



# Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects

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## Abstract

Conserving biodiversity in the face of ever-increasing human pressure is hampered by our lack of basic information on species occurrence, distribution, abundance, habitat requirements, and threats. Obtaining this information requires efficient and sensitive methods capable of detecting and quantifying true occurrence and diversity, including rare, cryptic and elusive species. Environmental DNA (eDNA) is an emerging technique that can increase our ability to detect and quantify biodiversity, by overcoming some of the challenges of labor-intensive traditional surveys. The application of eDNA in ecology and conservation has grown enormously in recent years, but without a concurrent growth in appreciation of its strengths and limitations. In many situations, eDNA may either not work, or it may work but not provide the information needed. Problems with (1) imperfect detection, (2) abundance quantification, (3) taxonomic assignment, (4) eDNA spatial and temporal dynamics, (5) data analysis and interpretation, and (6) assessing ecological status have all been significant. The technique has often been used without a careful evaluation of the technical challenges and complexities involved, and a determination made that eDNA is the appropriate method for the species or environment of interest. It is therefore important to evaluate the scope and relevance of eDNA-based studies, and to identify critical considerations that need to be taken into account before using the approach. We review and synthesize eDNA studies published to date to highlight the opportunities and limitations of utilizing eDNA in ecology and conservation. We identify potential ways of reducing limitations in eDNA analysis, and demonstrate how eDNA and traditional surveys can complement each other.

**Keywords** Biodiversity monitoring · Species detection · Conservation tools · High-throughput sequencing · Traditional surveys · Biological invasions

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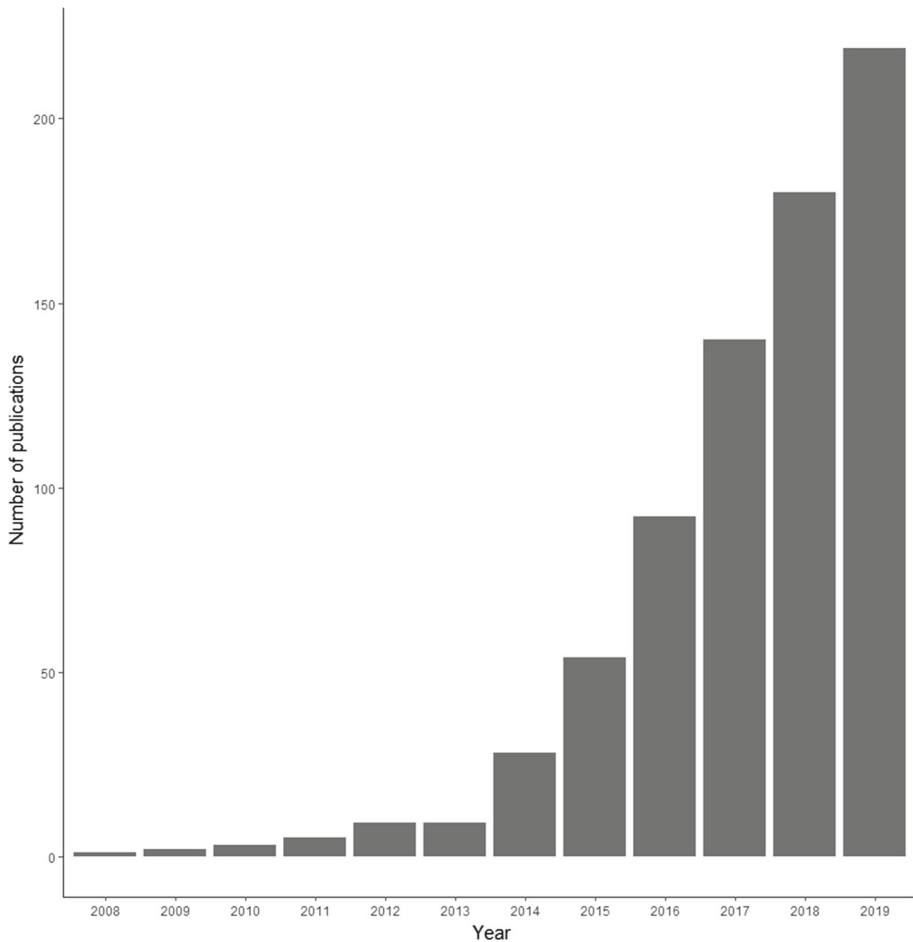
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## Introduction

Quantifying historical and contemporary biodiversity has traditionally relied on morphological and behavioral data collected using direct observations, microscopes, binoculars, traps, and more recently bioacoustics (Basset et al. 2012; Steenweg et al. 2017; Ovaskainen et al. 2018; Burivalova et al. 2019; Khelifa 2019; Rajan et al. 2019; Outhwaite et al. 2020). These techniques are often biased, invasive, destructive, and/or dependent on a declining pool of taxonomic experts for identifying specimens. Traditional surveys are also generally labor intensive and time consuming, and can be inefficient at detecting the true biodiversity present (van der Heyde et al. 2020; Basset et al. 2012; Gómez-Zurita et al. 2016; Stoeckle et al. 2016; Evans et al. 2017b; Rodriguez-Estrella et al. 2019; Zhang et al. 2020b). The advent of rapid and relatively cheap DNA sequencing techniques has significantly enhanced biodiversity research by overcoming some of the challenges of labor-intensive traditional surveys and offering the opportunity to efficiently characterize biodiversity in time and space, using standardized methods (Corlett 2017; Alexander et al. 2020; Cowart et al. 2020; Ji et al. 2020; Leempoel et al. 2020; Sales et al. 2020; Yang and Zhang 2020). Among these techniques, environmental DNA (eDNA) sampling has attracted worldwide attention, and interest in using this tool for biodiversity assessment has grown rapidly in the past few years (Fig. 1).

Environmental DNA (eDNA) is genetic material originating from the hair, skin, urine, feces, gametes, or carcasses of organisms that is present, in a more or less degraded form, in water, soil, or sediment (Taberlet et al. 2012a; Thomsen and Willerslev 2015). DNA can persist in the environment for periods from hours in temperate waters, to hundreds or thousands of years in cold, dry permafrost, allowing direct isolation without any other obvious signs of an organism's presence (Thomsen and Willerslev 2015). The utilization of eDNA has the potential to revolutionize conservation science and practice in several ways. First, eDNA techniques are fast, efficient and relatively cheap, thus providing the opportunity to monitor the dynamics of species, populations and communities, and to map their geographic distribution over long time periods and across large spatial scales (Ficetola et al. 2019; Itakura et al. 2019; Lecaudey et al. 2019; Preissler et al. 2019; Reinhardt et al. 2019; Sutter and Kinziger 2019; Sales et al. 2020). Second, eDNA sampling is simple, non-destructive, and non-invasive, causing no significant damage to the target species or its habitats (Antognazza et al. 2019; Mora et al. 2019; Leempoel et al. 2020). Third, eDNA can achieve high detection probabilities for rare, cryptic, and elusive species, even at relatively low densities (Carvalho et al. 2019; Franklin et al. 2019; Shelton et al. 2019; Takahara et al. 2020). Fourth, eDNA enables the early detection of biological invasions and their timely eradication before full establishment (Lin et al. 2019; Nardi et al. 2019; Schumer et al. 2019; Tingley et al. 2019). Fifth, eDNA allows accurate identification of target organisms using standardized, reproducible and auditable criteria that can be applied to different life stages and in different environments (Preissler et al. 2019; Takeuchi et al. 2019a). Sixth, eDNA sampling potentially offers a broad taxonomic breadth, allowing simultaneous biodiversity assessment for a wide range of organisms (Sawaya et al. 2019; Thomsen and Sigsgaard 2019; Zhang et al. 2020b).

However, despite the ecological and conservation significance of the questions that can potentially be addressed using eDNA, many challenges and limitations exist. eDNA does not always work, and even when it does 'work', the results are not always what are needed. We therefore review and synthesize eDNA studies published to date to highlight the opportunities and limitations of utilizing eDNA in ecology and conservation. Additionally,



**Fig. 1** Number of studies using environmental DNA (eDNA) recovered from a literature search with the words ‘environmental DNA’ OR ‘eDNA’ for the period between 1 January 2008 and 31 December 2019

we identify potential routes to addressing fundamental assumptions and reducing the limitations of eDNA (Table 1). We then propose a framework to discuss how eDNA can supplement traditional biodiversity surveys. Lastly, we highlight new areas where eDNA studies are well positioned to advance research in ecology, evolution and biodiversity.

## Literature search

We searched for peer-reviewed journal papers in the Web of Science using the keywords ‘environmental DNA’ and ‘eDNA’, and restricted the review to studies involving macro-organisms. The final literature search was conducted on 16th January 2020 and covered the period between 1 January 2008 and 31 December 2019 (2008 representing the year when eDNA emerged as a survey tool in macro-ecology; (Ficetola et al. 2008)).

**Table 1** Potential ways of reducing limitations in environmental DNA (eDNA) analysis

Challenge and limitation	Causes	Potential solution(s)	Reference (s)
Imperfect sampling of eDNA and false detection	Limited persistence of eDNA	Use multiple field and PCR replicates Estimate detection rates using occupancy or other models	Roussel et al. (2015), Valentini et al. (2016), Willoughby et al. (2016), Alberdi et al. (2018)
	PCR primer biases	Use multiple markers and primers, even when targeting the same taxonomic group	Alberdi et al. (2018), Collins et al. (2019)
	Inhibition of DNA amplification	Use inhibition-reducing assays	Jane et al. (2015)
	Sample contamination	Use negative and positive controls Use particle size-based selective capture/enrichment of target eDNA	Turner et al. (2014a), Bista et al. (2017)
	eDNA from dead organisms	Co-extract extracellular and intracellular DNA Co-extract DNA and RNA Amplify both longer and shorter DNA fragments	Bista et al. (2017), Laroche et al. (2017)
	Ancient DNA (aDNA) resuspension	Confirm the organism's presence with traditional surveys	Wu et al. (2018)
Difficulties in quantifying abundance and biomass	Variability in eDNA deposition and preservation	Quantify the relationship between eDNA release and biotic, and abiotic factors	Laramie et al. (2015), Sassoubre et al. (2016)
	Choice of eDNA sampling and processing protocols	Use fully integrated environmental DNA sampling systems	Thomas et al. (2018,2019)
	PCR primer and sequencing biases	Use PCR-free and capture-based approaches	Zhou et al. (2013), Wilcox et al. (2018), Ji et al. (2019)
	Variation in DNA copy number of target loci	Use multiple DNA markers	Ma et al. (2016), Bylemans et al. (2018)
	Sequence filtering stringency	Adapt workflows based on sequencing technology and library	Divoll et al. (2018)
Taxonomic assignment biases	Incomplete reference databases	Increase barcode efforts	Young et al.(2019)
Limited understanding of the ecology of eDNA	eDNA origin, state, transport, and fate	Use experimental validation in laboratory and natural settings	Barnes and Turner (2016), Maruyama et al. (2019), Murakami et al. (2019)
Inconsistencies in data analysis and interpretation	Minimum sequence threshold	Use relative thresholds (e.g. 0.01% of total reads) rather than absolute copy number thresholds	Bista et al. (2017), Alberdi et al. (2018)

**Table 1** continued

Challenge and limitation	Causes	Potential solution(s)	Reference (s)
	Chimeric sequence detection and removal	Predict in silico and remove using de novo delimitation approaches	Bista et al. (2017), Alberdi et al. (2018)
	Percent identity for OTU clustering	Use existing knowledge of intraspecific diversity for target taxa	Bista et al. (2017)
	Percent similarity for taxonomic assignment	Evaluate the completeness and accuracy of reference database used	Bista et al. (2017)
Lack of ecological information	Target organisms not sighted	Conduct eDNA and traditional surveys simultaneously	Biggs et al. (2015)

## Current ecological and conservation questions addressed using eDNA

Two broad approaches that have received the most attention in eDNA-based studies are barcoding and metabarcoding. The main difference between barcoding and metabarcoding is that barcoding uses species-specific primers to detect the DNA fragments of a single species within an environmental sample (Takahara et al. 2020; Franklin et al. 2019; Strickland and Roberts 2019; Akamatsu et al. 2020; Harper et al. 2020; Kessler et al. 2020; Togaki et al. 2020) while metabarcoding uses universal primers to simultaneously detect millions of DNA fragments from the widest possible range of species from multiple trophic levels and domains of life (Alexander et al. 2020; Cowart et al. 2020; Djurhuus et al. 2020; Yang and Zhang 2020; Zhang et al. 2020b). For eDNA barcoding, conventional PCR (cPCR) is used to detect the presence of a species (Jerde et al. 2011; Dejean et al. 2012; Thomsen et al. 2012b; Mahon et al. 2013; Piaggio et al. 2014; Fukumoto et al. 2015) and quantitative PCR (qPCR) is used to quantifying the relative abundance of DNA sequences (proxies for relative species abundance or biomass) or to improve the sensitivity of species detection (Takahara et al. 2012; Goldberg et al. 2013; Pilliod et al. 2013; Doi et al. 2015; Klymus et al. 2015; Laramie et al. 2015; Balasingham et al. 2017). eDNA barcoding has been particularly useful for detecting invasive, rare, and cryptic species, even in difficult to access habitats, map their distributions, and design management strategies (Levi et al. 2019; Qu & Stewart 2019; Reinhardt et al. 2019b). eDNA metabarcoding has been successfully used to characterize past and present biodiversity patterns (Edwards et al. 2018; Singer et al. 2018; Zinger et al. 2019), to understand trophic interactions and dietary preferences (Galan et al. 2018; Harrer and Levi 2018; Mora et al. 2019; Thomsen and Sigsgaard 2019), to study the spawning ecology of elusive species (Maruyama et al. 2018; Antognazza et al. 2019; Bracken et al. 2019; Takeuchi et al. 2019a), and to monitor ecosystem health and dynamics (Cordier et al. 2019; Evrard et al. 2019; Graham et al. 2019).

## Detecting rare, cryptic or endangered species

Detection and monitoring of rare, cryptic, and endangered species using conventional techniques is a difficult task that often involves huge amounts of time and effort (Qu and Stewart 2019). Repeated sampling (in space and time) with conventional surveys is expensive and can cause irreparable damage to the target organism or its habitat. eDNA analysis offers a cost-efficient approach to non-invasive monitoring of such species. Several studies have evaluated the methodological efficiency of eDNA versus conventional surveys in detecting rare, cryptic, and endangered species, and demonstrated that the probability of eDNA accurately detecting a target species is relatively higher than or comparable to that of conventional surveys (Deiner et al. 2017). However, most eDNA-based studies have focused on aquatic taxa, especially fishes and amphibians (Beauclerc et al. 2019; Deutschmann et al. 2019). Studies on other taxa and in terrestrial environments are scarce.

## Estimating species distribution

Although there is increasing global concern about declines in populations of wildlife (Jia et al. 2018; Saha et al. 2018; Sekercioglu et al. 2019), monitoring the population dynamics of some species remains a challenge, partly due to large uncertainties in their geographic distributions, limited understanding of their lifestyles, the complexity of their life histories, and methodological constraints (Riggio et al. 2018; Srinivasan 2019; Wineland et al. 2019). eDNA analyses have enhanced the monitoring of wildlife species distribution and abundance over large spatial and temporal scales using efficient, sensitive and standardized methods (Matter et al. 2018; Hobbs et al. 2019; Itakura et al. 2019).

## Biomonitoring ecosystem health and dynamics

Biological invasions, pests, and diseases constitute one of the most serious threats for global biodiversity and cause adverse environmental, economic and public health impacts (Sengupta et al. 2019; Tingley et al. 2019; Walsh et al. 2019). There is thus an urgent need to develop effective monitoring and management strategies to contain the spread and establishment of these harmful biological agents (Marshall and Stepien 2019; Orzechowski et al. 2019). However, such efforts are constrained by our limited capacity to efficiently detect biological threats, especially when these harmful agents are at low density (Manfrin et al. 2019). eDNA has proven to be a very effective and sensitive sampling method, capable of monitoring the spread and establishment of harmful biological agents through early detection, analysis of spread patterns, and evaluation of population dynamics (Amberg et al. 2019; Ardura 2019; Fernanda Nardi et al. 2019; Gomes et al. 2019; Rudko et al. 2019).

## Diet and trophic interactions

Understanding and quantifying biotic interactions, such as predator–prey and host-parasite relationships, are key components of ecological research. However, these important biological processes remain poorly investigated, primarily due to methodological challenges. eDNA is increasingly being used in diet analysis to estimate diversity, composition and occurrence frequency of prey items in predator feces (Galan et al. 2018; Jusino et al. 2019;

Mata et al. 2019; Mora et al. 2019). DNA deposited by pollinators on flowers, and by dispersers on seeds, also offers an opportunity to investigate plant-animal interactions and the role of these interactions in the maintenance of ecosystem functions and the provision of ecosystem services (Harrer and Levi 2018; Thomsen and Sigsgaard 2019).

### Spawning ecology

Most aquatic animals, except for aquatic mammals and reptiles, reproduce through the process of spawning. Identifying areas for spawning, as well as the spatial extent of spawning activities, is vital for the effective management and conservation of these species. However, understanding the natural reproductive ecology of these organisms have mostly relied on collections of eggs, larvae and spawning-condition adults (Tsukamoto et al. 2011; Antognazza et al. 2019). These techniques are often biased, invasive, destructive, and/or strictly dependent on a declining pool of taxonomic experts for identifying life history stages (Maruyama et al. 2018). Surveys of this nature are also generally labor intensive and time consuming, and can be inefficient at detecting certain life history stages (Antognazza et al. 2019; Fritts et al. 2019). For instance, kick-sampling for eggs is sometimes conducted in areas of relatively shallow waters or during the day whereas the adults spawn in deep waters or at night (Antognazza et al. 2019). eDNA enables the detection of a species regardless of its life stage or gender, and is transforming our ability to non-invasively quantify spawning activities, and identify the spatial extent of spawning, with limited resources (Maruyama et al. 2018; Tillotson et al. 2018; Antognazza et al. 2019; Bracken et al. 2019; Fritts et al. 2019; Takeuchi et al. 2019a, b).

### Monitoring biodiversity

Conserving biodiversity in the face of ever-increasing human pressure is hampered by our lack of basic information on past and present species occurrences, distributions, abundances, habitat requirements, and threats. Obtaining this information requires efficient and sensitive sampling methods capable of detecting and quantifying true biodiversity, especially in megadiverse regions with many cryptic and undescribed species (Kuzmina et al. 2018; Lacoursière-Roussel et al. 2018). eDNA has increased our ability to monitor past and present biodiversity, by overcoming some of the challenges of labor-intensive traditional surveys (Edwards et al. 2018; Fraser et al. 2018; Montagna et al. 2018; Cilleros et al. 2019). It is now possible and cost-efficient to assess the biodiversity of entire communities and infer diversity and assemblage patterns for a wide range of taxonomic groups simultaneously (DiBattista et al. 2019; Zinger et al. 2019).

### Challenges and limitations of eDNA

The application of eDNA in ecology and conservation has grown enormously in recent years, but without a concurrent growth in appreciation of its limitations. While there is evidence that eDNA can increase the precision and resolution obtainable from traditional biodiversity surveys (Thomsen and Willerslev 2015; Yamamoto et al. 2017), this is certainly not true in all circumstances, even with standardized and highly sensitive assays (Hinlo et al. 2017; Ulibarri et al. 2017). In cases where eDNA has been successful, it might not necessarily be the appropriate tool if information is required on the abundance or biomass of species (although this may be possible in some cases (Takahara et al. 2012;

Pilliod et al. 2013; Doi et al. 2015; Baldigo et al. 2017)), its ecology (life-history, sex ratio, breeding status), or its conservation status (Evans et al. 2017b; Trebitz et al. 2017). Presence/absence information from eDNA is useful in conservation for monitoring populations at large spatial scales and for identifying habitats that are of high value to species of conservation concern (Voros et al. 2017; Weltz et al. 2017). eDNA can also be used to detect the first occurrence of an invasive species or the continued presence of a native species that was considered extinct, sometimes at relatively low densities (Stoeckle et al. 2017; Trebitz et al. 2017). However, presence/absence can be misleading when eDNA is present in the environment in the absence of living target organisms or when eDNA is not detected but the target organism is present (Song et al. 2017). Abundance data provide far more information on the status of a population than presence/absence data and thus potentially allow for more robust assessments of the factors affecting populations.

To date, increased speed and reduced cost remain the key advantages of eDNA (Sigsgaard et al. 2015). Whether eDNA sampling is more sensitive and has higher resolution than traditional surveys remain controversial. For some species or taxa, eDNA performs better than traditional methods (Kraaijeveld et al. 2015; Deiner et al. 2016; Olds et al. 2016; Strickland and Roberts 2019; Tingley et al. 2019), for others, eDNA is as good as traditional surveys (Hanfling et al. 2016; Hopken et al. 2016; Yamamoto et al. 2017), while for some, eDNA provide little additional benefit to surveillance (Rose et al. 2019; Walsh et al. 2019; Wood et al. 2019). However, studies in which eDNA has been unsuccessful are much less likely to be published, so we inevitably know less about eDNA's failures than its successes. In addition to the taxa- or species-specific differences in sensitivity between eDNA and traditional surveys, the environment, time of the year, and biotic factors also play important roles (Dejean et al. 2011; Pilliod et al. 2014; Barnes and Turner 2016; O'Donnell et al. 2017; Lacoursière-Roussel et al. 2018; Anglès d'Auriac et al. 2019; Takeuchi et al. 2019a). In aquatic ecosystems, for example, eDNA can persist from a few hours to a month after release (Dejean et al. 2011; Pilliod et al. 2014). In addition, differences in eDNA persistence can occur even within the same environment, for example, between the surface and bottom layers of a water body (O'Donnell et al. 2017; Lacoursière-Roussel et al. 2018; Anglès d'Auriac et al. 2019).

Studies that have quantitatively assessed the cost-efficiency of eDNA relative to traditional methods suggest that eDNA sampling is relatively cheaper than traditional surveys (Biggs et al. 2015; Davy et al. 2015; Huver et al. 2015; Sigsgaard et al. 2015; Qu and Stewart 2019), although this can depend on the target taxa, site-specific detection rates, budgets, and other considerations (Smart et al. 2016). For instance, Qu and Stewart (2019) found that the cost of detecting and quantifying Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) populations using visual surveys was 1.41–1.88 times (monthly cost) and 4.22–5.64 times (seasonal cost) higher than using eDNA. Sigsgaard et al. (2015) found that using eDNA (\$4250) to detect the European weather loach (*Misgurnus fossilis*) was 1.9 times cheaper than using a combination of traditional methods (\$8100). Biggs et al. (2015) found that the cost of detecting newts (*Triturus cristatus*) was 10.4 times cheaper using eDNA (€140 per site) compared to traditional field sampling (€1450 per site). Davy et al. 2015 found that the cost of detecting nine sympatric freshwater turtles using traditional surveys was 2–10 times higher than using eDNA. However, Smart et al. (2016) evaluated the relative cost of eDNA and bottle-trapping for detecting the European newt (*Lissotriton vulgaris vulgaris*) and found that eDNA sampling was more cost-efficient than trapping under low setup costs but bottle-trapping was more cost-efficient than eDNA under high setup costs.



Qualitatively novel applications with actual conservation outcomes are still largely lacking, although researchers are now moving away from proof-of-concept research to studies that quantify population dynamics across organisms and environments (Stewart et al. 2017; Carraro et al. 2018). However, the ability of eDNA to detect the continuous presence of a species not sighted in its habitat for many years also raises questions about the mechanisms and processes by which eDNA is transported and the conservation implications of unexplained variability in eDNA transport (Sigsgaard et al. 2015; Jerde et al. 2016; Lim et al. 2016). Although methods and models to handle imperfect detection are increasingly being improved (Piggott 2016; Guillera-Arroita et al. 2017; Ji et al. 2019), it is not possible to simply ignore the presence of eDNA in the absence of living target organisms and/or the absence of eDNA in the presence of living target organisms without actual field surveys. Increased PCR replication can maximize eDNA detection and minimize false positives and/or negatives (Piggott 2016) but this cannot substitute for actual biological replicates and will increase cost (Ficetola et al. 2015; Roussel et al. 2015; Evans et al. 2017b). Detection of species using eDNA relies on DNA isolated from living and dead cells (characterized by low concentration and high degradation (Deagle et al. 2006)), and on PCR amplification (subjected to high variability and stochasticity (Kebschull and Zador 2015)), and is prone to imperfect detection (Pilliod et al. 2014; Ficetola et al. 2015). Increasing the number of DNA extracts per sample or the number of amplifications per DNA extract does not necessarily increase the probability of detection but will require more laboratory reagents, time, and effort. However, collecting biological samples from sites where the target species is most likely to be detected—based on knowledge of the target species' ecology—can enhance the detection probability (Ficetola et al. 2015; Akre et al. 2019; Wineland et al. 2019; Wood et al. 2019; Bedwell & Goldberg 2020; Vimercati et al. 2020).

Degradation of eDNA in the environment limits the scope of eDNA studies, as often only small segments of genetic material remain, particularly in warm, humid conditions (Strickler et al. 2015; Collins et al. 2018; Goldberg et al. 2018; Harrison et al. 2019; Moushomi et al. 2019; Murakami et al. 2019; Sirois and Buckley 2019). Additionally, the impacts of varying environmental conditions on time to degradation and the potential of DNA to travel throughout media such as water can affect inferences of fine-scale spatiotemporal trends in species and communities (Coissac et al. 2012; Taberlet et al. 2012a; Eichmiller et al. 2016; Goldberg et al. 2016; Deiner et al. 2017; Hering et al. 2018). However, eDNA workflows have been improving continuously, including the optimization of protocols for improved sample collection and preservation, library preparation, sequencing, and bioinformatics (Williams et al. 2016; Yamanaka et al. 2017; Ji et al. 2019; Jusino et al. 2019; Koziol et al. 2019; Muha et al. 2019; Singer et al. 2019; Thomas et al. 2019; Yamahara et al. 2019). For instance, Thomas et al (2019) developed desiccating filter housings that can automatically preserve captured eDNA via desiccation. These housings also reduce the amount of time (or steps) required to handle samples, and do not require the addition of chemicals and/or cold storage, thus minimizing the risk of contamination. Singer et al (2019) found that for the same eDNA sample, Illumina NovaSeq detected 40% more metazoan families than MiSeq and attributed this difference to NovaSeq's advanced technology.

Despite the important role that eDNA already plays in biodiversity assessment, diet analysis, and detection of rare or invasive species, we are concerned that it is being over-promoted as a standalone technique for ecological and conservation initiatives that may not fully benefit from it (Roussel et al. 2015). We emphasize, in particular, that it is challenging to distinguish between detection of eDNA and detection of a species, or to quantify

organismal abundance and biomass using eDNA, without a clear understanding of the challenges and limitations of the technique. Failure to address these problems may confound the interpretation of eDNA data.

### Imperfect sampling of eDNA and false detection

eDNA is prone to imperfect sampling and false detection, which can occur at various stages of the project, including field collection, sample storage, molecular analysis, and bioinformatics workflows (Ficetola et al. 2016; Deiner et al. 2017; Doi et al. 2019; Piñol et al. 2019). Cases where eDNA is detected in the environment in the absence of target organisms (false positives, (Ficetola et al. 2015; Ficetola et al. 2016; Lahoz-Monfort et al. 2016; Stoeckle et al. 2016; Guillera-Arroita et al. 2017)) or where eDNA is not detected but the target organism is present (false negatives, (Morin et al. 2001; Ficetola et al. 2008; Schmidt et al. 2013; Ficetola et al. 2015; Willoughby et al. 2016; Doi et al. 2019)) are common. Although site occupancy models have been proposed as a way to account for imperfect detection, they largely depend on the number of replicate samples per site and on the number of replicate amplifications per DNA sample (PCR), which vary considerably across taxa (Schmidt et al. 2013; Matter et al. 2018; Chen & Ficetola 2019; Doi et al. 2019; Strickland & Roberts 2019). Causes of false detections include.

### Limited persistence of eDNA in the environment

A key motivation for using eDNA is the fact that all organisms shed DNA into their environment, allowing direct isolation without any obvious signs of the organism's presence (Taberlet et al. 2012a). However, DNA released by aquatic or terrestrial organisms is not necessarily concentrated at the site of its release, but is transported across space and degraded over time (Deiner and Altermatt 2014; Jane et al. 2015; Sansom and Sassoubre 2017; Rice et al. 2018; Murakami et al. 2019). The eDNA release and decay rates depend on several biotic (e.g. life-history traits, species interactions, microbes) and abiotic (e.g. UV radiation, temperature, salinity) factors (Pilliod et al. 2014; Klymus et al. 2015; Lacoursière-Roussel et al. 2016; Stewart 2019). Our current understanding of how eDNA persist under different environmental conditions for different species is limited, but this information is critical for deciding on the most appropriate time window to conduct eDNA surveys. Environmental conditions are constantly changing and can be different in each location throughout the year. For example, Pilliod et al. 2014 detected eDNA after 11 and 18 days in water samples that were stored in the dark but eDNA was no longer detectable in samples that were exposed to full-sun after 8 days. Temperature directly affects the metabolic rate of some organisms (e.g. amphibians, invertebrates, reptiles, and fish) and consequently could strongly affect eDNA release rate (Clarke and Fraser 2004; Lacoursière-Roussel et al. 2016). For instance, Lacoursière-Roussel et al. 2016 showed that fish released more eDNA in warm water (14 °C) than in cold water (7 °C) and that the relationships between eDNA concentration and fish abundance or biomass were stronger in warm water than in cold water.

### Primer biases

The suite of molecular markers used in eDNA analysis is extremely important for the identification of species in both single taxa and multi-species samples. However, successful

amplification of eDNA depends highly on primer specificity, sensitivity, and efficiency (Stadhouders et al. 2010; Nichols et al. 2018). eDNA samples are characterized by highly heterogeneous DNA from mixtures of many different taxa or haplotypes, making it difficult to achieve full complementarity between primers and target sequences during PCR (Stadhouders et al. 2010; Nichols et al. 2018; Wei et al. 2018). These primer-template mismatches can affect both the stability of the primer-template duplex and the efficiency with which the polymerase extends the primer, potentially leading to biased results or complete PCR failure (Stadhouders et al. 2010). For instance, primer bias may lead to the preferential amplification of abundant sequences compared to rare ones, or of shorter fragments compared to longer ones, or of non-target organisms compared to target organisms (Nichols et al. 2018; Xia et al. 2018). Unlike metabarcoding, primer bias is not a major issue for barcoding. However, targeted PCR-based amplification of samples using species-specific primers, instead of universal primers, should be strongly encouraged in eDNA barcoding (Wilcox et al. 2013; Davy et al. 2015; Cannon et al. 2016). Conventional PCR (cPCR) methods may cross-amplify and provide false positive results but quantitative PCR (qPCR) methods are likely to be more sensitive (Wilcox et al. 2013).

### Inhibition of DNA amplification

eDNA analysis involves the collection of complex and heterogeneous mixtures from aquatic ecosystems, soils, sediments, or feces (Kozioł et al. 2019). The polymerase chain reaction (PCR) is the standard method for detection and characterization of organisms and genetic markers in these sample types. However, PCR is vulnerable to inhibitors, which are usually present in eDNA samples and which may affect the sensitivity of the assay or even lead to false negative results (Schrader et al. 2012; Nichols et al. 2018; Hunter et al. 2019). PCR inhibitors represent a diverse group of substances including bile salts from feces, polysaccharides from plant materials, collagen from tissues, heme from blood, humic acid from soil, urea from urine, and melanin and eumelanin from hair and skin (Watson & Blackwell 2000; Radstrom et al. 2004; Schrader et al. 2012). Although PCR inhibitors have different properties and mechanisms of action, they generally exert their effects through direct interaction with DNA or interference with thermostable DNA polymerases (Schrader et al. 2012). Direct binding of inhibitors to DNA can prevent amplification and facilitate co-purification of inhibitor and DNA (Schrader et al. 2012; Jane et al. 2015). Inhibitors can also interact directly with a DNA polymerase to block enzyme activity. Since some inhibitors are predominantly found in specific types of samples, matrix-specific protocols for preparation of nucleic acids before PCR are essential (Schrader et al. 2012; Hunter et al. 2019).

### Sample contamination

Contamination occurs when DNA from an outside source (exogenous DNA) gets mixed with DNA relevant to the research. For instance, if a frog is eaten at one pond, then the predator defecates at another, this may introduce the frog's DNA to a pond where the frog is not present. Because of the sensitivity of the technique, this is a serious issue in eDNA surveys and may result in false positive detections and subsequent misinterpretation of results (Goldberg et al. 2016; Wilson et al. 2016). eDNA analysis requires multiple steps of sample handling and manipulation in the field (collection, storage and transportation) and in the lab (storage, DNA extraction, amplification, library preparation and sequencing), so

contamination may occur at various stages of the research (Goldberg et al. 2016; Doi et al. 2017). In the field, contamination may occur when DNA from one or multiple samples is unintentionally transferred into another sample, either from another site in the same study or from an unknown locality. This usually occurs when the same field equipment (e.g. corers, filters, gloves) is used repeatedly for sampling different sites without thorough treatment (e.g. sterilization). In the lab, contamination may occur when remnant DNA from previous molecular experiments (e.g. DNA extraction, amplification, library preparation and sequencing) spreads into new samples or when the same lab equipment (e.g. tubes, pipettes, benchtops) is repeatedly used for conducting different experiments without thorough decontamination. Instead of standard autoclaving (Unnithan et al. 2014) or the commonly used 10% bleach (sodium hypochlorite) solution (Prince and Andrus 1992), treatment of field and lab equipment with 50% bleach solution and thorough rinsing can effectively destroy and remove unwanted DNA and PCR products (Kemp and Smith 2005; Champlot et al. 2010; Goldberg et al. 2016; Wilcox et al. 2016).

### eDNA from dead individuals

Both dead and live organisms release DNA into the environment and both contribute to the eDNA pool. For most purposes, the researcher is only interested in the former—DNA from live organisms—but distinguishing between them remains a challenge. Since DNA degrades with time, the longer DNA fragments in a particular environment are likely to represent the most recent DNA. Jo et al. (2017) compared changes in copy numbers of long (719 bp) and short (127 bp) eDNA fragments with time and suggested that the concentration of longer eDNA fragments reflects fish biomass more accurately once the effects of decomposition and contamination have been removed. However, removal of carcasses and avoidance of contamination in natural settings is almost impossible, given that birth and mortality are key processes in the dynamics of natural populations. The contribution of dead organisms to the eDNA pool can vary considerably in different environments. For instance, in the tropics and sub-tropics with relatively higher temperatures and faster degradation rates, carcasses do not persist long. Tsuji et al. (2017) found that ayu sweetfish (*Plecoglossus altivelis altivelis*) and common carp (*Cyprinus carpio*) eDNA degradation rates increased with increasing water temperatures.

### Ancient DNA (aDNA) resuspension

Environmental DNA may occur as particle-bound or free-living dissolved molecules (Turner et al. 2014a). Particles that bind DNA may settle over long periods and be resuspended through natural phenomena like erosion, turbulence caused by fast-flow hydrological events, wind, and wave action or bioturbation. In cases where the objective is to detect the continued presence of a native species that was considered extinct, aDNA resuspension can lead to false positive results and misinform management.

### Difficulties in quantifying abundance and biomass

One of the most important issues limiting the application of eDNA in environmental monitoring is the difficulty of quantifying species abundance and biomass. To date, results of most eDNA studies have been interpreted as presence/absence (occurrence) information. However, some studies have used mock communities with known and differing

assemblage structures or combined conventional surveys with eDNA in order to explore whether or not eDNA can provide quantitative information (Piñol et al. 2019). The outcomes of these studies are still fairly contentious, with strong, weak, and no quantitative estimates reported (Piñol et al. 2019). For instance, Pilliod et al. (2013) reported that eDNA concentrations of Rocky Mountain tailed frogs (*Ascaphus montanus*) and Idaho giant salamanders (*Dicamptodon aterrimus*) were positively associated with in-stream density, biomass, and proportion of area occupied by the two species. Takahara et al. (2012) showed that eDNA concentration in water samples correlated with the biomass of common carp (*Cyprinus carpio*) in artificial ponds, and Thomsen et al. (2012b) showed that eDNA concentration was correlated with the density of common spadefoot toads (*Pelobates fuscus*) and great crested newts (*Triturus cristatus*) in natural ponds. Evans et al. (2016) found a modest, but positive relationship between species abundance and sequencing read abundance for eight fish and one amphibian species in replicated mesocosms, while Deagle et al. (2013) reported that the proportions of fish sequences recovered from 39 seal scats did not match the proportions of the three fish species the seals consumed.

## Problems with interpreting relative abundance data generated from PCR-based techniques and metabarcoding loci

### Variability in eDNA deposition and preservation

The production and stability of eDNA [origin, state, decay, transport, persistence (Barnes and Turner 2016)] vary greatly among taxa, individuals, and even tissues within the same organism. The concentration of DNA in the environment is influenced by several complex processes, including movement and degradation, making it difficult to extract abundance information from eDNA signals. Furthermore, an organism's size, age, condition, or biological activity can influence the relationship between eDNA concentration and relative abundance (Spear et al. 2015; de Souza et al. 2016; Erickson et al. 2016; Stewart et al. 2017), interactions between a target species and closely or distantly related species can influence the amount of eDNA released (Sassoubre et al. 2016), and environmental conditions can influence eDNA release, persistence, degradation, transport, location, and settlement (Laramie et al. 2015; Erickson et al. 2016; Stewart et al. 2017). For instance, large-bodied, long-lived, year-round, and highly dispersed species are more likely to be detected using eDNA than small-bodied, short-lived, seasonal, and sedentary species (Andersen et al. 2012; Buxton et al. 2017; Dunn et al. 2017; Hemery et al. 2017; Rees et al. 2017; Nichols et al. 2018).

### eDNA sampling and processing biases

Key considerations in eDNA analysis are maximizing DNA capture in the field, minimizing degradation during transport and storage, and successful isolation and amplification (Pilliod et al. 2013, 2014; Turner et al. 2014b; Renshaw et al. 2015; Goldberg et al. 2016; Wood et al. 2019). The choice of eDNA sampling and processing protocols can significantly influence DNA yield, detection probability, and the resulting abundance and biodiversity estimates (Brannock and Halanych 2015; Deiner et al. 2015; Renshaw et al. 2015; Djurhuus et al. 2017; Thomas et al. 2018). Specific protocols used in each study vary with sample type (water, feces, soil, sediment), the ecosystem of interest (freshwater, marine, terrestrial), and the questions being investigated (Renshaw et al. 2015; Goldberg et al.

2016; Djurhuus et al. 2017). For diet analysis, individual fecal samples are collected and dehydrated immediately using either alcohol or silica gel or a combination of both (Deagle et al. 2009; Zeale et al. 2011; Galan et al. 2012; Pompanon et al. 2012; Clare et al. 2014; Mata et al. 2016). In terrestrial ecosystems, multiple soil cores are collected and analyzed separately or are pooled together, homogenized and a representative subsample is taken. DNA is extracted from the soil samples immediately after collection or samples are stored at  $-20\text{ }^{\circ}\text{C}$  or  $-80\text{ }^{\circ}\text{C}$  for processing at a later date (van der Heyde et al. 2020; Andersen et al. 2012; Bienert et al. 2012; Epp et al. 2012; Taberlet et al. 2012b; Yoccoz et al. 2012). In aquatic ecosystems, different protocols are being used to collect water, capture eDNA with filters, transport samples from the field, and to store water and/or filters prior to DNA extraction and amplification (Goldberg et al. 2011; Pilliod et al. 2013; Biggs et al. 2015; Renshaw et al. 2015; Majaneva et al. 2018). Some studies filter, precipitate or centrifuge water on-site, and preservation media (e.g. ice, sodium acetate, lysis buffers, and absolute ethanol) are used to stabilize eDNA for enough time (up to 24 h) to safely transport it for storage and processing (Ficetola et al. 2008; Goldberg et al. 2011; Pilliod et al. 2013; Biggs et al. 2015; Valentini et al. 2016). In other studies, water is transported in cold conditions and filtration or precipitation is done in the laboratory (Jerde et al. 2011; Thomsen et al. 2012b; Goldberg et al. 2013). Minimizing DNA degradation in these samples is challenging, especially in remote field sites with little or no access to cooling and in situations where samples need to be transported for several days (e.g. international flights with stop overs) before processing.

Various types of filters have been used to capture eDNA (Minamoto et al. 2012; Thomsen et al. 2012a; Goldberg et al. 2013; Jerde et al. 2013; Piaggio et al. 2014) and the efficiency of each filter type depends on its pore size, the volume and chemical properties (e.g. pH, organic and inorganic particles) of the water filtered, and the extraction method (Liang and Keeley 2013; Turner et al. 2014a; Renshaw et al. 2015; Eichmiller et al. 2016; Djurhuus et al. 2017; Majaneva et al. 2018). In general, filtration is relatively more efficient for eDNA capture than precipitation and centrifugation methods (Deiner et al. 2015; Renshaw et al. 2015; Eichmiller et al. 2016; Spens et al. 2017; Majaneva et al. 2018). Among filters, cellulose nitrate (CN) filters capture relatively more eDNA than polyethylene sulfone (PES), polyvinylidene fluoride (PVDF), and polycarbonate (PC) filters, while glass microfiber (GMF) filters capture relatively more eDNA than PC filters (Liang and Keeley 2013; Eichmiller et al. 2016).

In some aquatic environments (e.g. muddy water), the pore size of a filter can influence filtration rate, where larger pore size filters (e.g.  $5\text{ }\mu\text{m}$ ) or pre-filtration require less time than smaller pore size filters ( $1\text{ }\mu\text{m}$ ). However, larger pore size filters and pre-filtration are less efficient in DNA recovery than smaller pore size filters (Liang & Keeley 2013; Eichmiller et al. 2016).

## PCR primer and sequencing biases

eDNA species detection and quantification is usually accomplished using relatively short DNA fragments. These increase detection probabilities with highly degraded eDNA, but they are prone to high error rates and biases. Primers used to amplify these short DNA fragments may not perfectly match the target organism's DNA, leading to primer–template mismatches and differential amplification of target DNA (Leray et al. 2013; Elbrecht and Leese 2015; Bista et al. 2018). Primers can fail to detect low concentrations of eDNA, miss entire taxa or preferentially amplify the eDNA of non-target organisms. For example, short

DNA fragments are more likely to represent ancient DNA (aDNA) that has persisted in the environment for very long periods, bound to sediments, and represent historical biodiversity (Barnes et al. 2014; Barnes and Turner 2016). On the other hand, longer DNA fragments may represent more recent biological information, but are present at lower concentrations in the environment, are less likely to persist after release, and degrade (Lindahl 1993; Deagle et al. 2006; Hanfling et al. 2016; Bista et al. 2017). Jo et al. (2017) showed that the decay rate of eDNA varied depending on the length of the DNA fragment, while (Hanfling et al. 2016) found that smaller (~ 100 bp) fragments of 12S rRNA persisted longer in lake water than longer (~ 460 bp) fragments of cytochrome b (CytB). Olson et al. (2012) reported that primers targeting the mtDNA of the eastern hellbender (*Cryptobranchus alleganiensis*) had six orders of magnitude higher sensitivity than primers targeting the nuclear DNA. It has also been observed that polymerase choice can affect both occurrence and relative abundance estimates and the main source of this bias can be attributed to polymerase preference for sequences with specific GC contents (Fonseca 2018; Nichols et al. 2018). The addition of short indices to PCR primers can also introduce biases to the resulting sequence counts, especially in mixed-template eDNA samples, presumably via differential amplification efficiency among templates (O'Donnell et al. 2016; Leray and Knowlton 2017). PCR amplification strategies also influence species detection and abundance estimation, with quantitative PCR (qPCR) being relatively more effective for species detection and abundance estimation than conventional PCR (cPCR) (Takahara et al. 2012; Turner et al. 2014b; Piggott 2016; Harper et al. 2018).

### Variation in DNA copy number of target loci

Environmental DNA studies have mostly relied on mitochondrial (mt), chloroplast (cp), and nuclear (n) DNA sequences, but the gene copy number of these target loci may vary between taxa, individuals or tissues, even when the same number of cells is present in an environmental sample (Moraes 2001; Morley and Nielsen 2016; Minamoto et al. 2017; Nichols et al. 2018). This distorts the assumption that read abundance correlates with genic or taxon abundance, or that there is a constant copy number to individual relationship. For instance, Minamoto et al. (2017) found that the copy numbers for nDNA of common carp (*Cyprinus carpio*) in environmental samples were considerably higher for mtDNA, with the nDNA marker requiring much less survey effort than the mtDNA marker, while Piggott (2016) found that the 18S nDNA marker required relatively higher survey effort to achieve a 0.95 detection probability for Macquarie perch (*Macquaria australasica*) than two 12S mtDNA markers. These differences between molecular markers can greatly influence species detection and abundance estimation, yet many eDNA studies do not address this issue.

### Sequence filtering stringency

Sequence filtering is a routine process in eDNA analysis and occurs at multiple steps of the bioinformatics pipeline. For metabarcoding, raw sequence data are initially processed to filter and correct (where possible) low-quality and erroneous reads (Valentini et al. 2016; Evans et al. 2017a; Günther et al. 2018; Bakker et al. 2019; Rytönen et al. 2019; Cowart et al. 2020; Zhang et al. 2020a). This quality control step removes any phiX reads (common in marker gene sequencing) and chimeric sequences detected in the raw sequencing data. Other quality filtering criteria include trimming off the first *m* bases of

each sequence, and/or truncating each sequence at position  $n$  (Bakker et al. 2019; Cowart et al. 2020). The appropriate number of bases to be trimmed and the truncation length can be determined using read quality profiles. Filtering can also be performed on an OTU-table or a species-by-site matrix to remove samples with a total read frequency less than a given threshold and/or OTUs observed in less than a given number of samples (Bakker et al. 2019; Rytönen et al. 2019; Cowart et al. 2020; Zhang et al. 2020a). Filtering out OTUs that are detected in only one or a few samples is common, and this is based on the suspicion that these low frequency OTUs are PCR or sequencing errors (Bakker et al. 2019; Rytönen et al. 2019; Cowart et al. 2020; Zhang et al. 2020a). Taxonomy-based filtering is also being applied to retain target taxa and/or exclude non-target taxa from eDNA analysis (Bakker et al. 2019; Cowart et al. 2020; Zhang et al. 2020a). Although there are accepted thresholds, across studies, about which filtering criteria are suitable, differences in sequencing depth, marker region, primer specificity, and taxonomic breadth makes it difficult to reach a general consensus (Evans et al. 2017a). Sequence filtering stringency can affect species detection, abundance and biomass quantification (Rivera et al. 2020). More stringent thresholds might filter out true biological sequences from the dataset, whereas more flexible thresholds might treat artefacts as true biological sequences (Laroche et al. 2017; Alberdi et al. 2018). Amend et al. (2010) reported a tradeoff between sequence quality stringency and quantification by showing that read-quality based processing stringency profoundly affected the abundance estimate for one fungal species.

### Incomplete reference databases and taxonomic assignment biases

Environmental DNA of complex eukaryotic communities is increasingly being used to quantify biodiversity in terrestrial, freshwater and marine ecosystems (Civade et al. 2016; Andruszkiewicz et al. 2017; Gillet et al. 2018; Fujii et al. 2019; Thomsen and Sigsgaard 2019). Assignment of OTUs to species or higher taxonomic levels is a fundamental step in such studies. However, the incompleteness of reference sequence databases for most organisms is an important limitation for biodiversity studies using eDNA (Thomsen and Sigsgaard 2019). The taxonomic identification of taxa is as good as the reference database used (Thomsen and Sigsgaard 2019). Reference sequences for taxonomic assignment are only available for one or a few genes for most species and the targeted marker regions (e.g. COI, 12S, 16S) cannot accurately resolve most groups to species or higher taxonomic levels due to incompleteness of reference sequence databases (Deagle et al. 2014; Liu et al. 2017; Thomsen and Sigsgaard 2019). Consequently, eDNA studies are often interpreted using molecular operational taxonomic units (MOTUs) or higher taxonomic ranks (genus, family, order) instead of binomial species names (Thomsen and Sigsgaard 2019). This makes it difficult to associate eDNA data with existing biological and ecological knowledge. Although user-friendly and cost-efficient methods that generate full-length reference barcodes could improve future eDNA studies (Liu et al. 2017), unbalanced barcoding efforts across regions of the world, taxonomic groups, and molecular markers (Ratnasingham and Hebert 2007; Machida et al. 2017; Porter and Hajibabaei 2018) currently limit the application of eDNA in ecology and conservation.

### Limited understanding of the ecology of eDNA

We lack a clear understanding of the ecology of eDNA – its origin, state, transport, and fate. This information is critical for deciding whether eDNA sampling is the appropriate



technique to make robust inferences about an organism's presence, and to quantify abundance (Turner et al. 2014a; Barnes and Turner 2016; Stewart 2019). Environmental DNA originates as urine, feces, epidermal tissues, secretions, reproductive cells or carcasses and this source material enters the environment as particles of various sizes. These sources of eDNA may be rapidly transported from the site of release, including leaching into the soil, downstream flow and dispersion by water currents. Although particle size may be a major determinant of movement velocity, intact genomic DNA within living cells may be transformed into extracellular fractions too small to be detected (Barnes et al. 2014). Murakami et al. (2019) found that eDNA of striped jack was mostly detectable within 30 m of the source, Jane et al. (2015) found that eDNA of brook trout (*Salvelinus fontinalis*) could be detected 240 m downstream, Deiner and Altermatt (2014) found that eDNA of daphnia (*Daphnia longispina*) could be detected 12.3 km downstream, and eDNA of pelecypod (*Unio tumidus*) could be detected 9.1 km downstream. Despite the fact that eDNA reflects the source within a range of distances (10–150 m; (O'Donnell et al. 2017; Yamamoto et al. 2017; Murakami et al. 2019), the relationship between water current and eDNA transport is not well known. Besides distance, many interacting factors can also influence eDNA detection after leaving its source (Pilliod et al. 2014).

It is unlikely that all organisms release equal amounts of DNA into the environment and that DNA from different sources degrades at the same rate, even under similar environmental conditions. Therefore, the detection of a target species may be influenced by eDNA release and degradation, which are in turn related to a species' size, life history, biotic interactions, and abiotic conditions (Barnes et al. 2014). For freshwater fish, eDNA degradation rates vary from 10.5%/h in common carp (*Cyprinus carpio*; (Barnes et al. 2014)) to 15.9%/h in bluegill sunfish (*Lepomis macrochirus*; (Maruyama et al. 2014)), while for marine fish, eDNA degradation rates vary from 1.5%/h in three-spined stickleback (*Gasterosteus aculeatus*; (Thomsen et al. 2012a)), 4.6%/h in European flounder (*Platichthys flesus*; (Thomsen et al. 2012a)) to > 5.0%/h in northern anchovy (*Engraulis mordax*), Pacific sardine (*Sardinops sagax*), and Pacific chub mackerel (*Scomber japonicus*) (Sassoubre et al. 2016). These studies suggest that the degradation rate of eDNA in aquatic fish, for instance, exhibit both species and environment effects.

DNA released into any environment is subjected to dynamic biological, physical, and chemical processes that determine its fate (Levy-Booth et al. 2007). After release, DNA may be bound to organic and inorganic particles that settle, and are later resuspended through natural phenomena like erosion, turbulence caused by fast-flow hydrological events, wind and wave action or bioturbation. However, whether eDNA is most abundant in the upper layer close to the source (surface, (Moyer et al. 2014; Murakami et al. 2019)) or in the lower layer away from the source (bottom, (Turner et al. 2015)) needs further investigation.

## Inconsistencies in data analysis and interpretation

An important challenge in eDNA analysis is dealing with errors that occur during PCR amplification and sequencing in a consistent way. Researchers have attempted to ameliorate this issue using a variety of techniques including the deliberate and careful removal of erroneous sequences.

## Minimum sequence threshold

Setting a minimum sequence copy number below which sequences are discarded is the most widely used strategy for eliminating erroneous sequences (Alberdi et al. 2018). However, this minimum sequence threshold varies considerably across eDNA studies, with some researchers only discarding singletons (i.e. a read with a sequence that is present only once (Andruszkiewicz et al. 2017; Bista et al. 2017; Yamamoto et al. 2017)), while others only consider sequences represented by  $\geq 10$  identical reads for downstream analyses (e.g. (Fujii et al. 2019)). In any case, erroneous sequences must be removed with caution: more stringent thresholds might filter out rare biological sequences from the dataset, whereas more flexible thresholds might treat artefacts as true diversity (Laroche et al. 2017; Alberdi et al. 2018).

## Chimeric sequence detection

Chimeras are sequences formed when two or more biological sequences bind together during PCR (Judo et al. 1998; Edgar et al. 2011). Chimera formation is common in eDNA analysis, especially when DNA from closely related organisms is amplified (Edgar et al. 2011; Aas et al. 2017). Since chimeric sequences are very similar to their parent sequences (i.e. low divergence) and sometimes have identical sequences to valid genes, it is very challenging to distinguish chimeras from true biological sequences, even with dedicated software and complete reference sequence databases (Edgar 2016; Aas et al. 2017; Alberdi et al. 2018). Detection and removal of chimeras is of critical importance in eDNA studies because undetected chimeras can be misinterpreted as real biological entities or novel taxa, causing inflated estimates of true diversity and spurious inferences of differences in community composition (Edgar et al. 2011; Aas et al. 2017).

## Clustering strategy and percent identity cutoff for OTU assignment

eDNA metabarcoding typically clusters amplicon sequences into operational taxonomic units (OTUs) as an initial step in data processing. Many quality assurance and quality control approaches, such as denoising, also require sequence clustering prior to further analyses, including abundance and diversity estimation. Clustering groups sequences into OTUs based on percent identity thresholds that represent intraspecific differences and approximate species boundaries (Alberdi et al. 2018). The choice of clustering strategy for OTUs is crucial for estimating the true diversity of biological communities, so choosing the wrong strategy may result in either inflated or underestimated species richness and affect final conclusions (Alberdi et al. 2018; Xiong & Zhan 2018; Rytkonen et al. 2019). While OTUs are typically constructed using a percent identity cutoff of 97% (Bista et al. 2017, 2018), lower and higher thresholds (Fujii et al. 2019; Rytkonen et al. 2019) have also been used. Moreover, lineages evolve at variable rates, so no single cut-off value can accommodate the entire tree of life. Developers of other programs, such as Swarm, argue that a single global clustering threshold will inevitably be too relaxed for slow-evolving lineages and too stringent for rapidly evolving ones (Mahe et al. 2014, 2015; Andruszkiewicz et al. 2017; Sawaya et al. 2019).

## Taxonomic assignment threshold

Taxonomic assignment is performed using a wide variety of methods and programs, but in general, a search of reference sequence databases is conducted and query sequences (OTUs) within a predefined percent similarity to the reference sequence are assigned to the lowest possible taxonomic level. Taxonomic assignments may be considered valid if the percent similarity is above the predefined threshold, but some studies use different similarity thresholds to make assignments at different taxonomic levels, while some programs generate taxonomic predictions with confidence estimates specified by bootstrapping (Andruszkiewicz et al. 2017; Alberdi et al. 2018; Bista et al. 2018). Sometimes, OTUs are discarded because they do not match any sequence in the reference database (Laroche et al. 2017). This is problematic for accurate abundance and diversity estimation.

## Lack of ecological information

eDNA analyses mostly report presence/absence and/or recent occupancy. Information on the ecological status of target organisms, including the life history stages (adults, eggs, larvae) present, the sex ratio, body condition (sick or healthy), and activity (e.g. breeding or non-breeding) cannot be obtained, but may be crucial for making informed management and conservation decisions. For instance, amphibians have complex life cycles and live both on land and in water, as eggs, tadpoles or adults. Many amphibians are highly threatened and each threat operates on different, sometimes multiple, life history stages (Klein et al. 2017). Thus, knowledge of an organism's life history stages and their respective threats is critical for effective management of their population (Klein et al. 2017). Moreover, life history traits that cannot be assessed using eDNA can be key considerations for designing a successful eDNA-based study. For instance, a species' life history can influence how well (when, where, and how) it can be detected via eDNA surveys (Olson et al. 2012; Barnes and Turner 2016; Bylemans et al. 2017; Eiler et al. 2018; Takeuchi et al. 2019a; Wineland et al. 2019).

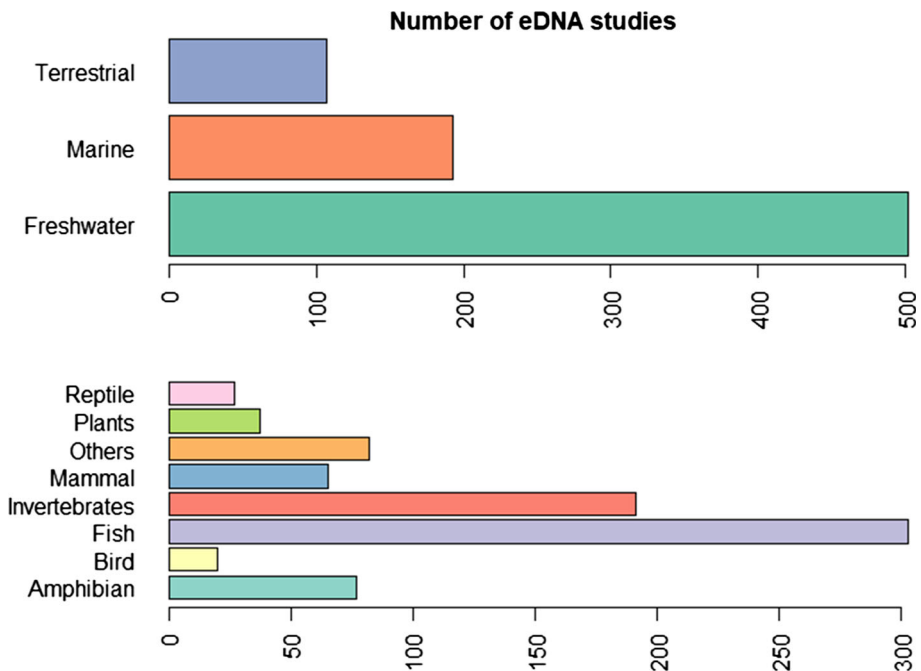
## Potential ways of reducing limitations in eDNA analysis

Researchers have long been focusing on the comparisons between the detection probability of eDNA and traditional survey methods (Ficetola et al. 2008; Jerde et al. 2011). But only recently have they begun to explore the origin, state, transport, and fate of eDNA and how these attributes influence species detection and quantification, data analysis, and result interpretation (Deiner and Altermatt 2014; Barnes and Turner 2016; Jerde et al. 2016; Collins et al. 2018; Lugg et al. 2018; Seymour et al. 2018; Seymour 2019). Most of the current limitations in eDNA analysis are directly or indirectly linked to technical aspects of the tool (Table 1). Developing improved techniques, optimizing current ones or combining eDNA with traditional surveys could overcome many of these limitations (Table 1).

## How eDNA and traditional surveys can complement each other

eDNA and traditional survey methods should not usually be considered as alternative methods for assessing and monitoring biodiversity, since they can give such different information (Ulibarri et al. 2017; Bailey et al. 2019; Rose et al. 2019; Leempoel et al. 2020; Takahara et al. 2020). Researchers must consider which of the two methods—or the

use of both— is most appropriate for addressing the questions they want to investigate (Qu and Stewart 2019). Information from eDNA often needs to be followed up with traditional surveys, but eDNA can help guide these surveys in the right direction (Rose et al. 2019; Ji et al. 2020; Sales et al. 2020). For example, Ji et al. 2020 found that leech-derived eDNA provides valuable information on the spatial distributions of vertebrate species and on the environmental and anthropogenic correlates of those distributions, making it a useful tool to efficiently measure the effectiveness of protected areas and to help optimize the deployment of management resources within reserves. The way in which eDNA and traditional surveys are implemented will largely be determined by the research questions, but will also be influenced by practical considerations, such as the availability of resources (including funding, time and the knowledge and skills of the persons undertaking the research), and sound methodology. Knowing when to employ eDNA techniques rather than—or in addition to—traditional sampling would enable practitioners to make more informed choices concerning data collection (Franklin et al. 2019; Qu and Stewart 2019). Based on the proportion of eDNA studies published (between 1 January 2008 and 31 December 2019, Fig. 2), eDNA might be the first choice for hard-to-collect aquatic species (e.g. marine macroinvertebrates) and would probably always be a useful supplement for fish and other cryptic aquatic species (Wineland et al. 2019). The complex nature of some projects can sometimes make it difficult for all aspects of a research question to be answered by a single method. In such cases, more than one method can be used to collect and analyze data, integrate the findings, and draw inferences (Harper et al. 2019; Jeunen et al. 2019; Knudsen et al. 2019; Wineland et al. 2019). eDNA can be an exceptionally



**Fig. 2** Number of studies using environmental DNA (eDNA) recovered from a literature search with the words ‘environmental DNA’ OR ‘eDNA’ for the period between 1 January 2008 and 31 December 2019 that utilized a different organismal group and ecosystem

useful ecological and conservation tool when used in combination with historical and other sources of data (e.g. citizen science) (Tingley et al. 2019). However, if conditions permit, traditional biodiversity surveys will usually still be the first choice, because of the additional types of information they can provide.

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