GUIDELINES
FOR
DESIGNING ENVIRONMENTAL DNA SURVEYS
FOR TARGET SPECIES

DRAFT

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Note: these guidelines were developed on Department of Defense lands in the coterminous U.S. and represent the state of knowledge at this date (December 2015). They are intended for stream and wetland systems and will require adjustment for larger river, lake, or ocean systems.
1.0 Deciding whether to use eDNA surveys for target species

Environmental DNA is a powerful tool for detecting aquatic species, and in many cases it can be more accurate and efficient than traditional field surveys. This may be especially true if the target species occurs in very low densities or is difficult to distinguish from similar species. Environmental DNA surveys may also be advantageous if current survey methods yield low detection probabilities, are destructive to the species or its habitat, or require extensive training or certification for personnel conducting surveys.

However, species detection with eDNA may not be better than conventional field surveys in all monitoring situations. If current survey methods provide high detection rates for a relatively low cost or time investment, eDNA methods are not needed. There may also be species or systems for which detection probabilities for eDNA surveys are low due to environmental conditions that contribute to movement or degradation of eDNA in the aquatic system.

The application of eDNA as a tool for detecting species will be different for every system and depends on the goals of the program, characteristics of the target species, and conditions of the systems to be sampled. The decision tool in Figure 1 can guide managers in deciding whether eDNA surveys may be beneficial as a replacement or supplement to current survey techniques.

Figure 1. Decision support tool for determining how environmental DNA sampling can complement or replace current survey methods.
2.0 Conducting a pilot survey

Once it has been determined that eDNA surveys can potentially be useful for monitoring of target species, a pilot survey should be developed and applied.

Environmental DNA detection rates depend on a variety of characteristics of the target species, conditions of the aquatic system, and sampling methods. By identifying the factors most likely to influence detection probabilities, managers can adapt sampling strategies to increase the probability of detecting the target species if it in fact occurs at the sampling site. The best way to do this is to conduct a pilot survey in which environmental factors are 1) measured at the same time eDNA water samples are collected, 2) analyzed to determine the most important factors in predicting species detection, and 3) used to modify sampling strategies to improve detection probabilities.

For example, eDNA detection probabilities for Chiricahua leopard frogs in Arizona were found to be strongly influenced by the size of the wetland that was sampled (Goldberg et al. *in prep*). By increasing the number of eDNA sampling locations as wetland size increased, detection probabilities for Chiricahua leopard frogs at large wetlands were improved. Similarly, eDNA detection of Sonora tiger salamanders was limited by the amount of sample water that could be filtered (Goldberg et al. *in prep*). Water samples from Sonora tiger salamander ponds was very silty and the filters tended to clog before the desired water volume was reached. Switching to eDNA filters with a larger pore size greatly improved detection probabilities for this species.

A pilot survey makes adaptive sampling strategies such as these possible. Ideally, the pilot eDNA survey is conducted simultaneously with conventional field surveys so detection probabilities for each method can be directly compared.

2.1 Designing the pilot survey

The pilot survey’s timing, sampling intensity, and environmental measurements are determined by the ecology of the target species and its habitat. This section provides guidance about when, where, and how much to sample.

2.1.1 Timing

The survey should be timed for the season with the highest species density in the water, as long as that coincides with the life stage of interest. For amphibians, this is likely to be during tadpole development if reproducing populations are the target of the surveys (as opposed to adults that may not be successfully reproducing).

Environmental DNA degrades fairly quickly in water, generally persisting for 1-3 weeks, though eDNA may persist longer in cold water bodies or degrade more quickly in very warm or acidic systems (Strickler et al. 2015, Barnes and Turner 2015). Environmental DNA surveys that are conducted more than a few weeks after the species occupied the site may fail to detect the species’ eDNA simply because the eDNA has degraded.
2.1.2 Environmental covariates

Identify characteristics of the aquatic system that are likely to affect eDNA concentrations in the water. These may include factors that may influence eDNA degradation (for example, water temperature, pH, or solar radiation), transport in streams (such as current velocity, discharge, or channel complexity), or diffusion in ponds, lakes, and wetlands (such as water body area, depth, or complexity). Select environmental covariates that are meaningful and can be measured in the field efficiently and accurately. Additionally, record data related to sampling methods, such as volume of water filtered or spatial arrangement of sampling locations, which may also affect eDNA detection.

2.1.3 Number of samples

Collect more than 1 sample at each site so that detection probabilities can be estimated. In streams, 2 samples per site has generally been sufficient for >0.95 detection of amphibians, but at least 8 samples are required for good detection probabilities of amphibians at acidic wetlands. During the pilot survey, consider collecting 4 samples per site. The occupancy modeling framework used to analyze pilot survey results can help calculate the relative value (in terms of improved detection) of each additional sample. Additionally, the optimal number of samples may be dependent on sample volume and spatial arrangement of samples.

2.1.4 Sample volume

In streams, eDNA water samples are generally 1 L, while in wetlands 250-500 mL is usually sufficient. The targeted sample volume is a balance between detection and efficiency: larger sample volumes may increase detection, but may be inefficient to collect and filter in the field.

The sample volume is often dependent on how much water can be filtered before the filter clogs, which is related to filter material and pore size. Generally, PES, cellulose nitrate, and mixed cellulose filters all perform equivalently for detecting eDNA of vertebrates (up to 5 µm filter pore size), but some sensitivity is lost for micro-organisms such as viruses.

2.1.5 Spatial arrangement of samples

Environmental DNA is not uniformly distributed in a water body, so it’s important to collect water samples where eDNA concentrations are likely to be high. Concentrations are highest close to the target organism, then decline as eDNA is carried away from the source by factors such as downstream transport in streams or diffusion in ponds and lakes.

Lotic systems: Much is still unknown about how eDNA moves in streams, but generally the thalweg, or deepest part of the stream, should have the most mixing of eDNA and may be a good sampling location. If animals tend to congregate in slower waters, higher detection may be obtained there. As with any sampling protocol, the most critical component for sampling streams is to be consistent about where samples are collected for each sampling replicate and across all sampling occasions.
**Lentic systems:** Diffusion is often a limiting factor for detection. In small ponds and wetlands, sampling at a single location may be sufficient, but in larger systems (e.g., > 1200 m²) samples should be collected from multiple locations (generally every 50 m around the perimeter). Samples should be replicates, so combining sample volumes from multiple spatial locations in equal volumes in each filter is more informative than using a single filter per sampling location at a site.

2.2 **Analyzing pilot survey results**

Detection probabilities can be estimated from replicate samples to determine if the pilot design is efficient enough for application or if changes in survey design need to be applied. Occupancy modeling can be used to determine limiting factors to detection (e.g., area, water quality) if those factors were measured at the same time pilot samples were collected. Concurrent field sampling can be helpful to confirm true occupancy at sampled sites.

3.0 **Implementing an adaptive sampling protocol**

Pilot survey sampling and analysis provide estimates of the probability of detecting the target species in eDNA samples as well as the environmental factors that affect those probabilities. If field surveys were conducted concurrently with eDNA sampling, error rates (sampling events in which the species was detected with eDNA sampling but not with field surveys, and vice versa) can also be compared.

Detection probabilities for the target species can be considered to be acceptable if they are above an agreed-upon threshold (often 0.75, but may be higher depending on monitoring requirements) or if they are consistently higher than field surveys. In these cases, the original sampling protocol can be used to continue eDNA monitoring with the same timing, sample volume, number of samples, filter pore size, etc.

However, if detection probabilities are low and are strongly influenced by environmental or sampling factors, it may be possible to improve detection probabilities by adapting the sampling strategy to address the influence of those factors. In the Chiricahua leopard frog example above, detection was influenced by the size of the wetland, and detection probabilities were improved by modifying the sampling intensity at larger wetlands.

Environmental factors and sampling approaches should continue to be measured and analyzed as part of a long-term monitoring protocol. It’s possible that the relative influence of different factors on detection could change over time if there are changes in environmental conditions, the target species’ distribution and abundance, or sampling methods or materials.
4.0 Lab protocol: designing species-specific qPCR assays

Species-specific surveys use quantitative polymerase chain reaction (qPCR) assays that are designed and validated for each species. Quantitative PCR provides estimates of the amount of the target species’ DNA in the eDNA sample. Conventional PCR, which simply screens eDNA samples for the presence of DNA from the target species, is not recommended because it is typically less sensitive than qPCR, and may cross-amplify and provide false-positive results.

Species-specific qPCR assays need to be validated to ensure that they are both sensitive (that is, detect the species’ DNA when it’s present in the sample) and specific (do not detect the DNA of closely related non-target species). It’s important that qPCR assays are designed and validated in adherence to the following steps:

1. Create an inclusive consensus sequence that incorporates all within-species variability for a species in a well-known region of DNA. Mitochondrial DNA is generally preferred because it is more abundant than nuclear DNA, and more sequence data are available. However, nuclear regions can also be used. Sequences can be from GenBank (National Center for Biotechnology Information, 2012), or can be obtained by sequencing tissue samples of target species. If closely related species co-occur in the area where the test will be applied, it is helpful if sequences for those species in the targeted region of DNA are also present in the database, so that cross-amplification can be avoided in the design stage. It is important that the data incorporated include adequate sampling in the geographic area where the test will be applied.

2. For the selected probe chemistry (e.g., MGB-NFQ), set appropriate qPCR primer software to design primers and probe. Alternatively, identify unique primers and probe from an alignment of target and co-occurring non-target species sequences, meeting quality-control criteria for the chosen chemistry (e.g., melting temperature, lack of GC-clamp, product length). Optimal probe length will differ by chemistry. These primers and probe will allow for amplification and detection of the target sequence.

3. Compare the resulting design to sequences in GenBank using PrimerBlast or other software to determine if the sequences are likely to cross-amplify with other species. Try to incorporate as many differences as possible (at least 2 on each primer and 2 on the probe, including 1 toward the 3’ end of the primers and 1 in the middle of the probe) between the primer/probe design for your target species and any other species occurring in the area where the assay will be applied.

4. For validation, test the resulting assay against tissues of target (at least 10 from across the area where the test will be applied) and non-target species (at least 5 of each species from across the area where the test will be applied). It is important that these tissues were collected with clean implements so that target species DNA does not appear in non-target species samples (this can be confirmed by sequencing any non-target species amplicons). Additionally, the assay should be validated against eDNA samples from known positive and negative locations in the study area.
5.0 Guidelines for selecting a laboratory to process environmental DNA samples

When laboratory analysis of eDNA samples can’t take place in house, samples will need to be sent to a commercial, academic, or governmental laboratory for analysis. Well-equipped eDNA laboratories should follow a set of practices and procedures designed specifically for handling eDNA samples. When selecting a lab for processing samples from eDNA monitoring, make sure the following practices are part of the lab’s operating procedures.

1. Environmental DNA samples are very low quality and quantity compared with DNA samples collected directly from organisms. They therefore need to be handled in a separate room (clean room) from high-quality samples and the products of polymerase chain reactions (PCR). This clean room needs to have dedicated equipment, including pipettors, centrifuges, and any other item that is needed for sample processing. Technicians should be required to shower and change clothes or go through equivalent decontamination procedures before entering this room after having been in a lab containing PCR product.

2. For assays to be specific enough to detect only target species in eDNA samples, quantitative PCR or next-generation sequencing is required. Conventional PCR tests are not adequate unless every sample is sequenced. Laboratories should be aware of this and have the skills and equipment to analyze samples appropriately.

3. Filter tips should be used at all times when handling samples or reagents are pre-PCR to prevent cross-contamination.

4. Fifty percent household bleach should be used to clean all items that come into contact with samples (e.g., forceps) between uses and at least 10% household bleach for regular decontamination by wiping, or UV directly in contact with surfaces. Autoclaving, ethanol, and products such as DNA-away are not sufficient to destroy DNA.

5. A negative control should be extracted with each batch of extractions and tested in all downstream processes.

6. A test for inhibition should be incorporated with each sample analysis. This consists of an assay that should always amplify at a known concentration, such as an added internal control (sold as IC or IPC by several companies). Environmental DNA samples are often inhibited and false negatives reported if this control is not included.

7. The laboratory should be able to archive samples after processing (preferably at -80°C) for future analysis, if that is requested by the agency (it is reasonable to expect an additional fee for this service).

8. The agency should collect a series of samples from known positive and negative sites and send them for a blind test to the laboratory (it is reasonable to expect to pay for this service, although some laboratories may waive this cost). All sites with the species should test positive and without should test negative. However, detection probabilities may not be perfect at positive sites and sometimes field crews can introduce small amounts of DNA into samples when first learning techniques (or if clean field practices are not kept to). This testing should be an iterative process that involves working with a lab to understand where errors are occurring and fix problems during a pilot phase.
Laboratories should be willing to work collaboratively with the agency during this phase and produce accurate data from blind samples to the satisfaction of the agency before embarking on extensive sample processing.