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Is it really there? Addressing Inadequate Sampling and False Detection in Environmental DNA Metabarcoding

RESEARCH NOTE

ABSTRACT

The use of environmental DNA (eDNA) for biodiversity assessment has garnered significant attention due to its cost-effectiveness, rapidity, accuracy, and non-invasiveness in acquiring data on organisms in various habitats. Despite its numerous advantages over traditional methods, there remains a pressing need for improved protocol standardization. This review synthesizes potential strategies to address false detection in eDNA metabarcoding, including inadequate sampling and limited persistence of eDNA, contamination, primer biases, inhibition of DNA amplification, differentiation of eDNA from deceased organisms, incomplete databases, and sequencing errors or poor-quality sequences. Best practices such as collecting 1 to 2 liters of surface water, replicating polymerase chain reactions (PCR), using multiple genetic markers and primers to mitigate PCR primer biases, and implementing stringent contamination controls at each analysis step are recommended. While no universal recommendations currently exist for the diverse applications of eDNA metabarcoding in species detection, adopting these measures can enhance the reliability and accuracy of results. Despite the present limitations in sequence databases and the necessity for improved primer quality and analysis pipelines, the eDNA approach is anticipated to evolve and become more standardized. This review provides practical guidance on eDNA technology for researchers, consultants, and managers, facilitating its effective use in biodiversity assessments.

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INTRODUCTION

Behavioral and morphological data obtained through visual observations, traps, binoculars, microscopy, and bioacoustics have traditionally been used to quantify biodiversity (*Beng and Corlett 2020; Hassan et al. 2022*). However, these methods are often damaging and intrusive, labor-intensive, time-consuming, and inefficient, making it difficult to accurately represent biodiversity in a region *(Thomsen and Willerslev 2015; Danovaro et al. 2016*). Additionally, traditional methods can introduce biases as they rely on experts for specimen identification. Advances in DNA sequencing techniques have significantly enhanced biodiversity research by overcoming these challenges and enabling the use of DNA barcoding to depict biodiversity across space and time (*Valentini et al. 2016; Grant et al. 2021*). Among these advances, environmental DNA (eDNA) sampling has gained considerable attention for its potential in biodiversity monitoring, as indicated by the growing body of research employing this technique over the past few years (*Beng and Corlett 2020*). eDNA, which includes any genetic material found in soil or water that originates from an organism's skin, feces, gametes, or carcasses, can persist for hours in temperate regions and for thousands of years in dry, cold permafrost environments (*Sahu et al. 2022; Stewart 2019; Deiner et al. 2017; Thomsen and Willerslev 2015*).

The advent of eDNA techniques has revolutionized conservation science worldwide. These methods are relatively non-invasive, objective, efficient, and fast, providing an opportunity to monitor biodiversity dynamics in both terrestrial and aquatic habitats without significantly harming the target species or their natural environments (*Huerlimann et al. 2020; Ruppert et al. 2019; Beng and Corlett 2020; Pascher et al. 2022; Rishan et al. 2023*). The application of eDNA can

yield a high detection probability for cryptic, rare, and elusive species even at low concentrations (*Barata et al. 2020;Diaz-Ferguson et al. 2014*).

Moreover, eDNA technologies enable the early detection of invasive species, allowing for timely eradication efforts before the species become fully established (*Martinez et al. 2020; Ardura et al. 2015*). According to *Takeuchi et al.* (*2019*), eDNA can accurately identify target organisms using consistent and repeatable criteria applicable across various habitats and life stages, and it allows for the simultaneous evaluation of multiple organisms (*Nagarajan et al. 2022*).

Despite the advantages of eDNA for monitoring biodiversity, it is prone to errors that can lead to false detections. Throughout the eDNA process, from sample collection in the field to molecular analysis and bioinformatics, errors can occur (*Burian et al. 2021; Evans et al. 2017*). False positives, indicating the presence of a target organism when it is absent, and false negatives, where the target organism is present but undetected, are common (*Willoughby et al. 2016; Doi et al. 2019*). These errors stem from factors such as the inadequate sampling and limited persistence of eDNA, variability in PCR primer efficiency, inhibition of DNA amplification, sample contamination, presence of eDNA from deceased organisms, and resuspension of ancient DNA (aDNA) (*Beng and Corlett 2020*). This study synthesizes potential strategies to address these challenges of false detection in eDNA studies. By exploring methods to minimize errors and improve the reliability of eDNA data, researchers can enhance the accuracy of biodiversity assessments using this technology. Additionally, the study discusses how traditional survey methods and eDNA approaches can complement each other in biodiversity monitoring projects. Integrating these methods offers a comprehensive approach to understand biodiversity dynamics, improving conservation efforts, and informing management strategies effectively across diverse ecosystems.

MATERIALS AND METHODS

Literature Search

The terms “environmental DNA” and “eDNA” were used to search the Web of Science, Scopus, and Google Scholar for peer-reviewed academic articles. All studies involving eDNA analysis in terrestrial and aquatic ecosystems were reviewed. The most recent search of the literature was done on the 20th September 2023, and it included the period between January 2008 and July 2023 (2008 was the year that eDNA became popular as an assessment tool) (*Ficetola et al. 2008*).

All the sampling locations of the reviewed papers (n=423) were identified and mapped. These locations were then categorized as either terrestrial or aquatic based on the primary habitat type where the eDNA samples were collected. Following this, relevant studies were presented that discussed the challenges associated with eDNA techniques as well as potential solutions (**Table 1**).

Table 1. Potential solutions for problems with environmental DNA (eDNA) analysis.

Problem	Potential Solution	References
Limited persistence of eDNA	Increase sample collection and employ numerous PCR replicates	*Evans et al. 2017, Yamamoto et al. 2017, Ficetola et al. 2015, Griffiths et al. 2020, Brys et al. 2021, Muha et al. 2019, Cantera et al. 2019, Doi et al. 2019*
Contamination	Follow standard sample collection and proper aseptic technique	*Miya et al. 2020, Goldberg et al. 2016, Shaw et al. 2016; Rees et al. 2014, Cowart et al. 2022*
Primer biases	Use multiple markers and primers	*Fraija-Fernández et al. 2020, McClenaghan et al. 2020, McElroy et al. 2020, Kumar et al. 2022*
Inhibition of DNA amplification	Use of inhibition-reducing assays	*Buxton et al. 2018, Sellers et al. 2018, McKee et al. 2015, Williams et al. 2016, Albers et al. 2013, Jane et al. 2015, Biggs et al. 2015, Takahara et al. 2015*
Differentiate eDNA from deceased organisms	Combine the extraction of DNA and RNA, amplifying both DNA segments of varying lengths	*Jo et al. 2017, Keer et al. 2003*
Incomplete reference sequence database	Develop a reference sequence database	*Nelson et al. 2016, Fernandez et al. 2021, Ruppert et al. 2019, Miya et al. 2020*
Sequencing errors and Poor-quality sequences	Improve bioinformatics pipelines	*Kunin et al. 2010, Thomsen and Willerslev 2015*

RESULTS AND DISCUSSIONS

The eDNA technique has revolutionized conservation science in numerous ways on a global scale (**Figure 1**). By enabling the monitoring of biodiversity dynamics of animals in both terrestrial and aquatic habitats (**Figure 2**), eDNA provides a non-invasive, efficient, and highly sensitive method for detecting and cataloging species. Despite its transformative impact, the technique faces challenges, but there are potential ways to address these issues.

Collection of more samples and the use of multiple PCR replicates

The poor permanence of eDNA in the natural environment presents a significant obstacle for eDNA studies. All living things spontaneously release DNA into the environment, enabling direct isolation without evidence of the target organism's presence. This is the foundation for the entire eDNA analysis process (*Senapati et al. 2019*). However, as the organisms

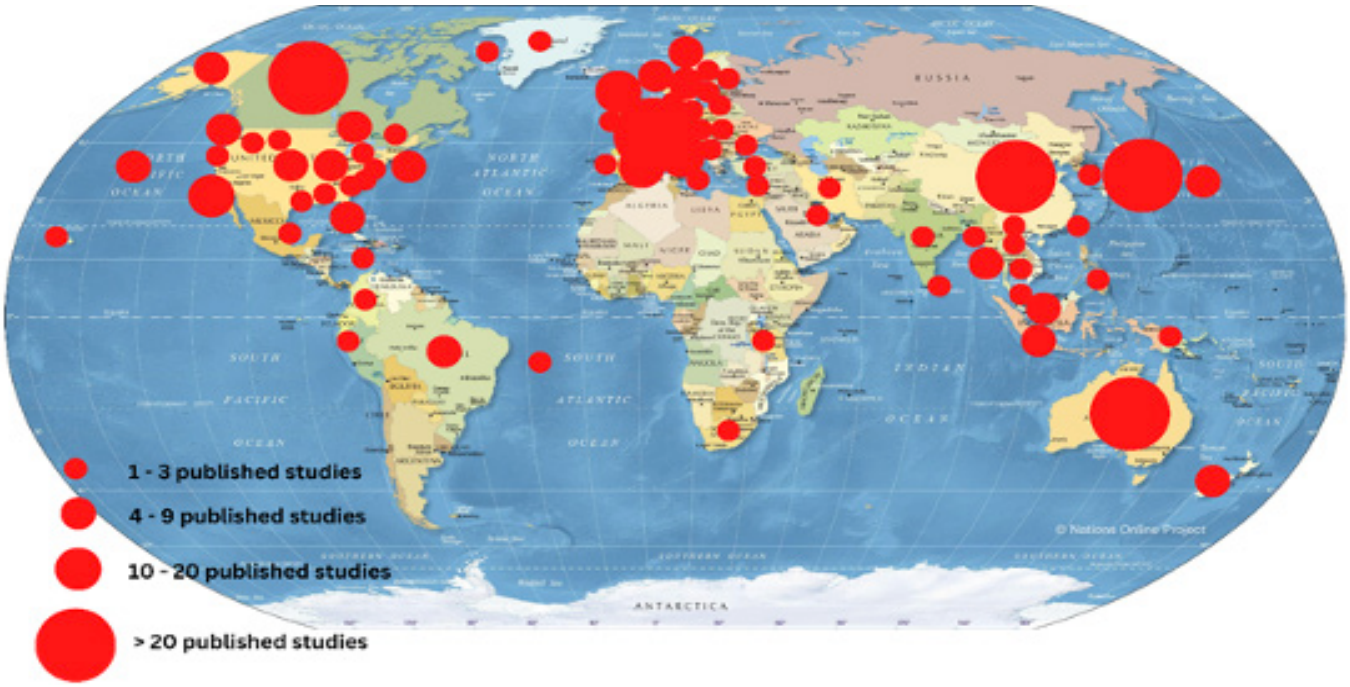

Figure 1. Worldwide distribution of published eDNA studies from 2008 to July 2023 (n=423).

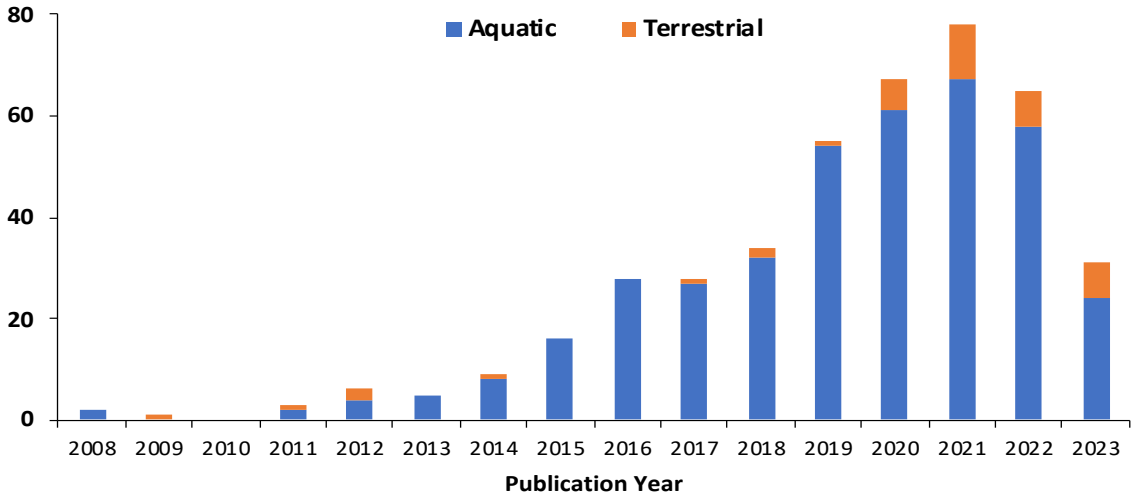

Figure 2. The number of terrestrial and aquatic assessment studies that used environmental DNA (eDNA) and have been published between 2008 and the present (n=423).

decompose over time, the DNA they shed is carried throughout space rather than automatically congregating in the location of release (*Yao et al. 2022*). Numerous biotic (such as species interactions, life-history features, and microorganisms) and abiotic (such as salinity, temperature, and UV radiation) factors influence how quickly DNA degrades in the environment (*Díaz-Ferguson et al. 2014; Stewart et al. 2019*). Thus, determining the most appropriate time to perform eDNA surveys is essential, given the limited knowledge of eDNA's persistence under various environmental circumstances. Every place has a variety of year-round environmental circumstances, and many species adapt their behavior to the season and to some of the same factors that slow down DNA degradation. For example, *Pilliod et al.* (*2014*) found that after 11 and 18 days, eDNA was still detectable in water samples kept in the dark. Nevertheless, water samples exposed to direct sunshine for eight days do not yield detectable amounts of eDNA. Conversely, the temperature directly influences the metabolic rates of some animals (such as fish, invertebrates, amphibians, and reptiles), influencing the pace at which eDNA is released (*Clarke and Fraser 2004*). Consequently, fish in warm waters (14°C) release more eDNA than fish in cold waters (7°C), according to *Lacoursière-Roussel et al.* (*2016*). Fish abundance and eDNA concentration correlate more strongly in warm than cold water.

DNA fragments in aquatic systems are likely to physically disperse away from target species due to huge habitat volumes and strong currents (*Salter et al. 2019*). The effects of fluctuating stream flow on eDNA concentrations and detectability were examined by *Curtis et al.* (*2021*). They discovered that floods caused false negatives or non-detections, which may have severe ramifications for detecting low-abundance organisms, and that increased stream flows reduced eDNA concentrations.

Water is the most collected environmental sample (79% of 423 publications) and the volume in most studies had a regular sample quantities ranging from 0.25 (*Evans et al. 2016; Olds et al. 2016*) to 2 L (*Griffiths et al. 2020; Brys et al. 2021*) (**Figure 3**). In order to study species diversity, distribution patterns, seasonal dynamics, and fish community conservation in rivers and lakes, researchers tend to gather huge volumes of water (>10 L) as sample equipment has advanced (*Civade et al. 2016*). It is widely acknowledged that greater volumes of water yield greater amounts of DNA and allow the detection of more eDNA-based organisms. In comparison to samples taken from two lentic and one lotic freshwater body in Wales, United Kingdom, containing 15 mL, 100 mL, 250 mL, and 1 L, *Muha et al.* (*2019*) found the highest level of eDNA in 2 L of water. *Evans et al.* (*2017*) determined that 5 to 20 L of water were needed to detect the entire fish ecology when assessing fish species richness in temperate lakes using spatial replicates. *Cantera et al.* (*2019*) examined trends in species richness by analyzing filtered eDNA samples of 34 to 340 L of water taken from tropical streams and rivers in French Guiana. Consequently, most species could be detected with sample sizes between 34 and 68 L, while larger than 68 L offered little further benefit. According to *Civade et al.* (*2016*), the biggest water sample volume for fish ecology research is now 45 L. As a result, while selecting the right water sample volume, one must consider whether the sample can provide sufficient taxonomic information and complete filtration in the allotted sampling period. According to *Muha et al.* (*2019*) sample duplicates from lakes, rivers, and offshore seawater seem to work best with a 2 L water volume because it can disclose more fish species than a ≤1 L volume while requiring less filtering time. On the other hand, sampling a larger amount of water could result in longer filtration times and a higher chance of DNA deterioration.

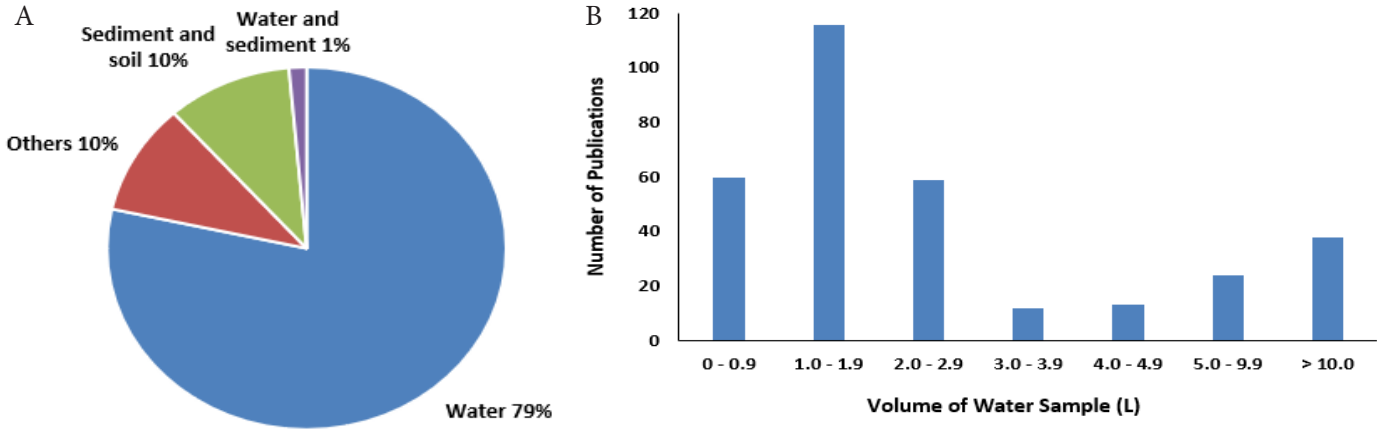

Figure 3. Relative frequency of the environmental samples (n=423, A) and the volume of water used in environmental DNA (eDNA) metabarcoding analysis in peer-reviewed academic articles published from 2008 to 2023 (n=322, B).

Multiple sample collection is advised for statistical replication as it improves spatial coverage and the likelihood of discovering target species (*Evans et al. 2017*). The number of species identified by DNA metabarcoding is also influenced by the number of biological replicates (number of samples) and technical replicates (number of PCRs performed on the same sample) (*O'Donnell et al. 2016; Yamamoto et al. 2017*). Using water from an aquarium tank with a known species composition, *Doi et al.* (*2019*) investigated the effects of the number of repetitions during filtration and PCR (repeated extractions and amplification for the same samples) on the detection probability of species. According to their data, there was a preference for increasing the number of PCR replicates over filter replicates, with consistently high detection rates at the filtration step and meager and variable detection rates at the PCR step. Thus, in order to retain a relatively high detection probability of the minor species contained in eDNA extracts, it would be a great idea to do PCR with numerous duplicates (e.g., eight replicates, for ease of experimental manipulations) in practice (*Ficetola et al. 2015*).

Due to eDNA's limited persistence, it may be possible to practically adjust for the insufficient sampling of eDNA by increasing the number of replicate amplifications per sample of DNA (PCR) (*Hansen et al. 2018*). Furthermore, there is mounting evidence that the density or activity of the target species in both space and time is positively correlated with the concentration of DNA recovered during a sampling effort (*Dougherty et al. 2016; Bista et al. 2017*). According to a study by *Macher and Leese* (*2018*) on three German rivers at four sample sites, communities varied considerably over time. That sampling location had an impact on the recovered communities. They concluded that not all of the eDNA found in rivers is always and everywhere present. In order to gather the eDNA of every species, sample sites' spatial layouts must, to the greatest extent feasible, encompass all water layers and diverse micro-habitats (*Hänfling et al. 2016; Zhang et al. 2020*). On the other hand, because species identification also depends on the number of reads used, data size is a limiting factor in DNA metabarcoding. More species can be identified the more readings there are.

Following standard sample collection and proper aseptic technique

Contamination happens when DNA of scientific interest combines with DNA from an external source. The DNA of the fish may be introduced to a pond where it is not present; for instance, if a predator at one pond kills a fish and is then brought to another (for example, anthropogenically) and excretes there (*Beng and Corlett 2020*). Exogenous DNA from catches, raw material processing, fish breeding, and fish feed can be found in sewage and wastewater from fishing ports, fishery processing industries, aquaculture ponds, and aquariums (*Yamamoto et al. 2016*). Non-resident fish may be found in the feces of piscivorous animals, including migratory marine birds, mammals, and fish. Dead fish can release significant eDNA into the surrounding environment (*Rees et al. 2014*). Furthermore, sites for water sampling should be carefully selected to prevent the collection of exogenous DNA from these sources during fieldwork. Furthermore, *Lafferty et al.* (2021) noted that eDNA-based habitat-specific analyses must consider eDNA spillover from one habitat to another brought on by tidal flows or ocean currents.

The handling and manipulating of samples in the field (such as collection, storage, and transportation) and in the laboratory (such as storage, DNA extraction, amplification, library preparation, and sequencing) are the first steps in conducting surveys utilizing the eDNA survey technique. Any of these stages could experience contamination (*Shaw et al. 2016; Rees et al. 2014*). The process may begin with sample collection in the field, for example, when DNA from one sample accidentally finds its way into another, whether the samples are from the exact location or a different one. According to *Goldberg et al.* (*2016*), contamination typically happens when the same field materials equipment- such as gloves, filters, and corers- is used repeatedly to sample different sites without adequate care. In the laboratory, there are two main pathways for contamination to occur. The first is when the same materials or equipment are repetitively used to conduct various experiments without systematic decontamination. The second is when DNA residue from previous experiments stretches out into the new samples. Standard decontamination methods include autoclaving, using 50% bleach solution, and using 10% bleach (sodium hypochlorite) solution (*Cowart et al. 2022*). Additionally, as thorough rinsing can effectively eliminate and destroy superfluous PCR products and DNA, it is strongly advised (*Evans et al. 2017*). According to *Miya et al.* (*2020*), at least one room should be dedicated to eDNA extraction, pre-PCR, and post-PCR steps, each with its equipment. There should also be rules for the personnel in charge of the experiments, such as the one-way rule to prevent contamination from the post-PCR room from spreading to other rooms and methods for decontaminating laboratory equipment, such as UV sterilization.

Use of alternative markers and primers

To address the problem of PCR primer biases, the use of alternative markers and primers must be taken into account, even when targeting similar taxonomic groups of species (**Figure 4**). In eDNA analysis, molecular markers must detect and identify species in single or multiple taxa. Primers' specificity, efficiency, and sensitivity are critical for successfully amplifying eDNA (*Furlan et al. 2016; MacDonald et al. 2017*). The many taxa or haplotype combinations in the eDNA samples contribute to their extreme heterogeneity. This nature makes complete complementarity between primers and target sequences during PCR more difficult (*Wang et al. 2021*). The consistency of the primer-template duplex and the effectiveness with which polymerase extends the primer are both impacted by primer-template mismatches, which may result in biased or unsuccessful PCR reactions (*Kalle et al. 2014*). As per *Xia et al.* (*2018*), mismatching can amplify shorter fragments more frequently than longer ones, numerous sequences more frequently than uncommon ones, or non-target species more frequently than target organisms. However, compared to metabarcoding, primer bias does not affect barcoding when species-specific primers are used instead of universal primers (*Collins et al. 2019*).

Given that there are more than 16,700 species of marine fish (*Eschmeyer et al. 2010*), no universal primer pair can amplify every target amplicon in all the different fish species (*Miya et al. 2020*). According to *Miya et al.* (2015), early eDNA metabarcoding using the original MiFish primers (MiFish-U) revealed an underrepresentation of certain large sharks and rays swimming in an aquarium tank. As a result, *Miya et al.* (*2015*) used the two primer sets (MiFish-U/E) simultaneously in PCR amplification (multiplex PCR) to optimize the MiFish-U primers to account for sequence changes in sharks and rays (*Elasmobranchii*). In the tank, all 18 elasmobranch species were identified with success using the multiplex PCR method. This method perfectly captures the problem of PCR dropouts, which is the inability of the fish DNA in the eDNA samples to be amplified, which invariably leads to false negatives during eDNA metabarcoding.

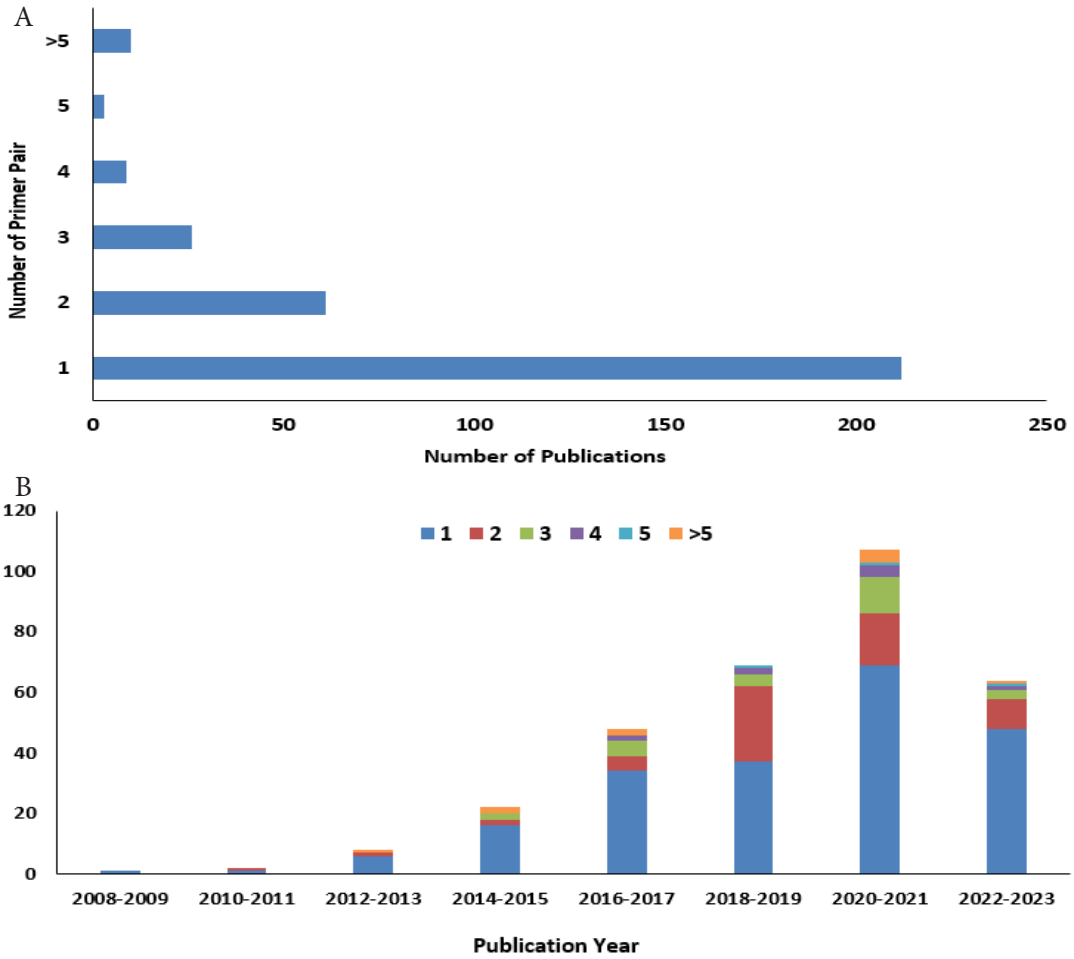

Figure 4. The number of publications along with the number of primer pair used (A) and The use of one or more alternative markers and primers in environmental DNA (eDNA) metabarcoding analysis from 2008 to 2023 (B).

Some research groups have employed multiple markers from different mitochondrial genes to avoid false negatives and underestimate species diversity (*Fraija-Fernández et al. 2020; McClenaghan et al. 2020*). According to *McElroy et al.* (*2020*), multimarker tests are necessary in ecosystems with substantial biodiversity to guarantee reliable species richness estimations. However, the accuracy and taxonomic coverage of reference databases used in multimarker assays vary, resulting in many taxonomic tables with diverse taxa at different taxonomic levels. Therefore, species-level ecological inference is challenging since it necessitates several assumptions to combine such disparate taxonomic tables into a single table. Accurately determining the species richness of fish communities in a study area requires knowledge of the sequence differences within priming sites and amplicons, regardless of the primers used.

Five primers that are often used to amplify fish rRNA barcoding genes were examined by *Kumar et al.* (*2022*). By evaluating water samples from the Indian River Lagoon in Florida, they could assess their accuracy and efficiency. A section of the mitochondrial 12S gene was amplified by three of the five primer sets (Riaz_12S, 106 bp; MiFish_12S, 171 bp; Valentini_12S, 63 bp); another amplified the nuclear 18S gene (MacDonald_18S, 217 bp), and one amplified the mitochondrial 16S gene (Berry_16S, 219 bp). The combined 12S primer sets detected 86% (65 species) of the 76 fish species identified across all datasets. By contrast, 93%(71 species) of the samples were detected using the combination of the Berry_16S and Riaz_12S primers. This underscores the significance of utilizing several primer sets and primers that target distinct genomic regions. Interestingly, their research revealed that there might be better options than the extensively used MiFish_12S primer set; the Riaz_12S primer set proved to be the most successful for eDNA-based fish surveys.

Several markers were evaluated by *Ficetola et al.* (*2016*) for the metabarcoding of freshwater macrobenthos. By combining *in silico* and laboratory testing to investigate the efficacy of several markers amplifying areas in the 18S rDNA (Euka02), 16S rDNA (Inse01), and COI (BF1_BR2-COI) genes, their research produced a complete database of benthic macroinvertebrates in France and Europe. The amplification success of several primer combinations was observed in the results, underscoring the difficulty in choosing the most appropriate markers and supporting the integration of many metabarcodes to provide a more thorough and precise understanding of the ecological effects on freshwater biodiversity.

Since any nonspecific amplification results in a false positive that may lead to the improper deployment of conservation or management measures, primer design becomes crucial. Primer specificity based only on *in silico* validation (*Wei et al. 2018*) or in conjunction with *in vitro* tests of one or a few nontarget species (*Yusishen et al. 2020*) are not advised because, according to *So et al.* (*2020*), their findings are imprecise when testing primer specificity using BLAST *in silico*. They advised performing validation tests *in situ* and *in vitro* to properly defend against deriving erroneous findings from eDNA research. As further assurance that the primers amplify the target gene from the studied organism, researchers should sequence a few false positives (*So et al. 2020*). In addition, the duration between sample collection and processing in the laboratory should also be evaluated as it may contribute to false negatives.

Use of inhibition-reducing assays

The central method to detect specific organisms from environmental DNA obtained from various sources (e.g., water, sediment or soils, feces) is Polymerase Chain Reaction (PCR). Nevertheless, PCR is highly susceptible to inhibitors, which may reduce the test's sensitivity and produce erroneous negative findings. According to *Nichols et al.* (*2018*), these inhibitors are found naturally in the eDNA. The compounds that make up these inhibitors encompass a variety of groups, including heme from blood, bile salts from feces, urea from urine, collagen from tissues, humic acid from soil, and plant components (*Watson and Blackwell 2000*). According to *Schrader et al.* (*2012*), these inhibitors usually work by interfering with thermostable DNA polymerases, obstructing enzyme activity, or directly interacting with DNA, which can halt amplification and make it easier to co-purify DNA and inhibitor. For instance, humic acid and tannin chemicals, which can inactivate DNA polymerase and hinder PCR amplification by reducing its efficiency or resulting in complete failure, are typically present in DNA extracts obtained from turbid water (*Albers et al. 2013*). Phenols can cross-link RNA and inhibit RNA isolation in oxidizing conditions (*Wilkins and Smart 1996*). Polysaccharides may also reduce the ability to resuspend precipitated RNA (*Sipahioglu et al. 2006*). For example, reverse transcription may be hindered by the enzyme's direct interaction with melanin (*Eckhart et al. 2000*). Because polysaccharides resemble nucleic acid structures, they may obstruct the enzymatic process. DNA polymerase and melanin combine to produce a reversible mixture (*Schrader et al. 2012*).

A study by *Harper et al.* (*2019*) on monitoring

freshwater ponds using eDNA, PCR inhibition, representative sampling, and eDNA capture was identified as their greatest challenge. False negatives may result from PCR inhibition. Therefore, a qPCR amplification of internal positive controls is recommended to test for it, for example, by employing Applied BiosystemsTM TaqMan Exogenous Internal Positive Control Reagents or spiking tests with control DNA that will not be in the sample (*Doi et al. 2017*). Some DNA extraction kits include Specific inhibitor removal techniques, which can be applied to complex pond eDNA samples (such as turbid, high algal content samples) (*Buxton et al. 2018; Sellers et al. 2018*). Stand-alone clean-up kits (e.g., Zymo® or Qiagen®) can be helpful for inhibited samples following DNA extraction (*McKee et al. 2015; Williams et al. 2016*). For example, bovine-serum albumen (BSA) can be added to PCR processes to reduce inhibition, according to *Albers et al.* (*2013*). The impact of inhibition can be minimized by optimizing thermocycling conditions, reagents, and protocols (*Jane et al. 2015*).

To circumvent inhibition, it was suggested to dilute eDNA extracts (*Biggs et al. 2015*) or reduce the PCR template (*Takahara et al. 2015*). *Harper et al.* (*2019*) would not recommend either strategy, as dilution may lower the target DNA concentration below the detection limit, and eDNA samples are known for having low target DNA concentrations. Despite diluting out inhibitory chemicals, this leads to misleading negative results (*Buxton et al. 2017*). The above limits on quantification and detection may be solved by using droplet digital PCR (ddPCR), particularly in waters with high PCR inhibitor concentrations. In ponds, ddPCR performed better than qPCR because of its reduced variability, particularly at low eDNA levels (*Doi et al. 2015*). As a result, estimating biomass or abundance may be more accurate (*Nathan et al. 2014*). Since the kind of material determines whether these inhibitors are present, it is imperative to prepare nucleic acids according to matrix-specific methods prior to PCR in order to address this issue (*Hunter et al. 2019*).

Combine the extraction of DNA and RNA, amplifying both DNA segments of varying lengths

Because all living things leak DNA into the environment, both living and deceased species add to the pool of eDNA. A cell must be metabolically active and reproducing to be considered alive (*Capo et al. 2021*). Depending on the goal of the monitoring activity, researchers may be interested in the DNA from either one living organism or from both dead and living (*Pochon et al. 2017*) for the biodiversity assessment. According to *Kamoroff and Goldberg* (*2016*), the ecological interpretation of eDNA data and the validation of the effectiveness of eDNA techniques in resource management and restoration depend on the knowledge that the animals discovered are alive. Hence, the problem of false positives must be addressed for eDNA to be used as a management tool. However, differentiating between dead and living organisms' DNA remains challenging (*Beng and Corlett 2020*). Longer DNA pieces in a particular environment most likely correlate to the most recent DNA because DNA naturally deteriorates with time. Therefore, *Jo et al.* (*2017*) proposed that comparing the changes in copy numbers of short (127 bp) and long (719 bp) eDNA fragments over time would help determine how well the concentration of longer eDNA fragments represents fish biomass after excluding the effects of contamination and breakdown. Aside from looking at DNA, RNA could be extracted to answer this problem. Analysis using RNA depicts the activities of living organisms at the sampling time. This is because the half-life of RNA is less than that of DNA, which is only about hours, or only a few minutes for messenger RNA and a couple of days for ribosomal RNA (*Keer et al. 2003*).

Removing the carcasses and preventing contamination in the natural environment is impossible since natality and mortality are vital in the natural population dynamics (*Beng and Corlett 2020*). Furthermore, the amount of dead organisms added to the pool of eDNA can vary significantly based on environmental conditions. In the subtropics and tropics, carcasses only persist briefly due to the relatively higher temperatures, contributing to a faster degradation rate. Degradation rates of eDNA for both common carp (*Cyprinus carpio*) and ayu sweetfish (*Plecoglossusaltivelis altivelis*) are reported to increase proportionally with increasing temperature of water (*Tsuji et al. 2017*).

However, according to *Turner et al.* (*2014*), eDNA molecules can either be free-living or attached to natural particles. Those particles that bind with the DNA might persist for extended periods. Moreover, it can be resuspended through natural phenomena such as wind, turbulence, wave action or bioturbation, and erosion. In circumstances like this, ancient DNA (aDNA) resuspension (together with DNA from dead organisms) could result in false positive results and misinform management, such as in cases of continued detection of extinct species (*Beng and Corlett 2020*). Therefore, verifying the organism's existence using conventional techniques is imperative.

Moreover, two studies (*Sønstebø et al. 2010; Jørgensen et al. 2012*) are especially interested in using

ancient DNA to rebuild ancient ecosystems. As a reference for conservation planning, they are using aDNA. This includes tracking the emergence of invasive species, testing for climate change models, and monitoring the impacts of humans on the past landscape and biodiversity (*Bellemain et al. 2013; Giguet-Covex et al. 2014*).

Completeness and accuracy of reference sequence database

According to *Miya et al.* (*2015*), some methodological issues must be fixed before the eDNA metabarcoding strategy is likely to become a standard technology in fish biodiversity research. According to *Ficetola et al.* (*2016*), mistakes can happen at any point in the process, from sample collection and storage in the field to molecular analysis and bioinformatics workflow.

One is the correctness and completeness of the reference sequence database; inaccurate or incomplete reference sequences might provide findings that are both falsely positive and falsely negative. The vast diversity of fish, which includes over 32,000 known species in aquatic environments worldwide, means that reference sequence databases are still far from satisfactory even after global gene databases, such as NCBI (www.ncbi.nlm.nih.gov/genbank), databases of DNA barcodes targeting specific biological taxa, such as World's Fish (www.fishbol.org), the mammal DNA database (http://www.mammaliabol.org), and the bird species DNA database (http://www.barcodingbirds.org), have been built (*Nelson et al. 2016*).

Improved reference database coverage is necessary before the eDNA approach may successfully supplement conventional census methods (*Fernandez et al. 2020*). *Ruppert et al.* (*2019*) also stressed the need to add recognizable sequences for all target biodiversity to reference databases. To accommodate the expanding data libraries, sophisticated infrastructure must be built, and more approachable data exploration techniques are required. Optimized bioinformatics pathways with intuitive interfaces and general enhancements to eDNA extraction and collection techniques—especially about accounting for degradation—would be beneficial for widespread use.

A collaborative effort between academic institutions and research centers in the California Current region has created a reference database for MiFish eDNA metabarcoding. This database comprises reference sequences belonging to 712 of the 864 identified species in the region (*Miya et al. 2020*). However, at present, the reference database for the MitoFish database- a comprehensive and standardized fish mitochondrial genome database created by *Miya et al.* (*2020*)- is 100% for order, 90% for family, 54% percent for genus, and 25% percent for species. Thus, the ongoing improvement of DNA reference databases will be essential for developing eDNA research, including DNA barcodes and perhaps whole mitochondrial DNA for broader applications.

Improvement of bioinformatic pipelines

Sequencing errors and low-quality sequences are inevitable despite the ongoing advancements in sequencing technology, and they significantly increase the likelihood of false positives when utilizing eDNA metabarcoding (*Kunin et al. 2010; Thomsen and Willerslev 2015*). Therefore, removing interfering sequences and increasing MOTU identification rates will be beneficial to improve bioinformatic pipelines and create practical computer programs for raw sequence filtering and quality control.

Traditional and eDNA surveys can complement each other

Information presented by traditional and eDNA survey methods differs. Accordingly, one should not be viewed as a replacement technique for determining and tracking the state of biodiversity (*Stat et al. 2019*). Adopting one approach or both must be considered depending on how well it fits the goal of the study they wish to conduct (*van der Loos 2021*). Traditional surveys must frequently be used with eDNA results, but eDNA data can assist in directing these evaluations in the proper direction (*Morisette et al. 2021*). Researchers could select what data can be collected by determining whether to undertake eDNA analysis alone or in addition to standard questionnaires (*Dickie et al. 2018*).

In order to evaluate estimates of fish community species richness between eDNA metabarcoding and traditional approaches (e.g., ocular census, netting, electrofishing), *McElroy et al.* (*2020*) synthesized 37 eDNA research in aquatic ecosystems. They contended that eDNA metabarcoding is dependable and offers an assessment approach for biodiversity that can surpass traditional techniques for determining species richness. However, several studies have also suggested using conventional methods as a complementary approach to eDNA. *Lee et al.* (*2022*) used environmental DNA metabarcoding and underwater visual census to evaluate fish diversity in the coastal waters near Nodaedo Island, Tongyeong, Korea. Accordingly, the UVC recognized 69 species, whereas eDNA metabarcoding discovered 68

species, concluding that these two approaches can work to shed light on the organization of the fish communities that live in different coastal habitats. Comparing eDNA and trawling studies of demersal fish communities in the continental slope depths of Southwest Greenland and the Gulf of St. Lawrence, Canada, respectively, *Thomsen et al.* (*2016*) and *Afzali et al.* (*2021*) also observed similar observations. When *Sato et al.* (*2021*) used qMiSeq and echo sounder survey to examine the distributions of marine fishes at artificial reefs and surrounding stations in the open oceanographic environment of Tateyama Bay, central Japan, they demonstrated the complementing use of both technologies. Thus, multiple methods can be used to gather and analyze data, incorporate findings, and draw conclusions, depending on how complicated the projects are (*Bruce et al. 2021*). Nonetheless, *Wee et al.* (*2023*) have established that eDNA is a valuable tool for assessment in both ecological and conservation contexts. Furthermore, it is more beneficial when combined with historical data, conventional biodiversity surveys, and other data sources (like citizen science) that offer more excellent details (*Tingley et al. 2019*).

CONCLUSION AND RECOMMENDATIONS

The complexities and variations inherent in eDNA protocols present significant challenges for researchers new to the field, potentially compromising the reliability of published findings. Despite its promising potential in biological research, the standardization of eDNA protocols remains essential. While international criteria exist, their adoption among researchers remains inconsistent. The review underscores the critical importance of adhering to established technological standards in eDNA research to minimize false detections. The authors advocate for adopting standardized field and laboratory procedures aligned with current eDNA sampling and experimental practices in ecological research. The analysis highlights that optimal species detection often requires collecting 1 or 2 liters of surface water for PCR replication as adopted from *Muha et al.* (*2019*). This approach not only enhanced the reliability of species detection across diverse environments but also underscored the protocol's efficacy in advancing the field of environmental DNA research for biodiversity monitoring. Furthermore, utilizing multiple markers and primers helps mitigate PCR biases, thereby enhancing accuracy in species identification and richness assessment. Stringent quality controls throughout the eDNA analysis process are crucial to reduce contamination and minimize false detections.

Looking ahead, it is recommended to advance eDNA research by implementing standardized protocols, enhancing comprehensive training initiatives, developing robust DNA databases, continuously improving technological advancements, and fostering collaborative efforts. These actions are pivotal in fully harnessing the capabilities of eDNA metabarcoding in ecological and conservation studies. By doing so, this innovative approach can become more accessible, reliable, and beneficial across diverse scientific communities, facilitating broader applications and advancements in biodiversity monitoring and conservation efforts globally.

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