Environmental DNA (eDNA)

Best Management Practices for Project Planning, Deployment, and Application

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Chapter 1

Introduction

Environmental DNA (eDNA) is defined as the DNA that is extracted from environmental samples such as soil, sediment, air, water, etc.; it is distinguished from other types of DNA samples by a methodology that targets DNA *in the absence of their source organisms*, rather than sampling that targets individual organism(s). The use of eDNA in a method to assess macro-organismal community diversity was first applied by Willerslev et al. (2003) for temperate sediments and permafrost cores 400,000 to 10,000 years old. However, despite being perceived as a modern methodology, eDNA has been utilized since at least the mid-1980's to detect bacterial communities in marine environments (Ogram et al., 1987). What constitutes an "environmental sample" is debatable, with studies that have targeted human DNA on the settled dust and bodies of small household insects (Toothman et al., 2008; Kester et al., 2010); DNA from preservative ethanol to assess benthic invertebrate biodiversity (Hajibabaei et al., 2012); and even DNA from bulk-collected carrion flies to assess local mammalian diversity (Calvignac-Spencer et al., 2013), to name a few. Despite debate on what may constitute an environmental sample, the fact that the utilization of eDNA in various applications has exploded in the past decade is undeniable.

Environmental DNA comes from many sources, including sloughed tissues or cells, waste products, gametes, saliva, blood, or other secretions. Its abundance can fluctuate in the environment as the abundance of different tissue types fluctuates (e.g., during fish spawning events, gametes are released and are highly abundant for a short period of time relative to non-spawning seasons). Environmental factors can influence shedding and degradation of eDNA (Barnes and Turner, 2016; Hansen et al., 2018; Strickler et al., 2015; Jo et al., 2019), and degradation of eDNA is affected by different water chemistries, temperature, and microbial activity (Barnes et al., 2014; Eichmiller et al., 2016; Jo et al., 2019; Seymour et al., 2018; Strickler et al., 2015). Most eDNA studies have targeted mitochondrial DNA (mtDNA) as a single mitochondrion has tens to thousands of copies of the mitochondrial genome vs. a single nucleus per cell. However, there have also been studies looking at the utility of nuclear DNA (nuDNA) markers (Minamoto et al., 2017; Dysthe et al., 2018; Gantz et al., 2018; Jo et al., 2019; Jo et al., 2020), including the potential ability for the ratio of mtDNA to nuDNA being able to provide age structure information for a population (Jo et al., 2020).

Environmental DNA is also found in nearly all environments, including aquatic, terrestrial, and even in the air itself. Aquatic environments include marine systems, from deep oceans to near-shore environments, coral reefs, estuaries, etc.; freshwater environments are varied as well, from large and swiftly moving river systems to small stagnant ponds. Terrestrial systems include extremely cold arctic regions to tropical jungles, caves, etc. Each of these environments requires careful consideration of sampling design and expected outcomes from a study. In addition, consideration must be given to understanding how an organism's DNA arrived in a location; while typically eDNA is found in areas an organism has occupied (including the distant past), it can also be transferred great distances by predation, physical forces (air, water), and human activity (sewage, ballast water, etc.). On the other end of the spectrum, when an organism's DNA is not detected, it does not necessarily signify that the

organism has not been in that location, as degradation, poor sampling design, laboratory procedures, and a host of other environmental factors can influence the ability to positively detect an organism's DNA.

Sanger sequencing was used primarily in the early period of eDNA method development, which involved sequencing fragments of DNA captured from an environmental sample. Methodologies quickly evolved to utilize real-time quantitative PCR (qPCR), as these methods were quicker and cheaper than traditional Sanger sequencing. Using qPCR is not without its limitations, however, as captured DNA is not sequenced with this method, therefore a sequence is not used to confirm the identification of the target fragment. Instead, qPCR assays rely on rigorous development, encompassing in silico (design using computational methods), and in vitro (laboratory) and in situ (field) testing, such that amplification within a certain number of cycles will be interpreted as a "positive" detection. Further development of eDNA methods have now included next-generation sequencing (NGS) methods (Illumina) and those also known as third generation sequencing technologies (PacBio, Nanopore, etc.). While the majority of contemporary eDNA studies still rely on qPCR methods, determining which method to use will depend on the study objectives. For instance, qPCR methods are typically very sensitive for single species detection and are not suitable for detecting communities of organisms. Metabarcoding using NGS technology is suitable for identifying a community assemblage, however, some of the limitations of this method are: failure to detect rare species, failure to detect species when universal primers are not compatible or overwhelmed with other species' DNA, etc. No matter which method is chosen for an eDNA study, it is important to understand the limitations and pitfalls for each of them.

The U.S. Fish and Wildlife Service (Service) has applied eDNA methodologies in a variety of studies within aquatic habitats for both conservation and aquatic invasive species (AIS) detection and management. However, while eDNA methodologies have demonstrated utility for monitoring aquatic environments in many different contexts, there is still considerable concern regarding potential sources of uncertainty associated with these methods. As pointed out by Darling and Mahon (2011) in their review of DNA-based detection methods for aquatic invasive species (AIS), to effectively deploy DNA-based monitoring tools as they become available, it is critical that multiple stakeholders participate in the informed and transparent discussions on the benefits and limitations of these various tools.

To provide guidance and insight into the complexities of the application of eDNA methods, and as part of a training course on the use and applications of eDNA at the Service's National Conservation Training Center, a best management practices guide was developed by eDNA practitioners (both field- and lab-based) from across the Service. The guide is intended to provide field and regulatory biologists with a more complete understanding of the broader aspects of eDNA methodologies in freshwater systems. While many of the aspects covered in this document are applicable to other systems, currently the Service focuses on aquatic systems, and to cover all possible environments is beyond the scope of this document. The six primary components of eDNA studies addressed herein include: marker validation, sampling design, field techniques, field and lab controls, data analysis and interpretation, and the role and mitigation of inhibition. Each of these areas is complex in its contribution to the interpretation of eDNA results, but given the increasing use of eDNA for management and conservation needs, it is critical

that managers and biologists have a general understanding of these concepts to improve both the interpretation of eDNA studies and the application of their results.

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Chapter 2

Laboratory techniques for eDNA studies

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Summary

A clear understanding of eDNA detection technology is necessary prior to processing samples. Additionally, marker validation is crucial prior to applying any kind of detection technique to field-collected eDNA samples. This includes a clear understanding of the performance capabilities of each marker so that the resulting eDNA data can be correctly interpreted. This chapter provides a summary and comparison of the most commonly used technologies for detecting eDNA in the laboratory including conventional PCR (cPCR), SYBR-based quantitative PCR, probe-based quantitative PCR (qPCR), digital PCR (dPCR), loop-mediated isothermal amplification (LAMP), and metabarcoding. Each technology is introduced along with the advantages, disadvantages, and typical applications. Targeted taxon-specific qPCR markers are currently the most widely used eDNA laboratory technique. Therefore, there will be a specific focus on the minimum requirements to properly validate qPCR markers for use with eDNA. Critical steps in marker validation are reviewed along with context and justification for why these steps are necessary prior to processing eDNA samples. These steps are specific to qPCR, but are also relevant for cPCR, dPCR, and metabarcoding markers with minor differences. LAMP marker validation is more complex than qPCR markers and are beyond the scope of this chapter.

Introduction

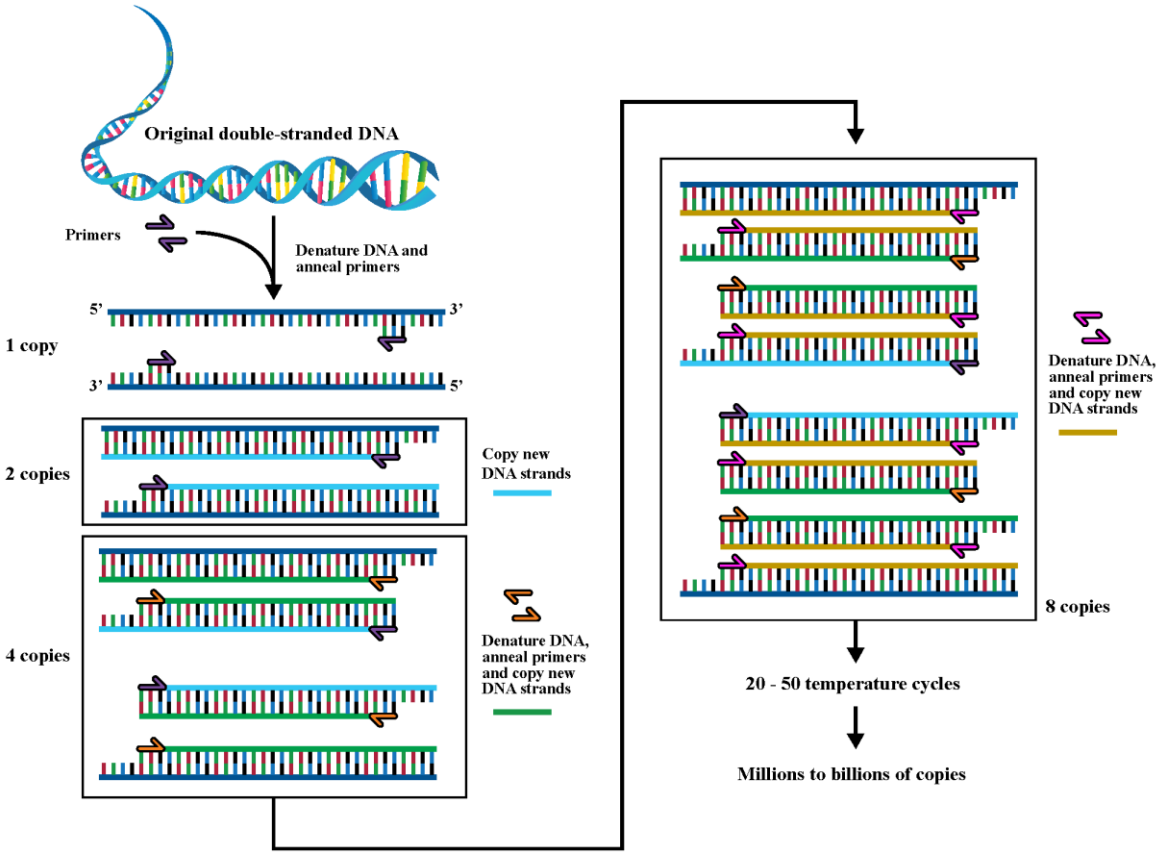
Environmental DNA is typically present at low concentrations in field samples. To detect the presence of target species DNA in a sample, it must be amplified using molecular techniques. Amplification is typically completed using an enzymatic reaction known as PCR (Polymerase Chain Reaction). The PCR is directed by a paired set of primers that bind to the target organism's DNA (Figure 2.1). Through a series of temperature cycles (thermocycling), the DNA is heat denatured into single strands and then cooled to the optimal temperature to facilitate binding of the primers to the denatured DNA (annealing temperature). Once the primers bind to the DNA the temperature is again raised to activate the enzyme Taq polymerase, which mediates the reaction process and assembles new copies of the targeted DNA. This process of heat denaturing, primer annealing, and DNA copying is repeated for 20-50 cycles depending on the application, resulting in hundreds of millions to billions of copies of the target DNA being obtained. This basic PCR approach forms the basis of nearly every laboratory detection methodology currently used to detect eDNA.

***Box 2.1.* Key terminology for eDNA laboratory techniques**

PCR – Polymerase Chain Reaction (Figure 1)
Amplification – Laboratory process where millions of copies of target DNA are made to allow for visualization and measurement
Primer – Short synthetic strand of DNA that is used to begin PCR amplification. Primers bind to target organism or taxon DNA and convey specificity to an eDNA marker
Marker – Region of DNA that the primers span. eDNA markers are generally short in length (< 150 base pairs)
Taxon – a taxonomic ranking of a group of organisms, for eDNA markers usually family, genus, or species

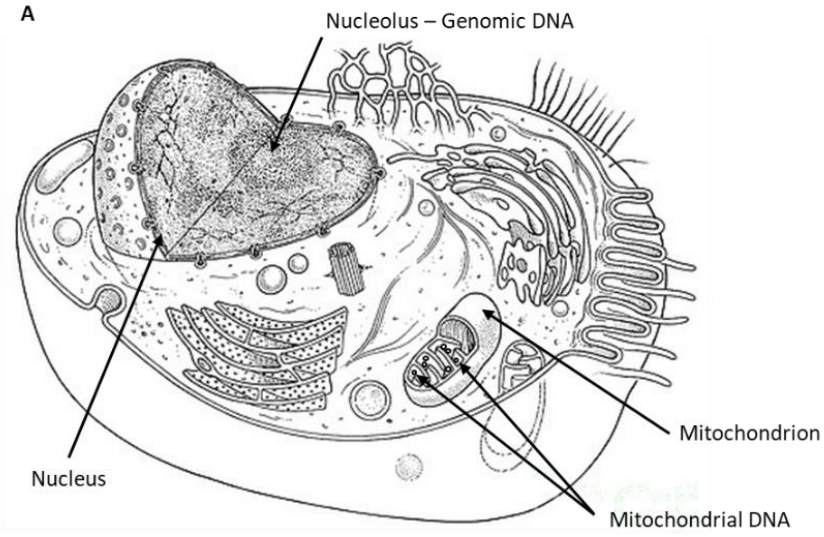
The region of DNA between and including the primer binding sites is known as a marker. Markers are used to identify either a taxonomic group or a specific species.

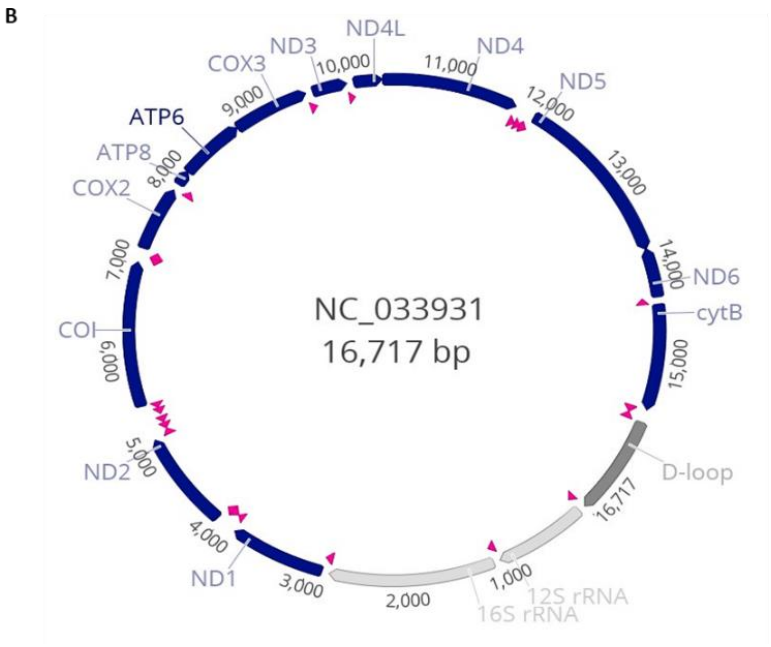
Figure 2.1. Schematic depicting the steps in the Polymerase Chain Reaction (PCR). Graphic: John Embrey, USFWS (adapted from National Human Genome Research Institute; www.genome.gov).



Environmental DNA collected from field samples can originate from two locations within the cell: the mitochondria or the nucleus (Figure 2.2). Many organisms have two distinct genomes, the nuclear genome (contained inside the nucleus of the cells) and the mitochondrial genome (contained inside the mitochondria of the cells). In animal cells, the mitochondria carry a greater number of DNA molecules than the nucleus for two main reasons; 1) there are multiple mitochondria within each cell and only a single nucleus and 2) there are multiple copies of DNA within each mitochondrion. Depending on the cell type, this can result in thousands of mitochondrial genomes to each nuclear genome (Miller 2003; Wolff and Gemmell 2008). Previous studies suggest that the eDNA detected in environmental samples is most often associated with the collection and concentration of DNA within cells (Turner et al. 2014). DNA residing inside the cells is less susceptible to microbial and mechanical degradation once in the environment. As a result, intracellular DNA is of higher quality than extracellular DNA (free DNA).

Figure 2.2. A) Depiction of a typical animal cell showing the nucleus which contains the nucleolus and genomic DNA. Membrane bound mitochondrion are located outside of the nucleolus and each contains many copies of mitochondrial DNA. B) Expanded view of the mitochondrial genome. Each mitochondrial genome is a circular DNA molecule typically around 16,500 base pairs in length. Each cell can contain hundreds to thousands of copies of the mitochondrial genome.





Understanding the differences between intracellular and extracellular DNA helps to inform sample collection strategies (covered in Chapter 3) as well as determine the appropriate laboratory detection methodology. Because eDNA is usually present at low concentrations in field samples, and most often originates from cellular material, one way to increase

the probability of eDNA detection is to develop markers that amplify mitochondrial DNA. Some organisms (i.e. bacteria and other prokaryotic organisms) do not possess mitochondrial DNA requiring alternative DNA detection targets, but similar principles apply.

Many field biologists and resource managers collect eDNA samples, use data in decision making and/or review eDNA funding proposals. For many of these end users of eDNA data, samples are sent for analysis at a specialized genetics laboratory. This often results in end users having little involvement in the initial assay design or detection methodologies adopted. This arrangement is increasingly necessary given the degree of technical knowledge necessary to design and implement eDNA laboratory analyses. To bridge any knowledge gap that may exist, we cover several critical questions to consider before contracting with a laboratory for sample processing. Addressing these questions will also help decide if an eDNA project proposal has a high chance of success, is meeting objectives, or is worthy of funding. It is difficult to answer these questions without a basic working knowledge of existing detection technologies. Likewise, it's important to understand what marker validation is and why it is critical for successful data collection in eDNA projects. This Chapter will discuss the basics of marker validation as well as the advantages, disadvantages, and suitable application of each detection method. While the fine scale details are important, an overall understanding is critical. In addition to providing information about the fundamentals of marker validation, we also provide resources for those interested in the finer details of the analytical methodology.

Finally, it is important to know that not all eDNA analysis laboratories possess the same detection technology or staff trained in all the available molecular detection instruments and data analysis techniques. Therefore, it is important to understand both the capability of the individual laboratory along with the type and quality of data the laboratory can produce.

Box 2.2. Critical questions to clarify prior to collection and processing eDNA samples

1. What type of data is required to accomplish project goals?
2. Presence data? Quantitative data? Single species? Multiple species?
3. Does a marker or markers exist for the target organism(s)?
4. Has the marker been validated for its intended use?
    - What level of marker specificity is required? Genus? Species? Other?
    - What level of marker sensitivity is required?
    - Is the analysis laboratory capable of designing and validating a new markers?
5. Does the analysis laboratory possess the necessary molecular detection technology to produce the required data for the project?
6. Is the analysis laboratory properly equipped with process controls to minimize sample contamination?

Laboratory detection technology used for eDNA analysis

As application of eDNA techniques have become commonplace in research and natural resource management, a number of different laboratory techniques have emerged and form the basis of nearly all eDNA work to date. Several techniques were common early in the development of eDNA science but have since been largely replaced by more reliable methods. Some are newly emerging techniques that are finding application in narrowly focused areas. Over time the techniques have evolved to incorporate increased sensitivity and specificity to the target DNA. This section is not intended to be an exhaustive review. There are some eDNA techniques that have been used in a research setting but have not yet found an application in applied management and will not be covered here.

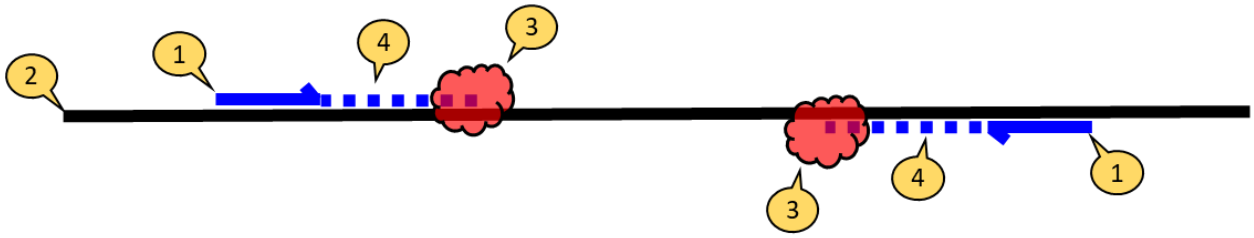
This section covers the five most common laboratory techniques used in eDNA and explores some of the similarities and differences between techniques including:

- the type of data produced
- the type of markers used
- marker validation approaches
- the appropriate eDNA application
- interpretation of resulting data

Conventional PCR (cPCR)

During cPCR (also known as endpoint PCR) Taq polymerase is responsible for assembling the new DNA fragments. cPCR works by first binding short fragments of DNA called primers to the template DNA (Figure 2.3). Primers are designed to be specific (exact match to the genetic sequence) to the desired

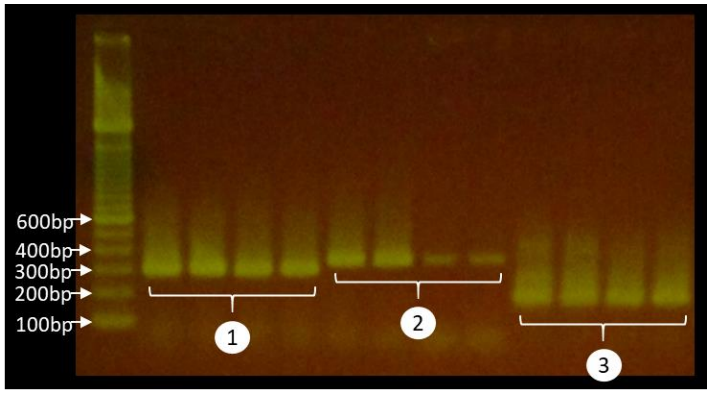
Figure 2.3. Stylized schematic of the conventional polymerase chain reaction process detailing the arrangement of forward and reverse primers (1), template DNA (2), Taq polymerase enzyme (3) and the newly forming PCR product (4).



target species or taxon. Enough mismatches between the primers and the template DNA will result in a failed amplification. Primers that bind to different species or a different location on the DNA produce non-specific products that confound data interpretation (see Figure 2.4). While primers convey marker specificity, the enzyme Taq polymerase is responsible for assembling the new DNA fragments. Taq polymerase uses the primer location as a guide to begin the replication process. Once enough copies are obtained, they can be visualized via gel electrophoresis (Figure 2.4).

Electrophoresis uses an electric current to separate PCR products through a gel matrix based on length (number of base pairs). A size standard is run on each gel and a detection is based on observing a product of the expected size. While this process is effective, PCR product size is not always clear

Figure 2.4. Image of gel electrophoresis results showing the different size fractions recovered relative to a standard of known size.



resulting in a subjective interpretation of the results. For example, the expected PCR product of the assay depicted in Figure 2.4 is 280 base pairs (bp) in length. Products in group 1 appear slightly smaller than the 300bp standard and are presumed to be positive detections. While groups 2 and 3 are a little too large and small, respectively, to be considered a detection and are assumed to be non-specific PCR products. Additionally, there has to be sufficiently large quantity of cPCR product generated during PCR to be visible on a gel, thus there must be relatively large quantities of target DNA present in a sample for visualization to be possible. As a result, cPCR assays are less sensitive than other molecular techniques. The subjective nature of the process often requires that PCR products be sequenced to confirm they originated from the target organism. Sequence confirmation of short PCR products is challenging and often results in low quality sequence data, preventing confirmation of short PCR products. Because of ambiguity in calling a PCR product a detection, non-specific primer binding, challenges in sequence confirmation, and the lack of detection sensitivity, cPCR and data collection via gel electrophoresis is not recommended for use in eDNA studies with management or conservation applications.

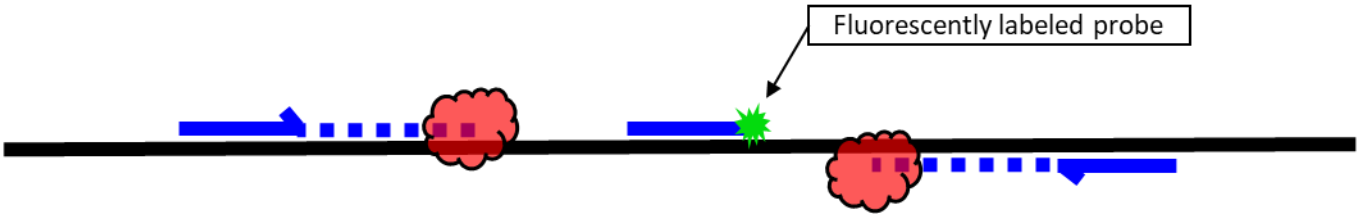
Quantitative PCR (qPCR)

Unlike cPCR, quantitative PCR results can be monitored in real-time during the amplification process. qPCR is common in two forms; one uses an intercalating fluorescent dye that binds to double stranded DNA and the other uses a fluorescently-labeled probe that binds to target DNA. Many existing cPCR assays can be converted to qPCR assays through the design of a specific probe or further optimization for use with SYBR green dye. The following section outlines the differences in qPCR assays (probe-based qPCR, intercalating dyes, and digital droplet PCR) and the pros and cons associated with each.

Probe-based qPCR

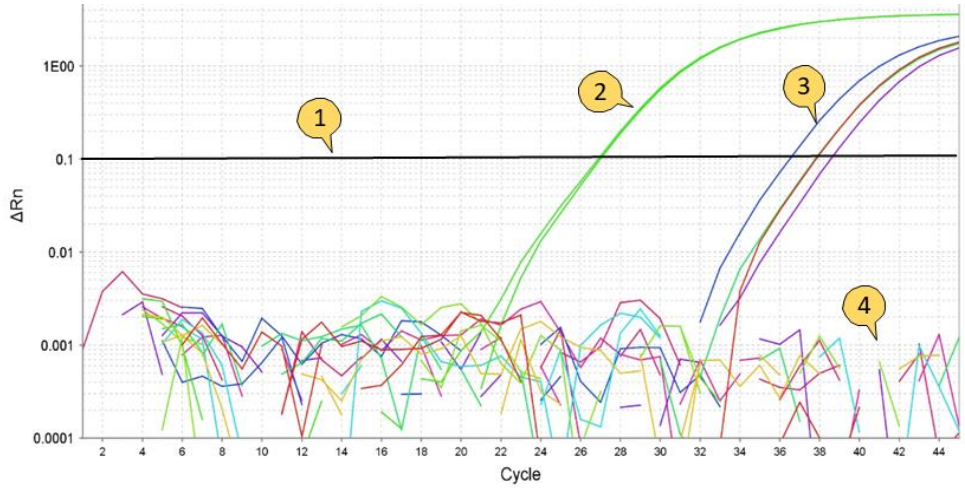
In probe-based qPCR, the PCR amplification steps are the same as cPCR. The element that distinguishes qPCR from cPCR is the inclusion of a fluorescently labeled probe that binds to a specific DNA sequence internal to the forward and reverse primers (Figure 2.5). This fluorescent probe allows for the visualization and measurement of fluorescence. During each PCR cycle, when the probe binds to the newly created PCR products, it's fluorescent label is released, and the resulting fluorescence is measured. As additional copies of target DNA are created, more PCR products are available for the probe to bind thus increasing the level of fluorescence. This allows for the increase in fluorescent signal to be monitored in real-time during qPCR (Kutyavin 2000). The probe is similar to primers but is not

Figure 2.5. Stylized schematic of the qPCR process showing the location of fluorescently labeled probe internal to the forward and reverse amplification primers



recognized by Taq polymerase and plays no role in amplification. It does however convey a greater level of specificity to the reaction than primers alone (Khan et al. 2018) and qPCR markers are less likely to produce false positive detections than cPCR markers. Because fluorescence is measured in real time, qPCR markers do not need additional end point analysis to determine if a sample is positive for the target DNA. A qPCR marker is also more sensitive than conventional markers since measured fluorescence detected by qPCR instruments can be detected at a lower level than by DNA visualization during agarose gel analysis (Khan et al. 2018). Because of this higher degree of specificity and the less subjective nature of the results interpretation (Figure 2.6), probe-based qPCR is currently the recommended method for eDNA analysis (see also digital PCR).

Figure 2.6. Amplification plot measuring the fluorescence (y axis) accumulation through each PCR cycle (x axis) in a probe-based eDNA assay. Plot features include cycle threshold line (1), amplification of lab controls (2), amplification in field samples (3), and no amplification (4).



Interpretation of qPCR data is based on measuring the fluorescent signal which increases proportionally to the amount of PCR product created. If the fluorescent signal crosses the cycle threshold line prior to a predetermined cycle number (usually

40 – 45 cycles), a sample is considered a detection. eDNA detections typically occur near the completion of PCR cycling (between 35 – 40 cycles) due to the low concentration of target DNA. Positive control samples usually cross the threshold line much earlier than eDNA samples due to a higher starting concentration. Samples that do not cross the threshold line are considered non-detections. By comparing the point where a sample crosses the threshold (referred to as cycle threshold or *Ct* value) to a series of known concentration standards, a relative quantification of DNA copy number can be calculated. This value estimates the number of target DNA molecules in the PCR reaction which are typically reported as copies per liter (i.e. the number of target DNA molecules detected in one liter of sampled water).

qPCR – intercalating dyes

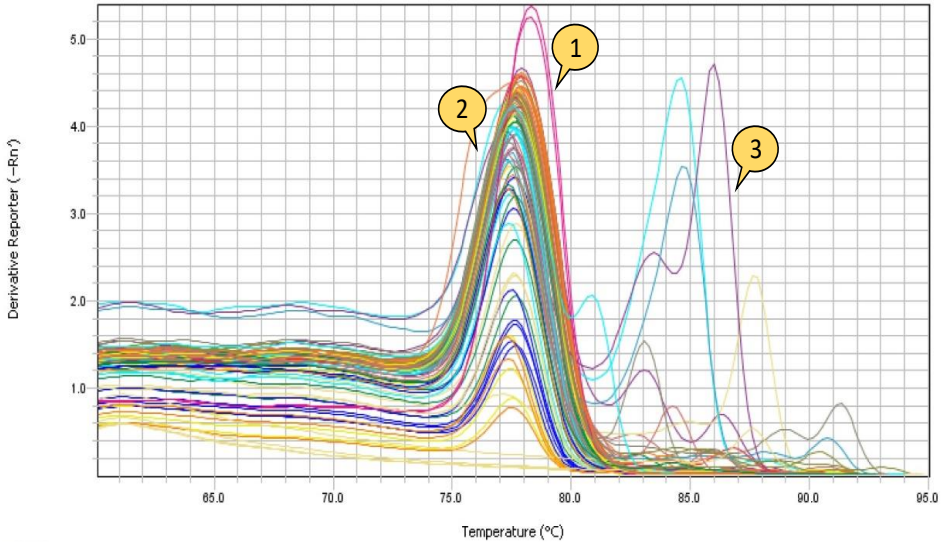
One strategy used in some eDNA studies is to convert a cPCR marker to a qPCR marker using an intercalating dye which binds exclusively to double-stranded DNA (Figure 2.7). Several intercalating fluorescent dyes are used, with SYBR Green and Eva Green being the most common. Incorporation of an intercalating dye increases the sensitivity of a cPCR marker. The fluorescent dye is added to each PCR reaction prior to reaction cycling. As the amplification proceeds, additional copies of PCR product are created, and more dye is bound to the newly forming double-stranded PCR products thus increasing the fluorescent signal. This allows for real-time measurement of fluorescence like probe-based qPCR.

Figure 2.7. Stylized schematic of the qPCR process showing the location of bound SYBR green fluorescent dye. SYBR green binds exclusively to double stranded DNA and is used to measure the accumulation of PCR product during amplification.

Interpretation of results using intercalating dyes is similar to probe-based qPCR in that a detection is based on the fluorescent signal increasing above the cycle threshold line. However, unlike fluorescently labeled probes, intercalating dyes do not convey additional specificity (So et al. 2020). Amplification of any non-specific products will cause an increase in fluorescence and potentially yield false positive detections, complicating the interpretation of eDNA results. Non-specific products may include:

- Primer dimers (PCR artifact formed when single stranded primers bind to each other forming a double stranded molecule)
- DNA amplified from non-target taxa
- PCR amplified products that are longer in length than the intended target
- PCR amplification products within the target species from a different type of DNA (i.e. nuclear DNA as opposed to mitochondrial DNA)

Figure 2.8. Melt curve analysis of SYBR green qPCR products measuring the fluorescence (y axis) and the melt temperature (x axis). Plot features include positive control samples (1), field samples (2) and non-specific products (3).



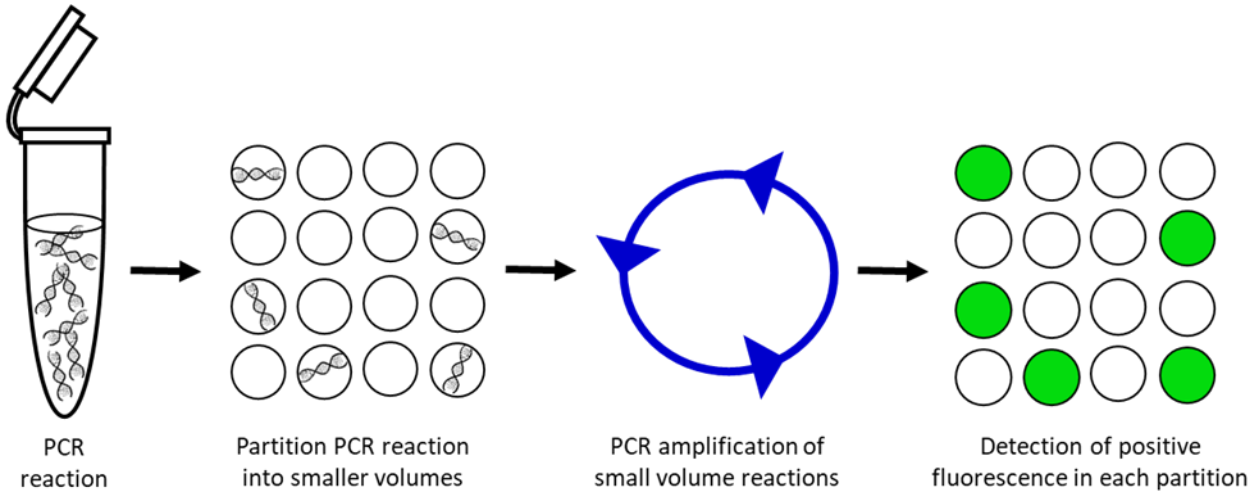
To determine the specificity of the reaction, a secondary post-amplification melt curve analysis is required for intercalating dyes (Figure 2. 8). The melt curve analysis is based on heating the PCR products until dissociation of the double-stranded DNA (referred to as melting) which results in a loss of fluorescence. Dissociation temperatures are largely based on PCR product length and the base pair composition of the DNA sequence. This allows the identity of PCR products to be confirmed by comparing the temperature at which the PCR product obtained from an eDNA sample melts relative to a positive control. Field samples are considered to be a detection if melt temperatures are within ±0.5°C of the positive control. While melt curve analysis works well in some applications, use with eDNA samples tends to be confounded by overlapping and shifting melt curves caused by non-specific PCR product amplification. eDNA samples often result in the production of multiple PCR products due to the presence of multiple amplified non-target DNAs. eDNA samples contain the DNA of many organisms and the slightest amplification of any non-target organisms' DNA has the potential for non-specific false positives when using a DNA intercalating dye. Ruling out this non-specific fluorescence is more difficult than probe-based qPCR and melt-curve analysis may also require additional end point analysis (agarose gel separation) and post-PCR sequencing of positive samples. In addition, intercalating dyes are also more prone to fluorescence quenching, which is a type of PCR inhibition (see Chapter 7) that can lead to false negative results (Sidstedt et al. 2020). As a result, intercalating dye-based methods are not recommended for processing of eDNA field samples outside of a research context.

Digital PCR (dPCR)

Reaction chemistry of digital PCR (dPCR) is the same as probe-based qPCR. The difference is in the reaction conditions, the laboratory instrumentation used, and how the data are collected (Figure 2.9). A typical PCR reaction takes place in a volume of 20 microliters (µl) and is analyzed as a single entity. In dPCR, the 20 µl reaction is partitioned into smaller volumes for thermocycling and analysis. Several different methods exist to achieve this partitioning, but the most commonly used in eDNA detection is digital droplet PCR (ddPCR). ddPCR works by partitioning each 20 µl reaction into 20,000 smaller reactions each containing 1 nanoliter (nl) encapsulated in an oil droplet. The reagents in each oil droplet

are then PCR amplified through the same series of temperature cycles common to all PCR methods. Although based on the same chemistry as qPCR, fluorescent measurements are not measured in real-time and data must be collected after the PCR amplification has completed.

Figure 2.9. Digital PCR workflow. The underlying PCR chemistry is the same as probe-based qPCR but differs in the reaction conditions and data collection.



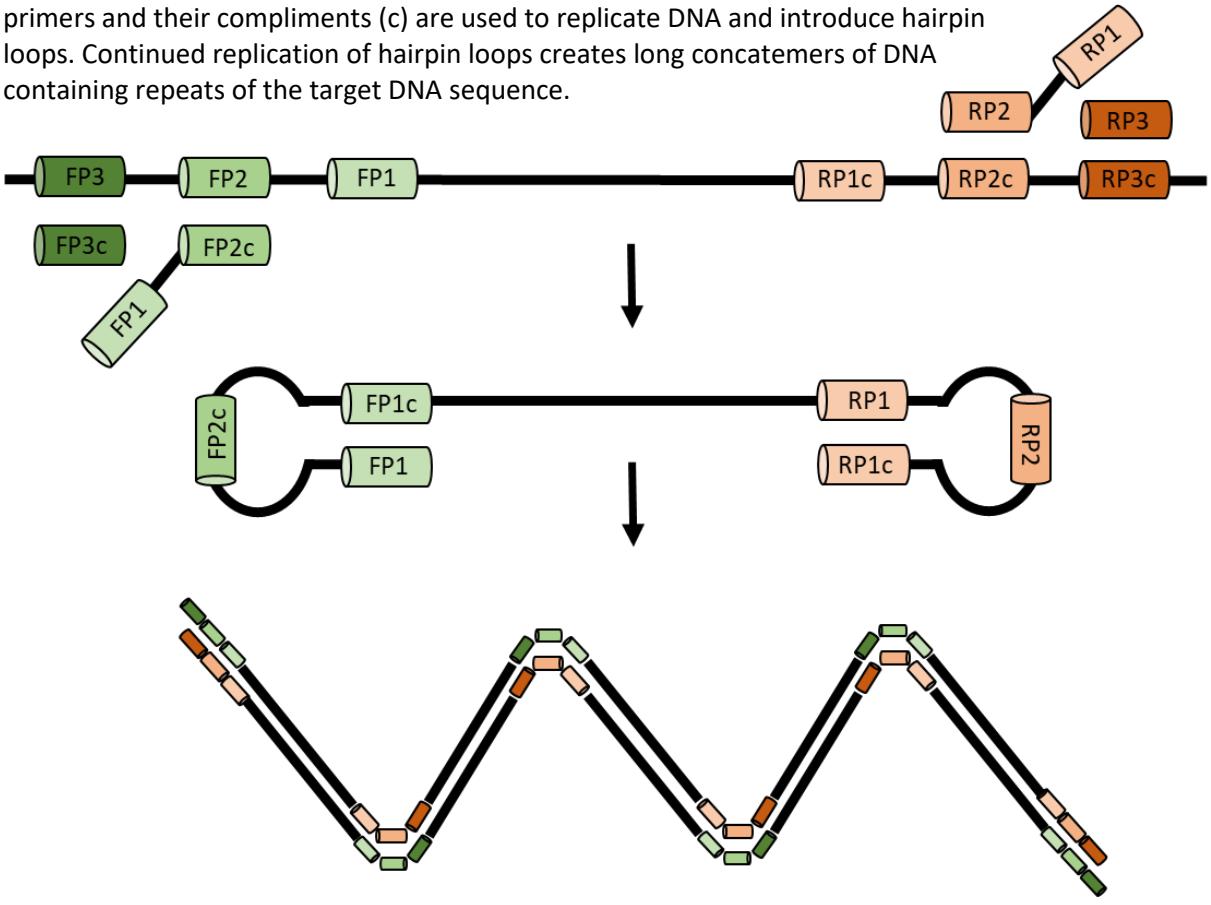
Collection of ddPCR data requires taking a fluorescent reading on each of the 20,000 droplets after completion of the amplification process. Each droplet is passed through a fluorescent detector (that acts much like a flow cytometer) and the proportion of fluorescent droplets (positive PCR reactions) counted. Similar to qPCR, the number of target molecules can be quantified as copies per liter in the original sample. However, ddPCR data does not require comparison to standard curves, instead the quantification of samples is based on Poisson's Law of small numbers (Dube et al. 2008) The use of a Poisson distribution to estimate copy number provides more accurate and reproducible data than is typically obtained with qPCR. Furthermore, ddPCR is considered more sensitive than qPCR due to its tolerance for PCR inhibitors (explained in more detail in Chapter 7). ddPCR is currently used to analyze eDNA samples in a relatively limited number of applications due to the higher cost and limited sample throughput relative to qPCR. Overall, ddPCR and probe-based qPCR are both recommended eDNA analysis approaches. The choice of one over the other is generally determined by funding, sample throughput necessary and if the sampling environment has unusually high levels of PCR inhibitors (see Chapter 7).

Loop-mediated isothermal amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is an eDNA detection method that is not based on the typical PCR temperature cycling process. LAMP uses a complex assortment of primers to direct the replication of DNA using an isothermal enzyme (Figure 2.10). The isothermal enzyme replicates the DNA at a single nominal temperature (ordinarily 60-65 °C) which eliminates the need for an expensive thermocycler making LAMP more amenable for use in the field. Efforts are underway to find faster eDNA detection methods that can produce results in the field and LAMP has several advantages in this respect. Not only is the thermocycler not needed, but results can also be obtained visually without the need for laboratory equipment. Assays produce a color change based on sample pH or form a cloudy

precipitate visible when enough amplification product is replicated within the sample. These features allow LAMP assays to be deployed in the field and offer quick turnaround time from sample to result. LAMP assays can also use a fluorescent reporter and allow for quantifying starting DNA concentrations; however, this application requires field-based fluorescence detection equipment (i.e fluorometer or qPCR platform). Currently, LAMP is seeing limited application in eDNA detection largely due to the difficulty associated with primer design and validation of LAMP assays. As with all the amplification methods discussed, primers are necessary to bind to the target DNA and direct replication. The LAMP process uses a combination of 4-12 primers (2-6 six primer sets) as opposed to the two primers (one paired set) used in most other PCR applications. The six primer sets create hairpin loops (DNA that folds over and binds to itself due to complimentary sequence structure) within the replicating DNA providing additional starting points for DNA replication as the reaction progresses. The replicating DNA forms long concatenated (repeating strands of DNA linked end to end) strands which accumulate faster and at greater quantity than is observed during each cycle of a typical PCR reaction (Figure 2.10).

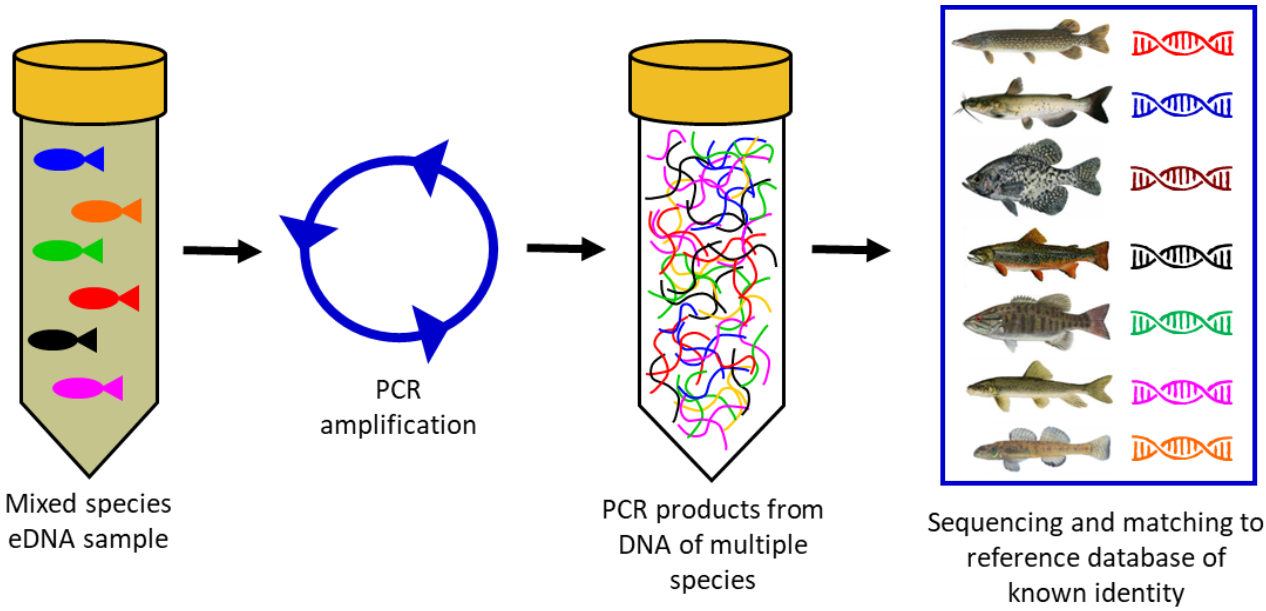
Figure 2.10. Stylized schematic of the loop-mediated isothermal amplification (LAMP) process using six primer sets. A set of three forward (FP1-FP3), three reverse (RP1-3) primers and their compliments (c) are used to replicate DNA and introduce hairpin loops. Continued replication of hairpin loops creates long concatemers of DNA containing repeats of the target DNA sequence.



DNA metabarcoding

Metabarcoding of field samples is based on cPCR amplification (Figure 2.11) and relies on direct DNA sequencing of PCR products for data interpretation. In most instances, generic primers are used to amplify a broad range of taxonomic groups simultaneously. These generic primers can be designed to target taxonomic groups such as fishes, insects, mammals, plants, etc. or target groups as broad as eukaryotes or prokaryotes. This flexibility of primer design allows metabarcoding to have broad application for eDNA biodiversity assessment. Design of generic primers that target broad taxonomic groups are never without some level of primer bias. Primer bias occurs when one species' DNA is preferentially amplified over that of another during PCR. While the problem of primer bias can be minimized through robust primer validation, no one set of generic primers is 100% effective at amplifying every species in the target group. Amplification of eDNA samples with metabarcoding primers result in PCR products that originate from the DNA of a mixture of species. Due to this mixture of products, the analysis of metabarcoding data is substantially different from that of cPCR or qPCR which generally target a single species.

Figure 2.11. DNA metabarcoding workflow. Metabarcoding utilizes generic primers to amplify multispecies assemblages simultaneously through cPCR. High Throughput generation sequencing is used to sequence the mixed PCR products which are in turn compared to a reference database of known species for identification.



Metabarcoding PCR products contain DNA fragments originating from a variety of species that differ slightly in their underlying genetic sequence. Many different molecular techniques have been used to separate and identify different sequence variants in the mixture, but over the last ten years efforts have coalesced around high throughput sequencing. High throughput sequencing allows individual PCR products to be sequenced as opposed to sequencing the consensus sequence of the PCR mixture. Depending on the sequencing platform, tens to hundreds of millions of sequences are produced. Processing millions of sequences and identifying which species they originate from is done using various data processing tools. In general, the process involves comparing each environmentally derived

sequence to a database of known sequences (sequences obtained from morphologically identified adults). This process requires that the reference database be relatively complete for the study area. Species lacking representation or species with significant genetic variation from those present at the study area will not be identified in the environmental sample. There is ongoing effort to build more robust reference databases that include regional and range-wide genetic variation for each species. Reference databases are relatively complete for organisms such as fish and mammals but are lacking for invertebrates and other less studied organisms. Continued development of reference databases and refinement of metabarcoding methods will continue to increase the quality and robustness of the data obtained. It is expected that metabarcoding will become an increasingly important tool for conservation

Table 2.1. Comparison of commonly used eDNA detection techniques and their application.

Method	Advantages	Disadvantages	Application
cPCR	Requires less specialized equipment and is generally less expensive	Difficulty in interpretation of data, risk of false positive detections, not quantitative	Taxon-specific detection, generally not recommended for eDNA due to possibility of false positives
Probe-based qPCR	Highly specific and sensitive, data interpretation relatively straight forward, quantitative, moderate to high sample throughput	Moderately expensive, can be prone to PCR inhibition, relative quantification is possible with proper marker validation	taxon-specific eDNA detection and large sample numbers, multiple species detection possible with further validation
Intercalating Fluorescent Dye	Slightly less expensive than probe-based qPCR, quantitative	Difficulty in interpretation of data, risk of false positive detections, quantitative application challenging due to non-specific PCR products	Taxon-specific detection, generally not recommended for eDNA due to false positive potential
dPCR	Highly specific and sensitive, absolute quantification, less prone to inhibition	Expensive, requires additional laboratory equipment, lower sample throughput than qPCR	taxon-specific eDNA detection when inhibition is problematic in sampling area, small to moderate sample sizes
LAMP	Less expensive, fast results, can be field-based	Difficult to design and validate primers, limited track record with eDNA	Potential application for taxon-specific field detection

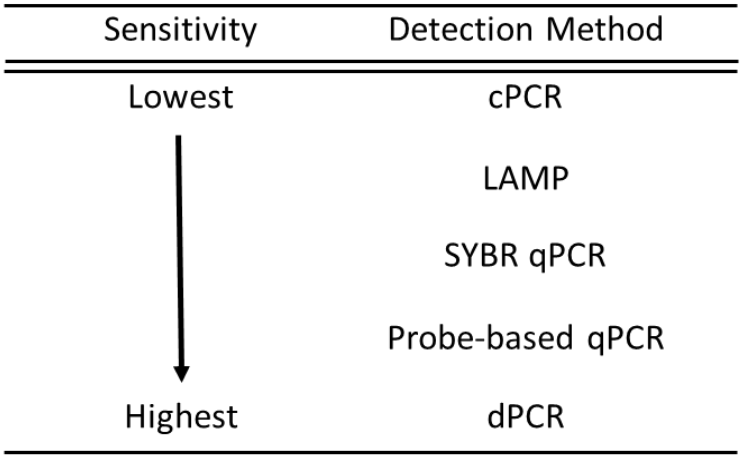
Metabarcoding	Multispecies detection, can achieve moderate sample throughput	Primer bias and limited reference databases can lead to non-detection error	Multispecies and biodiversity assessment of eDNA samples

and resource management in the future.

Each laboratory technique discussed above has advantages and disadvantages when applied to eDNA detection (Table 2.1). Choice of technique often comes down to the management or ecological question to be answered. Only metabarcoding offers multispecies biodiversity analysis. All other methods are primarily single species detection assays. While it is possible to combine multiple taxon-specific qPCR assays into a one PCR reaction (multiplex reaction), this often comes with a loss of sensitivity. Inclusion of additional primers and probes into a single reaction leads to more difficult optimization and higher complexity with assay performance. Most eDNA work is done with a single species assay, though examples of two or more species eDNA detection can be found in the literature(Farrington et al. 2015; Wozney and Wilson 2017; Tsuji et al. 2018; Rodgers et al. 2020).

The taxon-specific eDNA detection methods described above have inherently different levels of sensitivity (Hunter et al. 2017; Khan et al. 2018; So et al. 2020) as outlined in Figure 2.12. Although previous research has identified differences in the ability to detect target DNA, continuing efforts may demonstrate improvements for less sensitive methods such as LAMP. Additionally, although not specifically ranked in Figure 2.12, metabarcoding has been compared to qPCR in some laboratories and shown to be slightly less sensitive, albeit close, to qPCR (Harper et al. 2018) and depends on how the community sequencing data is scored and interpreted. Having a firm understanding of the capabilities of each method is crucial in terms of relative specificity and sensitivity. This also underscores the importance of validating eDNA markers regardless of the detection approach applied. When designing or evaluating an eDNA study, biologists and managers should work with a genetics laboratory experienced with eDNA to select the best detection method for the objective at hand.

Figure 2.12. Relative sensitivity of taxon-specific eDNA detection techniques



eDNA marker validation – probe-based qPCR

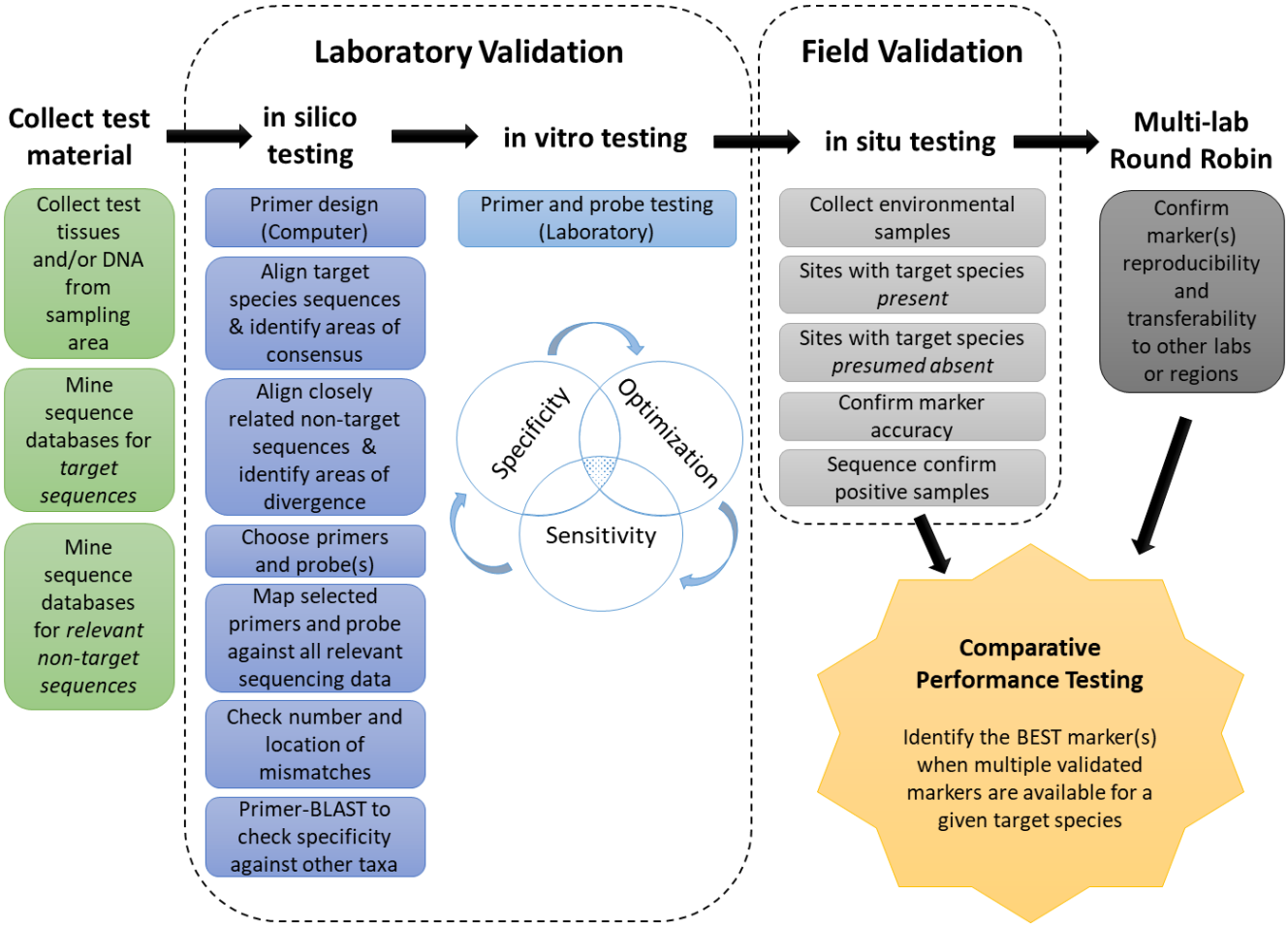
Marker validation is a critical step for successfully implementing eDNA projects (Raymaekers et al. 2009; Goldberg et al. 2016; MacDonald and Sarre 2017; Guan et al. 2019; Langlois et al. 2020; Thalinger et al. 2021). Validation methods are similar for cPCR, qPCR, and dPCR markers since all three techniques can share the same primers. However, the detection platforms used to collect data are considerably different. Since probe-based qPCR is the most commonly used eDNA approach and the following discussion of marker validation focuses on this application. The quality of the primers and probe used to bind to and amplify the target DNA impacts the quality of the data collected from eDNA samples. During eDNA project planning, it is necessary to verify a marker exists, is specific for the intended target, and is sensitive enough to detect eDNA of the target species at low concentrations. The following minimum requirements must be met before processing environmental samples collected in an eDNA project:

- the marker functions with an acceptable level of sensitivity and taxon specificity
- the marker amplifies target DNA in field samples where the presence of the target organism or taxonomic group is known
- the marker fails to amplify target DNA in field samples where the target organism or taxonomic group is presumed absent

A rigorous series of validation tests should be conducted in the laboratory prior to applying any marker to field samples (Hoorfar and Radstrom 2004; Bustin et al. 2009; 2010; Bustin 2010; Bustin et al. 2013; Nolan et al., 2013). Comprehensive guidelines for qPCR marker validation and reporting metrics when publishing can be found in 'Minimum Information for publication of Quantitative real-time PCR Experiments' (MIQE; Bustin et al. 2009, 2010; Bustin 2010). Many eDNA analysis laboratories follow the MIQE guidelines during testing and validation of eDNA markers although to date it has not been universally adopted.

Goldberg et al. (2016) identified many 'critical considerations' for eDNA methods proposing three main areas for probe-based qPCR marker testing: in silico testing, in vitro testing, and in situ testing; each of which have a series of validation steps (Figure 2.13.). In silico testing involves computer-assisted comparisons to identify the best areas for primer and probe placement. In vitro testing evaluates marker specificity and sensitivity. Collectively, in silico and in vitro testing constitute laboratory validation where all steps are carried out in the laboratory. Finally, field validation of qPCR markers is carried out during in situ testing where, for the first time, true eDNA samples are tested. Due to the many steps involved, it is common to find qPCR eDNA markers that have been carried through several, but not all, aspects of the validation process (Thalinger et al. 2021).

Figure 2.13. Example workflow highlighting each area and associated steps of qPCR marker validation



The minimum requirements for marker validation may be influenced by the marker's intended application and additional metrics measured and reported. For instance, if the intention is to gather presence data for the detection of a target species (i.e. early detection of invasive species or narrowing down survey locations for rare native species), validation should at a minimum determine the specificity and the detection limit of the marker to make sure it is functioning at an appropriate level of sensitivity. Alternatively, an eDNA investigation proposing to correlate a target species' abundance or biomass to a DNA strength of signal may require more rigorous quantitative testing to verify the marker is amplifying at a high efficiency across a wide cycling range of PCR. Other eDNA applications, such as large eDNA monitoring programs (U.S. Fish and Wildlife Service 2018), may require rigorous interlaboratory validation (U.S. Fish and Wildlife Service 2018; Guan et al. 2019) to verify the markers used are robust and accurate across a wide geographic range as well as in the hands of multiple laboratories with different personnel (Figure 2.13).

Although there are examples of validation efforts for markers in the primary literature these approaches are not standardized. Here we describe the minimum requirements for eDNA marker validation in an effort to produce reliable and interpretable eDNA data applied in appropriate regional context. The validation framework presented covers two main areas of qPCR marker performance evaluation: 1)

laboratory validation using both DNA standards and tissue-derived DNA samples and 2) field validation using environmental samples. Field validation is just as critical as validation in the laboratory. A marker may perform well under ideal conditions (in the lab) but may fail to detect target DNA at an acceptable level in field samples (Guan et al. 2019). This framework is presented to provide an example of minimum recommendations for qPCR marker validation.

Laboratory marker validation

in silico testing

in silico analysis constitutes computer-simulated testing of marker sequences for taxon specificity using both the target species and non-target species reference sequences. During this analysis, taxon-specific primer and probe sequences are designed and tested for specificity against DNA sequence data. The extent of the sequence data search is completely dependent on the geographic coverage area of the eDNA project. For instance, a project may be carried out in only one small stream and as a result only a limited diversity of species might co-occur with the target species. Alternatively, some projects may cover extremely large geographic areas. The critical difference in recommended validation testing between the two projects is the number of co-occurring species. Once a species assemblage list is developed (this is usually based on traditional survey data), sequence data is mined for both the target species and non-target co-occurring species from public databases (e.g. GenBank®; Benson et al. 2010), BOLD; Ratnasingham and Hebert 2007, MitoFish; Iwasaki et al. 2013; Sato et al. 2018). In some circumstances, it may be necessary to produce additional sequencing data internally if existing public data is lacking. As the list of co-occurring species increases, primer and probe design become progressively more challenging. Other complications with silico sequence comparisons can occur when intraspecific genetic variability is high or when genetically similar non-target species co-occur in the same sampling locations as the target species.

At minimum, in silico testing should include:

- Multiple representatives from the target species to account for potential genetic variation within a species at the target gene region
- Representation from as many closely related, co-occurring, non-target species as possible to ensure species specificity (e.g. marker is designed to detect DNA from darter species A, but darter species A co-occurs with 3 other darter species).
- Comparison against a large dataset of all known genetic sequences. This ensures that the primer and probe sequences are not unknowingly similar to a region of a non-target genome. For example, an assay designed for fish could inadvertently amplify a non-target gene in an amphibian or bacteria. While this is unlikely, the comparison is relatively straight forward and an essential step in the *in silico* testing process.

in vitro testing

There are three main components of *in vitro* testing during qPCR marker validation: (i) specificity testing, (ii) PCR optimization, and (iii) sensitivity testing. Following the *in silico* analysis, marker specificity is

expected, but this should still be confirmed through *in vitro* testing using DNA samples from both positively-identified target and non-target species.

Specificity testing

Specificity testing is used to verify specific amplification of tissue-derived DNA from as many individuals of the target species that can be obtained throughout the study area (minimum requirement) or native range. Tissue-derived DNA from all (or as many as possible) co-occurring non-target species within the study area is also tested and must fail amplification (Table 2.2). Trace levels of cross-contamination between tissue samples or DNA extracts can easily confound *in vitro* specificity testing (Brandl et al. 2015; Goldberg et al. 2016). This type of cross-contamination is common when multiple species are co-mingled in nets or collection buckets, especially when obtaining fin clip or other surface tissue samples. Rinsing samples or collecting internal tissues (i.e. blood, muscle, or other) can help avoid surface cross-contamination. Appropriate positive and negative controls (see Chapter 5) should be included in all tests to verify primers and probe are functioning appropriately under the standard test conditions. PCR products should be sequenced to confirm the target sequence from the target species is being amplified in the qPCR reaction.

Table 2.2. Potential outcomes of marker specificity testing

Outcome		Result
All target species DNA samples amplify efficiently and all non-target species fail amplification		Marker passes and moves on to next validation step
Both target and some non-target DNA amplifies with high efficiency		Result doesn't necessarily eliminate the marker from consideration, but information is critical to inform later interpretation of eDNA data. If species specificity is required for the project, sequence confirmation of any positive field results is required, otherwise marker is eliminated from further use
Target species DNA samples fail amplification but the control DNA shows successful amplification		Marker is eliminated from further consideration

Minimum requirements for *in vitro* testing:

- Marker specificity must be tested against DNA from target and non-target species.
- DNA from multiple individuals of the target species must be tested and the marker must successfully amplify for all of them.
- Multiple individuals of non-target DNA must be tested for as many co-occurring non-target species as possible and the marker must fail to amplify

- Both target and non-target organism DNA should be tested across a range of sampling locations to validate the geographic extent of marker suitability
- Positive and negative PCR controls must be included (see section 3: Field and Lab Controls)

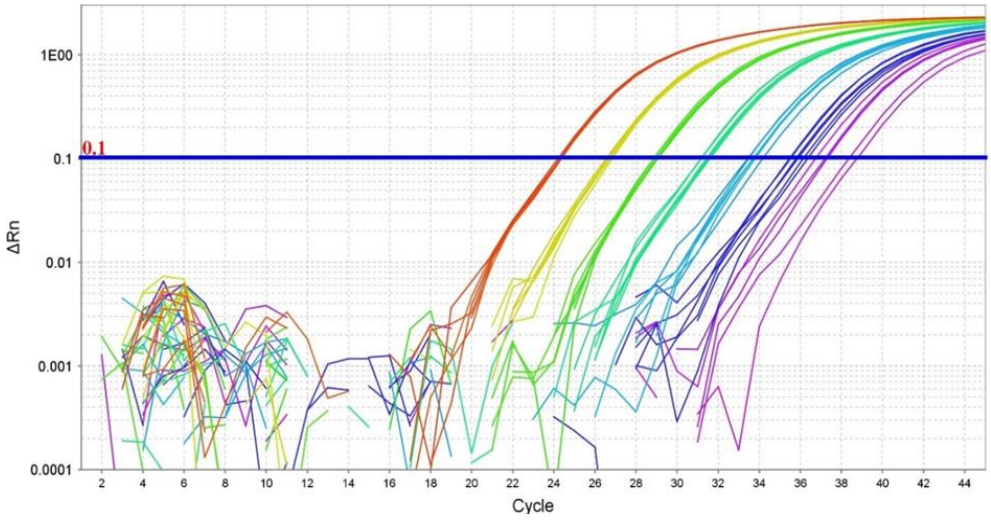
PCR optimization

Any marker that passes specificity testing must then be optimized for maximum PCR efficiency and assay sensitivity. Optimization is the process of determining the reaction chemistry and physical reaction conditions that convey specificity and ensure high levels of sensitivity and overall marker efficiency. At a minimum, optimal primer and probe concentration must be established along with the most appropriate PCR cycling protocol. All qPCR markers (primer and probe combinations) have different nucleotide (base pair) composition. As a result, efficiency can vary greatly among qPCR markers and can differ as primer or probe concentrations change or cycling conditions are altered (Mikeska and Dobrovic 2009; Raymaekers et al. 2009). It is therefore necessary to systematically optimize each marker used to ensure a high level of performance and reliability. The optimization process must also include the qPCR master mix. PCR master mixes are generally commercial products which all vary in their chemical composition. Reporting of optimized reaction conditions and thermocycling protocols must also include information regarding the specific master mixed and its working concentration.

Sensitivity testing

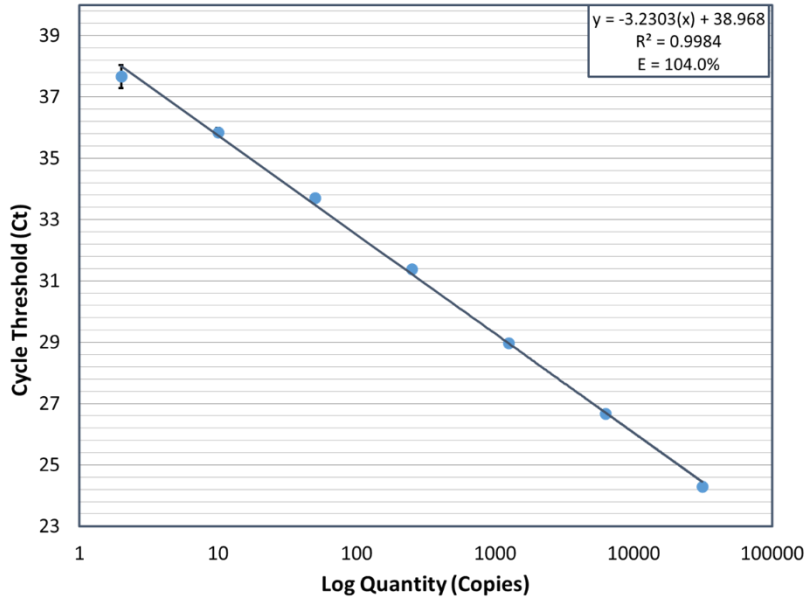
qPCR marker sensitivity testing must be performed after a given marker has passed *in vitro* specificity testing and has been optimized. The goal of sensitivity testing is to infer the relative performance of a qPCR marker and to evaluate the limit of detection (LOD) and limit of quantification (LOQ). This is achieved by analyzing multiple low copy standard replicates against a multiple point standard curve (Furlan et al. 2016; Klymus et al. 2019; Bustin et al. 2009; Johnson, et al. 2013). These standards are carefully constructed using fragments of commercially synthesized DNA that can be precisely enumerated. LOD and LOQ are mathematically determined after amplifying many replicates of a multi-point standard curve that commonly contains a high of

Figure 2.14. Sensitivity tests showing the raw fluorescence data from replicated serial dilutions (5x) of gBlock standard between 31250 copies (red curves) to 2 copies (purple curves).



31,250 copies of the target DNA to a low of just two copes (Figure 2.14). After collection of the raw fluorescence data, standard curve results can be plotted by placing log DNA quantity (in this case DNA copies) on the x-axis and cycle threshold (*Ct*) on the y-axis (Figure 2.15). The equation of this line (y = mx + b) provides most of the sensitivity information needed for a given assay, where y = cycle threshold (*Ct*), m = slope, x = log DNA quantity, and b = y-intercept. By plotting the standard curve data on the log scale, marker efficiency can be calculated (Rutledge and Stewart 2008; Raymaekers et al. 2009; Tellinghuisen and Spiess 2014; Bustin 2017) using the slope (m) in the following equation: $e = 10^{(-1/slope)} - 1$. The linear regression provides an estimate of error through the r^2 value. In qPCR analysis, this standard curve can also be used to extrapolate the number of copies of target DNA detected from a field sample by comparing the resulting *Ct* value to the standard curve. This copy estimate can then be used to determine the number of copies of target DNA per liter in the original environmental sample collected.

Figure 2.13. Raw standard curve data plotted as cycle threshold (Ct) values on the y-axis and log (copies) on the x-axis. PCR metrics associated with the plotted standard curve are denoted in the upper right-side of the figure.



For all the reportable qPCR sensitivity metrics listed above, the following list comprises minimum acceptable thresholds for each of the metrics:

- r^2 (linear regression correlation coefficient): ideally ≥ 0.990, but minimally ≥ 0.950
- m (slope): ideally between -3.20 and -3.50, minimally between -3.10 and -3.60
- e (PCR efficiency): ideally ≥ 90%, minimally ≥ 85%
- b (y-intercept): ideally 40 or less, minimally 45 or less
- LOQ (limit of quantification): ideally ≤ 10 copies, minimally ≤ 100 copies
- LOD (limit of detection): ideally ≤ 5 copies, minimally ≤ 20 copies

Not all qPCR markers will meet every minimum criteria based on sensitivity metrics. This does not mean the marker fails validation, but it is critical in these cases to accurately report the performance criteria above so potential limitations of the assay are known prior to interpretation of eDNA detection data (Furlan et al. 2016; Goldberg et al. 2016, Klymus et al. 2019).

Field validation – in situ testing

in situ testing of field-collected eDNA samples is the next step in the marker validation procedure. At this stage, the qPCR marker(s) is known to be specific to the target DNA (at least in ideal, less complex samples such as tissue-derived and positive control DNA), it is optimized, and the relative sensitivity is known. Therefore, the markers selected for *in situ* testing can be characterized as 'Laboratory Validated'. However, laboratory validated qPCR markers must also be 'Field Validated'. Marker(s) are tested against more complex samples that contain a mixture of DNA (i.e. bacterial, algal, human, aquatic vertebrates, pollen, plant, etc.) and where the DNA can range from high quality (intact/not degraded) to poor quality (fragmented). This is also the validation step where all aspects of the eDNA study (sampling protocols, transport, preservation, extraction, etc.) as well as the validated markers are put to the test.

The basic approach for *in situ* testing is to confirm positive marker amplification in eDNA samples collected in areas where the target species is present. In addition, it is critical to confirm a lack of amplification in eDNA samples collected from areas where the target species is presumed absent. Samples can be collected:

- from mesocosms where the density and distribution of the target species from the point of sampling is known
- in environments where the presence of the target species is either confirmed or presumed absent
- with as many sampling points that can be reasonably accomplished throughout the native range of the target species or target sampling areas where the target species is presumed absent

Collected environmental samples are then tested for amplification with laboratory validated markers. A good first place to start is to take samples where the target species is present or absent from aquariums or mesocosms. In this case, the performance of the lab validated marker(s) can be tested in smaller scale and controlled settings to confirm that the entire process is working as expected without potentially confounding complicated environmental factors. Full-scale field sampling can be time and budget intensive therefore this strategy is desirable for initial field validation of markers.

If positive amplification is found in positive source samples, the sampling location and the cycle threshold value is recorded. Ideally, all samples from positive source locations will amplify while samples from locations where the target species is presumed absent results in no amplification/no detection. If this result is found, the last step in field validation would be to sequence confirm any positive detections to verify the target species was indeed amplified.

Occasionally, a marker may fail to amplify a positive source eDNA sample. If that happens, additional scrutiny should be placed on sampling protocols (volume collected, time and/or location of sampling, type of filter used, etc.) or extraction protocols to maximize DNA capture. For instance, sampling may have occurred during a time when the target species was in low abundance, a lower state of metabolic

activity, or located a further distance from the point of eDNA sample collection. In this case, the most likely problem with detection has to do with the sampling or extraction protocols and not with the qPCR marker.

Minimum requirements for *in situ* testing

- Field samples are collected from locations where the target species/organism is known to be present and marker amplification is successful
- Field samples are collected from locations where the target species is known to be absent and marker amplification fails

Multi-laboratory round robin

Multi-laboratory round robin testing of validated markers is an additional validation step that can be incorporated for large-scale eDNA monitoring programs or where multiple labs may be involved in the production of data from samples of a larger study (Shanks et al. 2011; Guan et al. 2019). The steps in round robin testing typically do not cover the earlier phases of marker validation (i.e. in silico testing, in vitro specificity testing) but instead involve sensitivity testing and in situ testing of replicated environmental samples. The goals of round robin testing include:

- Confirmation of marker performance in replicated standard curves among laboratories
- Production of lab-specific performance metrics including PCR efficiency, LOD, LOQ, slope and y-intercept of standard curves
- Comparative performance evaluation of multiple markers for the same target species from both standard DNA as well as environmental samples
- Evaluation of inter-laboratory variability among biological (field samples) and technical (PCR) replicates

Multi-laboratory round robin testing is not a required step for validating qPCR markers, rather a good practice in cases where eDNA sampling programs cover large geographic areas, in projects involving multiple laboratories, or result in analysis of many thousands of environmental samples.

Marker validation recommendations

- Perform all validation testing in appropriate laboratory areas where separation of pre-PCR and post-PCR applications is carefully considered
- Handle all concentrated test DNA and tissue samples from the target species with care and follow pipetting protocols closely to ensure precision and accuracy
- Use standardized taxon-specific validation methods for qPCR markers
- Validation should include *in silico*, *in vitro*, and *in situ* components
- Primer and probe concentration along with annealing temperature must be optimized
- Cross–species amplification testing must be part of the validation testing and repeated whenever a marker is used in an area where different species compositions are expected.

- Sensitivity (PCR efficiency, LOD, and LOQ determination) and specificity testing must be conducted to understand marker performance, especially at low DNA concentrations since DNA of a target species is often in low abundance in eDNA samples.

Inhibition recommendations

PCR inhibition is common in eDNA samples and results from the copurification of substances that interfere with the PCR reaction. With the use of sound extraction methods and inhibition resistant *Taq* polymerase, most environmental samples can be processed without the negative effects of inhibition. However, to provide a higher confidence that false negative data are not being reported, screening of sample DNA for inhibition is necessary. With regard to marker validation, inhibition could play a role in the processing of the field samples. It is important that validation includes a means to monitor and detect the potential impact of inhibition on assay performance. Detailed methods for PCR inhibition testing are covered in Chapter 7.

Summary

This chapter outlines some of the available eDNA detection techniques along with the advantages, disadvantages, and appropriate application of each. A clear understanding of eDNA detection technology is necessary prior to processing samples. Additionally, marker validation is crucial prior to applying any kind of detection technique to field-collected eDNA samples. This includes a clear understanding of the performance capabilities of each marker so that the resulting eDNA data can be correctly interpreted.

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Chapter 3

Sampling Design for eDNA Studies

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Summary

Prior to field work or sample collection, an eDNA project should begin by developing a sampling design that accounts for the target species life history strategy and ensures statistically meaningful data collection. Sampling design is a method to connect the data collection to a research problem and objective. More specifically, sampling design is a procedure in which sample units (locations/habitats in the case of eDNA) are selected from a population of interest (Thompson 2012). Having a well-thought-out sampling design is important because what, where, and how you measure can have great implications for how data can be interpreted and used. This critical step of connecting the data to the collection ensures that the appropriate science is applied correctly, and that meaningful information is gained. An overlooked or rushed study design may result in an unfortunate scenario such as non-detection of a target that is actually present which has implications for aquatic invasive species or rare species detection. Potential consequences of skipping the sampling design process can include financial waste of both money and personnel time, damage to one's professional reputation and damage to the integrity of eDNA methodology as an emerging tool in natural resource management.

Here we describe and provide examples for 6 action elements that are critical to developing an eDNA sampling design: 1.) Define the Research Question, 2.) State the Statistical Objective, 3.) Describe the Ecological or Conceptual Model of the Target Species and Sampling Environment, 4.) Translate Ecological Model into a Quantitative Model, 5.) Define Sampling Design Strategy, and 6.) Determine Optimal Sample Size

Methods

Define the Research Question

The sampling design should clearly define the question that the proposed research will address. This is often addressed in a paragraph describing the background information, but clarity is brought to the table when the question and decision statement are explicitly stated. Reynolds et al. (2016) note that a robust question statement should define 5 elements (hereafter **RE's**): **RE1**.) the importance of the question, **RE2.**) temporal and geographic scope, **RE3.**) the decision maker, RE**4**.) information that is needed for the decision, and **RE5**.) potential stakeholders that would be interested or impacted by the decision. The latter can escalate quickly in a situation dealing with AIS or rare species detection.

Example: Elodea eDNA Research Question

Elodea spp. is an aquatic submerged plant that is native to much of North America and has recently been introduced to Alaska. **RE1.**) Invasive *Elodea* is an immediate threat in Alaska because dense stands degrade salmon spawning habitat and impede boat and float plane movement on lakes (Carey et al. 2016). **RE's 2.&3.) 5.**)The US Fish and Wildlife Service (USFWS) and many other supporting agencies and groups at local, state and federal levels are working to improve early detection of *Elodea* while eradicating it from Alaska where multiple invasions have been documented in waterbodies from south central AK to interior AK. **RE5.**)Private & small commercial pilots, and those who rely on salmon for commercial, sport and subsistence use in the state represent the main stakeholders in addition to the agencies managing resources for these groups. After qPCR assays were developed in Alaska, preliminary sampling events showed *Elodea* eDNA could be detected in waters sampled at the source of dense *Elodea* outbreaks. **RE4.**)To decide whether sampling lakes for the *Elodea* eDNA is a viable technique for monitoring early detection of this species, it is imperative to quantify the distance Elodea eDNA can be detected from a low-density outbreak in a natural cold-water Alaska lake within one year of introduction of this species. An estimate of the detection limits of *Elodea* eDNA would enable us to design a sampling plan for early detection if results from the pilot study were promising (if the eDNA could be detected at a reasonable distance from the source with high probability).

State the Statistical Objective

A specific, measurable and time-bound statistical objective is required for a sample design to inform and answer the research question. Specifics of the objective such as desired probabilities, confidence intervals, etc. will vary depending on the complexity of the research question and management application. Fine tuning a statistical objective utilizing the expertise of a statistician is highly recommended.

Example: Statistical Objective for Elodea eDNA Study

Our objective is to determine the distance *Elodea* eDNA could be detected from the introduced *Elodea* plants with a minimum of 80% probability of site occupancy (whether or not the eDNA can be collected at a sampling unit) and 95% confidence. The primary purpose of this objective is to evaluate the feasibility of sampling for eDNA as an early detection method for *Elodea* and answer the question "How far away from the source can eDNA be detected"?

Describe the Ecological or Conceptual Model of the Target Species and Sampling Environment

An understanding of the target species ecology and factors influencing eDNA occurrence should be fully explored in a literature search and described in a conceptual model during initial planning of the sampling strategy. A well-rounded study will consider both spatial and temporal aspects of the target species life history, and both physical and chemical components of the environment occupied by the species.

Target Species:

The probability of detecting eDNA is dependent on spatial factors such as home range size or distribution (in the case of plants) and micro-habitat usage. Detection rates are frequently positively correlated with density and proximity to the source organism (Takahara et al. 2012, Dunker et al. 2016). eDNA studies also must account for the temporal aspects of the target species that affect the rate at which eDNA is shed into the environment. For example, eDNA concentrations of salmonids fluctuate at fine time scales when affected by spawning, migration, and life stage (Tillotson et al. 2018, Levi et al. 2019). In order to capture temporal variation, a project may require repeated sampling over weeks, months or whatever timeframe is appropriate to evaluate the extent of variation. Special consideration also should be given for species with periods of dormancy in their life stages (e.g., crayfish, mussels, aquatic plants and many more spp. not listed) since they may not actively shed eDNA at detectable levels during dormant states.

Sampling Environment:

Physical parameters in the sampling environment that may affect eDNA detection include, but are not limited to, elevation, UV exposure, temperature, depth, discharge, turbidity, upwelling or inflows from groundwater, seasonal lake turnover and extreme weather or climate events. Chemical parameters include, but are not limited to pH, dissolved oxygen and tannin load and/or inhibitory compounds that can copurify with the eDNA. Microbial (enzyme) activity within the matrix where eDNA is collected also plays a role in the rate of eDNA degradation (Lance et al. 2017).

Pilot studies can inform future sampling by identifying the most influential variables driving detection rates in a particular system.

> *Example: Conceptual Model for Elodea eDNA Study*
>
> We will evaluate the life history of *Elodea* as a cold water adapted invasive in Alaska and combine this with known seasonal variables in our system to maximize eDNA detections for the study. Previous studies (Schrader et al. 2012, Lance and Guan 2020) have shown that the presence of tannins from foliage can create complications with eDNA detections in the form of PCR inhibition, so we will time our sample collection to minimize the presence of dead foliage from trees surrounding the study pond. We will also account for lake turnover and avoid taking samples during the expected “turnover time”. Accounting for variables proactively at the start of the study allows us to apply a model with reduced variability and therefore reduced statistical noise.

Translate Ecological Model into a Quantitative Model

It is important to hypothesize a quantitative model in the sampling design to estimate sample-size requirements, guide sampling design, and to estimate various parameters. It is possible that your model of the system will be incorrect, and an alternative model and analysis may better fit the data, however, a pre-emptive model will allow you to incorporate known bias and maximize precision.

It is important to identify key attributes (biotic and abiotic factors of the system we plan to measure) and evaluate the influence of those measurements on the response variable. Note the response variable is what your research question is addressing (ex. eDNA presence/absence of *Elodea)* and the biotic and abiotic factors presumably affecting the response variable are also known as explanatory variables. Attributes that are hypothesized to affect the response are important to consider (i.e., factors could influence the presence of eDNA (e.g., water depth, flow rate, Julian date, temperature). These abiotic and biotic factors should become apparent when you draw a conceptual model or outline an ecological model of the system. Abiotic and biotic factors that are best for modeling should have a high-quality measurement and should be hypothesized to affect the response of interest. If the factor is difficult to measure without error (e.g., categorical data), it may not be useful for reducing the environmental variation in the model and predicting the response.

Note it is not necessary to understand this model in every detail, but rather have a general concept of what eDNA models may look like and incorporate input from statistical experts in the study design process.

Example Elodea eDNA quantitative model:

We are interested in estimating probability of site occupancy as a function of distance from known *Elodea* source. We will use a multi-level occupancy model (Nichols et al. 2008, Mordecai et al. 2011, Schmidt et al. 2013), which distinguish 3 separate processes: 1) *Probability of site occupancy*—the occupancy or "state" process is our primary interest, because it is our estimate of whether a sample unit is occupied or unoccupied and we used distance from *Elodea* as a covariate (note that distance is high-quality attribute because it can be measured with low error rate), 2) *Availability Probability*—the water sampling process may cause some water samples at a given site to contain *Elodea* eDNA and others may not contain the eDNA, and 3) *Detection Probability*—the PCR sampling process results in detection of a species or not given that the eDNA was available for detection in the water sample. Although detection and availability probability are not our primary objective, these processes affect the bias of our estimate and should not be ignored.

***Note: the following is technical, and it was applied with the help of an experienced statistician. The point of including it here, is to emphasize that eDNA data can be modeled (with the right study design) to provide useful detection information which is commonly referred to in professional talks and in eDNA literature as "occupancy modeling" and this is an example of that.**

This state-space model estimates presence for our true state i.e., presence of *Elodea* eDNA in a water sample (equation 1 below). We modeled the observation process using equations 2 and 3 of the hierarchy by estimating probability the eDNA is available in the water sample (2) and probability of detection in the PCR sample.

$$z_{i,s} \sim \text{Bernoulli}\ (\psi_i) \qquad 1$$

$$\text{logit}\ (\psi_i) = \alpha + \beta * distance_i \qquad 1a$$

$$a_{ij} \mid z_i \sim \text{Bernoulli}\ (z_i \theta_{ij}) \qquad 2$$

$$y_{ijk} \mid a_{ij} \sim \text{Bernoulli}\ (a_{ij} p_{ijk}) \qquad 3$$

where, z is the true presence of a *Elodea* eDNA at site i (where i = 1, 2,...,25 sites) during season. If z = 1, then *Elodea* is present, whereas *Elodea* is absent when z=0.

ψ_i is the probability of occupancy at site i and,

logit (ψ_i) is the logit linear regression where distance at site i can be used to predict occupancy at a given location.

The availability of *Elodea* eDNA at site i, in water sample j (j = 1, 2, 3) when $a_{ij} = 1$ is a detection of *Elodea*, whereas $a_{ij} = 0$ is a non-detection, given the true presence of *Elodea* eDNA (z_i has availability probability $=z_i*\theta_{ij}$.

When the observation of *Elodea* eDNA is a non-detect (failed to amplify qPCR), $y_{ijk} = 0$ and $y_{ijk} = 1$ is the result of a positive PCR, for PCR sample k (k=1, 2, 3), water sample j, site i. This is conditional on the availability of the species DNA in the water sample (a_{ij}) has detection probability $a_{ij}*p_{ijk}$.

Define Sampling Design Strategy

We outline classical methods of probabilistic sampling as defined in Cochran (1977) and Thompson (2012). Probabilistic sampling allows for design-based inference which assumes the data are fixed (Ver Hoef 2002). With probabilistic sampling, each member of a population has a known probability of being selected in the sample, which allows us to make statistical inference, reduce bias, and provide reproducible results. Convenience sampling (which is sometimes called judgement or haphazard sampling), can provide biased results because the probability that any unit is selected is unknown and, therefore, the properties of the sample are unknowable. This can make it difficult to interpret results relative to the target population. Unfortunately, even well-intentioned field sampling can slip into convenience sampling as a result of either an incomplete or rushed sampling design process.

An exception to using a probabilistic design may occur when using model-based inference e.g., when using geostatistics. Geostatistics usually rely on infinite populations, whereas classical statistics usually use spatially discrete populations (Ver Hoef 2002). For example, spatial auto-covariance models and kriging (geostatistics) have been used to model and predict stream network spatial processes (Ver Hoef and Peterson 2010) and model-based inference is used. For these Spatial Stream Network Models (Ver Hoef and Peterson 2010), the best sampling designs may include clusters at headwaters or confluences depending on the intended inference and model specifics (Som et al 2014). Note that if model-based inference is used, it is imperative to select the model in advance to ensure the sampling design meets the study objectives. ***Note: this is simply included as a brief exception to probabilistic sampling ***

Cochran (1977) and Thompson (2012) provide detail to the most common probabilistic sample-selection approaches. It is helpful to be familiar with these approaches as they will ultimately determine what model (s) are appropriate to use for your study.

Simple Random Sample.—A sampling design strategy where n sample units are selected for measurement from the population (N) in a manner where every unit has an equal probability of selection (Thompson 2012). There are several advantages of a simple random sample: simplicity, it is robust to system changes, requires little knowledge of the population. Some disadvantages are that it is difficult to use when the sample unit is an individual organism, other designs may have lower sampling error if supplementary information is available, the sample may not capture variability in independent variables of interest, and it may be costly and logistically difficult to implement.

Stratified Sampling.—A sampling design strategy where the target population is divided into two or more sub groups (or strata) thought to be more homogenous to decrease the variability within groups. The two primary advantages of stratified sampling is that there is potential for greater precision of estimates if strata are classified correctly, and it provides mean and variance estimates among groups. The two main cautions to consider when using stratified sampling is it is much easier when strata doesn't change (e.g., elevation vs habitat) and it is important to minimize error in stratum definition.

Systematic Sample.—A sampling design strategy where a sample is taken at regularly spaced intervals (i.e., ordered in space or time). An initial location or start time is chosen at random and then samples are taken at regularly spaced intervals. Systematic samples are advantageous because they are easy to apply and usually have low sampling error if applied correctly. This sampling method may be inefficient if measuring something that exhibits a periodic response.

Spatially Balanced Samples.—A spatially balanced survey design generates samples that are spread throughout the sampled population to ensure that spatial coverage is obtained. The two most common spatially balanced designs are Generalized Random Tessellation Stratified (GRTS) sampling (Stevens and Olsen 2004) for two-dimensional space and Balanced Acceptance Sampling (Brown et al. 2015) for more than two dimensions.

Example: Elodea eDNA Sample Selection

Our ***target population*** is originally lakes in interior Alaska, however permits for research on invasive plants in a natural cold-water lake are difficult to obtain and the best we can do is sample the grid cells in a single lake. Our ***sampled population*** is therefore a lake (SACpond) located on a military base in interior Alaska and our inference is for this single lake. The lake is guarded by military personnel and the *Elodea* plants are contained in buckets that allow water to flow through.

We will place a 12.5m x 12.5m grid over the SAC pond map to guide our sampling design (Figure 1) and a list of the midpoint location of all grid cells is used as our ***sampling frame***. Our ***sample unit*** is defined as a grid cell. All grid cells within 50 m of each plant that can be sampled from the shoreline are sampled (shallow strata) and we will randomly select grid cells that do not touch the shoreline (deep strata). Our sample selection is therefore a ***stratified simple random sample***.

The grid cell is used as the sampling unit for several reasons: 1) we want a discrete sample unit to easily model spatial sampling error if it was apparent, 2.) our pilot study (done prior to actual study) indicated it was difficult to get to the exact point in the deep water due to wind blowing our boat and slow satellites, and 3.) a secondary objective of the study is to compare sampling methods, e.g., a transect sampler gradually pulling water from throughout the grid vs two one-liter samples collected at the midpoint of the grid cell.

Fig. 2.1. Locations of *Elodea* buckets (triangles) and planned sample locations (Black diamonds) for sampling *Elodea* eDNA in the Small Arms Complex Pond, Fort Wainwright Alaska, 2019. A 12.5 x12.5m grid is used as our sampling frame.

Determine Optimal Sample Size

It is important to consider sample-size requirements (number of sample units to be sampled) to ensure that you don't have Type II statistical errors (falsely accepting your null hypothesis) and that your parameter estimates have adequate precision for inference. For example, a project for a newly established invasive species may already be vulnerable to Type II error inherently from having a small

sample size to begin with and may require a higher sample size relative to a well-established invasive population. Sample-size requirements can be estimated using several approaches. The most effective methods require pilot data (or estimates of variability from similar datasets) and a hypothesized model of the system. Simulation methods can be used to estimate confidence interval coverage for parameters of interest or by conducting standard power analysis. These methods require some knowledge of statistics and a scripting language e.g., R (R Core Team 2019) and consultation with a biometrician may be required to save time and money, and to ensure sample sizes are adequate. If you do not have access to a biometrician or statistician, and do not have pilot data or an estimate of variance, carefully review literature on similar models and effective sample sizes. There are many resources for estimating sample size and power for common models, e.g., occupancy models (Mackenzie and Royle 2005, Guillera-Arroita et al. 2010, and Guillera-Arroita and Lahoz-Monfort 2012, Lugg et al. 2018).

Example: Elodea eDNA sample size calculation

We will simulate presence-absence data with an average 80% probability of site occupancy (site occupancy was simulated to decrease as a function of distance from *Elodea* source), 60% availability probability and 90% detection probability. The 80% site occupancy is our statistical objective, and we will select these availability and detection probabilities based on the literature and our preliminary results from Chena Lakes, near our study site, which have high detection probability of *Elodea* eDNA (due to the high level of invasion with *Elodea* visible everywhere). We will conduct the parameter estimates from each simulation and store results, then simulations will be repeated 1,000 times. Confidence intervals will be estimated for parameter estimates using the stored results. Our results from this indicate that we will need a minimum of 25 sampled grid cells, 2 water samples collected at each site, and these water samples will then be split into triplicates for the downstream PCR analysis in the lab.

Inhibition

For successful detection of target DNA, decisions must be made during the sampling design phase from field sampling protocols to DNA extraction protocols to PCR amplification protocols. There are many steps in each specific protocol that affect the probability of detecting target DNA, irrespective of whether the target DNA is captured in an eDNA sample. Failure to detect target DNA when the target DNA is present is characterized as a 'false negative'. False negatives may lead to a failure to act (or make a decision) and may provide a false sense of security in situations such as early detection of invasive species or environmental pathogen testing. Although there are several reasons why false negatives may occur during the analysis of eDNA samples, one common cause is PCR inhibition. Taking precautions to reduce the potential for inhibition when designing the study (seasonality, time of sampling) and having a plan in place to deal with PCR inhibition through the sampling design by incorporating replication or alternate sampling plans is essential for a successful eDNA detection project (Jane et al. 2014; Lance and Guan 2019). Decisions on the best strategy to handle testing for the presence of PCR inhibition is carried out by laboratory personnel and should be decided ahead of time, prior to the initiation of the project, so that a robust plan is already in place. See Chapter 7 for a detailed discussion on PCR inhibition.

Recommendations

Having a well-thought-out sampling design is prerequisite to producing data that ultimately address a scientific, conservation or management goal. Such a sample design will help prevent wasted resources and assure that collected data has a path to success by addressing the following 6 steps covered in detail above and listed again here for emphasis 1.) Define the Research Question, 2.) State the Statistical Objective, 3.) Describe the Ecological or Conceptual Model of the Target Species and Sampling Environment, 4.) Translate Ecological Model into a Quantitative Model, 5.) Define Sampling Strategy, and 6.) Determine Optimal Sample Size. In contrast, a poor sample design can result in significant lost time, lost money, poor conservation decisions that could have negative impacts on species or even foster distrust in eDNA as a tool when used haphazardly. This defeats the goal of eDNA practitioners to uphold eDNA and support its growth for continued application in the field of natural resource conservation.

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Chapter 4
Field Techniques for eDNA Studies

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Summary
A common challenge facing those new to eDNA work is the inability to collect samples using traditional genetic field techniques, which are inadequate for eDNA methods. Ideally, researchers will develop or adopt appropriate eDNA protocols prior to spending significant resources collecting samples that do not produce reliable results. The following chapter will cover the major facets of aquatic eDNA field sampling which are described in depth under the following topics: sample collection, abiotic and biotic factors that can impact sampling, metadata to collect with environmental samples, eDNA separation and concentration, volume of water to be sampled, sample preservation, sample storage and transport, sample labeling, equipment categorization and decontamination, and new technology. Following the guidelines presented here will substantially increase the likelihood that field samples collected from aquatic environments will produce meaningful eDNA results.

Introduction

The actions implemented during field sampling should be well researched and planned in advance for the best chance of project success (Goldberg et al. 2016). Methods should be well documented and care should be taken during all steps of the process to ensure sample viability and integrity (Dickie et al. 2018). The level of detail inherent in eDNA projects can be overwhelming, but these details are imperative to moving the eDNA field forward as a conservation tool that is both repeatable and reliable (Cristescu and Herbert 2018, Wilcox et al. 2018). The information presented is designed to provide general recommendations and to emphasize the importance of researching and trialing relevant methods to inform any new eDNA project. The project objectives, target species life history, and environmental factors will ultimately dictate the level of customization required for a given project beyond the general guidelines presented herein.

Sampling Design

Sampling design is handled broadly in Chapter 3. Below we describe general guidelines and considerations that are relevant to the field collection portion of a study. Environmental DNA research is a complex and rapidly evolving field, and the guidance below may need to be modified to accommodate specific environmental or study requirements or evolving best management practice guidance.

It is recommended that all eDNA practitioners consult with and work with a statistician or biometrician when determining the appropriate study design, sample and replicate numbers, and sample volume required to achieve a desired or optimized probability of detection. This determination should also take

into consideration the biology and life history of the target species and the hydrology of the target system, as these will also influence the probability of detection. Preliminary or pilot studies prior to project implementation are essential for determining the suite of spatial and temporal variables for a specific system that will maximize eDNA signal detection. Please see Chapter 3 for a discussion of statistical, biological, and environmental study design considerations as well as the importance of pilot studies.

Sample independence is another significant challenge faced in the field for aquatic study designs. A sample taken from a single lake, pond, or stream may or may not meet criteria for statistical independence. This will be determined by waterbody characteristics and the target species' home range size and/or life history characteristics. For example, independent samples will be difficult to obtain for large predatory fish that utilize most of a lake compared to samples for breeding amphibians that occupy the shallow nearshore. (Eichmiller et al. 2014, Moyer et al. 2014). In river and stream systems, depending on eDNA travel distances and degradation rates, downstream sample sites may not be independent of upstream sites (Wilcox et al. 2016). Practitioners should consider and be aware of the potential limitations of the sampling design and results when attempting to answer spatial objectives.

Geographic Sampling Considerations

Optimal eDNA sampling techniques often vary significantly between geographic regions. For example, the sampling techniques available to researchers in oligotrophic alpine lakes and streams in the American Northwest often vary from those that can be implemented successfully in the turbid, eutrophic waters common to the Southeastern United States (Robson et al. 2016, Coble et al. 2019). Within region differences are also often significant. Optimal eDNA sampling strategies in local systems frequently differ based on environmental factors discussed later in this chapter, and even within a small sampling area, sampling strategies can vary on the reach or microhabitat scale. A sampling technique used in a fast-flowing area, such as a riffle, may be ineffective or prone to contamination in nearby low-flow areas such as pools or side channel habitats (Carim et al. 2016). Logistical considerations also frequently vary between environments and locations, and can significantly influence the choice of sampling technique and study design. For instance, the amount of supplies that can be transported to remote backcountry locations can be significantly less than the number of supplies that can be transported to areas that are accessible via roads or boats. This limits the number of samples that can be collected and the types of sampling equipment that can be transported to backcountry sites (Duda et al. 2020).

We describe a variety of field techniques below which are applicable to a number of environments and geographic areas. When possible, we advise that researchers consult with local practitioners to see what sampling techniques have been successfully implemented within the study region. Pilot studies are also an invaluable method of assessing the applicability of potential field methods within the study area.

Contamination

Contamination is one the biggest challenges present during field sampling for any eDNA study. Negative field controls (hereafter field controls) should be included in all eDNA work in all systems. The purpose of field controls is to identify the source of any putative contamination, so that deficiencies in field procedures can be corrected and to ensure the accuracy of results. Field control surveillance schemes will vary depending on project-specific objectives, budgets, and study design, but the inclusion of field

controls is a standard for the successful publication of any eDNA study (Goldberg et al. 2016). For more information on field controls, please refer to Chapter 5.

Sample Collection

Aquatic eDNA sampling first involves the collection of samples from the studied water body. Optimal collection procedures vary between environments. For example, in clear lotic environments, inline filtration with a peristaltic pump is often possible, while in lentic and turbid or low-flow lotic environments, water is frequently collected using grab bottles or submersible containers such as Nansen, Niskin, or Van Dorn bottles. Research objectives and life history of the target species can also influence optimal collection procedures. When studying pelagic species, collecting samples from the surface or mid-water column may be advisable, whereas benthic-oriented samples may be more appropriate when studying benthic species. The collection protocol selected should maximize the likelihood of target eDNA detection while taking into account the biology and life history of the target organism within the system of interest. Collection protocols are available that outline commonly used sample collection techniques within headwater streams (Carim et al. 2016, Laramie et al. 2015), ponds and stagnant water (Goldberg and Strickler 2017, Laramie et al. 2015), and large rivers and lakes (Bergman et al. 2016, USFWS 2020). If a novel protocol is used, its applicability to research questions and effectiveness within the study environment should be assessed during a pilot study prior to collecting eDNA samples for the actual study.

Lotic Sampling

Water samples from lotic field sites can be collected from the river bank, by wading into a channel, or from motorized or non-motorized boats. When eDNA concentrations are expected to be homogeneous (e.g. in a turbulent, headwater streams) or when a specific microhabitat is being targeted, samples can be collected from a single location at each sample site (Carim et al. 2016, Bylemans et al 2017). When eDNA concentrations are expected to be heterogeneous at a sample site (e.g. in deep, wide, slow moving water), surveyors can collect samples from multiple depths and/or locations perpendicular to the direction of flow (Goldberg et al. 2013, Erickson et al. 2017) or use a mobile pump to sample along a transect (Thomas et al. 2019). The distribution of sample sites within a lotic waterbody are dependent on project objectives and the biology of the target species and should be determined before project commencement.

In any flowing system, the residence time of a particle, including eDNA, at a particular location may vary with site habitat characteristics and local water velocities (Paul and Hall 2002, Harrison et al. 2019). Collection location within lotic systems can therefore influence the probability that target DNA is collected and is an important study design consideration. Suggested eDNA collection locations can vary between protocols and often reflect protocol study objectives. Several protocols, particularly those describing headwater distribution assessments, detection of non-mobile populations, or where cross-site contamination is a concern, suggest collecting DNA from areas of laminar flow such as riffles, runs, or high-gradient pool terminuses (Carim et al. 2016). Other protocols that target low density eDNA concentrations in large rivers suggest targeting low-velocity depositional areas where eDNA may accumulate such as wind-driven scumlines and eddies or off-channel areas including shorelines, marinas, and backwaters (USFWS 2020 Section B1: eDNA Sampling in Lentic vs Lotic Systems). Environmental DNA concentrations are typically higher within depositional areas, and targeting accumulations in these areas can increase the likelihood of target DNA detection; however, due to the

distance that eDNA can travel and its ability to survive for long periods of time under certain conditions (e.g. buried and resuspended eDNA), eDNA detected at depositional areas can be less likely to be specific to that particular site or represent DNA from an organism that was recently present at or near the collection area (Shogren et al. 2017). The lotic collection locations selected for a given study should reflect protocol requirements, sampling priorities (e.g. detection likelihood maximization, contamination avoidance), research objectives, and the statistical and life history considerations discussed in Chapter 3.

Due to seasonal differences in flows within lotic systems, sampling timing relative to hydrograph stage is also an important component of lotic eDNA research. Increases in river flows can dilute eDNA concentrations and result in lower detection probabilities (Song et al. 2017, Curtis et al. 2021). It is also possible for older eDNA that was deposited and preserved within river substrate to be resuspended during a rising hydrograph, which is a concern when surveyors are attempting to collect novel eDNA (Shogren et al. 2017, Harrison et al. 2019). Low flow conditions can also negatively influence eDNA detection probabilities. If flows are sufficiently low within a lotic environment, eDNA may degrade or be deposited near its source, thus reducing the probability of individual sites containing detectable levels of DNA (Jane et al. 2015, Milhau et al. 2021). Optimal sampling strategies relative to local hydrographs are unique to each lotic system and research question, and hydrograph sampling considerations should be factored into the design phase of any lotic research project. Temporal study design and statistical sampling requirements should also be considered when optimizing collection timing.

The potential introduction of contaminant target eDNA into a sample site by researchers is a concern during all eDNA sampling activities (Rees et al. 2014, Goldberg et al. 2016, Cristescu and Herbert 2018). This risk can be mitigated during lotic sampling by implementing appropriate sampling strategies that often take advantage of directional flows within lotic systems. Whenever possible during lotic eDNA sample collection, researchers should begin sampling at the furthest downstream location and proceed in an upstream direction to avoid contamination of downstream areas prior to sample collection. This strategy is increasingly important as the distance between sites decreases (Pont et al. 2018). To avoid the unintentional detection of target DNA from potentially contaminated gear during sample collection, researchers should keep any submerged equipment (e.g., pump tubing, waders, kayaks, etc.) downstream of collection points, and consider collecting samples from areas of laminar flow instead of eddies or stagnant water whenever contamination risk from waders, boats, or other equipment is significant and cannot be mitigated (e.g. when boat disinfection is not possible between sites). When accessing sites from downstream is not possible, when transitioning from an area where target eDNA concentrations are likely high to an area where they are expected to be low (e.g. above and below a barrier), or when sampling strategies include targeting non-laminar eDNA deposition areas (e.g. eddies and stagnant pools), appropriate decontamination steps and risk mitigation strategies should be taken to avoid contamination (see decontamination section below).

Commonly used contamination avoidance strategies during lotic sampling include the following:

- When collecting samples from a motorized boat, the sample should be collected on the upstream side of the boat, so that the boat surface will not contribute contaminants to the sample. Care should be taken to avoid disturbing the sediment with the boat or motor (e.g., propwash).
- When accessing sites on foot, field personnel should avoid entering the water when possible.
- When wading is necessary, the individual collecting the sample should always be upstream of the rest of the team so no sediments are disturbed upstream of the sample collection. Even if disturbance of sediments isn't apparent, the sampler should pause to allow the sediment to settle before collecting a sample. Then, the sample should be collected upstream of their body

and from a location where contaminant DNA from waders or equipment cannot enter the collected sample. Low velocity habitat types and areas of circular flow (e.g. eddies) should not be sampled while wading unless stringent disinfection procedures are used (see below).

Note that these and similar strategies reduce relative contamination risk, but they do not completely eliminate the chance of surveyor induced contamination. All preventive strategies should be coupled with proper decontamination and field blank procedures (Chapter 5) to further minimize and test for contamination.

Lentic Sampling

One common sampling strategy in lentic systems is systematic sampling with grid spacing or logarithmic spacing for each point (Taberlet et al. 2018). If sampling points on a lake or pond are located along the shoreline, care should be taken to avoid stirring up sediments when accessing sampling locations from within the water or from a boat. If samples are to be collected by boat, operators should approach slowly and the sample should be collected before the motor is put in reverse, which may cause sediment upwelling or propwash to impact the sample area. If the lentic system is small, shoreline samples could be taken on foot from the bank in order to prevent stirring up sediment. Biological signal from eDNA in waterbodies has a limited lifespan; however, signal from sediment has been shown to persist much longer (Turner et al. 2015). Sediment can harbor historical signal from prior occupancy of the target species that could confound the results of a study seeking contemporary eDNA signal. It should be noted, that this can also be a factor in lotic systems, and there are some lotic systems, like lagoons, that can act like lentic systems with regard to flow rates and mixing within the water column.

eDNA distribution in lentic environments is frequently shown to be heterogeneous for many fish and amphibian species (Takahara et al. 2012, Bedwell and Goldberg 2020). The patchy nature of eDNA in lentic systems may be more pronounced in smaller ponds or wetlands where uneven organism distribution is coupled with stagnant water flow (Goldberg et al. 2018, Harper et al. 2019a). Environmental conditions such as wind direction may influence where DNA accumulates. Increasing the number of replicates sampled at each sampling point is one way to improve the efficacy in systems where eDNA is not well mixed or diffused in the environment (Schmidt et al. 2013, Ficetola et al. 2015). If detection rates from initial data sampling efforts are lower than expected (often the case in large lakes with low density of target species) (Lawson Hadley et al. 2019, Ruppert et al. 2019) some additional approaches to increase detection probabilities include repeated sampling over time and/or increasing the number of samples obtained. Some ponds and wetlands may be prone to seasonally higher levels of suspended particulates such as algae which may require the use of centrifuging or larger pore size if filtering (Klymus et al. 2017, Harper et al. 2019b).

Commonly Measured Abiotic and Biotic Factors

Both abiotic and biotic factors influence the production and the degradation of eDNA (Barnes et al. 2014, Pilliod et al. 2014). Many eDNA studies incorporate abiotic and biotic considerations into their study design and/or collect information on these factors and seek to relate them to observed eDNA results. The specific abiotic and biotic factors evaluated depend on the project goals, study design, characteristics of the study system(s), and biology of the target organism. Often, the covariates of interest are those that impact eDNA persistence or detection probability and can be efficiently and accurately measured. Common abiotic and biotic factors of interest include:

Water temperature: eDNA degradation rates increase with increasing water temperatures due to associated factors including increased denaturation rates and increased microbial uptake (Okabe and Shimazu 2007, Strickler et al. 2014, Barnes et al. 2014, Kumar et al. 2020). Water temperatures also impact the behavior and eDNA shed rates (metabolism) of target organisms. Even though higher water temperatures may lead to greater eDNA shed rates, the eDNA degrades more quickly. The inverse is true for colder water temperatures.

Turbidity: High levels of turbidity may clog sample filters and make extraction of centrifuged samples difficult. In addition, if the turbidity contains organic material, it can add large quantities of nontarget DNA to the sample and negatively impact detection sensitivity. See the inhibition section below for more information on this topic.

pH: The relationship between pH and eDNA persistence has been described by numerous researchers with variable results (Barnes et al. 2014, Tsuji et al. 2017, Collins et al. 2018). pH may indirectly impact eDNA degradation, but the magnitude of degradation effects are situationally dependent.

Microbial activity: Direct microbial consumption is consistently identified as a major DNA removal mechanism in aquatic systems (Nielsen et al. 2007, Strickler et al. 2014, Lance et al. 2017). Other abiotic factors (water temperature, pH, UV light) may up or down regulate microbial activity which can change eDNA persistence as these abiotic factors change.

Discharge and Velocity: Increases in discharge (volume) may dilute eDNA in aquatic environments decreasing the probability of detection (Shogren et al. 2017). Additionally, increased water velocity may increase the rates at which eDNA moves through a system and out of targeted sampling areas.

Solar Radiation: Ultraviolet (UV) light, and in particular UV-B spectrum light degrades eDNA by damaging its molecular (base pair) structure (Strickler et al. 2015). Aquatic environments are exposed to varying levels of UV light depending on factors such as season, aspect, climate, and overhead vegetative cover (Sridhar et al. 2004). Similarly, UV light penetration into aquatic environments varies, depending on factors including turbidity and the presence of aquatic plants and algae.

Other Factors: Other potentially important covariates that can be documented when meaningful and applicable to a study include dissolved oxygen, tannin load, waterbody depth, upwelling, lake turnover, channel complexity, habitat classifications, and extreme weather or climate events (Fremier et al. 2019, Harper et al. 2019, Harrison et al 2019, Littlefair et al. 2020).

Inhibition

During laboratory extraction of eDNA samples, various chemical compounds can inhibit the PCR amplification process (See Chapter 2 for information on PCR amplification). This process can delay PCR amplification, reduce target DNA detection probabilities, and cause PCR reaction failures (Acharya et al. 2017). Potential sources of inhibitors include organic compounds derived from the breakdown of organic matter such as leaf litter (Opel et al. 2010, Lance and Guan 2019), algae (Schrader et al. 2012, Stoeckle et al. 2017), and exogenous DNA from non-target species (Kainz 2000; Tamariz et al. 2006, Lance and Guan 2019). The presence, type, and concentration of PCR inhibitors in eDNA samples will

likely vary from site to site or even season to season (Gibson et al. 2012). It is important to be aware of potential local inhibitors when sampling, and when possible conduct sampling in a way that will limit their impact. Potential inhibitor avoidance strategies include adjusting seasonal sampling times, avoiding sites with high inhibitor concentrations, and selecting appropriate filtration or centrifugation techniques as described below. Optimal inhibition mitigation strategies vary between environments and sampling periods and should be assessed during pilot research. See Chapter 7 for a detailed discussion of PCR inhibition and a list of common inhibitors.

Metadata

Metadata are an essential component of field sampling and should be collected for every eDNA sample. Metadata often include covariate measurements such as water temperature or turbidity that are important for interpreting results. Other metadata are important for record keeping such as the sample location coordinates and collection personnel. Finally metadata are useful for quality assurance and quality control programs, and can be indispensable for explaining unexpected results. Some metadata are universal and should be collected for every study. Other metadata are situational and relate to specific aspects of the study design, field sampling environment, or collection equipment and will not be consistent among projects. Examples of universal and situational metadata are listed in Table 4.1.

Table 4.1.

Universal Metadata	Situational Metadata
Unique Sample ID	Turbidity
Geographical Location or Coordinates	Wind Direction
Sample Date	Water Depth
Water Temperature	Water Velocity
Collection Personnel	Sample Collection Depth
Sample Volume	Microhabitat Type
Sample Collection Start/End Times	Lake Turnover State
	Weather Conditions
	pH
	Salinity
	Dissolved Oxygen
	Solar Radiation
	Filter Brand and Pore Size
	Centrifugation RPM or Time Gravity

eDNA Separation and Concentration

Environmental DNA must be separated from water samples (often referred to as concentrating the DNA within the sample) prior to analysis. Regardless of whether collecting from a lentic or lotic system, the general methods for concentrating aquatic eDNA samples is the same across commonly employed options (e.g. filtration, centrifugation, and precipitation).

The most commonly used method to separate eDNA from a water sample is filtration through glass fiber, cellulose nitrate, or mixed cellulose ester filters (Manjaneva et al. 2018, Muha et al. 2019). In non-turbid lotic systems, in-situ filtration can be performed during collection by attaching a filter assembly to a peristatlic pump or other collection mechanism (e.g. Huver et al. 2015, Laramie et al. 2015, Carim et al. 2016, Thomas et al. 2018, Thomas et al. 2019a). Passive filtration has also recently been employed as an eDNA collection method in systems with adequate current (e.g. Sepulveda et al. 2019, Schabacker et al. 2020, Bessey et al. 2021). When water samples are collected using containers, vacuum filtration can be performed at a nearby location; or, when on-site filtration is not an option, samples can be transported to a laboratory for processing (e.g. Laramie et al. 2015, Spens et al. 2017, Goldberg and Strickler 2017). See the section about sample transport for further details.

Pore sizes used during filtration vary and can affect attainable filtration volumes and eDNA detection probabilities. Commonly used filter pore sizes during eDNA studies include 0.2-5.0 µm, although smaller and larger pore sizes have been reported (Turner et la. 2014, Evans and Lamberti 2018). Filters with small pore sizes generally increase eDNA retention rates, which can result in higher detection probabilities; although, overly small pore sizes can also lead to increases in inhibitor retention and reductions in sample volume due to filter clogging, resulting in lower detection probabilities (Turner et al. 2014, Kumar et al. 2020). Most previous filter pore size guidance suggested using the smallest pore size possible that did not result in excessive filter clogging or inhibitor build up within the study area (Kumar et al. 2020); however, there is emerging evidence that the use of larger filter sizes (>1.0 µm) can increase eDNA detection probabilities in certain situations. Jo et al. (2020) demonstrated that filters with larger pore sizes can retain higher relative concentrations of large DNA fractions (e.g. intracellular DNA and large extracellular DNA fragments) while reducing the clogging effects caused by small particulates including small fragments of non-target ambient DNA, thus facilitating larger sample volumes and potentially increasing detection probabilities. Specific filter pore size guidance is currently not available within the literature for most systems and target DNA (Kumar et al. 2020). Whenever possible, researchers developing new studies should consult local practitioners and available literature to determine if filter size guidance applicable to their research is available. When guidance is not available, researchers should include a range of filter sizes in a pilot study to assess optimal filter size for their project.

One alternative to concentrating eDNA via filtration is centrifugation. Centrifugation of water samples consolidates particulate matter including eDNA at the bottom of the collection container. A version of this technique was widely adopted by the USFWS Asian Carp monitoring program and is described in USFWS (2020) Section B2: Sample Collection Procedure. In contrast to filtration, where eDNA particle capture rates vary between filter pore sizes resulting in imperfect detection rates, centrifugation allows for the capture of all particulate eDNA present in the sample. The technique can also be rapidly deployed in turbid systems, whereas, filter clogging during filtration can result in decreased sampling efficiency when turbidity levels are high. A downside of centrifugation is that sample volumes are limited by the amount of water that can be transported to the centrifuge and processed within 24 hours of collection. When large water volumes are required to detect eDNA at low densities, filtration can allow for the collection of greater sample volumes when turbidity levels are not a limiting factor. Centrifuges also generate heat during operation, so a refrigerated centrifuge model is recommended when centrifuging eDNA samples. Mobile centrifuge units are available that can be used at field sites, in mobile trailers, or in laboratory settings; although, weight and power restrictions can restrict the applications of mobile centrifuges in some settings.

Another alternative to eDNA filtration is precipitation. Precipitation involves the addition of a precipitate agent, typically ethanol-sodium acetate, directly to water samples to separate eDNA from the water sample into a solid precipitate mass (e.g. Ficetola et al. 2008). Precipitation allows for the collection of particulate DNA as well as dissolved DNA within a sample (Tsuji et al. 2019). Due to volumetric restrictions during lab processing, this technique is typically limited to low sample volumes (≤15 mL) compared to centrifugation or filtration; therefore, precipitation methods are not recommended when expected eDNA concentrations are low and required sample sizes and volumes are large (Wilcox et al. 2018).

Flocculation is a technique similar to precipitation. In flocculation, an additive, such as lanthanum chloride, is combined with a water sample to separate DNA from the remainder of the sample into flakes called floccules (Braid et al. 2003, Kenward et al. 2018). Smaller amounts of additive are required when performing flocculation compared to precipitation, but the need to transport and store large flocculated samples to a laboratory prior to flocculate separation can limit the technique's applicability in some field settings (Schill 2020).

Sample Volume

The volume of water collected and/or filtered during aquatic eDNA sampling should optimize a project's detection probability and sampling efficiency. Optimal sample volume is therefore a product of each project's environmental conditions, logistical constraints, and processing methods.

Increased sample volumes typically increase eDNA detection probabilities, generally making larger volumes more desirable (Turner et al. 2014, Hunter et al. 2019, Song et al. 2019); however, environmental factors and field and laboratory processing constraints, such as filter clogging and limited centrifuge capacity, frequently limit sample volume. Sample volumes are also influenced by the eDNA concentration method used. For instance, methods that isolate higher relative proportions of eDNA from a sample (e.g. centrifugation) can improve analysis sensitivity and allow for smaller sample volumes (Furlan et al. 2015). Commonly reported total sample volumes range from 250-5,000 mL for filtered samples, 50-250 mL for centrifuged samples, and 15mL for precipitated samples (Goldberg et al. 2016, Evans and Lamberti 2018). Note that these total volumes may include several pooled samples or pooled replicate filters or vials. Sample volumes >1,000L have recently been reported by studies utilizing large filter pore-sizes and tow or passive filter apparatuses; although, the per-liter eDNA capture efficiencies of these devices are significantly lower than traditional techniques (Sepulveda et al. 2019, Schabacker et al. 2020). Whichever collection method is utilized, and accurate mechanism for measuring sample volume, such as a premeasured bucket or flowmeter, should be employed when recording sample volumes.

While increased sample volumes can lead to increases in the amount of captured eDNA and are generally more desirable across methodologies, increased sample volumes can also lead to increased inhibitor concentrations within the final sample (Hata et al. 2011, Sepulveda et al. 2019). When ambient inhibitor concentrations are a concern, sample volume and eDNA concentration techniques should be adjusted so that retention of inhibitors is minimized while maximizing target eDNA detection probabilities. Collection and concentration methods including multi-staged filtration, the use of larger filter pore sizes, and centrifugation can help reduce inhibitor retention rates while facilitating larger sample volumes and high relative eDNA detection rates (Hunter et al. 2019, Sepulveda et al. 2019, USFWS 2020).

Before selecting a sample volume for a new study, researchers should consult local practitioners and available protocols and assess expected environmental conditions. A pilot study can examine optimal sample volume relative to study objectives and regional environmental conditions including local inhibitor concentration levels.

Sample Preservation

Whenever possible, preservation steps should start at the moment of collection. At the time of collection, an eDNA sample is already progressing along a continuum of degradation in a limited lifespan (Strickler et al. 2015, Lance et al. 2017). The purpose of preservation is to stabilize the eDNA in a sample by countering the main forces of degradation that threaten eDNA integrity; namely nuclease enzyme activity and microbial activity (Barnes et al. 2014). Short-term preservation efforts starting at the moment of collection include keeping samples cool and out of direct light (UV) regardless of their final long-term preservation method. Whenever possible, a long-term storage method should be applied to samples as close to the time of collection as possible. Long-term preservation methods include the addition of chemical buffers including Longmire's buffer, benzalkonium chloride, and ethanol (Renshaw et al. 2015, Williams et al. 2016); desiccation with silica following filtration (Carim et al. 2016); and freezing filtered or chemically buffered samples at -80˚C (Jerde et al. 2011). However, several studies show that ethanol addition has mixed results when filtration is used to isolate eDNA from a water sample, which could limit the recovery of eDNA especially when the target DNA may be in low abundance (Hundermark et al. 2018, Majaneva et al. 2018). Exposing samples in long-term storage to unnecessary freeze-thaw cycles should also be avoided to prevent potential freeze-thaw related degradation (Takahara et al. 2015).

Long term preservation immediately following collection is not always possible, particularly for large water samples which are commonly transported to a laboratory for filtration or centrifugation. A cold cooler (water ice may be used if samples are in sealed containers) is sufficient for short-term storage (less than 24 hours), but long-term preservation methods must be used in addition to cooler storage when samples will be stored longer than 24 hours prior to analysis (USFWS 2020). Please note that freezing an entire water sample and thawing it out at a later date to filter/centrifuge etc. is NOT recommended. This process can lead to fractured cells and fragmented eDNA within the sample, which can compromise DNA fragments prior to analysis and lead to decreased filter eDNA retention (Kumar et al. 2020).

Following long term preservation and prior to analysis, samples should be stored in a clean, dedicated space where contamination from outside sources is not a concern (Goldberg et al. 2016). Shared, unregulated spaces, such as communal freezers, and other areas where contaminant DNA may be present should be avoided. If samples will be stored for long periods of time prior to analysis (e.g. multiple months or years), the chosen preservation method and storage infrastructure should be reviewed to ensure that samples will remain viable and safe until analysis occurs.

Research and pilot studies should be conducted to inform which combination of short-term and long-term preservation methods are best suited to the needs of each individual study. Remote and/or extended-period sampling may require extra planning for keeping samples cool during warm seasons. This can be accomplished using highly efficient cooler brands along with ice, disinfected freezer packs, or dry ice. Conversely, if collecting eDNA during extreme cold weather, it is advised to prevent water samples from freezing prior to filtering.

Sample Transport

Following sample collection and initial preservation, eDNA samples must be transported to a laboratory for storage, analysis, and/or eDNA concentration. During sample transport, precautions must be taken to avoid sample loss, DNA degradation, or sample contamination. Samples must always be transported in uncontaminated, secure, sealed containers that cannot spill or have contaminants inadvertently introduced. When transporting desiccated filters, precautions should be taken to avoid abrasion which can remove collected eDNA from the filters (Carim et al. 2016). If samples are frozen prior to transportation, they should be transported using a sufficient amount of dry ice or other methods that minimize freeze-thaw cycle exposure. If frozen samples are shipped through commercial carriers in the United States, there are no weight restrictions on domestic ground based dry ice shipments; however, most companies restrict the amount of dry ice in air shipments to 5.5 pounds or less. Once frozen samples arrive at their destination, they should be processed or transferred to a freezer for long-term storage as soon as possible.

Certain circumstances surrounding eDNA sample collection, such as the inclusion of federal or state listed species or legally contentious factors, may warrant the use of Chain of Custody (COC) forms to document how and by whom samples were handled throughout the collection and processing steps. Documentation typically includes each transfer of possession from sample collection to final disposition at the laboratory. For more information regarding COC utilization, see US Environmental Protection Agency (EPA) 2019 Appendix A Section 2.4.

Sample Labeling

Labeling systems used to identify samples and record metadata need to be clear and meaningful in both the field and laboratory. Samples should be labelled in a way that makes them uniquely identifiable and should incorporate a numerical reference that pairs a particular sample with the associated metadata. The following are recommendations for labeling systems:

1) Use waterproof labels (waterproof paper or stickers, water/ethanol proof markers on plastic bags, etc.) that are durable in wet field and storage (freezers) environments.

2) When writing on labels, use waterproof pens and/or pencils to ensure labels remain legible over the long term. Plan on using labeling media that are not easily obscured by water or preservatives (e.g., do not use pen to label samples preserved in ethanol). Writing on wet plastic, even with waterproof pens can result in smudged or distorted labels. Inclusion of alternate or duplicate labeling is recommended.

3) Test self-adhesive labels in advance to ensure they stay affixed in freezing (storage) or thawing (extraction) conditions. Adhesive labels may fall off and become separated from their associated samples when exposed to rapid temperature changes. There are options for chemical and temperature resistant adhesive labels available at most scientific supply retailers.

4) Pre-printed labels are helpful for standardizing data collection. They also help reduce contamination risks by reducing sample interactions and save valuable staff time, especially in larger studies or when large numbers of samples are collected in a single day.

Equipment Categorization and Decontamination

Decontamination approaches will vary from project to project depending on both the collection method (filter or bottle) and how sampling sites are accessed (boats, wading, etc.). The main goal of decontamination procedures is to prevent introducing target (and non-target) DNA accidentally into the eDNA sample. Non-target species are mentioned here because many eDNA programs archive unused sample material for future analysis and future species of interest may change. Therefore, decontamination procedures should be viewed as a way to prevent contamination by any exogenous DNA.

In discussing decontamination of eDNA materials/equipment/supplies, it is helpful to classify them into three tiers based on their proximity to the eDNA sample:

1) Level 1: Primary Equipment: Equipment that comes in direct contact with the eDNA sample (e.g. sample collection bottles, filters, filter funnels, filter cups, forceps, filter storage tubes, preservation additive, etc.).

2) Level 2: Secondary Equipment: Equipment that is used in every sample collection (coolers, bottle racks, pumps, pump tubing, "grabber arms", etc.) but that does not contact the sample directly.

3) Level 3: Tertiary Equipment: Equipment that is used to access the collection site or transport equipment to and from the collection site, but is not a part of the collection system (boats, backpacks, drybags, PFDs, waders and boots, etc.).

Contamination in any one of these equipment tiers can pose a sample contamination risk. However, in field data collection settings, it is logistically impossible and cost-prohibitive to work with 100% decontaminated gear 100% of the time. By classifying gear into tiers, we assume that gear in closest proximity to the eDNA sample poses the greatest contamination risk, and prioritize decontamination accordingly. Commonly used decontamination approaches are detailed below.

Single Use Supplies

Single use supplies are products used only once for collecting a single sample. The primary advantage of single use supplies is that they are free from target DNA at the time of purchase, and, if handled properly, remain so until used in sample collection. Proper handling measures include, keeping single use supplies in their original packaging until use when manufacturer contamination is not a concern. If manufacturer contamination is a concern and/or repacking is required, product handling and repackaging should be completed in a clean room, using clean gloves, and clean replacement packaging. Single use supplies are commonly used to limit contamination risk (Carim et al. 2016, Goldberg et al. 2016). A major downside of single use supplies is that they generate large amounts of waste; however there are recycling options in many areas for single use supplies. When possible, we recommend using single use supplies for Primary and Secondary sampling equipment. Examples of single use supplies include:

- Filters (glass fiber, cellulose, etc.)

- Nitrile/Latex gloves used to handle sampling equipment
- Plastic (Ziploc-type) bags, and packaging for organizing/transporting filter kits
- Distilled/deionized/target DNA-free water used to prepare field blanks
- Sterile vials/tubes used to store ethanol-preserved filters: for details on storage tube specifications, refer to USFWS (2020), section B1: Equipment Preparation

Reusable Supplies

Much of the equipment used to collect eDNA is either shared between multiple projects or used to collect multiple samples (ie: filter cups and nalgene bottles). These will need to be decontaminated prior to use on an eDNA project (all equipment), between sampling sessions (Tertiary Equipment, Secondary Equipment), or between individual sample collections (Primary Equipment). ***A major point of emphasis is that in order for decontamination to work, the previously-used gear must first be cleaned, and then decontaminated*.** The cleaning step is essential, as many decontamination agents will only work properly if they are applied to clean surfaces. The cleaning phase should involve removing any residual debris or material from the equipment surface: tissue residue, biofilms, vegetation, mud, dirt, or sediment. In addition to managing for DNA contamination, thorough cleaning will also help prevent the spread of aquatic invasive species (AIS).

Decontamination Protocols

Depending on the equipment (sample collection bottle vs motorboat), the extent of the cleaning process will vary widely. Pressure washers (especially when equipped with a heating element), brushes, gloves, and even clean water rinsing can all be used to effectively clean equipment prior to decontamination (USFWS 2020 Section B1: Equipment Preparation and Section B2: Field Equipment Needed).

Bleach: Commercially available concentrated bleach (6-9% sodium hypochlorite) is the most commonly-used DNA decontamination agent. For the purposes of this document, we refer to a starting (undiluted) bleach solution that is at least 8.25 % sodium hypochlorite. Many different protocols exist for the preferred dilution (10 – 50%), contact time (5 seconds – 20 minutes), and application method (spray, soak, wipe, swab) for successful bleach-based decontamination. Twenty percent bleach is a widely adopted standard (USFWS 2020); however some experts (Wilcox et al. 2016, Goldberg et al. 2016, Carim et al. 2016) recommend 50% bleach be used for all Primary Equipment to minimize the risk of low-level contamination. Tradeoffs exists for different bleach concentrations: stronger solutions are more effective, but also more damaging to gear, and require more rinsing to remove residue and the use of additional Personal Protective Equipment (PPE).

Additional important considerations for bleach:

- **Primary Equipment requires a thorough rinse with deionized/distilled/target DNA-free water following decontamination with bleach. The higher the concentration of bleach used, the more rinsing is required to remove any residue. If residual bleach contacts the sample, it can degrade new sample DNA. If resources are available, Secondary and Tertiary Equipment can also be rinsed.**
- Working with bleach requires proper PPE such as safety glasses, gloves, aprons, and potentially respirators depending on the concentration and application method (sprayers). Comply with all Material Safety Data Sheet (MSDS) instructions when working with this product.
- Many commercially available “disinfectant wipes” do not actually contain bleach, but use other cleaning agents instead. If purchasing and using wipes, make sure they contain the required concentration of bleach.

- Bleach is not a feasible decontamination option for all equipment. “Soft goods” such as Personal Floatation Devices (PFDs), waders, backpacks or equipment with metals that may oxidize/corrode (some Van Dorn samplers) should be decontaminated with alternative methods. Use of bleach on these items will drastically shorten their functional lifespan.
- Bleach solutions will degrade over time and with exposure to organic material, and will need to be made daily to ensure proper function.
- The bleach dilution guidelines discussed above assume a starting bleach solution that is at least 8.25 % sodium hypochlorite. If a lower-concentration (household) bleach product is used, the proportion of bleach should be increased accordingly. If a household bleach product is used, the selected product should be free of non-bleach additives. If large amounts of bleach are used during disinfection, local bleach disposal guidelines should be followed after disinfection, and neutralization agents such as sodium thiosulphate can be used as necessary.

Virkon is a trademarked, multipurpose disinfectant made by DuPont that is commonly used in aquatic invasive species (AIS) control protocols. While Virkon is widely-accepted for use decontaminating organisms (AIS application) its efficacy decontaminating genetic material (eDNA application) is not well documented. As such, using Virkon for AIS decontamination should not be confused with using Virkon for DNA decontamination. Peer-reviewed information on Virkon and DNA decontamination is limited: two studies have found that Virkon solution treatments are partially but not fully effective in destroying DNA (Bailey et al. 2014, Ballantyne et al. 2015) and more investigation is needed. An advantage of Virkon is that it is less corrosive than bleach, and can therefore be used to partially-decontaminate soft fabric goods such as PFDs and backpacks. When combined with heated pressure washing (Bailey et al. 2014), Virkon solution (1-2% with 20 minute contact time) may be an option for decontaminating soft-goods and metal Tertiary Equipment that might be damaged by bleach (Motorboats, Canoes, Waders, boots, etc.). However, Virkon should not be relied upon as the sole decontamination agent with Primary Equipment-- bleach and/or UV light should be used in those applications.

Additional important considerations for Virkon:

- Virkon solutions break down over time in the presence of mud/dirt. Virkon solutions are effective for up to one week and should be remade if needed for longer periods.
- Handling Virkon powder and solution requires proper PPE. Refer to the MSDS for instructions on handling this product.
- Repeated Virkon treatments will shorten the lifespan of fabrics and soft goods, but not as quickly as bleach-based decontamination options. Virkon-treated equipment, especially soft goods, should be rinsed thoroughly and allowed to air dry prior to eDNA sample collection. Extensive rinsing will help prolong the life of treated materials.
- Extended contact time of Virkon solution or powder with metal surface can cause corrosion, however Virkon is not as damaging as bleach. Metal surfaces should be decontaminated for no longer than 10 minutes and rinsed thoroughly.

DNA-Away is a sodium hydroxide-based DNA decontamination reagent made by Sigma-Aldrich. An assessment of DNA-Away (Fischer et al. 2016) found its decontamination effectiveness was more variable than bleach-based solutions, but that it outperformed several other name brand DNA removal products. DNA-Away is appropriate for use in situations where bleach is not an option (decontamination in the field, Van Dorn sampling bottles, etc.) or when used in combination with a second decontamination method. Proper PPE is required for use of this product; so refer to the MSDS for handling instructions.

Ultraviolet Light: UV light has a long history of use for decontamination and a mixed record of success (Champlot et al. 2010, Harrison et al. 2019) in DNA and PCR applications. UV decontamination doesn't leave residue that can damage future samples, but it does have limitations: chiefly that the target equipment needs to be placed physically close to the light source and that 100% of the equipment surface must be irradiated. Depending on the light box design, this means equipment may need to be flipped over and that 100% sterilization may not be possible on textured or structurally complex equipment. These limitations make effective UV sterilization of large and complex equipment challenging. UV decontamination is therefore not recommended for stand-alone decontamination of field equipment; instead UV should be paired with other decontamination methods.

Autoclaving and other heating methods are insufficient to fully decontaminate eDNA field sampling gear, and are not recommended (Goldberg et al. 2016, Unnithan et al. 2014)

Field Gear Decontamination

Dedicated eDNA Gear: To limit cross-contamination among shared project equipment, we recommend dedicating eDNA sampling gear to eDNA projects to the extent that your budget and resources permit. This is especially important when the focus of your eDNA study is present/common in areas where other field work occurs (USFWS 2020). For example, if a boat is used for invasive Northern Pike gillnetting in infested areas (and presumably gets covered with pike DNA), that same boat ideally should not be used for sentinel pike eDNA monitoring at the edge of the invasion. Dedicated rain gear, PFDs, waders, boots, coolers, backpacks, drybags, boats, storage totes, and even storage freezers are all helpful in reducing the risk of cross-contamination. However, even dedicated gear should be decontaminated, using the methods previously described, between sample collection events. For more information on dedicated gear, please refer to USFWS (2020), Section B2: Field Equipment Needed.

Decontamination Timing and Storage: Fully decontaminated equipment can become re-contaminated if it is exposed to target DNA prior to sampling. In addition, space limitations can make it difficult to store previously decontaminated gear in clean areas. For these reasons, we recommend that projects minimize the time between decontamination and sampling to reduce contamination risk. If decontaminated equipment (example filter kits) needs to be stored for an extended period of time prior to use, it should be kept in protective packaging and in a clean space that is free of PCR products and target DNA.

Boat Decontamination: Many eDNA sampling schemes rely on boats, including motor boats, rafts, kayaks, and canoes. Decontaminating these craft can be an extensive process, especially when boats are shared between projects or used in areas with high baseline levels of target DNA. Recommended boat cleaning and decontamination procedures include pressure washing with heated water and/or soap, followed by application of either bleach or Virkon solution and thorough rinsing. For more information on boat and trailer decontamination, refer to Bailey et al. (2014) and USFWS (2020), Section B2: Decontamination and Preparation of eDNA Processing Trailer and Appendix C Elimination of eDNA on Boats and Equipment.

Decontamination/Rinsate Blanks: Some sampling protocols require that field controls or "blanks" be collected when equipment is used, decontaminated, and reused in the field. The purpose of these blanks is to document that the decontamination approach applied in the field worked properly, and that

results in the corresponding sample are accurate. For more information on decontamination/rinsate blanks, please refer to Chapter 5.

Overall Decontamination Recommendations

As described above, all of the available decontamination approaches for field equipment are imperfect. Some agents will damage exposed equipment if used at high concentrations or extended contact time, others are not completely effective in decontaminating DNA equipment. 50% bleach solution provides the most consistent and complete decontamination, but is highly corrosive, and requires extensive PPE and rinsing. 20% bleach solution may be marginally less effective, but is also less damaging to equipment and has a broader range of applications. When using non-bleach alternatives, or bleach concentrations less than 20%, consider combining two approaches (for example Virkon and UV) to ensure equipment is sufficiently decontaminated. Use caution if using two approaches that involve chemicals and refer to MSDS information to avoid potentially hazardous combinations. Appendix C of the USFWS (2020) provides an excellent comparison of various decontamination approaches and is a good starting point for developing decontamination protocols.

DNA Extraction and Amplification in the Field

DNA extraction and amplification have traditionally been carried out in a formal laboratory setting; however, the recent development of portable extraction and amplification devices now allows on site DNA analysis (Marx 2015). Some portable devices have the ability to extract and amplify DNA using qPCR such as the handheld device manufactured by Biomeme (Thomas et al. 2019b). Other devices such as the LAMP system use a different type of amplification (loop-mediated isothermal amplification), circumventing the need for extraction altogether (Stedtfeld et al. 2014, Williams et al. 2017). Generally these portable devices are limited in the number of samples they can process at a given time and may be inefficient and cost-prohibitive for large-scale projects. Comparisons of handheld extraction and qPCR devices with traditional lab benchtop DNA extraction and qPCR suggest that portable qPCR devices may be more vulnerable to inhibition in the environment which can impact eDNA results (Sepulveda et al. 2018). Please see Chapter 2 for a detailed discussion of DNA extraction and amplification methods.

The above are by no means an exhaustive list or an endorsement for any particular field eDNA analysis equipment, but rather a representation of contemporary existing technology that has been reported or published with direct application to eDNA. It is worth noting that many portable devices are still in the process of research and development often deploying new versions, which may be challenging to keep up with on a limited budget.

Recommendations and Key Points

Recommendations regarding field techniques for eDNA studies vary between projects due to the many factors that influence project design. There are, however, general eDNA field technique recommendations and guidelines which are applicable to most projects. Several general recommendations are outlined in the following list. Note that the list is intended for quick reference, and readers should review corresponding sections in this document for further details on recommendations or summary points.

- Field study design considerations
    - Desired detection rates, target organism biology and life history, abiotic and biotic components of the aquatic system, and logistical constraints must all be considered when selecting field methods
    - Preliminary or pilot studies and consultation with statisticians and eDNA experts can be essential to project success
- Sample collection
    - Optimal collection procedures vary between environments and target organisms. Protocols are available that detail common collection practices
    - Samples should be collected in a manner that minimizes the chance of contaminant eDNA introduction
- eDNA separation/concentration
    - Filtration, centrifugation, and precipitation methods are used to isolate eDNA from water samples
    - 0.45-5 µm filters are the most commonly used separation medium but can clog in high turbidity environments. Refrigerated centrifugation is an alternative to filtration in turbid environments but requires shore or laboratory processing.
    - Precipitation is limited to small (commonly 15 mL) volumes and should not be used when ambient eDNA concentrations are low. Flocculation can necessitate the transport and storage of large sample volumes.
- Sample volume
    - Collection volume should optimize detection probability and collection efficiency
    - Environmental and logistical constraints limit maximum sample volume
    - Commonly reported total sample volumes include 250-5,000 mL for filtered samples, 50-250 mL for centrifuged samples, and 15 mL for precipitated samples
- Transport from field to lab and preservation
    - Samples should be preserved as soon as possible following collection.
    - Short-term preservation (in a cooler) is possible for samples returned to a laboratory, but long term preservation must occur within 24 hours
    - Long-term preservation methods include freezing directly, desiccation with silica, and addition of Longmire's buffer or ethanol. Use of multiple preservation methods (e.g. desiccation and freezing) is possible.
    - Chain of Custody (COC) forms should be used when transporting samples with legal restrictions; however, proper documentation of who, where, why, and how samples are collected is recommended for all projects.
- Abiotic and biotic factors:
    - Biotic and abiotic covariates that meaningfully influence eDNA concentrations and can be efficiently and accurately measured, should be recorded
- Sample labeling and required metadata:
    - Use water-proof, freeze-proof, and ethanol-proof pre-printed labels when possible. Record all necessary identification data.
    - Required metadata should include ID, location, date, volume, number filters/vials and relevant environmental covariates
- Decontamination standards and disposable equipment:
    - Gear **must** be thoroughly cleaned before and after decontamination

  - Equipment that directly touches/contacts the eDNA sample (primary equipment) should be single use or thoroughly disinfected between uses, ideally with 50% bleach.
  - "Secondary" and "tertiary" eDNA equipment (equipment that does not directly contact samples) should be disinfected between sample sessions whenever possible and potentially between samples depending on the project and sampling design employed
  - Multiple decontamination strategies (e.g. Virkon and UV) should be used when ≥20% bleach decontamination of secondary and tertiary equipment is not possible and when disinfecting primary equipment with <50% bleach
  - Strategies that reduce the risk of contamination, such as the use of dedicated eDNA gear, should be used in addition to decontamination methods
- New Technology
  - Methods for all facets of eDNA sampling have the potential to be innovated
  - Researching the latest available technology in the early stages of eDNA project design could have beneficial effects on project duration and cost

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Chapter 5

eDNA Field and Laboratory Controls

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Summary
Laboratory methods to detect eDNA are extremely sensitive, therefore controls are required at multiple points in both the field and laboratory processes to ensure results are accurate. Currently, there are no widely accepted standards for collecting and analyzing eDNA samples. This lack of standards means that different scientists using eDNA employ widely varying approaches for collecting, processing, and interpreting field and laboratory controls. This chapter will provide field and laboratory scientists with guidance on the use of field and laboratory controls to monitor for the presence of both false detections and false non-detections (also referred to as false positive and false negative detections, respectively). See Chapter 6 for more details on the interpretation of detections and non-detections. To obtain defensible results on which to base sound management, we recommend that all eDNA projects include field and laboratory controls, and that those projects document what controls were collected and why. We also anticipate that as public repositories are launched to warehouse eDNA data, the use of proper controls will be required for data submission.

Key Terms
Negative Controls: Samples lacking target DNA.

Positive Controls: Samples to which a known target DNA has been added.

Field Controls: Field controls are negative controls used to evaluate contamination during field sampling and transportation of samples to the lab. This includes surveilling equipment, identifying “dirty” collection and sample handling protocols, and screening for false detections. Throughout the BMP document, we refer to these as “field controls”; however, in the eDNA literature they are also commonly referred to as “field blanks” or just “blanks”.

Laboratory Controls: Lab controls are both negative and positive controls. Negative lab controls assess the presence of contamination during sample processing in the laboratory, and screen for sample inhibition when necessary. Positive lab controls help to provide confidence the target DNA, if present in

the sample, was successfully extracted and amplified. Positive lab controls minimize the possibility of false non-detections.

Introduction

A well-designed eDNA surveillance plan will help demonstrate that the results from the field and lab reflect reality on the ground and provide managers and researchers with accurate information to make informed decisions. If controls are not included, managers and researchers risk acting on imperfect or incorrect data, possibly with serious consequences due to faulty inference. For example, without negative controls DNA contamination could produce false detections of an invasive species, and valuable resources might be wasted responding to a threat that does not exist. Conversely, without positive controls, the efficiency of DNA extraction and amplification cannot be confirmed, and an endangered species that is present might go undetected, resulting in erroneous distribution information, and missed conservation opportunities.

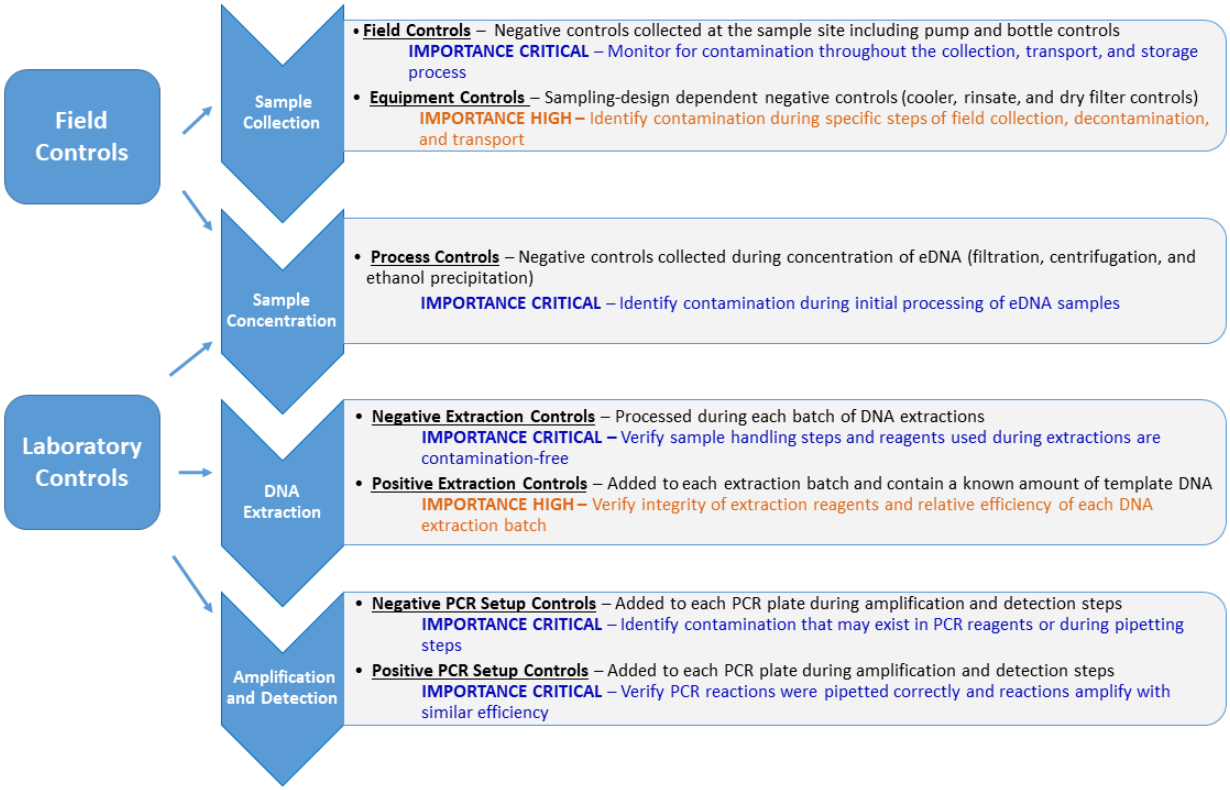
When used properly, field and lab controls can monitor for both false detections and false non-detections, thereby identifying deficiencies in the collection and analytical process prior to interpretation and release of results. Recent surveys indicate that negative and positive controls consistently rank among the top critical considerations for eDNA practitioners (Helbing and Hobbs 2019). Despite widespread recognition that controls are critical, there are no established standards for what controls are necessary in the field or in the lab, nor agreement on the number of controls needed to ensure confidence in the accuracy of eDNA detection results (Bustin et al. 2009, Rees et al. 2014). For example, some sampling protocols include extensive use of field controls (USFWS 2019), while others do not collect any (Carim et al. 2019). In practice, the extent to which eDNA programs use field controls is often related to the kinds of equipment being used: studies relying on factory-sterilized, single use-disposable supplies may have reduced control needs compared with studies where sampling gear is reused repeatedly and/or decontaminated in the field between collection events. The number and types of laboratory controls that are processed also varies across studies. As eDNA equipment and methods continue to evolve, so too will our understanding of how best to employ field and lab controls.

There are two key sets of processes that require controls to ensure that the methods and procedures used are accurately deployed: field collection and laboratory processing (Figure 5.1). In this chapter, we outline the methods for collecting and analyzing field and laboratory controls including both negative controls (blanks) and positive controls. Figure 5.1 illustrates the general workflow of eDNA sample collection, concentration, extraction, and amplification, and identifies where controls should be collected. We also provides guidance on the various types of controls, what each type of control is used for, and what it means if a control sample does not produce satisfactory results (i.e. a detections in negative controls, and non-detections in positive controls at a level consistent with expectations). Proper use and analysis of controls help provide robust eDNA data to support the management-based mission of the USFWS, and ensures consistency with the Code of Federal Regulations (CFR). The CFR section on Current Good Manufacturing Practice for Finished Pharmaceuticals 211.160 subpart (b) states that

> "laboratory controls shall include the establishment of scientifically sound and appropriate specifications, standards, sampling plans, and test procedures designed to assure that components, drug product containers, closures, in-process materials, labeling, and drug products conform to appropriate standards of identity, strength, quality, and purity" (EPA 2019).

Although the CFR does not specifically discuss field controls, it is important to have controls in both the field and laboratory for any kind of analysis. For example, the U.S. Environmental Protection Agency (EPA) provides guidance on both field and laboratory controls in their 2019 publication titled "Application of Quality Assurance and Quality Control Principles to Ecological Restoration Project Monitoring." The EPA document is geared toward ecological restoration projects; however, the detailed discussion of controls needed in each step of environmental monitoring can be applied towards eDNA studies. When collecting eDNA, it is critical to include controls for the accuracy of sample collection and subsequent analysis.

Figure 5.1. Flow chart depicting the process of collecting, concentrating, extracting, and amplifying target DNA in eDNA samples and where in the workflow controls should be incorporated. Blue text indicates critically important controls that are a priority for all studies. Orange text indicates highly important controls which are either study design-dependent or lack consensus among eDNA labs. To clarify terminology, we use the term negative controls rather than "blanks".



Rees et al. (2014) summarized the literature for field controls collected across several eDNA studies designed to detect the presence of aquatic species and suggest minimum controls required for field collection, including field (equipment) and lab controls. They note the importance of only using PCR and qPCR markers that have been thoroughly validated (See Chapter 2), and highlight the need for extraction and PCR controls, multiple PCR replicates, and documenting the criteria used to determine positive detections (See Chapter 6). A key recommendation is for studies to explicitly state both the type and number of controls included in the field sampling and laboratory processes. Hutchkins et al. (2021) reported that a minimum of 3-5 field controls prepared early in the sampling collection process is sufficient to reliably detect "systemic" contamination. They recommend increasing the number of PCR replicates processed per field control to improve contamination detection power rather than increasing the numbers of field controls.

Bustin et al. (2009) describes the minimum information necessary for conducting qPCR and reporting the findings from qPCR-based research. The majority of eDNA studies use qPCR detection techniques to determine if the target species' eDNA is present in a sample. The key controls outlined in Bustin et al. (2009) relate to laboratory control guidelines including analytical sensitivity, qPCR negative and positive controls, and measurements of PCR marker performance.

Types and Applications of Controls

Field controls: Field controls are samples that are prepared without collecting material from the environment (e.g. water, sediment, etc.) and should not produce positive detections for target DNA. Field controls are negative controls used to identify contamination issues that arise during the sample collection and DNA concentration process (if the samples are filtered or concentrated in the field).

Field controls can be used to assess contamination throughout some or all of the steps in the eDNA collection and storage process. The method used will depend on the intended goal of the field control. Some underlying principles of field controls include:

1) Regardless of which step in the eDNA collection protocol is being evaluated, the control must be exposed to the exact same process and conditions as a regular (non-control) sample.
2) The water used to prepare controls must be free of target species DNA. Double distilled/deionized water is a good option. Water from other sources (including tap water from wells and municipal sources) should be tested in advance to ensure it is free of target species DNA.
3) Controls should be taken in tandem with the next non-control sample and should not replace a non-control sample at that location.
4) Detection of target DNA in a field control indicates that all field samples collected on the same day or within the same sampling batch are compromised.

Different types of field controls are described in Olsen et al. (2012), Rees et al. (2014), Goldberg et al. (2016) and USFWS (2020), each evaluating a different aspect of the sampling process. The naming

conventions and level of detail describing field controls varies and is not always consistent among projects. For example, field controls taken at different stages of the sample collection process (and therefore monitoring different parts of that process) may be identified with the same term. When designing eDNA sampling plans and writing up final reports, it is important to clearly define and describe the controls collected. Likewise, it is important to identify what information is gained by collecting a specific type of control and what information might be missed if that control type is excluded. While every eDNA study should include field controls, not every type of field control should be included in every study. For these reasons, we recommend language clearly documenting which field controls were collected, the description of their purpose, and justification for omitting common controls. For more information on interpreting control results, see Chapter 6. Descriptions of commonly used field controls are listed below:

Pump Control (filters): Pump controls are collected at the same locations using the same procedures and equipment as field samples, but with "clean" water (such as deionized, distilled, or well water that is known to be free of target species DNA) poured onto and pumped through the filter. By replicating the exact same sample collection, storage, and transportation processes that are used for field samples, pump controls can surveil the majority of the field sampling process for contamination.

Dry Control (filters): Dry control filters are attached to the pump system while it is turned on, exposing the filter to the atmosphere and collection process, but no water is poured through the filter. It is important to replicate the exact same collection, storage, and transportation process, but without filtering any water. This type of control may be advantageous when sampling in remote locations where it is difficult to carry "clean" water into the field.

Bottle Control (bottles): Bottle controls are pre-filled bottles of deionized, distilled, or target DNA-free water that are opened to the air at the field collection site, re-sealed after 5 seconds, and then fully submerged into the field water (USFWS 2019). Bottle controls are stored with the other sample bottles both before and after being submerged. By replicating the exact same sample collection, storage, and transportation processes that are used for field samples, bottle controls surveil the majority of the field sampling process for contamination.

Cooler Control (bottles). Cooler controls are pre-filled bottles of clean (deionized, distilled, or target DNA-free) water that remain sealed in the cooler and are not exposed to the atmosphere at the field collection site, nor are they submerged in the field water supply. It is important to replicate the exact same storage, and transportation process used for field samples. Cooler controls are typically used in eDNA projects where sample bottles/tubes are filled at field sites, placed in a cooler, and taken to a different location for sample concentration (i.e. filtration/centrifugation).

Rinsate Control (bottles or filters): Studies that reuse sampling equipment in the field need to decontaminate gear between sample collections (often with bleach or DNA Away solution). Rinsate controls evaluate if field decontamination was successful. A rinsate control is collected using the decontaminated gear and deionized, distilled, or target DNA-free water, followed immediately by collection of the non-control sample using the same equipment but with water from the field.

Process Control: Not all eDNA samples are concentrated (excess water separated from the sample material) at the field collection site. Some programs collect water samples and later filter or centrifuge them at a vehicle or work trailer, before the samples are transferred to a lab for extraction and analysis. In these cases, process controls can be used to evaluate potential contamination during the eDNA capture and concentration process. The two main kinds of process controls are:

> *Filter Control*: A new filter is placed in the filter cup and clean, target DNA-free water is poured through it.
>
> *Centrifuge Control*: Negative control sample included during the concentration of eDNA samples using centrifugation. These can be empty 'dry' tubes or tubes that contain only sample storage solution. Centrifuge controls are exposed to the same environment as all other field-collected eDNA samples.

The number of process controls varies by project. For example, projects that combine subsamples may require that all consolidated samples be paired with process controls. This practice maximizes contamination surveillance, but also substantially increases the costs of analysis. Other projects rely on single use supplies (see Chapter 4) to mitigate contamination risks when concentrating samples, and do not prepare process controls for every sample. Similarly, some eDNA studies collect field controls and process controls, but only analyze a subsample of them. This is viewed as a cost-saving or sample optimization measure. The major issue with this approach is that field and process controls are necessary to demonstrate the validity of eDNA results. In the absence of field and process controls, positive eDNA detections indicate target DNA was present, but the likelihood of field equipment or technique-based contamination cannot be inferred. In addition, analyzing all field and process controls can help identify contamination issues that are not associated with positive samples. For these reasons, we recommend that all field controls and process controls be analyzed.

Field Control Limitations: While a well-designed collection of field controls can identify many forms of equipment and process-based contamination, not all contamination sources can be monitored using these methods. Field controls are unable to identify contamination that occurs when target species DNA from an external source (e.g. exogenous DNA from boats or boat motor cooling systems, fishing gear, wastewater outfalls, fish dropped by birds, migratory animal feces, etc.) is introduced into the sampling environment. eDNA assays can only confirm the presence of target DNA in a sample, not identify the DNA as originating from live specimens of the target species living in the study environment. For example, anglers commonly catch a target species of fish in one system (lake, ocean charters, etc.), and then travel home before cleaning their catch or disposing of the carcasses in local waters where that target species does not occur. DNA that is released from the introduced carcass could produce positive detections (target DNA was present in the sampled water) while the field controls would return non-detections (the contamination is in the sampled water, not on the equipment). This result would be wrongly interpreted as a "legitimate" positive detection even though the DNA detected was from an exogenous source and there were no live fish in the study environment.

eDNA collection teams can minimize the risk of introducing exogenous DNA into study areas by observing proper collection and decontamination procedures (see Chapter 4). However, because field controls cannot separate endogenous and exogenous DNA present in the sampled water source, it is

important to assess if the study environment is at high risk of containing exogenous DNA. High risk sampling locations include areas with high recreational use (boats, fishing, rafting, tubing), migratory wildlife corridors, and urban environments. When sampling in these locations, you should address the potential for exogenous DNA in the study design, data analysis and interpretation.

Laboratory Controls:

Laboratory controls serve two purposes in eDNA studies. First, negative controls (extraction and PCR blanks) are used to monitor for contamination potentially introduced during DNA extraction and PCR set up. These negative controls can be incorporated in a few different ways and should always result in non-detection of target DNA. If amplification of target DNA is observed in a negative DNA extraction control, it is assumed that contamination was introduced during the extraction process. This result would call into question the integrity of all field samples processed in the same extraction batch because DNA extraction reagents are shared across all samples. Negative PCR controls are used to monitor contamination in the PCR setup process and the PCR reagents. This takes place after DNA extraction and generally in a different physical location. If amplification is observed in a PCR negative control, it is assumed that contamination was introduced during PCR set up. Since PCR setup occurs after the DNA extraction and does not share reagents, the DNA samples are likely not contaminated. The PCR process can be repeated to ensure a contamination free set up and that field samples are not impacted.

Second, positive controls evaluate DNA extraction efficiency and ensure PCR reaction conditions were optimal, which is essential information for data interpretation and inference. For example, after qPCR screening a batch of field samples it is observed that all samples fail to amplify resulting in no detections of target DNA. These data could indicate that 1) the target DNA was absent from the field samples, or 2) the DNA extraction process failed (or was less optimal) and no amplifiable DNA was recovered, or 3) the PCR reaction failed resulting in no detections. To discern which of the three scenarios is correct positive control samples must be included to monitor the extraction and PCR set up process.

Types of laboratory controls:

DNA Extraction Controls

Negative Reagent Control: The negative reagent control monitors for contamination in the extraction process and the DNA extraction reagents. This control is processed in parallel with a batch of field samples and shares reagents with the rest of the field samples. If contamination is identified in the negative reagent control, it is assumed that all field samples processed in the same batch are compromised because the DNA extraction reagents are shared across all samples.

Negative Consumables Control: The negative consumables control monitors for contamination from consumable items used during eDNA sampling and processing. These controls provide insight into the source of potential contamination but does not change the end results as to which samples will be dropped from further analysis due to contamination. Consumable controls could include a representative filter, swab, centrifuge tube or other material used

during sample collection. The exact type of items included depends on the DNA collection process itself. Detection of target species DNA in this control (but not the negative reagent control) would indicate that the supply of consumables being used was contaminated. All field samples using the same supply of consumables would presumably be compromised.

Positive Extraction Control: Positive DNA extraction controls evaluate the success of the DNA extraction process. Positive extraction controls generally involve adding a known concentration of DNA at the beginning of the extraction process. Many sources of DNA are used including tissue-derived DNA, synthetic DNA or even cell culture material. The DNA is generally added to a stand-alone positive control (separate extraction tube), but some labs add this as an internal control to all samples. When added as an internal control, it is essential to use a surrogate species DNA that is not found in the sampling environment being investigated. Target species DNA is generally not used as positive extraction controls as their use increases the risk of cross contamination during the extraction process. By standardizing the amount of DNA used, a highly efficient extraction process will produce consistent and predictable qPCR amplification. Significant deviation from the expected amplification values indicates a less efficient extraction process that could lead to false negative detection data in field samples.

PCR Setup Controls

Negative PCR Control: The negative PCR control contains only the PCR reagents and no DNA. This allows for contamination of target DNA to be monitored in the PCR reagents and set up process. Target DNA detection in negative PCR controls would require all PCR results to be discarded. The setup area should be thoroughly cleaned, and a second PCR run carried out using new PCR reagents to eliminate any potential source of PCR contamination.

Positive PCR Control: The positive PCR control contains target-specific DNA (usually a synthetic DNA) of a known, low concentration that will amplify at a predictable cycle threshold. Failure to amplify or significant departure from the expected cycle threshold value indicates problems with PCR setup or reagent quality. PCR runs with failed or inefficient amplification of positive PCR controls should be rerun until amplification near the expected cycle threshold is obtained.

Standard curves: Standard curves are used in both marker validation and field sample analysis to calibrate qPCR assays. Standard curves are constructed using serially diluted standard solutions with known concentrations of target DNA that are processed in conjunction with the samples to be quantified. The standard curve estimates reaction efficiency and provides a conversion factor for quantifying the concentration (number of DNA copies/standardized volume) of target DNA in each sample. Inclusion of a qPCR standard curve is essential for measuring the effectiveness of PCR reactions and is covered in depth in Chapter 2 and Chapter 6.

Inhibition controls: PCR inhibition controls are used to monitor for compounds that can purify during DNA extractions and impact the efficiency of PCR amplification. PCR inhibition is an important topic that is covered extensively in Chapter 7.

Recommendations

In practice, the use of controls varies widely and is highly dependent on differences in sampling and processing protocols. In the case of field controls, budget or logistical constraints may limit the number and type of controls that are collected and analyzed. For example, when collecting samples in remote backcountry locations, it may not be feasible to pack in dozens of additional sample kits and gallons of distilled water for extensive field control collection. Whereas projects that re-use and decontaminate sampling equipment in the field should collect more field controls than projects relying on single use supplies. Considering this variability, we describe a minimum recommendation for controls below. Surpassing the minimum recommendation increases the QA/QC resolution for your project: more controls mean you have more precision in identifying, interpreting, and addressing contamination issues. Additional controls obtain more robust data sets, and limit the fallout from contamination incidents, such as discarded data, wasted field and lab staff time, and wasted materials. Finally, both peer-reviewed journal articles, agency reports, and data repositories need to include detailed information on the eDNA contamination control approaches used, the results from field controls, process and lab controls, and the implications (if any) those results have for interpretation of study results. Public-facing databases are increasingly requiring that control documentation be submitted for eDNA data. Without this information it is hard to assess the validity of these eDNA results and may provide a false sense of "good"-quality and reusable data when no assessment has been made.

Field Controls

All eDNA studies **MUST** include field controls. Studies need to describe how field controls were handled, identify the number of field controls collected and processed, and report the control detection results.

Minimum Recommendation:

1) A minimum of 10% of the total samples collected should consist of field controls (e.g. if 300 total samples are collected, 30 will be field controls and 270 regular samples). For projects with small sample sizes (fewer than 30 samples), an acceptable alternative is to collect a minimum of 3 field controls. Field controls can be arranged systematically or randomly throughout your sampling events to minimize the risk of contamination and ensure the results are robust.
2) Certain field controls (pump or bottle controls) are subject to collection, transportation, and concentration steps, and therefore surveil the majority of the eDNA process prior to extraction. Owing to their "broad" monitoring scope, pump and bottle controls should be prioritized. Additional controls that isolate specific aspects of collection, equipment, transport, or processing are strongly encouraged. After qPCR analysis of data, all field controls should fail to detect target species DNA. Detection of target species DNA in field controls should result in all field samples associated with the sampling day/batch being discarded.

Lab Controls

All eDNA studies **MUST** have lab controls. Studies need to describe how the lab controls were handled, identify the number of lab controls prepared, and report the control detection results.

Minimum Recommendation

1) Extraction Controls: Every batch of extractions should include at least one negative and one positive extraction control. When examining the negative extraction controls during qPCR analysis, there must be no amplification of target DNA. The negative extraction controls should be run with the same number of PCR replicates as were run with field samples. Please see Chapter 3 for assistance in determining the number of replicates for your study. At minimum, one positive extraction control should be included with every batch of DNA extractions to confirm integrity of DNA extractions in each batch. The positive control reactions must amplify with cycle threshold values consistent with laboratory expectations.
2) PCR Controls: Every PCR setup must include both negative and positive controls. At least 2 PCR replicates should be run for each negative and positive control. There must be no positive amplification in PCR negative controls. PCR positive controls must amplify with cycle threshold values consistent with laboratory expectations.

Standard curves

All eDNA studies must have target DNA standards and should report how the target DNA standards were handled and the number of standards included.

Minimum Recommendation:

1) Standard curves should meet a performance threshold of $R^2 \geq 0.95$ and efficiency values of at least 85% for each qPCR assay used to collect data. For more information on standard curve performance metrics, see Chapters 2 and 6.

Field Control Example Scenario

The following scenarios describe two potential approaches to field controls along with the different impacts (to both data and project viability) resulting from each in the event of field control contamination.

Your project is monitoring for the presence of invasive Northern Pike using eDNA sampling. You collect 50 samples and only 1 randomly ordered field control (a pump control collected after sample 14). The lone pump control produces a positive detection for pike DNA. Looking over your field notes, you realize that the first 20 samples were collected with equipment borrowed from a collaborator, but the remaining samples were all collected with gear from your office.

In this case, all 50 samples are suspect and any legitimate pike detections are indistinguishable from potential contamination. The positive sample detections cannot be reported, because the single field control doesn't provide enough information on when the contamination occurred and how long it persisted. Even though you have identified a potential contamination source (the borrowed equipment)

you don't have the control data to rule out contamination in the whole data set. The project is cancelled because the sampling and analysis will have to be repeated, budgets are tight, and you don't know what needs to change to prevent future contamination.

Alternatively, your project collects 50 samples and 5 randomly-ordered pump controls in accordance with the minimum recommendation for field controls. The first pump control (collected after sample number 14) detects pike DNA, but the next field control (collected after sample 22) does not. All remaining field controls are negative for pike. Looking over your field notes, you realize that the first 20 samples were collected with equipment borrowed from a collaborator, but the remaining samples were all collected with gear from your office.

In this case, the number of suspect samples is much smaller: the field control history suggests that the first 22 samples were potentially contaminated. Based on your field notes, the borrowed equipment may have only contaminated the first 20 samples. You decide to re-collect the first 22 samples, update your collection protocols to ensure that any borrowed equipment is properly decontaminated in the future, and notify your collaborator that they may have a contamination issue. You report the positive field blanks, and information about the recollected samples 1-22 in both the project database and final report.

Lab Control Example Scenarios

The following scenarios present situations where laboratory controls produce unexpected results. The impacts of the control results to the project data sets and database management are described as well.

Your project is monitoring for the presence of invasive Northern Pike using eDNA sampling. Your partners collect 50 samples with five randomly placed field controls (collected after samples 14, 22, 31, 36, and 41). You process your samples according to your protocols (including positive and negative extraction controls, and positive and negative PCR setup controls) and analyze the data. While reviewing the results you notice an issue: positive detections in 2 of 8 replicates of the negative extraction control for sample batch 1-25. The negative extraction control for sample batch 26-50 is clean (no detections) and there are no issues with the remaining positive extraction controls, PCR positive controls, PCR negative controls, and field controls. There are several samples with positive detections in the sample set: samples 7, 14, 27, 32 and 49.

In this case, samples 1-25 are suspect and any legitimate Northern Pike detections are indistinguishable from potential contamination. The positive detections (7 and 14) cannot be reported even though the associated field controls (collected after samples 14 and 22) are negative. The detections in the sample batch 1-25 negative extraction control indicate contamination is present, but it is unclear if the extraction batch was contaminated in the field, lab, or from sample-to-sample contamination. The reported data must include information about potential contamination of samples 1-25, but the sample detection/non-detection results cannot be reported. Conversely, the control results for samples 26-50 are as expected and don't indicate contamination or amplification issues. The positive detections for samples 27, 32 and 49 are therefore reportable. To complete the project, samples 1-25 need to be recollected which represents a substantial (but not insurmountable) duplication of effort and additional cost. You

report the contaminated extraction control data, and information about the recollected samples 1-22 in both the project database and final report.

*Alternatively, your partners collect 50 samples and while analyzing qPCR data for Quality Control checks and data reporting, you notice that a qPCR setup positive control failed to amplify (zero detections). All other extraction and qPCR setup control results are as expected, and samples 7, 14, 27, 32 and 49 are positive. You review your laboratory procedures (SOP's and/or QAPP's) and the established procedure for a failed PCR positive is to review plate notes and re-amplify all samples for the plate. While preparing the plate to re-amplify, you realize that you added water to the PCR positive reaction instead of template DNA, which would have resulted in the zero detections for the PCR positive control. You get your re-amplified data (with the correct template DNA) and all your controls pass. You can report all your samples with detections (7, 14, 27, 32 and 49) with confidence, and include information on the failed PCR positive in the project database.*2

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Chapter 6

eDNA Detections: Types of Detections, Where they Happen, and What They Mean

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Introduction

Although there is a growing number of methods used to detect species using eDNA surveys, qPCR remains the primary method for species detection and routine monitoring. Thus, this chapter mostly focuses on qPCR detections while briefly discussing detection data from metabarcoding and ddPCR efforts. Later iterations of these documents will expand on alternative detection methods and the detection data that is produced.

The eDNA project has been planned, samples have been collected, and the DNA isolated and analyzed. Now you are left with a list of samples with Cq values. Some are above zero, indicating the target DNA was present in the water sample. Other samples have no Cq value, indicating the target DNA was not detected in the water sample. These “positive” and “negative” detections do not necessarily mean the target taxa was present or absent in the environment sampled. In this chapter, we discuss what is a detection vs. non-detection, false detections and false non-detections, and what can contribute to each. Finally, we discuss how false detections and false non-detections impact data interpretation and eDNA surveys. We will focus on qPCR detections, but many aspects of qPCR detections are transferable to other eDNA detection methods (e.g., metabarcoding and ddPCR).

Detections and Non-Detections

A detection does not specifically indicate where, or when the target taxon was present. Moreover, a non-detection does not mean the target taxa is not present, especially at low population densities. All survey methods or assays have the potential for false detections (i.e., false positives) and false non-detections (i.e., false negatives) and eDNA surveys are no exception. For example, a species may not be observed during an electrofishing survey even when present at the location (false non-detection), and species can be misidentified during that same survey leading to false detections or false non-detections. In eDNA surveys, false detections and false non-detections are more complicated, and can be influenced by environmental factors as well as handling errors. Below is a brief overview of false detections and false non-detections in eDNA surveys.

Detections- A detection is when DNA of a target taxa is present in an environmental sample at a high enough abundance to be purified and detected through qPCR or other methods. Detections can result when the target taxon is present at the sampling location, contamination of target DNA is introduced to the sample (taxon is absent from the sampling location but its DNA is present in the sample), or if unintentional non-target amplification occurs (taxon and its DNA are absent from the sampling location). A detection when the target taxon is present is a true detection. A detection when the target taxon is absent (contamination in the field or lab, and non-target amplification) is a false detection. While it is not possible to say with 100% certainty that a detection correlates to a live specimen present

at the sampling location, the use of proper controls (see chapter4), robust study design (see chapter 2) and validated eDNA markers (see chapter 1) will provide a high degree of confidence in eDNA survey results.

1. **True Detection (Target DNA is present, and Target Taxon is present) –** A true detection is when the target DNA is present in the environmental sample and the target taxon is present in the general location where the sample was collected. Because eDNA surveys are mostly a detection only tool, there is no way to differentiate between target taxon DNA being present in the environmental sample because the target taxon is present at the sampling location, or if the target taxon DNA was introduced into the system without the target taxon being present. Ultimately, a single detection alone cannot confirm the presence of the target taxon, but repeated detections over time provide strong evidence that the target taxon is present.

2. **False Detection (Target taxon DNA is present, but target taxon is absent) –** The DNA of the target taxon is present at the sampling location, but the target taxon is absent (US Army Corp and US Fish and Wildlife 2014). Ultimately, positive detections that result from exogenous DNA (DNA that originates outside the system of study area) are still true DNA detections of the target species, but the detection of the DNA alone cannot be tied to the physical presence of that species at the sampled location. It is impossible to determine in every instance if a detection came from a live fish or from outside introductions in the environment. Thus, before deploying an eDNA survey, it is important to determine what a detection means for your study and what the next steps will be after a detection occurs (BMP Robust study design Chapter 2).

3. **False Detection (Target taxon DNA is present, but due to contamination) -** A detection can result from contamination through user introduction. During an eDNA survey, target taxon DNA can inadvertently be introduced into a sample in the field through contaminated sampling gear (e.g., Nalgene bottles, filter manifolds, sample racks), personal floatation devices, boats, and clothing. Contamination can also be introduced during laboratory processing (e.g., dirty gloves, reusing pipette tips, and cross sample contamination from positive controls). This results in a detection but neither the DNA nor target taxon is present in the environment.

4. **False Detection (Non-Target Amplification) –** An additional type of false detection is caused by a non-specific qPCR assay. A non-specific assay can inadvertently amplify and detect DNA from a source other than the intended target species. The proper validation of markers (Chapter 1) will test specificity prior to field use, but it is impossible to assess assay specificity against all possible sources of DNA encountered in an environmental sample. Therefore, it is critical to assess detection data in terms of expected versus unexpected results. A qPCR assay that produces unexpected results should be further scrutinized using a secondary method (e.g., DNA sequencing) prior to applying results.

Non-Detection – Non-detection, or a failure to detect, is when the target DNA is not present in the sample or the target DNA failed to amplify to a detectable quantity, resulting in a lack of detection data (e.g., Cq = 0). eDNA survey results can have many non-detection results, and it is difficult to

determine if these non-detections are a result of the target species not being present in the environment (true non-detection) or if there was a failure to detect the species when it is in the environment (false non-detection). Understanding the different non-detection scenarios and what causes each will help with the interpretation of eDNA results.

1. **True Non-detection** – A true non-detection results when the environmental sample does not contain DNA of the target taxon AND the target taxon is absent from the environment. It is difficult, if not impossible, to confirm the absence of the target taxon in the environment, especially at low population densities, even when adequate field replicates and water volumes are taken (Legendre and Legendre 2012, Erickson et al. 2019, Mize et al. 2019). Like other detection methods (e.g.: visual surveys, seining, telemetry, audio surveys), eDNA samples cannot prove a species is absent from a location. A non-detection is simply a failure to detect DNA of the target taxon. As with any detection tool, it is difficult to truly determine species absence at a location without taking a large number of samples over an ecologically relevant time period with repeated surveys over time.

2. **False Non-detection** – False non-detections result when the genetic assay fails to detect the target DNA even though it is present in the environmental sample. There are many reasons eDNA surveys can result in false non-detections. In the table 5.1 below, potential causes of false non-detections are listed alongside potential solutions or means of mitigation.

    a. ***False Non -Detection During DNA Extraction*** - DNA extraction is another element of eDNA surveys that can lead to loss of DNA and false non-detections. DNA Extractions are not 100% efficient and DNA will be lost in the process.

    Extraction methods have different efficiencies (e.g., Deiner et al. 2014 and Djurhuss et al. 2017) and sometimes there is a trade-off between convenience and DNA retention. For example, Phenol Chloroform extractions produce high quality, high quantity DNA but the process is tedious, time consuming, and requires the use of toxic and caustic chemicals. Alternatively, column-based DNA extraction kits use comparatively benign chemicals and are relatively quick, but DNA is lost through the column during extraction. Although DNA extraction kits can be convenient, and at times the only logistically feasible way to process large samples sizes, there is always going to be a chance that the target DNA is lost, leading to false non-detections.

    Extraction kits/methods are usually geared toward tissue or whole colony extraction, not designed to deal with low concentrations and potentially high levels of inhibitors. Using the wrong method leads to loss of DNA or retention of inhibitors which reduce PCR amplification. Overall, this leads to failure to detect. Testing needs to be done to determine what extraction method to use.

    DNA extractions will isolate ALL DNA in a sample, not just the DNA of the target taxa. This includes all animals, plants, and microbes contributing DNA to the environment. This can lead to DNA swamping of your extraction method due to presence of large portions of non-target DNA and can lead to failure to concentrate DNA of your target taxon.

b. **Inhibition and false-negative detection** - Inhibition results in a delay in DNA amplification and can lead to false non-detections if the DNA is in low quantity (like eDNA) and qPCR Cq values are shifted closer to 40-45 cycles, or if there is high concentration of inhibitors in the qPCR reaction to prevent DNA amplification. Inhibitors can be introduced through field collection (Chapter 2), during DNA extraction, and during qPCR analysis (Chapter 1). Recent work by Lance and Guan (2019) demonstrates that while inhibitory compounds may be present in many samples, concentrations of these compounds need to be high to have an impact on detection. While some eDNA monitoring and survey programs have inhibitors in samples (e.g., Burmese Python and Feral Swine) which causes concern for increasing false non-detections, inhibition is not a universally applicable concern and inhibition may not play as in all systems. In general, if inhibition is a potential concern, additional eDNA sampling and increased molecular replicates can alleviate the potential for false-non detections (See Erickson et al. 2019). If extensive testing for inhibition has occurred in eDNA samples that have been collected over many years in the same locations and inhibition is rare and/or has not affected detections, it is justifiable to disregard inhibition as a significant factor contributing to false non-detections. At a minimum, results should note if samples were tested for inhibition, if PCR inhibition was detected, and if inhibition was mitigated following MIQE (Minimum information for publication of quantitative real-time PCR experiments) standards (Bustin 2010).

Minimum criteria for calling a sample positive

The minimum criteria for calling a sample positive must be determined before data is collected. Ideally, multiple genetic markers are used to detect a target species so that there are multiple lines of evidence to suggest a positive detection is a true detection when more than one genetic marker detects the target species DNA. Unfortunately, for many species multiple genetic markers are not available or funds are too restricted for multi-marker usage. Here we discuss minimum criteria for detection and how each relates to detections and non-detections, both true and false.

1. **Single marker vs multi-marker reactions -** Even with thorough marker validation, it is good practice to sequence a proportion of positive detections observed from qPCR reactions when using a single marker. As described previously, false detections can result from non-target DNA binding and contaminations. Multiple markers will still detect contamination, but it provides multiple lines of evidence that the target DNA is present in the sample. A single marker does not provide this level of confidence, thus positive detections must be confirmed and identified to the appropriate taxonomic level through secondary Sanger sequencing.

2. **Single replicate vs multi-replicate detections -** eDNA is often low abundance, so single positive replicates will be a common observation. Setting criteria for the number of positive replicates needed to determine a sample is positive is important to do before data is collected and will be determined by how many markers are used in the reaction. If a single marker is used, sequence confirm the qPCR product for the single reaction. If multiple markers are used it is good practice to require multiple markers cross the threshold for the single reaction, thus providing more than

one data point for detection. If multiple markers are used, it is OK if a single replicate is positive as long as multiple markers are present for the single replicate.

3. **Detections after 40 cycles –** eDNA is typically low in abundance and it is tempting to allow a qPCR reaction to run as long as possible in order to detect target DNA, if it is present in the sample. In all PCR reactions, there is a point where the primers and reagents have mingled long enough that a false product can be generated, thus producing a false detection. This typically happens late in a qPCR reaction around 40-41 cycles. Thorough marker validation and testing can provide insight on the marker's behavior late in a qPCR reaction, but a good generalization is to disregard detections that occur after 40 cycles.

Table 5.1. Description of causes of false non-detection, and potential solutions or mitigation strategies that can be considered as part of the interpretation of data.

Cause of False Non-Detection	Potential Solution or Means of Mitigation	Relevant Citation
Target DNA is present in the environment but not collected with the water sample	Increase the number of replicate samples collected Target specific locations in the environment that eDNA may be more prevalent and consider the life history of the target species Increase the volume of water collected Collect from a wider area in addition to taking more samples Use of pilot studies and occupancy models	Erickson et al 2019 Mize et al. 2019 Guan et al. 2019 BMP Chapter 4
Target DNA was collected but the concentration is below the limit of detection	Use proper marker validation and optimization to ensure high efficiency assays Minimize DNA loss and degradation through proper sample storage and handling. Use of pilot studies and occupancy models	Pilliod et al. 2014 Lance et al. 2017 Guan et al. 2019 Erickson et al 2019 Mize et al. 2019 BMP Chapter 2
Sample storage and transport was not optimal for maintain sample integrity	Ensure proper handling and storage to maintain sample integrity Screen samples with qPCR assay for common species to assess sample integrity	QAPP 2020 BMP Chapter 4
There may be environmental inhibitors in the sample	Actively screen samples for inhibitors and treat as necessary	Lance and Guan 2019 BMP Chapter 7
eDNA sampling is not a suitable survey method given the biology of the target species	Establish pilot studies to assess eDNA suitability for each new species Use another survey method other than eDNA	Guan et al. 2019 Erickson et al 2019 Mize et al. 2019

Standard curves

Inclusion of a seven-point qPCR standard curve is essential for measuring the effectiveness of PCR reactions. As discussed in Chapter 2 (see sensitivity testing), standard curves are used to obtain the limit of detection (LOD), the limit of quantification (LOQ) and efficiency (R^2) of a qPCR assay. Concentration of the target taxon's DNA is often at the limit of detection in eDNA samples, especially for rare and newly introduced species. A highly efficient ($R^2 > 0.95$) assay with a low LOD provides increased detection sensitivity and more confidence in non-detection data. Assays with high LOD values can lead to false negative detections because the assay is unable to detect and amplify small quantities of DNA, thus, assays with a high LOD value are only suitable for the detection of abundant taxa. Assays that are efficient and have low LOD and LOQ values reduce the probability of false non-detections and give greater confidence in non-detection data.

Interpreting eDNA detections: I have detections, now what?

Once the data have been thoroughly vetted for false non-detections and detections, the question becomes: What do we do now? The answer to this question will depend on the goals of the study. For example, the follow up actions associated with a detection and the impact of those actions can vary depending on whether the target species is threatened and endangered or invasive.

1. **Threatened and Endangered Species (T&E):** When eDNA is used to assess presence of T&E species, a major assumption is that the species was present at some point in time where the eDNA samples were collected. Typically, historical records and/or contemporary data (e.g., habitat models) suggest a species should be present at a location, albeit in low density. Manual survey methods can be time consuming and because some species historical ranges are vast, range-wide sampling is challenging. Therefore, eDNA surveys can be used to pre-screen survey sites for evidence of the species presence before manual surveys are conducted. In these situations, contemporary data or other survey techniques can complement a positive eDNA detection. This approach to eDNA survey results has a profound impact on the reaction resource managers may have toward eDNA detections. Here, a detection is seen as evidence that the organism of interest is present in the survey area. The question that now needs answered is not IF the species is present, but WHEN or WHERE was the species present. Conversely, a failure to detect eDNA is assumed to mean that the population is too low to detect or not present in the survey area.
2. **Invasive and Non-Native Species:** Often, eDNA for invasive species detection is deployed when detection of a species is very difficult or cost-prohibitive with traditional survey methods. eDNA surveys for invasive species are typically used in one of two ways; 1) early detection of new introductions (e.g., introductions to the Great Lakes through ballast water or unintended transfer of dreissenid mussels between inland lakes), or 2) monitoring known populations (e.g., the USFWS Carp Monitoring Program). Detections and non-detections can mean different things depending on which approach is used. Strong evidence for eDNA detection of invasive and non-native species should rely on repeated sampling in the same area over a long period of time following an established protocol. With controlled methodology, a change in detections can be interpreted as an increase in eDNA deposition which could be correlated to an increase in population numbers.

Early Detection - With early detection, it is often assumed that the non-native target species is not present in an area being sampled. Given there is no contamination and an absence of allochthonous DNA, detections indicate the target species has been introduced into the area being surveyed. Non-detections suggest the target species has not yet been introduced to the sampling area or are too few to be detected using eDNA surveys.

Monitoring – With monitoring, a population has become established and the expansion of that population is being monitored. This involves sampling at the edge of, or just outside of the species range. Detections, non-detections, and the relative frequency of those detections are informative. An increase in detections can suggest the population is growing or its distribution has shifted. A decrease in detections could indicate the opposite. Alternatively, if sampling on the edge of a species range, non-detections could indicate that samples were taken too far from the established population or that individuals are too few in density to detect using eDNA surveys.

A non-detection does not necessarily mean the target species is absent. As stated throughout this document, there are many reasons that we get a non-detection even when the species is present. Because eDNA is a sensitive detection tool that can detect rare and low-density populations, it is assumed that eDNA can be used to determine the presence of only a few individuals. As is the case with many survey techniques, the population must be larger than a few individuals for eDNA to be detected. As of yet, there are very few studies that have examined the minimum number of samples required to detect eDNA (Willoughby et al. 2016, Erickson et al. 2019, Mize et al. 2019) and the minimum population size threshold for detection will vary widely from one species to the next and among habitat types. The strongest application of eDNA surveys for invasive species is repeated sampling in the same locations over time. It is the observed changes over time that are most informative for invasive species detection and monitoring (e.g., USFWS Asian Carp eDNA Monitoring Program).

Recommendation

The key to understanding and interpreting results is through determining what a positive detection is before data is collected and robust communication between all interested parties. A successful eDNA monitoring and surveillance program brings all the involved parties together as early in the process as possible. This is especially important early on in creating a program, where laying out a foundation for how results will be used to make management decisions is critical for programs looking at detecting both invasive as well as threatened and endangered species. The key to successful implementation of eDNA is ensuring that all parties understand the process used to collect samples, assumptions used to interpret results, and criteria used to apply results to make management decisions.

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Chapter 7

Characterizing and Treating PCR Inhibition in Environmental DNA (eDNA) Samples

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Summary

Interpretation of eDNA data can be confounded be PCR inhibition. Inhibition occurs when substances that interfere with the PCR amplification process are introduced to samples during the eDNA workflow. For successful detection of target DNA, decisions must be made during project planning regarding field sampling protocols, DNA extraction protocols, and PCR amplification protocols. There are many steps in each protocol that affect the probability of detecting target DNA, irrespective of whether the target DNA is captured in an eDNA sample. Disruption of the PCR amplification process can lead to false negative results – the failure to detect target DNA when present in the sample. False negatives may lead to a failure to act (or make a decision) and may provide a false sense of security in situations such as early detection of invasive species or environmental pathogen testing. Despite the challenges posed by PCR inhibition, collecting high quality eDNA data is possible with the use of a well thought out strategy for monitoring and remediation of PCR inhibitors. The following discussion will include a review of how PCR inhibition can occur, potential sources of PCR inhibition, and recommendations for detecting and minimizing PCR inhibition in eDNA samples.

Introduction

Environmental DNA (eDNA) is collected in a diversity of sampling environments under a wide array of changing conditions at different times of the year. Sampling environments can include headwater streams with cold clear water (Jane et al. 2014; McKee et al. 2015; Ikeda et al. 2016), bogs/swamps/wetlands (Doi et al. 2017), fast-flowing turbid rivers (Eva et al. 2016; Erickson et al. 2016), lakes with prolific algal blooms (Shaw et al. 2019), soil/sediments (Kyle et al. 2014; Stager et al. 2015; Monchamp et al. 2016), or even gut contents where the sample material is partially or completely digested (Brandl et al. 2015). In all cases, samples can be influenced by physical, chemical, and biological mechanisms that may compromise sample integrity (Cao et al. 2012). To some degree, loss of sample integrity can be overcome by using shorter length PCR markers (Lacoursière-Roussel et al. 2016; Klymus et al. 2017); however, this strategy is not useful for qPCR analysis of eDNA samples that contain chemical compounds that cause PCR inhibition.

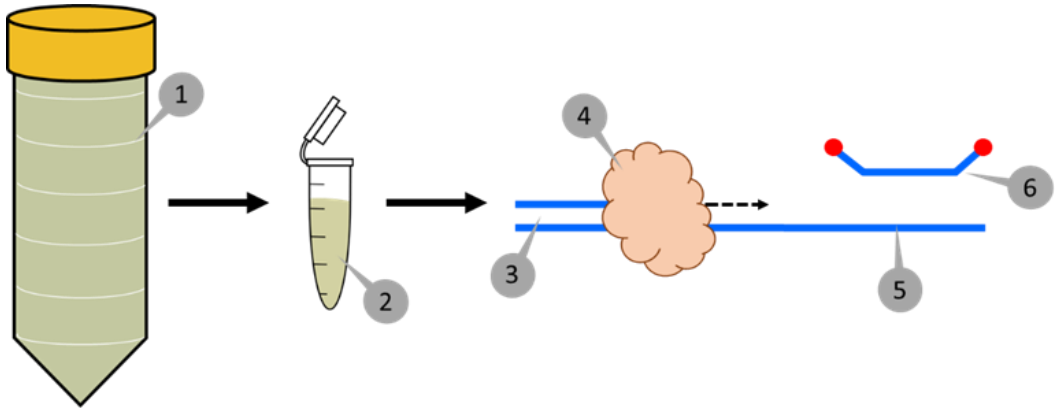
During DNA extraction of environmental samples, various chemical compounds can co-purify along with the DNA and interfere with (inhibit) the PCR amplification process. High levels of these PCR inhibitory compounds can cause complete disruption of the PCR reaction (causing a failed PCR reaction or complete inhibition) or can result in partial inhibition where the PCR amplification is delayed. This latter scenario can result in a loss of assay sensitivity (Acharya et al. 2017). PCR inhibitors can be introduced to eDNA samples during field sampling if the source of the inhibitor (Table 7.1 and 7.2) co-exists with the

target DNA (Schrader et al. 2012; McKee et al. 2015; Goldberg et al. 2016). Inhibitory compounds can also be introduced during laboratory procedures such as DNA extraction (Rossen et al. 1992; Green and Field 2012; Schrader et al. 2012), sample preservation, or post-extraction processing. Regardless of the source, PCR inhibitors can lead to false negative results (i.e. failing to detect the target DNA when it is present in the PCR reaction). Taking precautions and having a plan in place to deal with PCR inhibition is essential for a successful eDNA detection project (Jane et al. 2014; Lance and Guan 2019).

Environmental DNA sampling designs vary substantially and were covered in Chapter 3. Biotic factors (i.e. biology/physiology of target species, microbial activity/organic decomposition, algal growth) and abiotic factors (i.e. temperature, flow rate/velocity, turbidity) will vary based on geographic location, and/or the season(s) in which sampling is conducted. These biotic and abiotic factors are inherently difficult to control. The presence, type, and concentration of PCR inhibitors in eDNA samples will likely vary from site to site or even season to season (Gibson et al. 2012). For this reason, sampling protocols should be followed precisely, and site-specific physical data should be recorded (including qualitative observations such as clarity of water or flow). Sampling activity should be avoided during a rising hydrograph (U.S. Fish and Wildlife Service 2018) unless a rising hydrograph is targeted for specific reasons such as initiation of spawning activity or migration (Erickson et al. 2016). These factors make it necessary to test all eDNA samples collected for the presence of PCR inhibition (Gibson et al. 2012).

PCR inhibitors can originate from a broad array of chemical substances. Inhibitors act either directly on the enzymatic activity of *Taq* (*Thermus aquaticus*) polymerase, or indirectly by disrupting the primer/probe annealing dynamics or 'masking' the fluorescence generated during PCR amplification (Figure 7.1).

Figure 7. 1. Schematic outlining the various points where inhibitors can disrupt the normal PCR amplification process. PCR inhibitors may be collected with the environmental sample (1) and co-purify in the subsequent DNA extraction where they can react with the nucleic acids (2). Some inhibitory compounds disrupt primer annealing (3), directly interfere with Taq polymerase activity (4), degrade or modify the template DNA (5), or interfere with probe binding or their fluorophores (6). Schematic modified from Schrader et al. 2012.



Four different processes can cause PCR inhibition: 1) *Taq* inhibition, which affects the exponential amplification curve; 2) DNA binding, which produces changes in PCR cycle threshold (*Ct*) with no effect on amplification efficiency; 3) fluorescence quenching, where the inhibitory compound(s) mask any measurable fluorescence produced in the PCR reaction (Sidstedt et al. 2020), and 4) mixed mode inhibition, which affects both amplification efficiency and DNA template availability (Thompson et al. 2014). Different inhibitors have differing magnitudes of impact on PCR amplification which is related to both the mode of action and concentration of the inhibitor (Schrader et al. 2012). Removal and/or neutralization of inhibitors in environmental samples can be challenging given that samples may contain numerous inhibitors with varying chemical properties and concentrations (Figure 7.2). The compounds listed below are PCR inhibitors commonly encountered during field sampling (Table 7.1) or laboratory processing (Table 7.2). Many other compounds have been found to inhibit PCR and a more extensive review of the literature cited in this section will provide more examples.

Figure 7.2. Set of eDNA sample extraction tubes highlighting the color variation commonly observed in eDNA samples. Darker samples are more likely to show effects from inhibition than lighter samples. The coloration is caused by compounds found in the water sample that co-extract with the eDNA. Photo by Chris Rees.

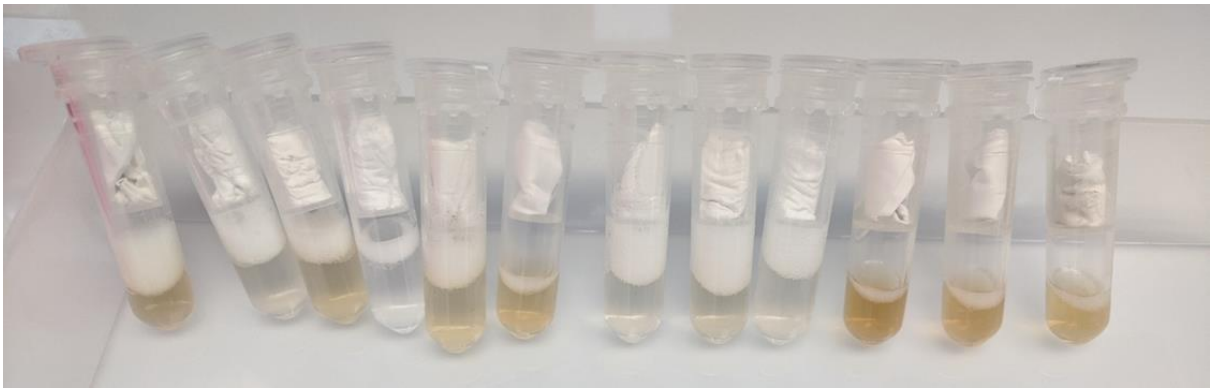


Table 7.1 Common inhibitors encountered in field samples

Inhibitor	**Source**	**Sampling Environment**	**Inhibitory Action**	**References**
Humic acid, Fulvic acid, Phytic acid	decomposition of organic material	wetlands and adjacent environments	disrupts *Taq* polymerase	(Matheson et al. 2010; Opel et al. 2010; Albers et al. 2013; Stoeckle et al. 2017; Lance and Guan 2019)
Tannic acid	decomposition of organic material	wetlands and adjacent environments	binds magnesium negatively impacting *Taq* polymerase efficiency	(Opel et al. 2010)
Algae	macroalgae and phytoplankton	aquatic systems with high nutrient loads	inhibits the *Taq* polymerase enzyme	(Schrader et al. 2012; Stoeckle et al. 2017)
Phenols/Polyphenols	berries, tomatoes, and other plant-based material	wetlands, croplands, farmlands	cross links DNA preventing amplification by *Taq* polymerase	(Abbaszadegan et al. 1993; Love et al. 2008; Opel et al. 2010; Schrader et al. 2012)

Polysaccharides	cellular structure of bacteria, fungus, plants, animals	samples with high cell counts for bacteria, fungus, or other cells	cross links DNA preventing amplification by *Taq* polymerase	(Opel et al. 2010)
Calcium	bone or samples rich in feces or quano	soil and fecal samples	binds magnesium negatively impacting *Taq* polymerase efficiency	(Opel et al. 2010; Schrader et al. 2012; Thompson et al. 2014; Orzechowski et al. 2019)
Exogenous DNA	any sample with very high DNA loads	samples with high cell counts for bacteria, algae, fungus, or other cells	impacts PCR efficiency through a variety of mechanisms including competitive binding	(Kainz 2000; Tamariz et al. 2006; Lance and Guan 2019)

Table 7.2 Common inhibitors encountered during laboratory processing of samples

Inhibitor	**Source**	**Inhibitory Action**	**References**
Ethanol/Isopropanol	used for sample preservation	disrupts *Taq* polymerase	(Rossen et al. 1992; Schrader et al. 2012)
EDTA (ethylenediaminetetraacetic acid)	used for DNA storage and preservation	competes with magnesium negatively impacting *Taq* polymerase efficiency	(Rossen et al. 1992)
Guanidinium thiocyanate	used for cell lysis and denaturing proteins in DNA extraction	negatively impacts *Taq* polymerase and binds to target DNA reducing amplification	(Rossen et al. 1992; Thompson et al. 2014)
CTAB (cetyltrimethylammonium bromide)	component of some DNA extraction buffers	binds magnesium and other metal ions negatively impacting *Taq* polymerase efficiency, can facilitate the copurification of polysaccharides	(Rossen et al. 1992; Corbisier et al. 2007; Demeke et al. 2009; Demeke and Jenkins 2009; Schrader et al. 2012)
SDS (sodium dodecyl sulfate)	ionic detergent used to disrupt cell membranes in DNA extraction	disrupts *Taq* polymerase	(Rossen et al. 1992; Peist et al. 2001; Demeke and Jenkins 2009; Schrader et al. 2012)
Phenol	organic compound used in DNA extraction	binds to and denatures *Taq* polymerase	(Rossen et al. 1992; Katcher and Schwartz 1994; Wiedbrauk et al. 1995; Wilson 1997; Peist et al. 2001; Demeke and Jenkins 2009; Thompson et al. 2014)

Process PCR inhibitors (those encountered specifically in the laboratory) can be easily avoided during eDNA sample processing through careful consideration of DNA extraction procedures, careful handling of samples during wash steps, or the addition of post-extraction purification steps. On the contrary, PCR inhibitors encountered during field sampling may not always be removed during DNA extraction. Regardless of the source of potential inhibitors, it is always good practice to 1) test for the presence and degree of PCR inhibition from all environmental samples, and 2) treat the sample(s) to remove the impacts of inhibition if inhibition is detected, or 3) eliminate inhibited samples from further data collection and interpretation.

Problematic environments and conditions

PCR inhibitors can be an intermittent problem in some sampling environments or during certain times of the year. Several studies have identified humic acid as the most problematic PCR inhibitor found in

environmental samples (Bej and Mahbubani 1992; Albers et al. 2013; Lance and Guan 2019; Sidstedt et al. 2020). Presence of humic acid is elevated in low lying wetlands, bogs, and swamps, as well as rivers and streams adjacent to these types of water bodies. But how does an eDNA practitioner decide when, where, and how to sample to mitigate the possibility of concentrating PCR inhibitors in any environmental sample? In Chapter 3 we discussed the different types of eDNA sampling designs. Here we will cover criteria to consider when devising a sampling strategy to help avoid concentrating high levels of inhibitors with the eDNA.

Sampling locations

Inhibition in eDNA samples varies widely with substrate, geographic location, environmental conditions, and site history, and the potential impacts need to be evaluated on a case-by-case basis. Some eDNA studies may be carried out without observable effects of PCR inhibition, and others may require substantial effort in primer design and DNA purification techniques to overcome the inhibitory effects. Given the complexity and variety of sampling environments, problematic areas are becoming more apparent, but continue to be difficult to evaluate. Inhibitors are more common in waters with darker coloration (often a sign of elevated humic or tannic acid levels), sewer treatment outflows, warmer waters (which typically increases bacterial and algal loads), areas with higher fecal material loads (possible presence of calcium and other inhibitors, Orzechowski et al. 2019), and areas with an increased algal biomass resulting from higher nutrient loads. These diverse and changing environments will continue to provide challenges for eDNA sampling related to the presence of inhibitors, and sampling strategies will evolve with those challenges.

Sample substrate, capture, and processing

Environmental DNA samples can be dramatically different in composition, the way the sample is captured, and how it is processed. For example, water can be passed through a single filter of uniform pore size to capture a sample, centrifuged to isolate a pellet, passed through a progressively smaller series of filters to capture a size fractionated sample (Hunter et al. 2019), or obtained through flocculation followed by centrifugation (Schill 2020). Some applications target very large volumes of water and may rely on plankton or larval fish tows obtained over long distances. Other approaches may target other sources of eDNA that include substrates such as gut/intestinal contents, feces, soil, sand, or even snow (Franklin et al. 2019). Despite where or how an eDNA sample is captured, all eDNA samples can potentially contain inhibitors which co-purify with the DNA during processing. Filtration volume is often increased to collect more eDNA to enhance detection rates, however larger filtration volumes of water can also increase the inhibitor concentration. In this case, if the location contains higher levels of inhibitors it may be appropriate to serially filter the water to potentially reduce the inhibitor load but not the DNA concentration (Hunter et al. 2019). The challenge is that DNA is often attached or bound to the inhibitory particles, making it difficult to separate the inhibitors from the DNA. Centrifuged water may come with a lower inhibitor risk, but the tradeoff is lower sample volume which often reduces DNA yield. Plankton/larval fish tows may encounter high levels of inhibitors due to the massive volumes of water that are passed through fine nylon mesh, however this can be overcome most of the time because DNA yields are ordinarily very high, and DNA sample dilution is possible and an effective

processing strategy to overcome inhibition. Soil, fecal, and gut content samples can contain the highest levels of PCR inhibitors. Depending on the study, sample substrate, and how the sample is captured and processed should be carefully evaluated for PCR inhibition potential.

Seasonality of sampling and the effects of changing temperatures

Seasonality of sampling also plays an important role in presence of inhibitors in eDNA samples. Sampling during times of elevated water temperatures (e.g. summer months, desert environments) may increase the abundance of bacteria, algae, or both. This situation warrants a higher level of caution regarding inhibition. However, depending on the location, spring sampling may also bring higher incidence of PCR inhibition from increased flow rates and turbidity due to precipitation and/or snow melt. Flows and turbidity should be monitored prior to planning sampling trips. Fall sampling brings a different concern with regard to presence of PCR inhibitors in samples. Forest leaf litter, particularly in locations where deciduous trees are in higher abundance, adds a source of organic matter directly to forested streams. Vegetative decomposition is a well-documented source of tannins, humic acid, and other inhibitory compounds that can enter streams, rivers, and lakes at higher concentration after deciduous trees begin losing their leaves. Because study design often precludes the ability to avoid a certain time period, it is essential to monitor for inhibition. However, if inhibition is known to pose a significant risk and other mitigation is unavailable, an alteration to the sampling strategy may be necessary to mitigate the risk of false negatives.

PCR Inhibition Mitigation Strategies

Having a firm understanding of the risks associated with PCR inhibition ahead of project implementation is particularly useful, but this does not mean it is altogether avoidable. As a result, there are many strategies that can be employed to minimize PCR inhibition and the risk of false negatives in eDNA data. For example, large quantities of algal cells and humic acid from decomposing organic matter are sources of known PCR inhibitors. A proactive measure would be to sample at times and locations where algal growth is limited and avoid sampling during leaf drop and the pursuant increase in organic decomposition. Mechanical methods such as a larger pore prefilter can be used to remove algal cells, and/or alternative DNA extraction and recovery methods can reduce the presence of inhibitors. Reactive measures are done by treating the DNA after extraction or using PCR reagents that are more tolerant to inhibitors. Many commercial DNA extraction kits include columns, chemicals/buffers, or post-extraction steps that aim to reduce or eliminate inhibitory compounds. It is important to remember that not all inhibitors have the same mode of action and may require a different strategy for mitigating PCR inhibition depending on the source of the eDNA sample. Most importantly, testing is needed to determine and characterize the optimal inhibition detection and removal strategy for each eDNA project.

Proactive Strategies

Changing Field Sample Volume

Water sample volume can vary widely from one eDNA study to the next. As a result, sample volume is an important factor to consider and may be appropriate to conduct a pilot study to determine if target volumes can be optimized in systems that contain higher levels of inhibitors. Volumes as small as 50 ml are sometimes used for concentrating samples by centrifugation or filtration with handheld syringe filters. At times, turbidity can limit filtration volume to only 50-200 ml, particularly when filter pore size is a critical factor. Filtration volumes in the 500 ml to 2L range are common, but some studies target sample volumes of 4L, 6L or larger. Filtering larger volumes increases the chance of collecting the target DNA, however, larger sample volumes run the risk of also concentrating higher levels of PCR inhibitors. If the volume of sample collected is sufficient to reach the desired detection probability, sample volume can be adjusted (smaller volume = lower inhibitor loads) to reduce the potential impact of PCR inhibition on collected samples.

Altering collection and concentration methods

The potential impacts of PCR inhibitors found in the sampling environment can also be reduced or mitigated by altering eDNA collection gear and method of sample concentration. At the current time, filtration is the most common collection and concentration method. Filtration protocols can vary dramatically in their application and both the volume filtered and filter type can be altered to help mitigate inhibition. Filters can vary based on filter membrane material and construction, the available surface area of the filter (e.g. column-based filters can be used to increase sample target volumes), the thickness of the filter, and the pore size of the filter. The main types of filter materials used for eDNA sampling are glass fiber, polyethersulfone, cellulose nitrate, and nylon mesh. In each of these materials, filter diameter, surface area, thickness, and pore size can vary. The critical decision to be made is whether the chosen filter is able to capture the target organism's cells or DNA at a detectable concentration without also concentrating high levels of PCR inhibitors that may result in false negative results. These are challenging criteria that can be addressed through protocol optimization and pilot studies that are used to determine the best volume and filter type for every eDNA study.

In general, smaller pore sizes (<0.45 µM) are used for the detection of organisms with smaller structures or cell sizes, i.e., viruses, bacteria, and other micro-organisms. For non-microbial DNA work, most studies have employed pore sizes between 0.45 µM and 5 µM. Still other studies use even larger pore sizes (10 µM and larger) of nylon mesh material. One example of this is plankton sampling in which very large volumes of water are processed through a comparatively large pore size. A trade-off exists with opting for a larger pore size. More volume can be sampled which increases the potential for collection and concentration of large amounts of target species DNA, although it also concentrates large amounts of non-target DNA as well. Larger pore size may allow suspended solids to pass through the filter membrane material, which can help to reduce PCR inhibitor loads in samples, but this may also come with a loss of target DNA captured since DNA is often bound to these suspended particles. There is no

right or wrong combination of volume and filter type, but this choice should be accompanied by an optimization study.

Alternatively, rather than collecting and concentrating target DNA using filtration, an eDNA practitioner can use precipitation, centrifugation, or flocculation. These methods tend to have some of the same issues with inhibitors co-concentrating with target DNA, but the complication of filtration rates and reduced filtered volume is eliminated. Particulate matter and suspended solids are still collected with the sample and optimization of sampling protocol is still necessary to ensure PCR inhibitors are not a source of false negatives during analysis.

Finally, magnetic beads can be used to selectively recover target DNA from various sample types including filtered, centrifuged, or flocculated water samples. Although this approach has been largely experimental to date, recovered DNA tends to be of higher purity and free from discoloration that commonly accompanies DNA extracted with other methods (see Figure 7.2). As a result, this strategy may show benefit of removing inhibitory compounds as well as discoloration that may be more prone to fluorescence quenching during PCR.

Increasing sampling intensity and coverage

Some studies have employed a strategy to reduce the potential effects of PCR inhibition by simply increasing sampling intensity in the study area. This is done by increasing sample replication, sample coverage area, or both. This strategy is particularly effective if pilot data suggests PCR inhibitors only effect a smaller percentage of samples or have less significant impacts on PCR amplification potential. Often this strategy is coupled with decreased sample volumes, (discussed earlier as an alternative PCR inhibition mitigation strategy). The decision to decrease sample volume may negatively impact detection probability, which could be compensated by increasing sampling frequency. However, increasing the number of samples will add to the cost of analysis for additional sample processing. This may be offset by the benefit of collecting individual samples that are less likely to contain PCR inhibitors.

Reactive Strategies

The impact of PCR inhibition in eDNA analysis can be overcome through reactive laboratory mitigation strategies. Inhibitor concentration thresholds differ based on inhibitor type so it is hard to predict how effective laboratory mitigation strategies will be. Once an inhibitor concentration threshold is reached, going from no observable effects of PCR inhibition to near complete inhibition happens within a tight inhibitor concentration range (Lance and Guan 2019). However, success has been found in using some of the reactive laboratory mitigation strategies listed below to reduce or remove the effects of PCR inhibition during eDNA sample analysis.

DNA Extractions

Regardless of the eDNA sample processing method used, important decisions have to be made regarding DNA extraction methodology. Many reports have characterized various quality parameters such as the efficiency of DNA isolation, the purity of the DNA, and/or the impacts the extraction has on

presence and concentration of PCR inhibitors (as reviewed in Rådström et al. 2004). Commercial DNA extraction kits and PCR reagents are available which are specifically designed to handle PCR inhibition and are quite effective at dealing with inhibitors commonly observed in environmental samples (Demeke and Jenkins 2009; Cox and Goodwin 2013). Consultation with eDNA analysis laboratory personnel is recommended if looking for suggestions on the best DNA extraction methodologies for dealing with PCR inhibition.

Post DNA Extraction Treatments

Dilution of extracted DNA

Dilution of DNA samples is a common method used to offset inhibition across many PCR applications, including eDNA. Generally, when inhibition is detected, samples are diluted with a pre-determined volume of diluent (Cao et al. 2012; Schrader et al. 2012; McKee et al. 2015; Acharya et al. 2017). 5x and 10x dilutions are commonly used (McKee et al. 2015), however, the exact volume of diluent needed depends on the level of inhibition (Gibson et al. 2012). Because inhibition tends to be concentration dependent, this strategy is often very successful, inexpensive, and efficient. eDNA samples are unique because target DNA is often at or near the limit of detection. Any sample dilution, although intended to relieve inhibition in the sample, may dilute the target DNA concentration beyond the limit of detection and result in a false negative. Dilution of eDNA samples should only be done when the target DNA signal is known to be high.

There are situations in which dilution of DNA is not desirable depending on the focus of the project. One example includes projects that target early detection of invasive species or detection of cryptic or rare species. The expectation in these studies is that the available target DNA for amplification will be near or below the limit of detection (LOD) of the assay. Therefore, any dilution of starting DNA quantity may risk the possibility of a false negative result. In studies where the target DNA is found or expected to be at higher concentrations, eDNA dilution may be the best and least expensive option to overcome inhibition in problematic samples.

Increasing DNA template volume or the number of PCR replicates

Although not generally recommended for early detection studies, there are instances when sample dilution is an appropriate reactive measure for dealing with PCR inhibition. In these instances, the number of PCR replicates (i.e. instead of 8 PCR replicates the lab may consider running 16 replicates) or DNA template volume could be increased (i.e. 4 µl instead of 3 or less). When the DNA is diluted in problematic samples, the expectation is that the inhibitors that may be present are diluted as well. The DNA is still present in the sample but it has now become less likely to be pulled in an aliquot from the DNA sample. By running more PCR replicates, a slightly higher volume per sample, or both, the overall percentage of the sample analyzed remains constant but the concentration of inhibitor in any given PCR reaction is reduced.

Taq Polymerase and Master Mix formulation

Commercial PCR master mixes or custom laboratory formulations are available that include PCR enhancers such as bovine serum albumin (BSA), betaine, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), and other reagents that can be used to overcome the impacts of residual PCR inhibitory compounds (Frackman et al. 1998; Ralser et al. 2006; Hedman and Rådström 2013). Most eDNA analysis laboratories either use commercial formulations specifically designed to overcome inhibition or custom formulations with some of the above compounds. Optimization is critical in these cases to verify efficient amplification in both ideal samples and those that may have differing levels of inhibitors. In some cases, custom enzyme formulations may be enough to counteract the impacts of inhibition for the sampling environment targeted, but this should be verified prior to analyzing large numbers of eDNA samples.

Inhibitor removal columns

Some DNA extraction protocols incorporate the use of inhibitor removal columns in the DNA extraction protocol. Given the danger of false negative results, we recommend using size exclusion chromatography columns to clean up samples showing signs of inhibition. This is beneficial particularly for eDNA projects where PCR inhibition is widespread and unavoidable. Studies have shown this strategy to be quite effective (Schrader et al. 2012; McKee et al. 2015). Many commercial vendors supply simple-to-use columns that provide a 'quick' and 'easy' method to remove inhibitory compounds. Some problematic samples may require passage through two or more columns to completely remove inhibitors. Treating every sample, however, can be expensive due to the extra cost of supplies and personnel time. An alternative strategy is to test all samples for presence of inhibition first and then treat only those samples that show signs of PCR inhibition.

Although these columns are very effective in removing inhibition, the danger of false negatives through sample dilution exceeds the risk in loss of DNA through size-exclusion chromatography (McKee et al. 2015). There are conflicting studies on this point, with some reporting inhibitor removal columns to have minimal loss of DNA after one pass, but subsequent passes in very problematic samples may lead to more significant losses of DNA from the sample (Hunter et al. 2019). Some studies have suggested large losses of DNA even after just one pass. More research is needed to adequately address this phenomenon because post-extraction inhibitor removal columns are one of the most widely used strategies for cleaning samples known to have high levels of PCR inhibitors.

Alternative technologies for inhibitor remediation

Droplet Digital PCR

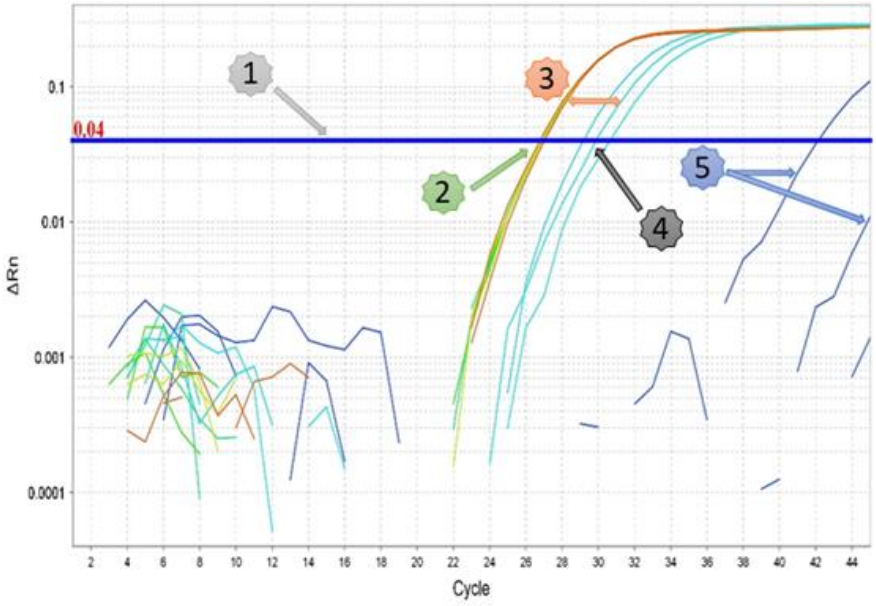
Finally, alternatives to qPCR detection can be used to combat the influence of inhibitors. Some sampling environments are inherently difficult to work in and have very high levels of inhibitors commonly present (e.g., swamps, bogs, ephemeral ponds, marshes, etc.). In those environments, an alternative could be to utilize droplet digital PCR detection technology (ddPCR, see Chapter 2). ddPCR has been shown to have higher resistance to PCR inhibition than qPCR (Hoshino and Inagaki 2012; Cao et al. 2015; Doi et al. 2015; Sidstedt et al. 2017). Although ddPCR is a potential solution for difficult sampling environments, the overall sample throughput is lower and equipment and sample processing cost are

higher than qPCR. While large-scale adoption is yet to occur, ddPCR is being used successfully in environments where qPCR has not performed as well (Taylor et al. 2017; Orzechowski et al. 2019).

Methods of Detecting PCR Inhibition

Detection of PCR inhibition can be conducted in several ways, each involving the addition of positive control DNA at various stages during the sample processing workflow. The basic premise involves the addition of a control DNA to the suspect field sample so that the detection of the control DNA can be compared to a laboratory control that is free from DNA inhibition. If the amplification cycle of a field sample is delayed relative to the laboratory control (known as the ΔCt/ΔCq method), the sample is characterized as inhibited and further DNA cleanup is necessary before eDNA analysis (Figure 7.3). Control DNA can be added to field samples prior to DNA extraction, to the extracted DNA, or directly to the individual qPCR reactions. These controls are generally referred to as internal positive controls (IPC). For eDNA samples, a standardized strategy has not yet been adopted, although the ΔCt/ΔCq method is most frequently used. Decisions on the best strategy to handle testing for the presence of PCR inhibition is carried out by laboratory personnel and should be decided ahead of time, prior to the initiation of the project, so that a robust plan is already in place.

Figure 7. 3. Raw data from a PCR inhibition test using an internal positive control (IPC) with the ΔCt/ΔCq method. Each sample was analyzed using 3 PCR technical replicates. Environmental DNA samples were evaluated by comparing the fluorescence data and the point the data crosses the cycle threshold (Ct) line (1). Control samples and eDNA samples showing no inhibition cross at nearly the same point within 1 Ct of each other (2). Environmental DNA samples that cross the line at a point ≥ 1 Ct later (3) are characterized as inhibited. In this example, two samples demonstrate inhibition, with the first showing a ΔCt of ~ 3 (4), and the second demonstrating near complete inhibition (5).



Goldberg et al. 2016 references a ΔCt shift of ≥3 as evidence of inhibition in eDNA samples. However, other reports suggest using more stringent ΔCt shifts of ≥1 with eDNA samples (Gibson et al. 2012; Wilcox et al. 2018). Given the high cycle thresholds typically found when detecting target DNA (>35), higher stringency is recommended for two reasons, 1) to reduce the probability of false negative reactions (especially for early detection of invasive species or detection of rare species) and 2) to increase the accuracy of inter-replicate estimates of DNA quantity, particularly in cases where target DNA abundance is needed for correlational studies to determine the relationship between eDNA quantity and target species populations or biological activity. At a minimum, PCR inhibition testing should be applied to all samples (Gibson et al. 2012). Detection of PCR inhibition and treatment should be reported following MIQE (Minimum information for publication of quantitative real-time PCR experiments) standards (Bustin et al. 2009; Bustin 2010).

Additional considerations

Previous research in the fields of microbial ecology, forensics, and ancient DNA has provided much information on the significance of PCR inhibition in a variety of sample types, both derived from specific organisms and those that are environmental in nature (Eilert and Foran 2009; Alaeddini 2012; Lorenz 2012). Despite this expansive literature bank, uncertainty still exists on the impacts of different inhibitor classes, particularly with regard to eDNA samples. There are several research papers that address the topic of inhibition in eDNA samples specifically (Jane et al. 2014; Sepulveda et al. 2018; Orzechowski et al. 2019; Lance and Guan 2019; Hunter et al. 2019). The issue is generally acknowledged in primary research articles with only a sentence or two providing limited detail. There is a need for more specific research on this issue, particularly if eDNA data is to be used to inform conservation-related

management decisions more broadly. Below are some additional considerations to consider before making a decision on the best strategy to reduce potential PCR inhibition in eDNA studies.

Assay Implications/PCR Efficiency variability among assays

Data exists in multiple eDNA studies that suggest specific quantitative PCR assays respond differently to different inhibitor classes (Lance and Guan 2019; Hunter et al. 2019). Lance and Guan (2019) tested 6 different markers against different inhibitors and found some responded more favorably than others. Test parameters such as GC content, amplicon length, and primer/probe melt temperature did not influence the effect of inhibition. Other studies have suggested PCR efficiency of each marker may be linked to differences in how well the marker overcomes inhibition. This is an important finding because it suggests that current inhibition testing protocols may not be adequate if an IPC follows a different amplification trajectory than an eDNA species-specific assay. As a result, it is recommended that optimization tests be conducted with both the IPC assay of choice as well as the species-specific assay in question. Given this uncertainty, it would also be beneficial to use multiple strategies to mitigate PCR inhibition in environmental samples.

Data and Interpretation implications

Knowledge about the potential for false negative results or even delayed amplification due to PCR inhibition is critical for several reasons. If engaged with early detection projects, complete or even partial inhibition could create a situation where the DNA of an invasive species is not detected. Alternatively, in projects directed at detecting rare, threatened, or endangered species, the same result may cause an otherwise important discovery to go unnoticed. Partial inhibition introduces higher variability into quantitative datasets and makes detecting differences in DNA copy numbers more difficult among samples. Without knowing if PCR inhibition is a problem for some sample types and environments, sound data interpretation is not possible.

Large programs with access to expansive historical datasets, widespread repeated sampling in similar areas from year to year, and large spatial coverage can entertain the idea of scaling back testing if previous results suggest inhibition is not a contributing factor to the possibility of false negative results for the locations being sampled and the sample types collected. In this case it would still be pertinent to screen a subset of samples periodically (several times each sampling season) to ensure that the frequency of sample inhibition is comparable to levels previously observed. Even in these circumstances, adopting one or more measures to mitigate the possibility of PCR inhibition in collected eDNA samples is recommended.

Accuracy of data to be included in public databases

Many organizations are currently exploring best practices for including eDNA data on public databases. These efforts have the task of ensuring data is of high fidelity and accuracy. To do this, decisions have to be made on data submissions to verify accuracy so that erroneous data points, whether they be positive results due to field-based or lab-based contamination or false negative results due to PCR inhibition, are not included for display. In one example, data is verified through pre-submission questionnaires that aim

to ensure data is produced using appropriate eDNA sampling and analysis protocols (https://www.sciencebase.gov/catalog/item/5e9db54982ce172707fb8ce0). Although not a requirement, whether PCR inhibition testing was done and how it was accomplished is included as a question to further verify the accuracy of the data submitted. This constitutes one additional reason why inhibition testing is a recommended step during processing and analysis of eDNA samples.

Conclusion

Despite extensive research to investigate causes and solutions for sample inhibition in qPCR, many unknowns remain and there is no 'one size fits all' remediation strategy. For instance, some studies have demonstrated clear differences in the potential for PCR inhibition issues strictly based on the inhibitor tolerance of the DNA polymerase itself (Matheson et al. 2010; Albers et al. 2013). Other studies have shown that the length of the qPCR marker, the nucleotide composition of the target DNA, primers and/or probe, or the probe's fluorophore may allow for differential impacts of PCR inhibition (Schrader et al. 2012; Lance and Guan 2019). These findings clearly demonstrate that if using IPCs, a clean inhibition test may not confirm the lack of inhibitory effects on the target assay. It is good practice to understand the potential of different inhibitory impacts on different assays, and to have a strategy to deal with inhibition if it occurs, i.e., with multiplex PCR or additional process controls, (Green and Field 2012). In the event of severe sample inhibition that is not effectively dealt with in pre or post reactive measures, it may be possible to use a ddPCR approach. ddPCR is still based on probe-based qPCR chemistry, but sample partitioning reduces the effect of inhibition on assay performance and may provide an alternative approach for environments with large quantities of inhibitors.

Newer technology exists that allows for field-based or near real-time eDNA detection using qPCR or loop-mediated isothermal amplification (LAMP). In this application, sample collection to data acquisition can be conducted entirely away from the laboratory in two hours or less. To accomplish this, DNA extractions are carried out in the field through rapid protocols. Several studies have shown these protocols have higher susceptibility to PCR inhibition (Sepulveda et al. 2018; Thomas et al. 2019). This type of technology has the capability to provide eDNA detection data in a very short time, however, caution must be exercised since the sample prep is more prone to carry over of PCR inhibitors. Inhibition is also more difficult to treat since the work is conducted away from a laboratory. Despite the potential shortcomings these new technologies pose, additional research being done in this area is likely to improve methods and results.

Recommendations

With the use of sound extraction methods and inhibition resistant *Taq* polymerase, most environmental samples can be processed without the negative effects of inhibition. To provide a higher confidence that false negative data are not being reported, screening eDNA samples for inhibition is recommended. Inhibition testing can be accomplished in many ways as outlined in this document, but given the wide diversity of sample types, markers and potential inhibitory compounds, there is no "one size fits all" solution. In the absence of specifics, it is essential that all eDNA studies are designed with inhibition monitoring and remediation in mind. Samples should be screened for inhibition using an approach that

is reasonable for the given sample type. Samples identified as inhibited should be processed using protocols that minimize sample loss and maintain maximal detection probability. This basic approach must be adaptive to effectively eliminate/reduce the negative impact on PCR inhibition. Use of a *Taq* polymerase that has been engineered for tolerance to inhibitory compounds is also recommended. There are currently a number of such products commercially available and empirical testing with specific sample types is recommended. Despite the challenges posed by PCR inhibition, collecting high quality eDNA data is possible with the use of a well thought out strategy for monitoring and remediation of PCR inhibitors.

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eDNA Glossary

3' end (three prime end) – Refers to the directionality of a DNA strand and is often used in the context of the amplification primer. Taq polymerase can only extend and synthesize target DNA in the 5' to 3' (upstream) direction. Therefore, amplification primers that mediate the DNA replication process are often carry a 5' and 3' notation to signify the direction of synthesis. For example, a forward primer would be denoted as 5'-ATGTCGACTGATCACC-3' and a reverse primer as 3'-TCGATCGATCTTCCAC-5'.

5" end (five prime end) – See 3' end.

A

Accuracy – Accuracy: a qualitative measure of the agreement between a measured value and a true, but generally unknown, value (see "bias", Mosher et al. 2019).

Amplicon – The short lengths of DNA obtained during the PCR process. These can be referred to as amplicons or PCR products. Amplicons for eDNA are usually 100-150 nucleotide base pairs (bp) in length, although at times are slightly smaller or larger, particularly for metabarcoding efforts where amplicons can be several hundred bp in length.

Amplification – Laboratory process where millions of copies of target DNA are produced through the process of polymerase chain reaction (PCR) to allow for visualization and measurement.

Annealing Temperature – The temperature at which primers bind to single stranded DNA template during PCR. Because the primers mediate replication of the DNA, it is essential that the annealing temperature be optimized for each primer set. Proper optimization will affect assay efficiency, sensitivity, and specificity.

Assay – An assay refers to the primers (forward and reverse) and fluorescently labeled probe that are used for species detection. Depending on the context, assay may also include the reaction chemistry (type of taq polymerase) and thermocycler conditions. Assay is often used interchangeably with the term eDNA marker.

B

Barcode genes – Refers to genes that can be used for species identifications. Different regions of DNA mutate at different rates and some change at just the right rate to be stable within a species but different between species. These are known as barcode genes. Most barcode regions are phylogenetically informative such that the higher taxonomic source of a sequence can be inferred even if the actual sequence has no exact match in a database of reference sequences. Environmental sequences can also be assigned to operational taxonomic units (OTUs), which may represent taxonomic

groups not yet recognized by systematics or not adequately represented in sequence databases (https://www.naturemetrics.co.uk/edna-glossary/).

Barcoding – Is a method of species identification based on the DNA sequence of a section of a specific gene or genes. By comparing the DNA sequence obtained from an unknown organism to that of a reference database, an individual sequence can be uniquely identified based on its similarity to sequences of known origin. This method is generally used to identify individual specimens from which a muscle, blood, hair or other tissue sample is obtained. This in in contrast to metabarcoding which allows for the simultaneous identification of many specimens from a single sample based on sequence similarity (see '*Metabarcoding*').

Bioinformatics – data processing science that takes the raw sequence data from high-throughput sequencing (often many millions of sequences) and transforms it into usable ecological data. Key steps for metabarcoding pipelines include quality filtering, trimming, merging paired ends, removal of sequencing errors such as chimeras, clustering of similar sequences into molecular taxonomic units (each of which approximately represents a species), and matching one sequence from each cluster against a reference database. The output is a species-by-sample table showing how many sequences from each sample were identified as each species (https://www.naturemetrics.co.uk/edna-glossary/).

Blank – an "empty" eDNA sample that is collected without filtering/centrifuging actual water from the sampling environment. Blanks are a type if field control used to evaluate whether there is contamination present in the samples/equipment and, by extension, if any positive detections are valid. Depending on the timing and location that the blank sample is prepared, these controls can evaluate different parts of the sample collection process. See Bottle/Field Control, Cooler Control, Pump/Equipment Control, Dry Control, Rinsite Control, and Process Control for details on specific blank collection procedures.

Bottle/Field Control – A negative field control. The control bottle is opened at the field collection site, re-sealed after 5 seconds, and then fully submerged into the field water (USFWS 2019). Bottle controls are stored with the other sample bottles both before and after being submerged. It is important to replicate the exact same storage, and transportation process used for field samples.

C

Centrifugation – Sample processing method utilizing a centrifuge whereby sample tubes (ordinarily containing a liquid) are spun at very high speeds that results in concentrating of particles suspended in the liquid to the bottom of the tube and subsequent separation of the particles from the liquid medium. For environmental DNA samples, centrifugation is an alternate concentration strategy to filtration.

Centrifuge Control – A 2-part negative field process control. The first control is created with a batch of tubes that are being centrifuged in order to collect the material at the bottom of the tube. The centrifuge process control should be labeled with the sample ID range that was included with the batch of tubes that were centrifuged at the same time.

The second centrifuge process control is created when tubes are set out to dry. Controls are prepared by placing a clean centrifuge tube in a clean rack in the drying area along with the range of samples that were placed in the hood to evaporate. The labelling for the control should contain the sample ID range that makes up the batch the control was associated with (example 1-46). Both the tube and lid should be labeled since they are separated during the evaporation process.

Community DNA – DNA extracted from a mixture of different organisms. Could be eDNA (environmental samples almost always contain DNA from a mixture of species) or organismal DNA (e.g. homogenized insect trap samples, plankton tows, larval fish samples, or gut content samples) (https://www.naturemetrics.co.uk/edna-glossary/).

Contamination – the transfer of genetic material (or derivative) from the target organism to samples or surfaces that did not naturally contain the organism's genetic material. Contamination can occur during field work (sampling) or in the laboratory if sampling and/or processing protocols are not followed (Mosher et al. 2019).

Cooler Control – A negative field control. Cooler controls remain sealed in the cooler and are not exposed to the atmosphere at the field collection site nor are they submerged in the field water supply. It is important to replicate the exact same storage, and transportation process used for field samples.

Conventional Polymerase Chain Reaction (cPCR) – See end-point PCR

Ct:(Cycle Threshold) – This is a metric used to determine a positive PCR/qPCR reaction which is measured by the accumulation of a fluorescent signal. Ct is the number of cycles required for the fluorescent signal to exceed background levels (the threshold).

D

Degradation – the process whereby DNA is broken down into smaller fragments. DNA obtained from eDNA samples is often 'degraded' and requires shorter length target amplicons for successful amplification. eDNA degrades through a variety of processes including exposure to chlorine bleach, ultraviolet light, higher temperatures, turbulence, or microbial activity.

Detection – A result that indicates a target taxon's DNA is present at the sample collected

Detection Probability – The probability of detecting an organism's DNA when present at a site, in a sample, or in a PCR replicate (also known as false negative probability; see "sensitivity" and "Type II error", Mosher et al. 2019).

Digital PCR (dPCR) – PCR assay in which a sample is partitioned into thousands of independent reactions that are each analyzed as end-point PCRs. The proportion of positive reactions can be directly related to the concentration of target in the original sample without need of a standard curve and may allow for a higher tolerance to PCR inhibitors present in the sample (see Quan et al. 2018).

Digital droplet PCR (ddPCR) – See also digital PCR. ddPCR is a method for performing digital PCR that is based on water-oil emulsion droplet technology. A sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet. ddPCR technology uses reagents and workflows like those used for most standard probe-based qPCR assays (https://seakfhp.org/wp-content/uploads/2019/04/eDNA_terminology.pdf).

DNA extraction – The process of separating DNA from an environmental sample, organismal tissues, cellular material, or any other type of biological sample that contains DNA. Many DNA extraction methods are available, and their use is primarily determined by sample type, cost, ease of use, laboratory proficiency, efficiency, and resulting DNA quality.

Dry Control – A negative field control. Dry control filters are attached to the pump system while it is turned on, exposing the filter to the field site atmosphere and collection process, but no water is used. It is important to replicate the exact same collection, storage, and transportation process, but without filtering any water.

E

Endogenous DNA – DNA that originates from the target species local to the collection location. See also exogenous DNA.

End-point PCR (epPCR) – A PCR assay in which the presence of the target amplicon is assessed after the reaction protocol has completed. Assessment of amplification is typically made by passing resulting PCR products through an agarose gel in which they are stained and visualized. Also referred to as conventional PCR (cPCR, see also Gel Electrophoresis).

Environmental DNA (eDNA) – DNA deposited in the environment through a variety of biological processes (e.g. excretion, shedding, mucous, slime, saliva, gametes, etc.) from single-celled (i.e. bacteria) or multicellular organisms (i.e. fish, amphibians, reptiles, mollusks, etc.). eDNA can be collected in environmental samples (e.g. water, sediment, soil, air, etc.) and used to identify the organisms from which it originated. Although eDNA is most accurately referred to as a sample type, it is also often referred to as a method, laboratory application, analysis, or technique.

As defined in Taberlet et al. 2018, 1st Edition, “Environmental DNA: For Biodiversity Research and Monitoring”, eDNA is defined as: Environmental DNA is a complex mixture of genomic DNA from many different organisms found in an environmental sample (Taberlet et al. 2012a). Soil, sediment, water, or even feces are considered as environmental samples, which can also include the material resulting from filtering air or water, from sifting sediments, or from bulk samples (e.g. the whole insect content of a Malaise trap). Alternatively, environmental DNA can be defined from another perspective (i.e., the objective of the study). In this case, eDNA corresponds to DNA extracted from an environmental sample with the aim of obtaining the most comprehensive DNA-based taxonomic or functional information as possible for an ecosystem under consideration. Total eDNA contains both intracellular and extracellular

DNA (Levy-Booth et al. 2007, Pietramellara et al. 2009). Intracellular DNA originates from living cells or living multicellular organisms that are present in the environmental sample. Extracellular DNA results from cell death and subsequent destruction of cell structures, and can be degraded through physical, chemical, or biological processes. For example, DNA molecules can be cut into smaller fragments by nucleases. After its release, extracellular eDNA may be adsorbed by inorganic or organic surface-reactive particles such as clay, sand, silt, or humic substances.

eDNA assay – Laboratory procedure to detect the presence of DNA from a target species in an environmental sample.

Exogenous DNA – DNA that originates from outside of the study system and is transported into the sampling environment and/or water supply. Examples of exogenous DNA sources include but are not limited to boats (bilge water, live wells, biofilms, etc.), fishing equipment, wastewater outfalls, feces from migratory or wide-ranging species like birds, anglers and hunters transporting then disposing of carcasses.

F

False Detection – A result that indicates that a species is present when it is not.

False Non-Detection – A result that indicates that a species is not present when it is.

False Detection Probability – the probability of detecting an organism when not present at a site, in a sample, or in a PCR replicate (see “specificity” and “Type I error”, Mosher et al. 2019).

Filtration – Passing water samples through a membrane that captures eDNA-containing particles. The pore size and membrane material are important considerations in selecting a filtration strategy.

Filter Pore Size – The size of the pores in filters used to separate eDNA from collected water, often measured in µm. Filer pore size influences the sizes and quantities of retained eDNA fragments and ambient particulate matter including inhibitors. Environmental DNA detections efficiencies can be significantly affected by filter pore size choice.

Field Control – negative controls used to evaluate contamination during field sampling and transport to the lab.

Filter Control – A negative field process control. A new filter is placed in the filter cup and clean, target DNA-free water is poured through it. The filter is immediately replaced, and the field sample is processed through the same filter cup.

Flocculation – The process of adding a chemical agent to a solution to cause a target substance to be deposited as a flakes or floccules to aid in physically removel. Following eDNA sample collection,

chemicals such as lanthanum chloride can be added to water samples to cause DNA along with other particulates to form floccules that can be collected and used during subsequent analyses. Flocculation allows greater sample volumes to be collected than precipitation and is an alternate eDNA concentration strategy to filtration and centrifugation; although, it requires the transportation and storage of large amounts of liquid compared to filtration and field centrifugation.

G

g-Block – Synthetic double stranded DNA created specific to each qPCR marker. g-Blocks include both the primer and probe binding locations and serve as positive control material for amplification and quantification of a given DNA target based on serial dilutions.

Gel Electrophoresis – technique commonly used in laboratories to separate charged molecules like DNA, RNA and proteins according to their size. Charged molecules move through a gel when an electric current is passed through it.

Grab Sample/Bottle – samples taken directly by hand with an open container, by skimming or dipping at or near the surface. A dip sample is a simple extension in that the container is affixed to a handle to extend the reach of the sampling individual. The grab sample may be in the form of a filter cup that is already attached by tubing to a pump/filter apparatus, or may also be taken at a defined depth through use of a submerged sampling device (i.e. Van Dorn, Kemmerer, etc.)

H

High-throughput sequencing (HTS) – Sequencing technology that produces millions of sequences in parallel. Enables thousands of different organisms from a mixture of species to be sequenced at once, so community DNA from a sample can be sequenced. Also known as Next-Generation Sequencing (NGS) or parallel sequencing (https://www.naturemetrics.co.uk/edna-glossary).

I

Internal Positive Control (IPC) – A small amount of amplifiable DNA that is added to each environmental sample to evaluate and measure whether samples demonstrate PCR inhibition and to what degree. DNA samples that display PCR inhibition must be purified further until confidence in PCR results can be obtained.

Inhibition – Also referred to as PCR inhibition. During DNA extraction of environmental samples, various chemical compounds can co-purify along with the DNA and interfere (inhibit) the PCR amplification process. High levels of inhibition can lead to false negative results in PCR reactions. Inhibition is measured and monitored through the use of internal positive control DNA.

Inhibitor – Substances that interfere with the PCR amplification process and can cause PCR inhibition. See Inhibition.

In-situ filtration – Filtration that occurs within the sampled body of water. In-situ filtration often involves in-line filtration of water through a filter assembly attached to a pumping device.

J

K

L

Lab Control – Lab controls are both negative and positive controls. Negative lab controls assess the presence of contamination during sample processing in the laboratory, and screen for sample inhibition when necessary. Positive lab controls evaluate whether the sample DNA was successfully extracted and the PCR reaction properly prepared.

Limit of detection (LOD) – the smallest quantity of a target DNA that can be identified using a particular technique, with some level of confidence (e.g., 95%). (Mosher et al. 2019 and https://www.sciencebase.gov/catalog/item/57e92b77e4b09082500c90ea).

Limit of quantification (LOQ) – the smallest quantity of a target DNA that can be accurately quantified using a particular technique, with some level of precision (e.g., 35% Coefficient of Variation). The LOQ is typically higher than the LOD, because at very low concentrations of target DNA, the PCR may not sufficiently approximate the expected exponential growth of the DNA target upon which quantification is based (Mosher et al. 2019 and https://www.sciencebase.gov/catalog/item/57e92b77e4b09082500c90ea).

Long-term preservation - preservation methods intended to preserve eDNA samples for >24 hours (often indefinitely). Examples of commonly employed long-term eDNA preservation techniques include freezing, desiccation, and the addition of buffer solutions. The expected shelf life and viability of stored samples can vary between storage methods.

M

Marker – A DNA sequence that is diagnostic of a taxonomic group or a specific species. Presence of the target DNA is detected by using specific primers, or primer/probe combinations, in PCR, or by sequencing. The term marker is generally used to reference the primer/probe combinations required to amplify a given DNA target. The term marker and assay are often used interchangeably.

Marker validation – Controlled experimental process by which primer/probe combinations are evaluated for use on eDNA samples. Throughout the process, a marker's sensitivity, specificity, and

optimal reaction conditions must be established. Validation includes sequence comparisons to existing sequence data for both target and non-target species, controlled laboratory testing, and testing of field samples.

Metabarcoding – Rapid biodiversity assessment that combines DNA barcoding and high throughput sequencing techniques. This combination allows identification of multiple species from community DNA. PCR is carried out with taxon-specific primers (e.g. all bivalves in a sample, all fish in a sample, all salmonids in a sample, etc.), followed by high-throughput sequencing and bioinformatics processing. The process can identify hundreds of species in each sample, and 100+ different samples can be processed in parallel to reduce sequencing cost.

Metagenetic sequencing – See metabarcoding. Metagenetic sequencing is often used interchangeably with metabarcoding. Some make the subtle distinction that metabarcoding targets a single barcode gene while metagenetic sequencing targets multiple barcode genes.

Metagenomic sequencing – Sequencing of random genomic fragments from complex environmental mixtures. Metagenomics can provide a less-biased assessment of community composition because taxonomically informative sequencing is not restricted to one or a few genetic barcodes but is much less efficient than metagenetics for this purpose because most randomly captured fragments will be uninformative. Metagenomics is more traditionally used to identify functional classes of genes present in a complex microbial sample, in order to predict metabolic capabilities of the community as a whole.

Minibarcodes – The traditional DNA barcode is a 650bp portion of the COI (mitochondrial cytochrome oxidase subunit I) gene and was suitable for Sanger sequencing. Newer high throughput sequencing technologies are restricted to shorter read lengths and required a redesign of the traditional 650bp barcode. These shorter (100-450bp) barcodes are referred to a minibarcodes. Shorter barcodes are also advantageous for eDNA work as they are more reliably amplified from degraded or fragmented DNA.

MIQE standard – A minimum information standard for quantitative PCR experiments adopted by some journals and often used as a standard in peer review. eDNA experiments have QC requirements that extensively overlap with the MIQE standard. For more info see, The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55(4): 611-622. doi: 10.1373/clinchem.2008.112797.

Mitochondrial DNA (mtDNA) – DNA derived from the mitochondria of a cell and ordinarily is the general target for most eDNA assays. Mitochondria are organelles located in the cytoplasm of a cell and produce energy. Because mitochondria play such an important role for the cell, ordinarily a single cell contains many dozens to many hundreds of mitochondria, each containing a mitochondrial genome. By default, mitochondrial DNA increases the probability of detection of an eDNA target due to the increased number of copies residing in each cell.

N

Negative control – Samples lacking target DNA. Negative controls are incorporated in both field and laboratory steps of sample processing to monitor for false positive detections resulting from contamination.

Next-generation sequencing – Catch-all term referencing modern high-throughput sequencing technologies and their applications. Common platforms for surveying environmental sequences are Illumina, Roche 454, and Ion Torrent, in various configurations. The specifications of these platforms differ in the lengths of fragments than can be sequenced, the number of independent fragments that can be sequenced per run, the duration and cost of the sequencing run, the error distribution of the data, and the number of independent samples that can be combined in a sequencing run. The key innovation shared by all classes of next-generation sequencing technology is that thousands to millions of independent reactions can be recorded in parallel.

Nuclease-free – Certified not to have nuclease (DNA-degrading) activity above a designated threshold. It is important that eDNA storage solutions be based on nuclease-free reagents.

O

Operational taxonomic unit (OTU) – A sequence that is classified as distinct at some threshold from other environmental sequences or known reference sequences and is postulated to derive from a phylogenetically distinct group of organisms. Environmental DNA sequences generated at a barcode locus are often clustered into distinct group (OTUs) that are assigned provisional taxonomic positions and then analyzed under the assumption that the clustering process approximates the genetic distinctiveness of species or higher-level taxa. OTUs are useful for estimating abundance and diversity metrics that are like what would be obtained if each species in a community sample were fully characterized at the barcode locus, which is rarely the case in practice.

Organismal DNA - DNA sampled directly from the target organism through whole organism collection, swabbing, blood sampling, fin clips etc. This practice generally results in highly concentrated and high-quality DNA sourced from a single individual. The species identity and collection location are definitively known. Overall, there are fewer uncertainties than for eDNA.

P

PCR assay – See **Assay**

Polymerase chain reaction (PCR) – An enzymatic reaction used to make copies of a specific DNA segment through a series of heating and cooling steps. This exponential amplification process is mediated by primers and generates thousands to millions of copies of a particular DNA target. Variants of a PCR assay include end-point PCR (or “conventional” PCR), in which the presence or absence of the

intended target is inferred only after the reaction completes; quantitative PCR, in which the amount of input target DNA is inferred by monitoring the change in DNA concentration as the reaction proceeds; and digital-droplet PCR, which also quantifies the amount of input target DNA by creating many random independent reactions from the input solution and determining the proportion of which produce an amplification product.

Pooled samples/replicates – Samples or replicates that are combined prior to analysis. An example of pooled sample is a group of grab samples collected along a transect at the same site and then filtered through a single filter.

Positive control – Samples to which a known target DNA has been added. Positive controls should be incorporated in both field and laboratory steps of sample processing to identify methodological issues contributing to false negatives. These issues might include degradation during storage, PCR inhibition, or sequencing bias. Internal positive controls are added directly to samples to be analyzed, whereas external positive controls are added to a DNA-free solution. Internal positive controls are typically used to provide evidence of inhibition during a qPCR reaction, whereas external positive controls are used as evidence of reagent quality, DNA extraction efficiency, and proper thermal-cycler function.

Precipitation – The process of adding a chemical agent to a solution to cause a target substance to be deposited as a solid that can be physically removed. Following eDNA sample collection, chemicals such as ethanol-sodium acetate can be added to water samples to cause DNA to form a precipitate that can be collected and used during laboratory analysis. Precipitation is an alternate eDNA concentration strategy to filtration and centrifugation; although, its application is typically limited to small sample volumes.

Preservation – The steps taken to stabilize the eDNA in a sample by countering the main forces of degradation that threaten eDNA integrity. Environmental DNA preservation methods often take the form of short-term or long-term preservation methods.

Primary Equipment – Field equipment that comes in direct contact with the eDNA sample (e.g. sample collection bottles, filters, filter funnels, filter cups, forceps, filter storage tubes, preservation additive, etc.).

Primers – Short sections of synthesized DNA that bind to either end of the DNA segment to be amplified by PCR. Can be designed to be specific to a particular species (so that only that species' DNA will be amplified from a community DNA sample), or to be very general so that a wide range of species' DNA will be amplified. Good design of primers is one of the critical factors in DNA-based monitoring.

Primer bias – An artifact of metabarcoding studies when generic or universal primers preferentially amplify one or a group of species over others. This preferential amplification can lead to false negative detections and or cause read count data to be skewed towards the species that amplify with a higher degree of efficiency.

Probe – An oligonucleotide that complements the intended target and is added along with primers to a qPCR. The probe matches a sequence in between the two primers and does not contribute to amplification of the target sequence. The probe produces a fluorescent signal that is measured by a qPCR platform. Several types of probes are successfully used in eDNA work. See Probe-based qPCR.

Probe based qPCR – Probe-based qPCR functions by recognition of a specific sequence on the desired PCR product. Unlike SYBR® Green qPCR methods, that use an intercalating dye to bind all double-stranded DNA, probe-based qPCR uses fluorescent-labeled target-specific probes. This technique yields increased specificity and sensitivity since only specific DNA molecules will be labeled. Other fluorescent dyes can also be combined with probe-based qPCR to label and quantify various sequences.

Process Control: – A negative field control. Process controls are collected when the sample concentration process (filtering or centrifugation) is not performed at the field sample collection site (such as back a vehicle or in an equipment trailer). The two main kinds of process controls are Filter Controls and Centrifuge Controls.

Pump/Equipment Control – A negative field control. Pump controls are collected at the same locations using the same procedures and equipment as field samples, but with "clean" water (such as deionized, distilled, or well water that is known to be free of target species DNA) poured onto and pumped through the filter. It is important to replicate the exact same sample collection, storage, and transportation process for water sampled in the field.

Q

qPCR assay – See **Assay**

Quantitative PCR (qPCR) – A PCR reaction incorporating a colored dye that fluoresces during amplification, allowing quantification of a given target in the reaction. Often used with species-specific primers where detection of amplification is used to infer presence of the target species' DNA in the sample. If the target DNA is not present in the sample, no fluorescence will be detected. The high specificity of the qPCR method makes it ideal for situations where a single target is required. See also real-time PCR.

R

Real-time PCR – A PCR reaction incorporating a colored dye that fluoresces during amplification, allowing a machine to track the progress of the reaction in "real-time." This term is often used interchangeably with quantitative PCR (qPCR).

Reference Databases – A collection of DNA sequences (usually from barcode genes) that have been obtained from species of known identity. Sequences from unidentified organisms – obtained either by

Sanger sequencing or high-throughput sequencing – are compared against a reference database to make species identifications. Databases can be curated (e.g. the Barcode of Life Database – BOLD – www.boldsystems.org (Ratnasingham and Hebert, 2007) or uncurated (e.g. GenBank – www.ncbi.nlm.nih.gov). In curated databases, identifications are scrutinized and verified; in uncurated databases they are not. GenBank is therefore far more extensive than BOLD but contains many more errors.

Replication – DNA replication is the process by which copies of DNA are made during cell division. This process is artificially achieved in the laboratory through the PCR process.

Rinsate Control – A negative field control. Studies that reuse sampling equipment in the field need to decontaminate gear between sample collections (often with bleach or DNA Away solution). Rinsate controls evaluate if field decontamination was successful. A rinsate control is collected using the decontaminated gear and clean (deionized, distilled, or target DNA-free water) water, followed immediately by collection of the actual sample using the same equipment but with water from the field.

S

Sanger Sequencing – Traditional DNA sequencing. Each reaction produces a single sequence, so it only works on amplified DNA of a single species. As such, Sanger sequencing is not appropriate for use on community DNA or most eDNA sample types.

Short-term preservation – preservation methods often taken immediately after sample collection that are intended to reduce DNA degradation rates for <24 hours following collection. Cooler storage is a commonly employed short-term preservation method when collecting water samples.

Shotgun sequencing – a strategy in which all DNA sequences in a sample are sequenced in an approximately random fashion without any PCR-based selection of genetic loci. Not commonly used for eDNA at present because techniques for identifying the source organisms are not well developed or validated for this use.

Secondary Equipment – Field equipment that is used in every sample collection (coolers, bottle racks, pumps, pump tubing, “grabber arms”, etc.) but that does not contact the sample directly.

Sensitivity – The minimum number of target copies in a PCR reaction that can be detected. Typically, sensitivity is expressed as the limit of detection (LOD), which is the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure.

Specificity – The ability of PCR primers and/or probe to detect the appropriate target sequence rather than alternative, nonspecific targets also present in a sample. In most instances a PCR assay is referred to as specific if it amplifies only the species for which it was designed.

Standard curve – Used to calibrate qPCR assays, serially-diluted standard solutions with known concentrations of the target template are processed in conjunction with the samples to be quantified.

The standard curve estimates reaction efficiency as well as a conversion factor for calculating target copy number from the primary measured variable for each sample.

SYBR Green – A fluorescent dye that binds with all double-stranded DNA in a sample or PCR reaction. SYBR Green is used in qPCR assays to measure DNA accumulation during the amplification process. Unlike probe-based qPCR assays, SYBR Green does not convey added specificity to the assay because it will bind to any double-stranded DNA in the reaction, potentially producing non-specific fluorescence. As a result, SYBR green a less appropriate choice for species-specific detection from eDNA.

T

Taq Polymerase – an enzyme critical for DNA replication through PCR amplification. Taq polymerase binds to single stranded DNA and adds complimentary nucleotides to the DNA strand to which it is bound, creating a copy. Taq polymerase also functions in DNA proof reading, where it finds mismatched nucleotides and replaces them with complementary nucleotides.

Tertiary Equipment – Field equipment that is used to access the collection site or transport equipment to and from the collection site but is not a part of the collection system (boats, backpacks, drybags, PFDs, waders and boots, etc.).

Thermocycling – a series of temperature cycles used in PCR reactions that allows for Taq Polymerase to activate, primers to anneal, and DNA replication to occur, resulting in target DNA replication. See Polymerase Chain Reaction.

Threshold cycle (Ct) – A unit of measure referring to the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction. Often used interchangeably with quantification cycle (Cq).

Type I Error – the probability of detecting an organism when it is not present in a sample (see "specificity" and "false positive probability", Mosher et al. 2019).

Type II Error – the probability of failing to detect the target organism when it is present in a sample (see "sensitivity" and "detection probability", Mosher et al. 2019).

U

V

W

X

Y

Z

Acronyms

cPCR - conventional end-point polymerase chain reaction
Ct – threshold cycle
Cq – quantification cycle
dPCR – digital polymerase chain reaction
ddPCR – digital droplet polymerase chain reaction
epPCR – end-point polymerase chain reaction
DNA – deoxyribonucleic acid
eDNA – environmental deoxyribonucleic acid
mtDNA – mitochondrial deoxyribonucleic acid
GenBank – Familiar name of a suite of biotechnology databases maintained by NCBI
HTS – high throughput sequencing
LOD – limit of detection
LOQ – limit of quantification
MIQE – minimum information for publication of quantitative real-time PCR experiments
NCBI – national center for biotechnology information
NGS – next-generation sequencing
OTU – operational taxonomic unit
PCR – polymerase chain reaction
qPCR – quantitative real-time polymerase chain reaction

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