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Environmental DNA Template Variation: Its Relevance for Species Detection and Conservation

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ABSTRACT

1. Accurate species monitoring is foundational for understanding and assessing species extinction risk. Environmental DNA (eDNA) based species detection methods have been proposed as fast and powerful biodiversity monitoring tools. Yet, these methods are susceptible to errors that might hinder the assessment of species extinction risk. Samples may contain low DNA concentrations of the target taxa and/or exhibit high levels of PCR inhibitors, which can yield false negatives.
2. We investigated how adjusting the input sample volume in the eDNA-based molecular assay improves detection of an endangered fish, *Anaecypris hispanica,* in highly eutrophic streams. Water samples were filtered and tested using a real-time PCR (qPCR) assay varying the input volume of eDNA samples (i.e., 0.5X, 1X and 3.3X). From the positive detections obtained with different eDNA input volumes, we built species occurrence maps and estimated geographic range metrics used in species extinction risk assessment.
3. Although the number of sites with positive detections was similar among the input eDNA sample volumes tested, positive detections were not spatially redundant. When comparing the pooled results from all eDNA-based trials to a fixed 1X eDNA volume, there was a nearly 75% increase in the number of sites with detections, consequently leading to increases in all geographic range metrics (i.e., extent of occurrence, area of occupancy number of locations).
4. Our results highlight that false negatives in eDNA-based surveys are not to be overlooked. The success of species detection will likely vary on a case-by-case basis, depending on the DNA concentration of the target taxa and the concentration of potential inhibitors in bulk eDNA samples, both of which are generally unknown. Improved species detection may be achieved by running, in parallel, qPCR assays with different input volumes of bulk eDNA samples.

Ana Veríssimo and Filipe Ribeiro contributed equally to this work.

5. As interest grows in integrating eDNA-based tools into species monitoring practices, it is essential to continuously refine protocols and carefully consider study design decisions to ensure robust results, advancing species management and conservation.

1 | Introduction

Biodiversity loss stands as one of the most pressing contemporary challenges in conservation, and addressing this issue requires strategic prioritisation of management efforts and efficient allocation of conservation funding (Albert et al. 2021; Ceballos et al. 2017; Pim et al. 2014). While conservation planning encompasses socio-economic, cultural, and ecological dimensions (Bennett et al. 2017; Herbert et al. 2023; Woinarski et al. 2017), consideration of species extinction risk remains indispensable in decision-making processes (Bennun et al. 2018; Betts et al. 2020; Cazalis et al. 2022). The International Union for Conservation of Nature's (IUCN) Red List of Threatened Species (hereafter Red List) assesses and classifies species in regard to their probability of extinction, based on a set of quantitative criteria related to population geographic range, trends and size (IUCN 2022). Among these criteria, extent of occurrence and area of occupancy are the most used in species assessments (Collen et al. 2016). Hence, accurate assessment and monitoring of species occurrences are paramount for assessing and updating species extinction risk, and to inform practical and theoretical efforts to conserve biodiversity.

Traditionally, biodiversity monitoring has relied on direct species observations and capture-based survey methods. Over recent decades, however, our ability to collect data on biodiversity has exponentially increased thanks to an array of innovative technologies (Amorim et al. 2023; Bolgan et al. 2023; Maasri et al. 2022; Tosa et al. 2021). Among these, methods based on environmental DNA (eDNA) have emerged as powerful, non-invasive, cost- and time-effective, highly specific and sensitive alternatives for monitoring biodiversity (Fediajevaite et al. 2021; Keck et al. 2022).

Environmental DNA refers to the traces of genetic material released into the environment by organisms, in the form of faeces, mucus, skin cells, organelles, gametes or extracellular DNA (Deiner et al. 2017). Depending on the primary focus of the study, eDNA-based workflows can either follow a metabarcoding approach (high-throughput multispecies taxonomic identification) or a targeted approach using species-specific real-time PCR (qPCR) assays. The latter approach is usually applied for detecting early signs of invasive species or for improving the detection of rare, endangered and cryptic native species (Duarte et al. 2023). Recently, the importance of eDNA methodologies has been recognised in the IUCN's guidelines for planning and monitoring corporate biodiversity performance (Stephenson and Carbone 2021). An extensive number of studies across multiple taxonomic groups have demonstrated that eDNA-based methods outperform traditional survey methods in species detection (Fediajevaite et al. 2021; Gehri et al. 2021; Seymour et al. 2021). However, similarly to any sampling method, eDNA-based methods are prone to errors, including both false positives and false negatives, which can arise from various sources (Burian et al. 2021).

False positives (type I errors) in eDNA-based methods are primarily attributed to contamination and inadequate assay specificity and have been extensively discussed (e.g., Darling et al. 2021; Ficetola et al. 2015, 2016; Hutchins et al. 2022; Sepulveda et al. 2020a). In contrast, false negatives (type II errors) are often perceived as less urgent in this context, despite their importance (Furlan et al. 2016; Song et al. 2020). It is understood that this type of error is mostly determined by the balance between release and degradation rates, eDNA dispersal patterns and the concentration of inhibitors within samples (Burian et al. 2021). However, the interplay between these factors and their influence on species detection is difficult to assess; thus, establishing effective mitigation strategies remains challenging. This is further accentuated by the absence of well-established protocols across the different stages of eDNA workflows, spanning from field sampling to laboratory procedures and bioinformatic analysis (Bunholi et al. 2023; Koziol et al. 2019; Shea et al. 2023). For instance, researchers conducting a species-specific qPCR can adopt multiple strategies to minimise false negatives, but the efficacy of many of these strategies in species detection is still incompletely known. This is, for example, the case when analysing water samples with varying levels of organic material, as both eutrophic and oligotrophic waters may result in false negatives, albeit for distinct reasons (Kumar et al. 2021). In eutrophic waters, false negatives are primarily attributable to the high concentration of PCR inhibitors, while in oligotrophic waters, they are mainly due to the low eDNA concentration.

Environmental samples inherently contain a myriad of potential PCR inhibitors, i.e., compounds affecting the efficiency of the amplification of a specific nucleotide sequence (Sidstedt et al. 2020). False negatives can generally be addressed by varying the amount of template input in the qPCR assay. By reducing the input amount of sample in the qPCR assay, the concentration of inhibitors is decreased in the reaction mixture, thus increasing PCR efficiency (Schrader et al. 2012). This is common practice across various applications of diagnostic qPCR, ranging from agricultural biotechnology (Demeke and Jenkins 2010) to clinical tests (Scipioni et al. 2008) and forensics (Imaizumi et al. 2005). In eDNA qPCR-based studies, however, diluting samples has rarely been practiced due to concerns that it would exacerbate the difficulty of working with already low quantities of highly degraded eDNA molecules. Nonetheless, McKee et al.'s (2015) has demonstrated that sample dilution can be an effective treatment to improve amplification in environmental samples which previously showed inhibition. Conversely, when eDNA and inhibitors are expected to occur in lower concentrations, as is often the case for mountain headwater streams, testing increased sample volumes in the assay might be a preferable strategy to enhance detection. Still, most eDNA-based species surveys do not experiment with different input volumes and instead rely on a single amount, potentially overlooking false negatives.

In this study, we examined how the input volume of eDNA samples affects single-species detection using qPCR on environmental samples and explored the implications of protocol selection in assessing species extinction risk. Our research was conducted within the context of a monitoring survey for *Anaecypris hispanica*, an endangered freshwater fish endemic to the Iberian Peninsula, occurring in highly eutrophic

Mediterranean-type streams (Collares-Pereira et al. 2021; Magalhães et al. 2023; Ribeiro et al. 2000). Specifically, we analysed whether reducing and increasing the overall concentration of bulk eDNA per qPCR reaction would reveal the incidence of false negatives in our survey. More specifically, we considered as evidence of a false negative if a sample initially tested negative returns a positive result after a change of template concentration. The effectiveness of each protocol is expected to vary depending on the composition of the sample, including factors such as inhibitor levels and ratios to target DNA, which are typically undetermined and likely unique to each sample or ecosystem. The concentration of eDNA in the qPCR reaction might, therefore, influence the likelihood of positive detections and directly impact assessments of species occurrences and geographic range, ultimately biasing evaluations of extinction risk. To fully demonstrate this effect, we estimated the extent of occurrence, area of occupancy and number of locations used in Red List extinction risk assessments based on the species detection results obtained using different input eDNA volumes. We further compared the results from our eDNA-based methods with those obtained using traditional survey methods, specifically electrofishing.

2 | Materials and Methods

2.1 | Study Species

Anaecypris hispanica (Steindachner, 1866) is an Iberian leuciscid, restricted to the Guadiana and Guadalquivir drainages in the Iberian Peninsula (Collares-Pereira et al. 2021; Figure 1). It is a small-sized and short-lived fish with low fecundity that occurs in small streams with seasonal flows (Collares-Pereira et al. 2021; De Miguel et al. 2010; Ribeiro et al. 2000). In Portugal, *Anaecypris hispanica* has experienced a severe reduction in distribution over the last 40 years (Cardoso 2022; Collares-Pereira et al. 1999; Magalhães et al. 2023), with recent electrofishing surveys failing to detect five out of its ten subpopulations, namely in the Xévora, Caia, Álamo, Degebe, and Carreiras subdrainages (Cardoso 2022).

2.2 | Field Methods

In May of 2022, we conducted water sampling at 70 sites (one sample per site) in the Lower Guadiana Basin (Figure 1), encompassing the ten subdrainages where *A. hispanica* had been detected at the turn of the century (Cardoso 2022; Collares-Pereira et al. 2000; da Costa and Collares-Pereira 2003; SIBIC 2017; Table 1). We have allocated our sampling effort differentially among subdrainages, contingent on the date of the most recent species record from electrofishing and stream length, assuring that the set of selected sites generally encompassed the historical distribution of the species in each subdrainage (Collares-Pereira et al. 1999). Specifically, fewer sampling sites (2–4 per subdrainage; total of 17 sites) were allocated to the sub-drainages with recent records of *A. hispanica* (Ardila, Chança, Vascão, Foupana & Odeleite; sensu Cardoso 2022), aiming only to confirm species presence through eDNA. In turn, increased sampling effort (6–13 sites per subdrainage; total of 53 sites) was allocated to the subdrainages with no records of *A. hispanica* since 2000 (Álamo, Caia, Carreiras, Degebe, Xévora; Ribeiro et al. 2000; Collares-Pereira et al. 1999). Sampling took place during the reproductive season of *A. hispanica* (Ribeiro et al. 2013) when discharge is low, to enhance the likelihood of species detection via eDNA released by active adults and gametes. We collected a total of 10 L of water per site along a transect of up to 1000 m (m), obtaining ten subsamples (1 L each) along a wide area and including different microhabitats per site, accounting for the fact that eDNA is heterogeneously distributed throughout the environment (Burian et al. 2021). The subsamples were collected while moving in the upstream direction with minimal sediment disturbance. The 10 subsamples per site were mixed in a single container on site, and the resulting 10 L of water per site were filtered within 6 h of collection. Up to filtration, samples were stored in the dark and on ice together with a blank consisting of a container with 4 L of bottled drinking water to account for possible contaminations during transport. All water samples, including blanks, were filtered using high-capacity GoPro filter capsules (0.45 μm pores, 700 cm^2, Proactive Environmental Products, FL, USA) with the aid of an EZ-Stream Pump (Merck Millipore, MA, USA). In case of filter clogging (5 out of 70 field samples), we registered the volume of water filtered (minimum of 3.5 L, average of 6.4 L; Appendix S2). Before each filtration event, 5 L of bottled drinking water were filtered as a filtration blank to account for contaminants associated with the equipment. Upon filtration, capsules were loaded with 50 mL of preservation buffer (mixture of 3:1 [v/v] of Lysis Solution to Water Lysis Additive; Sellers et al. 2018) and transported on ice before being stored at 4°C. Field procedures were performed under clean and rigorous protocols to avoid contamination, using single-use supplies or decontaminating any non-disposable equipment with 40%–60% commercial bleach.

2.3 | DNA Extraction of Environmental Samples

DNA extractions of environmental samples were carried out using the DNeasy blood and tissue kit (Qiagen Inc., Hilden, Germany), with modifications. Samples were chosen at random (i.e., regardless of subdrainage or site) in batches of 7 or 13, including an extraction blank per batch. Briefly, filter capsules were incubated at 55°C for 30 min to facilitate DNA elution from the filter and vortexed for 1 min to mix contents prior to the collection of 50 mL of filtrate into sterile falcon tubes, followed by a centrifugation step at 5000 rpm for 2 h. Next, we discarded the supernatant and kept the bottom 2 mL with the pellet, to which 200 μL Proteinase K was added, and digestion proceeded at 55°C overnight. Following digestion, 2 mL of AL buffer and 2 mL of absolute ethanol were added to each sample. Subsequent column purification steps followed the manufacturer's instructions, with a final elution step using 100 μL of AE buffer.

2.4 | Primer Development and Optimization of the Real-Time PCR Assay

The development and validation of species-specific primers involved *in silico* and in vitro testing of putative primers in the target and co-occurring fishes, as described in detail in Supporting

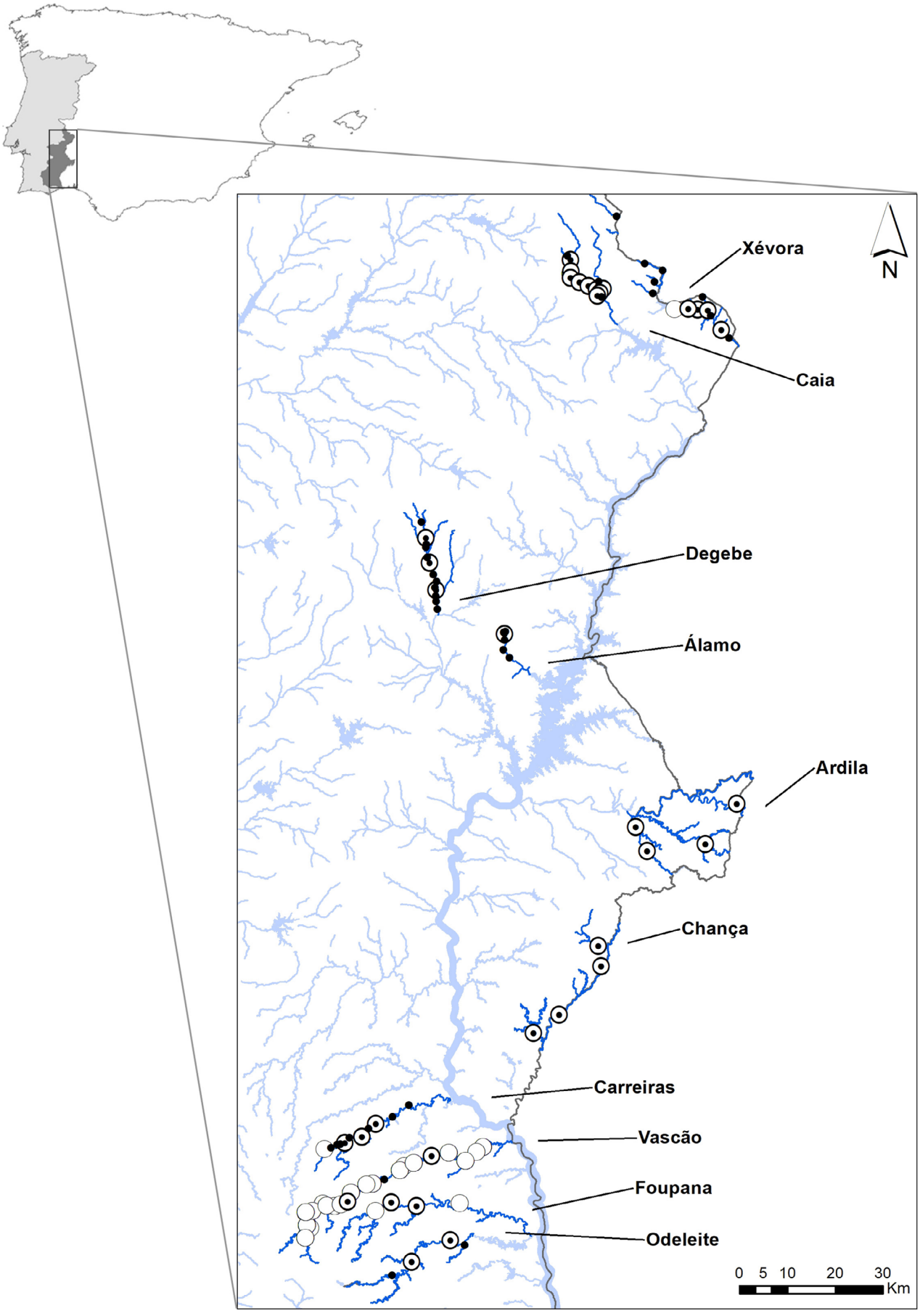

FIGURE 1 | Legend on next page.

FIGURE 1 | Map of the Lower Guadiana basin (in light blue) in southeastern Portugal showing sites from the historical distribution (white circles) reported in Collares-Pereira et al. (2000); sites from historical distribution where eDNA samples were collected (white circles with a central black dot); sites outside the historical distribution where eDNA samples were collected (black dots).

TABLE 1 | Overview of the positive detections of *Anaecypris hispanica* across the subdrainages where it occurred at the turn of the century, including the last detection prior to this study, and the summary of eDNA-based detections using 1X, 0.5X, and 3.3X eDNA concentrations and the pooled detections from all tested concentrations ("1X", "0.5X" and "3.3X" eDNA, "eDNA-all"). N: Number of sites sampled in each subdrainage in 2022.

Drainage	Last detection	N	1X	0.5X	3.3X*	eDNA-all
Xévora	2009[a]	12	2	1	0	3
Caia	2000[b]	12	1	1	0	2
Álamo	2003[c]	6	0	1	0	1
Degebe	1999[c]	13	2	2	0	3
Ardila	2021[d]	4	0	1	0	1
Chança	2021[d]	4	1	1	0	2
Carreiras	1998[b]	10	1	2	0	2
Vascão	2021[d]	3	0	0	1	1
Foupana	2021[d]	2	1	0	0	1
Odeleite	2021[d]	4	3	3	—	3
Total	—	70	11	12	1	19

*Only one randomly selected sample was tested per subdrainage, except for Odeleite, which was not considered for this analysis.
[a]SIBIC (2017).
[b]Collares-Pereira et al. (2000).
[c]da Costa and Collares-Pereira (2003).
[d]Cardoso (2022).

Information 1. The selected primers amplified a 101 bp region of the *cytochrome c oxidase I* gene and were as follows: *Ahis_COI-F* 5'-CGCAGTCAACTTCATCACC-3′ and *Ahis_COI-R* 5'-GGACCGCTGTTACCAATACG-3′. Calibration curves of the species-specific qPCR assay were generated by implementing a dilution series of 1:5, 1:25, 1:125, 1:625, 1:3125, and 1:15625 from a tissue-derived DNA solution (stock concentration: 10 ng/μl) of the target species. All reactions were conducted in a final volume of 10 μL containing 5 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., CA, USA), 0.5 μL of each primer (10 μM), 1.2 μL of template DNA solution, and 2.8 μL of water. The Supermix already contains additives to increase PCR inhibitor tolerance. All reactions were run in triplicates on a CFX96 Real-Time System (Bio-Rad Laboratories Inc., CA, USA) according to the following conditions: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 61°C for 30 s.

2.5 | Species Detection by Real-Time PCR

The effect of sample volume in the detection of *A. hispanica* was tested through three rounds of qPCR using different eDNA template volumes: 1.2 μL (1X), 0.6 μL (0.5X) and 4 μL (3.3X), while adjusting the final PCR reaction volume to 10 μL with autoclaved water. We tested for different eDNA volumes rather than varying total eDNA concentration based on the assumption that the total amount of eDNA in a sample is not informative of 1) the amount of PCR inhibitors present, nor of 2) the amount of target DNA, both of which can greatly affect species detection by qPCR. Thus, we expected to improve qPCR-based amplification and species detection by diluting putative PCR inhibitors in the samples (with 0.5X eDNA volume) and by increasing the amount of target DNA in samples (with 3.3X), compared to using a single standard 1X eDNA volume. All samples were tested at 1X and 0.5X eDNA concentrations. Anticipating that the increased eDNA volume (3.3X) would likely be ineffective due to the eutrophic conditions at the sampling sites (e.g., it may also increase the concentration of putative PCR inhibitors), we limited testing at this concentration to ten randomly selected samples to assess if additional gains in species detection could still be obtained. This included 1–2 samples from each subdrainage, except for the Odeleite subdrainage, which had already yielded nearly all positive detections in the other assays. Real-time PCR reactions were prepared to a total reaction volume of 10 μL containing 5 μL of SsoAdvanced Univ SYBR Green Supermix, 0.5 μL of each primer (10 μM), and the tested eDNA template volume. All reactions were run on a CFX96 Real-Time System (Bio-Rad CA, USA) according to the following conditions: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 61°C for 30 s. Melting curves were generated by measuring fluorescence from 65°C to 95°C at each 0.5°C increment. Each sample was run in triplicate for all concentrations tested. Field and extraction blanks were run in triplicate at 1X. Each qPCR run included two positive controls (tissue-derived samples of the target species) and a PCR blank tested in triplicate. Tests were considered positive if both an exponential phase was observed and the peak of the melting curve matched those of positive controls. The target species was considered present at a site if at least 1 out of the 3 replicates yielded a positive result.

2.6 | Geographic Range Metrics

To exemplify the potential consequences arising from initial sample volume and the subsequent occurrence of false negatives in eDNA-based methods, we derived Criterion B of IUCN (IUCN 2022) for *A. hispanica*. The Criterion B focuses on the geographic range of species in the form of Extent of Occurrence (EOO) and Area of Occupancy (AOO) and was designed to identify populations with restricted distributions that are also severely fragmented or have few locations, experiencing a continuing decline and/or extreme fluctuations (IUCN 2022). The EOO is defined as the area contained within the shortest continuous imaginary boundary that encompasses the known, inferred or projected sites of occurrence of the species, excluding cases of vagrancy, and is designed to measure the degree to which risks are spread across the species geographic distribution; the AOO is defined as the area within the EOO which

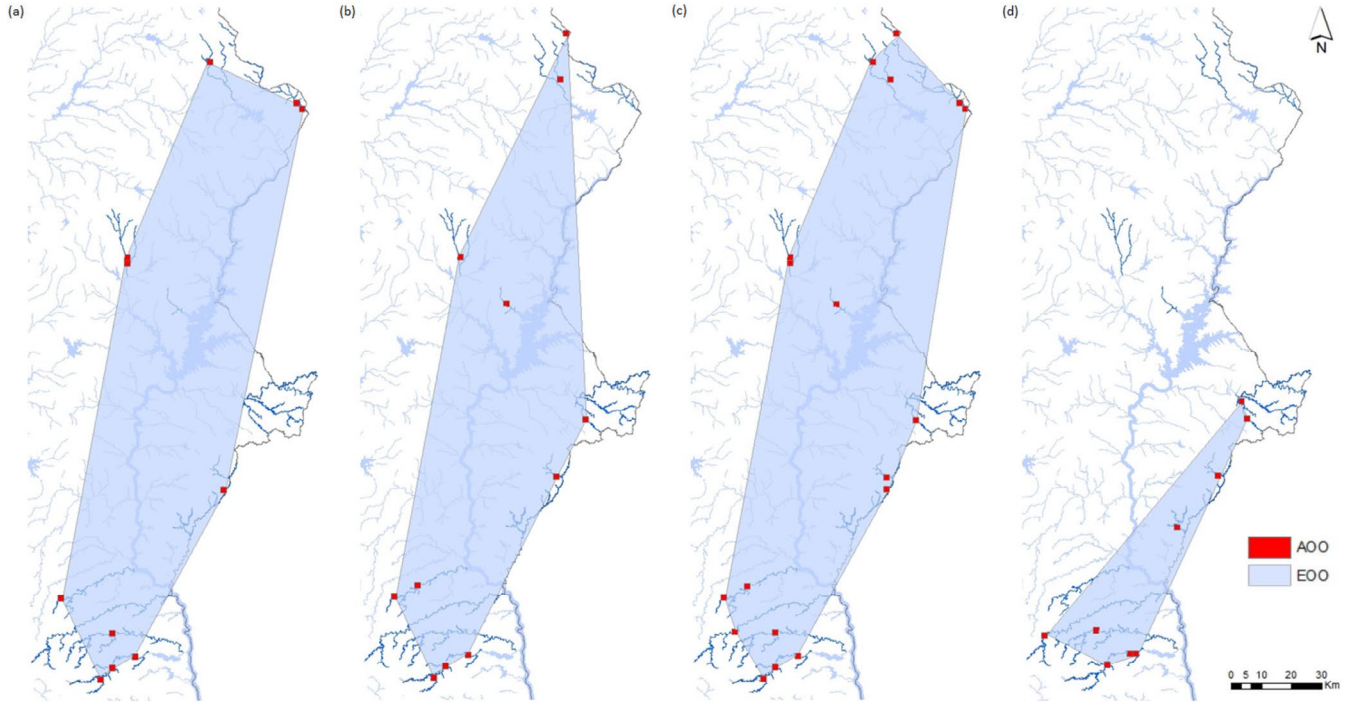

FIGURE 2 | Map of the Lower Guadiana Basin (Portugal) showing the sites with positive detections for *Anaecypris hispanica* using different survey methods, and representing the corresponding Extent of Occurrence (EOO) and Area of Occupancy (AOO) determined following IUCN (2021, 2022). Real-time PCR-based detections of environmental samples in 2022 using: A) 1X initial concentration; b) 0.5X initial concentration; c) pooled detections from all tested concentrations (1X, 0.5X and 3.3X); and d) electrofishing survey conducted in 2015 (Cardoso 2022).

is occupied by the species, excluding cases of vagrancy, and is designed to represent the area of suitable habitat currently occupied by the species (IUCN 2022).

We derived the EOO, AOO and number of locations (Locations) for *A. hispanica* following the current IUCN guidelines (IUCN 2022) for ease of interpretation and comparability. Specifically, the EOO was estimated from the area of the minimum convex polygon (i.e., the smallest polygon in which no internal angle exceeds 180°) which contains all sites with species detections; the AOO was estimated by the sum of the area of cells in a 2×2 km grid in which the species was detected; and locations were determined as the number of distinct geographic areas where the species was detected in which a single threatening event can affect all individuals present. All metrics were individually estimated based on species detections derived from qPCR results obtained under the 1X and 0.5X concentrations and the pooled results for all conditions (1X, 0.5X and 3.3X eDNA concentrations). Additional estimates were derived from species records from the last comprehensive electrofishing survey, conducted across 45 sites in the ten subdrainages where *A. hispanica* had been detected at the turn of the century (Cardoso 2022), to illustrate further how varying species detections can influence geographic range assessment.

3 | Results

The standard qPCR assay (1X eDNA concentration) detected *Anaecypris hispanica* in 11 of the 70 sites analysed, corresponding to approximately 16% of the sites sampled and encompassing seven of the ten subdrainages where the species has historically been recorded (Table 1; Appendix S2). All blanks showed no amplification. Using the 0.5X eDNA concentration, we detected *A. hispanica* in 12 sites from eight subdrainages, including seven samples which had no detections at the 1X eDNA concentration (Table 1). This result limited the spatial overlap between detected sites at 0.5X and 1X, resulting in some differences in the species' spatial distribution (Figure 2a,b) Specifically, the 1X concentration failed to detect the target species in two subdrainages (Ardila and Álamo), whereas the 0.5X concentration missed detection in one subdrainage (Foupana) (Table 1). Despite differences in positive detections, the assays were largely consistent, with results agreeing for 81.4% of the 70 samples tested, primarily due to shared negative detections. When pooling positive detections from the 0.5X and 1X concentrations, the overall percentage of sites in which *A. hispanica* was detected increased to 26%. In contrast, we obtained a single positive detection using the 3.3X eDNA concentration, albeit the only positive result in the Vascão subdrainage across all concentrations tested (Table 1). The pooled results of all assays provided a total of 19 sites (27%) with positive detections (Table 1) and appeared to converge into the historical distribution of the species (Figure 1). Performing only the standard qPCR assay (1X concentration) would have overlooked the detection of the highly endangered *A. hispanica* in eight sites and in three subdrainages, which corresponds to having a type II error in 11% of our samples. This is linked to the fact that around 74% of positive detections were specific to individual eDNA concentrations, underscoring that each assay contributed unique information.

The estimates of EOO, AOO and Locations resulting from using a single eDNA concentration for species detection were lower than those derived from the combined results with all eDNA volumes tested (Table 2). The impact of type II errors is made clear by the increases in all metrics between the 1X concentration and

TABLE 2 | Extent of Occurrence (EOO), Area of Occupancy (AOO), and number of Locations for *Anaecypris hispanica* estimated from detections obtained from real-time PCR detections (qPCR) of environmental samples under the standard conditions assay ("1X eDNA"), two-UIDfold dilution ("0.5X eDNA"), the pooled detections from all qPCR tested concentrations (1X, 0.5X and 3.3X eDNA, "eDNA-all"), and records from electrofishing carried out in 2015 (Cardoso 2022).

	EOO (km^2)	AOO (km^2)	Locations
1X eDNA	8871.6	44	7
0.5X eDNA	7626.7	44	8
eDNA-all	9432.8	72	10
Electrofishing	1803.2	36	5

the pooled results of all eDNA volumes tested. Specifically, EOO and AOO expanded by 6% and 64%, respectively, and Locations increased by 43% (Figure 2; Table 2). The estimates of EOO, AOO and Locations derived from detections obtained from the 1X and 0.5X eDNA-based assays were still higher than those using previous records from electrofishing surveys (Cardoso 2022).

4 | Discussion

Our study offers an empirical demonstration of the applicability of a straightforward approach to ameliorate qPCR inhibition in real-world conditions, suggesting a potential prevalence of false negatives in studies relying on a fixed eDNA concentration in qPCR-based species detections. Given the intrinsic variability in the concentration and types of PCR inhibitors among eDNA samples, as well as in the amount of target DNA, applying a standard, fixed volume of eDNA in a qPCR-based species detection assay will lead to variable success rates. Here, we show that by running qPCR assays under variable eDNA concentrations, we increased the number of sites with positive detections of the endangered *A. hispanica* by nearly three quarters compared to a standard PCR assay with a single eDNA concentration (1X). Notably, we detected our target species in the ten subdrainages where it historically occurred, including four where it had not been detected for nearly 20 years despite survey efforts using conventional methods (Cardoso 2022; Collares-Pereira et al. 2000; da Costa and Collares-Pereira 2003; SIBIC 2017). Instances such as these underscore eDNA's value in revitalising conservation efforts for threatened species, as seen in other cases where eDNA-based techniques have detected species previously thought to be locally extinct (Bonfil et al. 2021) or have strengthened monitoring programs by significantly reducing uncertainty about a species' presence after years of non-detection (Pfleger et al. 2016).

Thus far, research on the influence of PCR inhibitors and DNA concentration in single species detection has focused on experimentally controlled trials (Lance and Guan 2020; Mauvisseau et al. 2019; McKee et al. 2015). However, these experiments can overlook the complexity of environmental samples, where multispecies DNA exists in various cellular and molecular states and concentrations, continually interacting with biotic and abiotic factors, all of which might influence target species detectability (Jo and Minamoto 2021; Mauvisseau et al. 2022). The composition of bulk eDNA samples, particularly concerning the type and concentration of inhibitors, is typically unknown and likely highly variable among samples, as it is the case in the Guadiana basin, and would require significant resources for accurate estimation and mitigation. Several strategies to reduce false negatives in eDNA-based methods have been proposed, such as including more than one marker for the same target taxa (Brys et al. 2023), increasing the number of field replicates per site (Xia et al. 2018) or the number of qPCR replicates (Piggott 2016). However, these alternatives do not address the problem of inhibition directly. Inhibition in qPCR assays can be tested by including an internal positive PCR control (IPC) run with every sample (Goldberg et al. 2016). If there is not a positive signal from either the target species or IPC sequences, the reaction is deemed to have failed, and the sample is often discarded. However, for some inhibiting agents (e.g., off target exogenous DNA), the IPC results might not reflect the level of inhibition, leading to wasted resources and loss of information. The efficacy of conventional solutions like adding inhibition-counteracting compounds will depend on the class of inhibitor, and environmental samples may contain undetermined mixtures of inhibitors (Lance and Guan 2020; Schrader et al. 2012). Moreover, one of the most popular additives to counteract inhibition, bovine serum albumin, has been found to sometimes have the reverse effect (Albers et al. 2013). Further strategies, including the use of computational models, such as occupancy-detection models or process-based models, have been suggested to estimate false negative rates (Burian et al. 2021; Chen and Ficetola 2019; Strickland and Roberts 2019). However, the implementation and interpretation of these models require a solid understanding of the ecological context of the study, statistical and computational proficiency, as well as specialised software and computational resources. In light of our results, we argue that varying template bulk eDNA amounts in qPCR reactions may offer a more adequate approach to reduce type II errors, particularly because there may be high heterogeneity among eDNA samples.

Indeed, the number of positive detections for our target species would have been substantially reduced if we had not tested different template volumes, especially twofold dilutions (0.5X). The higher success of the dilution strategy over increasing the eDNA concentration in our experiment might be due to the eutrophic conditions of the Guadiana tributaries (Godinho et al. 2014, 2019), which potentiate the presence of PCR inhibitors. In such cases, the benefits of reducing inhibition through the dilution of eDNA samples in the qPCR reaction may outweigh the drawbacks of diluting the target DNA. This is a significant observation as it adds a nuance to the general assumption that eDNA is already present in such low copy numbers that additional dilution would be counterproductive. Yet, for oligotrophic waters (e.g., upstream reaches), where eDNA quantity and inhibitors are generally low, increasing the initial sample concentration in the qPCR assay may prove more useful (Kumar et al. 2021). Here, the single positive detection (out of the 10 samples tested) obtained with 3.3X eDNA pertained to one subdrainage—Vascão—where no other detection was made with 0.5X or 1X eDNA volumes. Thus, running samples with increased eDNA volume may still be a valuable strategy depending on the availability of time and resources. Based on our findings, we recommend that studies aiming at species detection using eDNA-based qPCR assays process all samples using different bulk eDNA volumes in the qPCR

step to enhance detection rates, especially in heterogeneous sampling networks with varying environmental conditions.

While eDNA-based methods are promising and fast-developing tools for species detection, their application warrants careful consideration along each step of the work to ensure both effective and robust results (Goldberg et al. 2016). Understanding the persistence of DNA in the environment is a critical subject for the interpretation of results (Barnes and Turner 2016). The dynamics that influence DNA persistence in lotic environments remain largely unknown but may be shaped by factors such as DNA composition and structure, and its complex interactions with biotic and abiotic factors (Jo and Minamoto 2021; Joseph et al. 2022; Shogren et al. 2017). Current research, however, supports that eDNA found in water from riverine systems mirrors species presence near real-time, whereas eDNA retrieved from sediments, particularly deeper layers, can persist for longer periods (Yao et al. 2022). Consequently, we conducted sampling in a manner that minimised sediment disturbance during water collection to reduce the risk of eDNA contamination from sediments. Furthermore, transport distances of eDNA have been found to correlate with stream discharge (Pont et al. 2018; van Driessche et al. 2023). Because we sampled small streams at low or no discharge, our sampling sites may have been close to the eDNA source. Nevertheless, if we considered the widest transport distance in the order of a few kilometres (Pont et al. 2018; van Driessche et al. 2023), the source of the eDNA would still fall within the subpopulation nuclei radius; hence, our conclusions would remain valid. Because high turbidity was observed in several sampling sites in our study, the inclusion of proxy measurements for microbial density might have benefited the analytical precision of our results.

Despite their limitations, eDNA-based methods have the potential to improve species detection and clarify their geographic range, which are basic yet key elements in species assessment and effective and targeted conservation actions. Such information is of utmost importance not only in the context of endangered species but also in the early detection of invasive species. The integration of these innovative survey technologies into decision-making and management responses is a current subject of discussion (Schenekar 2023; Sepulveda et al. 2020b, 2023; Stein et al. 2023). Here, we demonstrated the importance of tailoring laboratory protocols for testing eDNA samples to the specific ecological context of the study. We also emphasise the need for careful consideration when adopting molecular data in biodiversity monitoring and assessment, and for standardising methods and protocols to assure consistency and comparability. It is also key to contextualise these findings with data obtained through established survey methods or direct observation for accurate result interpretation. Given the higher sensitivity of eDNA-based methods for detecting organisms compared to traditional survey approaches (Fediajevaite et al. 2021), species records and population trends derived from these different methods should not be directly compared, nor used for the estimation of species extinction risk based on current IUCN's criteria and thresholds. Nevertheless, eDNA-based species detection should be implemented complementarily to traditional survey methods to help direct traditional monitoring efforts and improve biodiversity assessments. Specifically for *A. hispanica*, traditional electrofishing surveys should be continued or reinstated in the subdrainages where our eDNA-based assay produced positive detections of this species. In the future, as eDNA-based methods continue to be at the forefront of aquatic conservation advancements, further efforts need to be placed into methodological refinements, standardisation of best practices, and development of new metrics, which will ensure the robustness of results and conservation actions thereafter.

Author Contributions

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Primer sequences are available within the paper and procedures for primer development are available in Appendix S1. Field metadata and results for the qPCR assay for each sample are provided in Appendix S2 available online for this article.

References

Albers, C. N., A. Jensen, J. Bælum, and C. S. Jacobsen. 2013. "Inhibition of DNA Polymerases Used in Q-PCR by Structurally Different Soil-Derived Humic Substances." *Geomicrobiology Journal* 30, no. 8: 675–681.

Albert, J. S., G. Destouni, S. M. Duke-Sylvester, et al. 2021. "Scientists' Warning to Humanity on the Freshwater Biodiversity Crisis." *Ambio* 50, no. 1: 85–94.

Amorim, M. C. P., J. A. Wanjala, M. Vieira, et al. 2023. "Detection of Invasive Fish Species With Passive Acoustics: Discriminating Between Native and Non-indigenous Sciaenids." *Marine Environmental Research* 188: 106017.

Barnes, M. A., and C. R. Turner. 2016. "The Ecology of Environmental DNA and Implications for Conservation Genetics." *Conservation Genetics* 17, no. 1: 1–17.

Bennett, N. J., R. Roth, S. C. Klain, et al. 2017. "Conservation Social Science: Understanding and Integrating Human Dimensions to Improve Conservation." *Biological Conservation* 205: 93–108.

Bennun, L., E. C. Regan, J. Bird, et al. 2018. "The Value of the IUCN Red List for Business Decision-Making." *Conservation Letters* 11, no. 1: e12353.

Betts, J., R. P. Young, C. Hilton-Taylor, et al. 2020. "A Framework for Evaluating the Impact of the IUCN Red List of Threatened Species." *Conservation Biology* 34, no. 3: 632–643.

Bolgan, M., E. Parmentier, M. Picciulin, L. Hadjioannou, and L. Di Iorio. 2023. "Use of Passive Acoustic Monitoring to Fill Knowledge Gaps of Fish Global Conservation Status." *Aquatic Conservation: Marine and Freshwater Ecosystems* 33, no. 12: 1580–1589.

Bonfil, R., P. Palacios-Barreto, O. U. M. Vargas, M. Ricaño-Soriano, and P. Díaz-Jaimes. 2021. "Detection of Critically Endangered Marine Species With Dwindling Populations in the Wild Using eDNA Gives Hope for Sawfishes." *Marine Biology* 168, no. 5: 60.

Brys, R., D. Halfmaerten, T. Everts, C. Van Driessche, and S. Neyrinck. 2023. "Combining Multiple Markers Significantly Increases the Sensitivity and Precision of eDNA-Based Single-Species Analyses." *Environmental DNA* 5, no. 5: 1065–1077.

Bunholi, I. V., N. R. Foster, and J. M. Casey. 2023. "Environmental DNA and RNA in Aquatic Community Ecology: Toward Methodological Standardization." *Environmental DNA* 5, no. 6: 1133–1147.

Burian, A., Q. Mauvisseau, M. Bulling, S. Domisch, S. Qian, and M. Sweet. 2021. "Improving the Reliability of eDNA Data Interpretation." *Molecular Ecology Resources* 21, no. 5: 1422–1433.

Cardoso, A. C. 2022. "Update on *Anaecypris Hispanica* Distribution and Its Conservation Status in Portugal." *Fishes in Mediterranean Environments*: 1–12.

Cazalis, V., M. Di Marco, S. H. M. Butchart, et al. 2022. "Bridging the Research-Implementation Gap in IUCN Red List Assessments." *Trends in Ecology & Evolution* 37, no. 4: 359–370.

Ceballos, G., P. R. Ehrlich, and R. Dirzo. 2017. "Biological Annihilation via the Ongoing Sixth Mass Extinction Signaled by Vertebrate Population Losses and Declines." *Proceedings of the National Academy of Sciences* 114, no. 30: E6089–E6096.

Chen, W., and G. F. Ficetola. 2019. "Conditionally Autoregressive Models Improve Occupancy Analyses of Autocorrelated Data: An Example With Environmental DNA." *Molecular Ecology Resources* 19, no. 1: 163–175.

Collares-Pereira, M. J., M. J. Alves, F. Ribeiro, et al. 2021. *Guia dos peixes de água doce e migradores de Portugal continental*. Edições Afrontamento.

Collares-Pereira, M. J., I. G. Cowx, J. A. Rodrigues, L. Rogado, and L. M. da Costa. 1999. "The Status of *Anaecypris hispanica* in Portugal: Problems of Conserving a Highly Endangered Iberian Fish." *Biological Conservation* 88, no. 2: 207–212.

Collares-Pereira, M. J., I. G. Cowx, J. A. Rodrigues, et al. 2000. "Uma estratégia de conservação para o saramugo (*Anaecypris hispanica*), um endemismo piscícola em extinção. Programa LifeNatureza – contrato B4–3200/97/280, CBA-FCUL, Lisboa."

Collen, B., N. K. Dulvy, K. J. Gaston, et al. 2016. "Clarifying Misconceptions of Extinction Risk Assessment With the IUCN Red List." *Biology Letters* 12, no. 4: 20150843.

da Costa, L. M., and M. J. Collares-Pereira. 2003. "Programa de Monitorização para o Património Natural - Monitorização de Peixes Dulciaquícolas - Área de Regolfo de Alqueva e Pedrogão. Relatório Final, CBA-FCUL, Lisboa, 161 pp. + Anexos."

Darling, J. A., C. L. Jerde, and A. J. Sepulveda. 2021. "What Do You Mean by False Positive?" *Environmental DNA* 3, no. 5: 879–883.

De Miguel, R., E. Pino, A. Ramiro, et al. 2010. "On the Occurrence of *Anaecypris Hispanica*, an Extremely Endangered Iberian Endemism, in the Guadalquivir River Basin." *Journal of Fish Biology* 76, no. 6: 1454–1465.

Deiner, K., H. M. Bik, E. Mächler, et al. 2017. "Environmental DNA Metabarcoding: Transforming How We Survey Animal and Plant Communities." *Molecular Ecology* 26, no. 21: 5872–5895.

Demeke, T., and G. R. Jenkins. 2010. "Influence of DNA Extraction Methods, PCR Inhibitors and Quantification Methods on Real-Time PCR Assay of Biotechnology-Derived Traits." *Analytical and Bioanalytical Chemistry* 396: 1977–1990.

Duarte, S., L. Simões, and F. O. Costa. 2023. "Current Status and Topical Issues on the Use of eDNA-Based Targeted Detection of Rare Animal Species." *Science of the Total Environment* 904: 166675.

Fediajevaite, J., V. Priestley, R. Arnold, and V. Savolainen. 2021. "Meta-Analysis Shows That Environmental DNA Outperforms Traditional Surveys, but Warrants Better Reporting Standards." *Ecology and Evolution* 11, no. 9: 4803–4815.

Ficetola, G. F., J. Pansu, A. Bonin, et al. 2015. "Replication Levels, False Presences and the Estimation of the Presence/Absence From eDNA Metabarcoding Data." *Molecular Ecology Resources* 15, no. 3: 543–556.

Ficetola, G. F., P. Taberlet, and E. Coissac. 2016. "How to Limit False Positives in Environmental DNA and Metabarcoding?" *Molecular Ecology Resources* 16, no. 3: 604–607.

Furlan, E. M., D. Gleeson, C. M. Hardy, and R. P. Duncan. 2016. "A Framework for Estimating the Sensitivity of eDNA Surveys." *Molecular Ecology Resources* 16, no. 3: 641–654.

Gehri, R. R., W. A. Larson, K. Gruenthal, N. M. Sard, and Y. Shi. 2021. "eDNA Metabarcoding Outperforms Traditional Fisheries Sampling and Reveals Fine-Scale Heterogeneity in a Temperate Freshwater Lake." *Environmental DNA* 3, no. 5: 912–929.

Godinho, F. N., P. J. Pinheiro, J. M. Oliveira, and R. Azedo. 2014. "Responses of Intermittent Stream Fish Assemblages to Irrigation Development." *River Research and Applications* 30, no. 10: 1248–1256.

Godinho, F. N., P. Segurado, A. Franco, et al. 2019. "Factors Related to Fish Kill Events in Mediterranean Reservoirs." *Water Research* 158: 280–290.

Goldberg, C. S., C. R. Turner, K. Deiner, et al. 2016. "Critical Considerations for the Application of Environmental DNA Methods to Detect Aquatic Species." *Methods in Ecology and Evolution* 7, no. 11: 1299–1307.

Herbert, S., P. Bradley, and M. Everard. 2023. "Exploring the Prioritisation of Biodiversity Amongst Small-To Medium-Sized Enterprise Leaders With Strong Bigger-Than-Self Value Orientation." *Business Strategy and the Environment* 32, no. 8: 5121–6210.

Hutchins, P. R., L. N. Simantel, and A. J. Sepulveda. 2022. "Time to Get Real With qPCR Controls: The Frequency of Sample Contamination and the Informative Power of Negative Controls in Environmental DNA Studies." *Molecular Ecology Resources* 22, no. 4: 1319–1329.

Imaizumi, K., K. Noguchi, T. Shiraishi, et al. 2005. "DNA Typing of Bone Specimens - The Potential Use of the Profiler Test as a Tool for Bone Identification." *Legal Medicine* 7, no. 1: 31–41.

IUCN Standards and Petitions Committee. 2022. "Guidelines for Using the IUCN Red List Categories and Criteria. Version 15.1. Prepared by the Standards and Petitions Committee." https://www.iucnredlist.org/documents/RedListGuidelines.pdf.

Jo, T., and T. Minamoto. 2021. "Complex Interactions Between Environmental DNA (eDNA) State and Water Chemistries on eDNA Persistence Suggested by Meta-Analyses." *Molecular Ecology Resources* 21, no. 5: 1490–1503.

Joseph, C., M. E. Faiq, Z. Li, and G. Chen. 2022. "Persistence and Degradation Dynamics of eDNA Affected by Environmental Factors in Aquatic Ecosystems." *Hydrobiologia* 849, no. 19: 4119–4133.

Keck, F., R. C. Blackman, R. Bossart, et al. 2022. "Meta-Analysis Shows Both Congruence and Complementarity of DNA and eDNA Metabarcoding to Traditional Methods for Biological Community Assessment." *Molecular Ecology* 31, no. 6: 1820–1835.

Koziol, A., M. Stat, T. Simpson, et al. 2019. "Environmental DNA Metabarcoding Studies Are Critically Affected by Substrate Selection." *Molecular Ecology Resources* 19, no. 2: 366–376.

Kumar, G., E. Farrell, A. M. Reaume, J. A. Eble, and M. R. Gaither. 2021. "One Size Does Not Fit all: Tuning eDNA Protocols for High-and Low-Turbidity Water Sampling." *Environmental DNA* 4, no. 1: 167–180.

Lance, R. F., and X. Guan. 2020. "Variation in Inhibitor Effects on qPCR Assays and Implications for eDNA Surveys." *Canadian Journal of Fisheries and Aquatic Sciences* 77, no. 1: 23–33.

Maasri, A., S. C. Jähnig, M. C. Adamescu, et al. 2022. "A Global Agenda for Advancing Freshwater Biodiversity Research." *Ecology Letters* 25, no. 2: 255–263.

Magalhães, M. F., S. D. Amaral, M. Sousa, et al. 2023. *Livro Vermelho dos Peixes Dulciaquícolas e Diádromos de Portugal Continental*. FCiências. ID & ICNF, I.P.

Mauvisseau, Q., J. Davy-Bowker, M. Bulling, et al. 2019. "Combining ddPCR and Environmental DNA to Improve Detection Capabilities of a Critically Endangered Freshwater Invertebrate." *Scientific Reports* 9: 14064.

Mauvisseau, Q., L. R. Harper, M. Sander, R. H. Hanner, H. Kleyer, and K. Deiner. 2022. "The Multiple States of Environmental DNA and What Is Known About Their Persistence in Aquatic Environments." *Environmental Science & Technology* 56, no. 9: 5322–5333.

McKee, A. M., S. F. Spear, and T. W. Pierson. 2015. "The Effect of Dilution and the Use of a Post-Extraction Nucleic Acid Purification Column on the Accuracy, Precision, and Inhibition of Environmental DNA Samples." *Biological Conservation* 183: 70–76.

Pfleger, M. O., S. J. Rider, C. E. Johnston, and A. M. Janosik. 2016. "Saving the Doomed: Using eDNA to Aid in Detection of Rare Sturgeon for Conservation (Acipenseridae)." *Global Ecology and Conservation* 8: 99–107.

Piggott, M. P. 2016. "Evaluating the Effects of Laboratory Protocols on eDNA Detection Probability for an Endangered Freshwater Fish." *Ecology and Evolution* 6, no. 9: 2739–2750.

Pim, S. L., C. N. Jenkins, R. Abell, et al. 2014. "The Biodiversity of Species and Their Rates of Extinction, Distribution, and Protection." *Science* 344, no. 6187: 1246752.

Pont, D., M. Rocle, A. Valentini, et al. 2018. "Environmental DNA Reveals Quantitative Patterns of Fish Biodiversity in Large Rivers Despite Its Downstream Transportation." *Scientific Reports* 8: 10361.

Ribeiro, F., I. G. Cowx, and M. J. Collares-Pereira. 2000. "Life History Traits of the Endangered Iberian Cyprinid *Anaecypris Hispanica* and Their Implications for Conservation." *Archiv für Hydrobiologie* 149, no. 4: 569–586.

Ribeiro, F., M. F. Magalhães, and M. J. Collares-Pereira. 2013. "Spatial and Temporal Variation in Assemblage Structure of Fish Larvae in Mediterranean–Type Streams: Contrasts Between Native and Non-native Species." *Environmental Biology of Fishes* 96: 467–480.

Schenekar, T. 2023. "The Current State of eDNA Research in Freshwater Ecosystems: Are We Shifting From the Developmental Phase to Standard Application in Biomonitoring?" *Hydrobiologia* 850, no. 6: 1263–1282.

Schrader, C., A. Schielke, L. Ellerbroek, and R. Johne. 2012. "PCR Inhibitors - Occurrence, Properties and Removal." *Journal of Applied Microbiology* 113, no. 5: 1014–1026.

Scipioni, A., A. Mauroy, D. Ziant, C. Saegerman, and E. Thiry. 2008. "A SYBR Green RT-PCR Assay in Single Tube to Detect Human and Bovine Noroviruses and Control for Inhibition." *Virology Journal* 5: 1–8.

Sellers, G. S., C. Di Muri, A. Gómez, and B. Hänfling. 2018. "Mu-DNA: A Modular Universal DNA Extraction Method Adaptable for a Wide Range of Sample Types." *Metabarcoding and Metagenomics* 2: e24556.

Sepulveda, A. J., C. E. Dumoulin, D. L. Blanchette, et al. 2023. "When Are Environmental DNA Early Detections of Invasive Species Actionable?" *Journal of Environmental Management* 343: 118216.

Sepulveda, A. J., P. R. Hutchins, M. Forstchen, M. N. Mckeefry, and A. M. Swigris. 2020a. "The Elephant in the Lab (And Field): Contamination in Aquatic Environmental DNA Studies." *Frontiers in Ecology and Evolution* 8: 609973.

Sepulveda, A. J., N. M. Nelson, C. L. Jerde, and G. Luikart. 2020b. "Are Environmental DNA Methods Ready for Aquatic Invasive Species Management?" *Trends in Ecology & Evolution* 35, no. 8: 668–678.

Seymour, M., F. K. Edwards, B. J. Cosby, et al. 2021. "Environmental DNA Provides Higher Resolution Assessment of Riverine Biodiversity and Ecosystem Function via Spatio-Temporal Nestedness and Turnover Partitioning." *Communications Biology* 4, no. 1: 512.

Shea, M. M., J. Kuppermann, M. P. Rogers, D. S. Smith, P. Edwards, and A. B. Boehm. 2023. "Systematic Review of Marine Environmental DNA Metabarcoding Studies: Toward Best Practices for Data Usability and Accessibility." *PeerJ* 11: e14993.

Shogren, A. J., J. L. Tank, E. Andruszkiewicz, et al. 2017. "Controls on eDNA Movement in Streams: Transport, Retention, and Resuspension." *Scientific Reports* 7, no. 1: 5065.

SIBIC. 2017. "Carta Piscícola Española." Publicación electrónica (versión 02/2017).

Sidstedt, M., P. Rådström, and J. Hedman. 2020. "PCR Inhibition in qPCR, dPCR and MPS-Mechanisms and Solutions." *Analytical and Bioanalytical Chemistry* 412, no. 9: 2009–2023.

Song, J. W., M. T. Schultz, E. A. Casman, et al. 2020. "A Probabilistic Model for Designing and Assessing the Performance of eDNA Sampling Protocols." *Molecular Ecology Resources* 20, no. 2: 404–414.

Stein, E. D., C. L. Jerde, E. A. Allan, et al. 2023. "Critical Considerations for Communicating Environmental DNA Science." *Environmental DNA* 6: e472.

Stephenson, P. J., and G. Carbone. 2021. *Guidelines for Planning and Monitoring Corporate Biodiversity Performance*. IUCN.

Strickland, G. J., and J. H. Roberts. 2019. "Utility of eDNA and Occupancy Models for Monitoring an Endangered Fish Across Diverse Riverine Habitats." *Hydrobiologia* 826, no. 1: 129–144.

Tosa, M. I., E. H. Dziedzic, C. L. Appel, et al. 2021. "The Rapid Rise of Next-Generation Natural History." *Frontiers in Ecology and Evolution* 9: 698131.

van Driessche, C., T. Everts, S. Neyrinck, and R. Brys. 2023. "Experimental Assessment of Downstream Environmental DNA Patterns Under Variable Fish Biomass and River Discharge Rates." *Environmental DNA* 5, no. 1: 102–116.

Woinarski, J. C., S. T. Garnett, S. M. Legge, and D. B. Lindenmayer. 2017. "The Contribution of Policy, Law, Management, Research, and Advocacy Failings to the Recent Extinctions of Three Australian Vertebrate Species." *Conservation Biology* 31, no. 1: 13–23.

Xia, Z., M. L. Johansson, Y. Gao, et al. 2018. "Conventional Versus Real-Time Quantitative PCR for Rare Species Detection." *Ecology and Evolution* 8, no. 23: 11799–11807.

Yao, M., S. Zhang, Q. Lu, et al. 2022. "Fishing for Fish Environmental DNA: Ecological Applications, Methodological Considerations, Surveying Designs, and Ways Forward." *Molecular Ecology* 31, no. 20: 5132–5164.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.