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Active or passive? A multi-marker approach to compare active and passive eDNA sampling in riverine environments

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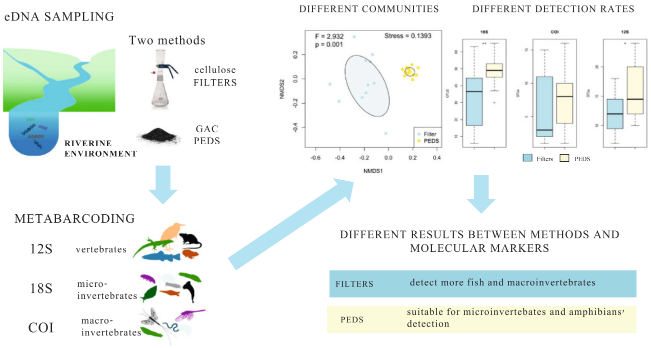
HIGHLIGHTS

- Passive and active eDNA samples have been collected in a riverine environment.
- Metabarcoding targeted micro/macro-invertebrates and vertebrates using 18S, COI and 12S markers from literature.
- Communities differed significantly in all assays. PEDS worked best for micro-invertebrates, filters for macro-invertebrates and fish.
- PEDS and filters both performed well for amphibians. PEDS had lower read variance than filters in the 18S assay.
- PEDS resulted more potentially sustainable and cheap.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental DNA (eDNA) is increasingly used in biodiversity monitoring with several collection techniques proposed. Those applied to aquatic eDNA can now be divided into two categories: active and passive sampling. Active sampling involves the deliberate and controlled collection of environmental samples, and the most common method is water filtration. Passive sampling is a more recent technique that involves capturing eDNA by relying on its adsorption to samplers, which can be fabricated from various materials, and submerged for minutes, hours or weeks. In this study, we compared the performance of water filtration and Passive eDNA Sampling (PEDS) with granular active carbon in terms of detected taxa collected from four different sites of the same river system. eDNA samples were amplified for three molecular markers for 18S rRNA, 12S rRNA and COI genes, with primers according to the literature that target invertebrates and vertebrates. The study revealed that PEDS detected on average more species in 18S rRNA and 12S rRNA assays, with 18S rRNA results presenting a significantly higher homogeneity of read variances between samples. Biological communities captured differed between PEDS and filters. The former method retrieved a significant number of microinvertebrates and chironomids (Chironomidae, Diptera), detecting a similar number of vertebrates to filters, but with lower

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performance in the detection of fish. Notably, both methods performed well with amphibians, successfully identifying all species linked to lotic environments in the studied area. Compared to PEDS, the eDNA capture protocol using filters yielded more sequences identified as ephemeropterans, trichopterans, and acarines. In addition, PEDS was more cost-effective and environmentally sustainable. These findings imply that there is no definitive superior eDNA sampling method. Consequently, in conjunction with studies proposing new methods of eDNA sampling, studies comparing their performance with a broad taxonomic representation will be pivotal.

1. Introduction

Environmental DNA (eDNA) metabarcoding is a well-established protocol to monitor diversity, especially in aquatic environments (Takahashi et al., 2023). Frontiers in this field are continuously pushed, with examples of successful eDNA extractions from the air (Lynggaard et al., 2022; Littlefair et al., 2023) and rain (Macher et al., 2023). Notwithstanding this, relatively few papers have attempted to compare different eDNA capture methods though the numbers have increased recently (Kirtane et al., 2020; Bessey et al., 2021; Bessey et al., 2022; Chen et al., 2022; Chen et al., 2024; Jarman et al., 2024). The majority of these papers share an ecological approach, applying eDNA to unveil biodiversity patterns (Beng and Corlett, 2020).

Among eDNA sampling methods proposed in the last years, two categories can be identified: active and passive eDNA sampling. The former, in aquatic environments, often corresponds to water filtration through vacuum pumps, the latter to the use of samplers, often built ad-hoc (e.g., Kirtane et al., 2020), to adsorb eDNA onto some kind of matrix for a longer time than active sampling. Some eDNA samplers may even be natural, such as filter-feeders like marine sponges (Mariani et al., 2019; Cai et al., 2024; Cai et al., 2024) and bivalves (Weber et al., 2023).

Many different variables may alter the taxa detected between active and passive technologies, including the material with which they are built (Kirtane et al., 2020; Chen et al., 2022), sampling times, and the kind of eDNA adsorbed (eDNA can either be present as a free molecule in the environment or situated inside cells and tissues) (Turner et al., 2014). By far the most common active eDNA sampling technique in aquatic environments uses filtration, with variation in the pore size, materials and volume of filtered water (Majaneva et al., 2018). In contrast, a wide spectrum of passive eDNA sampling techniques has been proposed in the last years, constructed in different forms and from different materials (e.g., Mariani et al., 2019; Kirtane et al., 2020; Maiello et al., 2022; Verdier et al., 2022). Examples of materials proposed to adsorb eDNA, which are therefore suitable for building passive eDNA samplers (PEDS) are porous materials, such as granulated active carbon (GAC) and montmorillonite clay (Kirtane et al., 2020), cellulose filters (Bessey et al., 2021), and glass fiber filters (Chen et al., 2022; Chen et al., 2024). It has been shown that the latter outperformed various other materials in capturing aqueous eDNA of vertebrates (Chen et al., 2022), but many other conditions, e.g., its use in different kinds of matrices and ability to detect different organisms, have yet to be reported.

The amount of captured eDNA is not the only crucial factor to consider. For one, eDNA sampling technologies may be selective for different kinds of eDNA, e.g., either intracellular or extracellular eDNA, (Nagler et al., 2022) and consequently may yield different representations of biodiversity. Moreover, the sustainability of the material, in terms of production and disposal is also a consideration, especially if the mitigation of climate change is taken into consideration (Chen et al., 2022), is something that should be considered, as well as the costs. Passive eDNA sampling has been effective in various environments (e.g., van der Van der Heyde et al., 2023) and at the same time less expensive and more user-friendly. These features promote passive environmental DNA sampling as more scalable, and therefore suitable for large-scale monitoring, including by citizen science participants (Biggs et al., 2015; Agersnap et al., 2022; Miya et al., 2022).

Like other studies that compared active and passive eDNA sampling for the same geographic location in this study, we attempt to compare active sampling through filtration and passive eDNA sampling with GAC-PEDS. As an economic, sustainable and better-performing material for eDNA sampling was indicated in Kirtane et al. (2020), we performed metabarcoding targeting three different markers for samples from riverine environments with this approach. Our study assesses if and how the selection of an eDNA sampling technology affects the outcome of a metabarcoding assay, and also provides a cost comparison for each method.

2. Materials & methods

2.1. Site selection and eDNA sampling methods

The study area, the Serchio River basin, is located in central Italy, Tuscany (Fig. 1). This 1.408 km^2 region is included in the Northern Apennines Hydrographic District (WFD, 2000/60/EC), and its main river, the Serchio, is the third longest in Tuscany. This basin is under-studied in terms of biodiversity monitoring compared to others in the same region. To maximize the retrievable diversity, we sampled eDNA from two areas inside the Serchio river basin, sampling from two water bodies each, the main stem of the basin and a primary tributary. The two water bodies belonging to each site are 4.3 km and 2.0 km apart, while the sites are 27.9 km far from each other (Fig. 1). Samples from the northern sites were collected from the Serchio River (44.07227°N, 10.44321°E; river width 15.6 m) and Fosso di Gragnana (44.18120°N, 10.29663°E; river width 4.2 m). Samples from the southern sites were collected from the Serchio River (43.99896°N, 10.55377°E; river width 28.9 m) and the Lima River (44.01167°N, 10.60453°E; river width 4.2 m). In June–July 2022, the regional hydrological service monitoring stations closest to the sampling sites recorded an average flow rate of 0.47–0.43 m^3/s in the northern area and 0.84–1.41 m^3/s in the southern area (https://www.sir.toscana.it/index.php). The site selection was chosen to provide a broad but representative picture of taxa communities within the river basin. We selected sites that collect water from different areas and environments within the study zone (the northern part drains from the Apuan Alps watershed, while the southern part drains from the Apennine watershed), with diverse characteristics, such as elevation and proximity to densely populated areas. Only four locations were sampled due to the methodological aims of this study, allowing more sampling replicates and molecular markers to be used.

Passive eDNA samplers were assembled in a PCR-free environment, following Kirtane et al. (2020) (granular activated carbon FILTERCARB provided by CARBONITALIA S.r.l.), sterilized under UV light for 12 h and stored in a sterile plastic bag. In the field, the samplers were completely submerged in triplicate inside a punctured PVC tube, to protect the samplers from UV DNA degradation and microalgal biofilm formation. Using a nylon rope, each PEDS was linked to the others. One week of submersion at the same level of water filtration was used following Kirtane et al. (2020), who didn't observe any increase of eDNA between 7 and 21 days of submersion. A submersion time of one week should ensure the capture of eDNA of a broad range of taxa that may differ in behaviors, such as their diurnal habits and home range, and improve time management of field sampling activities. A total of 24 samples were collected: 12 from the northern site (water filtered the 05/07/22, same day of PEDS submersion) and 12 from the southern site (06/07 and 08/07/22).

For efficient filtration that could be applied in different usage contexts (from citizen science to environmental monitoring by national agencies), and to maximize the number of site replicas, 0.75 L of water was filtered in three replicates in the field, together with a blank that filtered 0.5 L of deionized water. The filtration apparatus was composed of a manual vacuum pump linked to a funnel containing a cellulose filter (0.45 μm, Nalgene Single Use Analytical Filter Funnels, Thermo Scientific). Filtration occurred before PEDS were placed. All equipment used was previously sterilized by UV and also by 13 % sodium hypochlorite in the field to minimize contamination between samples. Afterwards, filters were wrapped in aluminum foil, kept in a cooler bag and transported to the Department of Biology of the University of Pisa, where they were stored at −18 °C for further analyses. PEDS were retrieved after seven days, placed in sterile plastic bags inside a cooler bag, transported to the Biology Department Laboratory and stored in the same manner as the filters.

2.2. DNA extraction, quantification, amplification and sequencing

eDNA samples were extracted using a QIAGEN DNeasy Blood & Tissue Kit, with a halved final elution volume of 100 μL. The protocol was performed under a biological safety cabinet, previously sterilized with UV light overnight. For each filter, one half was extracted, and the remainder was stored back at −18 °C. During every extraction an unused filter, or 0.25 g of granular activated carbon, was processed as a blank.

Concerning PEDS, two extraction replicates (0.25 g of GAC per extraction) were performed for each sample and merged after extraction. For passive samplers, two extraction replicates were performed, whereas a single replicate was used for filters. This approach was adopted based on methodological and practical considerations. Given that 50 % of the initial sample weight is extracted from the filters (corresponding to half of the filter), a single replicate of 0.25 g would have resulted in the extraction of only 16.7 % of the sample. To enhance this proportion to 33 %, thereby optimizing the balance between extraction representativeness and cost efficiency, two replicates were chosen.

Between the processing of samples during extractions gloves were changed and the instruments exposed to the samples, tweezers for filters and steel paddle for PEDS, were sterilized with 13 % bleach and flambéed with denatured alcohol.

Quantification of extractions was performed by means of a Qubit dsDNA HS Assay Kit (Invitrogen). Samples were then shipped to the company IGATech for amplification and sequencing.

Three sets of primers were chosen for metabarcoding: (1) the universal primer pair for vertebrates metabarcoding 12S—V5 (Riaz et al., 2011), forward 5′-TTAGATACCCCACTATGC-3′ and reverse 5′-TAGAACAGGCTCCTCTAG-3, also called Vert01 (Taberlet et al., 2018), which targets a short sequence (ca. 100 bp); (2) a 18S rRNA metabarcoding primer pair specific for soil metazoans, (Capra et al., 2016) M620F forward 5′-GCAGCCGCGGTAATTCC-3′ and M1260R reverse 5′-TCRGCTTTGCAACTATACTTCC-3′, which targets a ~609 bp long fragment of the V4-V5 region; and (3) the universal primer pair mlCOIintF, forward 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′ and jgHCO2198 reverse 5′-TAIACYTCIGGRTGICCRAARAAYCA -3′ (Leray et al., 2013), targeting a 350 bp long fragment of the COI gene.

Following amplification, performed in one technical replicate, libraries were prepared with the NExteraXT kit, and sequencing was performed by a NovaSeq 6000 sequencer to sequence the 250 bp paired-end libraries. Supplementary materials concerning PCR amplification protocols and library preparation have been uploaded in Zenodo (https://doi.org/10.5281/zenodo.15065548).

2.3. Sequence analysis

Raw sequences were processed using the DADA2 pipeline (Callahan et al., 2016). Quality profiles of the forward and reverse reads were first examined using *plotQualityProfile*. Reads were then filtered and trimmed with the *filterAndTrim* function, with sequences truncated to a maximum

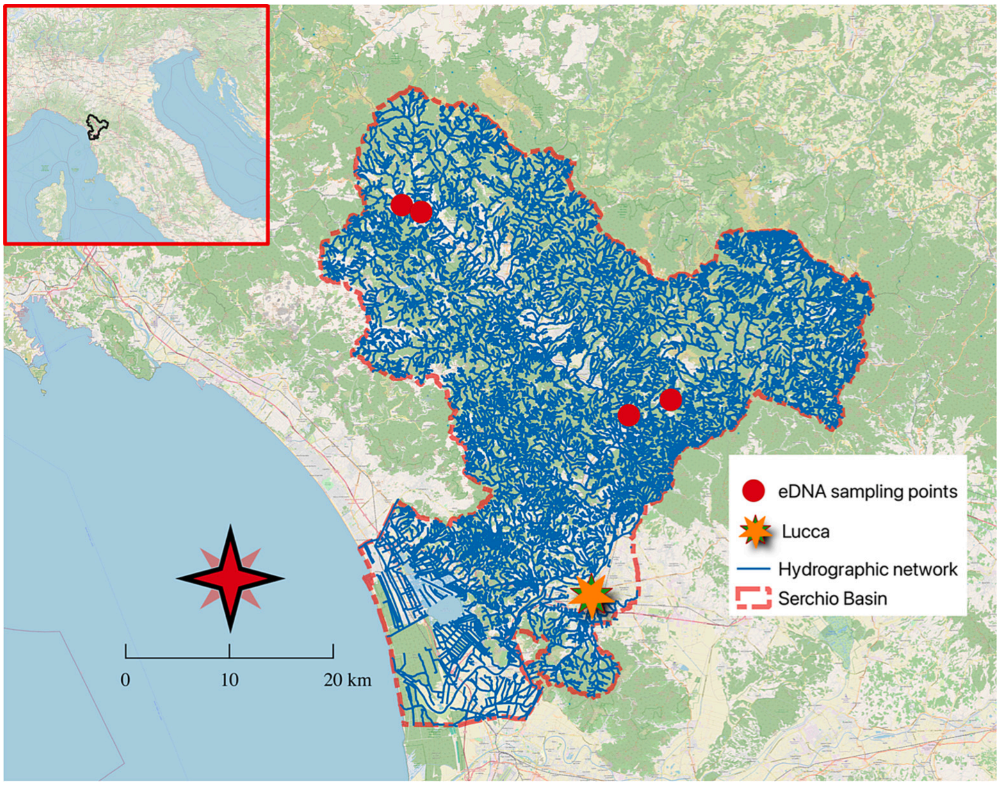

Fig. 1. QGIS generated map of the Serchio river basin. In the top-left its location in central/northern Italy is shown, while the higher magnification includes sampling points (in red) and the hydrographic network in light-blue.

length of 160 bases, and those with more than two expected errors or low-quality scores removed. Sequences containing 'N' characters were excluded, and contamination from *PhiX* was filtered out.

Forward and reverse reads were denoised and dereplicated using DADA2. The denoised reads were merged (for amplicons with overlapping forward and reverse reads) or analyzed as single-end reads in the case of 18S rRNA amplicons, where no overlap was present. Chimeric sequences were removed with *removeBimeradenovo* and a feature table of amplicon sequence variants (ASVs) was generated as the final DADA2 output.

Complete DADA2 script has been deposited in a GitHub repository https://github.com/gabmetabarcoder/Scripts/blob/main/DADA2%20denoising%20of%20NovaSeq%20sequences%20in%20R.

The ASVs found in the negative controls (field, extraction and PCR) were subtracted from their corresponding samples. The remaining ASVs were assigned to taxonomic units using QIIME2 (Bolyen et al., 2019) with the following reference databases: a vertebrate-specific database retrieved from CALeDNA (https://ucedna.com/reference-databases-for-metabarcoding) for 12S rRNA; GenBank for 18S rRNA; and BOLD (Barcode of Life Data System) for COI (Ratnasingham and Hebert, 2007).

For each molecular marker, the reference sequences were extracted from their respective database by performing an in-silico PCR with the *extract-reads* command from the *feature-classifier* plugin of QIIME2. With *fit-classifier-naive-bayes* a Naive Bayes classifier was trained on the reference sequences and a first unweighted classification was done with *classify-sklearn* (Pedregosa et al., 2011). The *clawback* plugin (Kaehler et al., 2019) was then used to assemble taxonomic weights from the unweighted classification to improve the taxonomic accuracy of *feature-classifier*. A weighted classifier was trained with *fit-classifier-naive-bayes* and a final classification was done with *classify-sklearn*.

The taxonomic assignments for 12S rRNA and COI were curated manually by using BLASTn (Altschul et al., 1990) against the GenBank NR database to check for mis-assignments and remove low quality assignments that had a BLAST match sequence identity lower than 97 %. Only hits with over 80 % query coverage and 97 % sequence identity were retained. If the top hits within 1 % of identity all matched a single species, that species was assigned to the ASV. If the top hits matched multiple species within the same genus, the genus was assigned. If they matched species within the same family, the family was assigned, and so on.

For each marker, a taxonomic table was generated by collapsing the taxonomy on the ASV table, and clustering ASVs with the same taxonomic assignment into a taxon (Singer et al., 2023). These tables were used for all subsequent diversity analyses except non-metric multidimensional scaling and PERMANOVA, where the ASVs table was used.

2.4. Statistical analyses

To assess if the sequencing depth was sufficient in retrieving diversity from samples, read-based rarefaction with 999 permutations was performed through the "specaccum" function of the Vegan R package (Oksanen et al., 2013) for the three molecular markers. This approach was chosen because species accumulation curves provide an effective way to evaluate whether sequencing depth captures most of the diversity present in the samples, helping to determine if additional sequencing effort would significantly increase the number of detected taxa. After the transformation of abundances in presence/absence data, accumulation curves were plotted through the R package iNEXT (Hsieh et al., 2016) to better understand if the number of samples collected is enough to represent the diversity.

Next, to compare species accumulation curves of PEDS and filter metabarcoding output from the three molecular markers used in this study, we ran the command "EcoTest.sample" of the R package RareNMtest (Cayuela et al., 2015) with 1000 randomizations. EcoTest was selected for its ability to statistically compare accumulation curves from different sampling methods, providing robust evidence of differences in taxon detection efficiency between approaches. A customized script for accumulation analyses with iNEXT and Ecotest has been deposited in GitHub (https://github.com/gabmetabarcoder/Scripts/blob/main/Accumulation%20Analysis%20with%20iNEXT%20and%20Ecotest).

ANOSIM and PERMANOVA (999 permutations) were performed with a Jaccard matrix with TaxonTableTools (Macher et al., 2021) and the vegan package ("adonis2" function) on the ASV tables respectively. MetaMDS from the Vegan package was used to perform and plot ASVs by non-metric Multidimensional Scaling. Ellipses in the nMDS plots were computed using the *ordiellipse* function from the Vegan package. These ellipses represent 95 % confidence intervals around group centroids, calculated based on the standard deviation of point coordinates within each group, using a weighted covariance matrix. Moreover, Vegan was used to perform SIMPER analyses, with 999 permutations, to understand which taxa contributed the most to patterns of diversity between methods. Eventual differences in dispersion between reads distribution in the two methods were checked with "betadisper", from the Vegan package, performed in 999 permutations with a Jaccard matrix.

To assess if the number of taxa detected varies between filtration and PEDS for each molecular marker a Shapiro-Wilk test was first performed to evaluate the normality of reads distributions across samples. Since the null hypothesis was rejected a Mann-Whitney test was performed. This non-parametric test was chosen because it does not assume normality and is suitable for comparing independent groups, ensuring robust statistical inference. The R package ggplot2 (Wickham et al., 2016) was used to generate boxplots showing the number of taxa detected with each set of primers and each eDNA capture method.

2.5. Comparison of costs between PEDS and filters

Since the extraction and analysis methods for eDNA were the same, the cost analysis includes the operator's time associated with preparation and field activities, considering the potential reuse of purchased materials. Regarding the costs of passive samplers, the expense for the materials of a single PEDS was calculated, considering the grams of GAC and the surface of mesh required. In each procedure, the operator used several pairs of nitrile gloves, as well as pre-sterilized plastic zip bags to store the PEDS, sterilized bottles for collecting water to be filtered, and aluminum foil to preserve the filters.

3. Results

3.1. Taxonomic results

The sequencing of 12S rRNA (https://www.ncbi.nlm.nih.gov/sra/PRJNA1214188), COI (https://www.ncbi.nlm.nih.gov/sra/PRJNA1214582) and 18S rRNA (https://www.ncbi.nlm.nih.gov/sra/PRJNA1214566) amplicons produced respectively 6,922,684, 6,259,223 and 11,496,624 raw reads, divided into ~12.9 million to "Filters" and ~11.7 million to "PEDS", which after denoising with DADA2 respectively became ~5 million; 566,652 and ~ 4,5 million, assigned to 1249, 1993 and 7260 ASVs. For both 18S and COI, the bioinformatic step which contributed to the most read loss was filtering by quality (Table S2-S4 provide details on reads per each DADA2 pipeline step).

ASV tables for each metabarcoding assay have been uploaded to Zenodo (https://doi.org/10.5281/zenodo.14803748), including a table with sample information. After taxonomic assignment, 187 taxa were assigned with the 18S rRNA marker, 59 with 12S rRNA, and 44 with COI. The three main phyla for the 18S rRNA marker were Arthropoda (45 % ca. of OTUs), Nematoda (14 % ca.) and Platyhelminthes (8 % ca.); for the 12S rRNA marker the main classes of vertebrates detected were Actinopterygii (32 % ca.), followed by Mammalia and Aves (29 % and 27 %, respectively); for the COI marker the main phyla detected were Arthropoda (52 % ca.), Chordata (20 % ca.) and Annelida (13 % ca.) (Fig. 2).

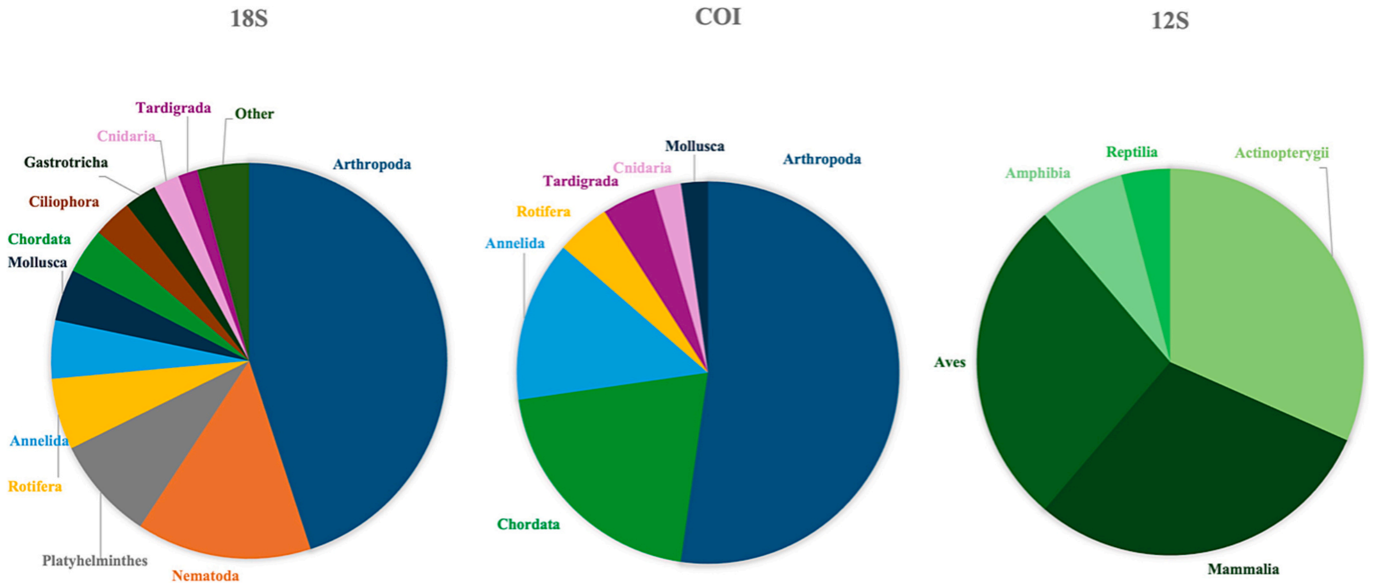

Fig. 2. Pie charts illustrating the taxonomic composition of metabarcoding results for the three molecular markers used in this study. To produce this graph, phyla were considered for 18S and COI markers, while classes were considered for the 12S marker.

Concerning taxonomic resolution, metabarcoding with 18S rRNA detected 54.5 % of taxa at species level, 14.3 % at genus level, 14.8 % at family level, 10.6 % at order level, 4.8 % at class level, and 1 % at phylum level. The 12S rRNA detected 77.5 % of taxa at species level, 13.3 % at genus level, 8.2 % at family level, and 1 % at order level. COI detected 81.8 % of taxa at species level, 15.9 % at genus level, and 2.3 % at family level. Taxonomic tables are provided in supplementary material (Table S1).

3.2. Comparison of metabarcoding with filters and PEDS

Read-based rarefaction revealed sufficient sequencing coverage for every sample (Figure S1), including COI results, represented by a low number of reads and rapidly plateauing. No significant difference has been detected between the accumulation curves of both methods in all three metabarcoding markers (18S: EcoTest, $p = 0.683$; COI: EcoTest, $p = 0.478$; 12S, EcoTest, $p = 0.245$). Within the COI dataset, iNEXT-generated plots with extrapolation show that both PEDS and filters plateaued at 35 samples, while in 18S only PEDS plateaued at 35 samples, and in 12S only filters (Fig. 3A).

The "betadisper" test revealed significant differences in read number variances within filters and PEDS in 18S rRNA metabarcoding results ($p = 0.001$; F = 28.31) (Figure S2). Since Shapiro-Wilk testing was statistically significant for all PEDS and filter reads distributions ($p < 0.001$), Mann-Whitney/Wilcoxon testing was performed to account for differences in the number of taxa detected by the different methods. The test was statistically significant for 18S rRNA ($p = 0.007$, W = 25) and for 12S rRNA ($p = 0.048$, W = 37.5) where PEDS detected a higher number of taxa per sample (Fig. 3B).

Concerning the nature of vertebrate sequences present, PEDS and filters shared 59.32 % of taxa, while COI shared 45 %, and 18S shared 46 % (Fig. 3C). The analysis of beta diversity by ANOSIM revealed a statistically significant, though slight, difference between the PEDS and filter communities for vertebrate metabarcoding ($p = 0.032$; $R = 0.11$ ca.), while stronger evidence has been observed for 18S ($p = 0.001$; $R = 0.30$). No difference emerged from COI ($p = 0.229$). To evaluate if other factors play a role in the difference between samples, an ANOSIM for both markers was performed for two factors that were selected as potentially influential: the type of water body (main stem or tributary) and the area of sampling. For the 12S and COI markers, the area of sampling was not statistically significant ($p > 0.05$), as well as the type of water body. For 18S, the area of sampling was statistically significant ($p = 0.026$, $R = 0.087$), while the typology of water body was not ($p = 0.377$).

PERMANOVA analyses performed on ASV table gave statistically significant results for 18S and 12S (18S: $p = 0.001$, F = 2.932; 12S: $p = 0.031$, F = 1.195; COI: $p = 0.08$, F = 1.111) (Fig. 3D). To account for other factors playing a role on clustering, PERMANOVA was also performed for location (differences between northern and southern sampling locations) and type of river (main river or tributary). For 18S only the location was statistically significant ($p = 0.013$, F = 1,5), and the nMDS (Fig. S3) shows a significant overlap between these two categories.

For vertebrates, SIMPER analyses, performed to identify taxa contributing the most to diversity between methods, revealed that the four main taxa were all fishes, namely: *Barbus* sp., comprised 13 % of the beta diversity between methods (p = 0.007), followed by *Padogobius martensii* (6.3 %, $p = 0.044$), *Telestes muticellus* (6 %, $p = 0.016$) *and Padogobius nigricans* (3.2 %, $p = 0.039$). Therefore, these species show a common pattern where most of their reads belonged to filter samples (Fig. 4A). Notably, PEDS detected some fish species which have not been detected from filters, such as *Thymallus thymallus*, *Pseudorasbora parva*, *Micropterus salmoides,* and *Lepomis gibbosus*. PEDS performed nicely in other classes of vertebrates though, such as amphibians, with two salamanders (*Salamandrina perspicillata* and *Salamandra salamandra*) and three anurans (*Bufo bufo*, *Pelophylax* sp., and *Rana italica*) detected by both methods (Fig. 5). The only amphibian uniquely detected with filters is *Speleomantes* sp., a plethodontid salamander which has no aquatic life stage.

For the 18S marker, the SIMPER analysis revealed the gastrotrich *Chaetonotus* sp., as the major contributor to diversity between methods (19.6 %, p = 0.001), followed by the copepod *Nitokra hibernica* (5.9 %, $p = 0.027$), the flatworm *Girardia tigrina* (2.6 %, $p = 0.035$) gand the chironomid *Tanytarsus bundini* (1.3 %, p = 0.001). The major contributors to the difference between PEDS and filters are invertebrates. The first four species contributing to the difference between methods for the 18S marker showed a similar, inverted, pattern as the 12S marker (Fig. 4B).

In addition, PEDS uniquely detected some families of micro-invertebrates, such as various nematodes (Bastianiidae, Sphaerulariidae, Thelastomatidae, and Tobrilidae), flatworms (Tobrilidae, Microstomidae, and Opecoelidae), and one protist (Gruberellidae). Despite

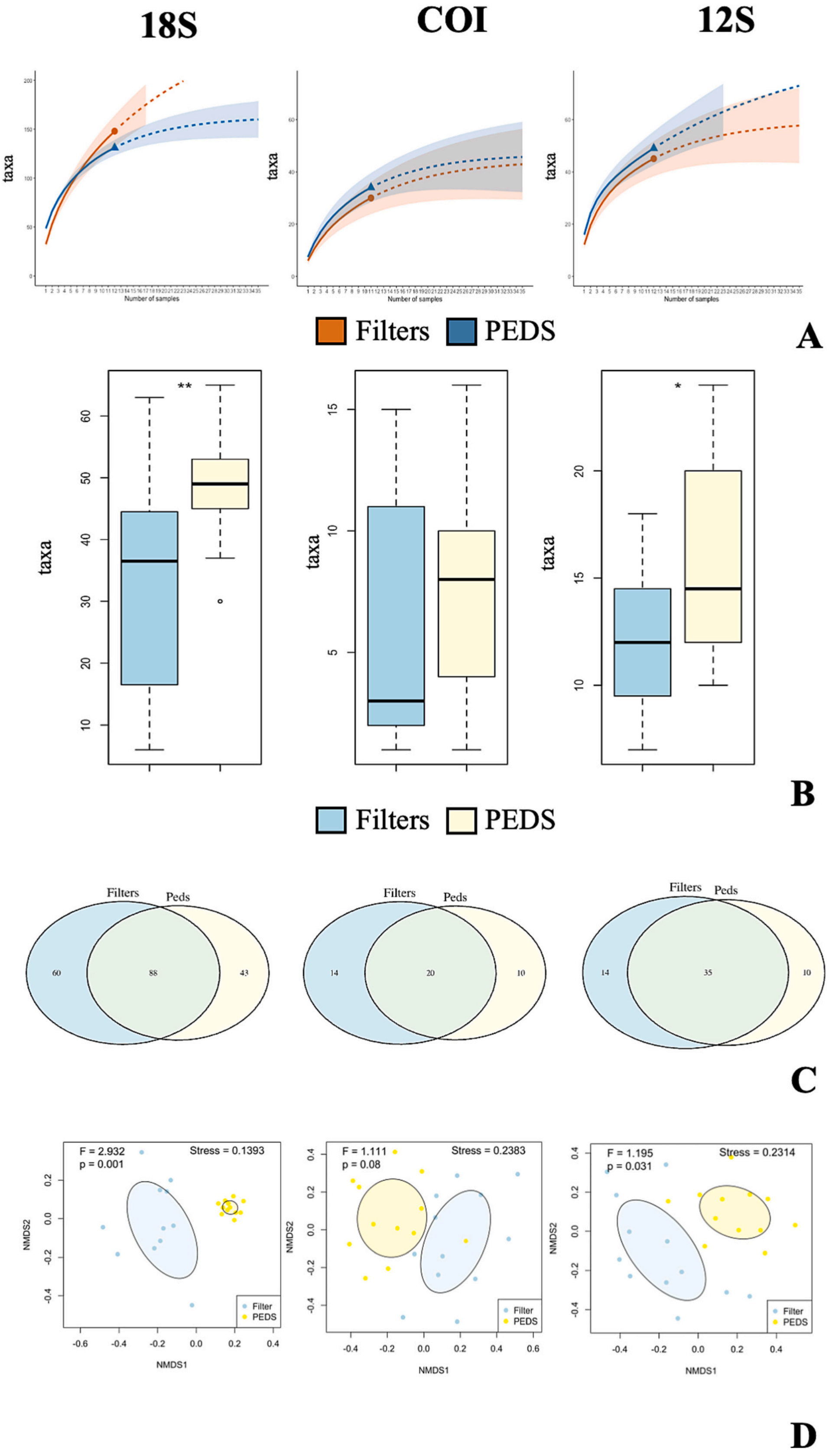

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Fig. 3. A) Accumulation plots generated with iNEXT. The x-axis indicates the number of samples considered in the generation of curves, while the y-axis indicates the number of species. Continuous lines show the real samples ($n = 12$ per method), while the dashed represent the extrapolation. B) Boxplot generated with ggplot2, showing the number of OTUs detected in the two different eDNA capture methods used in this study, with all three molecular markers (COI, 12S, and 18S). Asterisks above the boxplots reveal the significance of the Mann-Whitney test (* = <0.05; ** = <0.01; *** = <0.001). C) Venn diagrams produced with TaxonTableTools showing unique and shared OTUs for the three molecular markers analyzed in this study. Circumferences are proportional to OTUs number. D) nMDS plots produced with Vegan package from ASVs, showing PERMANOVA results for 18S, COI and 12S results.

PEDS, which detected just one family of the order Trombidiformes (Acari), namely Hygrobatidae, filters detected seven families (Brachychthoniidae, Eupodidae, Hydryphantidae, Johnstonianidae, Oppiidae, Tydeidae and Oribatulidae), suggesting a predilection for those species for filtration. Notably, filtration, and not PEDS, detected some families of macroinvertebrates with a crucial role in river ecosystems, and commonly used to calculate biotic indexes, such as Caenidae and Ephemeridae (Ephemeroptera), Ceratopogonidae and Tabanidae (Diptera) and Hydropsichidae (Trichoptera).

Finally, concerning COI metabarcoding, the annelid *Chaetogaster* sp., was the only taxon contributing significantly to the difference between methods (25.9 %, $p = 0.008$). Many species were unique species to either site: filters alone detected two species of ephemeropterans, *Baetis rhodani* and *Habroleptoides confusa*, while PEDS alone detected five species of insects of the Chironomidae family, namely *Tanytarsus brundini, Tanytarsus* *eminulus, Rheotanytarsus ringei*, *Rheocricotopus atripes,* and *Polypedilum convictum.*

3.3. Comparison of costs between PEDS and filters

The total cost for 12 PEDS and the materials required to use them in the field, rounded to €0,76 × 12, plus the costs of reusable materials, amounts to €34,68 for sample collection. For each PEDS, two extractions were performed and subsequently pooled together. Considering the kit used, the DNeasy Blood & Tissue Kit from QIAGEN S.r.l. (250 reactions, €969,60), the total cost rises to €127,76.

For the protocol applied to filter water in the field using ready-to-use filter cups were €54,48 for 12 samples (x50 enton filt analit 250 mL nc, 45 from Fisher Scientific, €227 in 2022). As for reusable materials, we followed the recommendations of a reference protocol (Laramie et al., 2015) and bought a polyvinylchloride hand-operated vacuum pump from the same company, however after a few uses it broke down, and we bought a metal vacuum pump on Amazon for €16 (a vacuum pump for brake unclogging). Considering the price of the manual metal vacuum pump, the cost for filtering and extracting eDNA from 12 samples is €170,31.

Finally, considering a number of operators needed equal for both protocols, the amount of time for the experimental set up and field activities resulted in 25 min for PEDS and one hour for filters (Table S5).

4. Discussion

Filtration of water and passive eDNA sampling are two promising methods to survey biodiversity. The former well-established method has been shown in the last number of years to be effective in detecting a wide spectrum of aquatic biodiversity in various environments. The latter, more recent method is an attractive alternative because it is cheaper, easier and potentially more sustainable. Moreover, filtration has shown various drawbacks in biodiversity assessments (Bessey et al., 2021; Kumar et al., 2022). Consequently, studies to standardize passive eDNA sampling may be vital for the future of environmental monitoring. In this study, we compared filtration using cellulose filters with Granular Active Carbon (GAC)-PEDS (Kirtane et al., 2020). Granular activated carbon has been chosen as the substrate because it is cheaper and more sustainable than other materials.

18S metabarcoding, compared to the other markers used in this study, 12S and COI, detected more OTUs, especially from invertebrates. SIMPER testing showed a bias of GAC-PEDS towards microinvertebrates, with a higher portion of reads assigned to a common genus of gastrotrich, a copepod species, flatworms and insects (Chironomidae). Furthermore, many microinvertebrates have uniquely been detected from PEDS.

In past studies, eDNA metabarcoding has shown limited ability to detect riverine invertebrates, particularly those of Nematoda, Platyhelminthes, Cnidaria and Nematomorpha (Poyntz-Wright et al., 2024). All these phyla have been detected in this work, especially with passive sampling. This may suggest the combination of GAC-PEDS and 18S (Capra et al., 2016) as a valid method to survey the invertebrate diversity of riverine environments, particularly microinvertebrates. For EPT (Ephemeroptera, Plecoptera and Trichoptera) insects, in this study filtration performed better than PEDS. For benthic macroinvertebrates, water filtration has not performed well (Gleason et al., 2021; Wang et al., 2021), while other techniques, such as sediment and biofilm, have shown better results (Ji et al., 2022; Rivera et al., 2021; Vourka et al., 2023).

Betadisper analysis revealed a minor dispersion of read numbers and typology (Jaccard matrix) per sample of PEDS compared to filtration. This result may indicate a minor stochastic bias of GAC-PEDS compared to filtration. Jaccard-based PCoA of Chen et al. (2024) showed a similar pattern of the nMDS performed in this study with 18S metabarcoding results, where graphically all PEDS samples are projected closer to each other compared to filters, which were more dispersed in the plot. Since the cited study uses a different PEDS based on glass-fiber filters, this common pattern may reveal a strong consistency of PEDS, probably due to submersion time. A higher similarity between sampling replicates may be used to reduce the number of samples taken per experiment, and lead to cheaper and faster protocols.

For vertebrates, PEDS detected more taxa compared to filtration (49 vs 45), but filters appeared to perform better with sampling fish biodiversity, as indicated by the higher number of reads assigned by this method. This may be problematic when monitoring fish biodiversity through eDNA metabarcoding with GAC-PEDS, as rare species may not be detected. In our assessments, the Etruscan goby, *Padogobius nigricans*, a species known to be rare in the Serchio river basin was detected in just one sample. Furthermore, the number of reads belonging to this species was low compared with other species listed, like the Padan goby *Padogobius martensii*, belonging to the same genus. On the other hand, PEDS did detect various unique fish species which is not congruent with the notion that this technique is inferior in detecting rare species. In agreement with Chen et al. (2024) this may be due to the prolongated time of submersion of PEDS, which would facilitate detection of species with nocturnal or transient habits.

As for other vertebrates, GAC-PEDS was efficient in detecting local amphibian diversity, retrieving eDNA from every species inhabiting lotic environments of the Serchio basin according to iNaturalist.org, namely the anurans *Bufo bufo* (common toad), *Pelophylax* sp. (green frog), *Rana italica* (red frog), salamanders *Salamandra salamandra* (fire salamander), and *Salamandrina perspicillata* (spectacled salamander). Only one amphibian species was detected solely by filtration, *Speleomantes* sp. (cave salamander), which has no life stage where it remains submerged in water. Its detection may be the result of an occasional crossings of the riverine environment, or due to the capacity of rivers to act as “conveyer belts”, catching eDNA not just from aquatic species but also from species living in the surroundings (Deiner et al., 2016).

Amphibians are facing a huge decline due to anthropic climate change (Hoffmann et al., 2010), and their detectability with standard

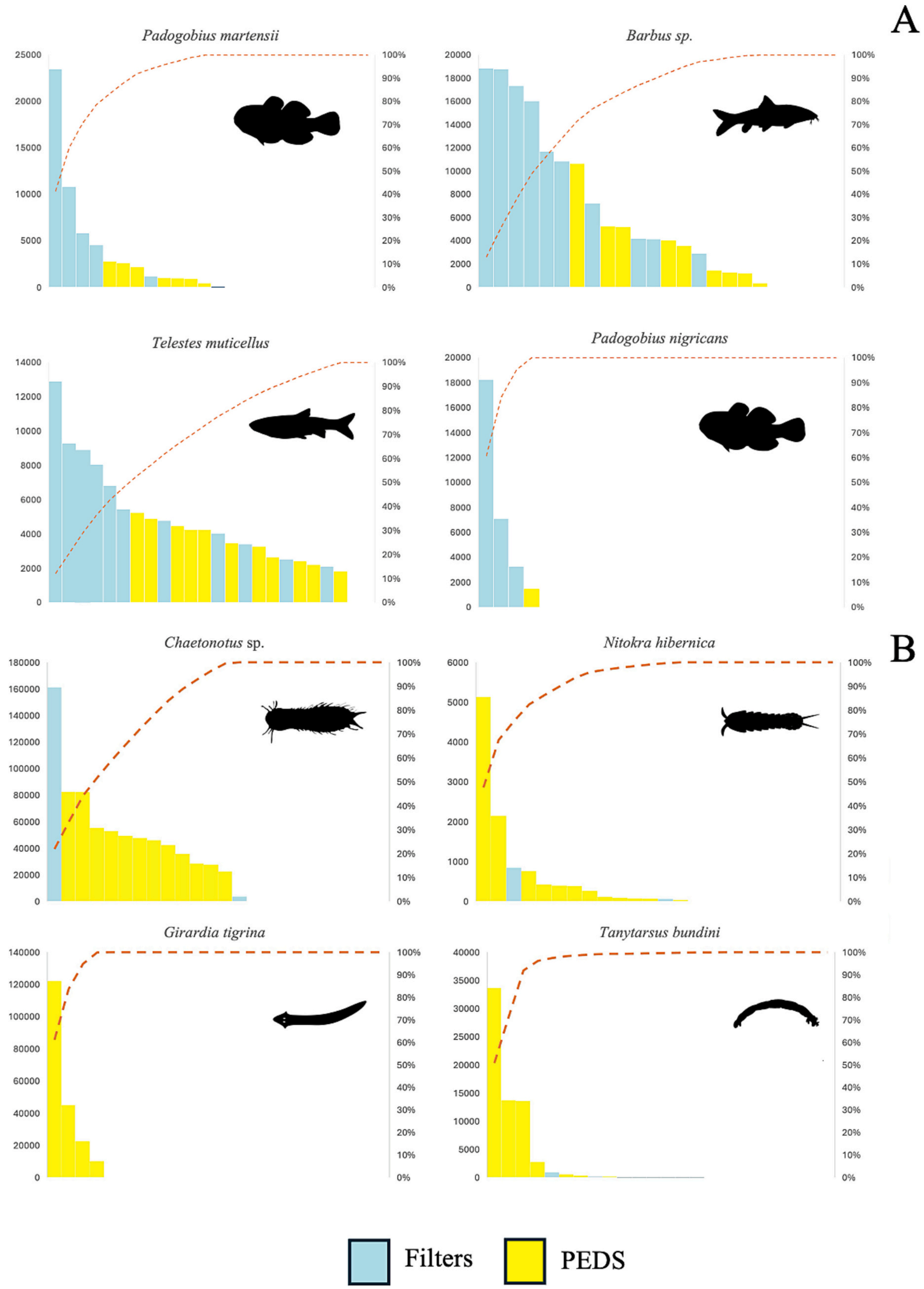

Fig. 4. This graph represents the distribution of reads belonging to the four species which contributed most to the difference between PEDS and filters with the 12S marker (first four plots from the top) and 18S marker (lowest four plots). Every column corresponds to an eDNA sample: yellow and light blue bars represent, respectively, PEDS and filters, in decreasing order of reads, while the Y-axis represent the number of reads (left) and the percentage (right). The orange dashed curve represents the accumulation of reads per sample. Silhouettes of species were retrieved from phylopic.org.

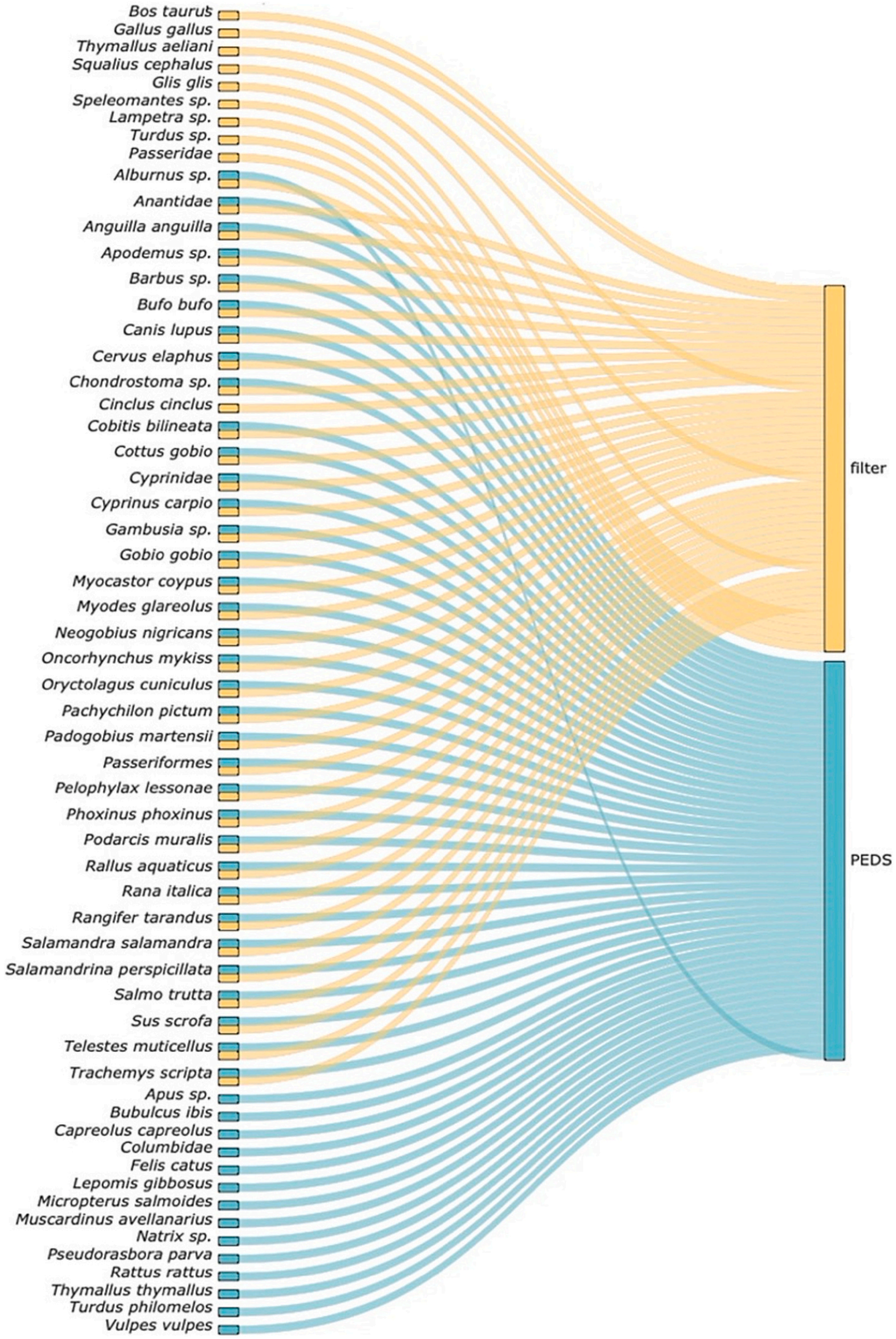

Fig. 5. ParCat plot, generated with TaxonTableTools, representing species of vertebrates detected with by the 12S marker divided into the two eDNA capture methods: filters in yellow and PEDS in light blue.

methods can be tricky (Mazerolle et al., 2007). eDNA has been proposed as an alternative monitoring method (e.g., Ficetola et al., 2019). eDNA capture has been identified as a “key aspect and challenge in eDNA monitoring of amphibians” (Sun et al., 2024) but only filtration has been reported as a technique used until now. Here we show that GAC-PEDS may be suitable for amphibian eDNA detection in rivers.

One possible explanation for the higher detection of microinvertebrates with PEDS compared to filters could be that, unlike filters, PEDS do not selectively retain eDNA based on particle size. Filters may preferentially capture larger eDNA fragments, potentially favoring taxa such as fish and macroinvertebrates, especially in active sampling where larger organisms might be the primary contributors to captured material. However, confirming these mechanisms would require dedicated studies investigating how different sampling approaches influence eDNA retention and taxonomic detection across various organismal groups.

One of the main limitations of this study is the number of rivers we could practically sample in the given time, which may restrict the generalizability of our findings to other riverine systems with different environmental conditions.

Nevertheless, our statistical analyses still reveal consistent patterns across the sites, suggesting that the findings are meaningful within the studied environmental conditions. We acknowledge, however, that the small sample size may limit the extrapolation of results to broader riverine systems with different hydrological or ecological characteristics. Future studies with a larger number of sampling sites across diverse river types will be necessary to confirm the generalizability of these findings. Another important limitation of this study is the use of a single PCR replicate per sample, which may reduce the detection of rare taxa. While our primary aim was to compare the performance of two eDNA sampling methods rather than to maximize biodiversity recovery, we acknowledge that multiple PCR replicates could have enhanced results. Future studies aiming for a more comprehensive biodiversity assessment should consider increasing the number of PCR replicates to improve the detection of low-abundance species. However, trading-off of the number of river sites sampled and technical replicates performed allowed us to maximize taxonomic coverage by employing three molecular markers, enabling the detection of a broad range of taxa from microinvertebrates to vertebrates. This approach provides valuable insights into the performance of PEDS and filtration across diverse taxonomic groups, although future studies incorporating a larger number of sites, technical replicates and environmental factors (such as water temperature and pH known to affect eDNA persistence in water; Strickler et al., 2015) would be needed to strengthen the reproducibility of these results.

5. Conclusions

Notwithstanding the high number of publications in the field, eDNA metabarcoding still has many unknowns to be assessed. Interactions of DNA molecules with different adsorbent materials has been described and tested in multiple studies, but often using a single marker targeting just one or a few groups, without an appropriate comparative framework.

In this study we opted for a wide taxonomic approach, revealing that one eDNA sampling technique can outperform the other depending on the nature of the biodiversity to be described. In this experiment, GAC-PEDS appeared to be a suitable method for detecting freshwater microinvertebates as well as amphibians, while filters outperformed the former, detecting more fish and macroinvertebrates. The use of passive samplers requires more time to prepare the material, to produce the PEDS and for sterilization. In addition, it requires two visits to the sampling site, which could have a major impact if the area to be reached is distant and inaccessible. However, the material prices and time to spend on the field site are extremely low compared to using filters. Moreover, the sustainability of filtration is lower compared to PEDS, since the use of plastic ready-made cups to reduce contamination risk is widespread.

The use of PEDS enables visiting numerous sites in a single day to quickly deploy the samplers, thus covering a vast area at once. Moreover, leaving the PEDS devices in water for several days enables the capture of DNA from more elusive species or those with heterogeneous behavioral or temporal habits. PEDS may also be useful in the case of turbid water or alge-rich water, which could clog filters.

A following step to help in deciding which protocol to apply, would be to deepen the understanding of possible relationships and differences between the capture rate of eDNA from different taxa, and have a more sustainable “ready to use” PEDS with disposable materials. As we showed, methods may vary not just in the species checklists they produce, but also in the distribution of reads between samples, which in turn influences the homogeneity of replicates and replicability. PEDS appeared to be more consistent compared to filters for 18S metabarcoding. eDNA is a promising method, but despite hundreds of publications, standardization seems to be still quite distant. With this study we aimed to bring this goal closer by comparing two eDNA sampling methods already developed.

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CRediT authorship contribution statement

Gabriele Cananzi: Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Irene Tatini:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Tianshi Li:** Writing – review & editing, Methodology, Formal analysis. **Matteo Montagna:** Writing – review & editing, Supervision, Project administration. **Valentina Serra:** Writing – review & editing, Visualization, Supervision, Funding acquisition. **Giulio Petroni:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giulio Petroni reports financial support was provided by Fondazione Cassa di Risparmio di Lucca. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All data is provided in the text.

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