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

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**DOI**
10.1007/s10531-020-01980-0

**Original publication date**
9 April 2020 (First Online)

**Document version**
Author's accepted manuscript version

**Published in**
Biodiversity and Conservation

**Citation**
Beng KC, Corlett RT. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation. 2020;29(7):2089-121.

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

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**Acknowledgments**

This study was financially supported by the Yunnan Oriented Fund for Postdoctoral Researchers (Grant No. Y7YN021B09) and the Chinese Academy Science (CAS) 135 Program (Grant No. 2017XTBG-T03). Neither funding bodies played any role in the design of the study, data collection and analysis, interpretation of results, or writing the manuscript. We are grateful to Zhang Xiaowei for his insightful comments and suggestions that improved the manuscript quality.

## 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

## 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.* ; 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 & 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 (Figure 1).

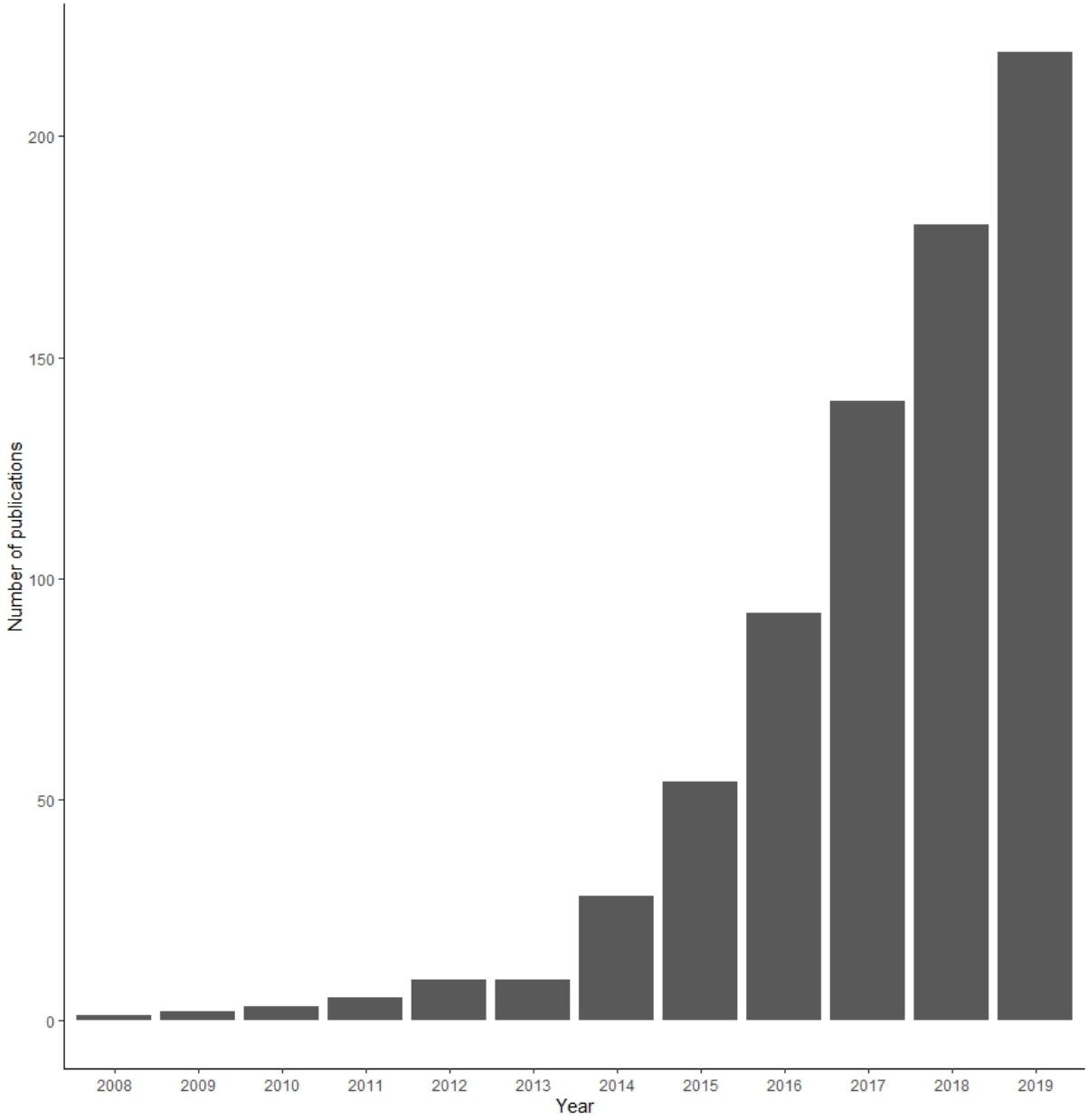


Figure 1 Number of studies using environmental DNA (eDNA) recovered from a literature search with the words 'environmental DNA' OR 'eDNA' for the period between 1 January 2008 and 31 December 2019.

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,

64 or sediment (TABERLET *et al.* 2012a; Thomsen & Willerslev 2015). DNA can persist in the
65 environment for periods from hours in temperate waters, to hundreds or thousands of years in
66 cold, dry permafrost, allowing direct isolation without any other obvious signs of an organism's
67 presence (Thomsen & Willerslev 2015). The utilization of eDNA has the potential to
68 revolutionize conservation science and practice in several ways. First, eDNA techniques are fast,
69 efficient and relatively cheap, thus providing the opportunity to monitor the dynamics of species,
70 populations and communities, and to map their geographic distribution over long time periods
71 and across large spatial scales (Ficetola *et al.* 2019; Itakura *et al.* 2019; Lecaudey *et al.* 2019;
72 Preissler *et al.* 2019; Reinhardt *et al.* 2019; Sutter & Kinziger 2019; Sales *et al.* 2020). Second,
73 eDNA sampling is simple, non-destructive, and non-invasive, causing no significant damage to
74 the target species or its habitats (Antognazza *et al.* 2019b; Mora *et al.* 2019; Leempoel *et al.*
75 2020). Third, eDNA can achieve high detection probabilities for rare, cryptic, and elusive species,
76 even at relatively low densities (Carvalho *et al.* 2019; Franklin *et al.* 2019; Shelton *et al.* 2019;
77 Takahara *et al.* 2020). Fourth, eDNA enables the early detection of biological invasions and their
78 timely eradication before full establishment (Lin *et al.* 2019; Nardi *et al.* 2019; Schumer *et al.*
79 2019; Tingley *et al.* 2019). Fifth, eDNA allows accurate identification of target organisms using
80 standardized, reproducible and auditable criteria that can be applied to different life stages and in
81 different environments (Preissler *et al.* 2019; Takeuchi *et al.* 2019a). Sixth, eDNA sampling
82 potentially offers a broad taxonomic breadth, allowing simultaneous biodiversity assessment for
83 a wide range of organisms (Sawaya *et al.* 2019; Thomsen & Sigsgaard 2019; Zhang *et al.* 2020b).

84 However, despite the ecological and conservation significance of the questions that can
85 potentially be addressed using eDNA, many challenges and limitations exist. eDNA does not
86 always work, and even when it does 'work', the results are not always what are needed. We

87 therefore review and synthesize eDNA studies published to date to highlight the opportunities
88 and limitations of utilizing eDNA in ecology and conservation. Additionally, we identify
89 potential routes to addressing fundamental assumptions and reducing the limitations of eDNA
90 (Table 1). We then propose a framework to discuss how eDNA can supplement traditional
91 biodiversity surveys. Lastly, we highlight new areas where eDNA studies are well positioned to
92 advance research in ecology, evolution and biodiversity.

93 ## 1. Literature search

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

99 ## 2. Current ecological and conservation questions addressed using eDNA

100 Two broad approaches that have received the most attention in eDNA-based studies are
101 barcoding and metabarcoding. The main difference between barcoding and metabarcoding is that
102 barcoding uses species-specific primers to detect the DNA fragments of a single species within
103 an environmental sample (Takahara *et al.* ; Franklin *et al.* 2019; Strickland & Roberts 2019;
104 Akamatsu *et al.* 2020; Harper *et al.* 2020; Kessler *et al.* 2020; Togaki *et al.* 2020) while
105 metabarcoding uses universal primers to simultaneously detect millions of DNA fragments from
106 the widest possible range of species from multiple trophic levels and domains of life (Alexander
107 *et al.* 2020; Cowart *et al.* 2020; Djurhuus *et al.* 2020; Yang & Zhang 2020; Zhang *et al.* 2020b).
108 For eDNA barcoding, conventional PCR (cPCR) is used to detect the presence of a species

(Jerde *et al.* 2011a; Dejean *et al.* 2012; Thomsen *et al.* 2012c; 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) (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 & Levi 2018; Mora *et al.* 2019; Thomsen & 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.* 2019b), 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 & Stewart 2019b). 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.

### 2.1 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).

### 2.2 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 & 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,

154 and evaluation of population dynamics (Amberg *et al.* 2019; Ardura 2019; Fernanda Nardi *et al.*
155 2019; Gomes *et al.* 2019; Rudko *et al.* 2019).

156 **2.3 Diet and trophic interactions**

157 Understanding and quantifying biotic interactions, such as predator-prey and host-parasite
158 relationships, are key components of ecological research. However, these important biological
159 processes remain poorly investigated, primarily due to methodological challenges. eDNA is
160 increasingly being used in diet analysis to estimate diversity, composition and occurrence
161 frequency of prey items in predator feces (Galan *et al.* 2018; Jusino *et al.* 2019; Mata *et al.* 2019;
162 Mora *et al.* 2019). DNA deposited by pollinators on flowers, and by dispersers on seeds, also
163 offers an opportunity to investigate plant-animal interactions and the role of these interactions in
164 the maintenance of ecosystem functions and the provision of ecosystem services (Harrer & Levi
165 2018; Thomsen & Sigsgaard 2019).

166 **2.4 Spawning ecology**

167 Most aquatic animals, except for aquatic mammals and reptiles, reproduce through the process of
168 spawning. Identifying areas for spawning, as well as the spatial extent of spawning activities, is
169 vital for the effective management and conservation of these species. However, understanding
170 the natural reproductive ecology of these organisms have mostly relied on collections of eggs,
171 larvae and spawning-condition adults (Tsukamoto *et al.* 2011; Antognazza *et al.* 2019b). These
172 techniques are often biased, invasive, destructive, and/or strictly dependent on a declining pool
173 of taxonomic experts for identifying life history stages (Maruyama *et al.* 2018). Surveys of this
174 nature are also generally labor intensive and time consuming, and can be inefficient at detecting
175 certain life history stages (Antognazza *et al.* 2019b; Fritts *et al.* 2019). For instance, kick-

176 sampling for eggs is sometimes conducted in areas of relatively shallow waters or during the day
177 whereas the adults spawn in deep waters or at night (Antognazza *et al.* 2019a). eDNA enables
178 the detection of a species regardless of its life stage or gender, and is transforming our ability to
179 non-invasively quantify spawning activities, and identify the spatial extent of spawning, with
180 limited resources (Maruyama *et al.* 2018; Tillotson *et al.* 2018; Antognazza *et al.* 2019b;
181 Bracken *et al.* 2019; Fritts *et al.* 2019; Takeuchi *et al.* 2019b; Takeuchi *et al.* 2019c).

182 **2.5 Monitoring biodiversity**

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

194 **3. Challenges and limitations of eDNA**

195 The application of eDNA in ecology and conservation has grown enormously in recent years, but
196 without a concurrent growth in appreciation of its limitations. While there is evidence that eDNA
197 can increase the precision and resolution obtainable from traditional biodiversity surveys

198 (Thomsen & Willerslev 2015; Yamamoto *et al.* 2017), this is certainly not true in all
199 circumstances, even with standardized and highly sensitive assays (Hinlo *et al.* 2017; Ulibarri *et*
200 *al.* 2017). In cases where eDNA has been successful, it might not necessarily be the appropriate
201 tool if information is required on the abundance or biomass of species (although this may be
202 possible in some cases (Takahara *et al.* 2012; Pilliod *et al.* 2013; Doi *et al.* 2015; Baldigo *et al.*
203 2017)), its ecology (life-history, sex ratio, breeding status), or its conservation status (Evans *et al.*
204 2017b; Trebitz *et al.* 2017). Presence/absence information from eDNA is useful in conservation
205 for monitoring populations at large spatial scales and for identifying habitats that are of high
206 value to species of conservation concern (Voros *et al.* 2017; Weltz *et al.* 2017). eDNA can also
207 be used to detect the first occurrence of an invasive species or the continued presence of a native
208 species that was considered extinct, sometimes at relatively low densities (Stoeckle *et al.* 2017;
209 Trebitz *et al.* 2017). However, presence/absence can be misleading when eDNA is present in the
210 environment in the absence of living target organisms or when eDNA is not detected but the
211 target organism is present (Song *et al.* 2017). Abundance data provide far more information on
212 the status of a population than presence/absence data and thus potentially allow for more robust
213 assessments of the factors affecting populations.

214 To date, increased speed and reduced cost remain the key advantages of eDNA
215 (Sigsgaard *et al.* 2015b). Whether eDNA sampling is more sensitive and has higher resolution
216 than traditional surveys remain controversial. For some species or taxa, eDNA performs better
217 than traditional methods (Kraaijeveld *et al.* 2015; Deiner *et al.* 2016; Olds *et al.* 2016; Strickland
218 & Roberts 2019; Tingley *et al.* 2019), for others, eDNA is as good as traditional surveys
219 (Hanfling *et al.* 2016; Hopken *et al.* 2016; Yamamoto *et al.* 2017), while for some, eDNA
220 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 & 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.* 2015a; Qu & Stewart 2019a), although this can depend on the target taxa, site-specific detection rates, budgets, and other considerations (Smart *et al.* 2016). For instance, Qu & 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.

244 However, (Smart *et al.* 2016) evaluated the relative cost of eDNA and bottle-trapping for
245 detecting the European newt *(Lissotriton vulgaris vulgaris)* and found that eDNA sampling was
246 more cost-efficient than trapping under low setup costs but bottle-trapping was more
247 cost-efficient than eDNA under high setup costs.

248 Qualitatively novel applications with actual conservation outcomes are still largely
249 lacking, although researchers are now moving away from proof-of-concept research to studies
250 that quantify population dynamics across organisms and environments (Stewart *et al.* 2017;
251 Carraro *et al.* 2018). However, the ability of eDNA to detect the continuous presence of a species
252 not sighted in its habitat for many years also raises questions about the mechanisms and
253 processes by which eDNA is transported and the conservation implications of unexplained
254 variability in eDNA transport (Sigsgaard *et al.* 2015a; Jerde *et al.* 2016; Lim *et al.* 2016).
255 Although methods and models to handle imperfect detection are increasingly being improved
256 (Piggott 2016; Guillera-Arroita *et al.* 2017; Ji *et al.* 2019), it is not possible to simply ignore the
257 presence of eDNA in the absence of living target organisms and/or the absence of eDNA in the
258 presence of living target organisms without actual field surveys. Increased PCR replication can
259 maximize eDNA detection and minimize false positives and/or negatives (Piggott 2016) but this
260 cannot substitute for actual biological replicates and will increase cost (Ficetola *et al.* 2015;
261 Roussel *et al.* 2015; Evans *et al.* 2017b). Detection of species using eDNA relies on DNA
262 isolated from living and dead cells (characterized by low concentration and high degradation
263 (Deagle *et al.* 2006)), and on PCR amplification (subjected to high variability and stochasticity
264 (Kebschull & Zador 2015)), and is prone to imperfect detection (Pilliod *et al.* 2014; Ficetola *et al.*
265 2015). Increasing the number of DNA extracts per sample or the number of amplifications per
266 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 & 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)(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.

289 Despite the important role that eDNA already plays in biodiversity assessment, diet
290 analysis, and detection of rare or invasive species, we are concerned that it is being over-
291 promoted as a standalone technique for ecological and conservation initiatives that may not fully
292 benefit from it (Roussel *et al.* 2015). We emphasize, in particular, that it is challenging to
293 distinguish between detection of eDNA and detection of a species, or to quantify organismal
294 abundance and biomass using eDNA, without a clear understanding of the challenges and
295 limitations of the technique. Failure to address these problems may confound the interpretation
296 of eDNA data.

297 **3.1 Imperfect sampling of eDNA and false detection**

298 eDNA is prone to imperfect sampling and false detection, which can occur at various stages of
299 the project, including field collection, sample storage, molecular analysis, and bioinformatics
300 workflows (Ficetola *et al.* 2016; Deiner *et al.* 2017; Doi *et al.* 2019; Piñol *et al.* 2019). Cases
301 where eDNA is detected in the environment in the absence of target organisms (false positives,
302 (Ficetola *et al.* 2015; Ficetola *et al.* 2016; Lahoz-Monfort *et al.* 2016; Stoeckle *et al.* 2016;
303 Guillera-Arroita *et al.* 2017)) or where eDNA is not detected but the target organism is present
304 (false negatives, (Morin *et al.* 2001; Ficetola *et al.* 2008; Schmidt *et al.* 2013; Ficetola *et al.* 2015;
305 Willoughby *et al.* 2016; Doi *et al.* 2019)) are common. Although site occupancy models have
306 been proposed as a way to account for imperfect detection, they largely depend on the number of
307 replicate samples per site and on the number of replicate amplifications per DNA sample (PCR),
308 which vary considerably across taxa (Schmidt *et al.* 2013; Matter *et al.* 2018; Chen & Ficetola
309 2019; Doi *et al.* 2019; Strickland & Roberts 2019). Causes of false detections include

310 **i. Limited persistence of eDNA in the environment**

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

331 **ii. Primer biases**

332 The suite of molecular markers used in eDNA analysis is extremely important for the
333 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).

### iii. Inhibition of DNA amplification

eDNA analysis involves the collection of complex and heterogeneous mixtures from aquatic ecosystems, soils, sediments, or feces (Koziol *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,

357 collagen from tissues, heme from blood, humic acid from soil, urea from urine, and melanin and
358 eumelanin from hair and skin (Watson & Blackwell 2000; Radstrom *et al.* 2004; Schrader *et al.*
359 2012). Although PCR inhibitors have different properties and mechanisms of action, they
360 generally exert their effects through direct interaction with DNA or interference with
361 thermostable DNA polymerases (Schrader *et al.* 2012). Direct binding of inhibitors to DNA can
362 prevent amplification and facilitate co-purification of inhibitor and DNA (Schrader *et al.* 2012;
363 Jane *et al.* 2015). Inhibitors can also interact directly with a DNA polymerase to block enzyme
364 activity. Since some inhibitors are predominantly found in specific types of samples,
365 matrix-specific protocols for preparation of nucleic acids before PCR are essential (Schrader *et*
366 *al.* 2012; Hunter *et al.* 2019).

367 **iv. Sample contamination**

368 Contamination occurs when DNA from an outside source (exogenous DNA) gets mixed with
369 DNA relevant to the research. For instance, if a frog is eaten at one pond, then the predator
370 defecates at another, this may introduce the frog's DNA to a pond where the frog is not present.
371 Because of the sensitivity of the technique, this is a serious issue in eDNA surveys and may
372 result in false positive detections and subsequent misinterpretation of results (Goldberg *et al.*
373 2016; Wilson *et al.* 2016). eDNA analysis requires multiple steps of sample handling and
374 manipulation in the field (collection, storage and transportation) and in the lab (storage, DNA
375 extraction, amplification, library preparation and sequencing), so contamination may occur at
376 various stages of the research (Goldberg *et al.* 2016; Doi *et al.* 2017). In the field, contamination
377 may occur when DNA from one or multiple samples is unintentionally transferred into another
378 sample, either from another site in the same study or from an unknown locality. This usually
379 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 & 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 & Smith 2005; Champlot *et al.* 2010; Goldberg *et al.* 2016; Wilcox *et al.* 2016).

### v. 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.

#### vi. 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.

### 3.2 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.* 2012c)

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.

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

#### i. Variability in eDNA deposition and preservation

The production and stability of eDNA (origin, state, decay, transport, persistence (Barnes & 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

445 sedentary species (ANDERSEN *et al.* 2012a; Buxton *et al.* 2017; Dunn *et al.* 2017; Hemery *et al.*
446 2017; Rees *et al.* 2017; Nichols *et al.* 2018).

447 ii. **eDNA sampling and processing biases**

448 Key considerations in eDNA analysis are maximizing DNA capture in the field, minimizing
449 degradation during transport and storage, and successful isolation and amplification (Pilliod *et al.*
450 2013, 2014; Turner *et al.* 2014b; Renshaw *et al.* 2015a; Goldberg *et al.* 2016; Wood *et al.* 2019).
451 The choice of eDNA sampling and processing protocols can significantly influence DNA yield,
452 detection probability, and the resulting abundance and biodiversity estimates (Brannock &
453 Halanych 2015; Deiner *et al.* 2015b; Renshaw *et al.* 2015a; Djurhuus *et al.* 2017; Thomas *et al.*
454 2018). Specific protocols used in each study vary with sample type (water, feces, soil, sediment),
455 the ecosystem of interest (freshwater, marine, terrestrial), and the questions being investigated
456 (Renshaw *et al.* 2015a; Goldberg *et al.* 2016; Djurhuus *et al.* 2017). For diet analysis, individual
457 fecal samples are collected and dehydrated immediately using either alcohol or silica gel or a
458 combination of both (Deagle *et al.* 2009; Zeale *et al.* 2011; Galan *et al.* 2012; Pompanon *et al.*
459 2012; Clare *et al.* 2014; Mata *et al.* 2016). In terrestrial ecosystems, multiple soil cores are
460 collected and analyzed separately or are pooled together, homogenized and a representative
461 subsample is taken. DNA is extracted from the soil samples immediately after collection or
462 samples are stored at -20 °C or –80 °C for processing at a later date (van der Heyde *et al.* ;
463 Andersen *et al.* 2012b; Bienert *et al.* 2012; Epp *et al.* 2012; Taberlet *et al.* 2012b; Yoccoz *et al.*
464 2012). In aquatic ecosystems, different protocols are being used to collect water, capture eDNA
465 with filters, transport samples from the field, and to store water and/or filters prior to DNA
466 extraction and amplification (Goldberg *et al.* 2011; Pilliod *et al.* 2013; Biggs *et al.* 2015;
467 Renshaw *et al.* 2015a; Majaneva *et al.* 2018). Some studies filter, precipitate or centrifuge water

468 on-site, and preservation media (e.g. ice, sodium acetate, lysis buffers, and absolute ethanol) are
469 used to stabilize eDNA for enough time (up to 24 h) to safely transport it for storage and
470 processing (Ficetola *et al.* 2008; Goldberg *et al.* 2011; Pilliod *et al.* 2013; Biggs *et al.* 2015;
471 Valentini *et al.* 2016a). In other studies, water is transported in cold conditions and filtration or
472 precipitation is done in the laboratory (Jerde *et al.* 2011b; Thomsen *et al.* 2012c; Goldberg *et al.*
473 2013). Minimizing DNA degradation in these samples is challenging, especially in remote field
474 sites with little or no access to cooling and in situations where samples need to be transported for
475 several days (e.g. international flights with stop overs) before processing.

476 Various types of filters have been used to capture eDNA (Minamoto *et al.* 2012; Thomsen *et al.*
477 2012a; Goldberg *et al.* 2013; Jerde *et al.* 2013; Piaggio *et al.* 2014) and the efficiency of each
478 filter type depends on its pore size , the volume and chemical properties (e.g. pH, organic and
479 inorganic particles) of the water filtered, and the extraction method (Liang & Keeley 2013b;
480 Turner *et al.* 2014a; Renshaw *et al.* 2015a; Eichmiller *et al.* 2016; Djurhuus *et al.* 2017;
481 Majaneva *et al.* 2018). In general, filtration is relatively more efficient for eDNA capture than
482 precipitation and centrifugation methods (Deiner *et al.* 2015a; Renshaw *et al.* 2015b; Eichmiller
483 *et al.* 2016; Spens *et al.* 2017; Majaneva *et al.* 2018). Among filters, cellulose nitrate (CN) filters
484 capture relatively more eDNA than polyethene sulfone (PES), polyvinylidene fluoride (PVDF),
485 and polycarbonate (PC) filters, while glass microfiber (GMF) filters capture relatively more
486 eDNA than PC filters (Liang & Keeley 2013a; Eichmiller *et al.* 2016).

487 In some aquatic environments (e.g. muddy water), the pore size of a filter can influence filtration
488 rate, where larger pore size filters (e.g. 5 μm) or pre-filtration require less time than smaller pore
489 size filters (1 μm). However, larger pore size filters and pre-filtration are less efficient in DNA
490 recovery than smaller pore size filters (Liang & Keeley 2013a; Eichmiller *et al.* 2016).

### iii. **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 & 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 & 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.* 2012b) 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

514 amplification efficiency among templates (O'Donnell *et al.* 2016; Leray & Knowlton 2017).
515 PCR amplification strategies also influence species detection and abundance estimation, with
516 quantitative PCR (qPCR) being relatively more effective for species detection and abundance
517 estimation than conventional PCR (cPCR) (Takahara *et al.* 2012; Turner *et al.* 2014b; Piggott
518 2016; Harper *et al.* 2018).

519 iv. **Variation in DNA copy number of target loci**

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

533 v. **Sequence filtering stringency**

534 Sequence filtering is a routine process in eDNA analysis and occurs at multiple steps of the
535 bioinformatics pipeline. For metabarcoding, raw sequence data are initially processed to filter

536 and correct (where possible) low-quality and erroneous reads (Valentini *et al.* 2016a; Evans *et al.*
537 2017a; Günther *et al.* 2018; Bakker *et al.* 2019; Rytkonen *et al.* 2019; Cowart *et al.* 2020; Zhang
538 *et al.* 2020a). This quality control step removes any phiX reads (common in marker gene
539 sequencing) and chimeric sequences detected in the raw sequencing data. Other quality filtering
540 criteria include trimming off the first *m* bases of each sequence, and/or truncating each sequence
541 at position *n* (Bakker *et al.* 2019; Cowart *et al.* 2020). The appropriate number of bases to be
542 trimmed and the truncation length can be determined using read quality profiles. Filtering can
543 also be performed on an OTU-table or a species-by-site matrix to remove samples with a total
544 read frequency less than a given threshold and/or OTUs observed in less than a given number of
545 samples (Bakker *et al.* 2019; Rytkonen *et al.* 2019; Cowart *et al.* 2020; Zhang *et al.* 2020a).
546 Filtering out OTUs that are detected in only one or a few samples is common, and this is based
547 on the suspicion that these low frequency OTUs are PCR or sequencing errors (Bakker *et al.*
548 2019; Rytkonen *et al.* 2019; Cowart *et al.* 2020; Zhang *et al.* 2020a). Taxonomy-based filtering
549 is also being applied to retain target taxa and/or exclude non-target taxa from eDNA analysis
550 (Bakker *et al.* 2019; Cowart *et al.* 2020; Zhang *et al.* 2020a). Although there are accepted
551 thresholds, across studies, about which filtering criteria are suitable, differences in sequencing
552 depth, marker region, primer specificity, and taxonomic breadth makes it difficult to reach a
553 general consensus (Evans *et al.* 2017a). Sequence filtering stringency can affect species detection,
554 abundance and biomass quantification (Rivera *et al.* 2020). More stringent thresholds might filter
555 out true biological sequences from the dataset, whereas more flexible thresholds might treat
556 artefacts as true biological sequences (Laroche *et al.* 2017; Alberdi *et al.* 2018). (AMEND *et al.*
557 2010) reported a tradeoff between sequence quality stringency and quantification by showing

558 that read-quality based processing stringency profoundly affected the abundance estimate for one
559 fungal species.

560 **4.3 Incomplete reference databases and taxonomic assignment biases**

561 Environmental DNA of complex eukaryotic communities is increasingly being used to quantify
562 biodiversity in terrestrial, freshwater and marine ecosystems (Civade *et al.* 2016;
563 Andruszkiewicz *et al.* 2017; Gillet *et al.* 2018; Fujii *et al.* 2019; Thomsen & Sigsgaard 2019).
564 Assignment of OTUs to species or higher taxonomic levels is a fundamental step in such studies.
565 However, the incompleteness of reference sequence databases for most organisms is an
566 important limitation for biodiversity studies using eDNA (Thomsen & Sigsgaard 2019). The
567 taxonomic identification of taxa is as good as the reference database used (Thomsen & Sigsgaard
568 2019). Reference sequences for taxonomic assignment are only available for one or a few genes
569 for most species and the targeted marker regions (e.g. COI, 12S, 16S) cannot accurately resolve
570 most groups to species or higher taxonomic levels due to incompleteness of reference sequence
571 databases (Deagle *et al.* 2014; Liu *et al.* 2017; Thomsen & Sigsgaard 2019). Consequently,
572 eDNA studies are often interpreted using molecular operational taxonomic units (MOTUs) or
573 higher taxonomic ranks (genus, family, order) instead of binomial species names (Thomsen &
574 Sigsgaard 2019). This makes it difficult to associate eDNA data with existing biological and
575 ecological knowledge. Although user-friendly and cost-efficient methods that generate full-
576 length reference barcodes could improve future eDNA studies (Liu *et al.* 2017), unbalanced
577 barcoding efforts across regions of the world, taxonomic groups, and molecular markers
578 (Ratnasingham & Hebert 2007; Machida *et al.* 2017; Porter & Hajibabaei 2018) currently limit
579 the application of eDNA in ecology and conservation.

580 **4.4 Limited understanding of the ecology of eDNA**

581 We lack a clear understanding of the ecology of eDNA − its origin, state, transport, and fate.
582 This information is critical for deciding whether eDNA sampling is the appropriate technique to
583 make robust inferences about an organism's presence, and to quantify abundance (Turner *et al.*
584 2014a; Barnes & Turner 2016; Stewart 2019). Environmental DNA originates as urine, feces,
585 epidermal tissues, secretions, reproductive cells or carcasses and this source material enters the
586 environment as particles of various sizes. These sources of eDNA may be rapidly transported
587 from the site of release, including leaching into the soil, downstream flow and dispersion by
588 water currents. Although particle size may be a major determinant of movement velocity, intact
589 genomic DNA within living cells may be transformed into extracellular fractions too small to be
590 detected (Barnes *et al.* 2014). (Murakami *et al.* 2019) found that eDNA of striped jack was
591 mostly detectable within 30 m of the source, (Jane *et al.* 2015) found that eDNA of brook trout
592 (*Salvelinus fontinalis*) could be detected 240 m downstream, (Deiner & Altermatt 2014a) found
593 that eDNA of daphnia (*Daphnia longispina*) could be detected 12.3 km downstream, and eDNA
594 of pelecypod (*Unio tumidus*) could be detected 9.1 km downstream. Despite the fact that eDNA
595 reflects the source within a range of distances (10–150 m; (O'Donnell *et al.* 2017; Yamamoto *et*
596 *al.* 2017; Murakami *et al.* 2019), the relationship between water current and eDNA transport is
597 not well known. Besides distance, many interacting factors can also influence eDNA detection
598 after leaving its source (Pilliod *et al.* 2014).

599 It is unlikely that all organisms release equal amounts of DNA into the environment and
600 that DNA from different sources degrades at the same rate, even under similar environmental
601 conditions. Therefore, the detection of a target species may be influenced by eDNA release and
602 degradation, which are in turn related to a species' size, life history, biotic interactions, and
603 abiotic conditions (Barnes *et al.* 2014). For freshwater fish, eDNA degradation rates vary from

604 10.5%/h in common carp (*Cyprinus carpio*; (Barnes *et al.* 2014)) to 15.9%/h in bluegill sunfish
605 (*Lepomis macrochirus*; (Maruyama *et al.* 2014)), while for marine fish, eDNA degradation rates
606 vary from 1.5%/h in three-spined stickleback (*Gasterosteus aculeatus*; (Thomsen *et al.* 2012b)),
607 4.6%/h in European flounder (*Platichthys flesus*; (Thomsen *et al.* 2012b)) to >5.0%/h in northern
608 anchovy (*Engraulis mordax*), Pacific sardine (*Sardinops sagax*), and Pacific chub mackerel
609 (*Scomber japonicas*) (Sassoubre *et al.* 2016). These studies suggest that the degradation rate of
610 eDNA in aquatic fish, for instance, exhibit both species and environment effects.

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

618 **4.5 Inconsistencies in data analysis and interpretation**

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

623 **i. Minimum sequence threshold**

624 Setting a minimum sequence copy number below which sequences are discarded is the most
625 widely used strategy for eliminating erroneous sequences (Alberdi *et al.* 2018). However, this

626 minimum sequence threshold varies considerably across eDNA studies, with some researchers
627 only discarding singletons (i.e. a read with a sequence that is present only once (Andruszkiewicz
628 *et al.* 2017; Bista *et al.* 2017; Yamamoto *et al.* 2017)), while others only consider sequences
629 represented by ≥ 10 identical reads for downstream analyses (e.g. (Fujii *et al.* 2019)). In any case,
630 erroneous sequences must be removed with caution: more stringent thresholds might filter out
631 rare biological sequences from the dataset, whereas more flexible thresholds might treat artefacts
632 as true diversity (Laroche *et al.* 2017; Alberdi *et al.* 2018).

633 **ii. Chimeric sequence detection**

634 Chimeras are sequences formed when two or more biological sequences bind together during
635 PCR (Judo *et al.* 1998; Edgar *et al.* 2011). Chimera formation is common in eDNA analysis,
636 especially when DNA from closely related organisms is amplified (Edgar *et al.* 2011; Aas *et al.*
637 2017). Since chimeric sequences are very similar to their parent sequences (i.e. low divergence)
638 and sometimes have identical sequences to valid genes, it is very challenging to distinguish
639 chimeras from true biological sequences, even with dedicated software and complete reference
640 sequence databases (Edgar 2016; Aas *et al.* 2017; Alberdi *et al.* 2018). Detection and removal of
641 chimeras is of critical importance in eDNA studies because undetected chimeras can be
642 misinterpreted as real biological entities or novel taxa, causing inflated estimates of true diversity
643 and spurious inferences of differences in community composition (Edgar *et al.* 2011; Aas *et al.*
644 2017).

645 **iii. Clustering strategy and percent identity cutoff for OTU assignment**

646 eDNA metabarcoding typically clusters amplicon sequences into operational taxonomic units
647 (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; Bista *et al.* 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).

### iv. 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.

### 4.6 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.* 2012a; Barnes & Turner 2016; Bylemans *et al.* 2017; Eiler *et al.* 2018; Takeuchi *et al.* 2019a; Wineland *et al.* 2019).

## 5. 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.* 2011b). 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 & Altermatt 2014b; Barnes & Turner 2016; Jerde *et al.* 2016; Collins *et al.* 2018; Lugg *et al.* 2018;

692 Seymour *et al.* 2018; Seymour 2019). Most of the current limitations in eDNA analysis are
693 directly or indirectly linked to technical aspects of the tool (Table 1). Developing improved
694 techniques, optimizing current ones or combining eDNA with traditional surveys could
695 overcome many of these limitations (Table 1).

696 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<br>Estimate detection rates using occupancy or other models | (Roussel *et al.* 2015; Valentini *et al.* 2016b; 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<br>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<br>Co-extract DNA and RNA<br>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) |
| | 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.*) |
| Limited understanding of the ecology of eDNA | eDNA origin, state, transport, and fate | Use experimental validation in laboratory and natural settings | (Barnes & 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) |
| | 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) |

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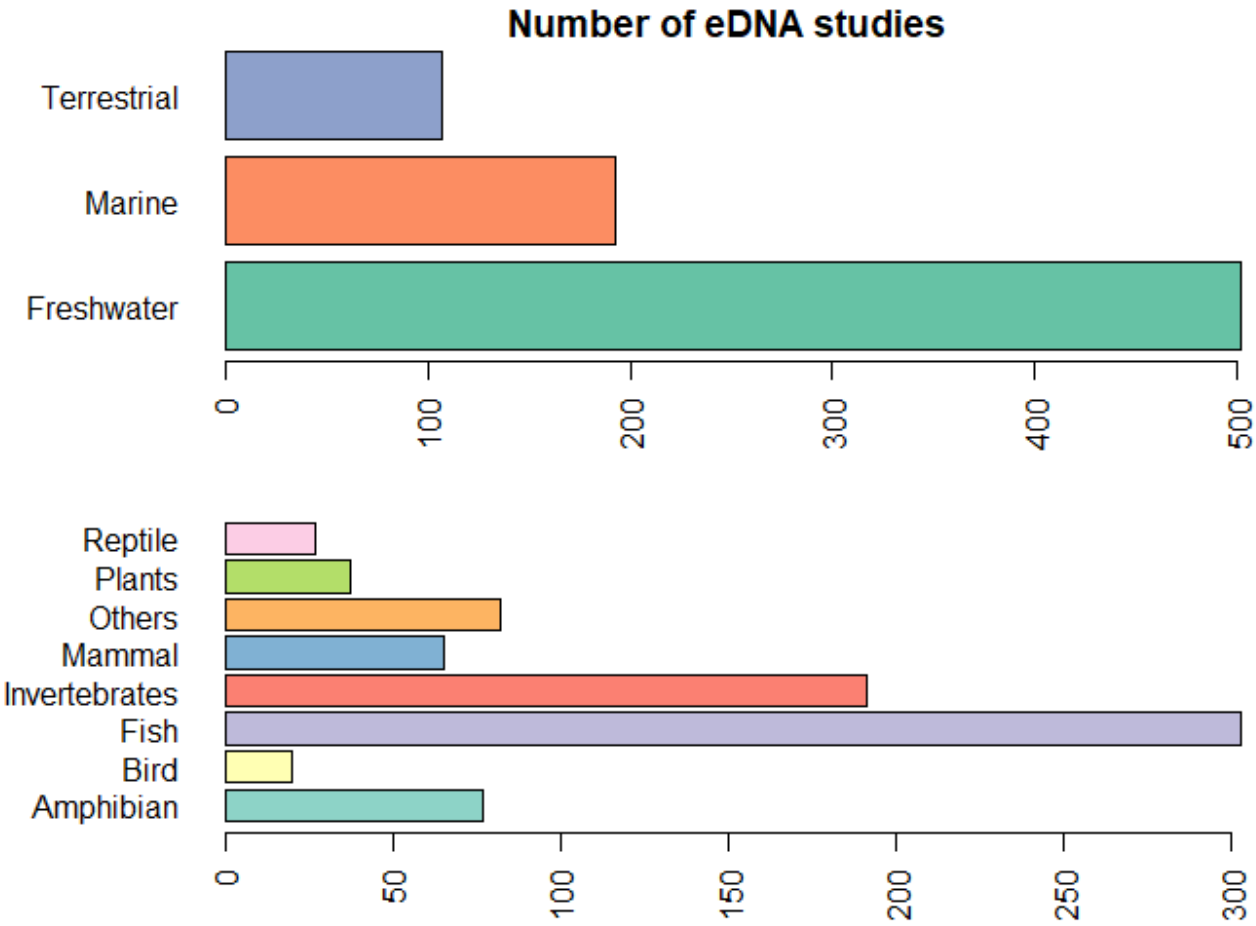
## 6. How eDNA and traditional surveys can complement each other

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 & Stewart 2019b). 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 & Stewart 2019b). Based on the proportion of eDNA studies published (between 1 January 2008 and 31 December 2019, Figure 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

722 one method can be used to collect and analyze data, integrate the findings, and draw inferences
723 (Harper *et al.* 2019; Jeunen *et al.* 2019; Knudsen *et al.* 2019; Wineland *et al.* 2019). eDNA can
724 be an exceptionally useful ecological and conservation tool when used in combination with
725 historical and other sources of data (e.g. citizen science) (Tingley *et al.* 2019). However, if
726 conditions permit, traditional biodiversity surveys will usually still be the first choice, because of
727 the additional types of information they can provide.

728




729

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

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