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Environmental DNA Standardization Needs for Fish and Wildlife Population Assessments and Monitoring

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Foreword

Information from fish and wildlife population assessment and monitoring projects inform important considerations during natural resource management planning processes. Conventional survey methods employed as standard tools are fraught with a high likelihood of missing target species of interest, particularly ones that live in sparse population densities, occur in habitats that are difficult to survey, or are inconspicuous. Each of these traits result in imperfect detection probabilities.

The application of genetic methods in applied field ecology studies began as early as 1987 with the use of genetic material, or deoxyribonucleic acid (DNA), to profile microbial communities in sediment samples (Taberlet et al., 2012). In the early 2000's, the term "environmental DNA" (eDNA) was coined to refer to DNA that can be isolated from environmental samples such as sediments, soil, water, or air without first isolating the target organism (reviewed in Taberlet et al., 2012). eDNA can also include DNA recovered from whole organisms during the extraction process (e.g., from microbes in sediment samples). Just over a decade ago, Ficetola et al. first applied eDNA methods in the context of wildlife inventory and assessment to detect an invasive frog species from water samples in a field-based ecological study in France (2008). Since its inception, eDNA methods have expanded and evolved rapidly to detect "at risk" and invasive species with an exponential rate of uptake in aquatic and terrestrial environmental studies worldwide. As of February 2019, there were 2,617 scientific papers within "environmental DNA" or "eDNA" topic areas in the Web of Science with 68,111 citations. Half of the papers were published within the last 4 years.

Since the first Canadian application of eDNA methods for wildlife inventory (Bronnenhuber and Wilson, 2013), its use has spread rapidly across the nation, promoted by collaborative engagement between US and Canadian academic laboratories and supported by privately-funded studies from practitioners within the environmental sector. This uptake is motivated by increased recognition, from practitioners and resource managers, noting gains in efficiency and accuracy with reduced negative effects conferred to the focal taxa and its environment.

While the fundamental principles of eDNA detection, project design, and workflow are the same, considerations for sample collection, data generation, and interpretation are dictated by individual project goals, needs, and constraints. As more and more opportunities arise for the application of eDNA methods, it has become evident that there are varying levels of rigour, transparency, and reporting of results. As such, multiple technologies and approaches contribute to the brisk pace of evolution and growth of this transformative method. This variability is normal for a new field of innovation, but may ultimately negatively impact acceptance of eDNA methods if not reconciled with development of appropriate guidance for uptake.

To analyze the opportunity for eDNA methods to augment or replace conventional surveying techniques, this report draws upon recent discussions/presentations at scientific meetings, published research, a questionnaire, and follow-up interviews. In 2018 these included:

- a regional eDNA workshop at the University of Victoria;
- a national workshop "Pathways to Increase Standards and Competency of eDNA Surveys (PISCeS)" at the University of Guelph; and
- targeted sessions at the 45th annual Canadian Ecotoxicity Workshop in Vancouver, British Columbia and the Society for Environmental Toxicology and Chemistry 39th Annual meeting in Sacramento, California.

While there are many published studies and reviews on eDNA, we regret that it was not possible to include all of them within the confines of the research paper. Rather, examples have been selected to illustrate key points. Interested readers are encouraged to further consult the many excellent in-depth reviews on aspects of eDNA methods and application (Coble et al., 2019; Cristescu and Hebert, 2018; Goldberg et al., 2016; Herder et al., 2014; Hering et al., 2018; Littlefair et al., 2017; Porter and Hajibabaei, 2018; Roussel et al., 2015; Taberlet et al., 2012; Thomsen and Willerslev, 2015).

In this research paper, relevant aspects of eDNA implementation are discussed, including:

1. Common practices (i.e., techniques) for sample collection, analysis, and interpretation;

2. Accuracy, repeatability, efficacy, and reliability (relative to existing conventional methods);

3. Areas that may benefit from standardization and further study.

This research paper describes current common practices for implementation of eDNA methods, identifies knowledge gaps and critical considerations during implementation, and evaluates the motivation for standardization of various aspects of these methods. This paper is intended to guide, solicit support, and encourage development of rigorous standards by providing a comprehensive overview of current understanding and anticipated challenges associated with widespread adoption and implementation of eDNA methods in applied biomonitoring within the environmental sector.

Executive Summary

Introduction

The effective management of natural resources requires accurate and timely information regarding the occurrence and distribution of 'at risk' and invasive species, information about ecosystem health, data on wildlife population trends, and the assessment of ecosystem responses to environmental perturbation and remediation (Kelly et al., 2014). For fish and wildlife taxa, conventional survey methods generally rely on direct observation of target taxa, which can be stressful and damaging to them and their habitat. Common sources of conventional survey bias may include variation in applied effort and observer skill, ease of sampling in different habitats, taxa-specific differences in detectability between seasons, taxa density and distribution, and sampling equipment.

A new approach to environmental assessments and monitoring involves sampling environmental DNA (eDNA) that is present in sediments, soil, water, or air through secretions, excretions, or exogenous sloughing of skin cells. It may also refer to whole organisms or parts of organisms that are present in environmental samples during the extraction process (e.g. benthic invertebrates). eDNA methods have the distinct advantage of using unique sequence segments of genetic material to identify taxa without coming into direct contact with the target taxa or performing onerous morphology-based taxonomic identifications. The high sensitivity of eDNA methods can dramatically increase detection rates, particularly for species that occur at low densities, have secretive ecologies, feature discontinuous distributions, or share morphological traits that confound accurate identification (Pfleger et al., 2016). Furthermore eDNA methods are more cost-effective, non-invasive, and more accurate than conventional survey methods.

Methods

Herein, a consensus-based synthesis of the current understanding of eDNA methods is presented with information drawn from available published literature; from expert-led interviews, discussions, and workshops; and from questionnaire responses obtained from experts, practitioners, end-user groups, and regulatory agencies across Canada, United States, and Europe.

eDNA Monitoring

eDNA methods require an effective synergy between field and DNA detection methodologies with several points of consideration that require attention during development, adoption, and implementation (Goldberg et al., 2016). Analytical techniques and technologies are rapidly evolving and the methods chosen depend upon the desired purpose to 1) detect taxa in a targeted fashion, or 2) assess community biodiversity. The former current approach utilizes some form of polymerase chain reaction (PCR) that specifically amplifies small amounts of target taxa DNA to enable analytical detection within an environmental sample. The latter utilizes various methods of high throughput sequencing such as metabarcoding (Porter and Hajibabaei, 2018).

Considerations Regarding eDNA Methods

Environmental samples are complex and the quality and quantity of DNA can vary substantially. As such, field and analytical components of eDNA methods face particular challenges that require a heightened awareness and attention to methodological requirements (Goldberg et al., 2016). Best practices strive to mitigate sources of false positive and false negatives, utilize eDNA methodology appropriate for study design objectives with appropriate regard to statistical power, include attention to quality control, and provide transparency in assessment of test performance (Cristescu and Hebert, 2018).

As eDNA methods are increasingly utilized for environmental assessment and monitoring activities, their results will have greater influence upon management decisions. Indeed, they have the potential to revolutionize environmental assessment and monitoring. Despite the power of eDNA detection methods, addressing specific issues to remove barriers to the adoption of eDNA methods and enhance confidence in their use is vital. These include quality issues with regards to accuracy and reliability, lack of accredited national standards for both sample collection and analysis, and the need for demonstrated competency and proficiency testing by practitioners.

eDNA Common Practices

Research efforts need to focus on the development of appropriately validated tests to address environmental and sampling factors that may affect eDNA detection, develop competency and proficiency testing for laboratory accreditation, promote inter-laboratory comparisons, and improve and enhance reference databases for DNA analysis.

Need for Standardization

The existence of appropriate standards will enhance confidence in the transformative use of eDNA detection methods in applications that inform environmental management decisions. It is increasingly relevant to assess and consolidate robust, reliable methods for eDNA implementation. Practitioners have a responsibility to support and, as appropriate, adopt a leadership role in bringing together multiple stakeholders nationally and internationally to facilitate discussion and harmonization of best practices for eDNA methods, both within Canada and internationally.

Fish and wildlife population assessment and monitoring requires timely and accurate detection of "at risk" and invasive species to make resource management decisions.

1. Introduction

Effective management of natural resources requires accurate and timely information regarding occurrence and distribution of "at risk" and invasive species, information about ecosystem health, data on wildlife population trends, and assessment of ecosystem responses to environmental perturbation.

Environmental assessment and monitoring support natural resource managers in making permitting decisions and assessing regulatory compliance. Conventional surveys of fish and wildlife populations generally rely on the direct observation of the **target taxa**, but these methods can be stressful and damaging to the focal species and their sensitive habitats. Methodological error or bias introduced during sampling are also primary concerns.

In contrast to conventional surveys, environmental DNA (eDNA) methods rely on the detection of the genetic material specific to the taxa of interest that are shed into, and can be isolated from, environmental samples such as water, sediments, or soil without direct contact with the target taxa. The high sensitivity of eDNA methods can dramatically increase detection rates (power and accuracy), particularly for species that occur in low densities, have secretive ecologies, feature discontinuous distributions, or share morphological traits that confound accurate identification (Pfleger et al., 2016).

The credibility of any survey, including eDNA survey data, however, depends on statistical validation and verification. Accurate results require rigour during assay design and validation, survey design, field sampling, sample processing, analysis, and interpretation. As the methods used to detect eDNA are rapidly evolving, understanding the benefits and disadvantages of various approaches and recognizing which method is best-suited for a specific project can be quite daunting. Indeed, understanding the likelihood of various approaches to mitigate false positive and false negative results is currently the most important aspect of eDNA research (Cristescu and Hebert, 2018).

Fish and wildlife assessments and monitoring...

- A **taxon** (plural **taxa**) is one or more populations of an organism put into easily classified groups. For example, sometimes a taxon can be a species and sometimes a taxon is a group of species.
- **Conventional survey methods** generally rely on direct observation of target taxa that can be stressful and damaging to them and their habitat.
- **Environmental DNA (eDNA)** methods rely on the detection of the genetic material specific to the taxa of interest found in water, sediment, or soil samples without direct contact with the target taxa.

An eDNA test can be performed on water samples taken from easily accessible areas to detect "at risk" and alien invasive species.

2. Research Objectives

Herein, a consensus-based synthesis of the current understanding of eDNA methods is presented. The main points of consideration that require attention during implementation, adoption, and development of eDNA methods are discussed. This research paper broadly reviews the status of eDNA techniques currently used for sample collection and handling, eDNA extraction, and current analytical DNA detection methods, including polymerase chain reaction (PCR) and high throughput or next generation sequencing-based approaches.

This research paper summarizes information drawn from available published literature; expert-led interviews, discussions, and workshops; and written input solicited from experts, practitioners, end-user groups, and regulatory agencies across Canada, the US, and Europe. This approach was employed to facilitate a fulsome discussion of previous, current, and anticipated challenges associated with the implementation of eDNA methods. It is hoped that this research paper will provide a valuable reference tool to promote alignment, synergy, and collaborative development of eDNA methods amongst practitioners by highlighting areas where standardization and collaborative future research may be beneficial.

Ultimately, this paper will serve to focus attention on key aspects of eDNA methods in the interest of increasing rigour and reliability during application and interpretation of eDNA methods and results. This research paper was prepared to promote recognition and adoption of shared responsibility, by key organizations representing all end-user groups, for standardization of recognized key aspects of eDNA methods in practice nationwide.

In order to achieve these objectives, an overview is provided of the current standard of practice for fish and wildlife monitoring and assessment using conventional survey methods. The fundamental eDNA concepts are then reviewed and current considerations, along with the most common practices for eDNA analyses, are discussed. Finally, the needs for standardization are articulated as well as the associated research activities required to meet those needs.

3. Methods

To provide focus on key aspects of eDNA methods that warrant specific attention, input was solicited through a questionnaire, email feedback, and follow-up interviews from leading practitioners, including academic researchers, consultants, eDNA testing laboratories, and regulatory and government agencies. The written questionnaire reflects the collective input of 40 individuals in Canada, the US, and Europe. More than one third of respondents had over five years of experience with eDNA and half had used eDNA in 10

or more projects to date. The most common role of respondents was in eDNA results analysis followed by field and lab practitioners and decision-makers.

To ensure inclusion of current views from multiple perspectives, the expertise was evenly distributed across field and lab practitioners, eDNA result analysis experts (including statistics and modeling), and decision makers.

A questionnaire was developed and distributed to 60 recipients to solicit input into all key areas of consideration in this research paper. This questionnaire was sent to pre-selected qualified, recognized experts to solicit their experience and perspectives in relation to practices and critical considerations currently being used during implementation of eDNA methods pertaining to fish and wildlife assessments and monitoring.

As a key component of the questionnaire's development, an independent advisory committee assembled by CSA Group vetted each of the 29 questions. The nine-page questionnaire was divided into four topic areas, including:

1. eDNA implementation

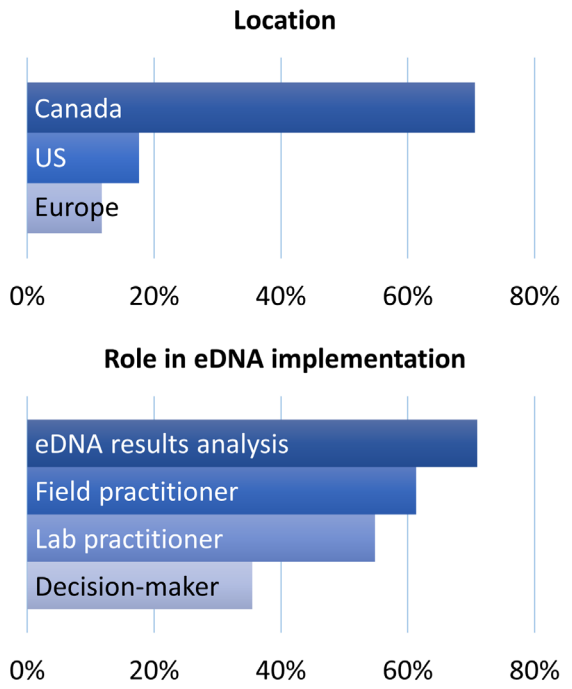
2. eDNA sampling and handling methods

3. eDNA common practices; and,

4. Need for standardization.

A list of candidate expert respondents was assembled based upon review of published literature, attendance at pertinent eDNA conferences, and consideration of peer recommendations and respondent affiliation. This approach was used to ensure representative feedback from academics, qualified environmental practitioners (with experience implementing eDNA projects), regulatory agencies, provincial and federal government staff, and eDNA service providers. Questionnaires were distributed electronically by email on January 27, 2019. Two follow-up emails were sent to encourage participation, as required, and the last accepted questionnaire was received on February 28, 2019.

Forty leading international experts responded to the eDNA questionnaire...



Of the 60 questionnaires sent out, 31 were returned by the requested deadline. Of these, five questionnaires were returned with collaborative completion by multiple individuals within an organization. Only one of the returned questionnaires was not used in the data analysis as the respondent did not yet have sufficient relevant applied experience with eDNA. In total, the collective viewpoints of 40 respondents were captured in the questionnaire responses; this provides a reasonable representation of current informed views on eDNA practice. When completing the questionnaire, respondents were asked to complete only those questions that they felt were relevant to their personal experience. This valuable input was incorporated into our synthesis for each of the perspectives afforded treatment in this research paper.

Responses were tabulated using the "form distribution" and "compilation" functions in Adobe Acrobat X Pro®; this program converts the responses into a comma-delimited output file to facilitate efficient analysis in Microsoft Excel®.

Two categories of questions were posed in the questionnaire:

1. **Objective Questions:** The majority of the questions posed in the questionnaire relied upon objective criteria rankings. Responses were enumerated and reported by percentage of each assignation or value. Where pertinent, the percentage of respondents with relevant experience were indicated to provide appropriate context.

2. **Subjective Questions:** Following the questions that applied an objective rank or score to an answer, respondents were given the opportunity to expand in free-form to provide additional information or context. All free-form answers were reviewed. Care was taken to ensure that all respondent viewpoints were accurately reflected in the current document, as were relevant and appropriate to the content of this research paper. To maintain confidentiality, quotes from individual respondents were avoided. Instead, respondents' viewpoints were accurately and carefully paraphrased. References to published literature were added to support the information provided, to explore perspectives, and to delve more deeply into the ideas and viewpoints presented by respondents.

This approach imparts a unique perspective to the research paper by employing a collective, considerate synthesis that includes and faithfully represents multiple perspectives. It was also deliberately employed to enhance the value of the research paper by fostering stimulation of discussion and to encourage sharing of innovative and diverse approaches, as employed by experts, to advance the highly technical and exciting field of eDNA practices.

4. Conventional Fish and Wildlife Population Assessments and Monitoring

Effective and responsive environmental management requires a consistent source of accurate information about the ecosystems or species under consideration (Kelly et al., 2014). This requirement often is challenged by imperfect detection rates from available methodologies. In addition, sources of bias may arise from differences in applied effort, observer skill, behavioural attributes of target taxa, population density, habitat niche requirements, timing of sample collection, and sampling conditions. Each of these factors influence the ease with which a given taxon may be detected during the survey. Inaccurate detection rates will ultimately compromise management objectives and may expose taxa and ecosystems to deleterious influence. Regardless of the applied method, sources of bias deserve and require attention to ensure accurate information informs management decision-making processes.

When variation between samples is high, the precision of the estimated population parameter (presence) is low. Variability in detection rates are improved with repeated sampling of the same population, but this additional effort is often cost-prohibitive, particularly when using labour intensive methods. Regardless of costs, consideration for the efficacy of sampling methods is required to minimize sources of error (bias) and reduce variation between samples when informing management of wildlife resources.

Measures to control bias resulting from observer skill, variable survey effort, and restrictions on appropriate survey conditions (including timing and weather) are often costly. In addition, as is true for any method, considerations also extend to equipment costs and worker safety. Conventional survey techniques used to collect and analyze species distribution are widely characterized as being labour intensive (Kelly et al., 2014). Successful implementation often requires skilled observers to design and implement the sampling program. Finally, direct and indirect disturbance to focal taxa are also important considerations that must be mitigated. Concerns regarding pathogen transfer,

disturbance to sensitive habitats, and the potential for direct mortality (e.g., inadvertent crushing, stress, etc.) warrant serious consideration during implementation of any fish or wildlife survey.

Common sources of conventional survey bias may include...

- **Applied effort and observer skill –** the number of individuals detected increases with sampling effort and observer familiarity. Effort and skill are difficult to standardize because increased experience results in constant improvement in observer skill.
- **Ease of sampling in different habitats –** some habitats are more conducive to surveying and this affects taxa detection rates. Different amounts of effort or survey methods may be needed.
- **Taxa-specific differences in detectability between seasons –** in addition to behavioural attributes that vary between taxa (e.g., active during the day versus night, burrows in sediments, hides in logs, etc.), many taxa will also exhibit differences in detectability between sex and age class. For example, adult males may be noisier and more conspicuous and easier to capture than females or juveniles.
- **Density –** variability in taxa density and distribution (evenly dispersed versus clumped distribution) has a pronounced influence on detection rates.
- **Sampling equipment –** choice must reflect best available information appropriate for target taxa.

As eDNA methods are largely focused on survey of freshwater aquatic systems, it is relevant to compare these methods with conventional methods currently used to survey amphibious and aquatic taxa in environments with flowing (e.g., rivers and streams) or "stationary" bodies of water (e.g., ponds, lakes, and wetlands). In that context, as application of eDNA methods continue to expand to new environments (e.g., marine and terrestrial environments), future comparative cost-benefit analyses (see Section 5) may be expanded to include additional conventional sampling methods to determine if eDNA might provide a more cost-effective and accurate solution for survey of these environments. In that context, to ensure relevance to the majority of the current applications of eDNA methods, eDNA is compared with conventional methods for survey of taxa associated with both types of water features; these include surveys of aquatic-breeding amphibious taxa and freshwater fish.

When conducting surveys for various ecosystem components, it is necessary to consider both geographic scale and inventory intensity – both factors influence decisions involving survey design and during selection of appropriate method(s) to meet the research objective.

Identifying an inventory scale...

- ...is important as some target taxa occupy discrete geographic areas or special habitat types while others are distributed over a large area.
- ...should include consideration of the extent, magnitude, duration, and severity of impact on the environment.
- ...is important as costs typically increase in direct proportion to the size of the study area, the intensity of survey information required, or the need for accuracy and precision between samples.

Scale: Inventory may be conducted on a continuum of scales. Inventories focused on small (localized) areas can often be conducted to a higher degree of survey intensity. However, as the geographic scale of application increases, survey methods should consider common practices and effort should be normalized where feasible to allow measurement of detection rates between multiple systems. Standardization allows consistent measurement of effort between sample locations or over time. Quantitation of survey effort is often achieved by accurate measurement of:

1. Time (e.g., person hours/system) (i.e., time constrained physical searches);
2. Amount of area surveyed (i.e., area constrained physical searches); or
3. Capture mechanisms (e.g., number of traps, number of eDNA samples collected per unit area, etc.).

Intensity: Inventory may also be completed at three broadly recognized levels of intensity, but the need for accurate information needs to be balanced with considerations regarding the impact of the inventory

The pacific giant salamander (Dicamptodon tenebrosus) is difficult to observe through time constrained physical survey techniques.

upon the target taxa and its environment. These categories are:

1. **Presence/not-detected[1]:** These inventory methods are arguably the most common inventory method used to inform species and ecosystem management. Ascertaining presence, or inferring low likelihood of presence based on failure to detect the target taxa, is a key component of required information in species management decision-making processes.
2. **Relative abundance:** Determining relative abundance over time or between different geographic areas is the next tier of effort, or intensity. Normalizing measures of effort, with repeated sampling or measurement of abundance of target taxa, is almost always recommended. The need for precision typically results in a proportional increase in survey costs. Relative abundance indices are often desirable when trying to assess long-term population trends in response to environmental perturbation; or when trying to assess effects to an environmental stressor.
3. **Absolute abundance:** This level of survey intensity is often destructive to the target taxa or the environment as it requires a closed system unit that is typically sampled intensively to ensure enumeration of all individuals. The habitat (or target taxa) needs to be sampled or trapped exhaustively, often with a lasting effect to the habitat at the survey site. Absolute abundance "counts" are often not considered an ethical option due to impacts on the focal taxa and sensitive habitats.

For survey of amphibious taxa such as frogs, toads, and salamanders, conventional survey methods commonly employed during survey of aquatic ecosystems are either area-constrained or time-constrained physical searches. Unfortunately, controlling observer and effort bias is challenging for taxa and ecosystems that have low levels of detection. The accuracy of the estimate is increasingly challenged for species that are cryptic, secretive, have evasive behavioral response to disturbance, or feature discontinuous distributions. Of paramount concern, conventional survey methods are widely accepted and applied despite a lack of consideration for concerns regarding known imperfect detection and the method's known deleterious effects on sensitive species or their habitats.

For surveying fish in natural systems, electrofishing is widely applied yet concerns have been raised regarding methodological effects. In addition, detection rates are generally low (Benejam et al., 2012; Lyon et al., 2014), particularly when compared with eDNA methods for taxa in rivers and streams. Benejam et al. (2012) compared estimates between four electrofishing crews; results suggested that capture probability depends heavily on a number of factors (such as species, size, and sampling site). Observer bias is negligible for assessment of species richness and composition, but considerable for fish abundance (Benejam et al., 2012). Peterson et al (2004) evaluated the accuracy of estimates of fish abundance between single-pass

[1] Often incorrectly referred to as "presence/absence". This point warrants emphasis as absence cannot be inferred unless conducting an absolute abundance inventory.

and multi-pass electrofishing methods for bull trout *(Salvelinus confluentus)* and westslope cutthroat trout *(Oncorhynchus clarki lewisi)*. In this study, capture efficiency was low for the first electrofishing pass (mean, 28%) and decreased considerably with successive passes (mean, 1.71 times lower). The authors suggested that fish were responding adversely to the electrofishing procedures. Again, this illustrates a source of bias discussed earlier. The same considerations should also be extended to species incidental to the survey, including tailed frog and coastal giant salamander larvae (and adults). These two listed species frequently occur as "by-catch" during electrofishing in the Pacific Northwest. However negative effects of electrocution are not quantified or considered despite federal laws that afford wildlife legal protection from harm and harassment.

Occupancy Modelling...

- ...is a statistical method for estimating the probability that a site is occupied by target taxa.
- ...yields improved accuracy in distribution estimate by understanding and correcting for variation between estimates.
- ...can only be conducted when particular assumptions are met through survey design.

Conventional surveys are still applied widely and accuracy can be improved through occupancy modelling techniques (Dorazio and Erickson, 2018). Fortunately, conventional methods are receiving increased scrutiny regarding their associated negative impact to target taxa and ecosystems, variability, and low efficacy. eDNA methods can address these shortcomings and are receiving increased attention as a replacement, or means to augment, conventional survey methods.

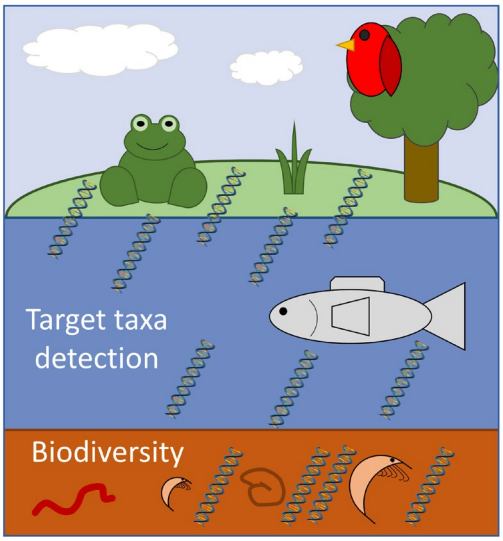
5. eDNA Monitoring

5.1 Definition

eDNA refers to genetic material (DNA) that is present in environmental samples such as sediments, soil, water, or air through secretions, excretions, or exogenous sloughing of skin cells. It may also refer to whole organisms or their parts that are present in environmental samples during the extraction process (e.g., benthic invertebrates) (reviewed in Taberlet et al., 2012).

While comprised of the same four fundamental building blocks collectively called nucleotides, the order in which the nucleotides are assembled is unique to an organism and, thus can be used as a means to identify taxa, populations, and even individuals. An environmental sample such as water, sediment, air, or soil, will contain a complex mixture of DNA from many organisms. However, the DNA can be isolated and separated through various analytical techniques for target taxa detection or determination of biodiversity.

Organisms constantly shed DNA into their immediate environments



Nucleotide building blocks make up DNA whose unique arrangements allow for the detection of target taxa or the determination of biodiversity in environmental samples.

Motivated by the desire for more efficient and timely results, a diversity of innovative and creative eDNA sampling and analytical tools have been used. These include portable pump systems, portable field-filtration units, portable (compact) thermocyclers, new collection and sample preservation techniques, and various

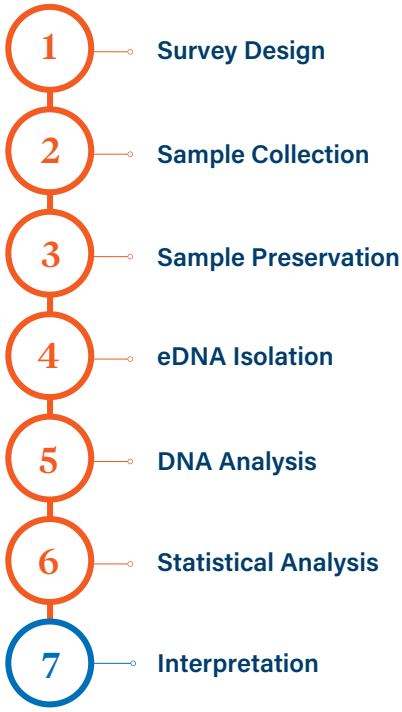
approaches to analytical methods and data interpretation (Coble et al., 2019; Goldberg et al., 2016; Herder et al., 2014; Veldhoen et al., 2016). The techniques and technologies are rapidly evolving. In general, detection of target taxa utilizes some form of polymerase chain reaction (PCR) that specifically amplifies small amounts of target taxa DNA to enable analytical detection within an environmental sample. Community assessment of biodiversity can also be accomplished through various iterations of high throughput sequencing.

The methods used for detecting eDNA from a wide range of complex environmental samples must rise to multiple challenges. Inappropriate consideration of critical sources of error introduced during the process may bias the inference of taxa presence. Thus, the use of eDNA methods requires a heightened awareness and attention to methodological requirements to reduce the potential introduction of error (Goldberg et al., 2016). In addition, interpretation and reporting of eDNA results requires appropriate consideration of multiple factors (Goldberg et al., 2016), including disclosure of assumptions, applied interpretive criteria, mitigation of potential sources of both Type 1 (false positive) and Type 2 (false negative) errors, and accurate conveyance of uncertainties relating to temporal and spatial characteristics associated with species detection using eDNA methods. Given fish and wildlife surveys are used to make regulatory and management decisions, the consequences of false positives and negatives can be quite costly in terms of time, money, or the environment.

Regardless of the choice of analytical method, the same fundamental sequential steps are followed within an eDNA survey (Figure 1). It is important to note that the field component is intimately connected to the subsequent eDNA analysis. Thus, an appropriate understanding of the limitations of the analytical techniques is essential. The best analytical practices cannot correct for poor sampling in the field and the best field practices cannot correct for poor analytical practices.

The vast majority of analytical methods are based upon the detection of DNA from the mitochondria within the target taxa cells. Cells have many mitochondria and each mitochondrion can contain from a few to thousands of DNA copies, each making this an abundant source of DNA (Clay Montier et al., 2009). All analytical methods identify target taxa through the unique arrangement of nucleotides, typically from mitochondrial DNA because it is present in high copy number relative to nuclear DNA.

Figure 1 – *eDNA method implementation steps.*



5.2 Methods for the Detection of Specific Taxa

For the detection of specific taxa, PCR is the method of choice. There are many versions of PCR-based techniques that have been used in the scientific literature, including semi-quantitative endpoint PCR, isothermal PCR, quantitative real time PCR (qPCR), and digital PCR. Endpoint PCR has inherent issues with sensitivity, specificity, and quantitation and is not recommended for eDNA detection. Digital PCR is highly sensitive and quantitative but is currently low throughput and there is limited access to specialized equipment. qPCR-based methods are the most accessible and scalable with many reasonably-priced thermocyclers

available. Methods based upon hydrolysis probe qPCR chemistry (e.g., TaqMan or equivalent) are the preferred analytical approach compared to endpoint-based PCR or the use of intercalating dye (e.g., SYBR Green) for qPCR in recognition of the ability to quantitate DNA and additional sequence-specific detection, respectively, that results from the use of the hydrolysis probe.

Quantitative PCR (qPCR)...

- qPCR results are easy to visualize, amenable to high throughput analyses, and allow reliable quantification of DNA molecules above the limit of quantitation.
- Methods based upon qPCR are preferred relative to endpoint-based PCR using gel electrophoresis.
- Hydrolysis probe-based techniques (using probes that bind specifically to the targeted DNA strand sequence) are preferred over the use of intercalating dyes that are not sequence-specific.

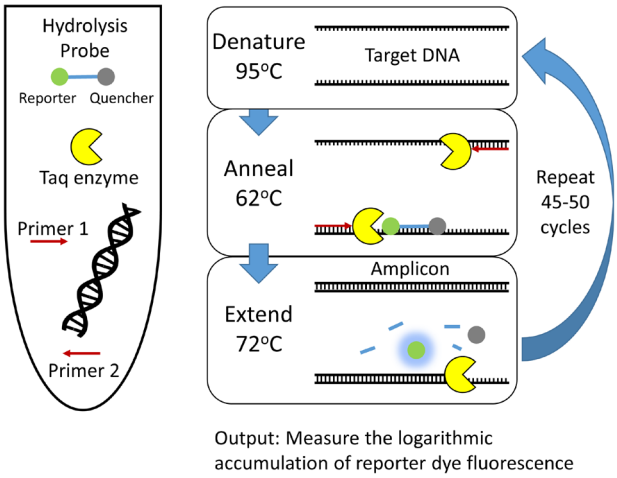
Specificity of the qPCR hydrolysis probe reaction is provided by two components: the locus-specific **primer** pair and the **hydrolysis probe**, as they pertain to a selected target **locus**. This target locus is most often derived from mitochondrial DNA and may be chosen from several candidates including genes or non-genic regions. The choice of locus is dictated by a variety of factors, including available sequence information for the target taxon and closely-related taxa that coexist in the sampling environment and suitability for primer/probe design.

The general principle of qPCR is as follows. A portion of the eDNA sample is mixed into a reaction tube and heated to **denature** the DNA into two separate strands. If the target DNA is present, the taxon-specific primers specifically **anneal** to it after denaturation and prime the synthesis or **extension** of a complementary DNA strand to create a new double-stranded piece of DNA called an **amplicon**. This effectively doubles the number of DNA molecules present in the sample. The hydrolysis probe allows for the actual detection of the amplicon produced through fluorescence. Amplicon amount is expressed as a $\mathbf{C_t}$ or $\mathbf{C_q}$ value and is directly proportional to the amount of original target DNA present. It is detected through successive cycles of denaturation, annealing, and extension by the accumulation of the reporter dye fluorescence. For very low amounts of DNA, detection is accomplished through increased sample replication and the presence of amplification by a binomial (detect/not detect) call.

qPCR at a glance...

- **Amplicon –** a segment of DNA that undergoes amplification. Its production requires the presence of target DNA.
- **Anneal –** to recombine single stranded DNA to double stranded form after separation of the strands by heat.
- **$\mathbf{C_t}$ or $\mathbf{C_q}$ –** the cycle where the accumulation of the reporter dye is reliably detected in a technical replicate of a DNA sample.
- **Denaturation –** to split apart double stranded DNA into two single strands. In qPCR, this is done with heat and is the first step in a PCR reaction.
- **Extension –** the third step in a PCR cycle that allows the complete formation of an amplicon and the release of the reporter dye.
- **Hydrolysis probe –** a short string of DNA that matches part of the amplicon sequence. It has a reporter and a quencher. The Taq enzyme releases the reporter from the quencher allowing it to fluoresce in proportion to the amount of amplicon produced.
- **Primer –** a short string of DNA nucleotides in a particular order that identifies DNA from a target taxon. Two primers are needed to synthesize an amplicon.
- **Target DNA –** a small piece of double stranded DNA that is derived from the target taxon. This piece of DNA is often a part of mitochondrial DNA and the area or locus that is chosen has desirable characteristics for test development. It is required to produce amplicons.
- **Test or assay –** refers to the combination of primers, probe, and reaction conditions to detect target taxon DNA.

Anatomy of a hydrolysis probe-based qPCR reaction...



The primers and hydrolysis probe confer specificity of DNA detection.

There are different types of reporter dyes that are commercially available and are compatible for concurrent use within a reaction for simultaneous detection of different targets. While the theoretical limit for multiplexing qPCR reactions is five tests, the multiplexing of assays can lead to reduced sensitivity and needs to be carefully validated prior to application. Practitioners may find that in reality three (including an internal positive control (IPC); discussed in Section 7) may be the upper limit for most reactions.

5.3 Methods for the Determination of Biodiversity

Another important aspect of fish and wildlife assessments is the determination and tracking of biodiversity, or species richness, within a given ecosystem. In contrast to the qPCR methods described above, where a single to a few taxa are examined at a time, an eDNA sample can be evaluated more broadly for composite taxa using methods that sequence isolated DNA fragments. While there are a wide range of techniques that can be employed for this purpose, the most frequently used is a **metabarcoding** approach (Porter and Hajibabaei, 2018).

Metabarcoding is a method of inferring taxa composition by comparing the DNA barcodes. DNA barcoding was introduced as a biological identification tool in 2003 using "universal primers" designed to a highly conserved region of the mitochondrial **cytochrome c oxidase I (COI)** gene (Hebert et al., 2003). These primers amplify DNA fragments from a wide range of taxa within a single sample. The amplicons are sequenced and organized into **operational taxonomic units (OTUs)**. The DNA sequences within the amplicons differ between taxa and act as identifiers or barcodes to infer the biodiversity within an environmental sample. Extensive reference nucleotide sequence databases such as the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Barcode of Life Data Systems (www.boldsystems.org) facilitate taxon identification. However, notable gaps for many species remain for various invertebrate taxa.

Metabarcoding...

- ...is a high throughput sequencing method of inferring taxa composition by comparing the DNA barcodes isolated and sequenced from an environmental sample to a standardized reference database for phylogenetic identification.
- ...is currently the most popular genetic method for biodiversity and biomonitoring of community samples (e.g., benthic invertebrates).

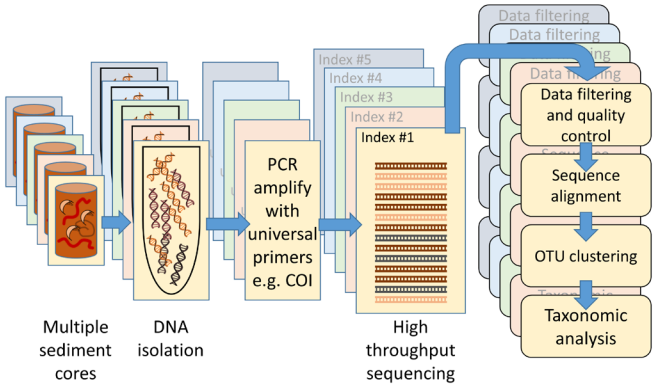
Adopting methods originally applied to studies on microbial populations in environmental samples, metabarcoding methods frequently combine eDNA isolation with **high throughput sequencing** methods. For community analysis, DNA is isolated from a sample and then amplified using universal primers. Each sample gets a unique tag or index that makes it possible to mix DNA from multiple samples together for high throughput sequencing in one sequencing run. The indices allow for subsequent bioinformatics separation of the DNA sequences post-sequencing. This ability to pool samples for sequencing greatly lowers the cost and facilitates high throughput sample analyses.

High throughput sequencing refers to non-Sanger-based high throughput DNA sequencing technologies. Up to billions of DNA strands can be sequenced in parallel. There are several types of high throughput sequencing, each involving different chemistries. These include Illumina®, 454 pyrosequencing, and Ion Torrent™ (Grada and Weinbrecht, 2013; Liu et al., 2012; Quail et al., 2012), but there already are new technologies such as nanopores on the horizon (Niedringhaus et al., 2011). The fact that the technologies are diverse and rapidly evolving presents a particular challenge in their application on eDNA samples.

Metabarcoding at a glance...

- **COI –** cytochrome oxidase I; a mitochondrial gene that is the most commonly-used gene for fish and wildlife DNA barcoding.
- **DNA barcode –** a portion of a specific gene (e.g., COI) or locus whose sequence is used analogous to a barcode to classify an organism into taxa.
- **Index –** an artificial DNA tag that is placed onto all amplicons from a given sample. This allows DNA from multiple samples to be pooled and sequenced together, but then bioinformatically be separated after sequencing.
- **Library –** a collection of indexed DNA fragments from an eDNA sample.
- **High throughput sequencing –** also known as next generation sequencing; is a term broadly referring to a variety of highly parallel sequencing technologies.
- **OTU –** operational taxonomic unit; a group of similar DNA sequences sometimes representing a species or otherwise definable group of organisms for taxonomic classification.
- **Read –** the actual portion of DNA that is sequenced; the fundamental data output of high throughput sequencing.
- **Universal primer –** a primer designed to amplify a highly conserved locus across taxa. It is used to simultaneously analyze the DNA from many organisms in biodiversity studies.

Anatomy of a metabarcode analysis...

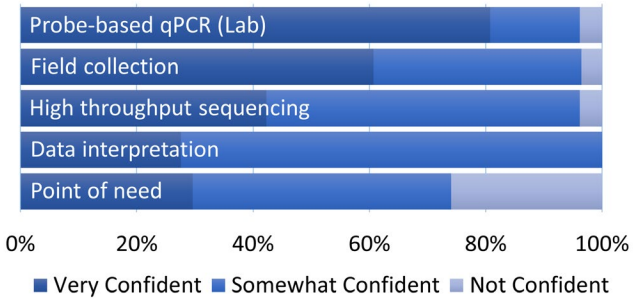


*DNA from multiple environmental samples (e.g., sediment cores) is PCR amplified with universal primers. Each sample of DNA is tagged with a unique index to create a sample library. Multiple sample libraries are pooled for high throughput sequencing. Bioinformatics methods separate the sequences by sample using the indices and align the sequences to reference databases or generated **de novo** to determine OTUs for taxonomic analysis. This creates a biodiversity profile for each sample.*

5.4 Applications

eDNA applications for fish and wildlife studies are rapidly expanding. The application of eDNA methods has grown exponentially since early applications in France in 2008 (Ficetola et al., 2008; Herder et al., 2014). The method has been successfully applied to different habitats (including terrestrial, marine, and freshwater) and to a variety of target taxa worldwide. As more and more people with a diversity of backgrounds and knowledge implement eDNA methods, the variation in technical rigour applied to studies increases and, as such, impacts the users' confidence in the results obtained (Ficetola et al., 2016; Roussel et al., 2015). The consequences of false negatives can lead to incorrect management decisions based upon the incorrect conclusion that "at risk" or invasive species are not present, thus causing a delay in appropriate mitigation strategies. The inconsistency of applied rigour in eDNA methods is a major barrier to the adoption for regulatory use.

Figure 2 – *Respondent confidence regarding the current application of eDNA methods*



Questionnaire respondents were asked to indicate their *current* degree of confidence regarding the application of eDNA methods. This is a reflection of their relative experiences, particular technical challenges encountered, and the varied reporting and rigour displayed thus far. The greatest degree of confidence is currently in probe-based qPCR (laboratory-based) methods followed by field collection (Figure 2). High throughput sequencing and data interpretation have a lower degree of confidence largely due to the greater number of choices in analysis methodologies and particular challenges associated with very low DNA abundances, respectively. The relatively new point-of-need eDNA detection methods, such as on-site DNA filtration/ extraction and handheld thermocyclers deployed in the field, provides an exciting augmentation to the eDNA detection arsenal but currently was ranked with lower confidence relative to the other DNA analysis methods.

6. Comparison of eDNA to Conventional Survey Methods

With the rapid adoption and uptake of eDNA methods, as applied to inventory of aquatic fauna (and flora) in freshwater and marine aquatic systems, practitioners are increasingly motivated to examine efficacies and cost-efficiencies associated with the application of eDNA versus conventional inventory assessment methods. Comparing eDNA methods to available conventional methods is an increasingly salient consideration when determining whether eDNA methods may confer benefit to a program, or research question, relative to the use of conventional methods. Indeed, eDNA methods can augment the generation of occupancy models. The R-package (eDNAoccupancy) allows users to select and fit multiscale occupancy models (with or without covariates) to generate more accurate occurrence estimates (Dorazio and Erickson, 2018) for studies with appropriate sampling design.

The power of eDNA

When appropriately applied, eDNA methods are superior to traditional survey methods. Benefits include:

- Non-invasive to the target taxa and their habitat
- Low risk of pathogen transfer between sites
- Highly accurate
- Very sensitive
- Ability to detect pathogens
- Generally more cost-effective for taxa that are difficult to detect using traditional methods

Survey responses indicated that 63% of participants have compared detection rates or efficacy between conventional and eDNA methods, but only 37% have compared implementation costs. However, several respondents indicated that projects evaluating these characteristics were currently underway.

Although it is impossible to precisely quantify all aspects that warrant consideration, a theoretical consideration of the major benefits and limitations of eDNA methods relative to conventional methods is feasible with the understanding that specific values will vary on a case-by-case basis. To accommodate this component of the objectives, a conceptual cost benefit analysis was applied to systematically compare eleven key aspects of fish and wildlife surveys.

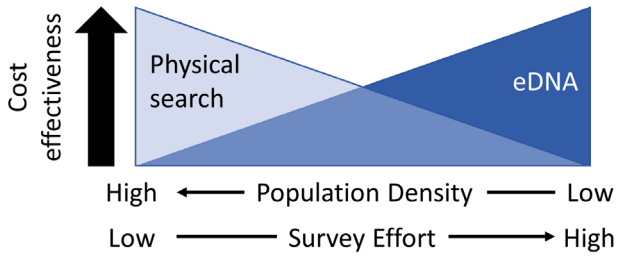
6.1 Efficacy

The most often touted gain associated with eDNA methods results from increased efficacy (detection probability) relative to conventional methods. Well-designed eDNA surveys (field sampling, DNA preservation, and analyses) are highly specific and sensitive and can have up to an 100% detection rate in locations where conventional surveys concurrently detect target taxa (Cristescu and Hebert, 2018; Goldberg et al., 2016; Herder et al., 2014; Hobbs et al., 2019; Littlefair et al., 2017). Metabarcoding studies can also detect multiple taxa; one study of benthic macroinvertebrates achieved identification of twice the number of taxa than conventional morphological identification (Elbrecht et al., 2017). This inherent gain results from the sensitivity of analytical techniques, the undocumented diversity of invertebrates, and the ability to detect exogenous DNA from target taxa in trace quantities from appropriate samples. While conventional physical search methods are most effective when the target taxa are found in high densities, eDNA is comparably most cost-effective when taxa are difficult to visually observe and/or identify (Cristescu and Hebert, 2018; Goldberg et al., 2016; Herder et al., 2014; Hobbs et al., 2019; Littlefair et al., 2017). In recognition of detectability and observer bias, conventional methods typically demonstrate greatly reduced efficacy (Herder et al., 2014). To achieve a parallel level of efficacy, or confidence in detection rate, conventional methods would typically require a degree of effort or multi-replication that is not feasible. An accurate cost benefit analysis should therefore include the cost of replication when using conventional methods in comparison to the effort required to achieve a parallel degree of detection probability using eDNA methods.

6.2 Simultaneous Study of Multiple Target Taxa

At a basic level, eDNA methods should be comparatively assessed between conventional methods recommended for the same target taxa (e.g., eDNA versus time constrained search methods for coastal tailed frog). Unfortunately, this approach may underestimate the utility of eDNA methods, as this approach invalidates one quintessential benefit of eDNA studies: the ability to rapidly assess presence/not-detected inferences for multiple taxa from the same sample. Failure to consider this aspect of eDNA studies eliminates a key efficiency (i.e., multi-species assessment).

When to choose eDNA over conventional surveys...



eDNA methods are best used when the population densities are low and survey effort is high.

6.3 Detectability

eDNA is arguably best suited for relatively inconspicuous species that are characterized by low population densities and/or occur with discontinuous distributions. As detectability increases, conventional methods may surpass eDNA for considerations strictly limited to cost-effectiveness during field application. Assuming a single species or taxon focus, the use of physical searches for species that are conspicuous, and that can be confidently detected quickly, negates the need and costs associated with eDNA sample collection, sample handling, and analysis. In that context, prudent study design can incorporate an adaptive decision-making structure that stipulates that eDNA methods will only be applied if the species is not detected at a site using conventional methods during the assessment (i.e., there is no need to collect and analyze eDNA samples unless the target taxa was not encountered during application of conventional methods). With this approach, the cost of eDNA sample collection and analysis is only undertaken when traditional methods fail. This approach ensures that, even for relatively conspicuous species, augmentation of conventional methods with eDNA collection will likely provide the most cost-effective solution during field implementation, and add confidence to occurrence estimates relative to the use of conventional methods alone. A caveat to this approach is that water samples for eDNA analyses must be collected before commencement of a conventional survey to avoid contamination of the eDNA sample.

Battling sampling bias...

In a recent eDNA project on great basin spadefoot in the grasslands of southern BC the researcher noticed that, on overcast days, spadefoot tadpoles became inactive and were not visible as they stayed concealed in the muddy substrate. On sunny days, the tadpoles were very conspicuous. eDNA samples were collected under both conditions; spadefoot DNA was detected regardless of the weather.

6.4 Restrictive Testing

Efficiencies can be gained in eDNA studies during qPCR analysis at the lab, subsequent to field collection. Testing of replicate samples from each site can be done hierarchically by testing only one replicate from each sample location at a time. Analysis of additional replicates can be ceased after the first positive detection to reduce the costs associated with laboratory analysis. The costs of collection and filtration are not entirely negated but are greatly reduced. Substantial savings can result from this adaptive testing approach. Although this approach may seem desirable, there is a reduction in power if replication is eliminated. As a result, the ability to better estimate detection rates is compromised. Prudent design may also assign test priorities to samples by collecting all samples whilst in the field but then following a prioritization schema for laboratory analysis to sequentially increase precision, or focus, within areas (systems) with detections.

6.5 Observer Bias

Conventional methods are heavily influenced by observer skill, as the ability of surveyors to detect focal taxa increases dramatically with experience; retaining skilled observers for any inventory program increases the associated cost. Conversely, eDNA sample collection can easily be completed by technicians with very little formal taxon-specific training[2]. Skill requirements associated with each method need to be considered for a meaningful or accurate cost benefit analysis. Disparity in the skill-level required during implementation can create considerable cost-efficiencies for eDNA methods relative to conventional methods.

6.6 Sampling Conditions

Weather can strongly influence detectability for both methods. However, with eDNA methods, the predominant concern for sampling conditions is the occurrence of recent heavy precipitation that may dilute the concentration of eDNA in a system or mobilize eDNA from more distant sources. With conventional methods, other subtler variables, including temperature, cloud cover, wind, and even ambient noise and light can dramatically influence detection rates.

6.7 Timing

With conventional methods, appropriate sample timing is often limited, as field studies need to be completed during appropriate life history phases. Red-legged frogs provide a relevant example; physical searches for egg masses or for adults can only be conducted during the breeding seasons (late February through early March).

[2] Some understanding of species ecology will benefit accuracy of micro-site selection during eDNA sampling when optimizing site selection to increase detection probability.

Pathogen transfer through traditional trapping methods is a cost that is mitigated through proper eDNA sampling methods.

Funding constraints, the availability of trained staff, and consideration of logistics (large survey scope) often hinder implementation of conventional methods for red-legged frogs. By converse, eDNA studies are much less restrictive and can be conducted anytime the species is present in the ecosystem, rather than being restricted to conspicuous life history phases. The cost savings that result from broader sampling windows are challenging to estimate, but this affords considerable benefit to eDNA methods.

6.8 Retro-Active Sampling

Sample preservation allows samples to be tested months, or even years, after they are collected – albeit, this aspect of eDNA methods would benefit from further controlled study to better quantify temporal degradation of stored samples. Regardless of duration, and accepting some loss of sample quality during prolonged storage, post-hoc analysis for the presence of additional species of concern not identified during the initial field survey is feasible for eDNA studies. This creates an intangible but substantial benefit to eDNA methods relative to conventional methods – with conventional methods when the survey is completed there is very limited ability to glean additional information from observations.

6.9 Logistics

It is impossible to broadly compare the costs associated with mobilization (i.e., time and cost of travel to site) as these costs will vary dramatically between projects. The costs associated with field implementation are not dramatically different between eDNA and conventional methods as, regardless of the method, field-staff still need to travel to the site to complete the survey. That said, eDNA methods are typically more sensitive than conventional methods, so fewer visits may be required to obtain the same degree of confidence in field results. In many cases the use of eDNA over conventional methods may confer cost savings to achieve the same degree of power in the data collected. Cost considerations associated with sampling programs should additionally include the following logistical considerations:

- access (e.g., helicopter, vehicle, hiking, boat);
- requirement for specialized equipment (e.g., electrofishing equipment, nets, minnow traps, etc. are often required during conventional methods compared to water collection and filtration devices);
- staff training in use of equipment (eDNA sample collection can be learned in a few hours); and
- staff safety – snorkel surveys, electrofishing each have inherent associated risks. eDNA is typically much safer as the field staff are not required to use potentially hazardous equipment.

6.10 Invasiveness

This aspect is often neglected in economic considerations, as it is challenging to accurately assign a monetary value to subjective parameters. eDNA methods are typically much less invasive relative to conventional methods, as they do not require capture or handling of often rare, vulnerable, or sensitive target taxa and the risk of pathogen transfer (e.g., ranavirus or chytrid) between separate systems is greatly reduced or eliminated. In contrast, conventional methods are typically more invasive.

6.11 Permitting costs

Conventional methods typically involve the capture and/ or handling of target taxa. eDNA methods do not. Permitting fees, including time associated with permit applications, are substantially reduced with eDNA methods.

In closing, it is overly simplistic and misleading to compare the cost associated with a single visit using conventional methods to the cost associated with an eDNA study. However, in one brief site visit, eDNA may afford a simultaneous high detection probability for multiple species and allow post-hoc analysis of preserved samples for additional species years later. An adaptive approach to restrict sampling or testing as described will lower eDNA costs even further. By comparison, to achieve the same efficacy with conventional methods for many difficult-to-detect taxa would require multiple site visits by skilled observers for each species. Analysis to assess the presence of any other species at a later date is impossible. Pathogen transfer risk is higher and the sensitive microhabitats are more likely to be deleteriously affected by the level of effort applied to achieve a less accurate result.

It is beyond the scope of this research paper to provide a fulsome comparison of the efficacy of eDNA methods, for multiple taxa and between multiple conventional methodologies. However, the synopsis of a recent study on the coastal tailed frog (Hobbs et al., 2019) provides a compelling illustration of comparative detection rates and effort between conventional physical search and eDNA methods.

7. Considerations Regarding eDNA Methods

7.1 Importance of Synergy between Field and Analytical Components

Regardless of the selected study method, reliable and accurate environmental studies generally require rigour during all stages of implementation to minimize and, where possible, eliminate potential sources of error. In any method, error may occur during assay design and validation, survey design, sample collection, preservation, transport, sample analysis, or data interpretation; some common potential sampling biases are discussed in Section 3. It is important to note that preliminary site assessments should be done to ensure appropriate knowledge and expectations regarding the survey sites.

Support for synergy...

It is worth noting one area of application that warrants additional research. With traditional methods it is not uncommon to use baited traps to increase detection rates. Similar approaches have been demonstrated to be effective for eDNA studies as well. Bait stations can be used to pre-emptively draw focal taxa to a subsequent detection site thus increasing detection rates even further for eDNA applications (Ghosal et al. 2018).

Ensuring rigour during field sampling to ensure accuracy in data interpretation is a fundamental tenet during environmental assessments. With eDNA, considerations need to be afforded not only to sample collection and preservation, but also to the particular DNA analysis method, test design, and quality control.

The synergy, or partnership, between the field and lab component is a fundamental difference that underlies eDNA methods from many other purely field- or lab-based assessment applications. While acknowledgement of this synergy is growing (Goldberg et al., 2016; Veldhoen et al., 2016), there is considerable room for improvement. Only 53 and 60% of questionnaire respondents have estimated the probability of false positives or negatives, respectively, in any of their projects.

A "tail" of two efficiencies...



*The coastal tailed frog **(Ascaphus truei)** is endemic to the Pacific Northwest of North America and is listed as a "species of special concern" under the Canadian **Species at Risk Act**, S.C. 2002, c. 29. Its range is limited to British Columbia, where it occurs widely west of the Coast Mountain Ranges extending north almost to the Alaskan Panhandle.*

A study was conducted within the Cayoosh, Bridge (Shulaps), Seton, Anderson, Carpenter, and Downton Lake drainages near Lillooet, BC (Hobbs et al., 2019). Data was compared to four years of previous traditional time-constrained search methods that documented the tailed frog to occur at 23/292 discrete sites (7.9% sites occupied) in seven watersheds.

Water samples were collected and filtered at 72 sites in duplicate within the study area in 2016 over five consecutive days. eDNA methods were applied using a two-step eDNA analysis approach. The first IntegritE-DNA step was used to screen for samples without viable DNA (e.g., due to residual bleach on sampling equipment or sample labeling issues) and to test all DNA samples for the ability to support amplification (i.e., no substantial inhibition or DNA degradation). This was achieved by testing endogenous plant chloroplast DNA as a measure of sample viability. Three samples did not pass the IntegritE-DNA test.

Coastal tailed frog eDNA was detected in 55/72 (76%) discrete stream reaches; nine sites with historical known occurrence were all eDNA positive. The false negative rate for traditional compared to eDNA methods was 58%. The results expanded known coastal tailed frog distribution to 24 watersheds, effectively more than tripling the number of known extant occurrences.

Most importantly, the results provide conclusive empirical support for the efficacy of eDNA methods and support rigorous application of eDNA methods to survey a cryptic, rare species within fast flowing stream systems that proved challenging to survey accurately using traditional methods.

While eDNA methods can be quite sensitive and accurate, these methods cannot distinguish between life stages (e.g., eggs versus adult), age of the target taxa, or whether organisms are alive or dead. Nor can eDNA abundance distinguish between one large fish versus several small fish.

Threats to the reliability and credibility of eDNA conclusions…

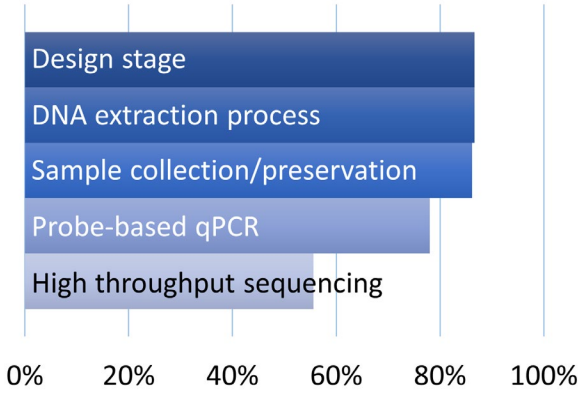
- Variable application of rigour in sample collection and preservation
- Variable validation and quality control of analytical tests

In order to gain a contemporary understanding of different approaches during eDNA sample collection and transport, and analytical methods for eDNA detection, analytical methods for eDNA detection, considerations were presented relevant to each aspect (Goldberg et al., 2016; Veldhoen et al., 2016) and respondents were asked to rate the importance of each aspect if relevant to their experience. The majority of respondents (86-87%) had experience with eDNA study design, DNA extraction process, and sample collection/preservation (Figure 3). Where experiences differed substantially was in the use of probe-based qPCR (78%) and high throughput sequencing (56%). Therefore, the responses presented in Figures 3 to 5 reflect this diversity of eDNA application and experiences.

7.2 Field Sampling Component – Design Considerations

The majority of eDNA studies have focused on obtaining water samples. This is reflected in the narrative accompanying the sample collection and preservation regimens (Goldberg and Strickler, 2017; Herder et al., 2014; Hobbs et al., 2017). However, there is a notable caveat in these viewpoints, as there is considerable variation in the rigour applied during sample collection and preservation and the validation and quality control of eDNA analytical tests. There is also considerable variation in the interpretation of sample analysis results.

Figure 3 – *Percentage of respondents with experience in the indicated eDNA methods.*



This variability may threaten the reliability and credibility of conclusions drawn from eDNA methods.

Design considerations are a critical component of pre-survey planning. During pre-survey planning, a well-designed eDNA study must consider at least four fundamental underlying design elements, detailed below in decreasing order of priority as determined by questionnaire respondents:

Project objectives: All experienced respondents unanimously noted that determination of the inferential goal (i.e., detected/not-detected versus a desire for measured relative or absolute abundance) is a critical consideration at the project design stage. While the techniques and knowledge are rapidly evolving, eDNA methods are currently not capable of measuring relative organismal abundance at either a temporal or spatial scale and should not be used where inference regarding relative abundance is required.

System characteristics: Once DNA is released from the target taxa, it can be transported from the area of release and be subjected to a variety of conditions that promote degradation. Environmental variables, such as UV, temperature, pH, and microbial/fungal content, affect degradation rates. As such, the relative concentration of eDNA in the system will diminish with the distance to the source and be deleteriously affected by degradation over time (Goldberg et al., 2018; Herder

et al., 2014). Water flow and volume will also affect the rate and distance of transported DNA as it diffuses passively or is transported actively from the source. It may seem obvious, but timing is particularly important when assessing ephemeral aquatic features. It is clear that there are many interactions between DNA released into the environment that influence eDNA detection. Understanding the ecology of eDNA, including the influence of origin, state, and transport is an important area of research and is integral in the interpretation of eDNA study results (Barnes and Turner, 2016).

Ecology: A comprehensive biological understanding of the target taxa is also essential to sound design of any eDNA study. Understanding the taxa's reproductive cycle and timing of all requisite life stages ensures sample collection can be conducted during peak eDNA output periods. An understanding of the use of different micro-habitats can also be applied, to increase the likelihood of detection. A novel new approach that involves using bait to lure the focal taxa toward eDNA sample collection locations (Ghosal et al., 2018) holds great potential for increasing detection probability, particularly where a poor understanding of the species ecology may limit detection rates (e.g., Pacific water shrew).

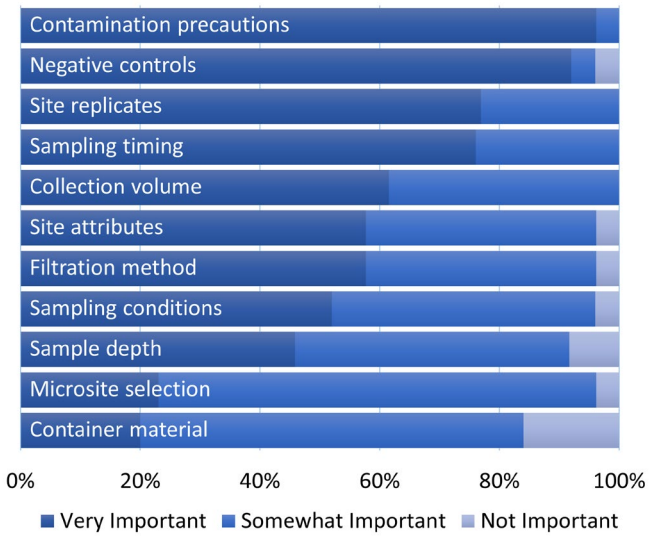
In many cases, it may not be feasible to consider all of these variables during the implementation of eDNA studies, but a fulsome understanding of system characteristics and species ecology will ensure sound design is adopted to the extent practicable.

Sample contamination: For sample collection, the most important considerations identified by respondents during study design and methodological considerations was the inclusion of appropriate precautions to reduce the risk of contamination of environmental samples during all phases of eDNA implementation (Figure 4). The use of appropriate negative controls for sample collection, handling, transport, and filtration allow assessment of the potential influence of these variables on the project results. In addition, appropriate study design with site replication, and inclusion of known positive and known negative sites can also add confidence during the interpretation of lab results. While considerations surrounding the potential for sample contamination are important, they are quaternary to the aforementioned design considerations regarding project objectives, system characteristic, and species ecology.

Contamination precautions include bleaching and rinsing equipment with water, planning the sampling sequence to allow prioritization of sample collection from areas furthest downstream, wearing gloves during sample collection (or, more importantly, exercising care when handing sampling containers[3]), and using single-use filtration units. Each serve to ameliorate the risk of sample contamination. Negative controls for filtration include filtering bottled water at the end of each filtration session. Site replication (i.e., taking multiple environmental samples from each site) also increases the likelihood of detection and adds confidence in results when estimating detection rates and assessing the potential influence of field contamination.

Collection volumes, site attributes (e.g., water flow rates, area, terrain types), and filtration methods were ranked next in importance, followed by sampling conditions (e.g., after a rainfall, drought) and depth, microsite selection, and container material (Figure 4).

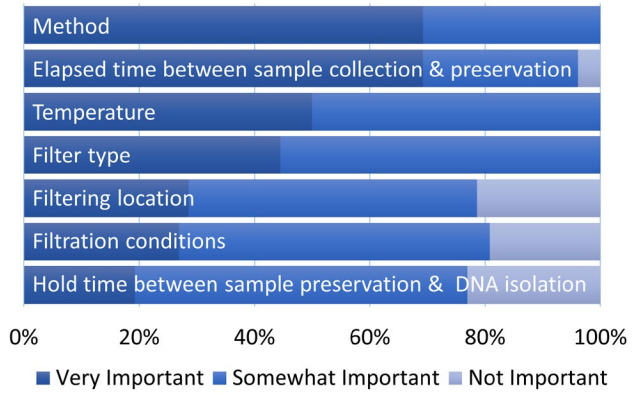
Figure 4 – *Respondent ratings of critical considerations pertaining to eDNA field sample collection.*



[3] The use of nitrile gloves during sample collection can impart a false sense of security against sample contamination as gloves are quickly contaminated during field samples (e.g., reaching into a pocket or using a contaminated pen will render the glove ineffective). It is likely more effective to exercise care when sampling to avoid any back-flow of site water over a hand (gloved or un-gloved) when filling the sample bottle on site.

Top considerations for sample preservation are preservation method (e.g., use of ethanol or silica) and elapsed time between sample collection and preservation followed by holding temperature and filter type (Figure 5). Filtering location (e.g., on-site, at a base camp, or after transport to a lab), filtering conditions (e.g., handheld versus automatic, positive versus negative pressure pumps), and hold time between sample preservation and DNA isolation were still considered very or somewhat important by most respondents, but about 20% did not regard these parameters as important.

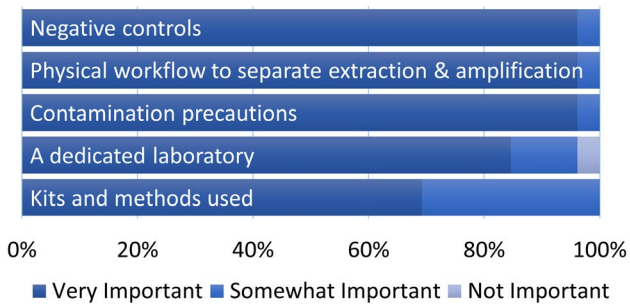
Figure 5 – *Respondent ratings of critical considerations pertaining to eDNA field sample preservation.*



7.3 Analytical Methods – Design Considerations

DNA isolation methods: DNA isolation methods are common to all subsequent analytical methods used. The vast majority of respondents deemed each of the considerations within this category very important (Figure 6). Negative controls (e.g., blank filters), physical separation of the workflow between DNA extraction and amplification areas (but not necessarily a dedicated eDNA laboratory), and strict contamination precautions (e.g., personal protective equipment, filter tips on pipettors, sample preparation in laminar flow hoods or biosafety cabinets, etc.), were each overwhelmingly deemed very important. The kits and methods used were least important within this group.

Figure 6 – *Respondent ratings of critical considerations pertaining to the DNA extraction process.*



Probe-based qPCR: These tests rely upon the ability of the primer/probe combination to selectively detect the target taxon DNA from a complex environmental sample. Test design relies upon knowing a portion of the genetic information of the target taxa and, critically, that of closely-related taxa that are anticipated to be present at the sampled site. There are three stages that must be considered including ***in silico***, ***in vitro***, and in the ***field***; these are discussed below.

Due to variable selective constraints and rates of molecular evolution among taxa, not all loci are suitable to satisfy the requirements for good primer and probe design. Careful design therefore includes the appropriate choice of DNA fragment during the *in silico* design stage that uses robust primer design software. Known sequences are compared between taxa and primer and probe sequences are chosen to only detect the target taxa.

In addition, even though fish and wildlife DNA tend to diverge from human DNA, human DNA should also be tested during the design phase. Although not closely related, humans are present in many natural aquatic systems and are involved in all aspects of implementation. All designs should include a comparison with this ubiquitous contaminant to ensure that human DNA is not amplified by the eDNA test.

Once candidate primers and a probe are selected, their specificity must next be rigorously evaluated ***in vitro*** against DNA from vouchered specimens of the target taxa and closely related sympatric species (i.e., species that may coexist in the sampled environment). Any possible detections should be noted to allow for appropriate interpretation of eDNA results. qPCR reaction conditions also influence the accuracy and performance of the test. The most common parameters that can be optimized are magnesium concentration, annealing and extension temperatures, as well as nucleotide, primer, and probe concentrations.

eDNA analytical test validation...

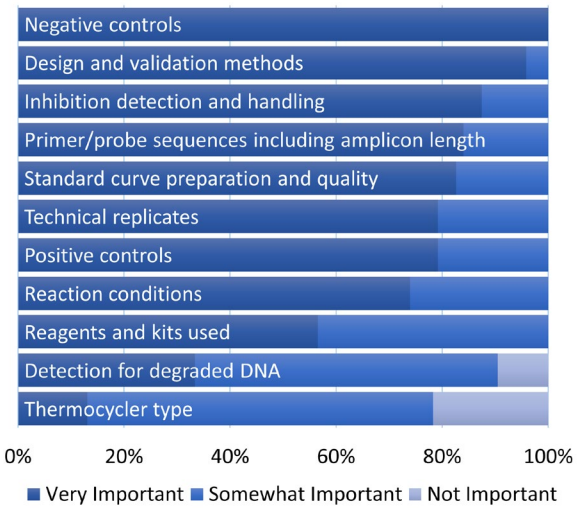
- ...should include verification of specificity by testing on vouchered specimens (specimens that are expert-confirmed) of the target taxa and all possible confounding taxa that coexist in the sample environment.
- ...should include sensitivity testing using double-stranded synthetic DNA derived from the target taxon amplicon sequence.
- ...should include performance validation on field samples from known positive sites.

Once specificity of the eDNA test is established, sensitivity should next be evaluated. This is done through the assessment of test performance at a range of target taxa DNA concentrations. While total DNA isolated from vouchered specimens is commonly used, differences in sample origin, age, preservation method, and even accurate sample species identification can each confound this aspect of the test design. As such, the most reproducible method that is most conducive to standardization and inter-laboratory replication is the use of double-stranded synthetic DNA (e.g., gBlocks, etc.) that is derived from the target taxon amplicon sequence. This approach is rapidly gaining traction in the practitioner community (Klymus et al., In preparation; Wilcox et al., 2016). This method also facilitates the determination of test performance characteristics, including limits of detection (LOD) and quantitation (LOQ). In addition, test validation should also include analysis to allow more informed estimation of associated statistical error during subsequent testing of environmental samples that will typically feature very low quantities of DNA from the target taxa (Klymus et al., In preparation; Veldhoen et al., 2016). The ability of the eDNA test to detect low concentrations of DNA that are typically found in environmental samples is greatly enhanced through a combination of technical replicates and binomial "detect/not detect" results. Logically, the sensitivity of the test is increased as the number of technical replicates is increased. While eDNA detection poses these additional challenges on classical qPCR, many considerations of good experimental design, as outlined by Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2010), still apply and should be incorporated.

The final stage in sound design includes a ***field*** validation component where test performance is evaluated against environmental samples collected from sites with known extant occurrence of the target taxa. Further validation using DNA isolated from sites with recent expert-verified sightings of the target taxa serves to validate the tests on actual field-collected samples.

The one critical standard consideration that was unanimously identified as very important by respondents is the use of negative controls (e.g., no template controls (NTCs)) that control for assay and lab contamination (Figure 7). Also receiving very strong support are robust design and validation methods.

Figure 7 – *Respondent ratings of critical considerations pertaining to probe-based qPCR.*



Various test attributes were also deemed very important, including primer/probe sequences that closely consider amplicon length, standard curve preparation and quality for determination of LOQ and LOD values, use of an appropriate number of technical replicates, use of positive controls (e.g., running known target DNA routinely on reaction plates), and clearly defining reaction conditions. Respondents also indicated that the reagents and kits used are very or somewhat important in equal proportions.

Isolating viable DNA for testing from environmental samples can be challenging, as impurities can be co-purified with the DNA. These can inhibit the qPCR reaction during the analysis of environmental samples. Inhibition must be identified when it occurs. If inhibition is missed (or not considered as a key component of sample test procedures), the sample may be falsely classified as a non-detection when the target taxon is actually present. A large majority of respondents also deemed this aspect very important. Spiking qPCR reactions with exogenous synthetic DNA that is not naturally occurring (commonly referred to as an "IPC") is often performed to determine whether inhibitors are present (reviewed in Goldberg et al., 2016). An additional processing step may be performed to attempt to remove the inhibitors and the sample retested to determine the success of this effort.

An alternative method that detects both inhibition and DNA degradation is to test for the presence of DNA from a ubiquitous source (e.g., plant/algae chloroplast DNA) in every sample (Hobbs et al., 2019; Veldhoen et al., 2016) before testing the sample for the presence of DNA from the target taxon. The failure to detect the endogenous eDNA source in an environmental sample provides a clear indication that inhibition or DNA degradation will confound the result when testing for the target taxon. The use of generic animal group tests (Furlan and Gleeson, 2017; Hobbs et al., 2019; Veldhoen et al., 2016) have also been proposed, but are dependent upon having suitable abundance levels within samples.

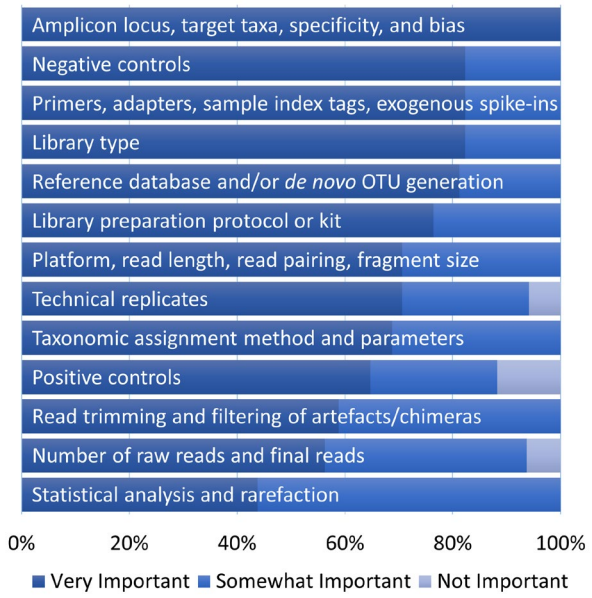
eDNA from environmental sources will always be degraded to some degree, as degradation begins immediately upon release from the source organism. As such, there comes a point at which the DNA will be too degraded to be detected. Sample degradation may also occur during collection, transport, or processing. While degradation of eDNA is a common point of discussion, 30% of respondents had no experience in this aspect of the qPCR process. This is reflective of the paucity of scientific investigation into this facet of eDNA detection (Goldberg et al., 2018). Degradation cannot be ascertained by measuring DNA content. For qPCR methods, IPCs also cannot provide information regarding the state of sample degradation. The incorporation of a test for sample integrity using endogenous DNA has been suggested as a powerful and robust method of detecting both inhibition and degradation (Furlan and Gleeson, 2017; Hobbs et al., 2019; Veldhoen et al., 2016). DNA samples that are not intact enough for a viable detection alert the potential for false negative results (i.e., failure to detect the target taxa during the qPCR process even when present in environmental samples).

High throughput sequencing: Metabarcoding has considerations that are distinct from qPCR-based methods, as the data output is based upon the generation of short **reads** that are then bioinformatically assembled using a variety of computational methods. Considerable focus is placed on generating quality sequence reads, alignments, and classifications.

Respondents with experience with high throughput sequencing techniques unanimously deemed amplicon locus (i.e., target gene fragment), choice of target taxa (e.g., benthic invertebrates), specificity, and amplification bias of the universal primers as very important considerations (Figure 8). Negative controls to ascertain background levels of OTUs, particularly from microbial sources, that can confound biodiversity determination are particularly important. Additional important considerations are the primers, sequencing adapters, sample index tags, and exogenous spike-ins; library type (e.g., amplicon or shotgun) and enrichment protocol; having a reference database or *de novo* OTU generation capability; and the library preparation protocol or kit. Platform (e.g., Illumina, 454, etc.), read length, read pairing, and expected fragment size were important considerations. The importance of technical replicates was mostly rated as high, but was mixed

within assignation by some respondents. This is likely in consideration of the associated cost and in recognition of the high fidelity of technical replicates of libraries, thus diminishing the necessity of this type of replicate.

Figure 8 – *Respondent ratings of critical considerations pertaining to high throughput sequencing.*



More than half of the respondents deemed the taxonomic assignment method and parameters; positive controls; read trimming and filtering of artefacts/chimeras very important. However, the utility of positive controls (e.g., a mixture of DNA from a defined set of taxa) had mixed support as did the number of raw reads and final reads. The read number (also termed "read depth") is directly related to the sensitivity of detection (i.e., the greater the read depth, the greater the sensitivity). It is also a measure of sample quality. For high throughput sequencing methods, excessively degraded DNA will produce poor quality reads. The statistical analysis and rarefaction (data complexity reduction) approaches were ranked as very important by slightly less than half of respondents and ranked as somewhat important by the rest (Figure 8).

Risk of sample alteration and sources of error

eDNA methods require identification and mitigation of sources of error. Assuming the use of carefully validated eDNA tests, general precautions include careful chain of custody of samples from field through to lab processing and analysis. Sample processing bias can be mitigated by running samples blind during DNA analysis and randomizing samples during analytical processing to reduce machine or technical bias.

Causes of false positives include:

- Improper organization of sampling sequence (e.g., starting sampling upstream and moving downstream)
- Improper cleaning of equipment (e.g., flaming forceps between samples, not sufficiently rinsing the bleach away)
- Processing water samples where target taxa have been handled
- Contact between filters during drying
- Lack of separation between eDNA samples and PCR products
- Mislabeling of samples during collection or processing
- Poor workflow practices
- Closely-related species confounder
- Incidental target taxa DNA disposition by predators
- Poorly curated databases used for metabarcoding

Causes of false negatives include:

- Improper removal of bleach from equipment
- DNA sample integrity is too poor for detection (e.g., excessive degradation)
- Distance from the source organism is too great
- Extensive sample hold times before sample preservation
- Mislabeling of samples during collection or processing
- Insufficient sample replication (e.g., technical and/or site)
- Ineffective inhibitor removal
- Sequencing errors
- Sequence divergence in the selected DNA fragment of target taxa
- Improper timing of sample collection relative to taxa (e.g., wrong season, directly after a large rain event, etc.)
- Poorly curated databases used for metabarcoding

8. eDNA Common Practices

eDNA detection is challenged by the complexity and variability of environmental samples. Samples taken from the same sites over time can have different impurities co-isolated with DNA depending upon the season. DNA within environmental samples will also suffer varying rates and degrees of degradation. In addition, dilution due to varying (unknown) distance from the source organism, local ambient environmental conditions, or with system volume and flow, each confound DNA detection capabilities in the eDNA analytical process. This is in contrast to DNA samples collected from defined tissues for which the analytical methods commonly applied were originally developed. Thus, special attention must be given to the validation of eDNA analytical tests and reliability is dependent upon mitigating confounding factors and routinely, rigorously, and appropriately testing for them.

Common practices...

- ...mitigate sources of false positives and negatives.
- ...choose eDNA methodology appropriate for study design objectives with appropriate regard to statistical power.
- ...include attention to quality control.
- ...provide transparency in benchmarks of test performance.

In recognition of the need for a systematic approach during the execution of eDNA studies, some groups have made efforts to provide guidance regarding best practices while others are in development (Carim et al., 2016; Goldberg and Strickler, 2017; Goldberg et al., 2016; Herder et al., 2014; Hobbs et al., 2017; Klymus et al., In preparation; MacDonald and Sarre, 2017; Porter and Hajibabaei, 2018; US Fish and Wildlife Service, 2018; Veldhoen et al., 2016). Unfortunately, these recommendations (Table 1) are not universally applied. There is a strong need for the development of a consensus-based approach to the implementation of standards that accommodate these critical considerations during all phases of eDNA implementation. There is also a strong need for consistency, transparency, and accuracy during the reporting of eDNA results. Shared common practice and the demonstration of attention to critical considerations as described herein are vital in promoting rigour, and ultimately acceptance by industry and regulators, during the implementation of eDNA methods.

Best practices mitigate false positives and false negatives at all stages of an eDNA detection protocol and provide transparency in methodological application. This ensures reproducibility and reliability of results — both key tenets of science. Important considerations include the appropriate choice of the techniques employed with an understanding of the methodological biases (Cristescu and Hebert, 2018). Analytical techniques require appropriate validation and verification of test performance, including the application of the appropriate number of technical replicates based upon the project needs. It is clear that for the detection of very low amounts of DNA, detection probability increases with an increase of technical replicates in probe-based qPCR methods (Goldberg et al., 2016; Hobbs et al., 2019; Veldhoen et al., 2016). The determination of DNA sample integrity (e.g., low inhibition, not excessively degraded) should be included to mitigate false negative results. Similarly, consideration of the number of biological replicates is also important.

Table 1 – *Examples of common practices to mitigate false positives and false negatives*

Mitigating false positives...	Mitigating false negatives...
Run negative field and lab controls	Run positive field and lab controls
Rigorously protect sample processing from exposure to PCR products	Evaluate sample inhibition and integrity
	Choose appropriate number of technical replicates (qPCR) or sequencing depth (metabarcoding)
Use rigorously validated eDNA tests	
Identify primer bias in metabarcoding methods	
Use good sampling practice	
Use good analytical practices	
Spatial and temporal site replicates	

9. Need for Standardization

As eDNA methods are increasingly utilized for environmental assessments and monitoring activities, their results will have greater influence upon management decisions. Indeed, eDNA methods have the *"potential to revolutionize biodiversity science and conservation action"* (Cristescu and Hebert, 2018).

eDNA needs...

Despite the power of eDNA detection methods, the following specific issues need to be addressed:

- Quality issues with accuracy and reliability
- Lack of accredited national standards
- Lack of competency and proficiency testing

Current legislation often requires species inventory to inform management: *"From the perspective of maintaining biodiversity, the importance of standardization cannot be overemphasized, as province-wide consistency and reliability will facilitate comparison among and between different studies to reveal landscape-level changes in distribution and population trends. In contrast to this, the continuance of the historic approach of using project-specific data collection methods will make broad scale comparisons difficult, if not impossible."* (British Columbia et al., 1998). As there is broader uptake, the need for the development of reasonable eDNA performance standards becomes increasingly important.

Barriers to the implementation of eDNA methods revolve around uncertainty and reliability issues. These include a lack of accredited national standards for eDNA methods, a lack of comparability between studies or methods, and quality issues with accuracy and reliability.

As mentioned in Section 8, several groups have addressed the issue of best practices for eDNA methods, but these practices need to be consistently applied. The development of key descriptors of eDNA test performance characteristics is highly desirable to instill confidence in eDNA methods. As the methods are diverse and evolving rapidly, fundamental parameters common to all methods must be distinguished from method-specific ones and applied appropriately. This requires an understanding of the features of eDNA and how these features relate to the application of analytical techniques.

The development of accredited national standards for eDNA approaches is a challenging but necessary proposition. It is challenging because of the inherent complexity in both field and analytical methods. It is necessary to usher in confident uptake of this transformative management tool. Initial focus should be on field collection and probe-based qPCR methods, as they are the most mature and conducive for standardization (Carim et al., 2016; Goldberg et al., 2016; Hobbs et al., 2017). Metabarcoding standardization is more difficult due to the nature of high throughput sequencing and is currently suggested to be a lower priority for standardization. However, several groups are forming initiatives to work towards laying the foundation for standardization in the future (Cristescu and Hebert, 2018; Elbrecht et al., 2017).

Proficiency testing...

- ...is commonly used for commercial laboratory accreditation.
- ...establishes the desired level of competency regardless of which DNA analysis method or protocol is followed.

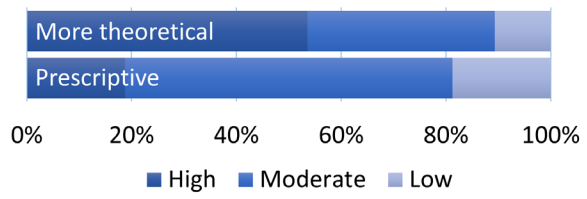
As adoption and application of eDNA methods continues to grow exponentially within Canada and worldwide, qualified environmental practitioners, academic bodies, government agencies, and private sector participants with interest in resource development each share a responsibility for the development of appropriate guidelines, standards, and best practices. This is necessary to ensure eDNA methods continue to gain support for widespread application in research, resource

development, and environmental conservation and management. As is true for any emerging and evolving practice, early development and acceptance of concept-based (more theoretical) and, where appropriate, prescriptive guidance for undertaking eDNA studies would benefit implementation and acceptance nation-wide (Figure 9). This is critical as the use of eDNA methods become more widespread for use in practical applications related to resource management decision-making processes.

While the implementation of eDNA methods present novel challenges, there is no need to "reinvent the wheel" with respect to adopting the concept of proficiency assessments. Proficiency testing is an important, platform-independent mechanism that is used by the Standards Council of Canada (SCC; www.scc.ca) and the international Organization for Standardization (ISO; www.iso.org).

There is currently no mechanism in place for the proficiency testing of eDNA methods. However the SCC has published proficiency testing requirements and guidance for testing and medical laboratories that can serve as a starting point for the development of appropriate proficiency tests suitable for eDNA applications (Standards Council of Canada, 2017). The current proficiency tests supported by the SCC meet ISO 17043: *Conformity assessment – General requirements for proficiency testing and ISO 17025: General requirements of the competence of testing and calibration laboratories.*

Figure 9 – *Respondent views on the extent to which eDNA standards should be more theoretical or prescriptive in approach.*



An example of an active relevant proficiency testing model is that used for commercial labs involved in animal DNA genotyping. Official member labs participate biennially in inter-laboratory proficiency testing administered by the International Society for Animal Genetics (ISAG). A set of extracted DNA samples are prepared by a "duty lab"; an ISAG member who volunteers to prepare the samples for the testing. Samples are sent to participating laboratories who apply their analysis methods. Results are tabulated by the participating laboratories and are uploaded to the ISAG website for evaluation by a certain deadline, where they are compiled and assessed for consensus results by an ISAG staff member. The results are sent out to all participating laboratories, and certificates are prepared designating the status of participating labs based upon the accuracy of their results. Proficiency testing and open release of test results facilitates laboratory method reviews and establishes a desired level of competency regardless of which DNA analysis method or protocol is followed.

10. Suggested Research Activities

A number of research activities are required to address development needs and promote continued innovation, expansion, and applicability of eDNA methods. These activities should occur in tandem with continued efforts to educate practitioners and regulators regarding the strengths and weaknesses and appropriateness of various eDNA techniques and include promoting inter-laboratory performance comparisons (Figure 10). eDNA analytical methods require careful validation and appropriate disclosure of critical performance characteristics (e.g., specificity and sensitivity) and run parameters to facilitate comparability and reliability.

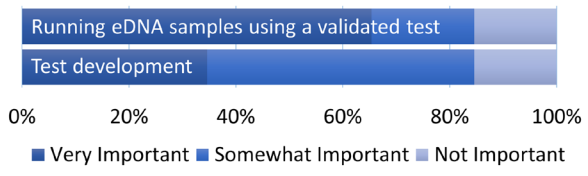
Research efforts need to...

- ...apply appropriately validated tests to understand environmental and sampling factors affecting eDNA detection.
- ...develop competency and proficiency testing for lab accreditation.
- ...promote inter-laboratory comparisons.
- ...improve and enhance metabarcode reference databases.

Additional research areas should focus on:

1. Developing a framework for routine proficiency testing.
2. Inter-laboratory comparisons of eDNA tests to ensure consistency and reliability.
3. Probe-based qPCR tool design to understand the LOQ and detection (Klymus et al., In preparation; Lamb et al., 2019).
4. Evaluating and quantifying inherent confidence limits with eDNA techniques (Hobbs et al., 2019; Klymus et al., In preparation; Veldhoen et al., 2016).
5. Transport (salt or dye tests in different gradients or stream order) to understand, by inference, how eDNA precipitation rate may be affected by flow and gradient (Goldberg et al., 2018).
6. Sample transport stressors (freezing water or holding preserved samples).
7. Quantifying temporal degradation of stored samples.
9. The effects of environmental factors (e.g., UV, temperature, inhibitors, etc.) on DNA degradation in natural systems (Goldberg et al., 2018).
10. Use of bait stations to increase detection probability (Ghosal et al., 2018).
11. Improving and enhancing metabarcode reference databases (Cristescu and Hebert, 2018; Elbrecht et al., 2017).

Figure 10 – *Respondent rankings of third party or inter-laboratory comparison of eDNA methods.*



11. Conclusion

The adoption of eDNA methods in Canada is an ongoing success story of collaboration between regulatory agencies, First Nations, academia, and industry in fostering the emergence and uptake of more cost-effective, non-invasive, and more accurate practices in resource management and conservation.

Many projects are being more efficiently advanced through the environmental assessment process. Species conservation efforts are more accurately and efficiently informed, and clients are realizing significant cost savings as they comply with environmental assessment and permitting conditions during resource development.

Through active training and education programs, regulators and new practitioners are gaining an improved understanding of eDNA methods and considerations for appropriate and reliable use.

The enthusiastic adoption of this transformative approach to environmental assessments and monitoring must be tempered by appropriate rigour, transparency, and reporting to promote information sharing as new techniques emerge. Communication should convey methodological limitations accurately and responsibly. Synergic development, to improve various aspects of eDNA methods, will also ensure cost-efficient evolution that will ultimately strengthen eDNA methods in practice. Ignoring these points will detriment rigour, negatively affect acceptance, result in cost-inefficiencies, and ultimately impact acceptance of eDNA methods.

As more projects employ eDNA methods at an encouraging rate, the need for appropriate checks and balances is great. The nature and promise of eDNA attracts collaborative efforts from multiple stakeholders. For example, a recent $2.6 million Genome Canada project was announced to use eDNA metabarcoding to generate and monitor biodiversity of benthic invertebrates in Canada's watersheds to better understand the impacts of natural resource projects like mines and hydroelectric dams. This involves an extensive

eDNA standards will enhance confidence in its transformative use for informing critical management decisions.

partnership including the University of Guelph, the World Wildlife Fund-Canada, Living Lakes Canada, and Environment and Climate Change Canada (ECCC). This supports wider adoption of technologies within existing environmental monitoring and assessment applications including ECCC's Canadian Aquatic Biomonitoring Network (CABIN) that engages over 1,400 users from all levels of government, academia, First Nations, industry, NGOs, and environmental consulting firms.

It is clear that now is the critical time to assess and consolidate robust, reliable methods for eDNA detection. Canada can play a leadership role in bringing together multiple stakeholders nationally and internationally to facilitate discussion and harmonization of eDNA approaches.

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