RESEARCH ARTICLE

Fine-tuning biodiversity assessments: A framework to pair eDNA metabarcoding and morphological approaches

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Abstract

1. Accurate quantification of biodiversity can be demanding and expensive. Although environmental DNA (eDNA) metabarcoding can facilitate biodiversity assessments through non-invasive, cost-efficient and rapid surveys, the approach struggles to outperform traditional morphological approaches in providing reliable quantitative estimates for surveyed species (e.g. abundance and biomass).

2. We present an integrated methodology for improving biodiversity surveys that pairs eDNA metabarcoding with morphological data, following a series of taxonomic and geographical filters. We demonstrate its power by applying it to a new spatiotemporal dataset generated on an Iberian-wide distributed aquatic mesocosm infrastructure that spans a wide biogeographical gradient.

3. By building upon the strengths that these two approaches offer, our framework improved taxonomic resolution for 30% of the taxa and enabled species' traits (e.g. body size) and abundance to be assigned to 85% of the taxa in hybrid datasets.

4. These results indicate that eDNA-based assessments can complement, but not always replace, conventional approaches. Integrating conventional and modern eDNA metabarcoding approaches, already available in the ecologist's toolbox, will greatly enhance biodiversity assessments.

KEYWORDS
biodiversity, environmental DNA, metabarcoding, morphological approaches

1 | INTRODUCTION

Quantifying species composition and richness is a fundamental step for assessing the status of local and regional biodiversity (Matias et al., 2017). However, required data for such quantifications are often unavailable at the relevant temporal, spatial and taxonomic resolutions (Altermatt et al., 2020). Complexities associated with varying detection probabilities and (mis)identifications further plague assessments, particularly when addressing finer levels of taxonomic resolution (McGill et al., 2015). Morphological identification to the level of species or genus can be difficult (Hajibabaei et al., 2011), especially for invertebrates requiring adult stage observations for correct identification to species level. This process generally necessitates specialized taxonomic expertise, which is scarce and time-consuming. For these reasons, biodiversity assessments often recur to classifications at coarse taxonomic levels (Elbrecht et al., 2017). Spatial differences in β-diversity can be detected using taxonomic classifications at genera or family level (Terlizzi et al., 2009), but

species within higher taxonomic groups may exhibit responses to stressors (Macher et al., 2016) that go unnoticed in studies with low taxonomic resolution.

Recently, molecular approaches based on environmental DNA (eDNA) metabarcoding have made it possible to generate large amounts of data with non-invasive, cost-efficient, rapid surveys (Deiner et al., 2017; Taberlet et al., 2012, 2018). In addition, metabarcoding could boost biodiversity data acquisition to unprecedented levels by making such efforts less reliant on increasingly scarce taxonomic expertise (Elbrecht et al., 2017; Taberlet et al., 2012). Nevertheless, eDNA metabarcoding is not without drawbacks. Among shortcomings are the sensitivity of results to differences in the DNA extraction method and marker choices, its dependency on often-incomplete reference databases, and the difficulties in providing quantitative estimates of abundance or biomass for the surveyed species (Deiner et al., 2017; Ruppert et al., 2019). Several studies have emphasized that biodiversity assessments conducted with eDNA metabarcoding may not always replace morphological approaches (Altermatt et al., 2020; Bush et al., 2019; Seymour et al., 2020) and proposed that eDNA-based biodiversity assessments should complement morphological approaches (e.g. Groendahl et al., 2017; Seymour et al., 2020). However, to our knowledge, there is no standardized method to fully combine the species lists from both approaches.

We present a framework to pair datasets from eDNA metabarcoding (hereafter eDNA) and morphological (hereafter morphology) approaches to improve the resolution of biodiversity assessments. The framework is based on three increasingly restrictive workflows (Figure 1), which can be applied independently of each other, according to the ecological question at hand. (a) Additive workflow, for identification of rare species, detection of endangered or invasive species, or for studies in areas less explored or remote locations. Here, taxonomic assignment match between eDNA and morphology is assessed, and the remaining taxa identified by each approach are added to create a hybrid dataset (i.e. containing taxa from the two approaches). (b)

Taxonomic Correction workflow, for use in monitoring programmes, where after performing a taxonomic match between eDNA and morphology, an assessment of taxonomic identification match at genus level is done, and the taxonomic assignment is corrected using the lowest level of identification (regardless of dataset has the lowest level of identification). And (c) Geographical Fit workflow, for studies where quantification of species' abundances and biomasses are needed (e.g. food webs). Here, following the previous steps, the taxa retained from eDNA are checked for their geographical fit to the studied regions through automated data-mining and targeted literature (e.g. check if taxa have been previously found in the same regions, biomes).

To demonstrate the power of the proposed framework, we used a novel dataset obtained through approximately 300-hybrid biodiversity aquatic surveys (simultaneous eDNA and morphology assessments) conducted across the Iberian Pond Network, a multi-region experimental facility of freshwater pond mesocosms (http://www.iberianponds.uevora.pt). Within it we surveyed freshwater communities of phytoplankton, zooplankton and macroinvertebrates over time and across a biogeographical gradient. The proposed framework seeks to improve the resolution of biodiversity assessments through improved species detectability, taxonomic resolution and community structure, by assigning abundance and biomass to as many species as possible. This will allow eDNA to be used not only in qualitative biodiversity assessments but also in quantitative descriptions of community structure.

2 | MATERIALS AND METHODS

2.1 | Iberian Ponds Network

The Iberian Ponds Network is a multi-region experimental facility using 192 freshwater pond mesocosms (1,000 L plastic tanks, Prebac
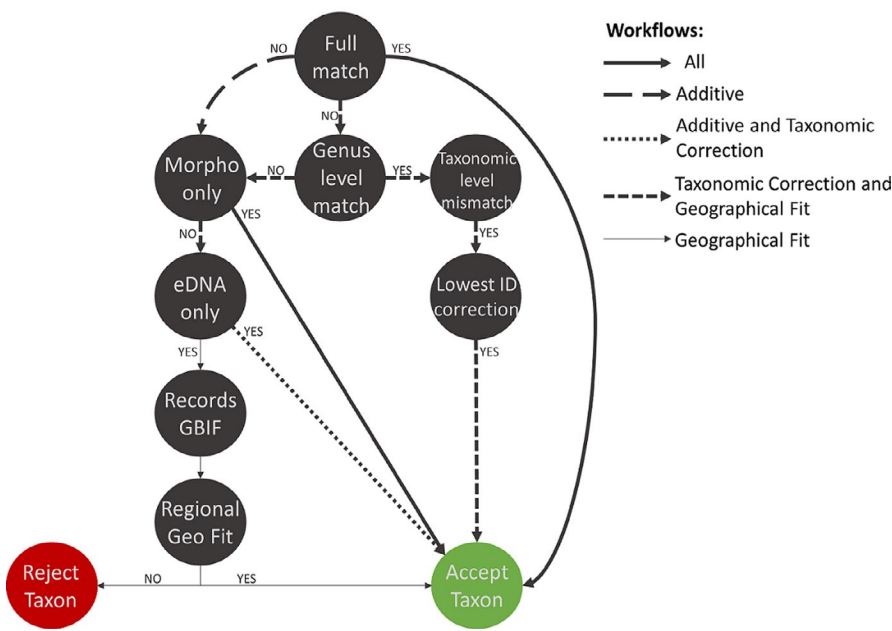
FIGURE 1 Framework to pair eDNA metabarcoding and morphology datasets. This framework comprises three different workflows: Additive, Taxonomic Correction and Geographical Fit

1000, La Buvette, France; hereafter ponds) distributed across six locations in the Iberian Peninsula, ranging from southern semi-arid (Murcia, Toledo), temperate (Évora, Porto) and alpine environments (Jaca and Madrid; Figure S1), varying in annual average temperature and total precipitation (more details on the climate of each region in Supporting Information). These ponds mimic natural ponds, having been originally inoculated and colonized by all sorts of organisms from bacteria to small vertebrates. In the southern sites, the ponds behave as temporary ponds, annually alternating phases of flood and drought. Since the dry season can have a duration of 3–8 months, usually during summer and autumn, fieldwork was carried out in the six locations every Spring from 2016 to 2018. Considering the climatic and environmental conditions of each location, fieldwork was done from the south (April) to the north (June) of Iberia to ensure sampling during the ponds' wet phase. Each pond was sampled once a year.

2.2 | eDNA metabarcoding

Water samples for eDNA analysis (500 ml) were filtered through enclosed 0.22 μm Sterivex unit filters (EMD Millipore Corporation) using a Waterra Easy Load II peristaltic pump (In-Situ Europe Ltd). All extractions were done with a modified protocol using the QIAGEN DNA Blood and Tissue Kit (Spens et al., 2017). Field and extraction blanks were added, to control possible contaminations during field handling, transport and DNA extractions. DNA was PCR amplified in duplicates from the extracts using two primer sets: (a) primers 1380F and 1510R (Amaral-Zettler et al., 2009) to amplify the V9 region of eukaryotic 18S rRNA gene targeting phytoplankton and (b) primers mlCOIintF and jgHCO2198 (Geller et al., 2013; Leray et al., 2013) were used to target the mitochondrial Cytochrome c Oxidase I (COI) gene for metabarcoding metazoan diversity (zooplankton and macroinvertebrates). PCR negative controls (molecular grade water) and PCR positive controls (DNA from species not present in the study system) were also added. PCR products from each primer set were subsequently combined into different amplicon pools, which were converted into Illumina sequencing libraries following the Blunt-End-Single-Tube (BEST) protocol (Carøe et al., 2018). Indexed amplicon libraries were sequenced using 250 bp paired-end on an Illumina MiSeq platform at the National High-throughput Sequencing Centre, Copenhagen, Denmark. Illumina sequences were then analysed using DADA2 (Callahan et al., 2016). Taxonomic assignment was performed using BLASTn and the NCBI nt database (Benson et al., 2005) at 97% similarity, and classification was attributed using the software MEGAN Community Edition (Huson et al., 2016). Taxonomic assignments and their associated amplicon sequence variants (ASVs) that returned incomplete taxonomy or unknown identifiers were excluded from further analysis. An additive strategy was used regarding the number of PCR replicates (Alberdi et al., 2017), that is, through combining the sequences of all PCR replicates from one sample to maximize diversity detection (e.g. Leray & Knowlton, 2015). Further details are shown in Supporting Information.

2.3 | Morphology

Three different trophic groups were surveyed using standard sampling procedures: phytoplankton, zooplankton and macroinvertebrates. For phytoplankton and zooplankton identification and enumeration, samples were concentrated in 100 ml by filtering 3 and 5 litres, respectively, of water through plankton nets with mesh size of 20 μm for phytoplankton and 53 μm for zooplankton. Macroinvertebrates were sampled using a 50 L core, which represented 5% of the total volume of the ponds, and a net with mesh size of 500 μm, and samples were preserved in 96% ethanol. Morphological identification was done to the lowest taxonomic level possible for all three trophic groups. Individuals' enumeration was done using a light microscope (phytoplankton and rotifers) and a stereo microscope (cladocerans, copepods and macroinvertebrates) using standard methods for each trophic group. Species' biomasses were calculated by their dry weights, which was estimated from published allometric relationships using individual measurements.

2.4 | Workflows to pair eDNA and morphology

All workflows require (a) an eDNA taxa*site matrix and (b) a corresponding morphological taxa*site matrix, both with unique taxa names (as rows), with the respective taxonomy (from Domain to Species) followed by their abundance (or number of reads for eDNA) across the studied samples (as columns). The first step to pair eDNA and morphology datasets is common to all three workflows (Figure 1) and consists of checking the taxonomic assignment match between morphology and eDNA, that is, check taxa shared by the morphology and eDNA, and abundances obtained through morphology are assigned to these taxa. Then the three workflows are implemented to combine both datasets with no restrictions (Additive), with taxonomic filters (Taxonomic Correction) and with both taxonomic and geographical filters (Geographical Fit; Figure 1). (a) Additive workflow: After the first step, the remaining taxa identified by each approach are accepted. Abundances estimated through morphological methods are retained, and in any sample where a taxon is identified with eDNA but not with morphology, the number of reads for that taxon is replaced by 1 to keep the information of its presence. (b) Taxonomic Correction workflow: Mismatched taxa after the first step are checked to select genera shared by both approaches. If there is a different level of identification between approaches within the same genus, the identification is corrected using the lowest level of identification, independently of which dataset has the lowest level of identification. When matching taxa from a genus identified with morphology (e.g. *Chironomus* sp.) and a species of the same genus identified by eDNA (e.g. *C. aprilinus* and *C. riparius*), it becomes necessary to assign the abundances from morphology to one or more species. To automate this procedure, we implemented the R function *redistributeAbundances* (Supporting Information section 1.4 and Figure S2), whose main purpose is to assign abundances from the genus identified with morphology proportionately using the relative

frequencies of the number of reads from each species. There are three possible routines to assign abundances depending on the number of taxa matched: if there is one single taxon of the genus; if there is one single taxon of the genus identified with eDNA and multiple taxa identified with morphology; and, if there are multiple taxa of the genus identified with eDNA. The function checks these numbers of taxa from the same genus and acts accordingly. If the taxon with the lowest level of identification is from morphology, it is accepted as well as its abundance. However, if the taxon with the lowest level of identification is identified with eDNA, its taxonomy is accepted, and the number of reads is transformed into relative frequencies. Then, the abundances from the genus identified with morphology are redistributed by the eDNA species proportionately by its relative frequencies using the *redistributeAbundances* function. The remaining taxa identified by each approach are accepted, keeping abundances for morphologically identified taxa, and 1 to eDNA identified taxa. And finally, (c) Geographical Fit workflow: After the first step (i.e. 'full match'), a match at genus level is also performed, followed by taxonomic correction using the lowest level of identification. Taxa abundances are treated in the same way as in the Taxonomic Correction workflow. Remaining mismatched taxa from the morphology are accepted, retaining its abundances. The geographical fit of the remaining taxa from eDNA is assessed by (3.1) checking if each taxa occurs more than once in ponds from the same region; (3.2) checking if these taxa have occurrences registered for the Iberian Peninsula in the Global Biodiversity Information Facility (GBIF) using the custom function *taxaOccurrencesIP*, which uses functions available in the ʀɢʙɪꜰ package (Chamberlain et al., 2020); and (3.3) checking regional geographical fit with species lists in available literature. To avoid false positives, taxa that have only one occurrence, and those that do not meet one of the other two conditions, have no records for the Iberian Peninsula on GBIF or are not present in species lists from literature, are rejected. The remaining taxa are accepted, and their corresponding number of reads is replaced by 1, to retain these taxa presence. Geographical fit of taxa identified using morphology is not assessed computationally because this step is part of the morphological identification protocol, where taxa are checked against regional classification keys and records in regional species lists.

2.5 | Data analysis

All analyses were performed using R version 3.6.3 (R Core Team, 2020). From a pool of 576 samples available from eDNA and morphology, a random selection was done using the function *sample* from base R to ensure balance across regions and years (18 samples per region per year). The resulting subset consisted of a total of 324 samples matching eDNA and morphology. Separate analyses were performed with either the (a) full dataset with the 324 samples, with all six regions analysed together or (b) six independent regional datasets, with 54 samples each.

To assess the performance of eDNA and of the three hybrid datasets in comparison to using morphology, or eDNA, alone regarding taxa detection, a log-ratio was applied to taxa richness (TR) of each dataset using the formulas $\log_{10}(TR_{dataset}/TR_{Morphology})$ and $\log_{10}(TR_{dataset}/TR_{eDNA})$, accordingly. A positive result indicates that the dataset used outperforms morphology, a negative result indicates that morphology outperforms the dataset used, and a result equal to zero shows an equal performance between morphology and the dataset being analysed. Similar log-ratios were used in other studies (e.g. Cardinale et al., 2006; Mayer-Pinto et al., 2016), and are widely used as they estimate a proportional difference between treatments that can be readily compared (Cardinale et al., 2006). To test for differences between datasets, an analysis of variance (one-way ANOVA) was used with 'Dataset' and 'Richness' as main factors. A two-way ANOVA was used with 'Region', 'Dataset' and 'Richness' as main factors, to test for differences between regions and datasets.

Taxa accumulation curves, which represent the number of taxa accumulated in an inventory correlated with the actual sampling effort, are a powerful tool to standardize the estimations of obtained richness and a direct expression of β-diversity, and the rate at which diversity increases from local to regional scales (Terlizzi et al., 2014). Sample-based taxa accumulation curves, that take account sample heterogeneity, were developed using the function *specaccum* from ᴠᴇɢᴀɴ package (Oksanen et al., 2019) with method random and 100 permutations.

Diversity profiles were calculated for each dataset using the function *div_profile* and visual represented using the function *div_profile_plot* from ʜɪʟʟᴅɪᴠ package (Alberdi & Gilbert, 2019a), a framework developed around Hill numbers (Alberdi & Gilbert, 2019b) that encompasses a group of diversity measures that quantify diversity and is expressed in units of effective number of taxa. Within this Hill numbers' framework, diversity profiles show the different components of diversity, that is, the number of taxa and their evenness. The sensitivity towards abundant and rare taxa can be modulated using the scaling parameter q value (order of diversity). The larger the q value, the higher is the importance attributed to abundant taxa. Three q values are particularly relevant, both for their significance, and their close relationship to popular diversity indices: (a) $q = 0$, it becomes insensitive to taxa frequencies, thus yielding a richness value; (b) $q = 1$, weights taxa proportionately by their frequency, and the value it yields is exactly the exponential of the Shannon index; $q = 2$, abundant taxa are overweighted, and it yields the multiplicative inverse of the Simpson index (Alberdi & Gilbert, 2019b). An abundances-based approach was used, where the unit used to compute diversity with eDNA was the relative number of DNA sequences assigned to each taxon while relative abundances were used to compute diversity with morphology.

3 | RESULTS

### 3.1 | Taxonomic resolution

A total of 443 taxa were identified across the 324 samples, using morphology (Table 1). Numbers of taxa per pond ranged between

TABLE 1 Contribution of each step of the framework to pair datasets from eDNA metabarcoding and morphology approaches to the final number of taxa in the hybrid datasets with Additive Workflow, Taxonomic Correction Workflow and Geographical Fit Workflow. N = Number of taxa. Percentages are related to the final number of taxa in each hybrid dataset

			All sites	Murcia	Toledo	Évora	Porto	Jaca	Madrid
Initial Datasets		Morphological	N	443	154	184	205	223	216	159
		eDNA	N	412	128	112	175	207	177	151
Hybrid Datasets	Additive	Full Match	N	59	17	11	19	23	22	16
			%	7.41	6.42	3.86	5.26	5.65	5.93	5.44
		Taxa present only in Morphological	N	384	137	173	186	200	194	143
			%	48.24	51.70	60.70	51.52	49.14	52.29	48.64
		Taxa present only in eDNA	N	353	111	101	156	184	155	135
			%	44.35	41.89	35.44	43.21	45.21	41.78	45.92
	Taxonomic Correction	Accepted Taxa	N	796	265	285	361	407	371	294
		Full Match	N	59	17	11	19	23	22	16
			%	8.43	7.14	4.23	5.81	5.99	6.38	6.02
		ID Correction: Total	N	216	14	24	45	46	22	25
			%	30.86	5.88	9.23	13.76	11.98	6.38	9.40
		ID Correction: From Morphological	N	101	1	7	15	21	7	8
			%	14.43	0.42	2.69	4.58	5.47	2.03	3.01
		ID Correction: From eDNA	N	115	13	17	30	25	15	17
			%	16.43	5.46	6.54	9.16	6.51	4.35	6.39
		Taxa present only in Morphological	N	261	130	159	154	171	181	126
			%	37.29	54.62	61.15	47.09	44.53	52.46	47.37
		Taxa present only in eDNA	N	164	77	66	109	144	120	99
			%	23.43	32.35	25.38	33.33	37.50	34.78	37.22
		Accepted Taxa	N	700	238	260	327	384	345	266
	Geographical Fit	Full Match	N	59	17	11	19	23	22	16
			%	9.32	8.17	4.87	6.71	7.17	7.56	7.27
		ID Correction: Total	N	216	14	24	45	46	22	25
			%	34.12	6.73	10.62	15.90	14.33	7.56	11.36
		ID Correction: From Morphological	N	101	1	7	15	21	7	8
			%	15.95	0.48	3.09	5.30	6.54	2.41	3.64
		ID Correction: From eDNA	N	115	13	17	30	25	15	17
			%	18.17	6.25	7.52	10.60	7.79	5.15	7.72
		Taxa present only in Morphological	N	261	130	159	154	171	181	126
			%	41.23	62.50	70.35	54.42	53.27	62.20	57.27
		Taxa present only in eDNA	N	97	47	32	65	81	66	53
			%	15.32	22.60	14.16	22.97	25.23	22.68	24.09
		Retained Taxa	N	633	208	226	283	321	291	220
		Rejected Taxa	N	74	30	34	44	63	54	46

5 and 61 (average = 26 ± 9.22). From the 443 taxa, 63% were identified to species level, 30% at genus level and 7% to coarser taxonomic resolution (Figure S3). With eDNA, a total of 412 taxa were identified after bioinformatic processing of the sequence data (Table 1). Taxa numbers ranged between 1 and 158 (average = 15 ± 21.13) per pond, of which 70% were identified to species level, 19% to genus level and 11% to coarser taxonomic resolution (Figure S3). Overall, eDNA yielded finer taxonomic resolution

than morphology. The same patterns were observed when analysing the datasets grouped by region, with taxonomic resolution from morphology varying between 54% and 61% (Madrid and Jaca, respectively) at species level, and 30 (Murcia and Porto) and 38% (Madrid) at genus level (Figure S4). With eDNA, the taxonomic resolution varied between 64% and 71% (Murcia and Porto, respectively) at species level, and 18% and 21% at genus level (Figure S4).
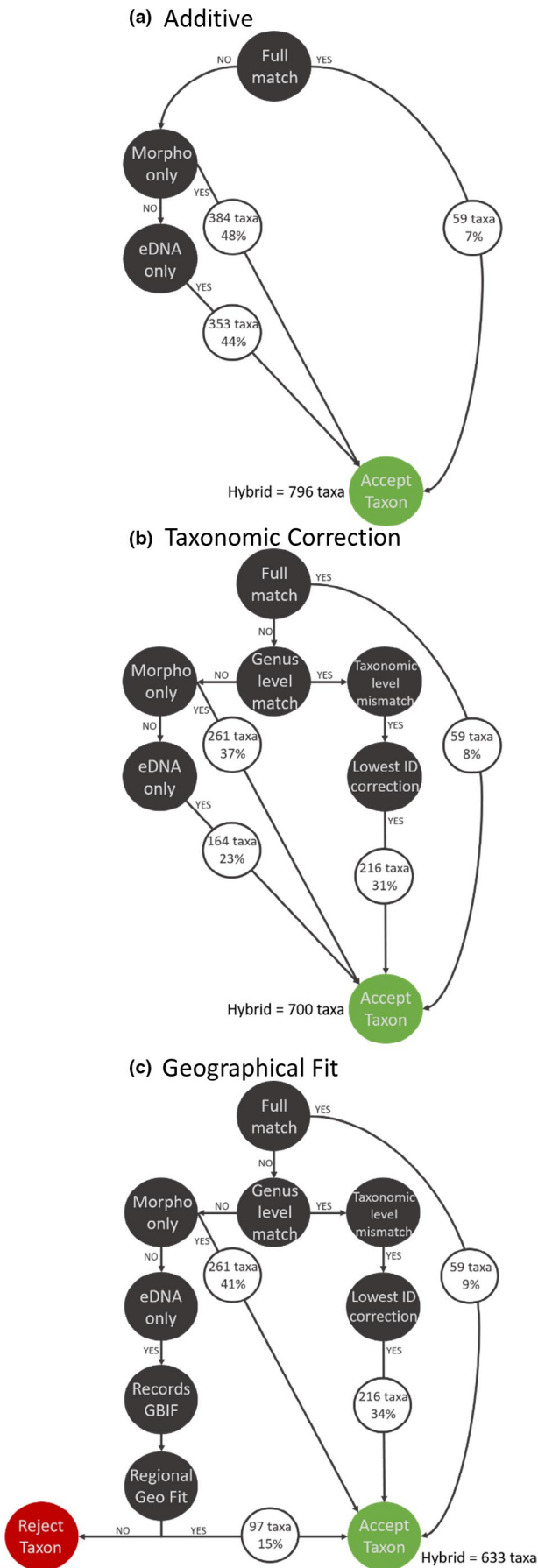
FIGURE 2 Contribution of each filter of the framework to pair datasets from eDNA metabarcoding and morphology approaches to the final number of taxa in (a) Additive workflow, (b) Taxonomic Correction workflow and (c) Geographical Fit workflow. Percentages are related to the final number of taxa in each hybrid dataset

Our three different workflows that pair the morphology and eDNA data use increasingly restrictive filters to retain taxa. Consequently, the more filters applied in the workflow, the smaller the number of species in the resulting hybrid dataset. As the Additive workflow contains no restrictive filters, it revealed a greater number of species (Figure 2a). The Taxonomic Correction (Figure 2b) yielded fewer taxa, and the most restrictive Geographical Fit workflow yielded the lowest number of species (Figure 2c; Table 1; Figures S5–S10). The number of taxa shared by both approaches was 59, which represents 7%–9% of the final number of taxa in the hybrid datasets (Additive: 7.49%; Taxonomic Correction: 8.43%; Geographical Fit: 9.32%; Figure 2, Table 1). Overall, regions with greater numbers of taxa showed greater taxonomic assignment matches (Table 1, Figures S5–S10), which varied between 11 and 23 taxa in Toledo and Porto, respectively. However, despite the low taxonomic assignment match, both approaches identified similar environmental gradients, with regions exposed to greater environmental filters, for example, higher temperatures in southern regions (Murcia and Toledo) and colder temperatures in mountain tops (Jaca and Madrid), being less diverse than temperate regions (Évora and Porto).

By implementing the genus-to-species correction step in the Taxonomic Correction and Geographical Fit workflows, it was possible to improve the taxonomic resolution of 216 taxa to species level, representing a total of 31% and 34% of the final number of species in these hybrid datasets, respectively (Figure 2b,c). This improvement decreased when analysing datasets within each region, varying between 6% and 14% in the Taxonomic Correction, and between 7% and 16% in the Geographical Fit hybrid dataset (Table 1, Figures S5–S10). In all sites, eDNA contributed most to correcting taxonomy from genus to species level, improving taxonomic resolution by up to 10% when compared to using morphology alone (Table 1). In some regions, for example, Murcia and Évora, taxonomic resolution was only marginally improved by up to 4% when compared to only using eDNA (Figure S4). Furthermore, the genus-to-species correction step allowed assigning traits from taxa (e.g. abundance and biomass obtained through morphology) to 12%–14% of the retained eDNA taxa in the hybrid datasets obtained with Taxonomic Correction and Geographical Fit, respectively. When analysing datasets by geographical region, these percentages increased by up to 41% in Toledo with the Geographical Fit workflow (Table S1).

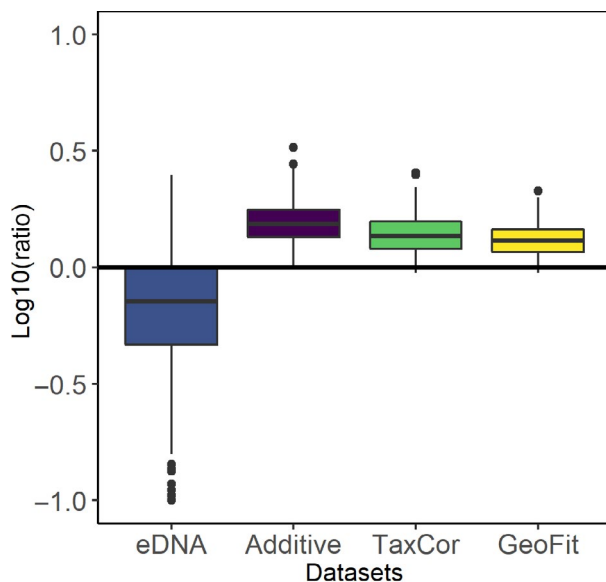## 3.2 | Approach performance assessments

To assess the performance of taxa detection when using either morphology (Figure 3), eDNA (Figure S11), or each of the three hybrid

datasets, a log-ratio was applied to taxa richness (TR) of each dataset. Except for Porto and Madrid (Figure S12), morphology consistently outperformed eDNA in relation to taxa richness (Figure 3; Figure S11), with significant differences between datasets (ANOVA: $p < 0.05$). Furthermore, all hybrid datasets yielded higher taxa richness than morphology and eDNA on their own (Figure 3, Figures S11 and S12), with significant differences between datasets (ANOVA: $p < 0.05$).

3.3 | Diversity analysis

Taxa accumulation curves revealed that, on their own, morphology and eDNA were either clearly approaching or reached an asymptote (Figure 4a); thus, when used independently, these approaches reached their ability to identify new taxa in these ponds. The taxa accumulation curves derived from the hybrid datasets showed faster accumulation rate of taxa (steeper slopes) and the Additive was still not approaching an asymptote, which indicates that morphology and eDNA are identifying different taxa. In the Taxonomic Correction and Geographical Fit hybrid datasets, the accumulation rate was slightly slower than in Additive, which also reflects the differences in taxonomic resolution obtained in the different datasets.

Diversity profiles using Hill numbers were used to show different components of diversity, number of taxa and their evenness (Figure 4b; Figure S13). Increasing the weight given to the most abundant taxa (higher q) revealed that the communities become more even (flat profile; Figure 4). The same was observed when

subsetting datasets by region (Figure S13). Overall, diversity profile analyses showed that the studied freshwater communities are dominated by few taxa independent of the approach used, and there are greater numbers of rare taxa.

4 | DISCUSSION

To improve the resolution of biodiversity assessments, we developed a framework that builds on the advantages of eDNA and morphology using a series of taxonomic and geographical filters. It has been proposed that eDNA can revolutionize biodiversity assessments given its ability to sample broad biodiversity in one stroke (Altermatt et al., 2020; Pawlowski et al., 2018). Although eDNA studies are often used in local conservation studies, a number of studies have used eDNA for ecological research and monitoring at broad geographical scales (Taberlet et al., 2018). We content, however, it that eDNA-based diversity assessments might not always substitute morphological-based ones, rather serving as a complement to it (see also Altermatt et al., 2020; Bush et al., 2019).

Our datasets highlighted some limitations of both morphology and eDNA approaches. eDNA presented a finer taxonomical resolution. It is difficult to identify all specimens to species level with morphology, even with the best taxonomic expertise available, since (a) many early life stages lack necessary diagnostic features (Elbrecht et al., 2017; Pawlowski et al., 2018), (b) some specimens may not be complete, (c) there is a need for complete identification keys and (d) human error plays an important role as well. Species are subsequently aggregated at higher taxonomic ranks, decreasing taxonomic resolution, obscuring species-level responses and constraining our knowledge of whether species' environmental preferences are conserved or variable (Bush et al., 2019; Macher et al., 2016). In the present study, morphology presented a slightly higher species richness, and the number of taxa shared by both approaches was low (only 59 out of 633–796 taxa, representing 7%–9%, depending on the workflow, for the dataset with all regions). While there is a plethora of evidence that eDNA can increase the precision and resolution of biodiversity surveys (e.g. Cahill et al., 2018; Serrana et al., 2019; Seymour et al., 2020, 2021), this is certainly not universally true, as several studies (e.g. Beentjes et al., 2019; Beng & Corlett, 2020; Hinlo et al., 2017) report morphology outperforming eDNA. Also, the low overlap of common taxa shared among the two approaches is not unusual; especially when studying invertebrates (e.g. Seymour et al., 2020, 2021), due to mixed class identification that is used for conventional invertebrate biomonitoring, which is often limited to family level (Morse et al., 2007). Low overlap can also happen when studying groups that are less represented in public databases, for example, diatoms, which have an extensive inconsistency of taxonomy names between existing studies and databases (Mann, 1999). Also, a crucial step for any metabarcoding study is the selection of primers used to amplify specific DNA sequence marker regions, as they determine the taxonomic groups under study and resolution of assignment (Gibson et al., 2014), and different primer sets for the same
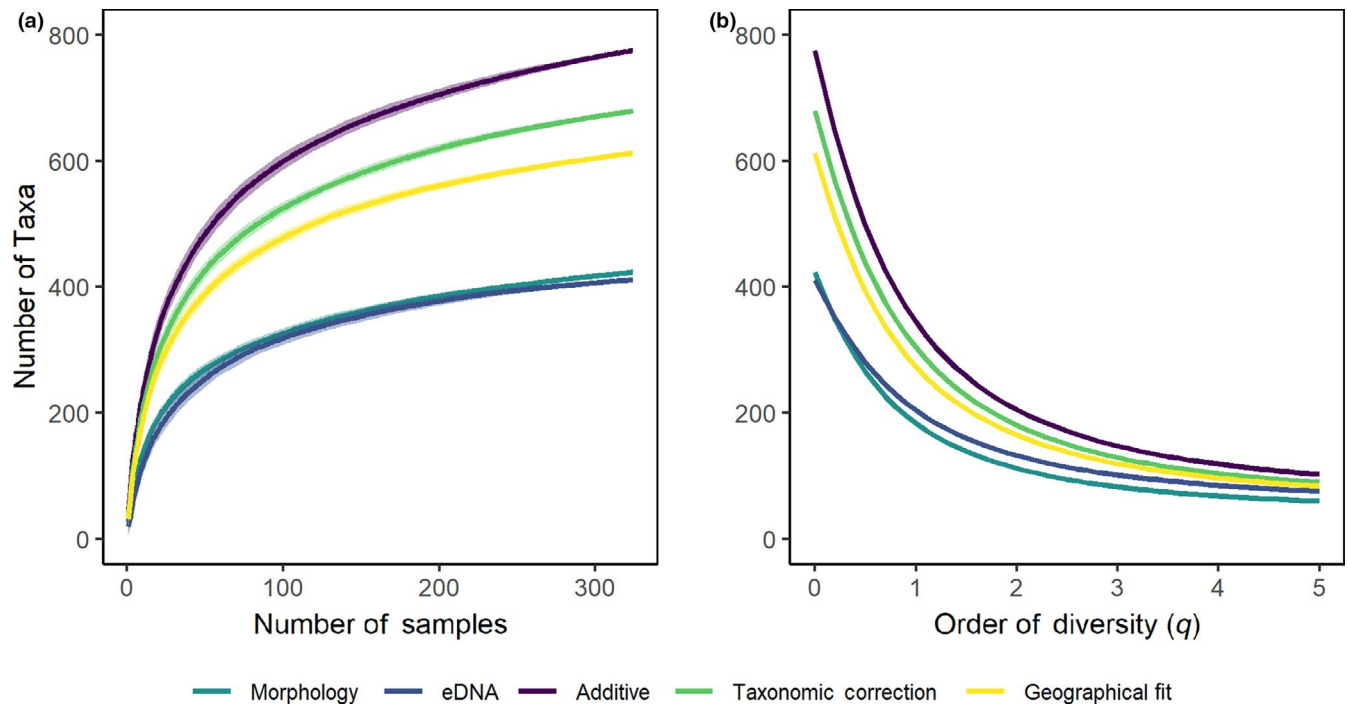

FIGURE 3 Performance of morphology approaches compared with eDNA and with the three hybrid datasets (Additive, Taxonomic Correction and Geographical Fit) to detect taxa, a log-ratio was applied to taxa richness (SR) of each dataset using the formula $\log_{10}(TR_{dataset}/TR_{Morphology})$. Morpho: Morphology, eDNA: Environmental DNA, TaxCor: Taxonomic Correction, GeoFit: Geographical Fit

FIGURE 4 Taxa accumulation curves (a) and Hill's diversity profiles (b) of each dataset. Taxa accumulation curves represent the number of taxa accumulated in an inventory in relation to the actual sampling effort (number of samples). Diversity profiles show the different components of diversity (i.e. the number of taxa and their evenness) and its sensitivity towards abundant and rare taxa is modulated using the scaling parameter q value (order of diversity)

taxa may present different results (Corse et al., 2019; Schenekar et al., 2020). Barcoding regions are well defined for some taxonomic groups (e.g. bacteria and fungi) while others are still under debate (e.g. microeukaryotes), because these regions often span across a large phylogenetic branch and do not always perform equally well for all involved taxonomic groups (Altermatt et al., 2020), and there is often primer bias (Elbrecht & Leese, 2015). These aspects hinder the equal amplification and thus detection of all targeted taxonomic groups in the same sample.

The framework we presented combines the species lists from both eDNA and morphology with increasingly restrictive criteria to retain eDNA detected taxa. The Additive workflow imposes no taxonomic filtering, resulting in complete retention of identified taxa. Although no standard method exists for combining the species lists from these two approaches, other studies have combined eDNA and morphology (like our Additive workflow), into complementary tools for comprehensive biodiversity assessments and more accurate ecologically effective management strategies (e.g. Groendahl et al., 2017; Harper et al., 2020). By simply checking the taxonomic assignment overlap between morphology and eDNA, and by adding the remaining taxa identified with both approaches, it was possible to recover a total of 796 taxa. However, it may increase the possibility of false positives (i.e. false presences), which can potentially arise from metabarcoding data through contamination during sampling or laboratory work, PCR and sequencing errors, and poor reference database coverage or quality (Ficetola et al., 2016; McClenaghan et al., 2020). Strict bioinformatic filtering helps to minimize the

inclusion of these errors in resulting datasets, but the possibility of false positives cannot be eliminated (Ficetola et al., 2016; McClenaghan et al., 2020). Since morphological-based identification depends on taxonomic expertise, it is prone to human error (Hajibabaei et al., 2011; Serrana et al., 2019) and it is not without the risk for false positives. Therefore, all morphology identifications were checked against regional classifications keys and in regional species lists. Nevertheless, combining morphology and eDNA approaches can be useful for detecting unexpected endangered or invasive species, taking advantage of the detection capability of eDNA (Bista et al., 2017) and getting data on community structure provided by morphology. Other studies that have successfully combined eDNA and morphology have increased the resolution of biodiversity assessments when monitoring invasive (e.g. Bylemans et al., 2016) or threatened (e.g. Harper et al., 2020) species distributions, and helped guide management decisions, in both areas from which little biodiversity data are available (e.g. Delabye et al., 2019), and complex communities (e.g. Groendahl et al., 2017).

As highlighted before, morphological identification of diverse taxonomic groups, such as invertebrates, is challenging, for example, non-biting midges (Chironomidae) are extraordinarily well-suited as environmental indicators in freshwater biomonitoring. Nevertheless, these organisms are excluded from many national monitoring programmes due to their complex taxonomy, insufficiently described early stages and resource demanding identification (Ekrem, 2019). A major advantage of eDNA over morphology identification is the ability to generate more accurate identifications

in a consistent manner (Elbrecht et al., 2017). However, if specimens are misidentified at the time of sequence deposition, reference library sequences become associated with an incorrect taxonomic name (Bush et al., 2019). Taxonomic Correction workflow allowed to rectify the taxonomy of more than 200 taxa to species level (>30% of the total taxa of the hybrid dataset). By increasing the taxonomic resolution to species level, Taxonomic Correction workflow can be of greater importance for biomonitoring, especially for freshwater biomonitoring programmes. eDNA contributed the most to rectify taxonomy from genus to species level, which may seem contradictory, since eDNA showed less diversity. However, within the genera shared among the two approaches, several were only identified at genus level with morphology (e.g. *Chironomus*, *Chlamydomonas*, *Oedogonium*), while with eDNA, several species were identified within the same genus (e.g. *Chironomus* and *Chlamydomonas* 4 each, *Oedogonium* 6). This happens mainly with phytoplankton and macroinvertebrates, where taxonomic resolution was greater with eDNA (Figure S14). Additionally, with other genera, even though both approaches identified the same number of species (e.g. *Agabus bipustulatus* and *A. nebulosus*), in some samples those were identified only to genus level with morphology, being corrected by eDNA to species level. The ability to recognize and genetically identify organisms in their early life stages and cryptic species, with potentially different environmental preferences, can increase the resolution in biological monitoring, especially with rich and widely distributed taxa such as chironomids. Thus, this workflow offers the potential to include a much wider range of taxa and indicator groups that are not currently included, which may allow for a finer-scale assessment, particularly in assessing differences among adjacent sites or in evaluating moderate changes in environmental conditions (Cordier et al., 2017).

Although progress has been made in recent years, DNA metabarcoding does not provide reliable data on species abundances (Elbrecht & Leese, 2015). Thus, for eDNA metabarcoding to be used regularly in freshwater biomonitoring, water quality indices must rely on presence/absence data. A strong correlation has been shown between abundance-based and presence/absence-based water quality indices (e.g. Beentjes et al., 2018), illustrating that it is possible to incorporate presence/absence metabarcoding data into water quality assessment methodology, and implement it into routine biomonitoring programmes (Beentjes et al., 2018; Bush et al., 2019). Nonetheless, regulators have remained hesitant to transition to monitoring with metabarcoding (Bush et al., 2019). While metabarcoding is being established as a monitoring technique and while water quality indices are improved (Pawlowski et al., 2018) by incorporating more species-level information on indicator taxa, a combination of morphology and eDNA and the application of the Taxonomic Correction workflow presented here represents a viable solution, since with the *redistributeAbundances* function, we were able to assign traits, such as body mass, and abundance, up to 76% of the final taxa in the hybrid dataset.

Metabarcoding is also being used for increasingly novel applications, such as the study of trophic interactions, either through direct analyses of gut contents, or via the reconstruction of networks of multi-trophic assemblages (Bohan et al., 2017), based on next-generation sequencing co-occurrence data. There have been efforts to improve DNA-based co-occurrence networks (e.g. Compson et al., 2018, 2019; Djurhuus et al., 2020) by reducing the putative interactions to more probable interactions using information about traits (e.g. body size, trophic feeding group; see for discussion Morales Castilla et al., 2015). However, using presence/absence data from metabarcoding to reconstruct trophic networks does not capture abundance changes in species populations and the dynamics of trophic interactions, as one cannot assume presence of interactions based on geographical co-occurrence of species (Araújo et al., 2011; Cazelles et al., 2016). By combining morphology and eDNA with the Geographical Fit workflow, besides improving taxonomic resolution comparing to morphology, it was possible to assign species' traits (e.g. body size) and abundance to 85% of the total number of taxa in the final hybrid dataset with all regions, and to 77%–86% of the total number of taxa in the final hybrid datasets when treating each site as an independent dataset. Obtaining measurements of species' traits (e.g. body size, population structure) across time and space would be more informative, and could reveal seasonal shifts in species interactions (González-Varo & Traveset, 2016).

Trait-based approaches are commonly used to explore and understand the diversity of forms and functions within an ecosystem, and they have been used to approximate some aspects of ecosystem functioning (Naeem et al., 2012), including trophic interactions (e.g. Albouy et al., 2019; Mendoza & Araújo, 2019). Identification of key traits associated with different types of interaction holds great potential for further understanding the strength of the evolutionary processes structuring the architecture of real-world networks (Hervías-Parejo et al., 2020). One example is species' body size (Ings et al., 2009; Morales-Castilla et al., 2015), as this trait is a determinant of consumer–resource interactions in food webs and can also determine interaction strength (Berlow et al., 2009). Occurrence of interactions between species (e.g. consumer–resource or plant–pollinator pairs) depends on the matching between the traits of the interacting pairs (Bartomeus et al., 2016; Morales-Castilla et al., 2015). Trait-matching approaches have been widely used to infer ecological networks across different systems, ranging from terrestrial (Laigle et al., 2018), to freshwater (Pomeranz et al., 2018), to marine ecosystems (Pecuchet et al., 2020). Morphology allowed also to distinguish the different life stages of each species, which is of great importance since it is possible that some trophic interactions are not persistent in time or spatially dominant (Olivier et al., 2019); for example consumers may undergo ontogenetic dietary shifts (McMeans et al., 2015).

In the Geographical Fit workflow, every taxon with only one occurrence and with no occurrences in GBIF or in literature in the studied regions were rejected, having greater loss of information and, consequently, lower uncertainty and lower probability of false positives. Thus, this workflow is suitable, not only for studies on ecological networks but also for conservation programmes, providing

efficient insights on the distribution of species, and estimation of abundance and population sizes, which all provides the basis of taking appropriate conservation actions (e.g. Anderson et al., 2020).

In conclusion, the proposed framework highlights and focuses on the best that both eDNA and morphology approaches have to offer. As DNA sequencing capacity continues to increase, there is a growing interest from the research community, as well as environmental managers, for guidance in how to apply these new tools and show their improvements over morphology. eDNA has been successfully applied to a plethora of different studies; however, it is important to highlight that comparisons between morphology and eDNA identifications are far from straightforward (Bush et al., 2019; Seymour et al., 2020). Our results support the view shared by several authors (e.g. Altermatt et al., 2020; Bush et al., 2019) that eDNA will complement rather than replace morphology approaches and, instead of highlighting the limitations of less established methods in areas that more conventional methods handle well, the focus should be on the strengths of the new methods in areas that conventional methods address inadequately. Since one of the advantages of eDNA approaches is their cost-efficacy, biodiversity surveys should aim towards paired and stratified sampling strategies (in space and time) using both eDNA and morphology (for quantification of species' traits and abundances) as a function of the question at hand. In the present scenario of global change, where accurate predictions about species' distributions and biological responses are needed to effectively conduct management and conservation of ecosystems (Araújo et al., 2019), quantifying biodiversity accurately is of the essence. Developing approaches, such as the one presented here, that integrate already available methods, will get us closer to enhanced biodiversity assessments.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

C.L.P., M.G.M., M.B.A. and M.T.P.G. designed the sampling design; C.L.P. and M.G.M. performed the biodiversity surveys; C.L.P. performed the laboratory work; C.L.P. and M.G.M. designed and implemented the framework; C.L.P. performed the analyses; C.L.P. wrote the first draft of the paper. All authors commented extensively on subsequent versions of the paper.

ETHICAL APPROVAL

The surveys performed comply with the current laws of Portugal and Spain.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

MiSeq raw sequence data are available on the NCBI sequence read archive (accession numbers: PRJNA757444, PRJNA757555, PRJNA757593, PRJNA757597, PRJNA757601, PRJNA757610). Data and scripts for data analysis and figure generation, as well as scripts for each workflow to pair eDNA and morphological approaches are available on Dryad Digital Repository https://doi.org/10.5061/dryad.k6djh9w71.

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