife export permit or a valid CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) permit[8]. The lead time required for an export permit may be substantial (e.g., weeks to months) and should be submitted for approval well in advance of the anticipated shipping date of tissue specimens.

Tissue samples should be securely stored as indicated in 4.1.2 until shipping. Samples should preferably be shipped via overnight courier at the beginning of the work week to minimize the risk of loss or damage during shipping. The tissue sample package should include a copy of the export permit (keep the originals) and a hardcopy of the tissue sample data, including:

- Species' scientific and common name
- Collection location (using gazetted names if applicable)
- Collection UTM (Zone/Easting/Northing)
- Collection date and time
- Sample ID (a unique number assigned to each different sample)
- Preservation method (usually ethanol, specify **molecular grade**)
- Specimen type (e.g., tail, eggs, scales)
- Collector name

[8] A CITES permit is required only for certain species (MOE 2005) and may be issued either through CITES Canada (for species listed on Appendix 1) or FrontCounterBC (species listed on CITES Appendix 2).

5.0 WATER SAMPLE COLLECTION PROTOCOL

It is recommended that eDNA sample collection wait until the species-specific assay has been developed and verified. However, if necessary given timing or other constraints, samples can be collected, filtered, extracted and stored so sample collection can precede assay development. For shorter term storage filters can be preserved and stored in molecular grade ethanol in the dark (e.g., a drawer or cabinet) and remain viable for at least six months in storage. Samples can also be folded inward and placed in a coin envelope in a bag with self-indicating silica beads until desiccated. Using self-indicating silica as a desiccant is showing promise as an alternative to ethanol-based preservation but requires further validation to determine the storage parameters (e.g. longevity). Once DNA is extracted from the filters by the lab, it can typically remain in storage indefinitely.

5.1 SURVEY CONDITIONS

While the potential implications on eDNA detection from sampling in the rain are not yet fully understood, it is recommended that sampling does not occur during or immediately after moderate or heavy rain events as there is higher potential for the eDNA concentration in samples to be diluted, and also potential for higher levels of suspended solids to be present in the sample which could lead to inhibition during analysis. Finally, sampling during high-flow events may lead to re-suspension of sediment-bound eDNA in the system which could result in a positive detection even though the species was not recently using the water feature (Turner et al. 2015).

5.2 REQUIRED EQUIPMENT

The following list of field equipment has been adapted from existing sampling protocols by Pilliod et al. (2012) and Goldberg and Strickler (2013). Refer to **Appendix A** for *additional* information on these items.

5.2.1 Sample Collection Materials

- Polypropylene Nalgene sample bottles (volume dependant on target species and system). Use of regular plastic sample bottles (i.e., not polypropylene based) may lead to eDNA adsorption and should not be used).
- Permanent ink marker (e.g., Sharpie®) to label sample bottles
- Non-powdered gloves
- Tote filled with 10% bleach water and scrub brush
- Field notebook and pencils
- Collection of water quality information using a YSI meter (optional) or thermometer (recommended) is not required but is generally advisable.
- GPS unit

5.3 COLLECTION PROCEDURES

Avoiding cross-contamination between samples is critically important for the prevention of false positive results. Each step of the sample collection protocol contains measures to prevent contamination of samples and should be strictly adhered to. The following additional practices are also recommended to minimize the potential for sample contamination:

- Do not leave the sample collection gear unprotected or exposed to the environment where it may come in contact with site water.
- When collecting site water ensure the collector does not enter the water upstream of the collection site in lotic environments or within 3 m of the collection site in lentic environments.
- When collecting water samples in lentic habitats an extendable pole is recommended to facilitate collection at least 3 m from the collector's location
    - If a pole is used to collect sample water the bottle clasping mechanism must be sprayed with bleach and rinsed with site water to remove bleach as bleach may destroy DNA if it enters the sample water during collection. The bottle clasping mechanism should be decontaminated at each collection site *prior* to collection.
    - An extensible pole is also recommended when collecting eDNA samples from a boat, particularly in lentic habitats although it should be noted that sample collection from a boat is not recommended for lentic habitats; boat collection methods are more feasible in lotic habitats. Collect each sample upstream from the boat to prevent contamination of an aquatic habitat with eDNA transported on the boat surface from other sites.
- Sample collection in both lentic and lotic habitats should precede other field studies where field personnel will be entering the aquatic habitats being sampled. Coordination with other studies is required.
- In addition to following the eDNA field protocol, crews should adhere to the *Hygiene Protocols for Amphibian Field Staff and Researchers*[9] (BC MOE 2008) to prevent the spread of infectious disease between water bodies if repeatedly entering aquatic habitats.

Upon arrival at the sample site, record the date, time, site name, UTM coordinates and weather conditions. Wait until after the samples are collected to collect the water quality data to prevent contamination. Recommended sample data are as follows:

- Date
- Time
- Site name

[9] Note that if this uses quat or other disinfectant instead of bleach, it may not decontaminate adequately to prevent transfer of eDNA between sites.

- UTM location (Zone/Easting/Northing) (NAD 83 UTM)
- Collection time
- Collector initials
- Weather (cloud cover, air temperature, precipitation)
- Sample volume
- Water quality data:
  - water temperature at surface (^{o}C)
  - pH
  - dissolved oxygen (mg/L)
  - conductivity (µs/cm)
- Additional habitat attribute data specific to the target taxa (e.g., substrate description, forest cover description for associated riparian and upland habitat, presence of emergent vegetation etc.)

Adhere to the following steps during sample collection:

1. Label the lid of the sample bottles with the sample site ID and sample letter (i.e., A, B, C). Label the side of the bottle with site ID, collection time, collection site UTM coordinates (on at least one of the bottles) and initials to identify collection staff. Use a 'permanent ink' marker (e.g., Sharpie®) to ensure labels are waterproof; labels can easily be wiped off after filtration is complete with ethanol or rubbing alcohol for bottle reuse. Ideally at least three 1 L samples (depending on wetland size) are recommended for lentic species when detection probabilities are unknown. For species where primer efficacy has been confirmed to be >90% (e.g., Rocky Mountain tailed frog, coastal giant salamander, chinook etc.) sample collection can be reduced to two 1 L samples if desired.
2. The collector must put on a clean pair of gloves at each independent collection site and should refrain from touching anything other than the collection bottles to prevent cross-contamination with eDNA from other sites.
3. Fill the sample bottle with water from the *surface* of the feature close to but slightly away from the specific location where water will be collected (for example, once situated the sampler does this step on one side of them and then takes the sample on the other side). This consideration is less important for lotic systems.
4. Place the lid loosely back on the bottle and shake the bottle (whilst holding the lid with a finger) to rinse the bottle with site water.
5. Empty the contents of the bottle away from the sampling site (either downstream (lotic habitats) or on the ground (lentic habitats) where the rinse water won't re-enter the sample site water.
6. Repeat steps 2 through 4 two more times to achieve a triple rinse of each replicate bottle. This step ensures there is no bleach residue, from previous bottle decontamination, remaining in the sample collection bottle.

7. Fill the sample bottle with water from the surface of the feature and securely tighten the lid.
8. Repeat steps 2 to 7 for the second and third sample bottles. To increase detection probability samples can be collected from different microsite locations at the sample site (typically all samples at a site should be collected within 10m of one another).
9. Place the sample bottles in a cooler filled with crushed ice, ensuring direct contact with ice, as soon as possible. It is important to keep samples cool during field collection as degradation rates are directionally proportionate to temperate.
10. Collect water quality data at the site using a water quality meter (e.g., YSI). It is important that this step be completed *after* water sample collection to prevent cross-contamination with eDNA from other sites *unless* the meter has been decontaminated.
11. If either member of the collection team entered the aquatic habitat and came into contact with site water, decontamination of clothing (particularly footwear) is required between sites. Follow the decontamination protocol outlined in the *Hygiene Protocols for Amphibian Field Staff and Researchers* (only if required). The water quality meter does not need to be decontaminated as it is used *after* sample collection however if disease transmission is a concern then decontamination is recommended.

6.0 WATER SAMPLE FILTRATION PROTOCOL

Filtration should take place within 24 hours of sample collection to reduce the likelihood of degradation of DNA in the sample. Samples should be filtered in the same order they were collected in the field. The filtration step should be carried out in a workspace dedicated to the filtration process (e.g., equipment room bench or field camp lab/kitchen area). Filtering samples manually in the field is logistically challenging and not recommended if other options are feasible; however, it can be done using a small hand-vacuum device (e.g., for brake bleeding) if absolutely necessary. Filtering is most efficiently completed in an interior space with access to a power source (possibly from a generator) to power a high-capacity vacuum pump and to power a refrigeration unit for samples waiting to be processed[10]. Access to treated water is also required to decontaminate sample bottles after filtration is complete to prepare for subsequent collection of new samples in the field. After cleaning, sample bottles can be re-used to collect subsequent samples.

During filtration, eDNA particles in the water sample will be captured by the cellulose membrane at the bottom of the filter funnel. This membrane is removed from the funnel after filtration is complete, and is preserved in a vial filled with molecular grade ethanol or a coin-envelope that is then stored in a bag with self-indicating silica beads[11].

6.1 SAMPLE FILTRATION MATERIALS

- 250 mL sterile polypropylene filter funnels with 0.45 µm cellulose membrane (although there are many available options Nalgene Polypropylene Analytical Test Filter Funnel (see **Appendix A**) are recommended).
- GAST high-capacity vacuum pump (Note: this requires access to electrical power supply, a portable power generator can be used at remote sites if in-field filtration is required (i.e., filtration in a remote field setting))
- If using a Masterflex peristaltic pump silicone tubing (masterflex) tubing is preferred. If using a GAST pump stiff tubing 3/8” (for flask tubation) and rigid re-inforced 5/8” diameter (for pump tubation) is recommended.
- A two-pronged Y-splitter is preferred to support concurrent filtration of two samples to reduce processing times
- 1 L vacuum flasks with tubulation (x2 for increased efficiency)
- #8 rubber stopper
- Two pairs of forceps (or stainless steel tweezers) are required

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[10] If using a refrigerator, check to make sure the temperature is set above 0^{oC} to prevent samples from freezing.

[11] Dry preservation with indicating silica bead desiccant is recommended where possible as DNA extraction rates are higher with this method. In addition, use of silica obviates cumbersome and costly permitting requirements associated with transport of ethanol as ethanol is classified as a dangerous good under the Transportation of Dangerous Goods Act.

- Plastic container with 50% bleach, 50% distilled water
- 1 L plastic measuring cup
- Distilled or deionized water for lab blank/control
- Three rinse containers for bleach and distilled water to sterilize forceps/tweezers
- Coin envelopes (for dry sample storage with silica desiccant (preferred method) **or** 2 mL volume cryogenic polypropylene vials (for sample storage in ethanol)
- Whirl-paks or Ziploc® Snack-bags
- Graduated 1L measuring container (to measure total volume of sample water filtered).

6.2 FILTRATION PROCEDURES

Individual equipment preference and availability may vary but the steps required are outlined below.

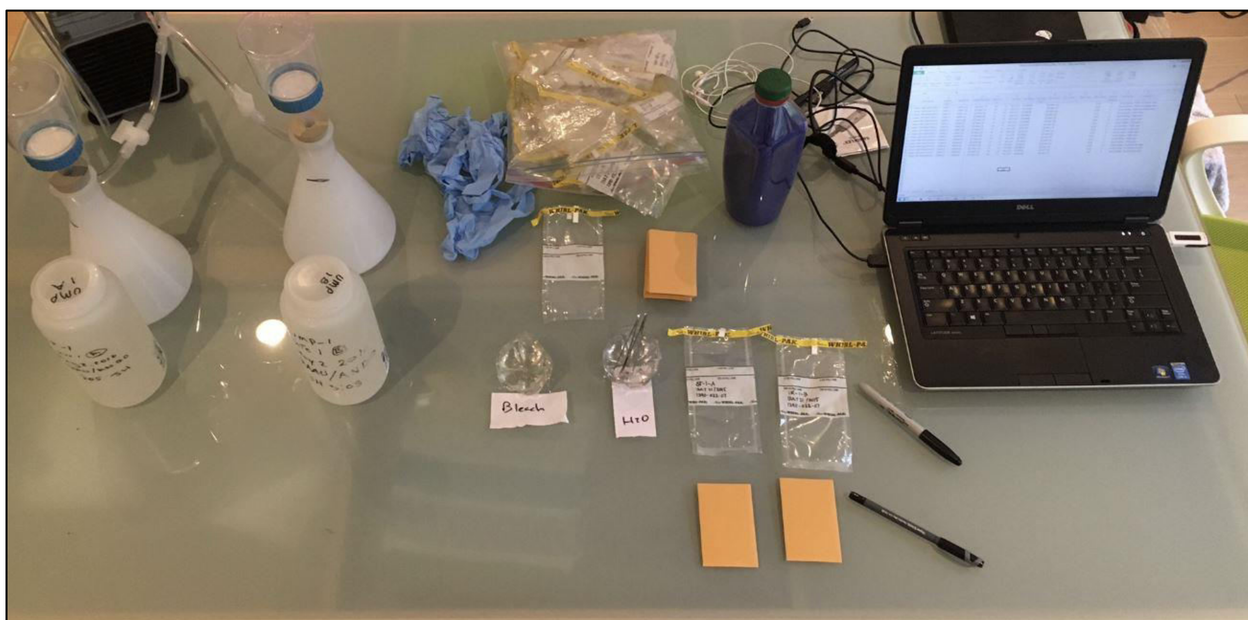

Figure 2 Typical filtering set-up.

- Plug the vacuum pump into the power source.
- Run the silicone tubing from the pump head and attach it to the tubulation of the vacuum (Erlenmeyer) flask(s). To expedite filtration a Y-splitter can be used to run tubulation to two flasks to allow simultaneous processing of two samples. A shut-off valve can be installed above the Y-splitter to provide control of suction applied to each flask independently.
- Attach the rubber stopper(s) to the top of the vacuum (Erlenmeyer) flask(s).
- Insert the plastic filter funnel adapter into the hole in the rubber stopper, creating an airtight seal.
- Remove disposable filter funnel from its sterile package by gripping the bottom of the funnel. **Do not to touch the inside of the funnel.**

- Firmly attach the funnel filter to the filter funnel adapter and make sure the funnel is sitting level.
- Gently turn the sample bottle upside down three times to mix the sample prior to filtration.
- Slowly pour the sample into the filter funnel and note the volume and filter start time.
- Turn the pump on to begin filtration.
- If necessary, place the sample bottle back in the cooler or refrigerator if more volume is required. Gently mix the sample bottle every time more site water is poured into the funnel.
- As filtration slows, avoid adding any additional water after 40 minutes has passed from the filter start time. Instead allow the suction to continue until all the sample water has all passed through so that the filter membrane is dried before preserving.
    - If filtration rate is slowing after the first 250ml of sample water any additional water added should be added in small increments (10-25ml) to avoid having to pour remaining unfiltered water out of the filter cup.
    - If the filter membrane becomes too clogged for any water to pass through the filter, any leftover water remaining in the filter cup should be slowly drained to allow the filter to be extracted and preserved. Make a note in the filter data to indicate if water had to be drained due to a clogged filter. Try to avoid having to pour out excess unfiltered water by adding only in small increments as filtration speed slows.
- When using silica as a desiccant ensure that suction is applied to the filter for a 3-5 minute period after all the water has passed through the filter (or been poured out of the filter cup) to allow the filter sufficient time to dry before proceeding with sample preservation.
- When filtering is complete, record the total sample volume that passed through the membrane. Filtering is typically completed when 1 L of sample water has been filtered OR when 45 minutes to one hour (for GAST pump at 20 mg/Hg) or 1.5 hrs (for Masterflex pump) has passed since the filter start time.
    - It is handy to have a pre-prepared spreadsheet open, on a laptop, during filtration, to ensure sample start and end times and sample volumes are properly assigned for each bottle of site water being filtered.
    - To avoid mixing up samples it is handy to use post-it notes, beside each bottle on the lab bench, so you can keep track of start and end times for each sample during filtration.

During filtering, make sure the vacuum pressure is sustained by monitoring pump gauge (on GAST pump models) or by monitoring the water level in the funnel to make sure the water is passing from the funnel to the vacuum flask. If suction is not occurring, check the silicone tubing in the pump-head mechanism, if using a peristaltic pump, for cracks and leaks and check to ensure that each rubber stopper is securely attached to the vacuum flask to create an airtight seal. If simultaneous filtration of two samples is conducted using two-pronged tubulation, ensure that airflow is cut-off from empty samples by closing a stop valve. For multi-directional peristaltic pumps, check the pump direction to ensure the pump is not being operated in reverse. It is recommended that the amount of time to filter each sample is recorded so that inefficiencies in the process can be identified and rectified in future programs.

Ensure that the vacuum flask volume is monitored and emptied when necessary to prevent back-flow of sample water from the flask into the funnel. If this occurs it can contaminate the membrane and damage the pump's internal mechanism. If using a GAST vacuum pump avoid getting water in the pump internal motor by keeping tubulation dry and empty of water droplets.

Filter either the entire sample volume (typically 1 L) or continue filtering until the filter membrane becomes too clogged and is no longer permeable (i.e., suction is not drawing sample water through the filter membrane). Generally, if no more sample water is being drawn through the filter after a period of one hour of filtration, filtration is ceased (see notes above). If less than 100 ml of sample water was filtered, the sample may be insufficient for PCR testing and omission of the sample should be considered. When the maximum volume of the sample has been filtered through the membrane, proceed to the sample preservation step. Measure the total volume filtered (collected in the Erlenmeyer flask) when filtering is complete by pouring the filtered site water into a graduated measuring cup..

For each independent sampling session a lab control, consisting of 1 L distilled or deionized water, must be run through the same filtration process under the same conditions (i.e. same personnel) as the samples to check for potential contamination of samples during filtration.

6.3 Sample Preservation

If using ethanol as a preservative, pre-label a 2 mL vial using an ethanol-resistant marker. If using silica as a preservative pre-label a coin envelope (for dry storage) using a pen or pencil. At a minimum, labels must include the project ID (#), site name and collection date.

When handling the filter:

- Decontaminate two pairs of forceps by resting them in a cup containing 50% bleach solution for at least 1 minute and then swirling them in a rinsing cup of deionized or distilled water before placing them a second cup of deionized water prior to use. Leave the forceps sitting in the second container of distilled water container for at least 30 seconds while preparing the funnel to ensure adequate rinse time has been achieved before using. A double-rinse process is recommended as bleach residue will accumulate in the first 'rinsing' cup of deionized water with successive sample treatment. Alternatively, or in addition, replace deionized or distilled rinse with fresh water after every tenth sample to prevent compromising sample integrity with diluted bleach.
- Remove the filter funnel from filter collar by firmly gripping the collar with one hand and gripping the top of the upper portion of the filter funnel with the other hand. Gently pull the upper portion of the filter funnel upwards and towards you until the seal is broken and the upper portion pops off. Do not twist the filter funnel as this could damage the membrane.
- Ideally the filter should be rolled and placed in the vial using forceps, without ever being touched by a hand. If that proves cumbersome you can use a glove. In this case put a glove on your non-dominant hand and do not touch anything except the membrane once the glove is on.

If preserving with ethanol:

- Pick up the disinfected forceps (two pairs) with your dominant hand. Using the forceps, gently grip one edge of the membrane and fold the membrane in half. Note that the filter funnel has a thicker base filter in addition to the membrane. Make sure you are preserving only the filter membrane and not the cardboard base.
- Use the second pair of forceps (or, if necessary, a finger from the gloved hand) to gently press and secure the folded filter membrane. Grip the open edge of the membrane with the forceps and fold in half again, using the forceps (or, if necessary, the gloved finger) to secure the fold, continuing the hold the membrane with the forceps (or the gloved finger) to prevent it from unrolling. Roll the membrane into a cone shape. Avoid tearing the filter membrane.
- Place the rolled filter into the labelled vial using forceps.
- Fill the vial with ethanol only up to the threading (if you fill it past the threads the vial will overflow when the cap is screwed on and the label could be compromised). Ensure the cap is screwed on securely.
- If ethanol is used as a preserving solution place each separate vial in an individual whirl-pak or Ziploc snack bag, labelled with the same information used to label the vial; this provides an additional level of sample identification in the event the vials leak during shipping.

If preserving by drying with self-indicating silica desiccant: (this method is currently being tested for effectiveness, use with caution[12])

- Pull air through the filter for three to five minutes after all the water has passed through the filter to dry the filter paper.
- Fold the membrane once in half, inward, with the disinfected forceps.
- Using forceps, place the folded filter into a labeled coin envelope. Put the envelope in a Ziploc bag with self-indicating silica beads. Seal the bag. Check the color of the beads periodically, especially in the first few hours, to be sure that they are not saturated. The beads will fade in color if they become saturated. In this case, more beads should be added until color is sustained.
- Remove and dispose of gloves.
- If silica is used as a desiccant, place all three samples (each stored in a separate coin envelope) from each site, together in a single Ziploc® snack bag with desiccating beads.
- Instruct the lab to extract DNA from the filter as soon as possible upon receiving the samples.

[12] For more information please contact Jared Hobbs.

6.4 Decontaminate Equipment for Future Use

- Labels on the sample bottles can be removed using rubbing alcohol and a soft cloth.
- Clean the vacuum flask, sample bottles and tubing in 50% bleach and 50% distilled or municipally treated water: let them soak for at least one minute in the solution.
- Rinse well in municipally treated water, (i.e., do not use water from well or stream sources that may have the potential to be contaminated with DNA from the target species).
- Sample bottles should not be put in direct sunlight to dry as UV rays will break down polypropylene material and decrease the material's ability to resist DNA adsorption.
- Allow bottles to dry out prior to the next collection event, preferably for 24 hours.

7.0 SAMPLE SHIPPING PROTOCOL

Samples should be shipped to the lab as soon as possible after filtration, and should be stored in a secure, cool, dark environment until shipment. Shipping the preserved sample vials does not require a wildlife export permit; however, if the samples are preserved in ethanol it is classified as a hazardous material and is subject to certain regulations and requirements. TDG permitting is mandatory.

A hardcopy of the filtration data, detailing: sample ID, project ID, collection date and time, collection and filter personnel, target taxa for qPCR, UTM for collection location, filter start, end and total time, and filter volume should accompany the preserved samples. The person receiving the samples at the lab should review the samples when they arrive to ensure no samples were lost or destroyed during transport. The receiver will sign off (or email) that the samples were received. In addition to sending hardcopies of the data with the filter shipment a digital copy should be emailed to the lab, to be used for subsequent data entry, at the time of shipment. See **Appendix B** for required data to be sent with the samples.

8.0 REPORTING

The following sections consist of recommendations for authors of eDNA reports including guidance on interpretation of results and suggestions to incorporate, address and describe study limitations. To ensure consistency in terminology between studies (**see Figure 3**) we define:

Location as an aquatic feature (lentic or lotic) where eDNA sample(s) will be collected and analyzed for one or more target taxa.

Site is defined as a specific area, within a selected sample location, where site water will be collected.

Water Sample Replicate is defined as an individually collected water sample (typically 1 L volume) that will be subsequently filtered. The filter will be sent to a lab for qPCR analysis.

qPCR (assay) Replicate is defined as a single qPCR reaction of the DNA isolated from the filter performed at a lab that specializes in analysis of eDNA samples.

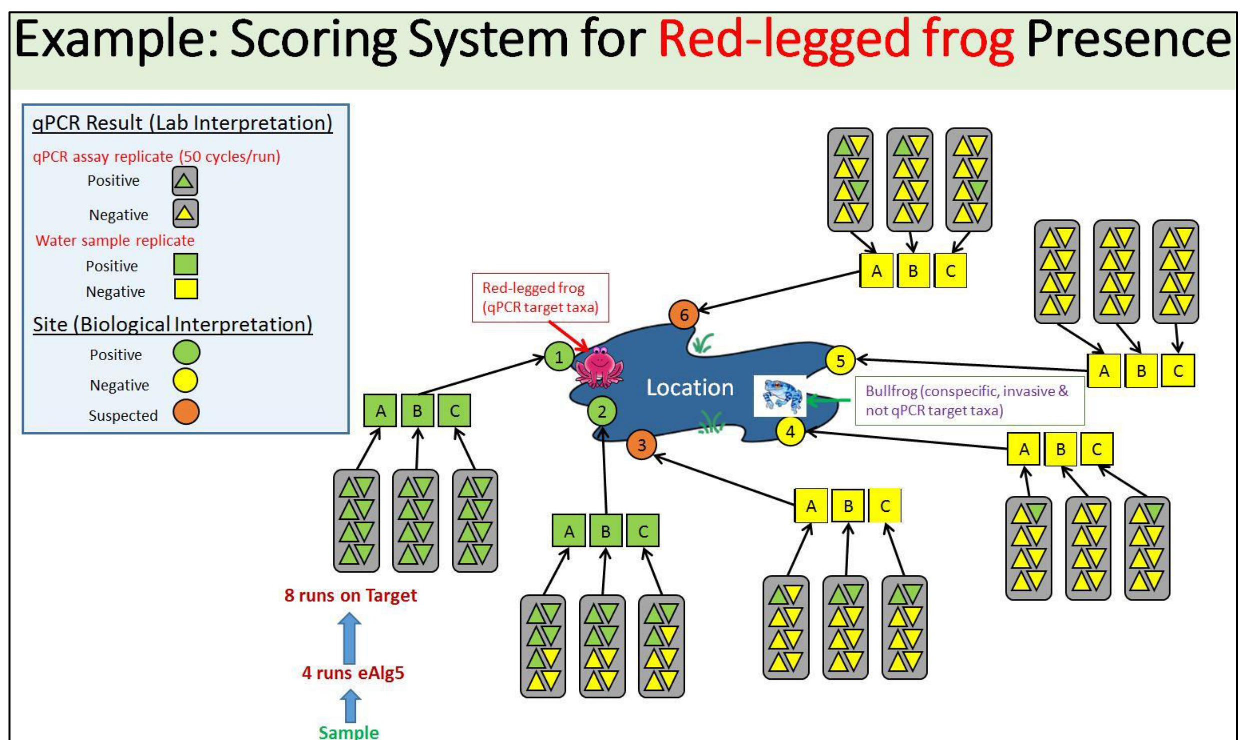

Figure 3 Terminology typically used during discussion and analysis of eDNA lab results.

Inference of species occurrence based on number of positive qPCR and water sample replicate ranges from high certainty (all or most replicates testing positive) to lack of detection (zero replicates testing positive). The level of evidence required will depend on the application, and transparency in the uncertainty of results will assist in communicating strength of evidence. Like any detection method, eDNA

detection of species is imperfect and variation among water and qPCR replicates is expected. The focus, therefore, is on the minimum level of evidence required to indicate species presence. Analysis of eDNA samples requires careful qPCR assay design (validated for the species of interest), clean consistent lab practices, and a dedicated lab space. In addition to these controls, two known negative samples and one known positive sample should be analyzed with each batch, or plate; if any of these negatives tests positive, the associated batch must be considered suspect.

In addition to these controls, and because qPCR reactions to detect eDNA are so sensitive, we recommend lab methods described in Veldhoen et al (2016) following interpretive guidelines outlined in **Appendix C**[13].

- Veldhoen et al (2016) recommends a hierarchical analytical approach, beginning with a test for amplifiable DNA using a primer targeting algae, before advancing to an eight-run qPCR process for the target taxa. Only samples returning a result ≥3/8 runs are treated as positive.
- Alternatively, Goldberg et al. (2016) recommends a three run qPCR process; accepting samples returning ≥1/3 runs as positive if the result is repeatable (i.e. consistent when repeated). Most labs have yet not adopted a requirement to ensure presence of amplifiable DNA as outlined by Veldhoen et al. (2016).

In summary, specific lab procedures, and specific protocols regarding interpretation of lab results, may vary based on lab procedures. Replicate-based results and interpretation should be provided by individual labs, along with a summary of lab precautions and validation procedures.

8.1 DISCUSSION OF LIMITATIONS

As eDNA is a relatively new inventory method it is appropriate to describe associated methodological limitations. As discussed in detail in **Section 2.1 and 2.2** many factors influence the ability of qPCR analysis to detect an organism's DNA from samples collected in natural systems. The effect of those factors on study results must be considered and explained during discussion of the results.

Negative results may represent a true negative (i.e., the species was not present in the habitat during or preceding sampling), or a false negative (i.e., type 2 error). If all replicates collected at one site are negative and control samples are negative, this provides evidence that the qPCR results are accurate and the species was not present at the site. Notwithstanding, presentation of this result requires express consideration that samples were taken with adequate spatial coverage and that enough samples were taken, given the per-sample probability of detection, to ensure that the probability of missing an extant occurrence of the target species is very low. In addition, and consistent with traditional inventory methods, failure to detect an organism in the habitat at a single point in time is not conclusive evidence that the

[13] **Appendix C** details eDNA analysis procedures conducted by the Helbing Lab at the University of Victoria Department of Biochemistry & Microbiology.

species is absent from the site throughout the year, or that the species does not or has not occupied that site in other years. Repetitive sampling in the habitat over multiple years and / or multiple seasons will increase confidence in inferences made regarding species presence using eDNA methods. Additional information regarding knowledge of site-specific habitat conditions can be used to support negative results (e.g., proximity of the sample site to documented species occurrence records or potential impacts from nearby resource extraction activities).

Positive results may represent either a true positive or a false positive (i.e., type 1 error). Careful qPCR assay design criteria better informs the eDNA status of target taxa within each water sample (Veldhoen et al 2016). At the site level, multiple water samples are considered to increase detection confidence. True positive results indicate the species' DNA was detected in the sample and the species is assumed to be present in the habitat during or shortly preceding sample collection. If all replicates at a site are positive, this provides compelling evidence to conclude the target species was present. For large lentic or lotic water bodies, it is possible to have uneven distribution of target taxa eDNA, even at the site level, leading to difference qPCR result assignations among water sample replicates. Therefore, any site where >1 water sample replicate tests positive should be considered a site where the target taxa is likely to have occurred during or just prior to sampling. However, the potential for contribution of location-associated confounding variables needs to be addressed during data interpretation as outlined below.

For every study, there are variables that have the potential to affect the eDNA assessment score from multiple sites sampled (see below and **Section 2.3**). Factors for consideration include:

- eDNA from the target species may have been introduced via other means, such as feces containing the DNA of the target taxa from a predator.
- eDNA from the target species may have been introduced by contamination during any stage of the process.
- Environmental conditions (e.g., high flows and subsequent dilution of DNA in the sample)
- Low densities of the target organism in the habitat (e.g., few individuals contributing DNA to the system)
- Large distance from the source organism (e.g., downstream from source in lotic systems (Deiner and Alternatt 2014))
- Inhibition arising from suspended materials in the samples causing reduced sensitivity of the qPCR (even after clean-up and/or sample dilution) and decreasing the probability of detecting DNA when it is present.
- Timing of sample collection relative to species phenology (e.g., sample collection in relation to the breeding season)

These factors should be considered when evaluating site results. Increasing the spatial sample replication at large lentic or high volume lotic sites, and increasing the number of replicates per sample location, will reduce the potential for false negative results. Inclusion of hierarchical lab-procedures that test for

amplifiable eDNA, before testing for eDNA from the target taxa (as described in Veldhoen et al. (2016)) are also strongly recommended to reduce potential for false negatives to occur.

Since eDNA is a rapidly developing field these protocols are anticipated to evolve as new processes and procedures are developed for both sample collection and analysis as well as QA/QC in both the field and lab. Thus, an open and communicative relationship between the field and the lab practitioners is essential to ensure appropriate interpretation of results. Ideally, this relationship is established during project planning, so that all parties can contribute to sampling design, including the number of replicates required, volume collected, filter type, spatial distribution of replicates, qPCR requirements and sample filtration and storage recommendations. When there are decisions to be made regarding assay design, including consideration of sympatric species in the study area, the practitioners conducting the assay design should be in discussion with the practitioners conducting the fieldwork and come to an efficient and consensus-based choice considering the desired application of the test, available data, and genetic differentiation among species. Any additional limitations identified within the study design (e.g., sampling outside of the species breeding window) or conflicting results (e.g., negative results at a previously known positive site) should also be discussed among the practitioners.

When results are returned from the laboratory, any caveats or sources of uncertainty should be identified (e.g., potential errors in sample handling or removal of inhibitors that may have reduced detection probability). Results that are unexpected should be discussed to ensure results are re-checked if necessary. In some cases the lab may re-analyze samples to ensure the result is consistent (QA/QC lab guidelines recommend storing half of the membrane for this purpose). Conclusions about the biological interpretation of unexpected results should be reached through discussion between lab and field personnel and ideally should be made following procedures for interpretation outlined in **Appendix C**.

9.0 REFERENCES

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APPENDIX A

eDNA Equipment and Materials

Description and Quantity of Required eDNA Materials and Equipment

Item / Description	Description	Purpose	Quantity Required
Field Collection Materials			
Nalgene bottles	• Material must be **polypropylene (PP)** • Volume can be 250 mL or 1 L (contingent on the species and the system)	Grab samples for offsite filtering	One bottle per replicate
Nitrile gloves	Non-powdered, disposable	Prevent contamination of samples	One set of gloves per site
Water quality meter	Capable of reading temperature, pH dissolved oxygen and conductivity	Collection of water chemistry data to facilitate calculation of detection probabilities	One
Cooler and ice	Re-useable ice packs are recommended	Keep water samples cool while transporting for offsite filtering	Various depending on quantity of sample bottles
Filtration Materials			
Sterile filter funnels (250mL capacity)	• Nalgene Polypropylene Analytical Test Filter Funnel with Grid and Sterile • 47 mm diameter membrane, 0.45 µm pore size	Filter the site water and capture DNA on the cellulose nitrate membrane	One filter per sample site (recommend ordering extras)
Gast vacuum pump (recommended option)	• Vacuum/pressure diaphragm pump with gauges, regulators, and relief valve, single head, 1.1 cfm, 115 VAC	Create vacuum to filter water through membrane	One each
250 mL Nalgene wash bottle	LDPE polyethylene wash bottles	Squeeze bottle with: (i) Ethanol (ii) Distilled water rinse (iii) Bleach rinse	Three
1L Vacuum flask with tubulation	Polypropylene 1L filtering flask	Create vacuum to filter water through membrane	Two (recommended)
Forceps/tweezers	Stainless steel, straight tweezers	Remove the filter membrane from the funnel and place in vials	One
#8 rubber stopper	#8 size fits snugly into the vacuum flask opening to create airtight seal	Connects filter funnel to the vacuum flask	One per vacuum flask
Silicone tubing	Tubing for pump to flask	Attach the vacuum flask and vacuum pump	10 ft length

Item / Description	Description	Purpose	Quantity Required
Y-splitter	For double pumping	Split line	1
Shut-off valve	One way shut off	To control suction	2
Nitrile gloves	Non-powdered, disposable	Prevent contamination of samples	At least one box
Filter Preservation Materials			
Silica beads	Self-indicating silica desiccant beads	Dries and preserve DNA present on membrane	Two tablespoons per sample site
Coin envelopes	3x5" small envelope	To store filters	As required
Whirl-paks	4 oz bags with white label are recommended	To transport vials individually	One whirl-pak per replicate
95% or 100% molecular grade ethanol	Not denatured	Preserve any DNA present on membrane	2mL per sample
mL cryogenic vials with o-ring	• Extra-thick walls for superior puncture and rupture protection, imprinted writing area, graduation marks, silicone rubber seal ring, and self-standing base	Storage of filter papers to preserve and ship to lab for analysis	One vial per sample

APPENDIX B

Sample Data Sheets

Appendix B – Table 1 eDNA Sample Data Collection Fields

Date:			
Site ID:			
UTM Location Zone:			
Easting:		**Northing:**	
Collector Initials:			
Collection Time:			
Weather Cloud Cover (%):		**Air Temperature:**	
Sample Volume:			
Water Chemistry			
Temperature (°C):			
Dissolved oxygen (mg/L):			
pH:			
Conductivity (µs/cm):			

Appendix B – Table 2 Example Data Collection Form to be Submitted to the Lab with Preserved Membranes

Site Name	Unique name (Gazetted Location) or number per collection site
Sample ID	Unique sample number per site
Collection Date	Date that samples were collected in the field
Collection Time	Time that samples were collected
Collector Initials	Initials of collector
Species to Test	Unique letter (a,b,c) per replicate
(sps code) Test Result	Target species to be tested for (repeat column for each species)
Zone	From GPS
Easting	From GPS
Northing	From GPS
Sample Contents	Do not include this column in the hardcopy going to the lab. This will ensure QA/QC of lab procedure.
Filtration Date	Time that samples were filtered
FiltrationStart Time (hh:mm)	Start time of filtration for each sample
Filtration End Time (hh:mm)	End time of filtration for each sample
Initial Sample Volume (mL)	Sample volume collected in the field
Final Sample Volume (mL)	Total volume filtered through the membrane

APPENDIX C
Analysis of Lab Results

Analysis of Lab Results

Analysis of eDNA samples may vary between labs depending on specific practices and procedures. The following information illustrates a step-wise approach in the design, validation and execution of qPCR-based analysis of eDNA conducted by the Helbing Lab at the University of Victoria Department of Biochemistry & Microbiology.

Each eDNA water sample replicate is analyzed using eight repeated qPCR analyses (hereafter referred to as qPCR replicates) as per methods described by Veldhoen et al. (2016). In experimental data from this lab, eight qPCR replicates were demonstrated to be appropriate, based on power analysis, to provide adequate confidence to reduce the potential for error in a standard binomial analysis (Veldhoen et al. 2016).

As a first step, before samples are tested for eDNA from the target taxa, each sample is tested for the presence of inhibitors of the qPCR assay and to ensure field filtration methods have effectively isolated eDNA (Veldhoen et al. 2016). To perform this initial screening, the lab runs an internal control to confirm that the sample contains amplifiable DNA (Veldhoen et al. 2016). If the sample is confirmed to have amplifiable DNA, further qPCR assessment for the target taxa is conducted. If the sample fails this initial screening, further evaluation is necessary to determine the cause and appropriate steps taken (such as sample clean up if inhibitors are suspected or resampling if an error in collection is suspected).

Assignation of results for each WATER SAMPLE REPLICATE

qPCR results are analyzed and interpreted for each **water sample replicate** as follows:

- A **positive water sample replicate** qPCR score indicates positive evidence of the species' DNA presence. It is defined as greater than or equal to three of eight qPCR replicates that result in a positive detection signal for the target taxa. (Note: this is higher level of evidence than that required by most other labs but is recommended, by Veldhoen et al. (2016), based on findings from test results).
- A **negative water sample replicate** qPCR score indicates no evidence of the species' DNA presence. It is defined as less than or equal to two of eight qPCR replicates that result in a positive detection signal for the target taxa.

The assignation, for reach **SAMPLE** replicate may be done by the lab as it is naïve to the results of the other replicates and does not consider site context (connectivity and adjacency to extant populations of the target taxa), habitat suitability or condition and/or species ecology (distribution, motility and dispersal capabilities).

Assignation of results for each SITE

The preferred approach for site level scoring is to collect three water samples per site; however, in cases where a species' is known to have high rates of detectability, using eDNA methods, and this understanding is based on consideration of prior eDNA studies for that specific target taxa (e.g. tailed frog or coastal giant salamander) under similar conditions two samples may be presumed adequate in study design[14].

Assignation of positive or negative values at the **SITE** level is based on consideration of all three (or in limited cases as described above) two filtered 1L water samples collected from each site. When assigning a result at the site level the principal investigator (i.e. a qualified biologist) will be aware of the results of each replicate collected at that site. In addition, the principal investigator should be well informed regarding the ecology (distribution, motility and dispersal capabilities) and habitat selection preferences (i.e. habitat suitability) of the target taxa. In addition, based on this knowledge and in consideration of existing information already known for the target taxa the principal investigator may also consider site context (i.e., connectivity and adjacency to extant populations of the target taxa). As such, the principal investigator should use the following decision criteria for assignation of result at the site level:

- If any of the replicate samples at a site yield a positive qPCR result for three or more of eight runs (≥3/8) the site is categorized as **positive**, regardless of the other sample's score.
- If at least one of the samples yield a positive qPCR result for two of eight runs (i.e. 2/8) and the other replicate(s) receive(s) a score >0 the site is categorized as **suspected**.
    - For studies with triple replicates qPCR results are assigned 'suspected' status if:
        - 2/8, 2/8, 2/8,
        - 2/8, 2/8, 1/8, or;
        - 2/8, 1/8, 1/8.
    - For studies with double replicates qPCR results are assigned 'suspected' status if:
        - 2/8, 2/8 or
        - 2/8, 1/8
- If all samples yield a positive qPCR results for ≤1/8 runs the site is categorized as **negative**.

In this case all replicates are being considered. During reporting, the eDNA site assignation should be presented and considered in the context of the species ecology with consideration of both habitat connectivity and quality and adjacency of extant sites.

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[14] Note that calculation of false negative rates will have reduced confidence and sites with low concentrations of eDNA are more likely to remain undetected.

APPENDIX D

Assay Status for Freshwater Species in BC

Appendix D Biological Assay Status and Conservation Rank for Freshwater Species validated by the University of Victoria Genetics Lab for use in British Columbia (unless otherwise noted). Note that this list will evolve as additional research leads to development of additional assays.

Taxonomic Group	Common Name	Scientific Name	SARA Status[1]	BC List[2]	Assay Status[3]	Access
Amphibians	Rocky Mountain tailed frog	*Ascaphus montanus*	1-E (2003)	Red	Complete	Veldhoen et al. 2016
	Coastal tailed frog	*Ascaphus truei*	1-SC (2003)	Blue	Complete	Veldhoen et al. 2016 or B. Murray
	Oregon spotted frog	*Rana pretiosa*	1-E (2003)	Red	Complete	C. Helbing
	Columbia spotted frog	*Rana luteiventris*	--	Yellow	Complete	C.Helbing
	Red-legged frog	*Rana aurora*	1-SC (2005)	Blue	Complete	C.Helbing
	American bullfrog	*Lithobates (Rana) catesbeianus*	--	Exotic	Complete	Veldhoen et al. 2016
	Great basin spadefoot	*Spea intermontana*	1-T (2003)	Blue	Complete	Hemmera / C. Goldberg or B. Murray
	Western toad	*Anaxyrus boreas*	1-SC(2005)	Blue	Complete	C.Helbing
	Blotched tiger salamander	*Ambystoma mavortium*	1 (2003)	Red	Not validated	Hemmera / C. Goldberg
	Coastal giant salamander	*Dicamptodon tenebrous*	1-T (2003)	Red	Not validated.	C. Goldberg
	Cascades Frog	*Rana cascadia*	--	Yellow	Complete	C. Helbing
Mammals	Pacific water shrew	*Sorex bendirii*	1-E (2003)	Red	Pending	C. Helbing

Taxonomic Group	Common Name	Scientific Name	SARA Status[1]	BC List[2]	Assay Status[3]	Access
Fishes	Bull trout	*Salvelinus confluentus*	--	Blue	Not Validated	C. Goldberg; United States Forest Service (USFS) Rocky Mountain Research Station (different assays)
	Chinook salmon	*Oncorhynchus tshawytscha*	--	Yellow	Not Validated	Laramie et al. 2015
	Rainbow trout	*Oncorhynchus mykiss*	--	Yellow	Not Validated	C. Goldberg; USFS Rocky Mountain Research Station (different assays)
	Lake trout	*Salvelinus namaycush*	--	Yellow	Not Validated	C. Goldberg, run by USFS Rocky Mountain Research Station
	Brook trout	*Salvelinus fontinalis*	--	Exotic	Not Validated	Wilcox et al. 2013
Fungi	Chytrid	*Batrachochytrium dendrobatidis*	--	--	Not Validated	Boyle et al. 2004

Note:
[1] *Species at Risk Act* (SARA) listing: 1 = schedule 1, 3 = schedule 3, E = Endangered, T = Threatened, SC = Special Concern
[2] BC List: Red = Species that are extirpated, endangered, or threatened; Blue = Species of special concern; Yellow = species and ecological communities that are secure.
[3] Complete = a specific qPCR assay for this species has been developed and validated against for use in BC by UVic.
[4] Pending= a qPCR assay has been developed, but there is evidence for cross-reactivity with the closely-related species.
[5] Not validated = a qPCR assay has been developed for this species but has not been validated for use in BC by UVic.