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Invited Review

# Environmental DNA and metabarcoding for the study of amphibians and reptiles: species distribution, the microbiome, and much more

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**Abstract.** In the last decade, eDNA and metabarcoding have opened new avenues to biodiversity studies; amphibians and reptiles are animals for which these new approaches have allowed great leaps forward. Here we review different approaches through which eDNA can be used to study amphibians, reptiles and many more organisms. eDNA is often used to evaluate the presence of target species in freshwaters; it has been particularly useful to detect invasive alien amphibians and secretive or rare species, but the metabarcoding approach is increasingly used as a cost-effective approach to assess entire communities. There is growing evidence that eDNA can be also useful to study terrestrial organisms, to evaluate the relative abundance of species, and to detect reptiles. Metabarcoding has also revolutionized studies on the microbiome associated to skin and gut, clarifying the complex relationships between pathogens, microbial diversity and environmental variation. We also identify additional aspects that have received limited attention so far, but can greatly benefit from innovative applications of eDNA, such as the study of past biodiversity, diet analysis and the reconstruction of trophic interactions. Despite impressive potential, eDNA and metabarcoding also bear substantial technical and analytical complexity; we identify laboratory and analytical strategies that can improve the robustness of results. Collaboration among field biologists, ecologist, molecular biologists, and bioinformaticians is allowing fast technical and conceptual advances; multidisciplinary studies involving eDNA analyses will greatly improve our understanding of the complex relationships between organisms, and our effectiveness in assessing and preventing the impact of human activities.

*Keywords*: cost effectiveness, detection probability, diet, DNA metabarcoding, high-throughput sequencing Microbiome, pathogens, qPCR.

## Introduction

Distribution records are among the most basic and pivotal data for many studies on species ecology, conservation and evolutionary biology. Unfortunately, obtaining robust distribution records is often challenging. Many species are elusive, live in difficult access environments and can be detectable only during specific periods or under specific weather conditions (Mazerolle et al., 2007). However, in the last decade a growing number of studies has demonstrated that the DNA of target organisms can be extracted from environmental samples (e.g. water, soil, faeces, ancient sediments, . . .) to obtain robust information on the presence-absence, and even on the abundance, of target species. This environmental DNA (eDNA) is revolutionizing our approach to biodiversity studies, as it greatly increases our ability to detect elusive species, and is also opening new research avenues, given that it allows obtaining information that was nearly inaccessible just one decade ago (Ficetola et al., 2008; Pompanon et al., 2012; Taberlet et al., 2012; Thomsen and Willerslev, 2015). On the other hand, as it occurs with any sampling technique, eDNA is not a perfect approach. The underlying assumptions and the technical issues of this approach are somehow different from the issues of traditional sampling strate-

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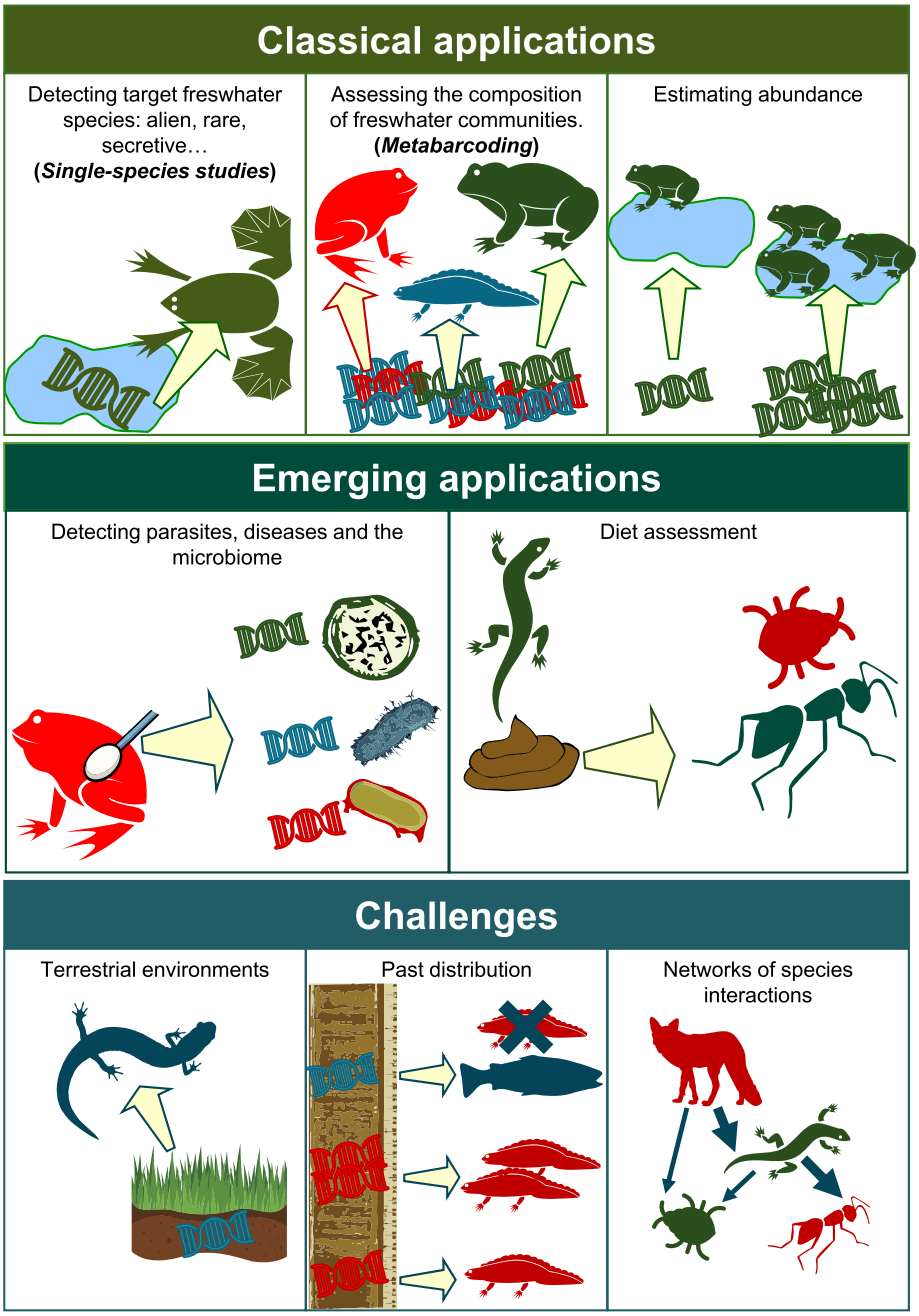


**Figure 1.** Examples of applications of environmental DNA and metabarcoding to the study of amphibians and reptiles.

gies, therefore special care is needed to obtain robust eDNA data.

Amphibians have been the first vertebrates for which eDNA has been extracted from water to successfully assess species distribution (Ficetola et al., 2008). Since then, impressive advancements of eDNA techniques have greatly broadened the application field, showing the usefulness of this approach for a huge range of research and monitoring questions (fig. 1).

In this review, we present different approaches through which eDNA can be used to study amphibians and reptiles (and many more organisms), we highlight some of its challenges, and describe additional aspects that have received limited attention so far, but can greatly benefit from innovative applications of eDNA.

## eDNA to assess the distribution of amphibians and reptiles

### *eDNA for the detection of macro-organisms from water samples*

In its early applications, eDNA was extracted from environmental samples to reconstruct the biodiversity of microorganisms (e.g. Venter et al., 2004). In microbiological studies, a large number of the target organisms (e.g. bacteria, fungi, . . .) are present in the environmental samples (water, soil, . . .), therefore DNA can be extracted directly from the target organisms within the environmental samples. Such a eDNA approach his boosting studies on the ecology and functioning of microbiological communities, revealing intriguing patterns in microbiome organization and leading to the emergence of a new theory of microbial community ecology (Goldford et al., 2018; see also the "microbiome" paragraph). The situation is somehow different from large organisms. When we haphazardly collect a water sample from a pond, it is unlikely it contains a frog or a turtle. However, all macro-organisms continuously interact with their environment. Urine, faeces, mucus, gametes or skin debris contain cells, cellular remains or extra-cellular DNA that flow into the environment, and all these sources of DNA can be used to detect species, either using approaches targeting one single species, either using the metabarcoding approach (Box 1).

Several analyses have shown that this approach can be more efficient than traditional monitoring. For instance, Dejean et al. (2012) assessed the distribution of invasive bullfrogs [*Rana* (*Aquarana*) *catesbeiana*] in ponds of Western France using both traditional monitoring (visual encounter surveys and calling surveys) and eDNA. Using traditional surveys, they detected bullfrogs in just 14% of sites. However, eDNA detected bullfrogs in many more ponds (78%), suggesting that the range of this invasive frog was broader than expected. This probably occurred because some life history stages (e.g. juveniles) have a lower detection probability than calling males, which are preferentially detected by traditional surveys. After obtaining the eDNA results, targeted and very intensive traditional monitoring was performed in ponds that were positive using eDNA (Dejean et al., 2012). Such new surveys detected bullfrogs at these sites, confirming the reliability and the higher sensitivity of the eDNA approach.

eDNA can be extremely useful to detect rare or secretive species, and to analyse difficult to explore environments. The olm (*Proteus anguinus*) is a remarkable example of secretive vertebrate. This cave-dwelling urodele inhabits deep groundwater in karstic environments. Vörös et al. (2017) have successfully used eDNA to detect olms from multiple caves of Croatia. Importantly, they have also detected olms in several caves where no records were available from traditional surveys, expanding the number of localities for this elusive species. Caves are among the less known environments on the globe, because of the complexity of access and exploration (Ficetola, Canedoli and Stoch, 2019), and eDNA is an extremely promising approach to understand the biodiversity and plan the conservation of these delicate environments.

eDNA is effective both in still and running water (Pilliod et al., 2014; Katano et al., 2017), even if in running water patterns and data interpretation are not identical to studies performed in still water. Some studies suggested that, in running water, the dilution effect can be strong. For instance, Jane et al. (2015) used caged fish to assess at which distance from the source eDNA can be detected. They found that the amount of eDNA quickly decreases far from the source, and detected the eDNA of target species

**Box 1. *eDNA*, *DNA Barcoding* and *DNA Metabarcoding***

The study of eDNA is strictly linked to DNA barcoding: interspecific differences for DNA sequences are used for the identification of species (Hebert et al., 2003b; Hebert, Ratnasingham and deWaard, 2003a). The Consortium for the Barcode of Life indicates the necessity of using standardized DNA regions for DNA barcoding, such as the mitochondrial cytochrome oxidase I gene for animals, and two plastid fragments (*rbc*L and *mat*K) for plants (Hebert et al., 2003a; Hebert et al., 2003b; Cbol Plant Working Group, 2009). However, the study of DNA from environmental samples requires the relaxation of this assumption. First, in most environments eDNA suffers degradation, thus eDNA studies generally retrieve DNA fragments with length 150 bp or shorter, and fragment length may be very short (below 100 bp) when dealing with DNA that remained for long periods in the environment (Murray et al., 2012; Bylemans et al., 2018, but see also Sigsgaard et al., 2017; Heeger et al., 2018). Furthermore, standard "barcoding" regions often have interspecific variation in the priming region, and this can cause heavy bias when multiple species are amplified in the same PCR run (Ficetola et al., 2010; Clarke et al., 2014; Taberlet et al., 2018). These issues hampers employing sequences used for standard DNA barcoding, such as the >600 bp region of mitochondrial DNA that constitutes the standard barcode for animals (Hebert et al., 2003b), and other DNA regions are routinely used in eDNA studies (Valentini, Pompanon and Taberlet, 2009a; but see also Baird and Hajibabaei, 2012).

Some of the first studies on eDNA from macro-organisms used specific primers amplifying a target species. However, high-throughput sequencing now allows the automated identification of multiple species from a single sample, such as an environmental sample containing the eDNA of multiple organisms: this approach is named ***DNA metabarcoding*** (Taberlet et al., 2012). "Universal primers" are used to amplify all the species within a single taxonomic group (e.g. amphibians, Testudines, fungi, bacteria…; Box 2) (Ficetola et al., 2010; Taberlet et al., 2018). The amplified DNA is sequenced using high-throughput sequencing, and the retrieved sequences are then compared to a reference database, to obtain the list of occurring taxonomic units (Taberlet et al., 2012).

at distances up to ∼240 m. Furthermore, very high rates of water flow may hamper detectability of eDNA from running water (Klymus et al., 2015). However, these findings have been challenged by studies performed at the drainage scale. Streams and rivers can convey eDNA from the whole hydrographical basin, thus it has been proposed that, in some circumstances, eDNA can be an indicator of ecological processes occurring at the drainage scale (Giguet-Covex et al., 2014; Deiner et al., 2017; Carraro et al., 2018; Ficetola et al., 2018b). More data are required to evaluate the relative importance of nearby and distant sources of eDNA in running water, and to ascertain the actual frequency of long-distance detections.

*Just one species or the whole community?*

The first eDNA applications to macro-organisms used specific primers, targeted at amplifying just one species during PCR (Ficetola et al., 2008; Jerde et al., 2011; Thomsen et al., 2012b). However, the advances of high-throughput sequencing now allow to simultaneously amplify, sequence and identify multiple species within a community (DNA metabarcoding; Box 1). In practice, it is possible to expand the aims of studies: instead of searching one single species, we can assess the composition of the whole community (Thomsen et al., 2012a; Thomsen et al., 2012b; Taberlet et al., 2018). The simultaneous assessment of a whole community requires primers that amplify all the species of a target taxon with the smallest bias, to avoid that PCR preferentially amplifies species with less mismatches in the priming region (Ficetola et al., 2010) (Box 2). Valentini et al. (2016) provide a particularly complete example of the challenges and of the advantages of community-wide eDNA studies. First, they developed primers for the amplification

**Box 2. Useful versatile primers for metabarcoding analyses of amphibians and reptiles.**

The selection of appropriate primers is an essential first step when planning eDNA and metabarcoding studies. We provide here a non-exhaustive list of versatile primers that can be useful for studying amphibians and reptiles. A first set of metabarcodes deals with the detection of amphibians and reptiles. Note that reptiles are a polyphyletic group, and as a consequence, it is virtually impossible to design a metabarcode targeting all reptiles and only reptiles at once. A second set focuses on the microbiome analysis. Finally, a short list of primers that can help analyzing their diet. We focus on primers amplifying relatively short sequences, which are particularly appropriate for the analysis of extracellular and degraded DNA. The primer sequences are compliant with the IUPAC nucleotide code. More information on all the primers (except the ones from Kartzinel and Pringle, 2015) are given in Taberlet et al. (2018).

| Aim | Target taxonomic group | Code | Forward primer (5'-3')<br>Reverse primer (5'-3') | References | Length (excluding primers) |
|---|---|---|---|---|---|
| Species identification | Vertebrata | Vert01 | TTAGATACCCCACTATGC<br>TAGAACAGGCTCCTCTAG | Riaz et al. (2011) | ~100 bp |
| | Batrachia | Batr01 | ACACCGCCCGTCACCCT<br>GTAYACTTACCATGTTACGACTT | Valentini et al. (2016) | ~50 bp |
| | Testudines | Test01 | AGACGAGAAGACCCTGTGGAA<br>TCCGAGGTCRCCCCAACC | Taberlet et al. (2018) | ~75 bp |
| Microbiome | Bacteria | Bact01 | GGATTAGATACCCTGGTAGT<br>CACGACACGAGCTGACG | Fliegerova et al. (2014) | ~250 bp |
| | Bacteria Archaea | Bact02 | GCCAGCMGCCGCGGTAA<br>GGACTACCMGGGTATCTAA | Taberlet et al. (2018) | ~250 bp |
| | Fungi | Fung01 | GGAAGTAAAAGTCGTAACAAGG<br>CCAAGAGATCCGTTGYTGAAAGT | Epp et al. (2012) | ~230 bp |
| Diet | Spermatophyta | Sper01 | GGGCAATCCTGAGCCAA<br>CCATTGAGTCTCTGCACCTATC | Taberlet et al. (2007) | ~50 bp |
| | Eukaryota | Euka02 | TTTGTCTGGTTAATTCCG<br>CACAGACCTGTTATTGC | Guardiola et al. (2015) | ~100 bp |
| | Oligochaeta | Olig01 | CAAGAAGACCCTATAGAGCTT<br>CCTGTTATCCCTAAGGTARCT | Bienert et al. (2012)<br>Taberlet et al. (2018) | ~120 bp |
| | Arthropoda | Arth02 | GATAGAAACCRACCTGGYT<br>AARTTACYTTAGGGATAACAG | Taberlet et al. (2018) | ~140 bp |
| | Insecta | Inse01 | RGACGAGAAGACCCTATARA<br>ACGCTGTTATCCCTAARGTA | Taberlet et al. (2018)<br>Taberlet et al. (2018) | ~160 bp |
| | Insecta | - | TGAACTCAGATCATGTAA<br>TTAGGGATAACAGCGTAA | Kartzinel and Pringle (2015) | ~110 bp |
| | Aranae | Aran01 | TTRYGACCTCGATGTTGAATT<br>CGGTYTGAACTCARATCATGT | Taberlet et al. (2018) | ~60 bp |
| | Opiliones | Opil01 | YTYAACTGTTTATCAAAAACAT<br>GCTACCTTAGCACAGTCA | Taberlet et al. (2018) | ~70 bp |

of amphibians (and also of bony fish). These primers are particularly effective; for instance, in-silico analyses suggested that primers designed for amphibians can be able to amplify up to 99% of amphibian species. When Valentini et al. (2016) used the metabarcoding approach to assess amphibian biodiversity, they consistently found that eDNA is able to detect amphibians better than traditional surveys, and provided more complete estimates of amphibian communities at all the study ponds.

Nevertheless, when analysing study systems where hybridization between species is possible, metabarcoding is generally unable to ascertain whether the retrieved sequences originated from parental species, or from their hy-

**Box 3.** ***Species identification vs. Molecular Taxonomic units***

It is important to remark that the molecular taxonomic units (MOTUs) identified through eDNA metabarcoding do not always correspond to traditional species. The used markers often amplify short DNA fragments (Box 2), thus closely related species can have the same barcode. An accurate correspondence between MOTUs and traditional taxa would require exhaustive databases, sequencing all the potentially present taxa (Parducci et al., 2017), but reference databases are limited in space and taxonomic scope. The growingly available public reference databases (e.g. GenBank) can be combined with curated, local databases containing the sequences of all the potential target species (Valentini et al., 2016; Bálint et al., 2018a) to ascertain species identity. For taxa or areas without exhaustive databases, it is also possible to work directly on MOTUs, as this allows extracting ecologically relevant information, with levels of details similar to the ones obtained with morphological species (Ji et al., 2013).

brids. Therefore, this approach can have limited usefulness in exploring biodiversity gradients across hybrid zones. Such an issue can affect metabarcoding studies aiming at detecting invasive species that can hybridize with the native ones, such as crested newts (*Triturus carnifex*, which is invasive in areas where the closely related *T. cristatus* is native) or hybridogenetic pool frogs (*Pelophylax*) (Herder et al., 2014).

Until now, the majority of eDNA studies has been performed in temperate areas, still biodiversity peaks in tropical countries (Hoffmann, Schubert and Calvignac-Spencer, 2016). Studying biodiversity in tropical areas is particularly challenging, for instance because of the very large number of species, because many species are poorly known, and because for many species we lack genetic information needed for primer developed and species assignment (Box 3). Nevertheless, metabarcoding studies can surpass traditional surveys in the estimation of amphibian biodiversity even in tropical areas (Lopes et al., 2016; Sasso et al., 2017; Bálint et al., 2018c). For instance, in the Atlantic forest of Brazil, four days of eDNA sampling were enough to detect nearly all the amphibian species recorded during a 5-year monitoring, and thus provide a particularly effective tool for the monitoring of these hyper-diverse communities (Sasso et al., 2017).

Overall, metabarcoding analyses seem to be more efficient than studies targeting just one single species, particularly because the costs of high-throughput sequencing are quickly decreasing. Disadvantages of studies focusing on one single target species also include the need of developing specific primers and the risk that primers amplify non-target sequences, both from related and unrelated taxa (e.g. bacterial sequences). Furthermore, community-wide data provide much more complete information on ongoing ecological processes and can be particularly useful for conservation.

*What about reptiles?*

The majority of studies using eDNA to analyse aquatic macro-organisms focused on amphibians and fish. eDNA works very well for these taxa, which account for ∼70% of all eDNA studies on aquatic animals, while reptiles account for just 6% of studies (Roussel et al., 2015). Water eDNA requires that organisms release DNA into the environment through mucus, faeces, urine, gametes and remains (Ficetola et al., 2008; Roussel et al., 2015), and physiological differences among species likely affect the amount of released eDNA. This can explain why some taxa have been the focus of less intense research. Aquatic reptiles have scales instead of epithelial cells or mucus, and their excretion systems produces much less urine compared to fish and amphibians. Consequently, the shedding rate of reptiles can be lower than the

one of fish and amphibians, reducing their detection rate (Raemy and Ursenbacher, 2018).

Despite these limitations, eDNA has allowed the successful detection of multiple reptile species. Burmese pythons (*Python bivittatus*) are invasive in Florida, and their detection probability using traditional methods is extremely low (0.05% per trap night; Hunter et al., 2015), as it is typical for many snake species (Ficetola et al., 2018a). As a consequence, the distribution of these snakes in the invaded range is largely unknown (Hunter et al., 2015). eDNA extracted from freshwater samples detected pythons with a much higher success than traditional surveys, thus eDNA can be an important resource to track the invasion of these snakes, particularly when combined with occupancy modelling (Piaggio et al., 2014; Hunter et al., 2015). Nevertheless, not all the studies on reptiles showed that eDNA has a better performance than traditional surveys. Studies on turtles successfully detected the eDNA of target species when animals were in artificial tanks or mesocosms (Davy, Kidd and Wilson, 2015; Raemy and Ursenbacher, 2018), but in some cases eDNA did not perform better than traditional surveys in natural wetlands, where densities can be low. In these cases, eDNA can complement, rather than replace, traditional approaches (Raemy and Ursenbacher, 2018). Given the functional importance of semi-aquatic reptiles for small freshwater environments, technical developments are probably needed to improve the efficiency of available approaches.

### *Not just freshwater: detection of terrestrial species from water and soil samples*

Most of waterbodies gather their water from complex drainages, therefore it has been proposed that DNA from terrestrial organisms living upstream or close to wetlands can be transported into water, and detected there. This might allow using water eDNA also to detect terrestrial species. Water samples from forest ponds allowed the detection of a large number of mammal species that probably live nearby ponds and use them (Ushio et al., 2017). Nevertheless, the detection rate of terrestrial species is likely much lower than in aquatic species. For instance, studies on streams of the Atlantic forest detected nearly all the amphibians using water for breeding or during adult life stages, while failed to detect strictly terrestrial species, even if they exploited environments not far from water (Sasso et al., 2017).

Furthermore, aquatic environments are just one of the potential targets of eDNA extraction. Most of eDNA studies performed so far on amphibians and reptiles focused on aquatic or semi-aquatic environments, and extracted the DNA from water. However, eDNA can be extracted from a variety of environmental media, including soil, and soil eDNA has proven to allow the characterization of macro-organisms living both underground (e.g. Earthworms; Bienert et al., 2012) and above the soil (e.g. mammals and birds; Andersen et al., 2012). A very large number of amphibians and reptiles are strictly terrestrial, and many species live in underground environments. The detection of underground species is often challenging, therefore soil eDNA might be used to improve knowledge of their distribution and ecology, still the usefulness of eDNA extracted from soil samples to detect amphibians and reptiles has received limited attention so far. Kucherenko et al. (2018) maintained snakes in small containers, and successfully amplified the DNA from the soil of most of containers. Snake DNA was also detected in the field, from the soil of burrows inhabited by snakes, supporting the usefulness of soil eDNA for the detection of terrestrial herps. Nevertheless, other studies obtained contrasting results. For instance, Walker et al. (2017) maintained plethotontid salamanders in mesocosms, but successfully amplified salamander DNA from just $\sim$1% of soil samples. The monitoring of terrestrial vertebrates from soil samples can be challenging, and probably eDNA detection is more complex than in aquatic species, nevertheless there is

room for methodological developments, to understand the reason of strong differences in success rate among studies, and to improve the detection rate.

*eDNA for species detection: costs, benefits, and affordability for field ecologists*

Despite we have shown that eDNA analyses improve the detection of rare and secretive aquatic amphibians and reptiles, field ecologists could rebut that the cost of molecular analyses would offset the advantage of such approach. However, studies comparing the costs and benefits of different approaches have revealed that eDNA is often generally cost-effective, when compared to traditional field surveys.

For instance, Davy et al. (2015) compared the costs of traditional surveys methods and eDNA for the detection of nine species of freshwater turtles in Canada. Turtle detection using visual encounter surveys or trapping required a large number of surveys, while less man-hours were needed for eDNA analyses. Therefore, when also considering the personnel costs, turtle detection was generally cheaper using eDNA. Similarly, Smart et al. (2016) compared traditional trapping method and eDNA detection for sampling a European newt species (*Lissotriton vulgaris*), recently introduced in Australia. Traditional trapping methods attained lower detection probabilities than eDNA detection, and were not necessarily cheaper. eDNA sampling can require high expenses depending on the costs of laboratory setup, developing primers/probes, of sample processing and on the number of qPCR assays required to define positive samples (Smart et al., 2016; Bálint et al., 2018c), and models for design surveys in order to minimize the total necessary budget are going to be developed (Lugg et al., 2018). Generally, eDNA has a relatively high starting price because consumables and sequencing are costly, but neither the time spent in the laboratory, nor the sequencing costs increase linearly with the number of sampling sites, thus the cost-efficiency of eDNA detection may be maximised by performing surveys on a large number of sites, and can be particularly high in studies analysing many species with the same metabarcodes (Bálint et al., 2018c). In broad-scale studies, it is possible to have high detection rates of aquatic amphibians for hundreds of ponds in less than two weeks, with a cost per pond of less than £50 (Harper et al., 2018). It must also be remarked that the cost of molecular analyses is expected to decrease in the next years, for instance thanks to the improvement of sequencing technologies, further increasing the advantages of eDNA-based monitoring. The growing accessibility of molecular tools will facilitate the integration of eDNA also in traditional monitoring programs that are currently performed using field surveys, and the high cost-effectiveness of this approach can allow the expansion of monitoring programmes over broadest spatial, temporal or taxonomic extents.

*Detection or abundance?*

Assessing presence/absence of species is just one of the possible tasks of biodiversity studies. In principle, measures of abundance can provide much more complete information for both ecological and conservation studies. For instance, abundant species generally have a strongest impact on ecosystem dynamics, and measures of abundance are also extremely important to assess species conservation status (Nichols and Williams, 2006; Leung et al., 2012). Therefore, there is growing interest on the possible use of eDNA to obtain measures of relative and even absolute abundance, still only limited consensus among approaches exists.

Several methods have been proposed to transform eDNA data in measures of relative abundance (fig. 2), and their application is somehow different between studies targeting one single species, and metabarcoding studies. First, qPCR studies can use standards to quantify the number of template eDNA molecules (Thomsen et al., 2012b; Goldberg et al., 2016). The average number of DNA molecules estimated through

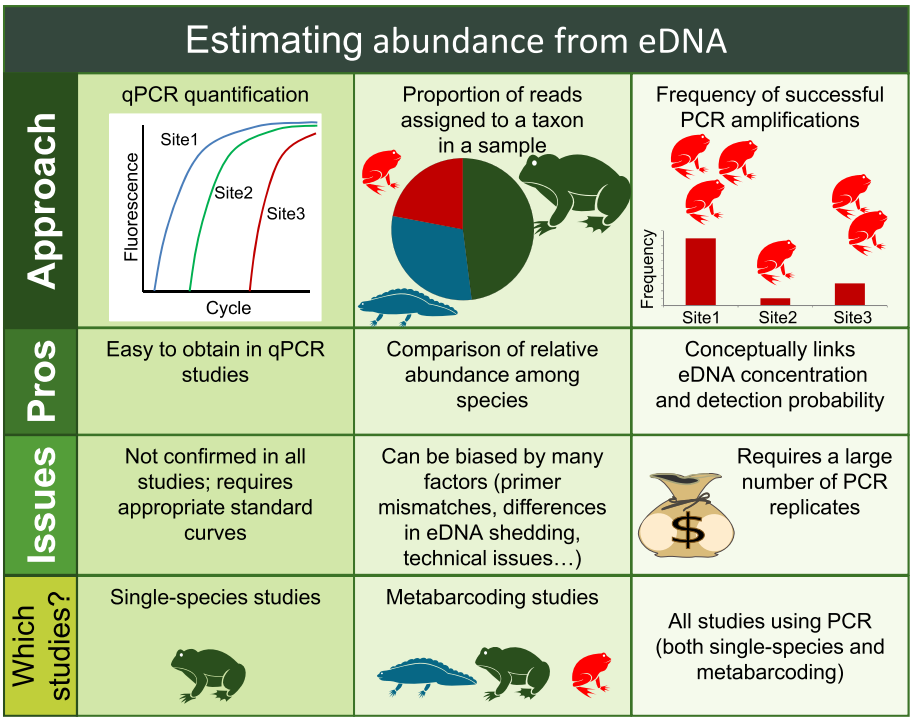


**Figure 2.** Estimating species abundance on the basis of environmental DNA: approaches and challenges.

qPCR has been correlated to species abundance in both mesocosms and field studies, suggesting usefulness of this approach to assess species abundance. However, this relationship can be affected by outliers, is not observed in all settings, and quantification though qPCR requires that the standard curve contains concentrations similar to those found in eDNA samples (e.g. 10 or less copies; Goldberg et al., 2016).

Measuring species abundance can be even more complex in metabarcoding studies. The relative abundance of eDNA of a given taxon (measured as the proportion of reads assigned to that taxon in a sample) is often positively correlated to the relative abundance (e.g. biomass) of species (Pansu et al., 2015a; Evans et al., 2016). However, such relationship can be biased by multiple factors, such as differences in eDNA shedding among species, different match with primers, and many technical parameters (e.g. polymerase mixes, GC content of the starting material, number of PCR cycles, . . .), therefore results need to be interpreted with cautions (Fonseca, 2018; Nichols et al., 2018).

Finally, the detection probability of species generally increases with their abundance (Tanadini and Schmidt, 2011). Similarly, the probability of detecting target DNA given it is present at a site increases with its concentration. When eDNA studies perform repeated analyses over the same samples, the frequency of successful PCR amplifications increases with species abundance, because detection probability increases with eDNA concentration (Furlan et al., 2016). If a large number of PCR replicates is performed, the frequency of successful PCR amplifications can be also viewed as a measure of relative abundance among samples, and can be used to estimate the probability of presence of the species (Ficetola et al., 2016; Lahoz-Monfort, Guillera-Arroita and Tingley, 2016). Measures of abundance, based on detection probability analyses of metabarcoding data (Lahoz-Monfort et al., 2016), correlated

positively with other measures of species abundance, obtained independently with traditional methods (Ficetola et al., 2018b). Furthermore, in qPCR studies, the number of PCR replicates per sample that successfully amplify is an additional approach allowing to estimate the concentration of target eDNA molecules (Furlan et al., 2016). The frequency of positive PCRs relies on the probabilistic nature of eDNA data, and can be useful to obtain abundance estimates in both single-species and metabarcoding eDNA studies.

Despite the availability of multiple approaches, all abundance estimates obtained using eDNA have their own limitations, and should be treated with caution (fig. 2). When eDNA is used to analyse a new system and a new set of species, it is therefore useful calibrating the relationship between eDNA and species abundance, for instance in a subset of sites for which abundance is also measured using traditional tools. All field researchers are well aware of the difficulties of obtaining reliable abundance estimates even when using traditional methods (Schmidt, 2004; Griffiths et al., 2015; Ficetola et al., 2018c). Finally, even if a positive relationship can exist between eDNA and species abundance, the predictive power of this relationship is not always strong (Thomsen et al., 2012b). When eDNA has a weak capacity to predict species abundance, its usefulness for conservation purposes can be limited. Collaboration among field biologists and molecular ecologists is thus essential to evaluate whether the abundance proxies are sufficiently robust to answer the study questions.

## Beyond the detection of present-day species

eDNA has been mostly used to detect the species that are currently present, particularly in aquatic environments, still its application field is potentially much broader. Until now, many of these applications have received limited attention in the herpetological literature, but can greatly expand our understanding of the biology of these organisms.

### *Species distribution in the past*

DNA molecules can persist in the environment for relatively long periods, thus a number of studies has successfully extracted ancient eDNA from appropriate paleo-environmental archives (e.g. soil, sediments, ice cores, ...) to understand long-term changes of ecosystems (Parducci et al., 2017; Bálint et al., 2018b). For instance, ancient eDNA has allowed to assess how human activities have determined vegetation changes in the Alps through the last seven millennia (Pansu et al., 2015b), and to identify the mammal communities associated with Neanderthal and Denisovan humans (Slon et al., 2017). Despite the existence of technical challenges, ancient eDNA has been applied to a very wide range of taxonomic groups, including plants, microorganisms, mammals and fish (Giguet-Covex et al., 2014; Pedersen et al., 2016; Parducci et al., 2017; Bálint et al., 2018b; Ficetola et al., 2018b), but surprisingly limited work has targeted amphibians and reptiles. Given that they constitute a substantial portion of the biomass, particularly in some typologies of wetlands (Gibbons et al., 2006), ancient eDNA has the potential to provide key insights on long-term dynamics. For instance, the eDNA of some amphibian species (*Bufo bufo*, *Rana temporaria* and *Ichthyosaura alpestris*) has been detected in 1000-years old sediments of a lake of the French Alps (Ficetola and Taberlet, unpublished data), suggesting that the eDNA stored in the sediments of lakes could be used to understand variation through time of the distribution of species; moreover, it could be combined with additional paleoecological data to understand species response to environmental variation (habitat and climatic changes, introduction of alien species, ...).

Nevertheless, ancient DNA studies pose specific challenges compared to traditional eDNA analyses (reviewed in Cooper and Poinar, 2000; Parducci et al., 2017; Bálint et al., 2018b). First,

the abundance of ancient eDNA is particularly low, thus enriching target DNA with PCR or DNA capture is a critical step. The whole eDNA can be also sequenced directly (shotgun sequencing) (Pedersen et al., 2016), but this poses challenges at the data analysis step (Bálint et al., 2018b). Second, ancient DNA fragments are very short and suffer nucleotide damages, increasing the complexity of taxonomic assignment. Finally, the very low DNA concentration determines a high risk of contamination, thus ancient eDNA require specific protocols to ascertain that detected sequences are genuine, and are not the result of contamination (Cooper and Poinar, 2000; Parducci et al., 2017; Bálint et al., 2018b; Zinger et al., 2019).

*Metabarcoding for diet analysis*

DNA metabarcoding has been extensively used for diet analysis, by extracting DNA from faeces and gut contents (review in Pompanon et al., 2012). Many studies have already been carried out for different groups of animals, particularly mammals, birds, fish, and insects. These studies included species with a wide range of diet typologies such as herbivores, carnivores, omnivores and even coprofagous, and allowed to describe diet with unprecedented resolution, revealing unexpected habits and dietary shifts (e.g. Valentini et al., 2009b; Kowalczyk et al., 2011; Shehzad et al., 2012; De Barba et al., 2014; Kerley et al., 2018) (Box 2). Surprisingly, to our knowledge DNA metabarcoding has not been used at all for amphibians so far, and only rarely for reptiles. Dietary assessment can be particularly challenging in reptiles, because stomach flushing can be harmful (Perez-Mellado et al., 2011), and some soft-bodied organisms can be poorly represented or difficult to identify morphogically in faeces (Pincheira-Donoso, 2008; but see also Perez-Mellado et al., 2011). Brown, Jarman and Symondson (2012) tracked earthworm consumption by slow worms (*Anguis fragilis*), for which the underground habits strongly limit the application of traditional approaches to diet analysis. They found that 86% of slow worms had eaten earthworms, and that all type of earthworms was consumed (epigeic, endogeic, and anecic species). Kartzinel and Pringle (2015) developed new 16S primers amplifying arthropods, but not reptiles, to study the diet of a lizard on Caribbean islands (*Anolis sagrei*). They demonstrated that dietary composition and richness were similar between size-dimorphic sexes, and that females had greater dietary richness per faeces than males. Accurate diet information is increasingly important in studies assessing the functional role of species, and the metabarcoding approach can allow obtaining high-quality diet information overcoming several limitations of traditional approaches. For instance, metabarcoding can allow species identification even in absence of diagnostic body parts and, if reference databases are available, can identify species with very high taxonomic resolution. A broadest application of this approach has the potential to boost our understanding of several aspects of herps biology, from the reconstruction of food webs to evolutionary and conservation issues. Finally, besides dietary studies, eDNA extracted from faeces can also allow the detection of parasites, thus opening new perspectives on questions related to parasites and their ecology (Bass et al., 2015).

*The microbiome*

The microbiome corresponds to a community of commensal, symbiotic, and pathogenic microorganisms found in and on multicellular organisms. It includes bacteria, archaea, fungi, protists and viruses, and metabarcoding is a powerful approach for cost-effective identification of the whole microbial diversity in both skin and gut. A complete analysis of studies on the microbiome of amphibians and reptiles is beyond the aims of this review. Nevertheless, given the importance of metabarcoding for microbiome analyses, we highlight key aspects that are emerging in the last years. A growing number of studies is focusing on different aspects of the microbiome, probably because of

the strong interest of pathogens and parasites for amphibian conservations (review in Bass et al., 2015; Jimenez and Sommer, 2017).

Most of these papers concerned the presence of pathogens, such as the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and the relationships between *Bd* and the microbiome (e.g. Kueneman, Weiss and McKenzie, 2017). If it seems that *Bd* has a strong effect on the skin microbiome (Jani and Briggs, 2014; Bataille et al., 2018; Ellison et al., 2019), the influence of the microbiome on the resistance to infection by this fungus is less clear. Furthermore, it is increasingly evident that the microbiome can be strongly affected by environmental variation, with possible cascading effects on hosts' immunity. For instance, it has been shown that pesticides could alter not only amphibian skin microbiome, but also increase *Bd* infections (Mccoy and Peralta, 2018). For frogs, host species identity was the strongest predictor of skin microbiome, with the locality explaining additional variation (McKenzie et al., 2012; Kueneman et al., 2014). Surprisingly, opposite results have been found when comparing multiple salamander species: the environment seems to play a more significant role than the species identity (Bird et al., 2018; Wolz et al., 2018). Skin microbiome also varies according to the developmental stage, and hatching and metamorphosis induce major changes in bacterial communities (McKenzie et al., 2012; Kueneman et al., 2014; Prest et al., 2018). The skin bacterial diversity of reptiles seems to be even higher than whose of frogs (Weitzman, Gibb and Christian, 2018), and a few studies assessed relationships between bacterial diversity and the pathogenic fungus *Ophidiomyces ophiodiicola* (Allender et al., 2018; Hill et al., 2018).

Very recently, a few papers are shedding light on the gut microbiome of both amphibians and reptiles. For amphibians, Chai et al. (2018) have shown that the life history transition occurring at metamorphosis determine major microbiome transitions in the toad *Bufo gargarizans*. For reptiles, Trevelline et al. (2018) suggest that lizards may acquire maternal microbiome in ovo, probably through the inoculation prior to shelling. Environmental contamination can have strong effects also on gut microbiome, with potential cascading effects at the organismal level. For instance, cadmium exposure modified the composition and reduced the diversity of intestinal microbiome of *Rana chensinensis* tadpoles (Mu et al., 2018), while exposition to glyphosate had a negative effect on the gut microbiome of green turtles (Kittle et al., 2018).

Analysing skin and gut bacteria relies on increasingly-well established protocols (but see also Pollock et al., 2018), therefore there is no doubt that many studies will be initiated in the near future to better understand the role of the microbiome, particularly in connection with conservation strategies.

## Technical challenges

There are two categories of environmental DNA analysis: single species identification and DNA metabarcoding, and each has its own technical challenges (Zinger et al., 2019). Concerning single species identification through specific primers, the challenges are (i) to implement an efficient sampling protocol that reliably capture the target species, (ii) to properly monitor the contamination risk, both in the field and in the laboratory, and (iii) to use a highly specific marker that only amplifies the target species, thus avoiding the risk of false positives. For this last point, it seems risky to only rely on a positive PCR to attest the presence of a species, and running quantitative PCR experiments with an internal probe provides more reliable results (e.g. Kim, Lim and Lee, 2013).

Challenges can be even more complex in DNA metabarcoding studies (Zinger et al., 2019). The experimental design for metabarcoding is apparently easy to implement according to the simplicity of its different steps, which consist of (i) sampling and extracting environmental DNA, (ii) amplifying a metabarcode, (iii) sequencing on a high-throughput sequencer, and (iv) analysing sequences on the

basis of published bioinformatics pipelines. Unfortunately, this simplicity is only apparent, and obtaining reliable metabarcoding results suffers from many difficulties due to several categories of experimental artefacts (Taberlet et al., 2018). First, the sampling protocol might not collect the expected biodiversity; eDNA availability can change with seasons, and the various available sampling protocols have very different ability to capture the different sources of eDNA. Furthermore, the ability of eDNA data to provide a correct representation of the environment depends on the use of appropriate standardization and randomization of sampling, and on respecting strict and repeatable field procedures (Dickie et al., 2018). Second, the amplification step might introduce a strong bias among the different target taxa, for instance because species have a different number of mismatches with the primers (Clarke et al., 2014; Deagle et al., 2014). Despite repeated calls for using just one standardized metabarcode for animals (Andújar et al., 2018), the low abundance of eDNA and the risk of amplification bias requires the accurate identification of highly conserved primers, optimized for the different target organisms (e.g. Box 2). Third, the "tag jump" problem can lead to false positives. Metabarcoding studies rely on the possibility to track tagged amplicons back to samples from which they originated (Schnell, Bohmann and Gilbert, 2015). False combinations of used tags can determine the assignment of sequences to the wrong samples and artificially inflate diversity, and this is particularly true for the most common taxa (Schnell et al., 2015). Fourth, the "index jump" problem can also erroneously show the presence of a taxon in a sample, if several libraries using the same primers are loaded on the same sequencing lane (Taberlet et al., 2018). Finally, bioinformatics filtering is essential to clean the data, and relaxed filtering thresholds during the bioinformatics analysis might also generate false presences. All these potential problems must be taken into account when designing an experimental protocol that will limit their impact and secure the final results.

To cope with sampling problems and to assess the variance of the results for each sample, it is necessary to include several biological replicates, e.g. collecting multiple samples from the same site, and analysing them in parallel. Furthermore, to limit the impact of the stochastic aspect of PCR, it is important to perform several technical replicates (e.g. multiple extractions of the same sample and/or multiple PCR replicates from the same extract). The setup of a large number of positive and negative controls allows to adjust the filtering steps, and to deal with several potential problems such as sporadic contaminations (Taberlet et al., 2018). Contamination can be a particularly strong issue if analyses are not performed in dedicated labs with strict conditions. In fact, not fulfilling the highest lab standards can result in the "detection" of species that were not present in the samples, and even the amplification of taxa analysed in the same lab during previous activities. To improve the quantitative aspect of metabarcoding, it is also possible to add an internal standard, either at the extraction (Smets et al., 2016) or at the PCR step (Saitoh et al., 2016; Ushio et al., 2018). Understanding the impact of these sources of bias is a key question of eDNA and metabarcoding analyses (Zinger et al., 2019), and we need more studies evaluating the effects of these sources, for instance by using different approaches on mock or on known communities.

Lastly, the use of appropriate statistical approaches for data analysis is a major challenge for all the studies. Even the best data can lead to misleading conclusions if analyzed incorrectly, thus the highest standards must be applied to statistical analyses of both single-species and metabarcoding analyses. Key issues include the use of models that can deal with false positives and false negatives (see following paragraph), the estimation of diversity measures from molecular operative units (Box 3), and the correct analysis of data characterized by complex levels of dependency and replication

(e.g. multiple sites with complex spatial structure; multiple samples per site; multiple technical replicates per sample).

Two decades ago, Cooper and Poinar (2000) concluded that researchers working on ancient DNA should "do it right or not at all", as failure to adhere to expensive and time-consuming good practices can lead to dubious claims and undermine advances. Even though eDNA and metabarcoding are certainly less challenging than ancient DNA, researchers must be aware of the existing complexity, and they should use the highest technical and analytical standards to ensure robustness and reproducibility of results.

*A matter of detection: false absences and false presences*

All researchers are aware that occurring species can remain undetected during biological surveys. Environmental DNA often allows a better detection probability, compared to traditional surveys, still species detection using this approach remains imperfect (Schmidt et al., 2013; Ficetola et al., 2015; Chen and Ficetola, 2019). eDNA analyses usually start from a limited amount of DNA, and stochastic processes determine whether PCR amplifies a given DNA molecule, thus it is essential to replicate analyses (biological and technical replicates; see above). The optimal number of replicates per environmental sample depends on the detection probability of the species. Even for the best detectable taxa, at least four PCR replicates can be needed to obtain reliable estimates of species distribution, and eight or more replicates have been recommended when detectability is limited (Ficetola et al., 2015). Given the pervasiveness of contaminations, it is generally suggested that only taxa confirmed by multiple, independent analyses should be considered to be genuine. Unfortunately, performing more replicates also intensifies the risk of false positives. Furthermore, dismissing taxa detected in just one or a few replicates increases the risk of missing rare species. When multiple samples are analysed with multiple technical replicates, site-occupancy detection models allow to estimate the true frequency of the species, its detection probability, and even the rate of false positives (Ficetola et al., 2015; Ficetola, Taberlet and Coissac, 2016; Lahoz-Monfort et al., 2016). Bayesian models are particularly promising in this respect, as they can allow to integrate prior information on the contamination rate of a given taxon, obtained for example from calibration experiments or from the analysis of controls (Lahoz-Monfort et al., 2016; Guillera-Arroita et al., 2017), and can even take into account confounding factors such as spatial or temporal autocorrelation, which are pervasive in ecological studies (Chen and Ficetola, 2019).

## Conclusions

In the last decade, eDNA and metabarcoding have opened new avenues to biodiversity studies, and further leaps forwards will likely occur in the future. eDNA and metabarcoding are greatly improving our assessments of species distribution, but researchers should not limit themselves to these aspects, as there is great potential of expansion in poorly explored directions, such as food web reconstruction or analyses of past biodiversity. We believe that field biologists should not consider eDNA-based approaches as a threat for their activities. Instead, exploiting a broader panel of techniques can improve the effectiveness of monitoring, and resources devoted to tedious activities could be redirected, with the possibility to obtain results that would have not been conceivable one decade ago. Integrated research, combining field activities with state-of-the-art molecular tools, could even increase success rates in grant and funding applications, given that such multidisciplinary studies can open new research fields.

However, eDNA and metabarcoding can be extremely challenging, because of the complexity of the techniques and of the underlying rationale, and because of the velocity of developments. The highest standard must be therefore applied through all the aspects of studies, from the experimental design, to lab work, to data analyses. This requires the contemporary presence of multidisciplinary competences, and can only be achieved through the collaboration among field biologists, ecologists, molecular biologists, and bioinformaticians. The community of herpetologists is showing a very lively response to the recent developments of molecular techniques, and we hope this will greatly improve our understanding of the complex relationships between organisms, and our ability to assess the impact of human activities.

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