GENETICS

Aquatic environmental DNA: Applications for assessment and monitoring vertebrate diversity

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Abstract

Environmental DNA from water samples (aquatic eDNA) is a noninvasive, cost-effective and high-throughput tool to conduct biodiversity assessment of both hydrobionts and terrestrial organisms that live nearby or frequently come into contact with a waterbody. Due to the exceptional importance of vertebrates in biomonitoring, a wide range of vertebrate taxonomic groups have been studied in recent years in various ecosystems using aquatic eDNA assays, including endangered, rare, secretive and elusive species that are often missed by traditional survey methods. Given that the potential uses of eDNA vary among different vertebrate groups, in this article we provide an overview of the use of aquatic eDNA for monitoring fish, amphibians, reptiles, mammals, and birds in small and large, marine and fresh water bodies from the tropics to the Arctic. We discuss the main applications of aquatic eDNA for single species detection, biodiversity assessment, genetic characterization, and biomass estimation.

Keywords: eDNA, metabarcoding, universal primers, fish, amphibians, reptiles, birds, mammals.

Citation: Galkina, S., Demina, I., Platonova, E., and Dyomin, A. 2024. Aquatic environmental DNA: Applications for assessment and monitoring vertebrate diversity. Bio. Comm. 69(4): 263–279. https://doi.org/10.21638/spbu03.2024.407

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Manuscript Editor: Maxim Vinarski, Laboratory of Macroecology and Biogeography of Invertebrates, Saint Petersburg State University, Saint Petersburg, Russia

Received: May 8, 2024;

Revised: June 21, 2024;

Accepted: September 11, 2024.

Funding: This research was funded by the Russian Science Foundation (grant no. 22-74-10043).

Ethics statement: This paper does not contain any studies involving human participants or animals performed by any of the authors.

Competing interests: The authors have declared that no competing interests exist.

Introduction

To conduct a thorough analysis and assessment of an ecosystem it is crucial to gather detailed information on biodiversity, ecological status, pollution presence, and invasive species. These elements collectively provide a comprehensive understanding of the ecosystem's dynamics and integrity. Over the years, a wide range of taxonomic groups from bacteria to vertebrates have been studied in various terrestrial and aquatic ecosystems. Research on species composition over space and time is consistently improving our understanding of ecosystems, which helps to predict their changes, establish management, conservation and restoration practices, particularly as human impacts increase.

Environmental DNA (eDNA) methodology has revolutionized biomonitoring techniques, particularly in identifying the presence and distribution of organisms and describing biodiversity in various ecosystems. Generally, eDNA refers to DNA isolated from environmental samples (soil, air, sea, or freshwater) as opposed to genomic DNA extracted directly from specimens (Miaud, Taberlet, and Dejean, 2012; Taberlet, Coissac, Hajibabaei, and Rieseberg, 2012; Foote et al., 2012). eDNA represents a mixture of DNA molecules of various types and origins (nuclear, mitochondrial, chloroplast) that enter the environment from cells or cellular material such as skin, feces, urine, and gametes of organisms inhabiting it. eDNA can be used to detect single or multiple species through polymerase chain reaction (PCR) amplification with species-specific primers or through metabarcoding using universal primers (Table 1) combined with high-throughput sequencing technologies. Due to the high copy number of mitochondrial DNA

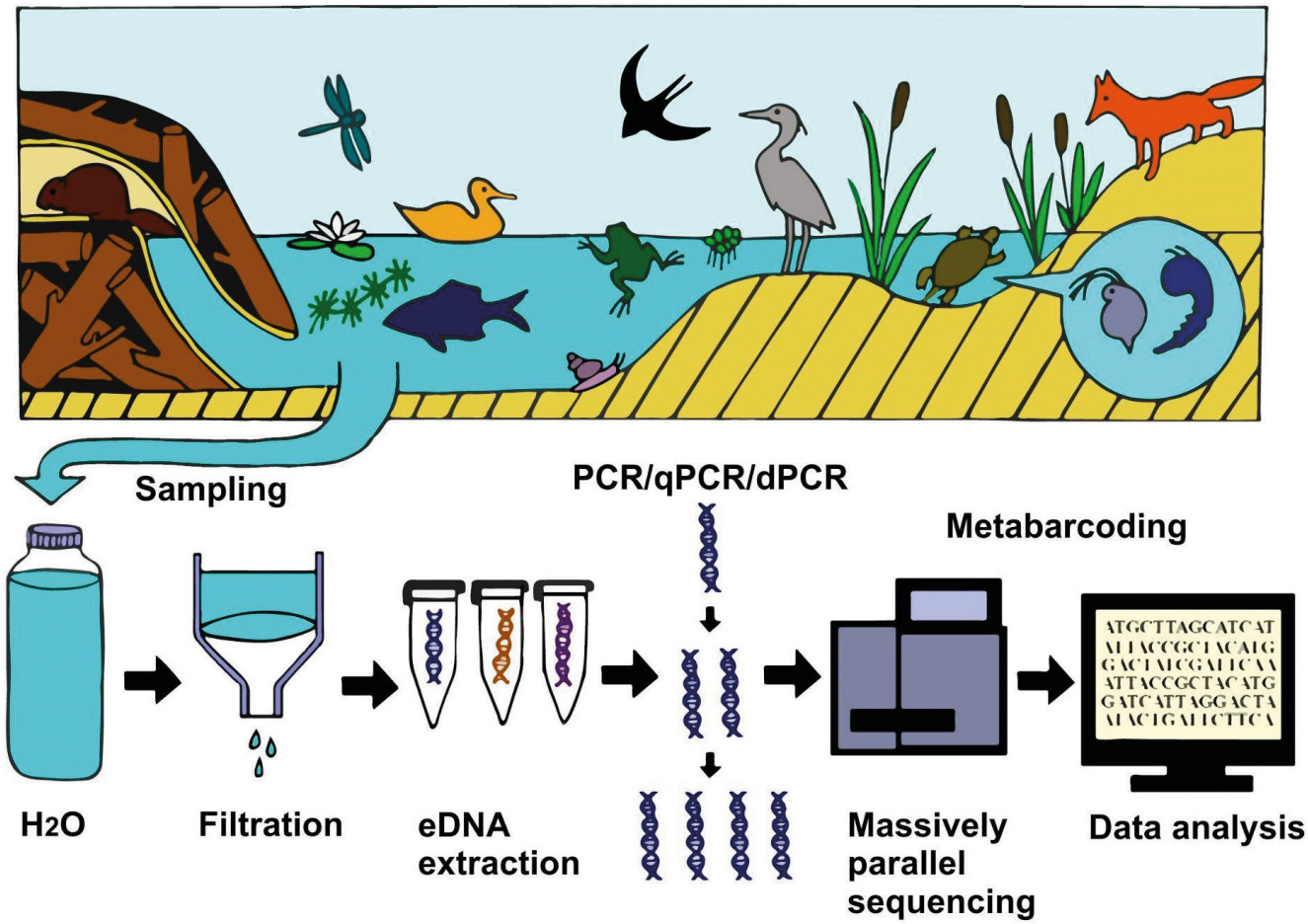

Fig. 1. Sources of aquatic eDNA data and a conventional eDNA workflow.

(mtDNA) in eukaryotic cells and the availability of annotated nucleotide sequence data from a wide range of species, primer sets are often designed to amplify short fragments (~60–250 bp) of the 12S and 16S ribosomal RNA (rRNA) genes, cytochrome C oxidase I gene (*COI*), cytochrome b (*Cyt b*) or control region (D-loop) (Thomsen et al., 2012b; Lacoursière-Roussel, Dubois, Normandeau, and Bernatchez, 2016; Valsecchi et al., 2020; Takahashi et al., 2023; Wang et al., 2023). Some eDNA studies have also targeted multi-copy nuclear DNA fragments, such as the rRNA gene cluster (Dysthe et al., 2018; Jo, Tsuri, and Yamanaka, 2022).

There are several benefits to analyzing eDNA from water samples (aquatic eDNA, Fig. 1). The sampling procedure is much easier compared to, for example, airborne eDNA (Clare et al., 2021; 2022; Garrett et al., 2023) or collecting intestinal contents of some insects, which are used for vertebrate monitoring (e. g., Gillett et al., 2016; Kerley et al., 2018). eDNA from aquatic environments potentially contains a wide range of molecular fingerprints since the DNA of both hydrobionts and terrestrial organisms can enter the water directly or indirectly through soil and rain. In contrast, eDNA from soil samples is less diverse as it primarily contains DNA from terrestrial organisms within the sampling area. Aquatic eDNA can provide valuable insights into species diversity in large ecosystems. Initially used to detect bacteria in marine sediments (Ogram et al., 1987) and developed for studies of fecal contamination in freshwater communities (Layton et al., 2006; Martellini, Payment, and Villemur, 2005), the detection of invasive species in freshwater bodies (Ficetola, Miaud, Pompanon, and Taberlet, 2008; Jerde, Mahon, Chadderton, and Lodge, 2011), and monitoring of marine mammals (Foote et al., 2012), aquatic eDNA has gained attention in the scientific community. It has the potential to become one of the most effective tools for basic biomonitoring (Taberlet, Bonin, Zinger, and Coissac, 2018; Takahashi et al., 2023; and Fig. 1 therein). The methodology of eDNA is continuously being improved to enable rapid and accurate biodiversity detection. For methodological details readers can refer to systematic literature reviews and technical notes (e. g. Taberlet, Bonin, Zinger, and Coissac, 2018; Takahashi et al., 2023).

The role of vertebrates in ecosystem functioning, where they contribute significantly to matter and energy turnover and influence ecosystem structure across various levels (Severtsov, 2013), underscores their critical

importance in biomonitoring efforts. Traditional methods for assessing and monitoring vertebrate biodiversity typically rely on direct visual or acoustic observation, trapping or sampling, species identification, and registration. However, these methods are limited by factors such as weather conditions, daylight hours, time constraints, the number of observations, and the need for specialized expertise. Technological advances have introduced a variety of tools like cameras, video systems, acoustic sensors, and robotic samplers, which can help to mitigate some of these issues but often increase the costs associated with fieldwork. In contrast, eDNA tools offer promising solutions by enabling cost-effective, non-invasive sampling with broad taxonomic coverage, high sensitivity, and potential for automation. In studies focusing on vertebrates, the effectiveness of eDNA methods has been particularly evident in research involving marine teleosts (Thomsen et al., 2012a), cetaceans (Foote et al., 2012), turtles (Davy, Kidd, and Wilson, 2015), sharks (Sigsgaard et al., 2016), and broader ecological studies (Bohmann et al., 2014; Garlapati et al., 2019; Saenz-Agudelo et al., 2022). For several vertebrate taxa, eDNA analysis has demonstrated sensitivity compared to traditional monitoring methods, with sensitivity levels reaching to 95 % or higher at target DNA concentrations of 11 molecules per liter or more (Furlan, Gleeson, Hardy, and Duncan, 2016; Sigsgaard et al., 2016). Importantly, widespread utilization of eDNA techniques that target biological communities rather than individual species is crucial for overcoming current challenges in ecosystem conservation and management, particularly those associated with the umbrella species concept (Miya, 2022).

Given that the potential uses of eDNA vary among different vertebrate groups, in this article we provide an overview of the use of aquatic eDNA for monitoring fish, amphibians, reptiles, mammals, and birds. We discuss the main applications of eDNA in this context, which include single species detection, biodiversity assessment, genetic characterization, and biomass estimation. We address the challenges and limitations associated with aquatic eDNA methodology when studying different vertebrate groups and highlight the opportunities and benefits that eDNA brings to the field of vertebrate monitoring.

eDNA analysis as a tool for smart fisheries management around the world

eDNA monitoring of individual fish species and populations, as well as the variety of fish species in aquatic ecosystems, is now considered as highly promising due to its ease of applicability and reproducibility (reviewed in (Wang et al., 2021)). Fish play a significant role in a wide range of aquatic habitats and serve as indicators of environmental quality changes. This suggests that fish communities are sensitive to changes within these systems, and fish abundance and species diversity may serve as reliable metrics for assessing impacts on specific ecosystems. Moreover, many fish species, particularly those in saltwater environments, are commercially valuable, highlighting the necessity for ongoing monitoring of their population dynamics (Rourke et al., 2022).

eDNA analysis offers several advantages compared to traditional methods of studying fish. These include lower costs, elimination of the need for prolonged field studies requiring specialized knowledge in fish taxonomy, and minimally invasive material sampling. Consequently, this approach is advancing increasingly, with studies on fish eDNA analysis currently being among the most numerous in vertebrate eDNA research (Nordstrom, Mitchell, Byrne, and Jarman, 2022). The reliability of fish eDNA analysis has been compared to traditional sampling methods (e. g., Sard et al., 2019; Piggott et al., 2021; Czeglédi et al., 2021; Gehri et al., 2021; Jacobsen et al., 2023; Wang et al., 2024). eDNA has demonstrated superior effectiveness in detecting fish taxa and functional traits (e. g. period of activity, vertical position in the water column) compared to traditional methods, enabling the identification of a broader range of species (Marquez et al., 2021; Piggott et al., 2021; Czeglédi et al., 2021; Gehri et al., 2021; Wang et al., 2024). Thus, eDNA may excel in metacommunity analyses compared to other traditional methods but may not significantly outperform them in overall biodiversity assessment (Wang et al., 2024). While no significant difference has been observed from complementing eDNA analysis with other survey data, the addition of supplementary data can enhance detection confidence and provide supporting evidence for unexpected or novel species detections (Piggott et al., 2021; Wang et al., 2021; Wang et. al., 2024). This underscores the importance of integrating both methodologies for accurate results. For instance, simultaneous eDNA analysis and hydroacoustic scanning in the Gulf of St. Lawrence were conducted to identify pelagic and hemipelagic fish species, which are crucial food resources for local marine mammals (Berger et al., 2020). In areas of the gulf where fish DNA was scarce in the water, traditional fish detection methods revealed fish presence, but accurate fish species identification was achieved through eDNA analysis. The combined use of these two methods enabled the description of fish population dynamics over several years (Berger et al., 2020). Noteworthy, the application of eDNA metabarcoding is particularly valuable for studying geographical areas or ecosystems that are challenging to access and describe using traditional methods. For example, a team of molecular biologists successfully conducted a biodiversity assessment of mesophotic marine ecosystems by sampling and filtering water down to depths of 200 meters using a submersible standalone pumping device (Muff

et al., 2023). They observed significant turnover in fish composition, taxa overlapping across depth zones, and a considerable number of species detected in samples beyond their known depth range, suggesting an underestimation of species' depth tolerances (Muff et al., 2023).

The substantial accumulation of information on fish eDNA has significantly enriched existing genetic databases. These databases include not only nucleotide sequences of all organisms (NCBI, http://www.ncbi.nlm.nih.gov; EMBL, http://www.ebi.ac.uk/embl; BOLD, http://v4.boldsystems.org/index.php/Public_BarcodeIndexNumber_Home) but also specialized fish databases (FISH-BOL, http://www.fishbol.org); MITOFISH, http://mitofish.aori.utokyo.ac.jp/). This wealth of data has streamlined the process of identifying promising genomic regions for primer design in eDNA studies. For example, Miya and colleagues (2015) developed universal metabarcoding primers targeting the mitochondrial 12S rRNA gene (MiFish-U/E, Table 1) suitable for amplifying specific DNA fragments from a wide range of cartilaginous and teleost fish species (Miya et al., 2015; 2022). This universal primer set has proven effective for metabarcoding eDNA from fish and other vertebrates (Thomsen et al., 2012a; Thomsen et al., 2012b; Thomsen et al., 2016; Kelly, Port, Yamahara, and Crowder, 2014; Port et al., 2016; Andruszkiewicz, Yamahara, Closek, and Boehm, 2017; Djurhuus et al., 2017; O'Donnell et al., 2017). Additionally, various species-specific primers are widely used for precise identification of rare, elusive, or invasive fish, as well as for population-level characterization. Notable examples include fascinating studies on marine megafauna, such as "eDNA haplotyping" of mitochondrial DNA to estimate intraspecific genetic diversity of the whale shark (*Rhincodon typus*) found in various locations in the World Ocean (Sigsgaard et al., 2016; Dugal et al., 2022).

Taking into account the specifics of fish research, eDNA analysis can serve as a quantitative tool for estimating biodiversity, particularly for assessing fish biomass and population density. An increasing number of studies have confirmed a positive correlation between eDNA concentration and biomass in aquatic environments in both experimental and field studies (e. g. Kelly, Port, Yamahara, and Crowder, 2014; Pont et al., 2023; Van Driessche et al., 2023). Quantitative real-time PCR (qPCR) and digital PCR (dPCR) are the primary eDNA methodologies for quantifying the abundance of fish DNA sequences amplified using universal primers. These methods enable the indirect estimation of absolute species abundance by quantifying the DNA concentration in samples (e. g. Van Driessche et al., 2023). In contrast, eDNA metabarcoding provides a semi-quantitative assessment of fish abundance, as it offers a count of reads per taxon that may not directly correlate to the amount of eDNA in native water samples. Pont and colleagues suggest that combining qPCR analysis to estimate the total concentration of amplified eDNA, eDNA metabarcoding with a high number of technical replicates, and employing appropriate spatial and temporal sampling strategies enable a more accurate assessment of species diversity and absolute abundance (Pont et al., 2023). This approach, based on taxon-specific DNA copy numbers per liter, is particularly useful for biomonitoring and bioassessment purposes.

eDNA also aids in tracking seasonal changes in fish communities, which is crucial for long-term environmental monitoring programs. Sigsgaard and co-authors collected eDNA samples over the course of a year in the waters of the Öresund, a strait connecting the Baltic and North Seas. The results revealed that the species composition in coastal waters varies throughout the year. Some fish were present/absent during specific periods; for example, species from the cod family (Gadidae) were absent in July-September, and lumpfish (*Cyclopterus lumpus*) DNA was only detected during the breeding season (Sigsgaard et al., 2017).

Amphibian eDNA: from initial use for vertebrate detection to searching for molecular fingerprints of rare, secretive and elusive species

Amphibians are among the most vulnerable groups of vertebrates. Populations of frogs, salamanders, caecilians, and their ranges have generally been declining, with approximately 40 % of amphibian species facing the threat of extinction due to habitat destruction, anthropogenic pollution, changes in environmental conditions, and the spread of infectious diseases (Svenningsen, Pertoldi, and Bruhn, 2022). The life cycles of most amphibians are closely linked to freshwater environments, whether for egg and larvae development, adult habitats, or temporary aquatic residence. Amphibians leave substantial amounts of DNA-containing material in their habitat, including various secretions (skin secretions, saliva, urine, feces, sperm), eggs, eggshells, tissues, and exfoliating cells. Therefore, it is not surprising that eDNA is recognized as an effective method for monitoring and assessing amphibian species diversity to complement traditional approaches (Moss et al., 2022; Sun, Guo, Gao, and Xiao, 2024).

The first amphibian eDNA study, exemplifying the initial use of eDNA for vertebrates, successfully applied species-specific primers designed for the mitochondrial gene *Cyt b* to detect the DNA of the invasive American bullfrog (*Lithobates catesbeianus*) in a French wetland (Ficetola, Miaud, Pompanon, and Taberlet, 2008). The results were entirely consistent with those obtained in the laboratory, where frog DNA was detected in aquariums with varying densities of animals, as well as with data from a four-year observation of bullfrogs in the area using traditional counts (Ficetola, Miaud,

Pompanon, and Taberlet, 2008). Later, the efficacy of eDNA was demonstrated in searching for "molecular fingerprints" of rare, secretive, and elusive amphibians. Among the initial successes were the detection of the Idaho giant salamander (*Dicamptodon aterrimus*) and the tailed frog (*Ascaphus montanus*) in the streams of central Idaho (USA) using *Cyt b* primers (Goldberg, Pilliod, Arkle, and Waits, 2011). Vörös and colleagues, using primers for the D-loop, identified the DNA of the red-listed olm (*Proteus anguinus*) in 15 caves surveyed in Croatia, with the species recorded for the first time in five of them (Vörös et al., 2017). In 2021, scientists from Brazil discovered DNA traces of four declining frog species (*Hylodes ornatus*, *Hylodes regius*, *Crossodactylus timbuhy*, *Vitreorana eurygnatha*), two locally extinct species (*Phasmahyla exilis*, *Phasmahyla guttata*), and the Bocaina big tooth frog (*Phantasmarana bocainensis*), last observed in nature in 1968 and considered extinct (Lopes et al., 2021). eDNA can be used to survey nocturnal amphibians that inhabit remote and inaccessible locations, as demonstrated with remnant populations of endangered Australian frogs *Litoria lorica* and *Litoria nannotis*, known to hunt in the splash zone of waterfalls at night and seek refuge in rock cracks in fast-flowing water during the day (Villacorta-Rath, Hoskin, Strugnell, and Burrows, 2021). Researchers sampled river water downstream from waterfalls and detected the eDNA of the species more than 20 km from its source. This study illustrated that small amphibian populations can be detected over considerable distances, exceeding previous estimates significantly (Olson, Briggler, and Williams, 2012). Importantly, numerous similar studies have been conducted concurrently with experimental eDNA research under controlled conditions and/or in combination with traditional monitoring methods in locations, where specific species distributions are known (e. g., Eiler et al., 2018; Wikston et al., 2023; Quilumbaquin, Carrera-Gonzalez, Van der Heyden, and Ortega-Andrade, 2023). These studies collectively validate the efficacy of eDNA analysis in environmental monitoring and species detection.

For amphibian eDNA metabarcoding, several effective assays have been developed based on universal primers that target mitochondrial genes, such as 12S rRNA, 16S, *Cyt b* and *COI* (Valentini et al., 2016; Svenningsen, Pertoldi, and Bruhn, 2022; Sun, Guo, Gao, and Xiao, 2024; Mu et al., 2024; Table 1). Their sensitivity allows for monitoring of amphibian biodiversity in tropical regions, despite high rates of DNA degradation at elevated temperatures. For instance, using eDNA metabarcoding, nine out of ten amphibian species observed during a five-year monitoring project in Brazilian Atlantic rainforest streams were successfully identified (Sasso et al., 2017). This highlights the strong agreement between eDNA metabarcoding and traditional methods of species identification. The cost-efficiency of eDNA metabarcoding, coupled with its ability to detect multiple target organisms simultaneously, has even spurred citizen science projects. In Denmark, for instance, researchers have engaged volunteers in characterizing amphibian diversity in various water bodies throughout the country (Knudsen et al., 2023).

The examples provided highlight the effectiveness of developed amphibian-specific eDNA assays for monitoring biodiversity, especially at low population densities. However, a study on the northern leopard frog (*Lithobates pipiens*) in Canada revealed that eDNA detection results were less reliable at low population densities (Randall, Goldberg, and Moehenschlager, 2023).

On the other hand, the relationship between the number of amplified DNA copies and the number of individuals in the environment permits the use of eDNA for biomass and population density analysis. This correlation has been examined in amphibians, where the number of eDNA sequencing reads correlated with results obtained through traditional population density measures for the Idaho giant salamander and the tailed frog (Pilliod, Goldberg, Arkle, and Waits, 2014). Nonetheless, caution should be exercised when interpreting such data, as eDNA concentration in water can be influenced by various factors such as temperature, water chemistry, flow rate. It is crucial to thoroughly analyze the acquired information and, where feasible, integrate eDNA screening with accepted methods for amphibian monitoring.

eDNA utility for reptile ecology

Reptiles are a paraphyletic group, comprising lizards, snakes, turtles, crocodiles, and the tuatara, whose representatives live in terrestrial and aquatic (freshwater and marine) habitats across tropical, arid and temperate environments. Approximately 21 % of 10,196 reptile species are classified as vulnerable, endangered, or critically endangered (Cox et al., 2022). Reptiles face threats similar to those affecting other tetrapods, including habitat loss due to agriculture, logging, and urban development, over-harvesting, disruption of trophic dynamics, and decline of native species due to invasive species (Böhm et al., 2013; Cox et al., 2022). The native range of many reptile species is often limited, making smaller populations particularly vulnerable to environmental pressures (Böhm et al., 2013). In contrast, certain introduced reptile species have become highly disruptive invasive species, such as the brown tree snake (*Boiga irregularis*) and the red-eared slider (*Trachemys scripta elegans*) (Lowe, Browne, Boudjelas, and De Poorter, 2000; Kraus, 2015; Nordstrom, Mitchell, Byrne, and Jarman, 2022). The use of eDNA tools could significantly enhance monitoring efforts for reptile distributions and population trends.

Table 1. Commonly used metabarcoding primers in aquatic eDNA studies targeting vertebrate taxa. Forward and reverse primers are designated by the letters "F" and "R" in their names

Primer name	Sequence	mtDNA gene	Amp. length bp (excluding primers)	Specificity	Reference
Teleo_L1848	5′-ACACCGCCCGTCACTCT-3′	*12S rRNA*	~63	Fish and other vertebrates	Valentini et al., 2016
Teleo_H1913	5′-CTTCCGGTACACTTACCATG-3′				
MiFish_U_F	5′-GTCGGTAAAACTCGTGCCAGC-3′	*12S rRNA*	~183	Fish and other vertebrates	Miya et al., 2015
MiFish_U_R	5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′				
Tele02-12S_F	5′-AAACTCGTGCCAGCCACC-3′	*12S rRNA*	~166	Fish and other vertebrates	Taberlet, Bonin, Zinger, and Coissac, 2018
Tele02-12S_R	5′-GGGTATCTAATCCCAGTTTG-3′				
Elas02-12S_F	5′-GTTGGTHAATCTCGTGCCAGC-3′	*12S rRNA*	~170	Sharks and rays	Taberlet, Bonin, Zinger, and Coissac, 2018
Elas02-12S_R	5′-CATAGTAGGGTATCTAATCCTAGTTTG-3′				
Fish16s_F/D	5′-GACCCTATGGAGCTTTAGAC-3′	*16S rRNA*	~202	Fish and other vertebrates	Berry et al., 2017
16s2R	5′-CGCTGTTATCCCTADRGTAACT-3′				
FishF1-COX1_F	5′-ACCAACCACAAAGANATNGGCAC-3′	*COI rRNA*	127	Fish	West et al., 2021
FishF1-COX1_R	5′-GATTATTACNAAAGCNTGGGC-3′				
FishCBL-CYTB_F	5′-TCCTTTTGAGGCGCTACAGT-3′	*CytB*	90	Fish	Thomsen et al., 2012b
FishCBL-CYTB_R	5′-GGAATGCGAAGAATCGTGTT-3′				
12S-V5_F	5′-TAGAACAGGCTCCTCTAG-3′	*12S rRNA*	~96–116	Vertebrates	Riaz et al., 2011
12S-V5_R	5′-TTAGATACCCCACTATGC-3′				
MarVer1F	5′-CGTGCCAGCCACCGCG-3′	*12S rRNA*	~179	Marine vertebrates	Valsecchi et al., 2020
MarVer1R	5′-GGGTATCTAATCCYAGTTTG-3′				
MarVer3F	5′-AGACGAGAAGACCCTRTG-3′	*16S rRNA*	~210	Marine vertebrates	Valsecchi et al., 2020
MarVer3R	5′-GGATTGCGCTGTTATCCC-3′				
Vert-16S-eDNA-F1	5′-AGACGAGAAGACCCYdTGGAGCTT-3′	*16S rRNA*	~251	Freshwater vertebrates	Vences et al., 2016
Vert-16S-eDNA-R1	5′-GATCCAACATCGAGGTCGTAA-3′				
Batra_L3541	5′-ACACCGCCCGTCACCCT-3′	*12S rRNA*	~55	Amphibians	Valentini et al., 2016
Batra_H3596	5′-GTAYACTTACCATGTTACGACTT-3′				
Amph_16S_1070F	5′-ACGAGAAGACCCYRTGGARCTT-3′	*16S rRNA*	~250	Amphibians	Sakata et al., 2022
Amph_16S_1340R	5′-ATCCAACATCGAGGTCGTAA-3′				
AqReptileF-degenerate	5′-AGACNAGAAGACCCTGTG-3′	*16S rRNA*	~212–275	Reptiles	West et al., 2023
AqReptileR	5′-CCTGATCCAACATCGAGG-3′				
Reptile_TURTLE COI_F	5′-GCMGGiACMGGiTGAAC-3′	*COI*	167	Turtles	Lacoursière-Roussel, Dubois, Normandeau, and Bernatchez, 2016
Reptile_TURTLE COI_R	5′-GATATiGCiGGRGMTTTTAT-3′				
Reptile_SNAKE COI_F	5′-GCYGGYACiGGiTGAAC-3′	COI	130	Snakes	Lacoursière-Roussel, Dubois, Normandeau, and Bernatchez, 2016
Reptile_SNAKE COI_R	5′-TRAAGTTRATTGCYCCiAGGA-3′				
MiBird-U_F	5′-GGGTTGGTAAATCTTGTGCCAGC-3′	*12S rRNA*	171	Birds	Ushio et al., 2018
MiBird-U_R	5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′				
BirdND2F	5′-CCATTCCACTTYTGRTTYCC-3′	*ND2*	229	Birds	Newton et al., 2023
BirdND2R	5′-GGGAGATDGADGARAADGC-3′				

Primer name	Sequence	mtDNA gene	Amp. length bp (excluding primers)	Specificity	Reference
BirT-F	5′-YGGTAAATCYTGTGCCAGC-3′	*12S rRNA*	267	Birds	Thalinger et al., 2023
BirT-R	5′-AAGTCCTTAGAGTTTYAAGCGTT-3′				
16Smam1	5′-CGGTTGGGGTGACCTCGGA-3′	*16S rRNA*	~92	Mammals	Taylor, 1996
16Smam2	5′-GCTGTTACCCTAGGTAACT-3′				
MiMammal-U_F	5′-GGGTTGGTAAATTTCGTGCCAGC-3′	*12S rRNA*	~171	Mammals	Ushio et al., 2017
MiMammal-U_R	5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′				

However, the application of eDNA to reptiles has been relatively limited compared to other vertebrate groups (Nordstrom, Mitchell, Byrne, and Jarman, 2022), largely due to methodological challenges. eDNA techniques have been more successfully applied to aquatic reptiles, particularly freshwater turtles, and to a lesser extent, sea turtles. In contrast, there have been relatively few studies focused specifically on eDNA analysis of crocodiles and snakes (Adams, Hoekstra, Muell, and Janzen, 2019; Rose, Fukuda, and Campbell, 2020; Nordstrom, Mitchell, Byrne, and Jarman, 2022). These reptiles leave abundant traces of their biological activity in water, such as excrements and skin fragments post-molting. However, according to the "shedding hypothesis", animals with hard, keratinized integuments do not shed as much DNA as organisms covered in mucus (Adams, Hoekstra, Muell, and Janzen, 2019; Reji Chacko et al., 2023; Mousavi-Derazmahalleh et al., 2023). Therefore, since eDNA degrades rapidly in water and PCR on environmental water samples is prone to inhibition, identifying reptiles using molecular methods in natural water samples presents a challenging puzzle. For example, in studies of European and North American pond turtles, some ponds known to have turtle populations did not yield eDNA, resulting in false negatives (Raemy and Ursenbacher, 2018; Adams, Hoekstra, Muell, and Janzen, 2019). This may suggest the potential limitations of eDNA-based methods in studying reptile species diversity and population size compared to traditional counts (Baker, Steel, Nieukirk, and Klinck, 2018; Nordstrom, Mitchell, Byrne, and Jarman, 2022; Rojahn et al., 2024). Monitoring introduced semi-aquatic snakes in California demonstrated a higher likelihood of detecting the banded water snake (*Nerodia fasciata*) and the common watersnake (*Nerodia sipedon*) through traps rather than eDNA analysis (Rose et al., 2019). However, a comparison of molecular and traditional reptile monitoring approaches showed either consistency or even greater efficiency for eDNA analysis for species such as the wood turtle (*Glyptemys insculpta*) (Akre et al., 2019), the red-eared slider (*Trachemys scripta elegans*) (Kakuda et al., 2019), and others (Nordstrom, Mitchell, Byrne, and Jarman, 2022). For instance, Piaggio and colleagues successfully identified the invasive Burmese python (*Python bivittatus*) in water samples collected in various areas in Florida (USA), while the probability of detecting this python using traps was extremely low (Piaggio et al., 2014).

Advancements in the development of specific molecular techniques, combined with the design of reptile-specific primer sets or metabarcoding assays (Table 1) and adherence to best practice work (considering species biology, activity/inactivity periods, appropriate sample replicates, and field sides for sampling) will ultimately enhance the efficacy of eDNA monitoring of reptile populations. Early research on reptile eDNA concentrated on monitoring rare, scarce, and invasive species using specific primers for quantitative and classical PCR (Adams, Hoekstra, Muell, and Janzen, 2019). These studies have confirmed the presence of several turtle and snake species in freshwater habitats in America, Canada, India, Malaysia, and Switzerland (Davy, Kidd, and Wilson, 2015; Kelly, Port, Yamahara, and Crowder, 2014; Wilson, Sing, Chen, and Zieritz, 2018; Adams, Hoekstra, Muell, and Janzen, 2019). The development of suitable eDNA metabarcoding assays for the comprehensive detection of aquatic and semi-aquatic reptiles has opened up new avenues for biodiversity studies. This includes targeted assessment of herpetofauna richness in North America (Lacoursière-Roussel, Dubois, Normandeau, and Bernatchez, 2016) and Australia (West et al., 2021; 2023), as well as species identification in studies of general vertebrate biodiversity in aquatic ecosystems (Lozano Mojica and Caballero, 2021). Additionally, dPCR technology may offer a robust alternative to current qPCR methods, since it can be more resistant to inhibition (Hunter et al., 2018; Orzechowski, Frederick, Dorazio, and Hunter, 2019; Adams, Hoekstra, Muell, and Janzen, 2019; Nordstrom, Mitchell, Byrne, and Jarman, 2022). Moreover, eDNA is promising for surveying terrestrial reptiles, as these animals visit water bodies, and their DNA can enter the water directly during activities such as foraging,

drinking, defecation, or through soil and rain (Nordstrom, Mitchell, Byrne, and Jarman, 2022). For example, the DNA of the common lizard (*Zootoca vivipara*) has been identified in rivers in the Swiss Alps (Reji Chako et al., 2023). However, currently, sampling from soil, feces, and even air proves to be more effective for this purpose (Kucherenko, Herman, Iii, and Urakawa, 2018; Matthias et al., 2021; Galbraith, 2022; Kyle et al., 2022). In summary, with one-fifth of reptile species considered endangered and another one-fifth lacking data on their status (Cox et al., 2022; Nordstrom, Mitchell, Byrne, and Jarman, 2022), advancements in eDNA research, including species-specific monitoring and DNA metabarcoding, will undoubtedly benefit reptile ecology.

eDNA for cost-effective single species detection, biodiversity assessment, and population characterization of mammals

The application of eDNA-based methods for mammal monitoring is predominantly linked to studies of cetaceans and other marine mammals, encompassing single species detection, biodiversity assessment, and genetic characterization. Traditional monitoring techniques necessitate highly skilled taxonomists and favorable weather and visibility conditions. They often involve vessels and aircraft, rendering them expansive. The potential of eDNA for mammal species detection was initially explored through the identification of harbor porpoises (*Phocoena phocoena*) DNA in water samples from controlled environments and from the open sea (Foote et al., 2012). In this study, qPCR and specific primers targeting the mitochondrial 12S rRNA gene were used, with acoustic monitoring data complementing the results (Foote et al., 2012). Similarly, the Yangtze finless porpoise (*Neophocaena asiaeorientalis*) was detected in both artificial and natural water reservoirs (Ma et al., 2016; Qu and Stewart, 2019). When primer sets targeting mitochondrial D-loop and *Cyt b* were used, in addition to the harbor porpoise, the DNA of the long-finned pilot whale (*Globicephala melas*) was also amplified (Foote et al., 2012), or D-loop primers intended for the humpback whale (*Megaptera novaengliae*) cross-amplified the mtDNA of the minke whale (*Balaenoptera acutorostrata*) and gray whale (*Eschrichtius robustus*) (Andruszkiewicz, Yamahara, Closek and Boehm, 2020; Suarez-Bregua et al., 2022). Ensuring the specificity of eDNA assays and avoiding off-target amplification necessitate validation of qPCR assays by testing the designed primers on vouchered DNA samples.

The accuracy of detecting marine mammal eDNA from open sea samples is influenced by factors including the distance from the animals during water sampling, water temperature, mixing rate, and animal behavior. For instance, eDNA of the deep-diving killer whale *(Orcinus orca*) was detected by dPCR in water samples collected up to 2 hours after visual encounters, despite water movement due to tidal currents (Baker, Steel, Nieukirk, and Klinck, 2018). Notably, the "surface effect", which reflects the density of organic substances near the water surface, resulted in exceptionally high DNA concentrations in surface water samples, enabling the sequencing of nearly complete mtDNA D-loop (Baker, Steel, Nieukirk, and Klinck, 2018). A successful strategy for obtaining ample eDNA yields from seawater involves collecting individual "flukeprints" left on the water surface by diving cetaceans (Baker, Steel, Nieukirk, and Klinck, 2018; Parsons, Everett, Dahlheim, and Park, 2018; Székely et al., 2021; Robinson et al., 2024). However, targeted qPCR and whole-genome enrichment capture followed by shotgun sequencing gave false-negative results for killer whale DNA in seawater samples from both inshore or offshore locations (Pinfield et al., 2019). This may be attributed to the behaviors of whales, such as infrequent molting in cold waters and low defecation frequency during resting and feeding periods (Pinfield et al., 2019). In contrast, experiments using dPCR and qPCR with genus-specific primers demonstrated high efficiency in detecting three manatee species (Hunter et al., 2018). These findings highlight varying assay sensitivities and environmental factors that influence eDNA persistence in marine habitats (Suarez-Bregua et al., 2022).

Over the past decade, eDNA metabarcoding has emerged as a primary technique for assessing biodiversity in marine mammal communities (review: Suarez-Bregua et al., 2022). Universal primer sets such as 12S-V5 and MiFish (Table 1) have successfully amplified the DNA of sea otters (*Enhydra lutris*) and other species including pinnipeds, cetaceans, and sirenians (e. g., Port et al., 2016; Andruszkiewicz et al., 2017; Djurhuus et al., 2017; O'Donnell et al., 2017). However, Closek and colleagues (2019) noted that eDNA metabarcoding is generally less accurate than traditional visual counts in detecting most marine mammal species, with the notable exception of bottlenose dolphins (*Tursiops truncatus*) (Closek et al., 2019). eDNA metabarcoding is particularly effective in detecting small individuals and species that exhibit secretive behaviors (Yamamoto et al., 2017; Fraija-Fernández et al., 2020). Primers targeting highly polymorphic mitochondrial 12S and 16S rRNA genes have been optimized for identification of cetacean or pinniped DNA (MarVer1 and MarVer3, respectively, Table 1), ensuring robust amplification of the corresponding mtDNA fragments across various vertebrates (Valsecchi et al., 2020; 2021a; 2022). By comparing sequencing read counts from target marine mammals and other vertebrates in different samples, hypotheses regarding target species' behaviors can be verified. This was demonstrated in a study on the Mediterranean

monk seal *Monachus monachus* (Valsecchi et al., 2021b). Notably, an eDNA metabarcoding investigation of offshore samples revealed higher read counts for bony fish and monk seals during nocturnal compared to diurnal samples, suggesting that monk seals primarily frequent deep waters at night, presumably for foraging purposes (Valsecchi et al., 2021b; 2022).

For highly mobile marine mammal species, determining population structure using traditional methods such as biopsy darting is often challenging and costly. eDNA approaches provide insights into intraspecific genetic variation and population differentiation (reviewed in Andres, Lodge, Sethi, and Andrés, 2023). For example, a study by Parsons and colleagues (2018) on harbor porpoises in inland waters of southeastern Alaska revealed significant genetic differentiation within a population previously considered homogenous. High-throughput sequencing of eDNA also uncovered two new mitochondrial haplotypes (Parsons, Everett, Dahlheim, and Park, 2018). Killer whale eDNA analysis accurately identified the ecotype of mammals present in the sampled area at the time of seawater collection (Baker et al., 2018). Analysis of bowhead whales (*Balaena mysticetus*) demonstrated that mitochondrial haplotype frequencies obtained from eDNA samples were consistent with those derived from biopsies taken over the years (Székely et al., 2021).

Another promising direction is the monitoring of terrestrial or semi-aquatic mammals through the analysis of eDNA from water samples. The story began in 2012, when genetic material from otters (*Lutra lutra*) was found in freshwater samples collected for invertebrate and vertebrate monitoring (Thomsen et al., 2012b). Subsequent studies have successfully monitored terrestrial and semi-aquatic mammals in small water bodies within zoos and natural settings. This includes the targeted detection of coyote (*Canis latrans*) DNA in stream water samples used by the animals for drinking (Rodgers and Mock, 2015). These studies either use qPCR with species-specific primers (e. g., Ushio et al., 2017; Williams, Huyvaert, and Piaggio, 2017; Seeber et al., 2019) or conduct eDNA metabarcoding (e. g., Klymus, Richter, Thompson, and Hinck, 2017; Harper et al., 2019; Sales et al., 2020; Coutant et al., 2021). While research on monitoring terrestrial, semi-aquatic, and arboreal mammals in large water bodies limited (Sales et al., 2020; Coutant et al., 2021), it highlights the benefits of eDNA analysis over transect surveys for identifying nocturnal semi-aquatic species (Coutant et al., 2021). One challenge in biodiversity studies using eDNA metabarcoding from water samples collected in the Neotropical zone is the scarcity of marker sequences in publicly available databases (e. g. GenBank). This scarcity limits the ability to identify specific species using Molecular Operational Taxonomic Units (MOTUs).

eDNA as a tool for avian species detection

Birds, due to their high visibility compared to other vertebrates, are commonly monitored using visual counts, which have proven successful in assessing bird species diversity. However, the eDNA method offers advantages in overcoming limitations such as low visibility at night and the need for field personnel to possess taxonomic identification skills. Publications focusing on the monitoring of waterfowl and shorebirds using eDNA began to appear in 2018, when it was proven that the DNA of birds frequently interacting with water persists in the environment, regardless of their main habitats (Ushio et al., 2018). Initial studies tested the eDNA approach on water samples from zoo enclosures and artificial ponds (Ushio et al., 2018; Schütz, Tollrian, and Schweinsberg, 2020). Later, eDNA from large natural water bodies was analysed to detect rare bird species (Neice and McRae, 2021) and assess bird species diversity in marine communities (Leduc et al., 2019; Gold et al., 2021), rivers (Lozano Mojica and Caballero, 2021; Polanco et al., 2021), and wetlands (Saenz-Agudelo et al., 2022). The effectiveness of eDNA for detecting aquatic and semi-aquatic bird species has been tested across diverse environments, including warm and cold waters in regions such as North Carolina (USA) (Neice and McRae, 2021), the coast of Los Angeles (Gold et al., 2021), the Canadian Arctic (Leduc et al., 2019), northwest Russia (Dyomin et al., 2024), and highland rivers of Latin America (Lozano Mojica and Caballero, 2021; Polanco et al., 2021; Saenz-Agudelo et al., 2022). Considering that sampling eDNA from birds is significantly more challenging than from aquatic species that live and continually shed DNA into landlocked water bodies (Takahara, Minamoto, and Doi, 2013), the golden standard is to complement eDNA field sampling with physical evidence of bird presence. This includes visual, acoustic, or camera monitoring, as well as identifying footprints, bird excrements, or food debris near water.

Similar to other vertebrates, a vide range of bird species can be detected using eDNA when appropriate primers are designed (Table 1). Universal primers originally developed for fish and mammals, such as MiFish/MiMammal targeting the mitochondrial 12S rRNA gene (insert length 171 bp), have occasionally detected 2–4 bird species per metabarcoding study (Thomsen et al., 2012a; 2012b; 2016; Port et al., 2016). Ushio and colleagues (2018) modified this universal set by designing MiBird primers, which were successfully validated for eDNA metabarcoding analysis on the Illumina MiSeq platform (Ushio et al., 2018).

When aiming to detect specific species, it is crucial that target sequences contain an adequate number of specific single nucleotide variants (SNV). Universal primers may not consistently produce satisfactory results.

For example, while universal avian primers for the *COI* gene have effectively distinguished over 260 bird species (based on variations in the 648 bp region) (Hebert, Stoeckle, Zemlak, and Francis, 2004), they have shown limitations in identifying certain species within Pelecaniformes, Charadriiformes, or Gruiformes (Schütz, Tollrian, and Schweinsberg, 2020; Neice and McRae, 2021). Therefore, species-specific eDNA diagnostic tests have been developed for endangered species such as the black rail *Laterallus jamaicensis* (Rallidae, Gruiformes) (Neice and McRae, 2021; Feist, Guan, Malmfeldt, and Lance, 2022) and shorebirds including the common spoonbill *Platalea leucorodia* (Threskiornithidae, Pelecaniformes), pied avocet *Recurvirostra avosetta* (Recurvirostridae, Charadriiformes), and common redshank *Tringa tetanus* (Scolopacidae, Charadriiformes) (Schütz, Tollrian, and Schweinsberg, 2020). In these species-specific bird eDNA studies, the detection limit is estimated to be between 109 and 300 copies of target DNA (Day et al., 2019; Neice and McRay, 2021).

While mitochondrial gene sequences such as *COI*, *Cyt*B, 12S rRNA, and 16S rRNA are effective for DNA barcoding, their variability among closely related species may sometimes be insufficient for designing species-specific primers and TaqMan probes. In such cases, the hypervariable regions (HVR1 and HVR2) of the mtDNA D-loop may be suitable. D-loop specific primers have been designed for several Anatidae species, including the pintail *Anas acuta*, the greater scaup *Aythya marila*, the barnacle goose *Branta leucopsis*, and the wigeon *Mareca penelope*, and validated for eDNA analysis (Dyomin et al., 2024). However, due to complexities in the speciation process, such as hybridization, specific mitochondrial haplotypes have only been identified for individual populations rather than for a species as a whole (e. g. in the genus *Larus* (Laridae, Charadriiformes)) (Dyomin et al., 2024 in press).

To date, targeted eDNA surveys in birds have been underutilized (Beng and Corlett, 2020), but avian-focused methods are emerging (Schütz, Tollrian, and Schweinsberg, 2020; Feist, Guan, Malmfeldt, and Lance, 2022; Dyomin et al., 2024). Bird species have been identified in biodiversity metabarcoding studies conducted across various ecosystems, including Greenland (Jensen et al., 2023), Australia (McDonald et al., 2023), North America (Palacios Mejia et al., 2021), South America (Lozano Mojica and Caballero, 2021; Polanco et al., 2021; Saenz-Agudelo et al., 2022), and Pacific islands (David et al., 2021; Roesma, Djong, Janra, and Aidil, 2021). In addition, birds have been detected as non-target taxa in "molecular bycatch" studies, where targeted markers used in aquatic eDNA biomonitoring also detect birds and mammals present in surrounding habitats (Macher et al., 2021; Mariani et al., 2021; Ritter et al., 2022). For example, in a study monitoring crocodiles in Cuba, genetic material from birds likely hunted by crocodiles was accidentally discovered (Pérez-Fleitas et al., 2023). Future eDNA monitoring programs will undoubtedly use this "molecular bycatch" as a valuable tool for assessing ecosystem-wide biodiversity at no additional cost.

eDNA for monitoring global biodiversity in aquatic ecosystems

Monitoring the global biodiversity of aquatic ecosystems provides crucial insights into their state and is fundamental for their management, conservation, and restoration. Traditional approaches require significant resources and distinct methodologies for different organisms. Aquatic eDNA offers an effective and versatile approach for detecting both aquatic and terrestrial animals. The potential of eDNA analysis was initially demonstrated in studies that assessed biodiversity by identifying various species of fish, amphibians, aquatic and terrestrial mammals, waterfowl, as well as dragonflies and scale insects in eDNA samples from 90 natural freshwater bodies across Europe (Thomsen et al., 2012b).

The real breakthrough occurred recently with the development of new universal primer sets that notably enhanced the efficiency of eDNA metabarcoding in both freshwater (Lozano Mojica and Caballero, 2021; Macher et al., 2021; Roesma, Djong, Janra, and Aidil, 2021; Ritter et al., 2022) and marine water bodies (Closek et al., 2019; Jensen et al., 2023). An intriguing study by Polanco and colleagues (2021) was conducted in the San Diego River Estuary (Colombia). The researchers showed a shift in community composition from marine to freshwater as they moved upstream from the river mouth. In addition to aquatic organisms (fish, amphibians, and some reptiles), eDNA analysis identified terrestrial, flying, and arboreal vertebrates (birds and mammals) living in the vicinity of the estuary, including rare species (Polanco et al., 2021). Jensen and colleagues demonstrated changes in marine fish and mammal communities across Greenland waters from the south to the northeast (Jensen et al., 2023). It is possible to identify not only spatial differences between species complexes but also seasonal variations in community composition (Lines et al., 2023). In the Rio Cruces Wetland of Chile, spatial variations in species communities with different salinity sensitivities were observed, despite complex reservoir hydrodynamics and diurnal tidal patterns. For example, the number of fish and amphibian taxa increased closer to the sea, potentially influenced by the specific patterns of eDNA accumulation in different areas of coastal wetlands (Saenz-Agudelo et al., 2022).

Due to differences in eDNA degradation rates between water and sediment, sampling both substrates in parallel provides more comprehensive information

on the species composition of organism communities (Sakata et al., 2020; Palacios Mejia et al., 2021). Increasing the number of biological replicates enhances species detection efficiency, particularly for small-sized species. For example, in a biodiversity study of the Mulde river (Germany), this approach significantly increased avian and mammalian species detectability by 68.9 % and 77.3 %, respectively (Macher et al., 2021). Moreover, analysis of eDNA in water samples from small natural ponds and artificial livestock water troughs in Australia has demonstrated the method's utility in assessing vertebrate biodiversity, including amphibians, birds, and mammals, across large drylands (McDonald et al., 2023).

eDNA is a driving force in the fields of ecology and population dynamics, primarily due to its ability to detect rare and elusive individuals across various taxa in diverse habitats. Recent studies have explored the potential of eDNA in elucidating complex biotic interactions, such as prey-predator dynamics. For example, Pérez-Fleitas et al., (2023) used eDNA methods to study the distribution of two species of crocodiles, identifying 55 vertebrate species, including previously unreported predators of crocodile hatchlings or consumers of their eggs (Pérez-Fleitas et al., 2023). In another study, Deeg and colleagues (2023) aimed to estimate the distribution and relative abundance of Pacific salmon (*Oncorhynchus spp*.) as well as their prey (such as copepods) in the Gulf of Alaska. They also assessed the distribution of squid and Myctophidae, which are potential prey and/or food competitors of salmon. Interestingly, eDNA analysis revealed the presence of salmon sharks (*Lamna ditropis*) and beaked whales (Ziphiidae), both predators of salmon, in the bay, despite not being observed using traditional survey methods (Deeg et al., 2023).

Conclusions

Aquatic eDNA methods have advanced sufficiently to detect individual vertebrate species of interest and study the taxonomic composition of aquatic communities, as well as terrestrial animals living nearby or in frequent contact with water bodies. This approach is particularly valuable for identifying endangered, rare, cryptic, and elusive vertebrate species that are often missed by traditional visual monitoring methods. Improvements in eDNA-based approaches are ongoing to determine biogeographic patterns in large water bodies, monitor temporal changes in communities, and characterize intraspecific genetic diversity. eDNA concentration, measured through qPCR or quantitative metabarcoding, can serve as a rapid and cost-effective indicator of abundance and/or biomass, particularly beneficial for assessing fish stocks. Advancements in quantitative eDNA assays are expected to enable accurate determination of the functional roles of species within an ecosystem (i. e., functional diversity) and facilitate rigorous assessment of anthropogenic impacts using this parameter. It is important to note that eDNA serves as an indirect genetic marker released by host organisms, and therefore, any eDNA-based assessment inherently includes errors such as false negatives and false positives. Ultimately, obtaining high-quality biomonitoring data is essential for developing effective environmental, political, and social programs aimed at mitigating human impacts on ecosystems.

Acknowledgements

The analytical facilities were provided by "Chromas" Resource Centre of the Saint Petersburg State University Scientific Park.

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