

## REVIEW

# Critical considerations for the application of environmental DNA methods to detect aquatic species

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## Summary

1. Species detection using environmental DNA (eDNA) has tremendous potential for contributing to the understanding of the ecology and conservation of aquatic species. Detecting species using eDNA methods, rather than directly sampling the organisms, can reduce impacts on sensitive species and increase the power of field surveys for rare and elusive species. The sensitivity of eDNA methods, however, requires a heightened awareness and attention to quality assurance and quality control protocols. Additionally, the interpretation of eDNA data demands careful consideration of multiple factors. As eDNA methods have grown in application, diverse approaches have been implemented to address these issues. With interest in eDNA continuing to expand, supportive guidelines for undertaking eDNA studies are greatly needed.

2. Environmental DNA researchers from around the world have collaborated to produce this set of guidelines and considerations for implementing eDNA methods to detect aquatic macroorganisms.

3. Critical considerations for study design include preventing contamination in the field and the laboratory, choosing appropriate sample analysis methods, validating assays, testing for sample inhibition and following minimum reporting guidelines. Critical considerations for inference include temporal and spatial processes, limits of correlation of eDNA with abundance, uncertainty of positive and negative results, and potential sources of allochthonous DNA.

4. We present a synthesis of knowledge at this stage for application of this new and powerful detection method.

**Key-words:** biodiversity, eDNA, invasive species, non-destructive sampling, quantitative PCR, reporting guidelines

## Introduction

The discovery that species can be detected using environmental DNA (eDNA) in water samples has enormous potential for gaining insight into the ecology and conservation of aquatic species (Goldberg, Strickler & Pilliod 2015). Specifically,

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eDNA methods have the potential to greatly increase the data available on occurrence of rare or endangered species, allow for early detection of invasive species and estimate biodiversity. Research over the past two decades established that micro-organism communities can be described from water samples (e.g. Venter *et al.* 2004) and that macroorganism DNA can be retrieved from sediments (e.g. Hofreiter *et al.* 2003). However, it was the recent detection of bullfrog (*Lithobates catesbeianus*) DNA from water samples (Ficetola *et al.* 2008) that brought this method to the forefront for detecting aquatic vertebrates in contemporary ecosystems.

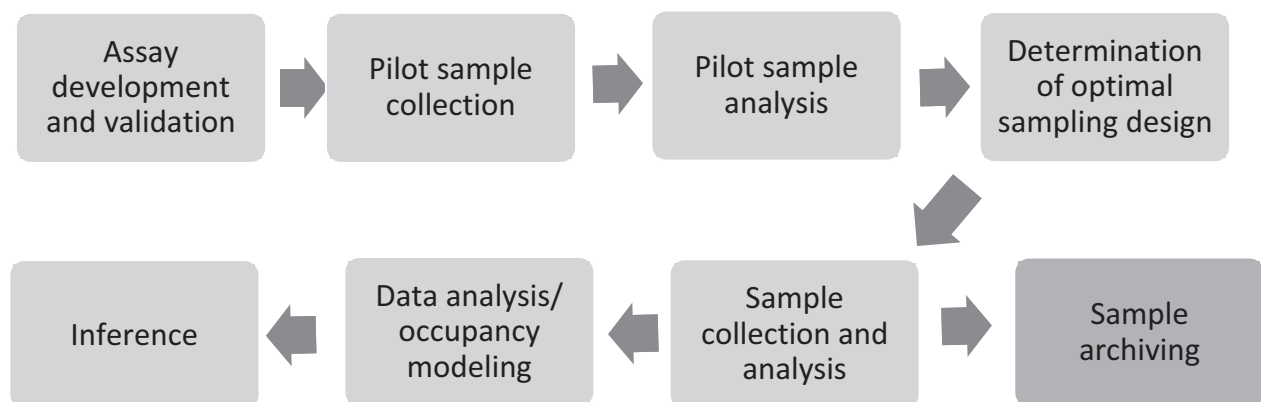
Since 2008, multiple independent research groups have developed eDNA analysis techniques, leading to a variety of protocols for eDNA detection of aquatic macroorganisms across various taxa and environments (Table S1). Currently, there are a number of diverse approaches for sampling and interpreting eDNA data. This is characteristic of emerging scientific fields, and we believe it is essential at this juncture to develop minimum reporting and quality assurance standards. Here, we bring together the knowledge of an international group of leading scientists to lay the foundation for a unified framework of current practices for implementing eDNA methods in conservation applications. Recommendations given in this paper are meant as guidance for applying eDNA methods; exact protocols used in each study will vary with the study site and questions addressed, and will progress with the development of this technology. Our goal here is to facilitate the growth of the field by establishing guidelines for preventing contamination, reporting methods and interpreting results.

### Field sampling considerations for eDNA studies

Aqueous eDNA from macroorganisms generally occurs at very low concentration (e.g. <200 pg/L) and can be heterogeneously distributed throughout a water body (Takahara *et al.* 2012; Pilliod *et al.* 2013). Species detection using eDNA sampling is therefore contingent upon detection probability (MacKenzie *et al.* 2002; Schmidt *et al.* 2013; Ficetola *et al.* 2015; Schultz & Lance 2015). This probability depends not only on the presence and concentration of eDNA in the water

sample (which reflects sampling in the right place and time for the ecology of the organism), but also on capture efficacy, extraction efficacy, sample interference (e.g. inhibition) and assay sensitivity. We recommend conducting a pilot study for each new application to assess detection probabilities for target species given sampling and analysis protocols (e.g. spatial sampling design, sample volume and collection method; Fig. 1) and site characteristics that may influence detection, such as water chemistry and temperature.

Due to the high sensitivity of eDNA methods, it is paramount to establish clean and consistent field collection protocols that minimize the probability of contamination. Protocols must include negative field controls, and studies should report details of precautions (Table 1). Field equipment, supplies and personnel should be separated from high copy number DNA settings (i.e. polymerase chain reaction (PCR) laboratory, tissue handling, organism capture) prior to sampling and analysis. Decontamination of equipment, including boots, boats and other field gear, and use of single-use disposable supplies are essential for maintaining sample independence. Typical field decontamination methods (e.g. 10% commercial bleach solution, quaternary ammonia) may be insufficient (Kemp & Smith 2005; Wilcox *et al.* 2016). Single-use supplies for eDNA collection can significantly reduce contamination risk; however, if supplies (e.g. grab bottles) are used more than once, they should be cleaned with a 50% commercial bleach solution (see following section for details) and rinsed thoroughly before reuse. Negative field controls (clean water collected using the same protocol and equipment, preserved and processed in exactly the same way as field samples) are critical for detecting contamination. Including separate negative controls at each stage can help identify the source of contamination when it occurs. Alternatively, initiating negative controls at the first stage (e.g. equipment decontamination) and handling them in the same manner as samples through all stages provides comprehensive negative control at reduced cost (but provides less information on where potential contamination occurred). For conventional PCR, when negative controls test positive for a species, all associated samples testing positive for that species must be considered suspect and should be discarded.



**Fig. 1.** Example workflow for designing and conducting eDNA studies.

**Table 1.** Minimum recommended reporting for environmental DNA studies

Stage	Information
Design	Inferential goal (presence/absence, quantity)
Water collection	Contamination precautions including negative controls Collection volume, container material, replicates, depth Site descriptions (flow rate, area, etc.)
Sample preservation	Method, temperature, duration Filter type (if applicable), filtering location (e.g. in field)
Extraction process	Contamination precautions (including dedicated laboratory), negative controls Methods including kit protocol adjustments
Probe-based qPCR	Design and validation methods Primer/probe sequences, amplicon length Positive and negative controls Inhibition detection and handling Reaction concentrations, thermal profile Technical replicates and their interpretation Standard curve preparation and quality
High-throughput sequencing	Library type (shotgun or amplicon) and any enrichment strategy Library preparation protocol or kit Platform, read length, read pairing, expected fragment size Primers, sequencing adapters, sample index tags, exogenous spike-ins Amplicon locus, target taxa, specificity and bias Read trimming and filtering of artefacts/chimeras Reference database and/or de novo OTU generation Taxonomic assignment method and parameters Statistical analysis and rarefaction Positive and negative controls and their interpretation, if applicable Technical replicates and their interpretation Number of raw reads and final reads

However, when quantification is the purpose of the study, very low or rare amplification in negative controls can have a negligible impact on results (e.g. Barnes *et al.* 2014 and Strickler, Fremier & Goldberg 2015). The number of negative controls necessary at each stage needs to be determined based on the number of samples and required confidence in inference.

Environmental DNA begins to decay immediately after shedding (Thomsen *et al.* 2012a,b); this process continues after sample collection due to mechanical forces, microbial activity (nucleases) and spontaneous chemical reactions (oxygenation, Lindahl 1993; Nielsen *et al.* 2007). Therefore, samples should be preserved using a standardized protocol as soon as possible after collection [up to 24 h on ice prior to preservation may not compromise detection (Pilliod *et al.* 2013), some protocols require filtering within 16 h [U.S. Fish and Wildlife Service (USFWS) 2015]]. Precipitation and filtration are the common methods for concentrating eDNA from water samples (Table S1). Precipitation consists of collecting a small volume of water (e.g. 15 mL, Ficetola *et al.* 2008) which is preserved

by the addition of a salt (typically sodium acetate) and absolute ethanol in the field followed by storing of the sample at  $-20^{\circ}\text{C}$ . A closely related protocol is to centrifuge the water sample (stored on ice) shortly after collection and then preserve the pellet in 95% ethanol or other DNA preservative (U.S. Fish and Wildlife Service (USFWS) 2015). The filtration method can process larger volumes of water (typically 250 mL – 5 L, but up to 100 L, Goldberg *et al.* 2011; Jerde *et al.* 2011; Valentini *et al.* 2016), which are filtered on-site or stored on ice for travel to filtration facilities. Benefits of filtering on-site include immediate preservation, which is critical for remote field locations. Alternatively, filtering in a laboratory can reduce field time as well as processing time when samples can be filtered simultaneously. Filters (and collected materials) can then be preserved by freezing (Jerde *et al.* 2011), immersion in ethanol (Goldberg *et al.* 2011), drying, or immersion in cell lysis buffer (Renshaw *et al.* 2015). Samples of eDNA have been effectively collected with cellulose nitrate (Goldberg *et al.* 2011), glass fibre (Jerde *et al.* 2011), polycarbonate (Takahara *et al.* 2012), nylon (Thomsen *et al.* 2012b), polyethersulfone (Renshaw *et al.* 2015) and cellulose acetate filters (Takahara, Minamoto & Doi 2013). While most free DNA molecules are lost during filtration (DeFlaun, Paul & Davis 1986; Liang & Keeley 2013), the majority of macroorganism eDNA may be captured by pore sizes 1–10  $\mu\text{m}$  (Turner *et al.* 2014). In some cases, filtration can provide higher detection than precipitation of the same volume (Deiner *et al.* 2015), but filter material, pore size and DNA extraction method interact to produce final detection rates (Deiner *et al.* 2015; Renshaw *et al.* 2015).

Comparisons of collection and preservation methods are underway to identify maximum efficiency, which may differ by system and target species. Suspended particulate matter (e.g. organic matter and sediment) can clog filters and may increase the concentrations of PCR inhibitors (Tsai & Olson 1992). If filters clog before a sample is completely collected, investigators should record volume sampled and consider swapping in new filters. Careful notes and sample records are critical to avoid treating multiple filters from one sample as independent samples. If clogged filters are a frequent occurrence, larger pore size filters may be required for the study (Turner *et al.* 2014).

### Clean practices in the laboratory

To obtain accurate and reproducible results from eDNA samples, stringent ‘clean laboratory’ protocols must be in place at all stages of the process, from water processing (if not done in the field) until sample plate wells containing the analysis reaction are sealed. Environmental DNA samples present the same contamination challenges as other low-quality, low-quantity DNA samples, such as ancient, forensic and non-invasive genetic samples. As with these types of samples, eDNA samples should be handled and stored in a dedicated room that is physically separated from rooms where high quantity DNA extraction and PCR products are handled (Taberlet, Waits & Luikart 1999). All equipment and supplies necessary to process eDNA samples should not leave this clean laboratory (e.g.

laboratory notebooks and pens) nor should researchers travel from laboratories where PCR product or high-quality DNA is handled to this clean laboratory without undergoing decontamination procedures (e.g. shower and clean clothes).

The reagent used for decontamination of equipment and surfaces is critical to maintaining clean standards. For example, standard autoclaving is inadequate for destroying nucleic acids (Unnithan *et al.* 2014). Of the generally applied decontaminating solutions, sodium hypochlorite (bleach) is the most effective for removing DNA and PCR products (Prince & Andrus 1992; Champlot *et al.* 2010). A dilution of 10% commercial bleach (typically ~6% sodium hypochlorite) is the standard in many laboratories (Prince & Andrus 1992); however, treatment with 50% commercial bleach is the minimum to remove extraneous DNA and PCR products (Kemp & Smith 2005; Champlot *et al.* 2010) and should be used on any surface coming into direct contact with eDNA samples that cannot be UV sterilized [note that bleach is a hazardous chemical that reacts with guanidinium thiocyanate (commonly used in lysis buffers) and produces a highly toxic component]. After using the clean laboratory, all bench-top work surfaces should be cleaned by wiping with bleach and the room should undergo thorough periodic cleaning, where all surfaces (including refrigerator handles, centrifuges, pipettes, heat blocks and floors) are wiped with bleach. Positive air pressure, air filtration and UV treatment for benchtops (such as PCR workstations with UV hood and HEPA filter) are additional measures that can help prevent contamination.

All PCRs should be assembled in the clean laboratory and brought to a separate laboratory for amplification; this separate room is where all post-PCR work is carried out. Along with positive controls, negative controls (lacking target DNA) should be created and analysed with each set of extractions and set of polymerase chain reactions. Filter pipette tips should always be used for handling samples and reagents during extraction and PCR set-up and gloves changed whenever they come into contact with a potential contaminant. In order to minimize contamination risk, it is important that every new person who starts eDNA work receives thorough and appropriate guidance on both theoretical and practical aspects of appropriate procedures to avoid contamination.

### eDNA sample extraction

For eDNA extraction, many studies have used the Qiagen DNeasy Blood and Tissue Kit (Table S1). Few direct comparisons of eDNA extractions have been published, but this approach has been shown to work better than the UltraClean® Soil DNA isolation kit for cellulose nitrate filter samples (MoBio Laboratories, Carlsbad, CA, USA), which yielded no target DNA (Goldberg *et al.* 2011), and the PowerWater® DNA Isolation Kit for glass fibre filters (Amberg *et al.* 2015) and generally provided larger numbers of detections eDNA of macroorganisms from precipitated and glass fibre filter samples (Deiner *et al.* 2015). However, phase separation and precipitation methods of DNA

extraction (e.g. CTAB–chloroform and phenol–chloroform) generally yield more DNA than silica column methods (e.g. MoBio and Qiagen kits) (Niu *et al.* 2008; Yuan *et al.* 2012), and data support this for animal eDNA as well (Renshaw *et al.* 2015; Deiner *et al.* 2015). For studies requiring eDNA quantification, it should be noted that net recovery of eDNA may vary within, as well as across, methods. Additionally, the comparative performance of these methods in removing different classes of inhibitors has yet to be established. To benefit future analyses, extraction methods should result in preserved samples archived at –20 or –80 °C.

### eDNA amplification and quantification

Initial eDNA protocols for the detection of aquatic macroorganisms used fragment analysis of conventional PCR products (Table S1). More recently, probe-based quantitative PCR (qPCR) methods have been used for single-species detection due to improved sensitivity, specificity and ability to quantify the eDNA in the sample (Pilliod *et al.* 2013; Wilcox *et al.* 2013; Amberg *et al.* 2015). Digital droplet PCR can also be used when quantification is the goal of the study, and may be more cost efficient for large numbers of samples (Nathan *et al.* 2014). If probes are not included (e.g. conventional PCR, SYBR Green PCR), the probability of cross-amplification, leading to false positives, requires subsequent confirmation of positive samples (e.g. by sequencing). Therefore, probe-based qPCR is currently the most efficient tool for eDNA detection of single or few target species, and we focus on the complexities of this method in subsequent sections. When target organisms are not *a priori* identified or there are more than a few target species in a study, high-throughput sequencing (HTS) can quickly become advantageous (Thomsen *et al.* 2012a,b; Mahon, Nathan & Jerde 2014; Evans *et al.* 2016). **Although still at the early stage for conservation application, the use of HTS on water samples holds enormous potential for future studies of aquatic biodiversity (Valentini *et al.* 2016).**

### qPCR assay design and validation

Quantitative PCR is used to test an environmental sample for the presence of a species' DNA through the use of a species-specific primer probe set targeting a small fragment (typically 50–150 bp). Assay performance for species-specific detection relies strongly on the specificity of oligonucleotide hybridization, a phenomenon that is well studied but not completely predictable (Wilcox *et al.* 2013). DNA sequence databases (e.g. GenBank) for designing species-specific assays are also far from complete (Kwong, Srivathsan & Meier 2012); thus, qPCR assay design may require additional sequence database development. Target loci are typically within the mitochondrial genome because of its greater biological abundance and higher level of coverage in genetic databases. However, this may limit inference in cases of introgression. **Assays should be designed and tested for application in an explicitly defined geographic area, thereby constraining the number of closely related, co-occurring species whose DNA may be present in**



**environmental samples.** To increase detection or specificity, multiple assays for a target species can be multiplexed. Assay testing should proceed through three general stages: (i) *in silico*, (ii) *in vitro* and (iii) *in situ*. *In silico* testing involves computer-aided assay design by searching DNA databases for possible non-specific oligonucleotide hybridization and by predicting primer and probe performance. Multiple alignments of a target species' DNA sequence with sequences from closely related, co-occurring non-target species are useful (e.g. Takahara *et al.* 2012), as are assay design programmes (e.g. Ye *et al.* 2012). For *in silico* validation, multiple unique bases on each primer and probe are required to prevent cross-amplification when target species are rare or absent (Wilcox *et al.* 2013), including  $\geq 1$  near the 3' end for primers (Stadhouders *et al.* 2010, Wright *et al.* 2014). *In vitro* testing involves applying the assay to tissue-derived DNA from target and non-target species, to empirically demonstrate specificity. It is important to note that trace levels of cross-contamination between tissue samples or DNA extracts can easily confound *in vitro* testing (Brandl *et al.* 2014). *In situ* testing involves applying the assay to eDNA samples from environments where the target species is absent and environments where it is present, to empirically demonstrate sensitivity and specificity under natural conditions. *In situ* testing requires confirmation of assay performance (e.g. by qPCR amplicon sequencing).

### Inhibition in eDNA samples

Inhibition of the PCR process commonly occurs in eDNA samples (McKee, Spear & Pierson 2015) and results in failed or delayed amplification of target species' DNA. For HTS, this process is evident during library preparation; however, with qPCR, consequences of misinterpreting results from an inhibited eDNA survey may include incorrectly inferring that a target species is absent or in low abundance. One method for identifying PCR inhibition involves the addition of a foreign DNA and a matching assay into all samples; these are known as internal positive controls (IPC) or internal amplification controls (IAC). For eDNA samples, using a low amount of IPC that matches expected eDNA concentrations (e.g. 100 copies) may best reflect the degree of inhibition affecting samples, as these will result in non-amplification at approximately the same level of PCR inhibition. Alternatively, a quantification cycle  $C_q$  shift of  $\geq 3$  cycles in the IPC in the environmental sample relative to the IPC in negative controls is considered evidence of inhibition (Hartman, Coyne & Norwood 2005). Prior to application, multiplexes with IPC must be tested to confirm that multiplexing does not, of itself, reduce amplification of the IPC. Methods based on the shape of sample amplification curves have also been proposed for detecting inhibited samples (Bar, Kubista & Tichopad 2011). Diluting samples has been shown to alleviate inhibition (Tsai & Olson 1992); however, this will codilute target DNA and potentially result in non-detections. Solid-phase kits such as the OneStep<sup>TM</sup> PCR Inhibitor Removal kit (Zymo Research, Irvine, CA, USA) can also be used to address this issue, but may also result in losses of

target DNA (McKee, Spear & Pierson 2015). Alternatively, modifications can be made to the PCR recipe, such as using bovine serum albumin and inhibitor-resistant polymerases (Wilson 1997; Hedman & Rådström 2013).

### eDNA-positive criteria

**There are currently no set criteria for minimum proportion of positive eDNA samples nor positive replicates of individual samples necessary to infer species presence.** The strength of evidence depends on the frequency and consistency of positive eDNA samples from a location, in the context of what is known about species distribution, habitat and behaviour, along with information about the ecology, hydrodynamics and other salient features of the system. For example, a single positive sample provides weak evidence of species presence relative to multiple positive samples collected over a multiyear time span (Jerde *et al.* 2011); however, When initial concentration of target DNA is extremely low ( $< 100$  copies/reaction – common with eDNA samples), inconsistency between qPCR replicates is expected (Ellison *et al.* 2006). In clinical diagnosis, Van der Velden *et al.* (2007) suggest a single positive qPCR replicate is sufficient to determine whether cancer cells are still present in patients in remission, even if the positive result is not reproducible. In contrast, Bustin and Mueller (2005) emphasize that results must be consistent among replicates, Kriger, Hero & Ashton (2006) required independent replication of triplicate reactions when results of qPCR tests for *Batrachochytrium dendrobatidis* were inconsistent. The number of PCR replicates (technical replicates) in eDNA studies has varied from one (Minamoto *et al.* 2012) to twelve (Tréguier *et al.* 2014). We recommend that inference from samples testing positive in only one well where results are not replicated (through repeat sampling of the same site or repeat analysis of the sample) be interpreted with caution. In practice, considering the strength of evidence rather than a dichotomous approach may better convey eDNA results.

### eDNA minimum reporting guidelines

Fields that use low copy number DNA (e.g. forensics, non-invasive genetics and paleogenetics) experienced periods of disagreement as they developed best practices and evidentiary standards (Taberlet, Waits & Luikart 1999; Cooper & Poinar 2000). Progress through such periods accelerates when scientists publish detailed information about their field, laboratory and bioinformatic procedures (Taylor *et al.* 2008). General recognition of the importance of these details led to the creation of many minimum information (MI) guidelines, currently consolidated through the MIBBI project (<http://www.mibbi.org/>). To this end, we have created a set of MI guidelines specific to analysis of aqueous eDNA samples (Table 1); these are in addition to standard details required for publication of molecular analysis. Including this level of detail in published studies will enhance the development of the field by increasing communication about techniques and quality control.

### Challenges of eDNA studies I: Inference across space and time

Quantifying the uncertainty in detection of eDNA and the scale of inference requires understanding the processes that produce, transport and degrade eDNA in the environment.

The concentration and distribution of eDNA is influenced by the physiology and space use of organisms (Eichmiller, Bajer & Sorensen 2014; Laramie, Pilliod & Goldberg 2015), but also by water movement, be it by diffusion or advection in streams, ponds or oceans (Ficetola *et al.* 2008; Pilliod *et al.* 2013; Deiner & Altermatt 2014). These factors need to be incorporated into eDNA study designs and considered when inferring the scale of results. For example, a water sample collected at one point along the shore of a wetland may not be representative of the wetland as a whole. To account for this, samples can be collected from multiple locations at a site and pooled for analysis, or known habitat components can be targeted. In stream reaches, flowing water may result in eDNA concentrations that do not correlate with local species presence over hundreds of metres (Pilliod *et al.* 2013).

Concurrent with transport, eDNA also degrades with exposure to the environment (Strickler, Fremier & Goldberg 2015). Degradation has been shown to limit the detectability of eDNA in water to between 1 day and 8 weeks (Dejean *et al.* 2011; Thomsen *et al.* 2012a,b; Pilliod *et al.* 2014) and can thus result in fine-scale temporal inference about the species' presence. Conversely, if there is a time lag between the species' presence and eDNA sampling event, degradation can lead to false conclusions of species absence. Finally, DNA bound to sediment can remain detectable far longer than DNA in the water column, so samples that include sediments have unknown temporal inference (Turner, Uy & Everhart 2015). These issues underscore the importance of fundamental knowledge of the species' phenology and eDNA degradation rates in a particular environment to inform effective sampling strategies and interpretation of results.

The degradation and transport of eDNA in water make drawing of fine spatial inferences complicated (Deiner & Altermatt 2014; Wilcox *et al.* 2016). By quantifying these processes and emergent patterns, however, an eDNA species detection approach has the potential to reduce the time it takes to find rare organisms over large areas (Thomsen *et al.* 2012a). As eDNA is transported through the watershed, wetland, lake or bay, the sampling area to detect it becomes larger, given the concentration of eDNA remains at detectable limits. Continuing to further investigate this uncertainty will improve the application of eDNA methods for species detection.

### Challenges of eDNA studies II: Inferring presence versus viable populations

Environmental DNA provides information on species occurrence, which is vital for detection and monitoring. For invasive species, detection can provide an early signal of presence, and may also be useful for monitoring established populations

(Smart *et al.* 2015). For rare or declining species, detection of individuals may only provide partial information, as individuals in sink habitats may not be distinguishable from a reproducing, stable population through presence/absence information. Additionally, data such as sex, body condition and directional hybridization (where the mitochondria is of the native species) can be missed using eDNA techniques. Given the ability of eDNA methods to detect species at low densities, solely using eDNA detection for monitoring rare species (when it is possible to observe the species directly) could mask actual declines and delay species conservation actions. However, eDNA surveys could be incorporated into an occupancy modelling framework to detect landscape-level population trends (MacKenzie *et al.* 2002).

Recent studies have found correlations between eDNA quantities and organismal abundances in experimental settings (Takahara *et al.* 2012; Thomsen *et al.* 2012a; Goldberg *et al.* 2013; Doi *et al.* 2015; Klymus *et al.* 2015) and some field sites (e.g. Pilliod *et al.* 2013), but this relationship is not always found in field settings (Spear *et al.* 2015) and can be influenced by outliers (Biggs *et al.* 2015). Additionally, using qPCR for quantification requires that the standard curve contains concentrations similar to eDNA samples (10 or lower copies). There are many features that can influence species eDNA concentrations in a sample, including eDNA dilution, dispersion and transport in different types of aquatic systems, temporal and spatial variability in eDNA degradation due to different factors (e.g. microbial activity, water chemistry and temperature, UV exposure) and variation in eDNA shedding rates among species, sexes, ages, seasons and habitat characteristics. Also, the treatment of non-detections (C<sub>q</sub> of zero) in qPCR replicates for absolute quantification of DNA molecules is a subject of debate, but results indicate that zeros should be included in calculations for increased accuracy (Ellison *et al.* 2006; Bustin *et al.* 2009). Additionally, emerging technologies such as digital droplet PCR and laser transmission spectroscopy (Hoshino & Inagaki 2012; Nathan *et al.* 2014; Doi *et al.* 2015) are likely to advance our ability to quantify eDNA, especially at low quantities that are an issue for standard qPCR (Ellison *et al.* 2006).

### Challenges of eDNA studies III: Confounding sources of eDNA

Because it is essentially impossible to observe an organism shedding DNA molecules or to track the movement of particular DNA molecules through an aquatic system, eDNA samples are basically 'blind' samples, and interpretation of eDNA data must rely on inference. Presence of a species' DNA in an environmental sample does not necessarily mean that the species is currently present in the system. A potentially critical factor is the possibility for eDNA originating outside of a system or sampling area – allochthonous eDNA – to be transported and deposited in the sampling area (Darling & Mahon 2011; Mahon *et al.* 2013) via faecal deposition by wide-ranging predators (Merkes *et al.* 2014), transport of carcasses by

**Table 2.** Recommendations for conducting environmental DNA studies

## Recommendations for eDNA sampling, analysis and reporting

## Pilot study

- Implement field sampling protocol and evaluate detection rates with sampling and site data (e.g. filter material and pore size, sample volume, number of samples, spatial distribution of samples)
- Test extraction and analysis protocols
- Validate eDNA assays *in silico*, *in vitro* and *in situ*

## Field

- Collect negative controls
- Employ strict decontamination protocols for all equipment and clothing that is reused
- Collect multiple samples at each site to address false negatives and estimate detection probabilities

## Laboratory

- Process samples only in a dedicated clean laboratory (completely separated from PCR products) with restricted access, regular decontamination (bleach, UV) and use of filtered tips
- Use probe-based qPCR if target is a few well-characterized species; for many target or unknown species, use high-throughput sequencing
- For qPCR, use technical replicates ( $\geq 3$ ), and internal positive control to test for inhibition
- Archive samples at  $-20$  or  $-80^{\circ}\text{C}$

## Reporting

- Report quantification values as copy #/volume sampled
- Acknowledge challenges inferring: across space/time, presence vs. viable population and confounding sources of eDNA
- Maintain archived database with collection date and exact geographic location

predators, scavengers or human traffic (e.g. fish that have leapt onto barges and died), or relocation of sediment containing eDNA (e.g. soil and sediment reclamation projects). Additionally, disturbance of the sediment within a system could potentially introduce DNA of extirpated or seasonally absent species into the water column (Turner, Uy & Everhart 2015). While the expectation is that these signals would be weak or inconsistent through time, a more robust body of research with regard to the potential influence of alternative sources and their vectors on the abundance and distribution of a focal species' eDNA will be critical to realizing the full potential of eDNA as a reliable ecological monitoring and surveillance tool.

## Recommendations

We present a summary of recommendations that are of critical importance for practical application when collecting and analysing samples and interpreting eDNA results (Table 2). For eDNA studies or monitoring with high ecological or economic stakes, contentious sociopolitical environments, and/or the possibility of legal challenges, additional quality control and quality assurance measures may be advisable (e.g. USFWS 2015). We hope that the guidelines we have provided will help the field as it develops, and contribute to providing robust, defensible studies using eDNA detection of species for conservation and management.

## Conclusions

Environmental DNA techniques are most advantageous when conventional survey methods are logistically difficult to apply, have negative impacts on individuals or populations, have low probabilities of detection or are very costly. Integration of eDNA into surveys therefore also depends on cost as well as the risk levels for false positives and false negatives (Darling & Mahon 2011). When species are easily detected with non-destructive traditional sampling (e.g. visual surveys), managers should keep in mind that eDNA methods of species detection may not be more efficient than trained observers; how eDNA can complement, rather than replace, field surveys should be considered. We believe that, with the application of robust field and laboratory protocols, this new field has much to offer for improving understanding of ecological systems, enabling efficient and effective conservation actions.

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## Data accessibility

This manuscript does not include data.

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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Examples of published studies using a diversity of extraction, PCR and sequencing methods for detecting aquatic macroorganisms with environmental DNA (eDNA).