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Guidance on the Use of Targeted Environmental DNA (eDNA) Analysis for the Management of Aquatic Invasive Species and Species at Risk

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Foreword

This series documents the scientific basis for the evaluation of aquatic resources and ecosystems in Canada. As such, it addresses the issues of the day in the time frames required and the documents it contains are not intended as definitive statements on the subjects addressed but rather as progress reports on ongoing investigations.

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ABSTRACT

Analysis of DNA from environmental samples (i.e., environmental DNA or eDNA) is increasingly being used as a non-intrusive, sensitive, and often cost-effective biological monitoring approach, either on its own or to complement other methods. Due to the numerous promising applications of eDNA, there has been significant recent growth in eDNA research and development; however, the complexity and rapid evolution of related methodologies has created challenges for resource managers when deciding how to apply eDNA technologies to inform decision making.

This document was prepared in response to a request from Fisheries and Oceans Canada's Aquatic Invasive Species (AIS) and Species at Risk (SAR) Programs for science advice on eDNA to support decision making on aquatic species and ecosystems. The need for this science advice document also was identified by the National Aquatic Invasive Species Committee (NAISC).

This document contains two main components to support AIS and SAR managers: 1) guidance on eDNA that includes definitions and considerations related to eDNA sampling, detection, and analysis for AIS and SAR managers; and 2) a reporting template that identifies reporting requirements for eDNA service providers delivering results to AIS and SAR managers. This document focuses on targeted eDNA approaches that selectively detect the DNA of a single species or taxon, often using quantitative polymerase chain reaction (qPCR).

This research document supports the science advice, which is the Department's first step towards providing national guidance on eDNA by promoting more consistent reporting and communication between eDNA service providers and AIS and SAR managers. We recommend that AIS and SAR managers use the science advisory report, guidance, and reporting template as part of their communication plan for eDNA projects both before a project commences and while interpreting results. Consistent reporting of eDNA results is intended to improve AIS and SAR managers' confidence in the use of eDNA to support the implementation of federal, provincial, or territorial legislation (e.g., *AIS Regulations*, *Species at Risk Act*).

INTRODUCTION

Environmental DNA (eDNA) approaches increasingly are being used to monitor species of conservation and management concern, including Aquatic Invasive Species (AIS) and Species at Risk (SAR). Environmental DNA is defined in this guidance document as DNA extracted from environmental samples (e.g., water, biofilms, air, sediment, gut contents, feces) and analyzed for biological monitoring and surveillance.

Fisheries and Oceans Canada (DFO), through the AIS and SAR Programs, has identified the need for guidance on the use of eDNA to support decision making for the management of aquatic species and ecosystems. A need for eDNA guidance also was recognized by the National Aquatic Invasive Species Committee (NAISC), a collaborative platform that promotes national coordination on AIS issues across federal, provincial, and territorial governments. AIS and SAR surveillance and monitoring programs have started to include eDNA approaches as a rapid, sensitive, and often cost-effective method to detect and monitor a variety of species or taxa in all seasons and environments, including remote and disparate locations. Analysis of eDNA can provide monitoring data to complement conventional monitoring methods (e.g., netting, electrofishing) generally without the need to collect or stress living organisms, making it ideal for the study of AIS and SAR that are rare, elusive, and/or have cryptic life stages. The genetic information that is generated can contribute to a better understanding of population dynamics, population size, distribution, and, in some cases, the abundance of species or taxa.

Environmental DNA analysis is a powerful tool for many resource and ecosystem management programs, including the AIS and SAR Programs. For AIS applications, the sensitivity of eDNA methods permits early detection of novel bioinvasions and bioinvasion fronts at large spatial scales, which can allow for rapid or timely management responses and control efforts to be undertaken before populations become established. The sensitivity of eDNA methods is equally valuable for the SAR Program, where eDNA is used as a non-intrusive approach to detect and monitor SAR and other species necessary for the survival or recovery of SAR (e.g., host species, keystone species). Environmental DNA also can be used for SAR to: identify potential risk factors for SAR (e.g., competitor species); detect illegal trade activities; and provide evidence of poaching, species presence (e.g., to delineate habitats that require protection such as critical habitat), and species absence in a given area (e.g., evaluating permit applications). Environmental RNA (eRNA) may also be used to detect species and due to its more rapid degradation compared to eDNA, it may be a better proxy for detecting living organisms and monitoring their health (see other possible applications of eRNA in Cristescu 2019). However, eRNA is challenging to work with and more research is required to support this new field. Estimates of biomass and relative changes in abundance can also be inferred in some eDNA studies (Spear et al. 2020), though these inferences require vigorous validation and ground-truthing for each new application (e.g., different species, habitat, season). All together, these new technologies can help generate population-level biomonitoring data that can be used to inform different management options or recovery efforts and support decision making for AIS and SAR.

Like any other monitoring method, eDNA detection has limitations, including: 1) eDNA is an indirect approach to monitor the occurrence of species and cannot provide biological or demographic data on target organisms (e.g., length, condition, recruitment); 2) samples can become contaminated (e.g., target DNA accidentally transferred between sites), which could produce a false positive or misinterpretation of results; 3) physical and chemical properties of aquatic environments can influence eDNA concentration and detection probability (note that detection probabilities can be determined only after a study); 4) detection of eDNA does not in itself confirm the presence of a living organism in the sampled area (e.g., DNA could have been transported into the system, or the DNA could be from a transient species no longer present); and 5) inconsistencies with estimating

species biomass or abundance from eDNA concentrations. Despite these limitations, numerous strengths of eDNA detection make it a valuable addition to researchers' and managers' toolboxes, and worthy of consideration by managers as a detection and monitoring method.

In response to calls for standardization of eDNA reporting and methods, guidelines and laboratory standards increasingly are being published in the primary literature (e.g., Goldberg et al. 2016; Shu et al. 2020). Additional standardization efforts are underway through the Canadian Standards Association (CSA 2019), Pathway to Increase Standards and Competency of eDNA Surveys (PISCeS) coordinated out of the University of Guelph, and many international groups. While the scientific community continues to advance towards standardization of eDNA practices, guidance is urgently needed by DFO managers who currently use, or are considering using eDNA results to support decision making on AIS and SAR.

There are many different methods that can be used to detect eDNA, each with different applications or desired outcomes. These can be divided broadly into two categories: targeted (single-species or taxon-specific) and semi-targeted (multi-species or community). Targeted approaches, which are the focus of this guidance document, detect and potentially also quantify the DNA of a single species or taxon; targeted assays are sometimes combined to detect multiple targets simultaneously (i.e., multiplexing). Semi-targeted (or community) approaches, often referred to as metabarcoding, encompass a wide range of high-throughput sequencing (HTS) techniques that are used to assess the biotic composition of ecosystems (see Bylemans et al. 2019 and Ruppert et al. 2019 for a comparison of targeted and community approaches). These approaches differ from targeted techniques; instead of using species- or taxon-specific primers in the polymerase chain reaction (PCR; to amplify DNA) to detect and quantify target DNA, they use generalized or "universal" primers and then simultaneously sequence diagnostic barcoding markers from multiple species or larger taxon groups. These sequences are then matched to reference sequences in online genetic databases (or in-house reference databases) to identify the community of organisms present in the sample. Semi-targeted approaches are newer, methodologically and analytically more complex, and are evolving very quickly, whereas targeted approaches are more established; thus targeted approaches are the focus of this document.

Currently, quantitative polymerase chain reaction (qPCR) is the standard analytical technique used to determine absolute or relative quantities of a known DNA sequence in a sample. Targeted eDNA approaches that use qPCR, when properly designed and thoroughly validated, have proven to be effective at reliably detecting eDNA of aquatic organisms (see Jerde et al. 2011 and Sigsgaard et al. 2015). Field-portable qPCR platforms have been developed and are currently being pilot tested by environmental scientists for on-site detection of eDNA (Nguyen et al. 2018; Thomas et al. 2019). These novel platforms are designed to form a complete field eDNA sampling and detection process, and are capable of delivering rapid results (some in < 60 min). Advancements in portable eDNA systems may allow for more effective early detection and monitoring of species, enabling AIS managers to adjust their actions in near real-time in response to field detections of eDNA from AIS.

In addition to qPCR assays, other apparatus or approaches may be used for eDNA detection. While conventional polymerase chain reaction (cPCR or PCR) can be used for eDNA studies, its detection sensitivity is less than qPCR and therefore is not recommended for eDNA analysis. Droplet digital PCR (ddPCR) also is used to detect eDNA. This technology benefits from increased precision and sensitivity in detecting low amounts of DNA (Mauvisseau et al. 2019); however, the higher costs associated with ddPCR have limited the widespread use of this relatively new technology. Research has shown that ddPCR results are acceptable for eDNA studies (Capo et al. 2019; Mulero et al. 2019), and will likely be utilized more as costs continue to drop. CRISPR/Cas technology (Williams et al. 2019) is being explored, but demonstration of proof-of-concept is needed before it can be recommended for eDNA studies. Testing also is underway for autonomous

eDNA sampling instruments (e.g., underwater vehicles, drones, sample processors) to determine if eDNA collection options can be enhanced beyond what is currently possible with conventional monitoring methods (Yamahara et al. 2019; Sepulveda et al. 2020a).

PURPOSE OF THIS DOCUMENT

Rigorous and defensible eDNA-based protocols that produce repeatable, reproducible, and accurate results are needed for regulators and managers to accept and have confidence in eDNA evidence to support decision making. To begin to address this need, a previous DFO effort identified the state of knowledge on eDNA through publication of a literature review report (Baillie et al. 2019). The current research document (this guidance document) expands upon Baillie et al. (2019) by providing a model for enabling consistent and transparent communication and reporting of eDNA results aimed at AIS and SAR managers as the primary end users. The aim is that this document will increase their confidence in the data provided and how to use eDNA data relative to data from conventional monitoring methods. This document may be applicable more broadly to government managers and other end users.

This document focuses on targeted eDNA approaches as these are the most well-understood, reliable, and credible. As such, semi-targeted approaches will not be addressed specifically. While semi-targeted approaches have vast potential to assess community biodiversity, they are less established and well-understood than targeted approaches, and methodological complexities (e.g., potential species detection bias, reference database errors) make it challenging to develop standard guidance at this point in time. However, eDNA sample collection, filtration, and extraction are similar for both approaches, therefore reporting guidance given here on those components also may be applicable to eDNA metabarcoding studies. Another focus of this document is the use of targeted eDNA approaches for qualitative assessments of species presence. Currently, the state of knowledge on using targeted approaches to estimate relative abundance and biomass is limited. Similar to other conventional monitoring methods, it is challenging to infer the absence of a species using eDNA approaches and requires extensive and repeated sampling often in combination with other sources of biological information and expert opinion.

The lack of reporting standards for eDNA and inconsistency in reporting among eDNA studies (Nicholson et al. 2020) creates challenges for end users and eDNA service providers (i.e., DFO Science or other third party service providers) when communicating eDNA results, leading to confusion about appropriate management responses. To address these challenges, we provide two pieces of guidance on targeted eDNA approaches:

1. General guidance on targeted eDNA approaches, including a glossary of terminology, that provides essential information for designing, conducting, reporting, and interpreting eDNA studies; and
2. An accompanying eDNA reporting template that identifies key methods and results to be reported in the template or appendices by eDNA service providers to allow for eDNA result interpretation (found in Annex 1; fillable PDF version available on the CSAS website).

The purpose of this document is to encourage more consistent reporting and communication of eDNA results between end users and eDNA service providers. The components of this document are intended to be adaptable to a broad range of targeted eDNA sampling and analysis protocols. Together, these components aim to increase confidence and reliability in eDNA detection of AIS and SAR by providing the resources and tools to improve the reporting and communication of important aspects of eDNA studies to support the implementation of federal, provincial, or territorial legislation (e.g., *AIS Regulations*, *Species at Risk Act*). However, this document also may be useful to other end users, researchers, and eDNA practitioners.

SUMMARY OF CONTENTS AND INSTRUCTIONS

This document, which supports the science advice, contains two main components: a guidance document and an eDNA reporting template. Details on the contents of each are provided below. The guidance document is predominantly aimed at AIS and SAR managers wishing to use eDNA testing as a tool to help them make sound, science-based decisions, and the reporting template is predominantly aimed at eDNA service providers delivering results to AIS and SAR managers. The two documents dovetail throughout and are designed to be complementary, such that managers or other end users receiving eDNA results via the reporting template have all the necessary information in the guidance to derive sound information to confidently assess the quality of results. Likewise, service providers will find instructions needed to complete the reporting template within the guidance document. While many fields in the reporting template are self-explanatory, some fields will require reference to the guidance for detailed instructions. The guidance document is divided into sections that parallel the sections in the reporting template.

Guidance document: Provides essential information for designing, conducting, reporting, and interpreting eDNA surveys, including study design, eDNA sampling of various substrates, post-collection sample processing, laboratory methods, and strategies for interpreting qPCR results to determine presence/absence of target DNA. At the top of each section of the guidance, general considerations for each stage of eDNA study design and implementation are given for AIS and SAR managers to facilitate understanding of the importance of the information being requested in the reporting template, followed by instructions for eDNA service providers filling out the reporting template. Text boxes are used throughout to explain fundamental eDNA concepts.

While the aim of the guidance and the reporting template is to promote more consistent communication and reporting of results, any inferences about species presence or absence should be done on a case-by-case basis, taking into account any other available information (e.g., known species distributions, suitable habitat, life cycles, variability in environments, results from previous surveys) and involving discussions between managers and eDNA service providers. The guidance document is best used by both end users and science experts working together to design studies and interpret findings in a way that best meets study aims.

The glossary defines the terms and concepts associated with targeted eDNA approaches to promote more consistent understanding and communication of eDNA results.

Metadata appendices: Some required information is to be appended to the eDNA reporting template. This includes four mandatory appendices found in Annex 2.

- Appendix 1 - Maps;
- Appendix 2 - Contamination prevention procedures;
- Appendix 3 - qPCR protocol; and
- Appendix 4 - Metadata and qPCR data.

Other project-specific information can be appended to the eDNA reporting template as needed.

eDNA reporting template: Reporting requirements are presented in the eDNA reporting template. The function of the eDNA reporting template is to encourage consistent reporting of eDNA results by providing a comprehensive overview of an eDNA project and identifying information that is crucial to demonstrate scientific integrity and establish confidence in the eDNA project and associated results. The template does not replace a detailed protocol but rather is a tool to highlight and compile the critical aspects of a protocol and its implementation so managers can more easily interpret and evaluate results. The reporting template should be filled out using simple language and sufficient detail for an AIS or SAR manager to understand what was done. Detailed information

related to individual samples is captured in the metadata appendices; the template is predominantly for capturing summary information and descriptions as well as brief additional details. If individual recommended elements are not applicable, write N/A in the reporting template and provide as much detail as possible for an end user to understand study elements and results.

SECTION I: eDNA TESTING– SAMPLE SUBMISSION INFORMATION

Section summary: This section provides information on important basic eDNA project elements and protocols. There are four appendices found in this section that describe sampling techniques and procedural practices in the field and the laboratory (see Annex 2). Each appendix presents important information that encourages consistent reporting and communication of eDNA results, making them more reliable. Appendix 1 outlines sampling sites on maps, and the information that must be included on any map included in an eDNA study. The information found on maps should illustrate sampling effort in the geographic region of the study and provide insight on any factors that could affect eDNA detection. Appendix 2 outlines contamination prevention procedures and measures that were taken to reduce contamination in the field and laboratory. Contamination prevention procedures are especially important when dealing with eDNA samples, as it can affect the interpretation of eDNA results. Appendix 3 outlines the guidelines and protocols used in an eDNA qPCR assay (following Bustin et al. 2009). These protocols ensure that important assay elements are being communicated to end users. Appendix 4 outlines the metadata and qPCR data that must be included for the tracking and traceability of samples. It is important to report on these data because they contain key details on the analysis of the eDNA samples that are used to interpret results.

Submission information: This section includes the report title, project number, laboratory accreditation and certification information, and identifies the eDNA service provider and end user, providing contact information for both.

Executive summary: The executive summary should be a brief description of study objectives including rationale and main findings derived from both eDNA samples and controls. Inferences about target eDNA presence or absence should reflect the level of validation and interpretation described in Sections E, F, and G.

Metadata appendices: Confirm that the four mandatory appendices described in Annex 2 have been provided. If applicable, list any additional appendices that describe important project elements, as indicated in this guidance document (e.g., complex experimental approaches, analytical techniques, and/or results outside the scope addressed in the guidance).

SECTION II: STUDY DESIGN AND eDNA SAMPLING

Section summary: This section provides information on the main elements involved in developing a robust eDNA study to allow meaningful interpretation of results. The methods chosen to collect DNA from the environment (i.e., field methods) are critical to results interpretation and should be chosen thoughtfully to meet the goals of the study. Results interpretation relies on proper sampling design and method selection; therefore any methods chosen should consider previous studies, logistical constraints, biology and habitat preferences of the target species, and water characteristics. Many of the study design and sampling considerations were developed with sampling of ecosystems in mind; however guidance and tools could be adapted for use in other types of situations where eDNA may be used (e.g., testing ballast, biofouling, bait fish).

Within this section are three subsections: study information, study design, and eDNA sample collection. The study information subsection outlines six elements that should be mentioned and explained in every eDNA study, such as the study objectives and the geographic region of the study. The study design subsection outlines the critical steps of designing an eDNA study. In order

to properly communicate the significance of these steps, important sampling terms have been defined clearly in this subsection for the service providers and end users. This subsection also discusses eDNA detection probability and the various factors that can affect it, such as the shedding rate of the target organism and weather events. Controls are important to include in the study design in order to detect any false negatives or false positives and validate results or highlight sources of error. The final subsection, eDNA sample collection, outlines and defines the information needed to report the methodology for eDNA capture, such as the sample depth(s) and the sample processing method.

As with other areas of the eDNA workflow, it is important for managers to consult an eDNA expert (e.g., DFO Science) on a case-by-case basis to optimize study design, especially for studies that require developing new sampling design or laboratory methods. It is recommended that a communication plan be developed *a priori* to establish the expected flow of information between all parties involved in an eDNA study from study design to the notification of eDNA results (Box 1).

Box 1. Communication plan

eDNA end user: a manager, client, or requestor of eDNA services who ultimately uses or is intended to ultimately use eDNA results.

eDNA service provider: it is recognized that eDNA services may involve more than one entity. For the purpose of communication and reporting, the eDNA service provider is the overall project manager who is responsible to communicate results to the end user.

- Communication plans ensure that project outputs are reported in a comprehensive and transparent manner that meets the end user's needs, allowing for timely, evidence-based decision making.
- Communication plans identify how, when, and what information is relayed among end users and the eDNA service provider(s).
- Communication plans should be adjustable, such that they can evolve to accommodate shifting needs and opportunities.
- At the onset of a project, a communication plan should be developed jointly with the end user and the eDNA service provider that includes:
    - the points of contact for both end user(s) and service provider(s);
    - the reason for testing and, if necessary, the potential impacts, consequences, and/or actions of results:
        - this information may influence field sampling and laboratory analysis design, and results reporting schedules; and
        - it is critical to describe how unexpected amplification of negative controls will be treated prior to initiating the study.
    - Estimated timelines for results reporting by the eDNA service provider and how to advise the end user of any subsequent changes to schedules or sampling plans/design.
    - For example, for projects where detection can have important impacts, such asearly detection of AIS, it is recommended to report results as they are obtained (i.e., presumptive detection of an AIS), whereas others can be reported at project completion.
    - If applicable, plan for how results and uncertainty can be communicated to the public.

A. Study information

General considerations: This section provides basic information of an eDNA study. The following considerations may influence the methods chosen to collect eDNA, impact the species detection probability, and/or affect the validity of results.

A.1 Species targeted: Provide common name and Latin name of the species targeted by the study.

A.2 Study objectives: Provide the reason for testing (e.g., species detection for conservation, early detection of AIS, secondary spread of AIS, testing effectiveness of AIS eradication measures, SAR population size estimation). Additional information may be necessary to elaborate on the reason for testing (e.g., pre- versus post-eradication sampling, SAR detection to identify candidate Marine Protected Area [MPA] sites, concurrent AIS and SAR sampling).

A.3 Geographic location and/or region: Identify and describe the geo-ecological location(s) or study area(s) (e.g., province, National Park, Fishery Management Area, Bay Management Area). See Box 2 for more information. Maps should be added in Appendix 1.

A.4 Sampling date (range): Indicate the start and end dates of the project in mm/dd/yyyy. Dates for individual sample collection should be captured in metadata.

A.5 Sample types: Indicate substrate sampled using the dropdown menu (i.e., water [freshwater, brackish, marine], sediment, stomach / gut contents, bulk zooplankton, or other). For 'other', provide details. Select 'bulk sample' if DNA was extracted from a fraction of a larger mixture of organisms (e.g., zooplankton and microorganisms).

A.6 Mapping databases: Indicate whether data generated here have been archived in any open-access mapping databases (e.g., Aquatic eDNAtlas Project) and include details, if applicable.

Box 2. Defining geographic regions, sites, stations, and replication

An eDNA sampling plan needs to be clearly defined, and the proposed definitions here are meant to facilitate the communication between eDNA service providers and end users.

Geographic location and/or regions are broad units that describe the geographic area and/or context of the study (e.g., province, National Park, Fishery Management Area, Bay Management Area).

Sites are physical places where samples have been collected; sites should be relatively independent of each other, such as different systems and habitats (e.g., different lakes, rivers, ponds, marine areas, order of tributary, marinas).

Stations refers to spatially distinct sampling locations within a site (i.e., spatial replicates) and are typically used to improve species detection or evaluate the eDNA variation within systems or habitats (e.g., samples distributed using a grid or transect design surrounding an aquaculture site, upper and lower reach of a river, locations within large open water environments).

Field sample replicates are separate sample units collected as close as possible to the same point in space and time, stored in separate containers, and analyzed independently. The number of field sample replicates collected can vary based on the logistics and goals of a study.

Technical qPCR replicates are qPCR reactions that are repetitions from the same DNA extract.

Technical filter replicates are obtained by cutting filters into pieces and testing each piece separately.

Depending on the objectives, some studies have a single site with multiple stations, or multiple sites with one or multiple stations. Replicates increase the probability of detection and are used to evaluate the variables that affect detection probability (e.g., research on the ecology of eDNA) or to model where a species may occur (e.g., occupancy modeling). They provide an idea of the detection repeatability, which is important to evaluate if the sampling effort should be increased to improve species detection probability. More sites and stations may be required when the target organism is rare. Sampling several stations within a site and several field replicates is often recommended, as eDNA may not be distributed homogeneously (e.g., eDNA can be very patchy in sediments) in the environment.

In a large body of water, taking and combining multiple water samples (e.g., composite sampling using a grid pattern) is a way to reduce the total number of samples and increase replication.

B. Study design

General considerations: This section provides information on eDNA study design and planning, in order to achieve AIS or SAR manager objectives, and how to describe or report key information that can influence the likelihood of detecting an organism(s). It is advisable to perform a preliminary or pilot study to determine if eDNA methodologies (e.g., sampling effort, qPCR assay) will be effective in addressing study objectives as the cost of false negatives might be very high when inappropriate management actions are triggered (e.g., eradication efforts, monitoring aquatic invader introduction).

B.1 Type(s) of ecosystem: Indicate habitat type(s) sampled (e.g., lake, river, stream, wetland, pond, estuary, coastal, marine, or other). If 'other' is selected, provide a brief description. For AIS in particular, add detail specific to the domain (e.g., Ports of Entry, along vectors) if it is important to the study goal.

B.2 Sampling design: Explain how sampling design and sampling effort contribute to meeting the study objectives.

Describe sampling design (i.e., transect, grid, random) and how the samples are distributed (e.g., stations every 100 m over a 1000 m transect; 24 stations within a grid design covering 1,500 m^2). Show the station location(s) on a map (Appendix 1).

Indicate how the sampling plan was developed to optimize species detection, including ecological (e.g., species biology) and environmental factors, and describe the rationale of selecting sampling locations (i.e., sites, stations; see Box 2).

Indicate how the spatio-temporal sampling design was selected to optimize the detection of target species or taxa. For example, the incorporation of knowledge of population distribution and migration timing, daytime or nighttime sampling due to vertical daily migration, sampling depth selected to align with habitat preference, seasonal and reproductive periods to target a specific life stage, specific tide selected due to freshwater inputs or reduced potential variation can help improve detection probabilities (see Box 3 for more examples).

Indicate if the field method was selected due to a need to follow a standard for comparison purposes, based on a previous pilot study, or because the study is part of a larger study with prescribed methods. Provide references and report any deviations from the reference, original or a previously established study and sampling design.

If the study design was modified to account for sub-optimal sampling conditions (e.g., weather considerations, unforeseen circumstances, equipment malfunction), indicate what changes were made and the rationale for this change. Sample-specific departures from the above sampling plan

and rationale for any adjustments should be recorded in the metadata. Any important aspects of a sampling plan that can clarify how sampling was carried out should be provided. An additional appendix should be added if a longer text is needed to understand how the sampling design has been developed and for explaining any follow-on adjustments from the original design.

B.3 Number of sites sampled: Provide the total number of sites sampled. For the definition of sites, see Box 2.

The rationale for how sites have been selected to meet the study objective(s) should be provided in the sampling design section (see B.2). Site-specific GPS coordinates are to be provided in the metadata.

B.4 Number of stations sampled within sites: Provide the number of stations sampled within each site and briefly explain reasons for any variability among sites (e.g., lake size, accessibility, coverage of various environments [upstream, downstream, lake intake]). For the definition of stations, see Box 2.

B.5 Number of field sample replicates: Indicate the number of replicates collected at each station or each site. Indicate whether a predictive survey design software tool was used to inform sampling design (e.g., SIBYL™). See Box 4 for the importance of replication, repeatability, and reproducibility.

B.6 Time series: If applicable, report the number of times that stations and/or sites were sampled (i.e., if repeated sampling was done), and provide sampling dates.

B.7 Environmental conditions, relevant observations, and additional field data: Report any environmental factors that may affect the amount, transport, and fate of eDNA (see Box 3), including, but not limited to, algal blooms, noteworthy weather events / conditions, strong currents, and eutrophic or oligotrophic conditions. List any additional environmental data and how they were collected (e.g., measurement, frequency and equipment used).

B.8 Field blanks and field controls: Describe how many blanks and any other field controls were used per site/station visit or day. Indicate if any sites were used as controls (i.e., location with target eDNA present used for *in situ* field / project positive control or location understood to have no target and used as an *in situ* field negative control). General information on control types can be found in Box 5. Report results of controls in Section F.2–F.4.

Box 3. Ecological and environmental processes relevant to eDNA study design

The probability of detecting eDNA is directly proportional to the rate at which eDNA from the target organism enters the environment, and is inversely proportional to the rate at which the eDNA signal is lost from environmental samples (e.g., through sedimentation, degradation, excessive dilution). There are many ecological and environmental factors that may affect DNA release, and DNA dilution and persistence in the environment.

DNA release

The rate at which DNA is shed or released from an organism into the environment (via feces, urine, epithelial cells, mucus) varies depending on the organism's size, life-stage, and metabolism (Maruyama et al. 2014; Klymus et al. 2015; Sansom and Sassoubre 2017). Shedding rate also varies with the species ecology, seasonal patterns, and environmental conditions. For example, detection probability may be higher for some organisms:

- during reproduction, due to the release of gametes (Tillotson et al. 2018) and the degradation of dead larvae;

- at higher water temperatures (Lacoursière-Roussel et al. 2016a; Jo et al. 2019);
- during increased stress, such as acclimation to a new environment (Takahara et al. 2012);
- for mucus covered species (e.g., fish, molluscs; Lacoursière-Roussel et al. 2016b);
- at higher population density (e.g., Schloesser et al. 2018);
- during some periods of higher animal activity (e.g., migration, nesting; de Souza et al. 2016 and Sevellec et al. 2020); and
- after a storm or disturbance has resuspended sediments; however this signal should be interpreted with caution, as eDNA can persist for long periods in sediments. A detection following a storm may therefore not reflect current species occurrences (McNair et al. 2012; Wilcox et al. 2016; Shogren et al. 2017, 2018).

DNA dilution / persistence in the environment

The dilution and persistence rate of eDNA varies depending on specific ecosystem characteristics, such as transport and the water matrix (i.e., components of a sample other than the eDNA; Harrison et al. 2019). Both are subject to significant alteration during extreme events (e.g., storms) or recurrent processes (e.g., tides). For example, eDNA detection probability may be higher:

- near the source (as distance increases, dilution effects make the eDNA signal weaker);
- downstream of a source (tissues, cells, and free DNA flow downstream);
- during drier / lower-water periods (heavy rains and high water levels decrease the detection probability due to dilution effects);
- when sediments may be resuspended following a storm or disturbance events and release ancient eDNA; therefore eDNA detection following a storm or disturbance may not reflect recent and local species occurrence (McNair et al. 2012; Wilcox et al. 2016; Shogren et al. 2017, 2018);
- in mid-river (higher flow in mid-river can carry DNA farther downstream of the source);
- in estuarine systems influenced by tides, the surface layer of water contains DNA from upstream;
- in the stratification layer where the organism resides (alternatively, consider sampling when lakes are mixed and/or sample the full water column);
- at the lake outflow / discharge, or intake, to capture eDNA from upstream;
- where water has fewer inhibitory compounds (Schrader et al. 2012); and
- where eDNA degradation rates are lowest (e.g., colder temperature, low UV irradiation, low microbial activity, and specific water chemistry [e.g., presence of the pesticide diazinon; Pourmoghadam et al. 2019]).

Box 4. Increasing reliability of eDNA results using replication, repeatability, and reproducibility

Replication is recognized across eDNA studies as being essential for establishing the reliability of results to address potential uncertainty of eDNA detections and increase confidence in results. Replication can occur through multiple ways and the type of replication needed in a project is not yet standardized (but see Ficetola et al. 2015 and Erickson et al. 2019). The type of replication

required in a project depends on a multitude of study-specific factors, including the hypothesized abundance of the targeted organism, end user risk tolerance, costs, and sampling accessibility. Whether it is more effective to add qPCR replicates, increase sample replicates, repeat sampling over time, or reproduce a project with a different eDNA service provider will vary across studies and influence costs. Additionally, the accessibility of sampling sites can change the cost-benefit trade-off of sampling less intensively initially and returning to re-sample if needed versus sampling intensively at the outset.

There are two main purposes of replication. One purpose is to determine the *repeatability* of test results, which the World Organization for Animal Health defines as the level of agreement between results of replicates both within and between tests of the same method in a given laboratory (OIE 2019). A second purpose is to determine the *reproducibility* of test results, defined as the ability of a test method to provide consistent results when aliquots of the same samples are tested in different laboratories using the *identical* assay (OIE 2019). While reproducibility often is not tested for eDNA assays, it is nonetheless an important metric for establishing assay reliability and its general 'technical soundness' (see Bustin and Nolan 2017 and Sepulveda et al. 2020b).

Replication is especially important for eDNA detection when the concentration of target DNA in the environment is low, as this can generate high variance among field and qPCR replicates. In contrast, when the concentration of target DNA in the environment is high, variance amongst sample replicates can be expected to be lower. The detection of low concentration eDNA will be increasingly reliable by repeating eDNA tests and/or reproducing eDNA projects. Different avenues can be considered for replication in eDNA studies, such as: collecting multiple field replicates across various locations at each site during a single visit (spatial replication) or at different times (temporal replication), testing multiple qPCR replicates on the same DNA extract, using multiple genetic markers, and reproducing the laboratory test in a different laboratory.

Box 5. Controls to assess possible sources of error

Results obtained from negative and positive controls are a crucial element when analyzing and interpreting eDNA results. Controls are incorporated throughout the eDNA workflow, providing information on the validity and reliability of results, and feedback where eDNA processes need improvement or may have failed. Increasing the number of controls at different processing steps to detect potential sources of error during sample processing will generally increase confidence in results. A general rule of thumb is that the number of controls should reflect error tolerance based on the objectives of the project. Criteria used to determine whether or not positive or negative controls have passed or failed must be reported.

Negative controls often are referred to as negatives, blanks, or no template controls (NTC). They are samples that go through the same procedures as eDNA samples but do not have the target analyte (eDNA) included or added to them. Negative controls are included at various stages of the eDNA workflow to identify sources of contamination, which can lead to detecting **false positives**. Negative controls typically are included during field sampling and filtration (to detect contamination during the collection and eDNA capture of the sample), DNA extraction (to detect cross-contamination between samples during eDNA extraction), and qPCR (to detect contamination during qPCR and to establish a fluorescence background baseline for improved data interpretation). If the negative controls are detecting DNA, the analysis should be repeated, if possible (e.g., using fresh qPCR reagents, or extracting the backup portion of a filter). The threshold used to determine that a negative control passed or failed should be defensible and set *a priori* to result analyses.

Positive controls refer to a control in a treatment that is known to produce results. They are used to assess the validity and reliability of eDNA results by ensuring that procedures in the eDNA workflow performed as expected and / or as a standard to identify deviations from expected results. Positive controls can be made from synthetic DNA fragments (e.g., gBlocks, exogenous DNA), PCR amplicons, a tissue slurry, endogenous DNA from target organisms, etc. Processed as independent samples or added into eDNA samples (i.e., 'spiked-in'), positive controls identify potential sources of error that can impact data interpretation and that can contribute to reporting **false negatives**. Common positive controls include but are not limited to extraction positives (to indicate success of eDNA extraction) and qPCR positives to indicate success of the qPCR reaction.

C. eDNA sample collection

General considerations: This section provides an overview on how to report the methodology for eDNA sample collection. Contamination prevention measures are essential for eDNA sample collection (see Box 6 for more information on contamination prevention measures) and should be reported in Appendix 2. Sample-specific metadata are to be reported in Appendix 4.

C.1 Environmental sample collection method: Indicate the container or device used to collect the sample (e.g., water bottle, plankton net, kick sample, automated or field-portable eDNA sampling system, bilge pump, sediment corer, Van Veen Grab sampler, or other [if other, provide details]). Provide the manufacturer and model.

C.2 Volume / weight sampled: Indicate the sample volume or weight measured or targeted during eDNA capture. Record any additional details (e.g., missing samples, incomplete filtration due to clogging, individual samples that deviated from the target volume / weight) directly in the template or in the appended metadata (Appendix 4), as appropriate.

C.3 Sample depth(s): Provide a detailed explanation of the depth(s) at which the sampling was performed (e.g., surface water sampling, 3 m above seabed, 10 cm below water surface, core from sediment surface to 2 cm depth).

C.4 Field sample storage and time before processing: Indicate how and for how long samples were stored between collection and processing, if applicable. An example entry could read, "coolers with ice, and filtration within 24 hours". Any deviation from the described protocol must be noted in the metadata. For solid samples (e.g., sediments) provide relevant details. Cold storage slows the degradation rate of eDNA, but does not stop it; to minimize possible sample degradation and reduce the risk of false negatives, the time between sample collection and processing should be kept at a minimum, and ideally should occur before 24 hours and not exceed 48 hours (Hinlo et al. 2017).

C.5 Sample processing method: Describe the method used to recover or concentrate eDNA from field samples, including equipment used (e.g., filtration: peristaltic pump, vacuum pump, syringe, automated eDNA filtration). Specify if any equipment used was disposable or previously used and sterilized (e.g., filter housing may be sterilized and reused for a limited number of times). For solid samples, describe processing steps prior to DNA extraction (e.g., DNA preservation method). Provide the manufacturer and model for equipment; provide the manufacturer and catalogue number for reagents.

C.6 Filter type and pore size: Indicate diameter, material, pore size, and manufacturer catalog number of filters used, if applicable.

C.7 Sample preservation: Describe preservation conditions of processed field samples (e.g., filters), including preservative type (e.g., desiccant, buffer, freezer conditions, etc.) and quantity /

concentration, and storage conditions. The quality of the sample and DNA is a key factor in the success of subsequent steps (extraction, PCR, etc.).

Box 6. Contamination prevention

Since eDNA analysis targets trace DNA, even very low levels of contamination can complicate interpretation. Contamination prevention measures are essential throughout the workflow and must be followed strictly (Thomsen and Willerslev 2015; Goldberg et al. 2016).

The two main sources of contamination are external DNA (e.g., DNA introduced from vessels, wastewater, hatcheries, or laboratories that have had contact with the target organism) and cross-contamination (e.g., DNA moved from one sampling site to the next, or from one sample to another).

It is highly recommended to choose an eDNA service provider that already has formal standard operating procedures (SOPs) and Good Laboratory Practices (GLP; OECD 1998) or equivalent policies in place specifically for contamination prevention, or laboratory accreditation.

Several approaches used together will significantly reduce the risk of contamination throughout the entire workflow:

- disposable gloves that are changed frequently to prevent cross-contamination between eDNA samples, minimize external DNA contamination of supplies and equipment during lab or field processes, and prevent sample degradation via means introduced by the eDNA practitioner (e.g., enzymes, bacteria);
- training is crucial in limiting contamination in all projects, including those involving public participation / citizen science; and
- thorough cleaning of laboratory and field equipment using diluted commercial bleach (sodium hypochlorite) and / or other approved methods, ensuring that there is no residual disinfectant on equipment (e.g., bleach residual), as this can lead to false negatives.

Essential contamination prevention strategies used during field sample collection include but are not limited to:

- sampling for eDNA before conducting other tasks that may perturb the environment (water) being sampled;
- sampling a set distance away from the vessel for boat-based sampling;
- carefully planning sampling locations to minimize contamination from upstream sources (e.g., collecting samples facing the current, sampling in an upstream direction, moving from sites of low probability to higher probability); and
- opting for shore based sampling when possible and ensuring sterilization of waders, boots, and other equipment between sites.

The physical layout of specialized PCR laboratories is designed to prevent contamination, and such laboratories should have operating procedures in place for effective reduction of cross-contamination. Characteristics of high-quality eDNA laboratories include:

- dedicated eDNA laboratory space with restricted access and prescribed allowable movements between rooms/spaces within the laboratory;
- physical separation of critical processes (e.g., separated DNA extraction and PCR set up space);

- use of PCR workstations or hoods, unidirectional air flow, and equipment specifically dedicated to eDNA; and
- GLP, SOPs, careful laboratory technique, and stringent lab cleaning protocols.

SECTION III: eDNA SAMPLE ANALYSIS – LABORATORY METHODS

Section summary: This section provides information on reporting crucial information from DNA extraction and qPCR assay protocols for the detection of AIS and SAR. The detection of AIS and SAR often is challenging because there may be a low concentration of target DNA in the environment (i.e., the species is rare) and the target may occur in a variety of habitats.

Reporting on DNA extraction methods is important because using different methods may affect the amount of eDNA extracted. The DNA extraction subsection reports on the methods used to isolate and purify DNA. It outlines and defines six critical elements that are important to include when reporting on DNA extraction, such as the reference protocol and the extracted eDNA storage conditions.

It is equally important to report qPCR assay protocols to understand the properties of the assay and to identify if the methodology of its application were altered from a previous project, which affects comparability of results and may impact the level of validation of the assay (Box 7 and 8). Section E, qPCR assay, outlines the different qPCR methods and the importance of qPCR validation. This section also defines nine elements that must be included in any qPCR assay report, such as the technical replicates per sample. A key part of this subsection focuses on detecting PCR inhibition which is especially important since an inhibited qPCR sample can lead to reporting a false negative.

An assay validation scale is presented to enable managers to determine the utility of an eDNA assay's application in a study area. Establishing an assay's validation level will increase transparency in laboratory methods, contribute to understanding of limits in result interpretation and the general confidence in eDNA results. The assay validation scale presented includes properties of the eDNA assay that allow the assay to be evaluated at all stages of the eDNA workflow. The suitable level of validation will depend on the ultimate goal and use of results. Exploratory studies could require less validation, whereas eDNA studies for enforcement purposes will require a more robust level of validation.

Box 7. What is a qPCR assay?

qPCR is a molecular biology laboratory technique based on polymerase chain reaction (PCR). PCR techniques are used to increase copies of, or amplify, target DNA sequence so that they can be visualized and measured. In qPCR, the amplification of a targeted DNA sequence, or amplicon, is continually measured throughout the PCR reaction process. This is different from conventional PCR that simply amplifies the target DNA sequence which is measured (quantified), using various methods, after the amplification process is completed. qPCR detects lower concentrations of DNA more accurately than conventional PCR since data are collected in real time, providing information when the amplification of target DNA starts, and when the doubling of DNA is most efficient and accumulating at a steady rate. In contrast, DNA concentrations measured when the amplification process has completed may show greater variability between replicates as a result of differences in kinetic properties (e.g., depletion of PCR reagents) among PCR reactions during the final stages of the amplification process.

To visualize and measure the amplified DNA, a qPCR assay uses either a fluorescent intercalating dye (e.g., SYBR green) or a fluorescence-emitting probe (e.g., Taqman) that binds to the target DNA. For various reasons, eDNA detection using probe-based qPCR assays usually is favoured over dye-based qPCR assays. qPCR involves multiple reaction cycles (commonly 40-50) of temperature shifts. With each qPCR cycle, the amount of amplicons double, and consequently, the fluorescent signal intensifies (Figure 1). When the eDNA concentration is high (Figure 1, red line), the number of qPCR cycles necessary to reach the fluorescence detection threshold (Figure 1, dotted blue line) and the corresponding Cq is low. When eDNA concentration is low (Figure 1, yellow line), the corresponding Cq value is high, indicating DNA is being detected later in the reaction.

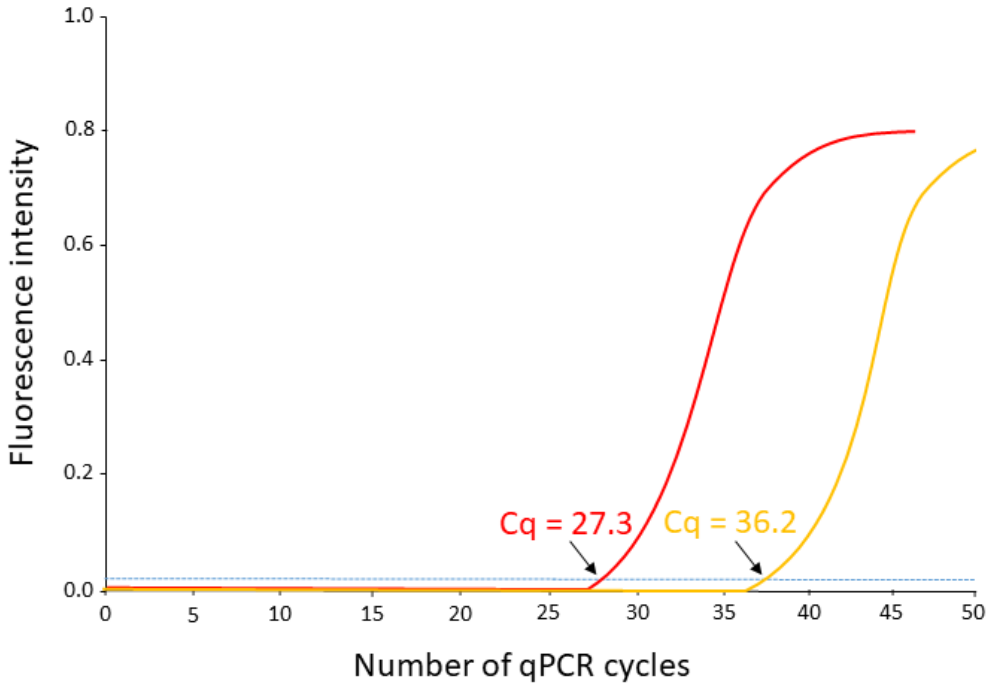

Figure 1. Schematic diagram showing the relationship between fluorescence intensity and the target DNA concentration (higher and lower target DNA concentration, red and yellow lines, respectively).The fluorescent signal intensity (vertical axes) increases above the detection threshold (blue dotted line) at a lower number of qPCR cycles (horizontal axes) in samples with greater DNA concentration. The number of reaction cycles and fluorescence signal intensity (correlating to DNA concentration) are inversely proportional.

Quantifying unknown concentrations of target DNA from eDNA samples usually is done using a standard curve (Figure 2, blue line) which consists of a series of standards or solutions containing a precise and increasing concentration of DNA (Figure 2; STD 1 to 9). The Cq result of the eDNA sample then is reported on the standard curve to determine its DNA concentration (Figure 2, black star). Note that Cq values are relatively variable for standards at low DNA concentrations compared to those at high DNA concentrations; which means that measurements for DNA samples of low concentration (high Cq) values are not as precise as low Cq values for both DNA standard replicates and eDNA samples. Increasing the number of DNA replicate samples can decrease the Cq value variance, which is crucial for reliable detection at low eDNA concentrations.

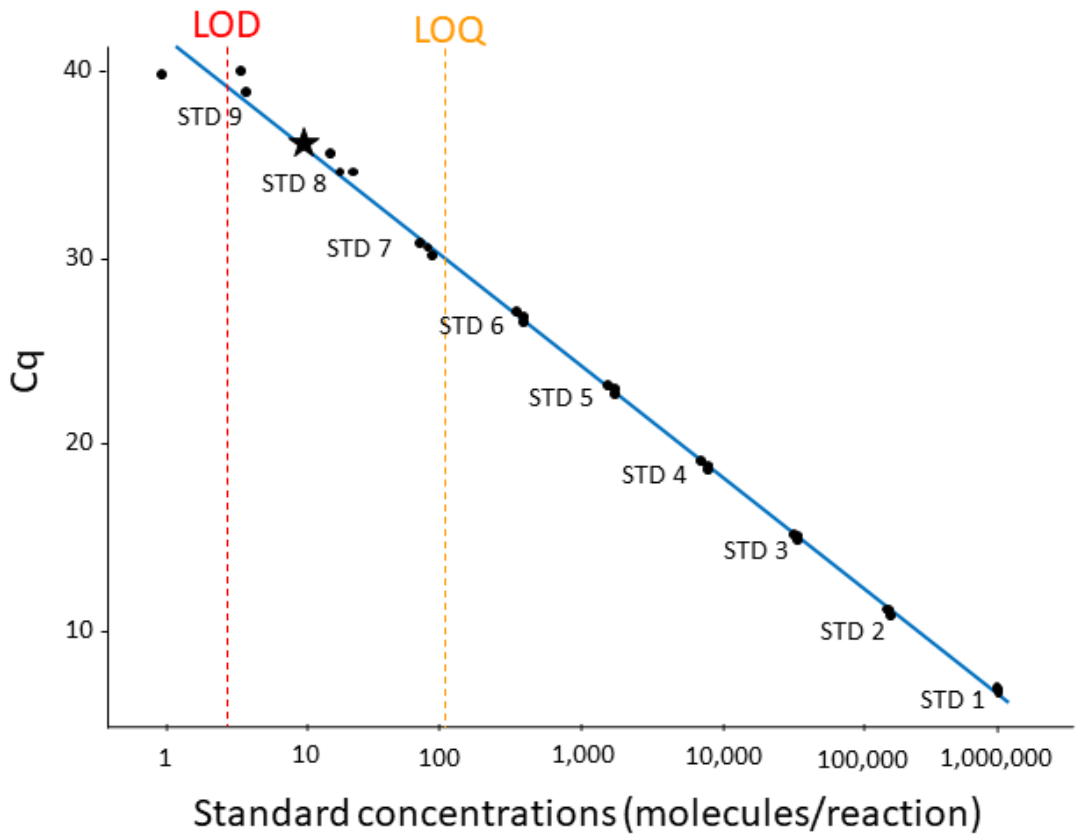

Figure 2. A qPCR assay standard curve showing results of solutions with known (circles) and unknown (star) DNA concentrations. STD 1 through STD 9 represents a dilution series of known DNA concentrations from high to low, respectively. The Cq (quantitation cycle) on the vertical axis is the cycle at which the fluorescent signal is detectable by the qPCR instrument, and is plotted against DNA concentration (in log scale) on the horizontal axis. Up to 40 reaction cycles is typical; when additional cycles are used, errors in qPCR amplification may become more frequent. The limits of detection (LOD) and quantification (LOQ) are estimated statistically based on the variance between replicates of standards (see glossary for LOD and LOQ definitions and Klymus et al. 2019 for an example calculation). The sample with unknown concentration (denoted by a black star) has a Cq value of 36 and its standard concentration would be 10 molecules per reaction. Note that detections below the LOD may be considered as acceptable qualitative data in some projects with low risk tolerance for false negatives (see Box 10).

D. DNA extraction

General considerations: Many methods exist to extract DNA (Goldberg et al. 2016), including commercial extraction kits and published protocols based on phase separation and DNA precipitation (e.g., CTAB / chloroform, phenol / chloroform). Net recovery of eDNA may vary within and across extraction kits or methods. A DNA extraction method is selected based on its efficacy to extract and concentrate nucleic acid from a sampling medium (e.g., environmental sample, gut contents), its ability to alleviate PCR inhibition, and appropriateness for the specific application. Cost, throughput capacity, laboratory infrastructure, and analyst experience also are taken into consideration when selecting DNA extraction methods. It is recommended to use and reference standardized operating procedures (SOPs) for DNA extraction protocols and to minimize contamination in the laboratory (see Box 6 for more information on minimizing contamination).

D.1 Name of commercial kit or protocol: Report the name of the extraction method used (i.e., commercial kit, published or in-house protocol).

D.2 Reference protocol: Provide the protocol for the extraction method used. If a commercial kit was used, report any alterations from the manufacturer's protocols. If a published or in-house protocol was used, append it to the report. If applicable, report any deviations that occurred for any samples in Appendix 4.

D.3 DNA extraction controls: Indicate the types of DNA extraction controls used and their frequency (e.g., one negative and one positive extraction control per batch of 24 extractions).

D.4 Proportion of total sample: Indicate what proportion of the collected environmental sample medium was used in the DNA extraction process (e.g., half of filter membrane, weight of sediment).

D.5 DNA elution volume: Indicate the volume and type of buffer used to collect the extracted DNA.

D.6 Extracted eDNA storage conditions: Describe storage conditions for DNA extracts. This is typically between -20 and -80ºC to prevent DNA degradation.

E. qPCR assay

General considerations: An eDNA service provider may use either published qPCR assays or proprietary assays for which the assay protocols are not disclosed. In either case, it is required that critical assay elements be included in the reporting template and Appendix 3 to allow objective review and interpretation of results. Published assays may not have been thoroughly validated, therefore the validation level of *all* assays (including published assays) needs to be assessed prior to implementation. It is important to recognize that the level of assay validation is determined using a specific lab and field workflow in a particular geographic area and environmental sample type; if any of these change, existing assay validation data will not be entirely applicable. It is imperative that assay specificity is verified in the geographic area in which an assay is being used and reported on here. Furthermore, even if an assay protocol is followed precisely, assay performance can change when it is used in a new environment or over time in the same laboratory facility.

E.1 Assay name: Provide the assay name and reference, if published.

E.2 Assay type: Provide the type of assay (i.e., intercalating dye or DNA probe). See Box 7 for more details on types of assays. Combining multiple assays using multiple genetic markers in a single qPCR reaction (i.e., a multiplex qPCR assay) can be an efficient approach to detecting one or more organisms by shortening the processing times and reducing the use of reagents. Multiplexing assays require validation to limit competition between assays, which could decrease assay sensitivity (Gingera et al. 2017). Multiplexed assays should be rigorously tested through all stages of assay validation presented in Box 8. If a multiplex qPCR assay is used, information for each assay should be included in Appendix 3.

E.3 Level of assay validation: Provide the level of assay validation using the dropdown menu in the eDNA reporting template (see Box 8). Ensure the level of assay validation reported here accurately reflects the validation level appropriate for the geographic region where it is being applied (i.e., if a Level 4 assay is being used in a new environment where extensive field testing and *in vitro* testing on co-occurring non-target species has not been completed, it should be reported as Level 3). eDNA service providers should justify the level of validation by presenting how they have assessed the minimum criteria found in Table 1. It is recommended here that managers implement assays validated to at least Level 4, when available.

E.4 Specificity data: Describe assay specificity data generated *de novo* or already available that validates use of this assay in the study area. This usually involves testing closely-related species

that may co-occur with the target organism to ensure no cross-reactivity of the assay with non-target taxa.

E.5 Dilution and volume of DNA used: Report if the DNA extract was diluted prior to qPCR analysis and the dilution factor used. Report the volume of the DNA sample used in the qPCR reaction. Different dilutions and volumes may be used within one study to limit qPCR inhibition or to increase the detection in specific areas. If different dilutions or volumes were used among samples, provide the range of dilutions and volumes used and include the specific information for each sample in the raw data results in Appendix 4.

E.6 qPCR positive and negative controls: Indicate the types of control and their frequency (e.g., one qPCR positive control in triplicate in a 96 well qPCR plate).

E.7 Technical replicates per sample: Provide the number of technical replicates (i.e., qPCR replicates analyzed from the same eDNA extract) per sample.

E.8 Inhibition tests: Provide the details about the approach used to detect qPCR inhibition (Box 9). Detecting possible inhibition is important in a new sampling area or when using a new sampling approach (i.e., take account of substrate types such as clay and other environmental factors that may potentially alter the amount of DNA available in eDNA surveys).

E.9 Number of qPCR cycles: Provide the number of cycles used in the qPCR reaction.

Box 8. Level of assay validation and results interpretation

The importance of validating eDNA methods prior to implementation is well-recognized (Sepulveda et al. 2020c); however, the term 'validation' is used very broadly. It requires further elaboration to enable managers to evaluate whether a particular assay meets their needs and how to interpret results. To this end, an eDNA assay validation scale and a guide to interpretation of results has been developed by European experts within DNAqua-Net (Leese et al. 2016; Thalinger et al. 2021) are presented (Figure 3).

The eDNA assay validation scale encompasses the entire workflow used to detect a species' DNA. As such, assay validation does not involve only the qPCR step but all assay components from sampling to interpretation of results. Thus, it is essential that the field components of assay validation are completed in an appropriate geographic location, ecosystem type, and substrate given the intended use of the assay for AIS or SAR detection. This means that even if an assay has been validated to a particular level in a specific geographic location/region, it may not have the same level of validation in another region. In these cases, additional validation may be required, such as experimental verification of assay specificity and sensitivity in a new geographic location and/or substrate. Successful achievement of a level of assay validation is possible only with strict adherence to this workflow by an eDNA service provider in a specific geographic region. This highlights the importance of SOPs and appropriate interpretation of results given the level of assay validation.

The eDNA assay validation scale (Figure 3) enables the classification of assays into five levels based on their accuracy and sensitivity for targeted eDNA detection, and guides interpretation of results for each level of validation. Confidence in an assay's performance and thus the ability to conclude presence or absence of target eDNA improves along the scale. For example, Level 1 validates the qPCR detection of the target species' DNA, while Level 2 confirms that the detection is species-specific. However, for these two levels, only *in silico* (i.e., computer-based testing of specificity; Level 1) and *in vitro* validation (i.e., testing the qPCR with a DNA extract of the target species and closely-related species; Level 2) have been done; no environmental samples have been tested to validate the performance of the assay under natural conditions (i.e., *in situ*). Consequently, it is impossible to conclude that target eDNA is present or not using a Level 1 or 2 assay; however, Level 2 provides initial insight on specificity. Assays with Levels 3 to 5 have been validated from the sampling stage to the interpretation of results and therefore can be used to conclude the presence of target eDNA in the sample. DNA sequencing may be used to confirm the presence of target DNA for assays at Levels 3 through 5, but cannot distinguish whether that DNA originated from the sample or from contamination, hence proper use of negative controls is vital. While the absence of target DNA cannot be concluded with a Level 3 assay, it is possible to conclude eDNA absence with a Level 4 or 5 assay. Replication during sampling can be used to increase confidence in results for concluding the absence of eDNA at Levels 4 and 5. The minimum criteria needed to achieve a given level of validation are presented in Table 1 (Thalinger et al. 2021).

Determining the level of assay validation is usually iterative, with confidence in the assay's performance increasing over time with accumulated evidence and broad-scale use. It may take a matter of months to achieve a Level 2, multiple years to achieve a Level 3, and longer to achieve a Level 4 or 5. As such, a given level of validation is not necessarily a static endpoint - the more an assay is used, the more confidence one develops as to how it performs. Assays may be constantly tested as technologies and sampling strategies continue to improve.

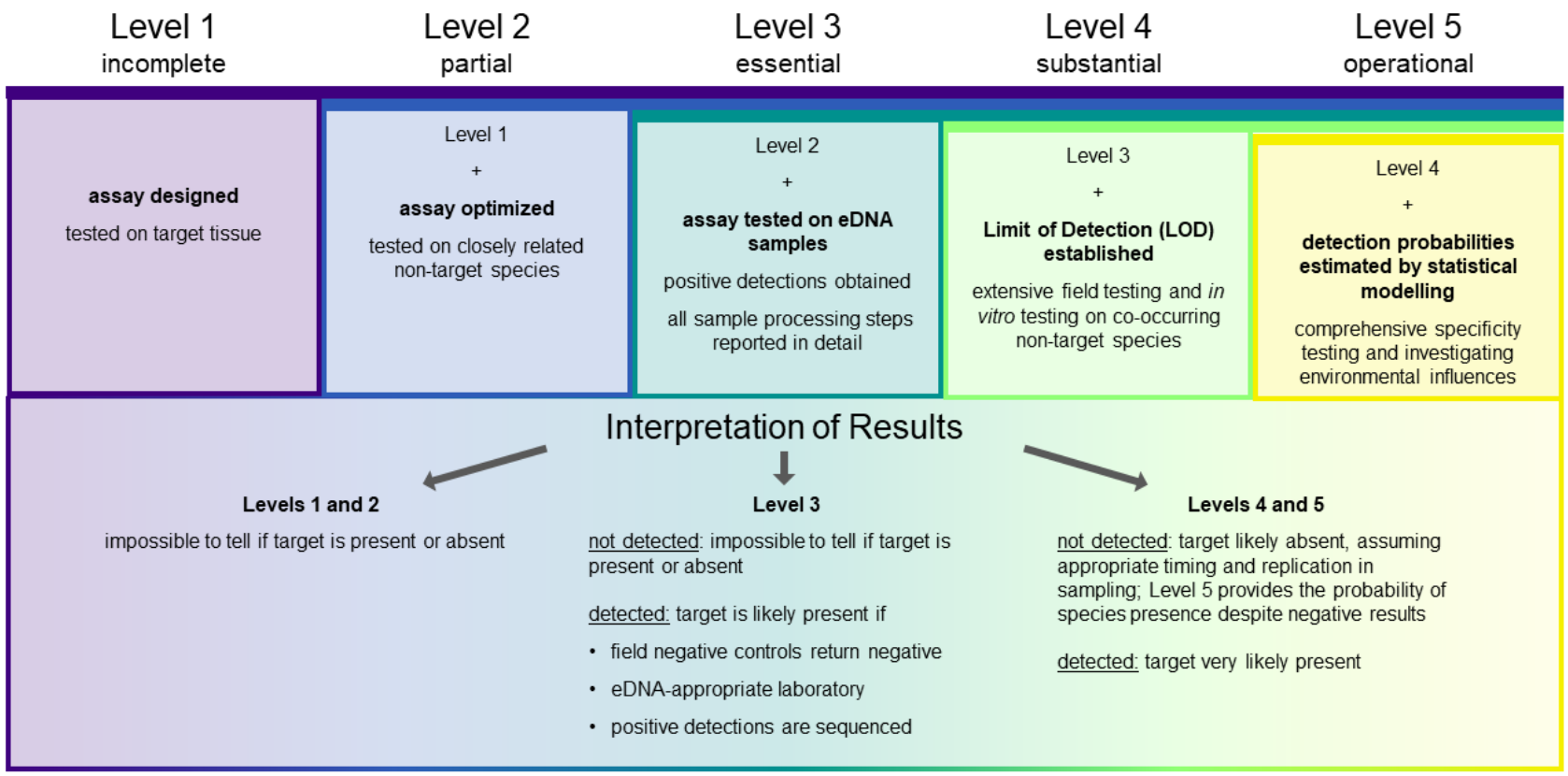

Figure 3. A 5-level validation scale developed by Thalinger et al. 2021 to aid evaluation of eDNA assays and appropriate interpretation of results. For each of the levels, the main accomplishments in the validation process and appropriate interpretation of results are provided. See Table 1 for the minimum criteria for each level.

Table 1. Minimum criteria for each of the 5 levels of assay validation (presented in the 'variable blocks' in Figure 3) that need to be fulfilled for an assay to reach a given level of validation.

Validation level	Variable blocks	Minimum criteria
Level 1	*in silico* analysis	target species
	target tissue testing	target tissue
	target tissue PCR	primer (and probe) sequence
Level 2	comprehensive reporting of PCR conditions	DNA extract volume in PCR
	in vitro testing on closely related non-target species	any *in vitro* non-target testing
Level 3	extraction method performed on eDNA samples	method of extraction
	concentration of eDNA from environmental sample	filter type or precipitation chemicals
	detection obtained from environmental samples	detection from an environmental sample (artificial or natural habitat)
Level 4	Limit of Detection (LOD)	LOD determined
	extensive field testing of environmental samples	multiple locations or multiple samples
	in vitro testing on co-occurring non-target species	any advanced *in vitro* testing
Level 5	comprehensive specificity testing	non-co-occurring/closely related species checked from *in silico*
	detection probability estimation from statistical modelling	any effort made towards detection probability estimation
	understanding ecological and physical factors influencing eDNA in the environment	any factor influencing eDNA in the environment tested

Box 9. PCR inhibition

PCR inhibition occurs when chemicals or substances present in the eDNA sample prevent amplification or lower the efficiency of DNA amplification during PCR. PCR inhibition can lead to the conclusion of a negative detection from a sample containing the target DNA (i.e., false negative). PCR inhibition can be problematic in aquatic and terrestrial samples due to known (e.g., humic substances; see review by Schrader et al. 2012) or unknown factors.

To test for PCR inhibition, a known concentration of a DNA sequence, different from the assay target sequence, called an internal positive control (IPC; see glossary), is added to the sample (i.e., 'spiked-in') to determine whether amplification progresses as expected. If there is evidence of inhibition (i.e., qPCR results indicate lower IPC concentrations than expected given the amount of IPC added to the qPCR reaction), it needs to be addressed before eDNA samples can be analyzed and interpreted reliably. Numerous options exist to mitigate inhibition problems, including diluting eDNA extracts, changing DNA extraction kits, changing qPCR kits, or using commercial inhibitor removal kits.

SECTION IV: SUMMARY OF eDNA RESULTS

Section summary: This section provides information on how to report and interpret eDNA results. Within this section are two subsections: reporting control results and reporting eDNA results. It is important to report the results of controls and eDNA samples because open communication between eDNA service providers and end users allows for clearer understanding when interpreting these results, which increases the credibility of using eDNA. Currently, there is no set criteria to assess controls, so transparency in communicating results is vital to interpreting results. To assess the quality and credibility of the results from samples, multiple positive and negative control types must be assayed and assessed. In the first subsection, there are four key elements outlined that must be included when reporting control results. Any indications of contamination must be reported. The second subsection, reporting eDNA results, outlines six elements (three that are optional) that must be included when reporting eDNA results, such as calculated LOD and quality-assured and quality-controlled (QA/QC) qPCR results. It is noted in this subsection that all sections preceding this one must be completed to meet reporting requirements. Should all sections be completed, qPCR can be interpreted for eDNA detection.

Two methods are commonly used to increase the reliability in interpretation of eDNA detections: detection decision trees and statistical approaches. Decision trees are currently more widely used than statistical approaches; thus for the purpose of this document only an example of a decision tree will be presented. Currently the use of statistical approaches is relatively new and therefore there are fewer examples of their use in environmental DNA studies. However, these approaches are developing rapidly and likely will become more routine. The method(s) used must be clearly defined in the reporting template for AIS or SAR managers so the interpretation of eDNA detection is more easily understood. Since data interpretation is species or ecosystem specific, end users are advised to consult eDNA experts, ecologists, and other relevant experts on a case-by-case basis to optimize species detection and discuss uncertainties in data interpretation of eDNA results.

F. Reporting control results

General considerations: The aim here is for transparency and clear definition of how results and controls were assessed, in order to facilitate interpretation and comparison of results while not restricting new ideas in this fast-evolving field of research.

Prior to interpreting results from eDNA samples, positive and negative controls need to be assessed to determine the reliability and quality of results from the samples. There is not yet a clear consensus on criteria to assess controls. Multiple positive and negative control types will have

been assayed in a project (see previous sections). Any indication of contamination must be reported, and if interpretation of eDNA samples proceeds, this needs to be justified fully and all potential impacts to the project objective(s) must be explained (see Box 6 for more details).

F.1 Criteria to determine if controls passed or failed: Provide the criteria used to determine whether positive and negative controls have passed or failed. If criteria differ among controls, provide information on each control separately and indicate any differences among different types of controls (e.g., field, filtration, extraction, assay, inhibition).

F.2 Positive control results: Report the number (and total) of positive controls that passed. If different positive control types were used, report each of these separately.

F.3 Negative control results: Report the number (and total) of negative controls that passed. If different negative control types were used, report each of these separately. Note that negative control detection thresholds should be defensible and set *a priori*.

F.4 Failed controls: For all failed controls, explain why results still may be considered including what type of control failed and the effect on the level of certainty and considerations in interpreting the results.

G. Reporting eDNA results

General considerations: The completion of all sections up to this point is required to meet the reporting requirements of this guidance document. The eDNA service provider may not be reporting beyond the delivery of QA/QC qPCR results (G.2) and the provision of qPCR data (Appendix 4), *in which case they would not complete G.4 to G.6*. However, if the eDNA service provider is aiding in the interpretation of eDNA results, these sections are required.

G.1 Calculated LOD: Report the experimentally-determined assay LOD and describe how it was derived (i.e., give the Cq value and concentration [preferably target copies per reaction or per volume of water sampled] associated with the LOD).

G.2 QA/QC qPCR results: Report the overall total number of qPCR replicates and how many generated a Cq *at or above* the LOD (see G.1) after controls were evaluated (see Box 10). For example, 10 sites with 2 stations each, 1 sample per station and 2 qPCR replicates per sample would give 40 overall qPCR replicates to report.

G.3 Other qPCR results: Report the total number of qPCR replicates and how many generated a Cq *below* the LOD (see G.1) after controls were evaluated (see Box 10).

G.4 Determination of sample-level results (optional): Define the criteria or statistical approach used to determine if eDNA was detected at the sample level (see Box 10). Report the total number of samples and how many returned a ‘detected’ result.

G.5 Determination of station-level results (optional): Define the criteria or statistical approach used to determine if eDNA was detected at the station level (see Box 10). Report the total number of stations and how many returned a ‘detected’ result.

G.6 Determination of site-level results (optional): Define the criteria or statistical approach used to determine if eDNA was detected at the site level (see Box 10). Report the total number of sites and how many returned a ‘detected’ result.

Box 10. Interpretation of qPCR results for eDNA detection

Interpretation of eDNA results is challenging because the eDNA of rare species may be detected in a low number of samples from a site or a low number of qPCR replicates from a study area. There

is no consensus on how to assess qPCR results from controls and samples, but assessing both controls and samples should reflect the risk tolerance of the study. Different end users will have different risk tolerances, thus they may use different approaches to translate raw qPCR results into final results of eDNA detection. These approaches use replication at several hierarchical levels (e.g., qPCR, sample, station, site) to increase the reliability of eDNA detection (see Box 4).

Figure 4 illustrates *an example* of how one might analyze qPCR results using a decision tree. It shows the key components of a decision tree and is not intended to be a standard for any given eDNA detection study. Different thresholds from those used in Figure 4 can be used to determine if eDNA was detected in a sample, station, and site; indeed, the criteria used in any given eDNA study will depend on the study design, objective(s), and target organism(s) being considered. In this example, the assay needed to be validated to at least Level 4 (because LOD is required, see Figure 3), and only qPCR replicates with results at or above the LOD were considered in the decision tree.

When reviewing qPCR results, a difficult situation is presented when the only non-negative results in an eDNA study do not meet minimum detection criteria. One example of this is where eDNA samples have only a low number of sample replicates that return a Cq value, and the minimum detection criteria has been set higher than this. Another example is when the only non-negative results found in a study are all below the LOD, and LOD has been used as a minimum detection criterion. Importantly, results below LOD mean those signals are not highly repeatable, it does not necessarily mean those signals are false. Determining how to interpret such results should take into account: study goals; the weight of evidence based on sampling/monitoring effort and other biological and ecological information; management risk tolerance; and should include consultation with experts. Results that are challenging to resolve should not be disregarded, as they could be indicative of a true finding or they may provide valuable insight that could direct future study design and/or revision of detection criteria.

As an alternative to decision trees using adaptable detection criteria, statistical approaches provide a quantitative value for inferring the reliability of eDNA detections. Simulations using these models show how the statistical power to detect eDNA varies as levels of replication vary (Ficetola et al. 2016; Lahoz-Monfort et al. 2016). Note that detection probabilities can only be calculated once a project has been completed; as such, they can be used to inform project design for subsequent resampling efforts or to indicate the need to run a higher number of qPCR replicates.

Whether a decision tree and/or statistical modeling approach is used in detecting eDNA, clearly describing and communicating the detection method(s) and criteria used in study design and analysis is crucial for AIS and SAR managers to understand how eDNA results were generated and interpreted. The generation and provision of defensible and reliable eDNA information and advice is crucial for ensuring end users have the necessary information for evidence-based decision making while at the same time fostering confidence in the use of eDNA as a valuable tool for monitoring aquatic organisms of management interest. Furthermore, depending on the study, corroboration of eDNA results may be sought; for example, by an alternative sampling method focused at the site(s) of eDNA detections, resampling, use of an additional qPCR assay(s), and/or DNA sequencing of a subset of qPCR products.

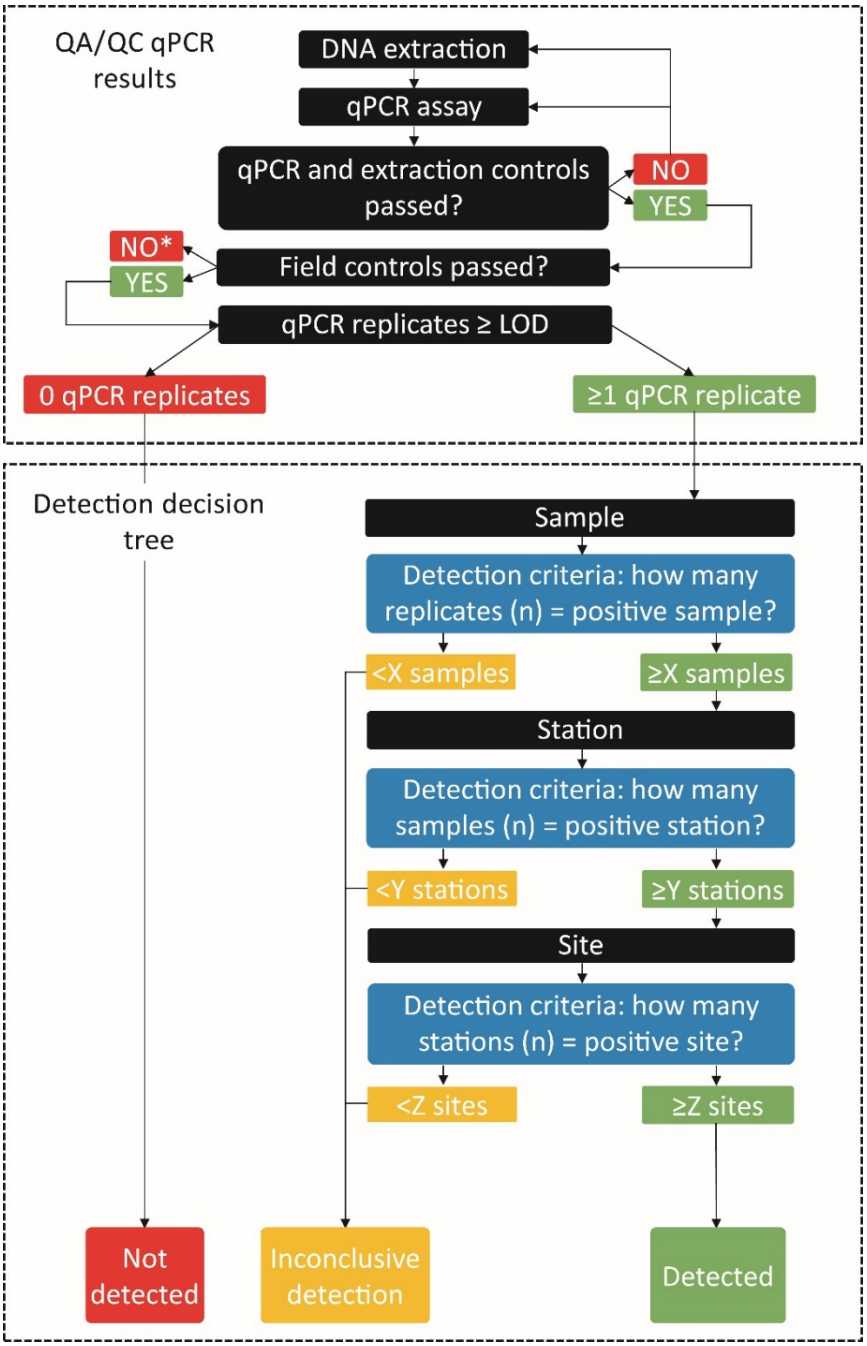

Figure 4. Example of interpretation of eDNA qPCR results. In the upper dotted box, final qPCR results are generated by verifying controls. The lower dotted box is a detection decision tree, within which the criteria applied are variable (as denoted by X, Y, and Z) and may or may not differ for each level, as they will be set according to study objectives and the risk tolerance of the end user. Note that the format of the detection decision tree or the detection criteria should by no means be used as standards for any eDNA detection study. This example is valid only for Level 4 and Level 5 validated assays (Figure 3) since LOD is required. In this example, the term "inconclusive" is used since some qPCR replicates have values above LOD, but result in no sample, station, or site level criteria being met.

***Note:** the * associated with field controls that did not pass indicates the need for a discussion between the end user(s) and eDNA service provider(s). There are many possible outcomes of how to proceed in this case and it will depend on project objectives and end user risk tolerance.*

H. Closing statements

H.1 Disclaimer: Provide additional information not already provided that may be relevant to results and downstream interpretation. For example, describe any issues encountered during sampling, eDNA sample collection, preservation, transport, storage, and laboratory work and their potential impact on results.

H.2 Summary of eDNA detection: Provide an overall summary of eDNA results. This could be either a table or an appended map, and should be summarized to whatever level is appropriate for the study (e.g., site, river, time series) and associated sampling design. This should help the end user to assess the number of eDNA detections (as reported in Section G) and how they were distributed spatially and/or temporally.

H.3 Future recommendations: Provide recommendations or modifications for future work, including methods to: reduce logistical or experimental challenges; improve lab efficiency; safeguard against contamination; optimize selection of equipment or reagents, etc.

Concluding remarks

This guidance document provides essential information for designing, conducting, reporting, and interpreting eDNA surveys, including study design, eDNA sampling of various substrates, post-collection sampling processing, laboratory methods, strategies for interpreting qPCR results to determine presence/absence of target DNA and what the end users will gain to repeat the study over time, space and labs. The accompanying reporting template allows managers to identify information that is crucial to demonstrate scientific integrity and establish confidence in the eDNA project and results. Ultimately, these documents are intended to improve eDNA techniques and enhance the use of results by AIS and SAR managers. It is important to recognize that the reliability of detections is related to the sampling design and how well suited it was to successfully categorize the detection of the eDNA of the organism(s) of interest.

Despite the benefits of eDNA methods, a main drawback is that positive eDNA detections do not directly indicate the presence of a local living organism(s) and eDNA non-detections do not confirm the absence of an organism(s). As such, eDNA results interpretation needs to be done in the context of the management objective(s) as risk tolerances can differ in magnitude and direction depending on study objective(s). Despite the increasing evidence that eDNA is a strong tool to detect species presence/absence, unfortunately there is no ‘silver bullet’ approach to interpreting eDNA qPCR results, given varying objectives, sampling designs, and levels of assay validation, the lack of broadly-recognized standards or guidelines for the generation or interpretation of eDNA data, and the wide variety of target organism(s). It is recommended that interpretation of eDNA results include a discussion between managers and scientists with consideration of all available biological information on the species, in consultation with other relevant experts, as appropriate. Consistent reporting of eDNA results can help increase confidence and reliability in eDNA detection of AIS and SAR; therefore we recommend that SAR and AIS managers use the eDNA guidance document and reporting template and work closely with eDNA service providers and other relevant experts, following an established communication plan, both before a project commences and during the project and interpretation of results.

ACRONYMS

AIS: aquatic invasive species

BHQ: black hole quencher®

bp: base pair

CRISPR/Cas: clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins

cPCR: conventional PCR

Ct: threshold cycle

Cq: quantification cycle

CSA: Canadian Standards Association

CTAB: cetrimonium bromide

ddPCR: droplet digital PCR

DNA: deoxyribonucleic acid

eDNA: environmental DNA

eRNA: environmental RNA

GLP: good laboratory practices

HTS: high throughput sequencing

IPC: internal positive control

LOD: limit of detection

LOQ: limit of quantification

MGB: minor groove binder

NAISC: National Aquatic Invasive Species Committee

NGS: next-generation sequencing

NTC: no template control

PISCeS: Pathway to Increase Standards and Competency of eDNA Surveys

QA/QC: quality assurance/quality control

PCR: polymerase chain reaction

qPCR: quantitative PCR

RNA: ribonucleic acid

SAR: species at risk

SOP: standard operating procedure

STD: standards

GLOSSARY

Abiotic: physical, chemical, and other non-living environmental factors.

Absolute quantification (qPCR standard curve method): quantification method to determine the exact concentration of a target DNA product by relating the quantification cycle (Cq) value to a standard curve to extrapolate a value.

Aliquot: a portion of a total amount of a solution or suspension.

Amplicon: a piece of DNA or RNA that is the product of replication events. The result of using amplification methods such as PCR.

Amplicon length: the length of a specific amplicon provided in terms of total number of base pairs.

Amplification bias (primer bias): differences in PCR amplification efficiencies between different DNA targets (e.g., different species) such that during each PCR cycle, different species will amplify at different rates based on their affinity to the PCR primers being used. This can be a common problem in metabarcoding when dealing with universal primers.

Amplification curve: a plot of fluorescence signal versus qPCR cycle number. Plots are characterized by the point in time during cycling when amplification of a PCR product is first detected and how it accumulates during an experiment.

Assay: an investigative procedure (test) for qualitatively assessing or quantitatively measuring the presence, amount, or functional activity of a target entity (e.g., a qPCR assay).

Barcode / barcoding marker: a short and standardized gene region used to identify an organism to a taxonomic category (e.g., species).

Base pair (bp): a pair of complementary bases in a double-stranded nucleic acid molecule, consisting of a purine in one strand linked by hydrogen bonds to a pyrimidine in the other. Can be used as a unit of measure to describe the length of DNA.

Biodiversity: the variability among living organisms from all sources including terrestrial, marine, and other aquatic ecosystems; and the ecological complexes that they form including the diversity within and between species, and ecosystems.

Bioinformatics: the development and use of software to study genetic and protein sequence data.

Bioinformatic pipeline: bioinformatic analyses involving shepherding large files through a series of transformations, called a pipeline or a workflow.

Biotic: relating to or resulting from living things, especially in their ecological relations.

Blank: a negative control sample that is inserted into the eDNA workflow. The primary purpose of blanks is to trace sources of artificially introduced contamination. The source of contamination introduced in the field or laboratory can be deduced by comparing different blank results (e.g., field blank, extraction blank).

- **Extraction blank**: a type of negative control sample that is included during the DNA extraction process. An extraction blank is manipulated in the same way as all the other samples during the extraction process. Results from an extraction blank show laboratory method sources of contamination.
- **Field blank**: a type of negative control processed in the field that is theoretically free from any DNA. A field blank commonly consists of DNA-free water that is manipulated in the same way as all the other samples (with the exception of collecting eDNA) in an attempt to characterize

any potential contamination occurring. Results from a field blank, if positive, highlight potential sources of contamination.

- **Laboratory filtration blank:** a type of negative control used when samples are filtered in the laboratory, enabling differentiation between contamination that occurred in the field (i.e., during sample collection or while manipulating samples from various stations and sites) from that which occurred during the filtration process.
- **qPCR blank**: a type of negative control used in qPCR that includes all PCR reaction components but no DNA. Also referred to as a no template control (NTC). Amplification in a qPCR blank indicates contamination occurred at the qPCR step.

Bulk sample: a sample resulting from the planned aggregation or the combination of sample units.

Community approaches: a study design method that uses universal primers for detecting a given focal group of organisms, typically achieved through DNA metabarcoding.

Contamination (cross-contamination): accidental introduction of undesired material in a sample that may make eDNA results uncertain in terms of the absence or presence of a species. Contamination can occur at any stage of the eDNA workflow (e.g., sample collection, filtration, preservation, extraction, or analysis).

Controls:

- **Positive control**: a control in a treatment that is known to produce results. They are used to assess the validity and reliability of eDNA results by ensuring that procedures in the eDNA workflow performed as expected and / or as a standard to identify deviations from expected results.
- **qPCR positive control:** a sample typically containing target DNA of interest that is known to amplify in a qPCR reaction. A qPCR positive control determines whether or not the PCR reaction conditions are optimal. This control is expected to produce a value within a predetermined range.
- **Internal positive control (IPC)**: consists of a unique DNA template (i.e., one not found in a test sample) and a pair of specific primers added to the isolate or purified sample. An IPC can be incorporated at any stage in the eDNA workflow (during extraction, purification and/or amplification) and is amplified with the target using its own unique primers/probes. An IPC is used to detect technical problems during DNA extraction, purification, and/or amplification and is used to detect inhibition in PCR. Also called an Internal Amplification Control (IAC) when used during qPCR, it is critical component used to detect PCR inhibition.
- **Negative control**: a sample that contains all essential components of an experimental treatment except the analyte being tested. Also see blanks.

Conventional monitoring methods: species monitoring that relies on physical identification of species by visual surveys, counting of individuals in the field or using distinct morphological characters. This can at times require taxonomic expertise to identify organisms difficult to classify using non-genetic methods.

Copy number: the number of copies of a DNA fragment.

CRISPR/Cas: a genome editing system derived from bacteria that allows genetic material to be added, removed, or altered at a particular target sequence in the genome.

Cq (quantification cycle): the qPCR cycle number at which the fluorescence generated within a reaction crosses the arbitrary fluorescence threshold value. A lower Cq correlates with a higher amount of target DNA in a sample. Also referred to as threshold cycle (Ct).

Decontamination: the process of removing any potential external DNA / contaminant from any eDNA equipment, typically involves bleaching materials.

Detection: an eDNA result that meets the detection criteria parameters, indicating the presence of target eDNA.

Detection probability: a statistical method that utilizes repeated sampling of the same site to estimate the true occupancy of a particular species / target in a given site. Detection probabilities can only be determined after a study.

Detection threshold: the point at which a qPCR reaction reaches a fluorescent intensity above background levels (see quantification cycle).

DNA extraction: a process of isolating and purifying DNA from any given sample using a combination of physical and chemical methods.

DNA metabarcoding: a molecular method allowing for mass DNA sequencing and simultaneous molecular identification of multiple taxa from a complex sample. Typically involves one or more sets of universal PCR primers to amplify DNA barcodes from mass collections of organisms or from eDNA.

Droplet digital PCR (ddPCR): a method for performing PCR that is based on water-oil emulsion droplet technology enabling the partitioning of a single PCR reaction into thousands of independent amplification reactions. Methods allow for the absolute quantification of target DNA.

eDNA end user: a manager, client, or requestor of eDNA services who ultimately uses or is intended to ultimately use eDNA results in various contexts, such as conservation and management.

eDNA service provider: is the overall project manager responsible for communicating results to the requester (the end user). A service provider may include government or non-government researchers or practitioners, or third-party consultants.

eDNA shedding rate: the rate at which an organism releases its genetic material (DNA) into an environment. Shedding rates can be impacted by seasonality, growth rates, reproduction and metabolic rates.

Environmental DNA (eDNA): DNA extracted from environmental samples (e.g., water, biofilms, air, sediment, gut contents, feces) and analyzed to infer the presence or absence of DNA from target organism(s).

Environmental RNA (eRNA): RNA extracted from environmental samples (e.g., water, biofilms, air, sediment, gut contents, feces) and analyzed to infer the presence or absence of RNA from target organism(s).

Elution: to extract one material (e.g., DNA) from another, usually by means of a solvent (e.g., water, buffer).

False negative: failing to detect the eDNA of a species within an environmental sample taken from an area where that species actually is present. Potential causes include sampling error (i.e., the eDNA was not captured in the environmental samples collected), degradation of eDNA from improper handling and/or storage, inefficient DNA extraction from environmental samples, failure of the qPCR assay to be as sensitive as required. Also known as Type 2 error.

False positive: recording the detection of eDNA of a species within an environmental sample taken from an area where that species actually is absent. Potential causes include misinterpretation of qPCR results, undetected contamination, or failure of the qPCR assay to be as specific as required. Also known as Type 1 error.

Fluorescence: emission of light in the presence of target DNA that is produced by DNA intercalating agents / dyes or fluorescently labelled probes.

gBlock: synthetically made double-stranded DNA fragments that are used as qPCR standards.

Genome: the entire DNA of an organism.

Good Laboratory Practices (GLP): is a quality system of management controls and conditions for research laboratories and organizations to ensure the uniformity, consistency, reliability, reproducibility, quality, and integrity of products. It is intended to promote the quality and validity of test data and improve the acceptance of data generated in adherence to its principles (OECD 1998).

High throughput sequencing (HTS): see next-generation sequencing

in silico: conducted or produced by means of computational modeling or simulation.

in situ: in its original place.

in vitro: performed or taking place in a test tube, culture dish, or elsewhere outside a living organism.

Inconclusive detection: an eDNA detection that does not meet the detection criteria parameters, thus indicating that further testing may be required.

Inhibition / inhibitor: non-target substances from the environmental system (e.g., total suspended solids) that remain present in the sample at collection and through DNA extraction. Inhibitors are typically co-extracted with the target DNA and inhibit the PCR/qPCR reaction. Failure to test / characterize the presence of inhibition in samples can result in false negatives. PCR inhibition is identified using an Internal Positive Control (IPC).

Intercalating dye / agent (also called DNA binding dye): molecules that insert themselves between double stranded DNA. In qPCR, the agent releases a fluorescent signal during each reaction cycle proportional to the concentration of any DNA present in the reaction (including but not limited to the target DNA sequence). The strength of this fluorescent signal is compared to a standard curve to estimate DNA concentration. Examples of DNA intercalating dyes are SYBR-green and Eva-green.

Limit of detection (LOD): the lowest concentration of analyte (i.e., target DNA) that can be detected with a defined level of confidence (with a 95% detection rate as the standard confidence level). The LOD can be determined using discrete threshold methods or curve fitting methods (Klymus et al. 2019).

Limit of quantification (LOQ): the lowest concentration of analyte (i.e., target DNA) in a sample that can be quantitatively determined with acceptable precision and accuracy, under stated experimental conditions. For qPCR assays, precision can be assessed using the coefficient of variation (CV) of measured concentrations of standards. The LOQ is critical in studies determining predictive relationships between eDNA concentration and target species biomass or relative abundance.

Marker: a DNA sequence that can be used to identify individuals or species where a variation is observable.

Master mix: a premixed concentrated solution that has all of the components for a PCR or qPCR reaction but not containing assay primers (and probes) and target DNA.

Metadata: a set of data that describes and gives information about other data.

Metabarcoding: see DNA metabarcoding.

Multiplex / multiplexing: Combining multiple assays using multiple genetic markers in a single qPCR reaction to detect one or more organisms. Multiplexing shortens processing times and reduces the use of reagents, but can be more difficult to optimize than single assay reactions.

Negative detection in a qPCR replicate, sample, station, or site: level of detection that does not meet the limit of detection parameters.

Next-generation sequencing (NGS): also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern (non-Sanger-based) sequencing technologies (e.g., Illumina [Solexa] sequencing).

Not detected: a test result indicating eDNA of a targeted species was determined to not be present in an eDNA sample.

Polymerase Chain Reaction (PCR): a molecular technique that replicates and amplifies target DNA sequences to produce sufficient copies suitable for genetic analyses. It involves a process of heating and cooling (i.e., cycling), where primers bind to a matching DNA sequence during each cycle and a polymerase replicates the DNA sequence in between these primers. At the end of the PCR reaction, the target sequence is present in the reaction in millions of copies (amplicons). In order to visualize these short fragments of DNA, the PCR product must be run on gel electrophoresis. PCR is often referred to as conventional PCR (cPCR).

Positive detection in a qPCR replicate, sample, station, or site: level of detection that meets or exceeds the limit of detection parameters.

Primers: short single strand DNA sequences manufactured to a match specified sequence of an organism or a taxonomic group, which are used to initiate the PCR process.

- **Species-specific primer**: a primer designed to amplify a unique gene region specific to a single species.
- **Universal primer**: a primer designed to amplify a highly conserved gene region across multiple taxa.

Probe: a fluorescent-labeled oligonucleotide (short sequence of DNA or RNA) that is added to the qPCR reaction which recognizes a specific sequence on the desired PCR product. qPCR probes offer an alternative to DNA-binding dyes (intercalating agents), as they typically increase specificity and sensitivity since only target specific DNA molecules will be labelled. In a probe-based qPCR, the probe releases a fluorescent signal during each reaction cycle, proportional to the concentration of the target DNA sequence. The strength of this fluorescent signal is compared to a standard curve to estimate the target DNA concentration.

Quantification cycle: see Cq.

Quantitative Polymerase Chain Reaction (qPCR): the real-time quantification of amplified DNA fragments during the PCR reaction. qPCR makes use of the same principles of PCR involving primers and a DNA polymerase, but employs a DNA binding dye that intercalates with DNA or a probe that hybridizes with target sequence allowing for real time visualization of amplification of the target sequence (amplicon). Depending on the approach, this method can result in absolute quantification within a sample or relative quantification between samples.

qPCR efficiency: the fraction of DNA molecules that amplified (i.e., thus doubling the concentration of DNA as expected) in one PCR cycle. It is an indicator of the performance of a qPCR assay. It represents the ratio of the number of target gene molecules at the end of a qPCR cycle divided by the number of target molecules at the start of the same qPCR cycle. Typically, acceptable qPCR efficiencies are in the range of 90-110%.

Relative quantification: analyzes changes in a given sample relative to another sample (e.g., an untreated control sample).

Repeatability: the level of agreement between results of replicates both within and between runs of the same test method in a given laboratory.

Replicate: repeated collection or analysis of a sample to reduce error in results caused by high levels of variation among repeated measures.

- **Field replicate**: separate sample units collected as close as possible to the same point in space and time, stored in separate containers, and analyzed independently.
- **Filter replicate**: filter replicates are obtained by cutting filters in pieces and testing pieces separately.
- **Lab replicate / technical replicate**: a PCR replicate, where the same DNA is tested in separate reactions.

Reproducibility: defined as the ability of a test method to provide consistent results when applied to aliquots of the same samples tested in different laboratories using the identical assay (including reagents and controls).

Sample degradation: when the DNA or RNA in a sample decays as a result of improper preservation and storage due to biotic factors (e.g., fungi and bacteria activity in the presence of excess moisture and air for an extended period of time) and procedural factors (e.g., repeated freezing and thawing of DNA samples). Degradation negatively impacts sample quality. As a result it is important to filter and preserve eDNA samples as soon as possible after collection to prevent sample degradation.

Sampling effort: number of sites, stations, and field replicates. The level of sampling effort required varies with the eDNA assemblage in the ecosystem and is determined based on the minimum level of effort necessary to reach the required level of confidence in the assessment.

Sanger sequencing: the traditional method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication.

Semi-targeted approaches: see community approaches.

Sensitivity: refers to a qPCR assay's ability to detect it's intended target. The ability to detect a true positive.

Single-species approach: see targeted methods.

Site: a specific area, within a selected sample location, where water (or other environmental substrate) will be collected.

***Species at Risk Act* listed species**: species listed on the List of Wildlife Species at Risk as set out in Schedule 1 of the *Species at Risk Act* (2002). A species at risk means an extirpated, endangered, or threatened species or a species of special concern.

Species monitoring / biomonitoring: the process of making reliable observations from nature to detect, measure, assess and draw conclusions about how species are changing through time and space.

Specificity: in terms of a qPCR assay, is the ability to discriminate or differentiate between species that are genetically similar. The ability of an assay to detect a true negative.

Standard operating procedure (SOP): a set of written instructions that provide a detailed description of how to perform a laboratory process or experiment safely, effectively, and consistently.

Station: refers to spatially distinct sampling locations within a site.

Target / target sequence: a key DNA sequence, gene or region of interest that once amplified allows for the identification of the target taxonomic group.

Targeted methods: a study design method that uses species-specific primers in an attempt to detect a single species from a sample, typically achieved through qPCR.

Taxonomic groups / taxon: a grouping of organisms assigned to a particular category of classification (e.g., species, genus, order).

Thermocycler parameters / cycling parameters: each cycle of PCR reaction involves a step for template denaturation, primer annealing and primer extension. Thermocycler parameters / cycling parameters correspond to the temperature and time required for each of these steps. The cycling parameters also include the number of times the PCR reaction needs to be repeated to achieve maximum amplification (typically 35-40 cycles).

Validation / validated methods: the process used to confirm that an analytical procedure employed for a specific test is suitable for its intended use. Validated methods yield quality, reliability and consistency of analytical results.

Water matrix: refers to the components of a sample other than the eDNA. The matrix can have a considerable effect on the way the qPCR analysis is conducted and the quality of the results obtained.

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ANNEX 1. eDNA REPORTING TEMPLATE*

I. eDNA Testing Sample Submission Information

Report Title:				
Project Number:			**Date of Final Reporting:**	
Service Provider Information	Type:	Select From Dropdown	Requesting Organization Information	Organization Name:
	Contact Name:			Contact Name:
	Address:			Contact Phone:
				Contact Email:
	Contact Phone:			
	Contact Email:			
	LAB ACCREDITATION / CERTIFICATION:			

Executive Summary - Study Objectives, Rationale, and Main Finding(s) derived from both eDNA samples and controls

Appendices (Required)	Check to confirm inclusion		**Appendices (Additional)**
Appendix 1: Maps of the study sites and sampling locations	☐		*Appendix 5:*
Appendix 2: Contamination prevention procedures	☐		*Appendix 6:*
Appendix 3: qPCR protocol	☐		*Appendix 7:*
Appendix 4: Metadata and qPCR data	☐		*Appendix 8:*

II. Study Design and eDNA Sampling

A. Study information	*A.1 Species targeted (common and Latin):*		
	A.2 Study objectives:	Select From Dropdown	
	A.3 Geographic location and/or region:		
	A.4 Sampling date (range):	Start:	Finish:
	A.5 Sample types:	Select From Dropdown	
	A.6 Mapping databases (list all):		
B. Study design	*B.1 Type(s) of ecosystem:*	Select From Dropdown	
	B.2 Sampling design (how does sampling optimize species detection for study goal?):		
	B.3 Number of sites sampled:		
	B.4 Number of stations sampled within sites (add explanation for variation among sites):		
	B.5 Number of field sample replicates:		
	B.6 Time series (number of times sites and stations were sampled):		
	B.7 Environmental conditions, relevant observations, and additional field data:		
	B.8 Field blanks and field controls (describe and give numbers):		
C. eDNA sample collection	*C.1 Env. sample collection method:*	Select From Dropdown	
	C.2 Volume / weight sampled:		
	C.3 Sample depth(s):		
	C.4 Field sample storage/time to processing:		
	C.5 Sample processing method (list disposable equipment; preservative used):	Select From Dropdown	
	C.6 Filter type and pore size:		
	C.7 Sample preservation:	Select From Dropdown	

*A downloadable version of this form can be found here.

III. eDNA Sample Analysis - Laboratory Methods			
D. DNA extraction	*D.1 Name of commercial kit or protocol:*		
	D.2 Reference protocol:		
	D.3 DNA extraction controls:		
	D.4 Proportion of total sample:		
	D.5 DNA elution volume:		
	D.6 Extracted eDNA storage conditions:		
E. qPCR assay	*E.1 Assay Name:*		
	E.2 Assay Type:	Select From Dropdown	
	E.3 Level of assay validation:	Select From Dropdown	
	E.4 Specificity data:		
	E.5 Dilution and volume of DNA used:		
	E.6 qPCR positive and negative controls:		
	E.7 Technical replicates per sample:		
	E.8 Inhibition tests:		
	E.9 Number of qPCR cycles:		
IV. Summary of eDNA Results			
F. Reporting control results	*F.1 Criteria to determine if controls passed or failed:*		
	F.2 Positive control results (report each type separately):		
	F.3 Negative control results (report each type separately):		
	F.4 Failed controls (report and explain):		
G. Reporting eDNA sample results	*G.1 Calculated LOD:*		
	G.2 QA/QC qPCR results:		
	G.3 Other qPCR results:		
	G.4 Determination of sample-level results:		
	G.5 Determination of station-level results:		
	G.6 Determination of site-level results:		
H. Closing statements	*H.1 Disclaimer (any additional information to help explain results for any samples, stations, or sites):*		
	H.2 Summary of eDNA detection:		
	H.3 Future recommendations:		

ANNEX 2. METADATA APPENDICES

APPENDIX 1. MAPS OF STUDY SITES AND SAMPLING LOCATIONS

Include maps that convey the spatial and temporal sampling design used in the eDNA study. Maps should include sampling locations (locations, sites, and stations; see Section II), scale bar, and any details about water movement between stations (e.g., water flow direction).

APPENDIX 2. CONTAMINATION PREVENTION PROCEDURES

Provide information on the measures used to prevent eDNA sample contamination and cross-contamination. Include decontamination methods and procedural practices during pre-field, in-field, and laboratory processes. Report any deviations from procedure encountered during the duration of the study.

APPENDIX 3. qPCR PROTOCOL

Essential information about the qPCR assay used are to be provided directly in the reporting template (Section E). Additional details listed in Table 2 are to be provided in an appended protocol as Appendix 3; note that the same Appendix 3 can be used across studies and through time if those details remain unchanged. Despite the fact that the primer and probe sequences are essential information to ensure the reproducibility of the study, they are considered optional given that some third-party eDNA service providers will not release proprietary or confidential business information to clients.

Table 2. List of eDNA qPCR assay properties. Information that is essential (E) or optional (O) for inclusion in the appended qPCR protocol (Appendix 3) is indicated (following Bustin et al. 2009). Information provided in this table may be more constant between eDNA projects from the same eDNA service provider.

1. qPCR target information	
a) DNA sequence for the targeted species. Provide the gene name and, if available, the GenBank accession number	E
b) If the assay is published, provide the reference to the publication	E
c) Amplicon length expressed in base pairs (bp)	E
d) Fluorescent probe, quencher, and any probe modification (e.g., MGB, BHQ) or type of DNA intercalating dye / agent	E
e) Primer and probe sequences	O
2. qPCR protocol	
a) qPCR Master Mix. Indicate whether a commercial or in house mix was used for amplification. For commercial products, indicate the brand and type of Master Mix	E
b) Thermocycler parameters. Provide the qPCR parameters including denaturation, annealing, elongation, and number of cycles	E
c) qPCR instrument. Indicate the instrument brand and model	E
d) Cq method determination. Provide details on Cq determination such as software, threshold determination, etc.	E

APPENDIX 4. METADATA AND qPCR DATA

These mandatory tables provide full tracking and traceability of samples through processing and analysis. All samples must be properly catalogued for their sampling information, processing (e.g., filtration), and qPCR analysis results. The format of the metadata and qPCR data template provides an example of how essential and optional data associated with eDNA results could be presented. Table 3 provides a list of mandatory and optional metadata but is not intended to be exhaustive. The suggested format for this appendix information is a Microsoft Excel file.

Table 3. List of information to provide in the mandatory metadata and qPCR data tables. Essential (E) and optional (O) information to include in Appendix 4. This table provides information that will or may vary within or between eDNA projects.

1. Metadata	
a) Geographic region	E
b) Site	E
c) Station	E
d) Field sample	E
e) Spatial coordinates (decimal degrees)	E
f) Collection date (mm/dd/yyyy)	E
g) Collection time (hh:mm; 24 hour clock or military time) including time zone	E
h) Collector name	E
i) Environmental conditions (see B.7 for more details)	O
j) eDNA processing (e.g., filtration date and time, sediment aliquoting, sample-specific departures from a sampling plan, rationale for any adjustment, and other information which is study dependent, see Section C for more details)	E
2. qPCR data	
a) Cq for each qPCR replicate	E
b) Assay efficiency for each run (standard curves: R^2, slope equation, and y-intercept)	E
c) Result for each sample (e.g., detected, not detected, or inconclusive; see Section G)	O