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PERSPECTIVE

The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories

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Abstract

Considerable promise and excitement exist in the application of environmental DNA (eDNA) methods to environmental monitoring and species inventories as eDNA can provide cost-effective and accurate biodiversity information. However, considerable variation in data quality, rigor, and reliability has eroded confidence in eDNA application and is limiting regulatory and policy uptake. Substantial effort has gone into promoting transparency in reporting and deriving standardized frameworks and methods for eDNA field workflow components, but surprisingly little scrutiny has been given to the design and performance elements of targeted eDNA detection assays which, by far, have been most used in the scientific literature. There are several methods used for eDNA detection. The most accessible, cost-effective, and conducive to standards development is targeted real-time or quantitative real-time polymerase chain reaction (abbreviated as qPCR) eDNA analysis. The present perspective is meant to assist in the development and evaluation of qPCR-based eDNA assays. It evaluates six steps in the qPCR-based eDNA assay development and validation workflow identifying and addressing concerns pertaining to poor qPCR assay design and implementation; identifies the need for more fulsome mitochondrial genome sequence information for a broader range of species; and brings solutions toward best practices in forthcoming large-scale and worldwide eDNA applications, such as at-risk or invasive species assessments and site remediation monitoring.

KEYWORDS

eDNA assay improvement, mitochondrial genome, quantitative real time polymerase chain reaction, robust assay design, targeted eDNA assay, test development and validation

1 | INTRODUCTION

Effective management of lands, waters, mineral wealth and natural resources, and protection of biodiversity and ecological productivity in a changing climate requires thorough knowledge of the distribution and relative abundance of at-risk, sentinel, invasive, and pathogenic taxa. Advances in molecular technologies have greatly improved our ability to survey planet Earth's biodiversity in natural ecosystems and anthropogenically impacted areas. There are many examples in the scientific literature of the detection of environmental DNA—genetic material isolated from environmental samples

without obvious signs of biological source material—providing more sensitive and cost-effective species detection of low-density, cryptic species than conventional ecological survey methods (Deiner et al., 2017; Goldberg et al., 2016; Jerde et al., 2011; Thomsen & Willerslev, 2015). High-throughput sequencing and targeted qPCR analyses have empowered us to detect eDNA molecules in water, soil, sediment, and air (Barnes & Turner, 2016).

There is tremendous potential for the application of eDNA methodology for environmental impact assessments, species inventories, monitoring, and biosurveillance worldwide. When conducted diligently, eDNA-based monitoring could substantially increase cost-effectiveness and robustness of at-risk or invasive species detection to inform environmental risk assessments (ERAs) and environmental surveying. For example, ERAs are required for permitting natural resource development projects such as mining, pipelines, or hydroelectric dams. Environmental surveying is required during such operations and following project completion to evaluate operation impacts or site remediation effectiveness. Indeed, eDNA methods are actively used in many locations internationally (Africa, Elberri et al., 2020; Asia, Lee et al., 2020; Europe, Brys et al., 2020). However, considerable variation in data quality, rigor, and reliability has eroded confidence in eDNA application and is limiting regulatory and policy uptake (Nicholson et al., 2020). Specifically, there is a need to effectively mitigate false positives and negatives from field and analytical sources (Goldberg et al., 2016; Helbing & Hobbs, 2019; Nicholson et al., 2020; Thalinger et al., 2020). Considerable effort has gone into promoting transparency in reporting and deriving standardized frameworks and methods for eDNA workflows (Baillie et al., 2019; Goldberg & Strickler, 2017; Hobbs et al., 2017; Laramie et al., 2015; The eDNA Society, 2019; US Fish & Wildlife Service, 2018).

Of the many methods used for eDNA detection, including metabarcoding and polymerase chain reaction (PCR), the most accessible, cost-effective, and conducive to standards development is targeted quantitative real-time polymerase chain reaction (abbreviated as qPCR) analysis (Tsuji et al., 2019). As this method forms the heart of eDNA analysis, it deserves particular attention in forming a robust eDNA workflow (Figure 1), especially with respect to the design and implementation of novel assays (Loeza-Quintana et al., 2020; Nicholson et al., 2020). Some vital issues remain to be addressed regarding qPCR-based assays that impact specificity, sensitivity, and reliability. Robust eDNA assays include abiding by fundamental principles of qPCR best practices (e.g., MIQE; Bustin et al., 2010), while also requiring special considerations in their relationship within the eDNA survey workflow and application to often highly dilute and complex environmental samples. In the present perspectives, we aim to critically examine qPCR assay components and how they affect validation of novel assays and variation within eDNA data. We also identify critical limitations for eDNA assay design that can be addressed to enhance confidence in eDNA techniques.

2 | THE HEART OF EDNA DETECTION—THE ISSUE OF RELIANCE ON PUBLISHED EDNA ASSAYS

There is a wide range in quality of qPCR-based assays among the published literature and in practice. An example of a thorough quality assurance/quality control (QA/QC) validation pipeline that we have employed while developing over 30 successful qPCR-based eDNA detection assays is depicted in Figure 2, yet remarkably few eDNA assays have addressed all elements of this pipeline. A typical qPCR requires the isolated DNA sample containing the target taxon DNA, a thermostable Taq DNA polymerase, a DNA primer pair, a

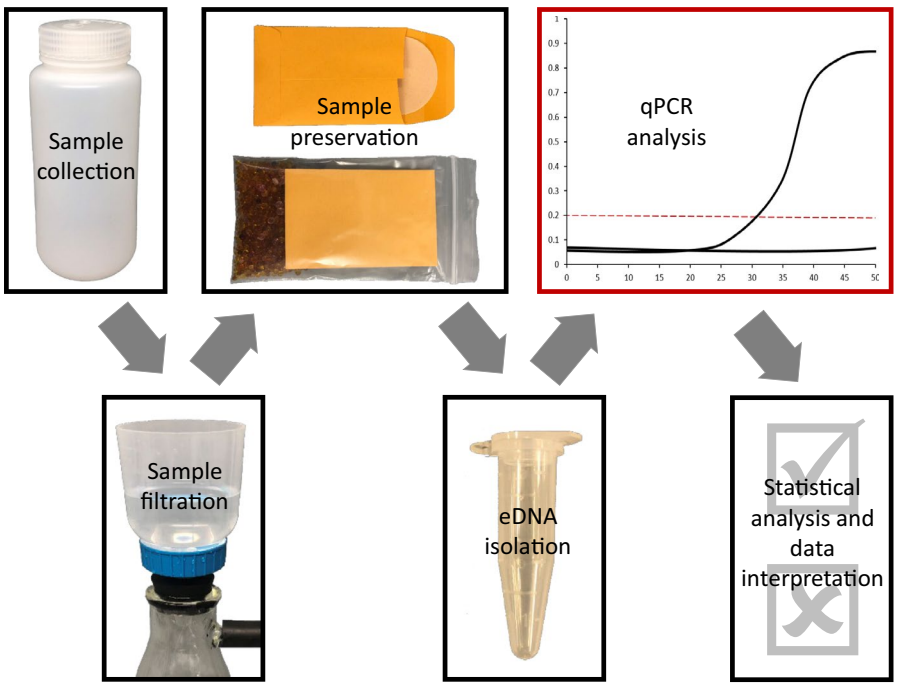

FIGURE 1 Overview of a general targeted eDNA workflow using water samples. eDNA in a water sample is captured on a filter membrane that is often preserved prior to DNA isolation and qPCR analysis [Colour figure can be viewed at wileyonlinelibrary.com]

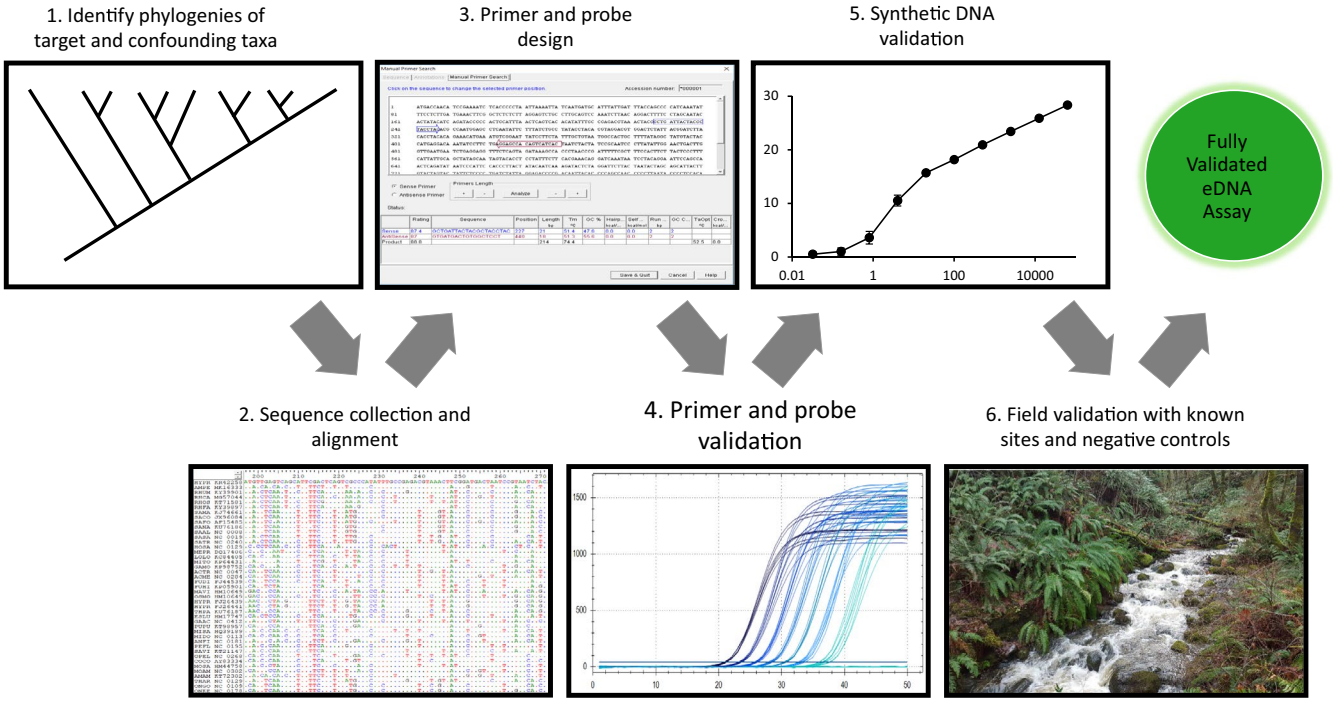

FIGURE 2 An example of a multistep qPCR-based assay design and validation workflow. The present perspective examines each step leading to a fully validated eDNA assay in sections 2.1–2.6. The numbered steps correspond to these sections [Colour figure can be viewed at wileyonlinelibrary.com]

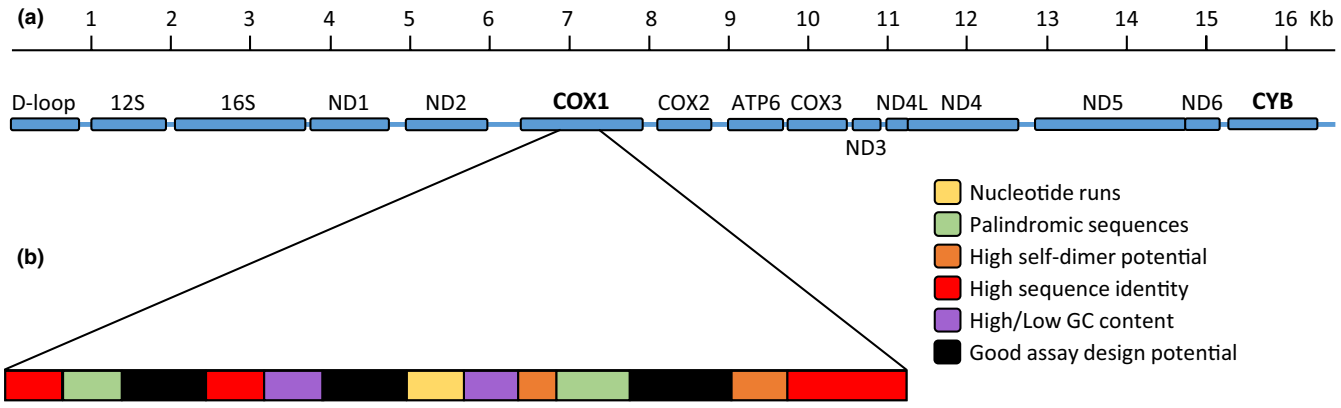

FIGURE 3 Limitations for eDNA assay design and implications for assay quality. (a) This schematic represents a generalized mitochondrial DNA gene structure. A circular mitochondrial genome is depicted in linear fashion to scale with all genes indicated (blue boxes) except the numerous tRNA-encoding genes. Typical animal mitochondrial genomes are ~16 Kb in size. The gene names are shown above the schematic along with a bp scale. Many taxa have no mitochondrial DNA sequence available. Very few have full mitochondrial sequences. Most common are partial gene sequences from the COX1 or CYB genes (bold). (b) A portion of the 650 bp COXI gene is detailed to demonstrate the design challenges, including satisfying the requirements for good primer/probe set performance, while using a region that effectively discriminates the target taxon from other species. The regions with good assay design potential may or may not be able to discriminate between taxa [Colour figure can be viewed at wileyonlinelibrary.com]

DNA hydrolysis probe with a fluorophore and quencher, deoxynucleotide 5′-triphosphates (or dNTPs), $MgCl_2$, other salts, and a buffer (for more details, see Helbing & Hobbs, 2019). Careful consideration of reaction components and how they interact with each other in the context of an environmental sample is key, but provides particular challenges when assessing eDNA.

2.1 | Identification of target and confounding taxa phylogenies

First, the phylogenies of the target taxon along with a search of potential confounding taxa should be established. These include closely related sympatric taxa and hybrids. In addition to the more

obvious related and co-occurring taxa, special attention should be given to any other DNA that is likely to be introduced to the samples during the workflow. In particular, human, dog, and any other taxa DNA that may be found in the laboratory setting should be considered as possible confounding DNA and therefore included in the design and validation phases of assay development. Also, taxa that co-occur in high abundance compared with the target taxon should also be identified. This step is critical to ensure the downstream workflow targets only the desired taxon. As we will see below, the limited sequence information available for target and sympatric taxa presents a particular problem in the design of robust eDNA assays.

2.2 | Sequence collection and alignment

The robustness of eDNA detection heavily relies on the design quality of the primers and probe. The choice of appropriate genes and sequence quality is crucial. The most common genes used to date for eDNA analysis are mitochondrial genes. These gene fragments are preferred over nuclear gene fragments since there are more copies per cell and portions of mitochondrial genes, particularly *cytochrome c oxidase I (known as MT-CO1, COI, or COX1)* and *cytochrome b (CYB)* are used extensively for DNA barcoding and DNA metabarcoding (Deiner et al., 2017; Thalinger et al., 2020) (Figure 3a).

By far, the most frequently used sequence is the 650 bp *COXI* sequence (Elbrecht et al., 2019), as this has been embraced as a useful barcode for taxon identification using DNA sequencing techniques (Deiner et al., 2017; Hebert et al., 2003). It is worth noting that some groups have obtained better eDNA metabarcoding coverage by using *12S* and *16S* mitochondrial rRNA genes rather than *COX1* (Clarke et al., 2014; Deagle et al., 2014). The extensive Barcode of Life Data Systems (https://www.boldsystems.org/) and International Barcode of Life (https://ibol.org/) databases have been truly transformative in our ability to identify and classify our planet's organisms. Despite these valuable DNA sequence resources available in publicly available databases, such as Genbank (National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/) and BOLD (https://www.boldsystems.org/), the sequences are often too short and limited in appropriate DNA sequence uniqueness to generate robust qPCR primer/probe sets capable of discriminating between taxa for use in eDNA detection (Figure 3b).

Typical eDNA assays amplify gene fragments (amplicons) that range between 50 and 400 bp in length (Freeland, 2017; Hernandez et al., 2020; Klymus et al., 2019; Thalinger et al., 2020). The forward and reverse primers are usually between 18 and 30 nucleotides (nt) long, and the probe is usually between 20 and 30 nt depending on dye chemistry (Hernandez et al., 2020; IDT, 2020; Klymus et al., 2019). The size of available sequences therefore has significant impacts on assay design potential. Unless the sequence is highly amenable to reasonable quality primer pair and probe designs, and all sympatric taxa exhibit low sequence identity to the target taxon at regions coinciding with the primer/probe designs, using sequences shorter than 1 kb to design selective assays which satisfy all of the demands of good qPCR can be very limiting and often result in a compromise of either selectivity or reaction efficiency (Wilcox et al., 2013). In addition to issues related to sequence length, careful scrutiny is required of each publicly available sequence to ensure it is not classified incorrectly. Several cases of mislabeled published sequences have been identified, particularly relating to poorly designed and utilized universal mitochondrial primer sets (Mioduchowska et al., 2018).

When designing a novel qPCR assay, all available mitochondrial gene sequences for the target, sympatric, and other confounding taxa should be compiled and aligned. The choice of sequence to use for assay design should consider the number of confounding taxa with corresponding sequence available, the level of sequence conservation, and how well-suited the sequence is for high-quality primers and probe. If a target taxon has closely related sympatric taxa, the assay design should be limited to those gene regions for which both taxa have sequences available. Once aligned, the sequences should be appraised for sequence differences between the target and confounding taxa. If possible, it is advisable to include multiple independent sequences of the target taxa to ensure this region is adequately conserved between target specimens and not a region subject to allelic variation (Wilcox et al., 2015). After the gene sequences have been narrowed down based on availability and sequence divergence, primer design software can be employed to assess their suitability for robust assay creation. If insufficient target and confounding taxa sequences are available to aid in assay design, then extensive qPCR testing must be done with DNA from multiple voucher specimens from target and possible confounding taxa to ensure that the assay candidates are selective for the target taxa (Thalinger et al., 2020).

2.3 | Primer and probe design

There are fundamental rules regarding good qPCR assay design that need to be considered. Superimposed on this are additional demands that eDNA application places on the qPCR assay given the complex matrices of environmental samples and the often low amount of target DNA within the sample. Several PCR-based methods exist (semiquantitative PCR, qPCR, and a variant of qPCR—droplet digital PCR). Of these, qPCR-based assays have the best combination of being the most accessible, cost-effective, and reliable (reviewed in Helbing & Hobbs, 2019). In addition, the primer pair and hydrolysis probe combination has proven to provide the most specific chemistry to increase the QA/QC of eDNA detection as it is far superior to primer-only-based methods relying on end-point (gel-based) amplicon evaluation or sequence-independent fluorescence (Wilcox et al., 2013). In contrast to nonspecific detection performed by double-stranded DNA-binding dyes (e.g., SYBR green; Ponchel et al., 2003), the primer pair/hydrolysis probe combination provides necessary additional sequence discrimination capability and specificity (Wilcox et al., 2013). The

best types of hydrolysis probes use a combination of fluorophore/quencher chemistry to reduce background fluorescence. Intact probes do not emit fluorescence at the wavelength of the liberated fluorophore, so no fluorescence should be observed when their emission wavelength is measured. Probes should be specific to their complementary sequences within a correctly synthesized amplicon to which they will selectively hybridize. During elongation, the 5′ → 3′ exonuclease activity of the DNA polymerase hydrolyzes (degrades) the probe only when the probe is bound with its complimentary sequence, releasing the quencher from the fluorophore and enabling fluorescence emission and detection (IDT, 2020). The use of the primer pair/hydrolysis probe strategy also reduces the interference of unwanted primer dimers as the mode of detection will ignore their presence, despite their still being created and impacting assay efficiency.

Despite the substantial body of literature that informs good assay design practices, many researchers still use and publish nonspecific and nonsensitive primer sets that cross-react with off-target organisms or else fail to reliably amplify target DNA. This is often due to the researchers using limited available sequence data to design primers, the running conditions are not stringent enough, and/or because they do not fully validate their simplex qPCRs, which is costly and time consuming. There are plenty of examples of this in the literature, but for courtesy reasons, none of these papers will be named within the present perspective paper. Rather, we will provide hypothetical examples that depict issues observed in the scientific literature.

When designing primer pair/hydrolysis probe sets, several QA/QC steps are needed in order to maximize qPCR efficiency and robustness. Using primer design software (e.g., Primer3, NCBI/Primer-BLAST, Primer Premier), selected primers need to be tested in silico to prevent primer dimer and hairpin formation, which could result in reduced reaction efficiency and detection artifacts. These issues are more likely to surface with sequences containing runs of 3+ nucleotides (nt) and guanine–cysteine (GC) content >60% (Dieffenbach et al., 1993). Primer design software will identify these characteristics and provide a free energy score (ΔG) for detected primer dimers and hairpins. A negative ΔG is to be avoided as this indicates that there is a high likelihood of formation. Runs of four or more consecutive Gs should be avoided. Thus, the general design benchmark is avoiding runs and choosing a GC content range of 40%–60% (Lorenz, 2012). Base-pair mismatches between target and nontarget sequences should be maximized to avoid cross-reaction, and particular attention should be given to the 3' ends of primers and the 5' ends of probes, as these regions are most critical for reducing off-target amplification (Freeland, 2017; Wilcox et al., 2013).

Other features, such as melting temperature (T_m) must be considered in choosing an appropriate primer set/probe combination. This has a direct bearing on the choice of annealing temperature (T_a) which, along with magnesium chloride ($MgCl_2$) concentration, influences reaction stringency (Lorenz, 2012). Stringency describes the set of conditions under which the qPCR occurs. Conditions of high stringency (high T_a with low $MgCl_2$ concentration) make it more difficult for the primers and probe to bind to the target DNA sequence and amplify it. Low stringency conditions make it more likely that primers/probes bind imperfectly to DNA and are more likely to result in spurious, nonspecific amplification and detection.

The primers/probe T_m, annealing and elongation temperatures, cycle duration, and concentrations of reaction components, including primers/probe, deoxynucleotide 5′-triphosphates (dNTPs), $MgCl_2$, and DNA polymerase can also influence the qPCR's stringency (IDT, 2020; reviewed in Lorenz, 2012). Briefly, optimal T_m approximated as $T_m \approx 4(G\text{-}C) + 2(A\text{-}T)$ should range between 52 and 58°C for primers, but a higher T_m will improve stringency and both primers' optimal temperature should not differ by more than 2–5°C (Lorenz, 2012). The T_m for probes should be about 6–8°C higher than the primers. Generally, the T_a should be no more than 5°C below the T_m of the lower primer T_m (Bustin & Huggett, 2017; Freeland, 2017).

In published eDNA papers, the most commonly used temperature regime is a two-step cycle (95°C denature, 60°C annealing/elongation) rather than a three-step (denature, annealing, elongation) cycle to save overall run time (Bustin, 2017). However, *Taq* DNA polymerase has an optimal elongation temperature of 70–80°C (Lorenz, 2012) and a three-step cycling regime (e.g., 95°C denature, 60–64°C annealing, 72°C elongation) may provide greater reaction specificity by providing optimal primer/probe annealing followed by optimal polymerase activity conditions. The three-step cycle approach can be considered if the two-step cycle approach is suboptimal (Jin et al., 2014). The dNTPs need to be added in equal concentrations; otherwise, this will affect the PCR efficiency. DNA polymerases require Mg^{2+} as a cofactor for activity, but too high Mg^{2+} concentrations will decrease the specificity of the reaction process. In general, a final reaction concentration of 0.5 to 5.0 mM Mg^{2+} is recommended (Lorenz, 2012). Most commercially available reaction mixes use 3.0 mM Mg^{2+}. It is important to choose the appropriate DNA polymerase from the variety that are available, as they are not all equal in their processivity and performance characteristics (Miura et al., 2013). *Taq* polymerase is the gold standard in qPCR analysis and comes with its optimal buffer and Mg^{2+} concentrations (Ricardo et al., 2020. These are excellent starting points for assay optimization.

Primer dimers can also occur if the primers and DNA polymerase function at room temperature (e.g., on the bench while preparing the samples); therefore, it is recommended to use DNA polymerases designed to work at high temperature only or to use a solution of $MgCl_2$ that is only released from its chemical matrix at high temperature (IDT, 2020).

2.4 | Primer pair and probe validation

Once a novel eDNA assay has been designed in silico according to the stipulations stated above, it must be empirically validated (Figure 2). Since environmental samples often contain substantially higher concentrations of nontarget DNA relative to target which is often at exceedingly low concentrations, the assay must have high

specificity and sensitivity to avoid type I and type II errors. A thorough validation program provides confidence in assay function and results and is a powerful tool in assay selection, application, and data interpretation by end users.

A panel of target and confounding taxa total DNA should be prepared from voucher specimens for assay validation. Multiple individuals of the target taxa should be surveyed to assess the effect of individual genetic variation on assay performance. DNA from individuals representing close genetic relatives and potential sympatric taxa should also be obtained to further assess assay specificity. Note that members of isolated subpopulations of the target taxon and novel confounding taxa present in unique ecosystems should be assessed in vitro before application of assays to new regions in order to confirm assay performance in highly localized populations. In addition, DNA from taxa that represent contamination risks should be included (e.g., human and dog). No template controls are included throughout the assay validation to ensure that no nonspecific amplification occurs (Freeland, 2017; Veldhoen et al., 2016).

Initial specificity testing of the forward and reverse primers is assessed for the taxa panel using nonspecific DNA intercalating dyes, such as SYBR Green and high total DNA concentration (e.g., 0.5–5 $\times$ 10^{-3} ng/μl) ideally using DNA from multiple voucher specimens and few technical replicates (e.g., Carim et al., 2016; Currier et al., 2018; Hobbs et al., 2020). The use of higher DNA concentrations at this stage provides a good assessment framework from which to evaluate assay specificity as tissue samples contain mitochondrial and nuclear DNA.

Reactions containing target DNA should demonstrate strong, exponential amplification within 40–50 cycles and a single strong peak in the melt curve at the predicted T_m for the amplicon (D'Haene et al., 2010; Dieffenbach et al., 1993; Wilcox et al., 2013). When reaction products are run on an agarose gel, a single band should be observed corresponding to the predicted amplicon length in reactions containing target template DNA. Nontarget reactions should not show amplification within 40–50 cycles, and not have visible bands when reaction products are run on an agarose gel. Limited off-target amplification and primer dimer products can be tolerated at this stage due to additional discrimination introduced when using a sequence-specific hydrolysis probe. When using a probe, off-target amplification will not result in fluorescence, but substantial production of by-products can greatly reduce reaction efficiency reducing overall assay sensitivity. Amplicon identity can be further confirmed via sequencing for increased confidence.

Once assay specificity has been preliminarily confirmed, the primer/probe combination can be assessed. Reactions containing target template must consistently show fluorescence above a standardized threshold. If these results confirm assay specificity, then additional technical replicates for target and possible confounder taxa can be run using multiple voucher specimens. This can provide further information regarding the possibility of low probability, off-target detection, and the ability of the assay to correctly identify a broad range of target taxa individuals.

Analytical assay sensitivity could be preliminarily assessed using a dilution series of target total DNA (e.g., 10^{-6} to 10^{-3} ng/μl Hobbs et al., 2020; Tréguier et al., 2014). Due to high degrees of variation in mitochondrial DNA concentration relative to total DNA in tissue samples, using total DNA as a basis for describing assay sensitivity is fraught with considerable variation between tissue and species types. Purified amplicons (e.g., Currier et al., 2018) and synthetic DNA have been used to address this issue. Of the two, synthetic DNA is the most standardizable across laboratories (discussed in Section 2.5).

When characterizing a novel assay design, sufficient technical replicates are required to provide a reliable degree of interpretive power. This is especially important at low concentrations and when observing off-target detection probabilities. These data provide the basis for deriving assay confidence intervals and inform recommended cycle threshold cut-off values and technical replicates required for high confidence data interpretation. For this reason, a high number of technical replicates is recommended during validation (e.g., up to 12–30 have been reported in Currier et al., 2018; Hobbs et al., 2020; Tréguier et al., 2014). Though more costly than running fewer technical replicates, an effective validation program can lead to significant cost savings and provides a reliable basis for eDNA development and expansion.

2.5 | Synthetic DNA validation

Due to variability in the proportion of mitochondrial and nuclear DNA, it is challenging to estimate template concentration in total DNA preparations derived from tissues. This variation also precludes interlaboratory comparison of assay performance. A solution to this problem is the use of readily available double-stranded synthetic amplicon, such as gBlocks™ (Klymus et al., 2017). Using a synthetic DNA fragment that exactly matches the theoretical amplicon produced by a given assay reacting with its target DNA sequence provides an objective measure of assay efficiency, which relates the qPCR cycle threshold (Ct or Cq) values to starting DNA copy numbers. As such, a synthetic amplicon approach is advantageous to determine an assay's sensitivity. This allows for the creation of a standard curve, from which one may determine the limit of detection (LOD) and limit of quantification (LOQ) for the assay (e.g., Hobbs et al., 2019, 2020; Klymus et al., 2019; Lesperance et al., 2020 and references therein). These metrics provide statistical error margins that may be translated to field samples to improve the robustness of project data interpretation.

2.6 | Field validation

Several studies have demonstrated the usefulness of qPCR-based eDNA assays; however, like any other technique, there are also limitations associated with type I and type II errors. False positives can occur when the assay does not succeed in detecting the targeted

taxon, but instead detects other taxa present in the collected samples. Similarly, false negatives result in not detecting the target taxon present in the ecosystem. Both types of errors can originate from poor qPCR assay design. To minimize these errors, it is of paramount importance to conduct preliminary robust primer/probe set optimization, but once a qPCR assay is determined to be both specific and sensitive in vitro for a given taxon, it should be tested in situ against well-characterized environmental samples. In addition, eDNA samples from real-world sites where the targeted taxon is known to be present and active should be tested to assess the false-negative rates. Likewise, sites where the targeted DNA presence is extremely unlikely should be scrutinize to establish associated false-positive rates. Field validation provides a basis for establishing specific scoring schema and setting a decision threshold for recommending detection/nondetection of a given taxon. This robust validation process provides detailed information about assay performance to future users, a basis for interlaboratory proficiency and reliability, and valuable insight into assay selection, study design, and troubleshooting. One initiative that is beginning to address these issues is the introduction of a "validation scale" (DNAqua-net, 2020, https://edna-validation.com/) that is being developed as a standardized way to communicate the extent to which a targeted eDNA assay has been validated.

3 | CONCLUSIONS

End users representing provincial and federal governments, First Nations, environmental consultancies, natural resource operators, and environmental not-for-profit organizations are pushing for investment in addressing and mitigating eDNA application components that are currently preventing acceptance of eDNA as a rigorous and defensible method for species inventories and monitoring. The present perspective is meant to assist in their development and evaluation. In the next years, the eDNA research community should concentrate their efforts to develop the required resources and approaches for creating robust and accessible validated eDNA assays and break the barriers to acceptance of eDNA methods. The reduced cost and the simplified data analysis of qPCR-based eDNA testing will democratize the qPCR approach to several groups of interest. The final perspective's recommendations are that the eDNA research community includes best practices (or standards) for minimal required information when using qPCR-based assays and work together in increasing the full mitochondrial genome sequencing for many more taxa to allow robust primer/probe test designs.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All the authors have contributed in writing this manuscript. MJA has made the figures.

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REFERENCES

Baillie, S. M., McGowan, C., May-McNally, S., Leggatt, R., Sutherland, B. J. G., & Robinson, S. (2019). Environmental DNA and its applications to Fisheries and Oceans Canada: National needs and priorities. *Canadian Technical Report of Fisheries and Aquatic Science*, *3329*, xiv+84.

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*, 1–17. https://doi.org/10.1007/s10592-015-0775-4

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M., & Mergeay, J. (2020). Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). *Journal of Fish Biology*, *10*.

Bustin, S. A. (2017). How to speed up the polymerase chain reaction. *Biomolecular Detection and Quantification*, *12*, 10–14. https://doi.org/10.1016/j.bdq.2017.05.002

Bustin, S. A., Beaulieu, J.-F., Huggett, J., Jaggi, R., Kibenge, F. S. B., Olsvik, P. A., Penning, L. C., & Toegel, S. (2010). MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology*, *11*, 74. https://doi.org/10.1186/1471-2199-11-74

Bustin, S., & Huggett, J. (2017). qPCR primer design revisited. *Biomolecular Detection and Quantification*, *14*, 19–28. https://doi.org/10.1016/j.bdq.2017.11.001

Carim, K. J., Dysthe, J. C. S., Young, M. K., McKelvey, K. S., & Schwartz, M. K. (2016). An environmental DNA assay for detecting Arctic grayling in the upper Missouri River basin, North America. *Conservation Genetics Resources*, *8*, 197–199. https://doi.org/10.1007/s12686-016-0531-1

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, *14*(6), 1160–1170.

Currier, C. A., Morris, T. J., Wilson, C. C., & Freeland, J. R. (2018). Validation of environmental DNA (eDNA) as a detection tool for at-risk freshwater pearly mussel species (Bivalvia: Unionidae). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *28*, 545–558. https://doi.org/10.1002/aqc.2869

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: Not a perfect match. *Biology Letters*, *10*, 20140562.

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*, 5872–5895.

Dieffenbach, C. W., Lowe, T. M., & Dveksler, G. S. (1993). General concepts for PCR primer design. *Genome Research*, *3*, 530–537. https://doi.org/10.1101/gr.3.3.s30

D'Haene, B., Vandesompele, J., & Hellemans, J. (2010). Accurate and objective copy number profiling using real-time quantitative PCR. *Methods*, *50*(4), 262–270. https://doi.org/10.1016/j.ymeth.2009.12.007

Elberri, A. I., Galal-Khallaf, A., Gibreel, S. E., El-Sakhawyb, S. F., El-Garawania, I., El-Sayed Hassab ElNabia, S., & Mohammed-Gebaa, K.(2020). DNA and eDNA-based tracking of the North African sharptooth catfish *Clarias gariepinus*. *Molecular and Cellular Probes*, *51*, 101535.

Elbrecht, V., Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J., Hajibabaei, M., Wright, M., Zakharov, E. V., Hebert, P. D. N., & Steinke, D. (2019). Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ*, *7*, e7745.

Freeland, J. R. (2017). The importance of molecular markers and primer design when characterizing biodiversity from environmental DNA. *Genome*, *60*(4), 358–374.

Goldberg, C., & Strickler, K. (2017). *eDNA protocol sample collection*. Washington State University. https://s3.wp.wsu.edu/uploads/sites/686/2017/01/WSU-eDNA-sampling-protocol-Jan2017.pdf

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

Hebert, P. D., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1512), 313–321.

Helbing, C. C., & Hobbs, J. (2019). *Environmental DNA standardization needs for fish and wildlife population assessments and monitoring*. CSA Group. https://www.csagroup.org/wp-content/uploads/CSA-Group-Research-Environmental-DNA.pdf

Hernandez, C., Bougas, B., Perreault-Payette, A., Simard, A., Côté, G., & Bernatchez, L. (2020). 60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. *Environmental DNA*, *2*(3), 373–386. https://doi.org/10.1002/edn3.89

Hobbs, J., Adams, I., Round, J. M., Allison, M. J., Goldberg, C. S., & Helbing, C. C. (2020). Revising the range of Rocky Mountain tailed frog, *Ascaphus montanus*, in British Columbia, Canada, using environmental DNA methods. *Environmental DNA*, *2*(3), 350–361.

Hobbs, J., Goldberg, C. S., Helbing, C. C., & Veldhoen, N. (2017). *Environmental DNA protocol for freshwater aquatic ecosystems Version 2.2*. https://www.hemmera.com/wp-content/uploads/2018/08/171115-eDNA-protocol-V2.2.pdf

Hobbs, J., Round, J. M., Allison, M. J., & Helbing, C. C. (2019). Expansion of the known distribution of the coastal tailed frog, *Ascaphus truei*, in British Columbia, Canada using robust eDNA detection methods. *PLoS One*, *14*, e0213849. https://doi.org/10.1371/journal.pone.0213849

IDT (2020). *Integrated DNA Technologies' site consulted on June 2020*. https://www.idtdna.com/pages/education/decoded/article/designing-pcr-primers-and-probes

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*, 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jin, H. X., Seo, S. B., Lee, H. Y., Cho, S., Ge, J., King, J., Budowle, B., & Lee, S. D. (2014). Differences of PCR efficiency between two-step PCR and standard three-step PCR protocols in short tandem repeat amplification. *Australian Journal of Forensic Sciences*, *46*(1), 80–90.

Klymus, K. E., Marshall, N. T., & Stepien, C. A. (2017). Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. *PLoS One*, *12*(5), e0177643.

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2020). Reporting the limits of detection (LOD) and quantification (LOQ) for environmental DNA assays. *Environmental DNA*, *2*, 271-282. https://doi.org/10.1002/edn3.29

Laramie, M. B., Pilliod, D. S., Goldberg, C. S., & Strickler, K. M. (2015). *Environmental DNA sampling protocol— Filtering water to capture DNA from aquatic organisms: U.S. Geological Survey Techniques and Methods* (book 2, chap. A13, 15 p.).

Lee, A. H., Lee, J., Hong, S., Kwon, B.-O., Xie, Y., Giesy, J. P., Zhang, X., & Khim, J. S. (2020). Integrated assessment of west coast of South Korea by use of benthic bacterial community structure as determined by eDNA, concentrations of contaminants, and in vitro bioassays. *Environment International*, *137*, 105569.

Lesperance, M., Allison, M. J., Bergman, L. C., Hocking, M., & Helbing, C. C. (2020). A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. Submitted.

Loeza-Quintana, T., Abbott, C. L., Heath, D. D., Bernatchez, L., & Hanner, R. H. (2020). Pathway to Increase Standards and Competency of eDNA Surveys (PISCeS)—Advancing collaboration and standardization efforts in the field of eDNA. *Environmental DNA*, *2*(3), 255–260. https://doi.org/10.1002/edn3.112

Lorenz, T. C. (2012). Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments: Jove*, *63*, e3998.

Mioduchowska, M., Czyż, M. J., Gołdyn, B., Kur, J., & Sell, J. (2018). Instances of erroneous DNA barcoding of metazoan invertebrates: Are universal cox1 gene primers too "universal"? *PLoS One*, *13*(6), e0199609. https://doi.org/10.1371/journal.pone.0199609

Miura, M., Tanigawa, C., Fujii, Y., & Kaneko, S. (2013). Comparison of six commercially-available DNA polymerases for direct PCR. *Revista do Instituto de Medicina Tropical de São Paulo*, *55*(6), 401–406. https://doi.org/10.1590/S0036-46652013000600005

Nicholson, A., McIsaac, D., MacDonald, C., Gec, P., Mason, B. E., Rein, W., Wrobel, J., de Boer, M., Milián-García, Y., & Hanner, R. H. (2020). An analysis of metadata reporting in freshwater environmental DNA research calls for the development of best practice guidelines. *Environmental DNA*, *2*(3), 1–7. https://doi.org/10.1002/edn3.81

Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T., Douglas, S. H., Field, S. L., Bell, S. M., Combaret, V., Puisieux, A., & Mighell, A. J. (2003). Real-time PCR based on SYBR-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnology*, *3*, 18.

Ricardo, P. C., Françoso, E., & Arias, M. C. (2020). Fidelity of DNA polymerases in the detection of intraindividual variation of mitochondrial DNA. *Mitochondrial DNA Part B*, *5*(1), 108–112.

Thalinger, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., Traugott, M., Goldberg, C. S., & Bruce, K. (2020) A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *BioRxiv*. https://doi.org/10.1101/2020.04.27.063990

The eDNA Society (2019) *Environmental DNA Sampling and. Experiment Manual. Version 2.1* (published April 25, 2019). www.ednasociety.org

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18.

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., & Roussel, J.-M. (2014). Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, *51*, 871–879.

Tsuji, S., Takahara, T., Doi, H., Shibata, N., & Yamanaka, H. (2019). The detection of aquatic macroorganisms using environmental DNA

analysis—A review of methods for collection, extraction, and detection. *Environmental DNA*, *1*, 99–108. https://doi.org/10.1002/edn3.21

US Fish and Wildlife Service (2018). *Quality assurance project plan eDNA monitoring of bighead and silver carps* (Bloomington, MN: US Fish and Wildlife Service, USFWS Midwest Region). 168 pp. https://www.fws.gov/midwest/fisheries/eDNA/documents/QAPP.pdf

Veldhoen, N., Hobbs, J., Ikonomou, G., Hii, M., Lesperance, M., & Helbing, C. C. (2016). Implementation of Novel design features for qPCR-based eDNA assessment. *PLoS One*, *11*(11), e0164907.

Wilcox, T. M., Carim, K. J., McKelvey, K. S., Young, M. K., & Schwartz, M. K. (2015). The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of *Oncorhynchus*. *PLoS One*, *10*(11), e0142008. https://doi.org/10.1371/journal.pone.0142008

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare taxa using environmental DNA: The importance of primer specificity. *PLoS One*, *8*(3), e59520.

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