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Performance of eDNA Filtration Methods for Monitoring Fish Diversity in a Hyper-Tidal Estuary

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ABSTRACT

Environmental DNA (eDNA)-based monitoring has become an established and efficient method for surveying biodiversity in aquatic systems. However, there is a need to compare and standardize sampling methods across different ecosystem types, particularly complex ecosystems such as estuaries, where unique challenges exist for monitoring fish populations due to fluctuating environmental factors. Here, we compare species richness obtained from eDNA metabarcoding data using four different eDNA filtration methods: three manual filtration methods with different pore sizes (0.45, 1.2, and 5 μm) and a newly established passive method, the metaprobe. The study was applied across a salinity gradient in a hyper-tidal estuarine ecosystem. Overall, 44 fish species were detected across the four methods used. The 0.45 μm filter recovered the highest richness (39 species), then the metaprobe method (35), followed by the 1.2 μm (34) and 5 μm (33) filters. Filter performance between salinity gradients revealed that the 0.45 μm and the 1.2 μm methods recovered the highest species richness across all sampled zones. The 0.45 μm also had the most consistent detection probabilities using representative species from each zone. While the 0.45 μm method appeared to be the optimal method, each of the methods can be considered a viable and comparable option for biomonitoring in dynamic ecosystems such as estuaries and rivers. In particular, the passive metaprobe (used in a freshwater system for the first time here) performed well in comparison to the manual filtering methods despite a short deployment time. This study provides critical insights for optimizing fish diversity assessments using eDNA metabarcoding in estuarine ecosystems, providing a valuable framework for future monitoring efforts in similar systems worldwide.

1 | Introduction

Monitoring organisms in complex environments, such as fishes in estuarine systems, poses challenges associated with different sampling methodologies (Alenzi 2024). Traditional fish monitoring relies on the capture, visual identification, and counting of specimens (Radinger et al. 2019; Franco et al. 2022), using approaches such as gill/seine-netting, electrofishing, beam trawling, and recreational angling (Magaju

et al. 2023; Baldino et al. 2018), and less harmful methods such as stow net fishing (Collas et al. 2021). However, these methodologies are invasive and can be expensive to undertake. The application of DNA-based approaches, particularly environmental DNA (eDNA) metabarcoding—the simultaneous amplification/sequencing of DNA from multiple species—has become an established and efficient method for monitoring biodiversity (Taberlet et al. 2012; Ogden 2022). The value that eDNA metabarcoding can provide as a monitoring

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tool has been well evidenced in a range of aquatic ecosystems worldwide (Rees et al. 2014; McDevitt et al. 2019; Valentin et al. 2020; Sales et al. 2021).

Nevertheless, eDNA monitoring is not without its challenges, particularly when sampling in estuaries, which are characterized by complex mixing dynamics and density gradients (Sanches and Schreier 2020; DiBattista et al. 2022). In contrast to oceans, lakes, and rivers, where the water chemistry tends to be more homogeneous, estuaries encompass both marine and freshwater. This results in strong, variable horizontal salinity gradients and high levels of resuspended sediments (Williams et al. 2017). The most common tools used to capture eDNA are filter membranes enclosed within a plastic cartridge, attached to a manual or mechanical syringe/pump, which forces water through the membrane (Deiner et al. 2015; Miya et al. 2016; Capo et al. 2020). These filters come in various pore sizes, that is, the size of microscopic holes across the surface of the membrane, measured in micrometers (μm). The diameter of these pores allows water to pass through while capturing genetic material on the surface of the membrane. The pore sizes commonly selected for eDNA studies range from 0.22 to $5\mu\text{m}$ (Turner et al. 2014; Thomas et al. 2018). A complication of sampling eDNA in estuaries is the level of resuspended sediments, which can cause these filters to clog quickly (Williams et al. 2017; Sanches and Schreier 2020). Larger pores are expected to become less clogged and hence filter larger volumes of water. However, this does not necessarily result in higher DNA yields (Kumar et al. 2022), as smaller DNA fragments may not be retained (Jo et al. 2022).

While standardization guides for eDNA sampling exist (e.g., Bruce et al. 2021), they are somewhat limited (Hirsch et al. 2024), and challenges persist in adapting these protocols to environments where filter clogging remains a key limitation (Sanches and Schreier 2020). Current solutions, such as automated probes or pooling multiple filters (Hunter et al. 2019; Hendricks et al. 2023), may be cost-prohibitive for large-scale or resource-limited monitoring programs and may limit the uptake of eDNA-based monitoring. To circumvent the challenges associated with manual filtration and filter clogging, the use of passive samplers has been proposed (Bessey et al. 2021). This involves absorbent materials being placed within the water source that then passively accumulate eDNA particles over varied time frames (Jeunen et al. 2022; Verdier et al. 2022; Chen et al. 2024). Recently, a new passive sampler, the metaprobe, has provided comparable results to more conventional filtration methods (e.g., $0.2\mu\text{m}$ filters; Maiello et al. 2022, 2024). However, its efficiency has not been evaluated in estuarine or freshwater environments and has exclusively been deployed within marine environments, typically placed inside nets and cast out from fishing vessels (Maiello et al. 2024; Sbrana et al. 2024).

This study aims to optimize eDNA metabarcoding as a monitoring tool for assessing fish biodiversity in estuarine ecosystems, using the Mersey Estuary as a case study. Located in Northwest England (Figure 1), the Mersey Estuary is characterized by strong tidal forces (Bowden 1963), leading to high turbulence and sediment resuspension. Historically, it supported thriving populations of ecologically and economically

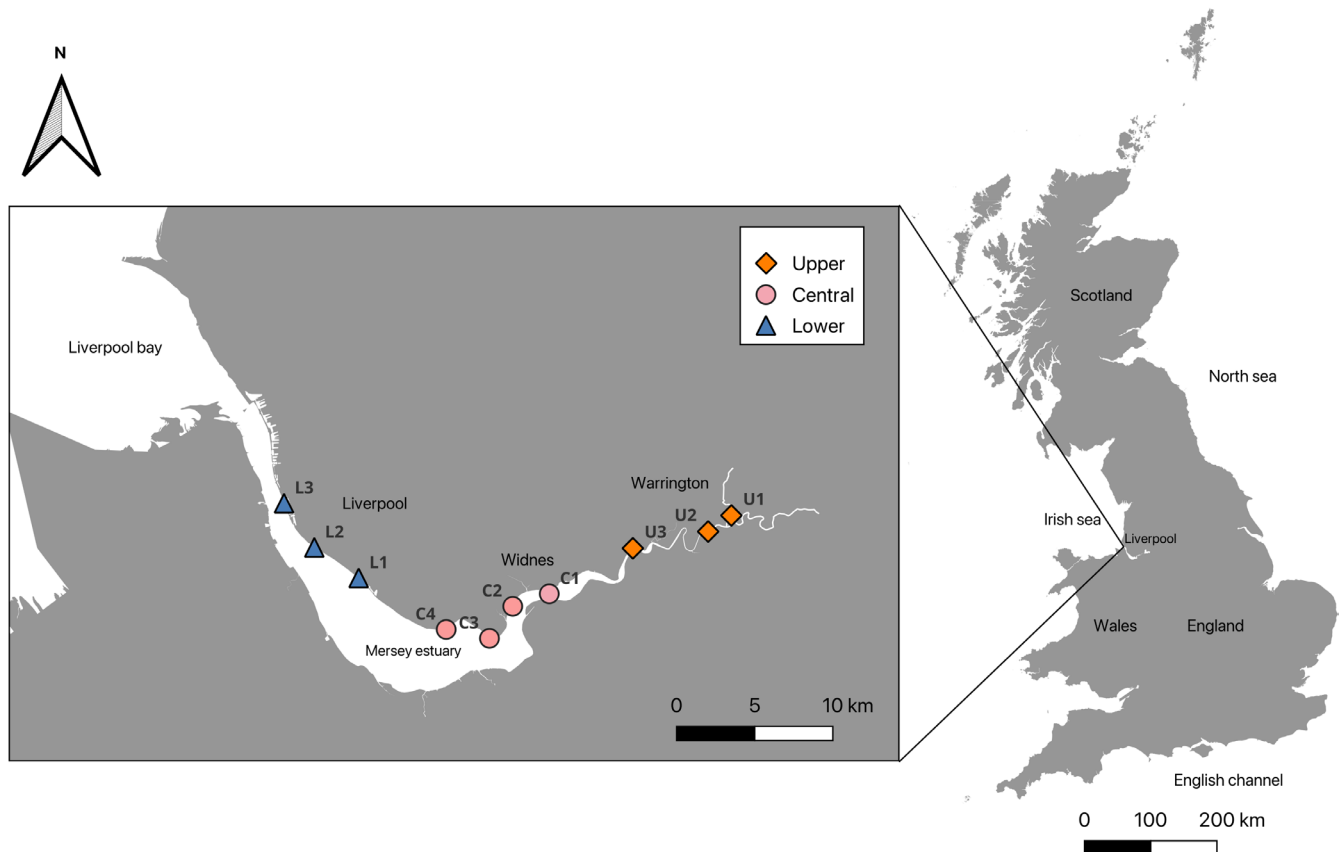


FIGURE 1 | Sampling sites on the Mersey estuary: Three in the upper zone (orange diamonds), four in the central zone (pink circles), and three in the lower zone (blue triangles).

significant, now protected species, including Atlantic salmon (*Salmo salar*), European eel (*Anguilla anguilla*), smelt (*Osmerus eperlanus*), lampreys (*Lampetra* sp. and *Petromyzon marinus*), and brown trout (*Salmo trutta*) (Joint Nature Conservation Committee 2007). However, industrial pollution during the 19th and 20th centuries led to severe habitat degradation, rendering it biologically barren for decades (Jones 2000, 2006). Recent restoration initiatives have led to significant ecological improvements (Kim and Batey 2021), with notable sightings of Atlantic salmon and other species after prolonged absences (Mawle and Milner 2003; Jones 2006; Buysse et al. 2008; Ikediashi et al. 2012). Here, we compare four filtration methods (0.45, 1.2, 5 μ m, and the metaprobe) across 10 sites spanning the estuary's lower (marine), central (brackish), and upper (freshwater) zones. These zones exhibit distinct physicochemical gradients, allowing us to assess how filtration performance varies with environmental conditions and will inform future eDNA sampling strategies for estuarine biodiversity monitoring worldwide.

2 | Methods and Materials

2.1 | Study Area

The Mersey estuary extends ~60 km from the upper tidal limit near Warrington to Liverpool Bay in the Irish Sea (Figure 1). The estuary is hyper-tidal with a spring tidal range of ~10 m at the mouth (~4 m during neap tides) and is semidiurnal with two similar tidal cycles per day (Lane 2004). Saltwater from the Irish Sea mixes with freshwater inputs, primarily from the River Mersey, which has a long-term mean discharge of 37 m³/s (cubic meters per second). Low-discharge conditions, represented by the Q95 (discharge exceeded 95% of the time), average 9.4 m³/s, while high discharge conditions (Q5, exceeded 5% of the time) reach 93 m³/s (National River Flow Archive 2024). These dynamics create a highly energetic, well-mixed estuary that structures species distributions along the river-coast continuum (Elliott and McLusky 2002).

For this study, 10 sampling locations on the northern banks of the estuary were selected, spanning the three distinct zones within the system. In the upper limits of the estuary, which are predominantly freshwater locations, three sites were sampled (U1, U2, and U3). The central areas of the estuary form an estuarine turbidity maximum (ETM), a zone of the highest turbidity resulting from the turbulent resuspension of sediment (Geyer 1993). This zone creates a large mixing section as the marine water converges with the freshwater. In total, four sample sites were selected here (C1, C2, C3, and C4) as this area is more prominent (~5 km in width) and has added complexity resulting from the ETM. The lower estuary, which is primarily a marine zone, had another three sample sites designated (L1, L2, and L3; Figure 1).

2.2 | eDNA Filtration Methods

Four distinct methods were evaluated: syringe filters with pore sizes of 0.45 μ m (Sterivex), 1.2 μ m (Whatman), 5 μ m (KX Nylon), and a passive method, the metaprobe (Figure S1).

Samples were collected during November and December 2022 and January 2023, across three sampling days per month, from 3 to 4 sites each day during high tide due to multiple sampling locations being frequently dry at low tides. Each month, three replicates for each method were taken at each of the 10 sites, for a total of 360 eDNA samples collected across the experiment. For the three methods using syringe filters, water samples were collected in individual 1 L sterilized buckets, using one bucket per replicate. A single 1 L field blank water replicate was taken at the beginning of each sampling day using sealed bottled water. Each of the syringe-based filters used a 60 mL syringe to manually pass the water through the filter membrane. Filtration of the water samples was performed on-site immediately following collection. Once each syringe filter replicate became clogged to the point when no more water could be pushed through, the volume filtered was recorded (Tables S4–S6), and the filter was sealed in an individual sterile air-tight bag and stored at –20°C in the laboratory until processed. The metaprobe method was prepared in a dedicated eDNA clean room at the University of Salford before fieldwork. Individual rolls (three rolls representing three replicates) of identical size (10 × 10 cm) medical-grade gauze were attached to the inside walls of the metaprobe sphere, with sterilized cable ties. For each metaprobe, three 50 mL Falcon tubes containing 99% ethanol were prepared for storage of the gauze after sampling. The prepared metaprobe and Falcon tubes were sealed in air-tight sterile bags until deployment at their respective sampling locations, which was immediately after completion of the syringe filtering method. The metaprobe was attached to a 20 m rope using a carabiner (Figure S2) and cast out into the water for 5 min at each site. Prior to the metaprobe deployment, a single field blank gauze replicate was placed in a Falcon tube containing 99% ethanol at the beginning of each sampling day. Immediately following deployment, the metaprobe was carefully cut open, and each of the rolls of gauze was placed in a preprepared individual Falcon tube and stored at –20°C until DNA extraction. The 5-min duration was determined based on the average time to filter one replicate for each of the other methods, therefore creating an equivalent sampling duration across all methods tested.

2.3 | DNA Extraction and Amplification

All DNA extractions were performed in a dedicated eDNA clean room and decontaminated via UV for at least 6 h before extractions by personnel wearing full Personal Protective Equipment (PPE, e.g., overalls, gloves, hair nets, masks, and disposable boots). DNA extractions followed the Mu-DNA protocol (Sellers et al. 2018) tailored for water samples for the syringe-based filtration methods and soil samples for the metaprobe filtration method. The choice of the soil extraction method was made to account for the unique characteristics of the metaprobe, as it collects and accumulates particulates, commonly suspended sediments, creating a sample composition more akin to soil. In total, 360 eDNA samples and 36 negative controls, including field and laboratory blanks, were processed.

Two different primer sets were tested: MiFish-U/E and Tele02. The MiFish-U/E primers target a hypervariable region of the

12S rRNA gene (163–185 bp) and have been used extensively in freshwater settings (Miya et al. 2015); the Tele02 primers amplify a ~167 bp fragment of the 12S gene, specifically targeting teleost fishes (Taberlet et al. 2018). Although both primers successfully amplified the target gene regions, the MiFish primers, when used on brackish and marine water samples (i.e., samples collected in the central and lower zone), amplified a predominant nontarget fragment of unknown origin (Figure S3A), whereas the Tele02 primers mainly amplified the fish-specific target fragment (Figure S3B). Due to the lack of consistency with the MiFish primers across all sampled zones, extracted DNA was amplified using the fish-specific Tele02 primers (Taberlet et al. 2018). Both a positive (*Hoplias malabaricus*, a neotropical freshwater species absent in the UK) and a negative PCR control (i.e., PCR reaction using nuclease-free water instead of eDNA) were included to account for possible tag jumping and contamination.

PCRs were prepared across six equally balanced libraries (90 rolls of gauze—15 per library, 270 syringe filter samples—45 per library, 12 field blanks—2 per library, 12 extraction blanks—2 per library, 12 PCR negative controls—2 per library, and 12 PCR positive controls—2 per library; 68 samples total per library), and PCR was performed in triplicate for each library. Each sample was amplified using the Tele02 primers, including a unique 8 bp oligo-tag attached to the forward and reverse primers and a variable number (2–4) of leading Ns (fully degenerate positions) to increase variability in amplicon sequences. PCR amplification was conducted using a single-step protocol to minimize bias in individual reactions. The PCR reaction consisted of a total volume of 20 μ L, including 10 μ L AmpliTaq Gold 360 Master Mix (1X; Applied Biosystems), 0.16 μ L of BSA (20 mg/mL), 1 μ L of each of the two primers (5 μ M), 5.84 μ L of ultrapure water, and 2 μ L of eDNA template. All PCR amplification of libraries was performed under the following thermocycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s, and a final elongation of 72°C for 5 min.

Replicates were then pooled, and samples were visualized on a 1.2% agarose gel stained with GelRed to check for successful amplification of target fragments. PCR products were then purified with HighPrep PCR Clean-up System magnetic beads using a 1.1 \times ratio for a left-sided size selection. The purified libraries were visualized on the Agilent 2200 TapeStation using High Sensitivity D1000 ScreenTape (Agilent Technologies). This indicated secondary non-target products on the right side of the target fragment, which were removed by right-sided size selection (0.8 \times ratio for all libraries).

Size-selected DNA was quantified using a Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Invitrogen). Based on the total DNA concentration, each library was diluted to 20 ng/ μ L at a volume of 50 μ L for library preparation. End repair, adapter ligation, and library PCR amplification were performed using the KAPA HyperPrep Kit according to the manufacturer's protocol. Libraries were quantified using quantitative PCR (qPCR) on a MIC qPCR system (Bio Molecular Systems) with the NEBNext Library Quant Kit for Illumina (New England Biolabs). Libraries 1 & 2, 3 & 4, and 5 & 6 were pooled in equimolar concentrations to create a final

three pools. All libraries were sequenced at 9 pM (3 sequencing runs total) on Illumina MiSeq Reagent v2 (300-cycle) kits (Illumina Inc.).

2.4 | Bioinformatic Analysis

The bioinformatics analysis was carried out using OBITools 1.2.11 (Boyer et al. 2016). Read quality was checked with *fastqc* (Andrews 2010), and low-quality ends were trimmed for downstream analysis. We used *illumina-paired-end* to merge all paired reads with a quality score > 30, and *ngsfilter* to demultiplex samples based on their unique barcodes. Sequences were filtered via *obigrep* to remove singletons and reads out of the expected length range (129–209 bp), and dereplicated via *obiuniq*. We removed chimeras with *uchime-denovo* (Edgar et al. 2011) and clustered the remaining sequences into Molecular Operational Taxonomic Units (MOTU) with *swarm* (Mahé et al. 2015), setting the threshold to $d=1$. Sequences were assigned taxonomy information using a DNA reference library dataset for fish species of the UK, derived from the NCBI GenBank and Barcode of Life BOLD databases (Meta-Fish-Lib v255). The reference dataset includes species for both freshwater and marine UK species (Collins et al. 2021).

The final dataset was manually curated to improve taxonomic assignments by checking MOTUs identified at the genus level. This was due to a small number of species being poorly annotated or not being distinguished correctly. Poorly annotated MOTUs were defined as those with ambiguous assignments, such as sequences that were only classified to high taxonomic ranks (e.g., only to family or order) and other sequences which had been assigned to species with low thresholds. Any expected species detections with low ID thresholds or MOTUs assigned only at the genus level were manually checked against the latest records on the NCBI nucleotide database to confirm their taxonomic assignment (Altschul et al. 1990). The final taxonomic assignment was conducted according to current fixed general thresholds: MOTUs were assigned at the species level when matching the reference sequence with > 98% in line with other UK fish studies (Hallam et al. 2021; Rourke et al. 2022). Overall, manual curation of the data revealed the correct identities of seven previously misassigned species or species in which the ID thresholds were unusually low. These were: the common roach *Rutilus rutilus*, dace *Leuciscus leuciscus* and common carp *Cyprinus carpio* in the Cyprinid order; the European flounder *Platichthys flesus*, common dab *Limanda limanda* and plaice *Pleuronectes platessa* in the Pleuronectiformes; and the whiting *Merlangius merlangus* in the Gadiformes.

Stringent filtering steps, performed in R v4.3.1 (R Core Team 2023), were applied to the final dataset to remove MOTUs/reads originating from sequencing errors or contamination to avoid false positives for the library using the “tidyverse” (Wickham et al. 2019) and “dplyr” (Wickham et al. 2023) packages. All non-fish reads were removed from the dataset, including non-target species (e.g., human and domestic species reads) and MOTUs that were likely to have been carried over from contamination. To remove contaminants, the maximum number of reads recorded in the controls (field collection blanks, DNA extraction blanks, and PCR blanks) was removed from all samples. To

address the potential of tag jumping, the sample data were examined to assess whether any positive control-specific MOTUs were present in any other samples, and all reads associated with the positive control were subtracted from the sample data. Finally, all MOTUs with <5 reads were removed from the final dataset (Sales et al. 2019; Murría et al. 2024).

2.5 | Statistical Analyses

All downstream analyses were performed in R v4.3.1 (R Core Team, 2023). The presence/absence data of species detected in each of the three sampling months was combined to create an overall dataset of species richness obtained from the total duration of the study sampling campaign.

To determine the overall richness obtained by each eDNA filtration method and the number of species detections shared, and exclusively detected by each sampling method, a box plot and Venn diagram were created using the R-packages: “ggplot2” (Wickham et al. 2016) and “VennDiagram” (Chen and Boutros 2011). Additionally, a Kruskal–Wallis test was performed to assess if there were significant differences in species richness among the four sampling methods, followed by Dunn’s post hoc pairwise comparisons. Rarefaction and extrapolation curves with 95% confidence intervals were used to compare the overall number of species richness recovered by each method and sampling effort for the 10 sites sampled (R-package: “iNEXT” v2.0.20; Hsieh et al. 2016).

To account for richness differences, which may be due to non-standardized volumes of water filtered by each method, a Pearson’s correlation test between the volume of water filtered and species richness was performed (Figure S4) scaling ranked subsampling (SRS; Beule and Karlovsky 2020) was used to correct the species richness based on a normalized volume of water filtered across all samples. The normalized value (C_{\min}) was selected to be the lowest volume of water filtered between the 0.45, 1.2, and 5 μm syringe filtration methods ($C_{\min} = 180\text{ mL}$). The metaprobe was excluded from this test, as the amount of water that had been processed by this method could not be quantified.

The data was visualized, including both the non-corrected and the corrected SRS values, using a combined grouped boxplot and violin plot showing overall richness by filtration method within the three zones present within the study system (Lower, Central, and Upper). Additionally, richness for the non-corrected values was also assessed on an individual monthly scale. Statistical comparisons were then performed to distinguish meaningful increases or decreases in species richness between the four methods (overall non-corrected richness) and the three methods (overall corrected richness) across the three zones with a Generalized Linear Mixed Model (GLMM) using the function *glmer* from the R-package “lme4” (v1.1-35; Bates et al. 2015). The GLMM was fitted using Maximum Likelihood Estimation (MLE) with Laplace Approximation (Raudenbush et al. 2000). The response variable, “Species Richness,” was modeled using a Poisson distribution with a logarithmic link function. “Site” was included as a random effect in the model to estimate the variability in Species Richness among different sites, beyond what

can be explained by the nested fixed effects (Zone/Method). The model was formulated as follows: *Species Richness* ~ *Zone/Method* + (1|*Site*) (Schielzeth and Nakagawa 2013). The GLMM was implemented using a nested approach to determine if species richness significantly increases or decreases when using a specific method inside and among the different zones (Method: filtering, designated by pore size of membrane: 0.45 μm , 1.2 μm , 5 μm , or passive metaprobe). The GLMM requires a baseline for comparisons to be made (intercept); here, the intercept was set as the species richness recovered in each zone using the 0.45 μm pore size. Therefore, all comparisons are made against the 0.45 μm method (e.g., the values associated with “Central: Method 1.2 μm ” are compared to “Central: Method 0.45 μm ”). The same GLMM was then also applied to the corrected richness values.

To compare the performance and consistency of the four eDNA filtration methods for selected species detections, we conducted two statistical analyses following Sales et al. (2020): (1) species detection probabilities (the likelihood of detecting a given species if it is present using a specific filtration method) and (2) species cumulative detection probabilities (the cumulative probability of detection with increased sampling). A single-season occupancy model (MacKenzie et al. 2002) was applied, where detection histories were constructed with each eDNA replicate as a sampling/observation event. As the objective was to directly compare the filtration methods, we did not consider any other competing models (Sales et al. 2020). Accumulation curves were computed as per MacKenzie and Royle (2005) to assess the number of replicates/sampling events that would be required to reach a detection probability of 0.95 (Sales et al. 2020) for each filtration method. We performed these analyses on three representative species: European eel *Anguilla anguilla*, European sprat *Sprattus sprattus*, and the common bream *Abramis brama*. These species were selected as representatives of species known to inhabit all three zonal gradients (European eel), only the lower zone (marine; European sprat), and only the upper zone (freshwater; common bream). Analyses were conducted using the R-package “Unmarked” (Fiske and Chandler 2011).

3 | Results

The resultant dataset obtained after quality checking and filtering consisted of 6,114,309 reads in total (Table S1), which allowed the detection of 44 unique fish species (Table S2). We recovered 36 species (2,924,358 reads) in November 2022, 38 species (2,333,900 reads) in December 2022, and 33 species (856,046 reads) in January 2023.

The overall species richness detected varied significantly across the filtration methods (Figure 2A; Kruskal–Wallis test: $\chi^2 = 16.58$, $df = 3$, $p = 0.0009$). Although ca. 60% of the species (26 out of a total of 44 species) were common across methods (Figure 2B), the 0.45 and 1.2 μm filters detected ca. 89% and 77% of the total species richness and also provided significantly higher per sample richness estimates compared to the 5 μm filters ($Z = 2.63$ – 2.91 , $p < 0.05$) and the Metaprobe ($Z = 2.83$ – 3.11 , $p < 0.05$; Figure 2). Despite their lower per sample richness,

the 5 μm filter and the Metaprobe were still able to detect ca. 75% and 80% of the total species, respectively (Figure 2; and see Table S2 for details on which species were detected by each method).

Rarefaction and extrapolation curves show a higher projected asymptotic species richness for the 0.45 μm method, which plateaus at 40 sites, followed by the metaprobe, 1.2 μm , and 5 μm methods, which plateau at 30 sites (Figure 3). Finally,

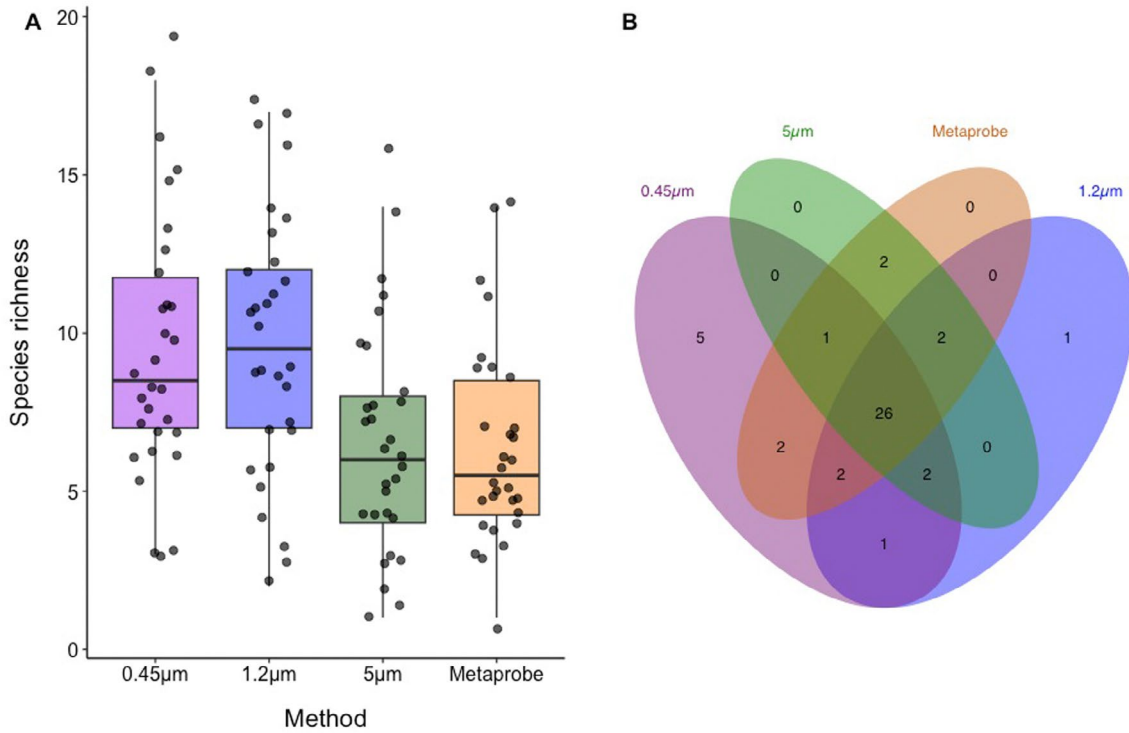


FIGURE 2 | Overall species richness detected by each eDNA filtration method (designated by filtering pore size for the filtration methods; A) and the total, shared, and exclusively detected numbers of species by each method (B).

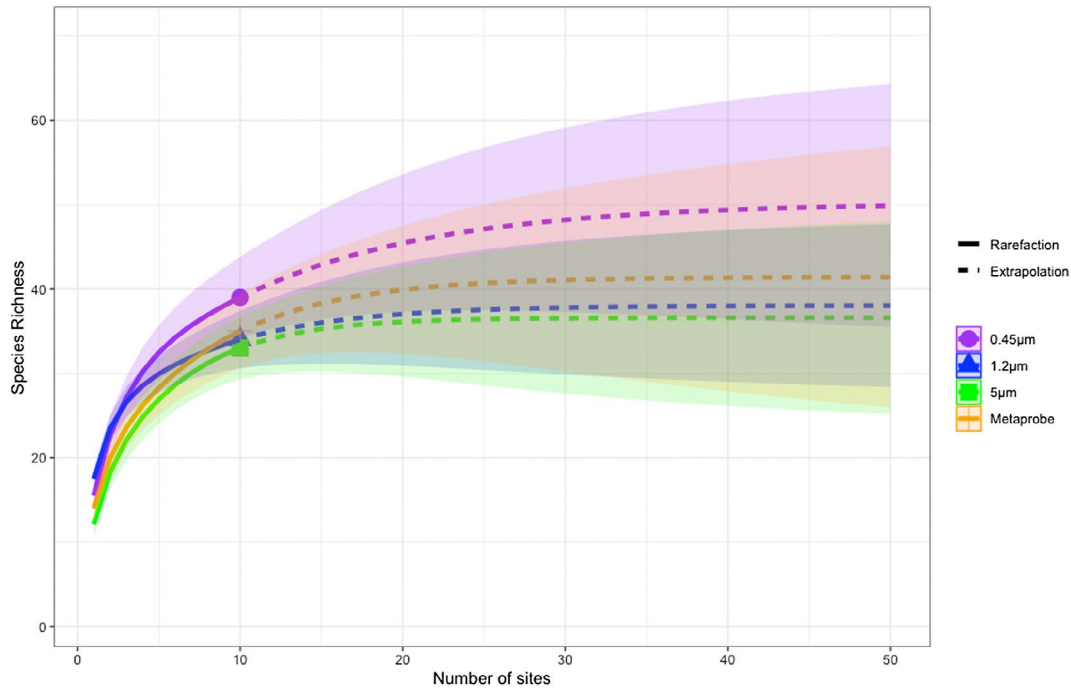


FIGURE 3 | Rarefaction and extrapolation curves of species richness detected by filtration method according to the number of sampled sites. Solid “Rarefaction” lines represent the richness detected, and dotted “Extrapolation” lines represent the estimated sampling effort required based on sampling sites to reach a sampling plateau.

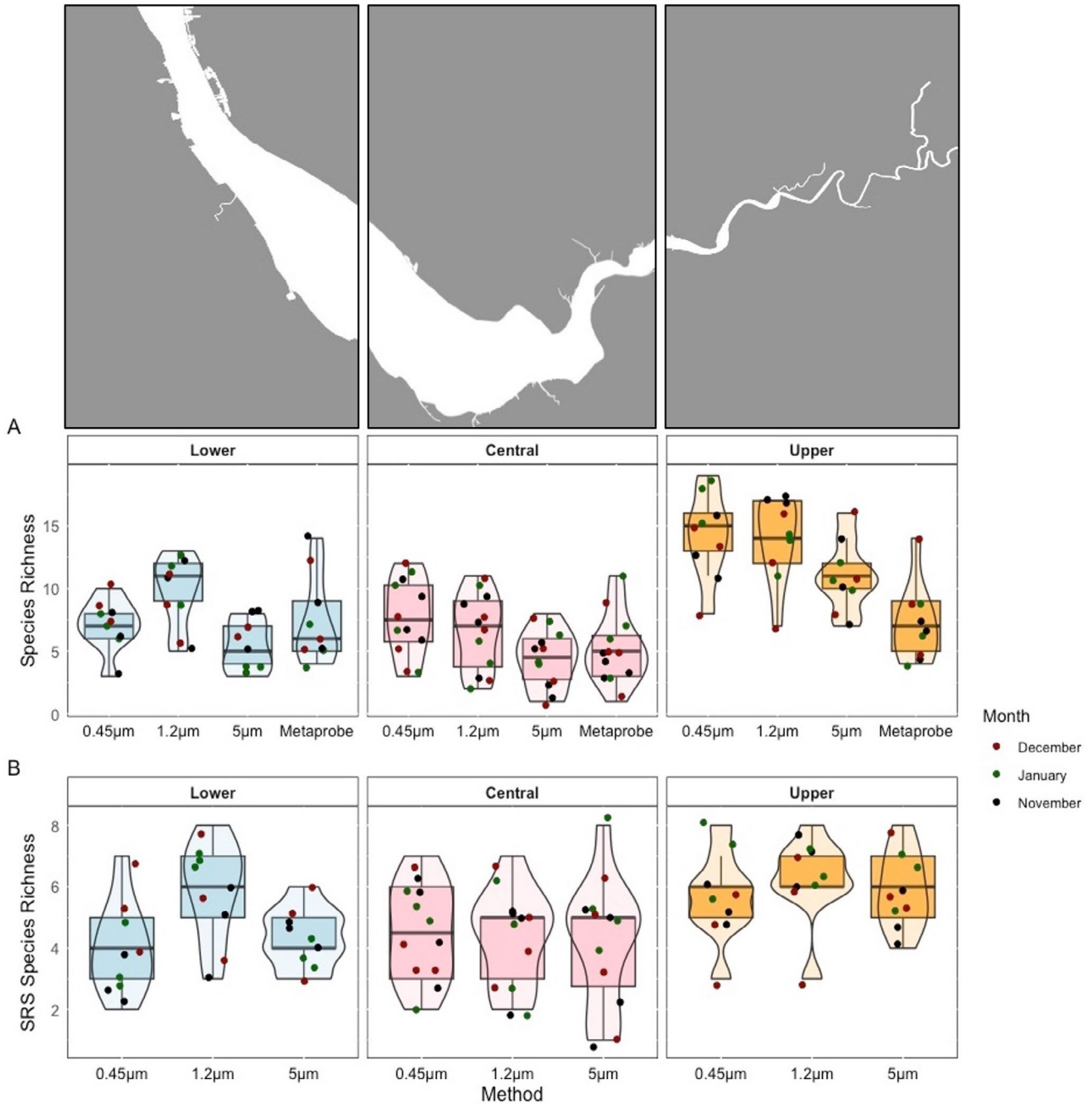


FIGURE 4 | Grouped boxplot and violin plot of overall species richness for each replicate recovered across November 2022, December 2022, and January 2023 by each filtration method within a specific zone (Panel A). Jittered points overlaid showing the monthly variation in species richness detection. Panel B shows the richness corrected using the SRS function described above, correcting the richness based on a filtered volume of 180 mL (Metaprobe excluded, as there is no recorded volume for this method).

species richness and volume filtered were significantly correlated (Pearson's $R=0.73$, $p < 0.001$; Figure S4).

When analyzing the overall non-corrected species richness per filtration method between habitat types, the best performing filtration methods were the 0.45 and 1.2µm filters (Figure 4A). Across the central and lower zones, the 5µm filter detected the lowest overall richness. The metaprobe detected the lowest overall richness in the upper zone, while detecting slightly more in the central and lower zones compared to the 5µm. It is worth noting

that there are differences in species composition among monthly replicates, possibly due to environmental heterogeneity, methodological variability, or stochasticity influencing detection rates.

The same pattern was observed in each month, with higher richness obtained from the 0.45µm and the 1.2µm methods within the upper zone compared to the other zones (Figure S5). Once the volumes filtered are scaled ($C_{\min} = 180$ mL), no significant differences were observed between the three syringe filtration methods (Figure 4B).

The GLMM was applied to the overall non-corrected data following goodness of fit assessment using Akaike Information Criterion (AIC: 600.9) and Bayesian Information Criterion (BIC: 637.1). Overall, the greatest declines in species richness were observed with the 5 μm and metaprobe methods, while the 0.45 and 1.2 μm methods showed the lowest declines, with no statistical difference between these two methods (Table S7). The 0.45 μm filtration method in the upper zone showed significantly higher baseline species richness (estimate = 2.649, $p < 0.001$), detecting approximately 2.6 more species per site on average than the other method-zone combinations, after accounting for site-level variation. Species richness significantly decreases in the central and lower zone (central: estimate = -0.6227, $p = 0.0001$; lower: estimate = -0.6937, $p = 0.0001$). Additional significant decreases in species richness were observed when comparing the 5 μm method in the central zone (estimate = -0.5706, $p = 0.0009$), the metaprobe in the upper zone (estimate = -0.6776, $p = 0.000009$), and the metaprobe in the central zone (estimate = -0.3947, $p = 0.0162$). Minor, nonsignificant increases in species richness were found using the 1.2 μm method in the lower zone (estimate = 0.3184, $p = 0.0523$) and the metaprobe in the central zone (estimate = 0.0458, $p = 0.7930$).

The GLMM was then applied to the overall corrected SRS richness data (which excludes the metaprobe) following the AIC (369.7) and BIC (394.7) assessment. These results indicate that

while the 0.45 μm method in the upper zone provided robust detections, alternative methods showed neither significant improvements nor substantial declines in species richness across the study area (Table S8). Comparisons revealed no significant differences in species richness between zones overall (Central: estimate = -0.231, $p = 0.238$; Lower: estimate = -0.348, $p = 0.110$). Method-specific analyses showed that the 1.2 μm filtration approached significance for increased richness in the lower zone (estimate = 0.387, $p = 0.073$) compared to the intercept, while demonstrating no significant effects in upper (estimate = 0.094, $p = 0.629$) or central zones (estimate = -0.038, $p = 0.846$). The 5 μm method showed no significant differences from the intercept in any zone (Upper: estimate = 0.038, $p = 0.845$; Central: estimate = -0.077, $p = 0.695$; Lower: estimate = 0.080, $p = 0.729$).

The 0.45 μm filter shows more consistent and higher detection probabilities across the four methods and three representative species (Figure 5A). In particular, the detection probabilities for European sprat (marine—lower zone) and common bream (freshwater—upper zone) were higher for the 0.45 μm filter, while it was comparable to the 1.2 μm filter for the migratory European eel (across all zones). The cumulative detection probability for the 0.45 μm (Figure 5B) suggests that as few as three (for common bream) and a maximum of five (for European sprat) replicates would be required to reach a 0.95 detection probability, consistently fewer replicates than any of the other three filtration methods.

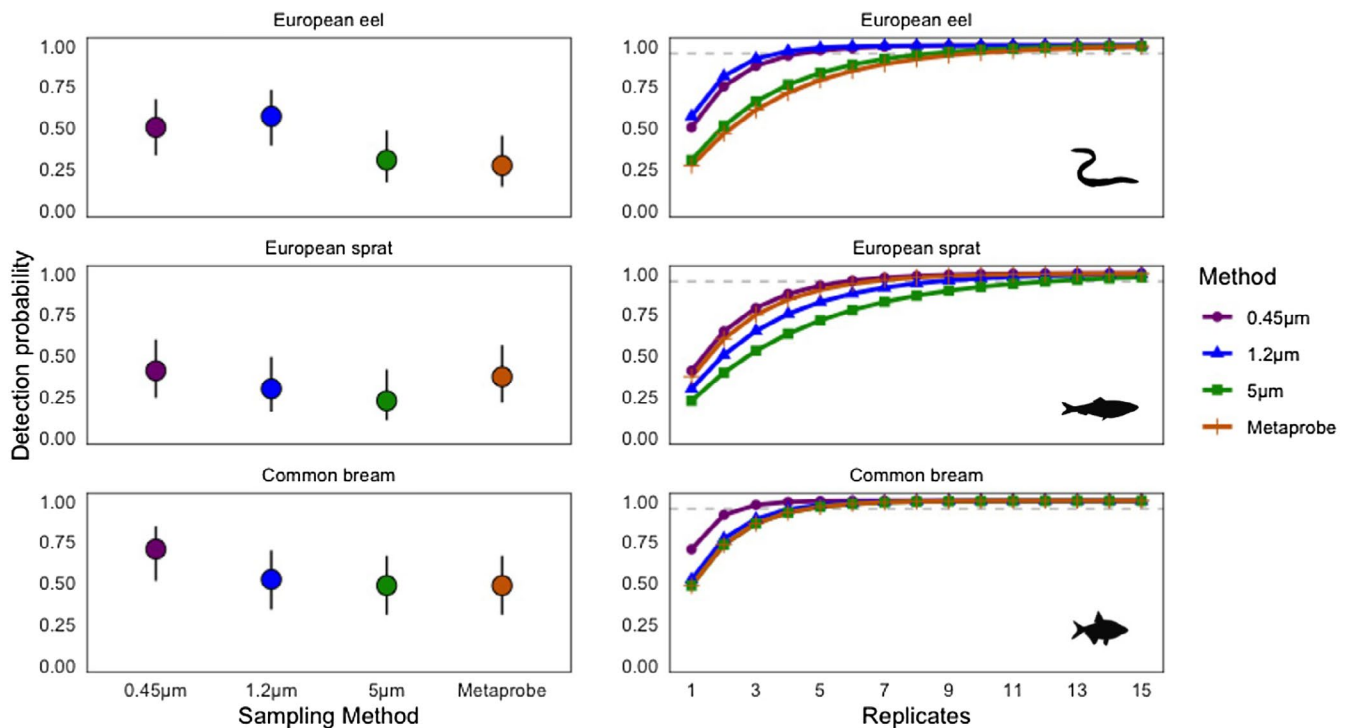


FIGURE 5 | Estimated detection probabilities of each filtration method for each of the three focal species (top: European eel, *Anguilla anguilla*; middle: European sprat, *Sprattus sprattus*; bottom: Common bream, *Abramis brama*) with 95% confidence intervals (A panels). Species-specific cumulative detection probability with an increasing number of sample replicates; the horizontal dashed line shows a probability of 0.95 of species detection based on the number of replicates collected (B panels).

4 | Discussion

This study evaluated the performance of four eDNA filtration methods (three manual, one passive) for monitoring fish diversity in the Mersey estuary, a hyper-tidal system with pronounced spatiotemporal variability in water chemistry, hydrodynamics, and turbidity (Blott et al. 2006). Along a ~60 km estuarine gradient, we compared their efficacy and applicability for routine fish monitoring, focusing on method performance in recovering species richness across zones, detecting sensitive species, and addressing practical implementation challenges. In general, the 0.45 μm filters detected more species: 87% of the overall fish species found across all four methods, with the other three methods detecting > 70% of the observed fish community.

4.1 | Performance of the eDNA Filtration Methods

Water samples at the upper zone (freshwater; sites U1–U3) appeared less turbid than the seaward zones, which aligns with both common descriptions of estuarine hydrodynamics (Uncles et al. 2002; Dyer et al. 2004) and personal observations that each sample replicate had less visible sediment present. This potentially explains the lower filter clogging rate observed during the filtration process in this zone (Wittwer et al. 2018) and the larger volume of water filtered at each of these sites compared to the sites in the other zones (central and lower; Tables S4–S6). Overall, there was a higher species richness found in the freshwater upper zone (22) than in the mixed central (13) and marine lower zones (14) and a consistently higher richness obtained by the 0.45 μm method. This is in agreement with previous studies suggesting that higher volumes of filtered water will likely yield higher species richness estimates (Lopes et al. 2017; Tsuji et al. 2019; Bessey et al. 2020; Schabacker et al. 2020). This is further substantiated by a clear correlation between volume filtered and species richness (Figure S4) and pairwise comparisons (Table S3).

The SRS correction altered the species richness values recovered by each manual filtration method, making it more consistent across zones. However, some disparities in species richness between the environmental gradients remain, particularly in the upper (freshwater) zone, where a higher richness was still found. This highlights that the volume of water filtered might not be the sole cause of this (Stoeckle et al. 2017; Barnes et al. 2021; Altermatt et al. 2023). Ecological factors such as habitat structure, salinity gradients, and environmental connectivity (Leibold et al. 2004; Lin et al. 2024), and methodological factors such as sampling effort, the accuracy of primers, and the completeness of reference databases (Ruppert et al. 2019) can all influence species detections.

When using the metaprobe, any issues experienced with filter clogging are eliminated (Maiello et al. 2022, 2024). In this study, the metaprobe was deployed for only a short period of time (5 min) to coincide with the manual filtration time frames, yet it detected a similar number of species in comparison to the other methods (35). The results obtained with the metaprobe were not as consistent as with the 0.45 μm , yet this approach presents several advantages. Moving towards passive eDNA

collection methods reduces field time, manual labour, and removes the need for specialized equipment such as filters and pumps (Mariani et al. 2019; Bessey et al. 2021), making it a potentially more accessible approach (Bessey et al. 2022). Passive samplers have been utilized effectively in marine environments in recent years (Chen et al. 2022; van der Heyde et al. 2023; Cai et al. 2024), with this being the first study highlighting the metaprobe as an effective passive eDNA filtration method within estuarine and freshwater river systems to our knowledge. Pore size appears to affect detection rates, with the 5 μm filter detecting significantly fewer species. This could be due to smaller DNA fragments becoming lost during filtration (Turner et al. 2014; Barnes et al. 2021; Jo et al. 2022). The metaprobe and 1.2 μm filter showed slightly higher (though non-significant) richness in the lower zone, potentially reflecting methodological advantages in certain environmental conditions (e.g., less impediment from high sediment loads). These findings emphasize that filter selection should consider both ecological context (e.g., zone-specific DNA distribution patterns) and practical constraints, rather than absolute performance alone.

Previous studies have demonstrated that increased sampling effort can enhance species detections in aquatic environments, but the effect depends on how effort is allocated, either through expanding spatial coverage (more sites; Willoughby et al. 2016; Perry et al. 2024) or increasing replication at the same site (Mauvisseau et al. 2019). This is further confirmed by our results, which highlight that while increased sampling effort spatially with additional sites may enhance species richness estimates, the gains may not justify the substantial increase in effort required (e.g., marginal increase in richness with the 0.45 μm through significant additional effort). Furthermore, access restrictions or difficult terrain (as was the case in this study) can limit the number of sampling sites. Therefore, increasing the number of replicates at a site (five instead of three; Furlan et al. 2016; Szitenberg 2019) or using longer deployment times in the case of the metaprobe (e.g., 30 or more instead of 5 min) could yield more robust and efficient richness estimates (Erickson et al. 2019; Yates et al. 2023).

While no obvious patterns emerge of why certain methods fail to detect certain species, some species detections associated with certain methods are noteworthy. The application of eDNA monitoring approaches for rare species detections has been widely documented (Jerde et al. 2011; Beng and Corlett 2020; Duarte et al. 2023). Within the data, four UK Biodiversity Action Plan (BAP) priority fish species are present: the European eel, brown trout, Atlantic salmon, and the European smelt (Joint Nature Conservation Committee 2007). Of these species, it is estimated that the Atlantic salmon population numbers in the Mersey are extremely low (Ikediashi et al. 2012), and the population numbers of the European smelt are currently unknown but also assumed to be low, as there are currently no catch records to generate a population estimate. Out of the four eDNA filtration methods, only the 0.45 μm successfully detected all four species. The European eel and brown trout were detected by all methods, whereas the Atlantic salmon was detected by the 0.45 μm and metaprobe, and the European smelt was exclusively detected by the 0.45 μm method. Additionally, an invasive species of concern, the pink salmon (*Oncorhynchus gorbuscha*), was also exclusively detected using the 0.45 μm method. Detecting rare,

elusive, or newly invading species may indicate that the 0.45 μm method is a more effective approach, especially if the detection of such species of concern is a key aspect of the study's goals (Sepulveda et al. 2019).

4.2 | Challenges, Limitations and Practicality of Each Filtration Method in Estuarine Systems

Although the filtration method pore size increased to 1.2 and 5 μm , this increase did not result in larger volumes of water being filtered. One potential explanatory reason for this was a noticeable difference between the filter designs. The Sterivex 0.45 μm filters have a cylindrical form, with excess space for around ~3 mL of water to be held within the filter cartridge, whereas the Whatman 1.2 μm and the KX Nylon 5 μm filters used here are disc form filters, with no excess space in the filter cartridge. The extra space inside the Sterivex filters may allow some sediments to stay in suspension, unlike the disc form filters, which trap all particles directly on the membrane. This could explain why the disc filters tend to clog more quickly. In addition, it was found that laboratory processing of the 0.45 μm filter cartridges was more simplistic than the other filters, due to the design of the product (the presence of an easily removable cap, not present in the 1.2 and the 5 μm filters used here). The 1.2 and 5 μm filter cartridges had to be cut open with excessive force using wire cutters, which added effort and time to the process, thus making the 0.45 μm easier to use.

A drawback of using the metaprobe over the more commonly used syringe/filter-based methods is that the volume of water that has passed through the metaprobe is challenging to quantify and would need further investigation. Assessing the water flow and discharge rate within a study system before metaprobe deployment could allow for comparisons between flow/discharge rates and species richness estimates. For example, periods of higher flow/discharge rates could be targeted, in which data via tide gauges can be freely accessed (Hibbert et al. 2015). In addition, this method has some potential barriers to its more widespread use, as it requires 3D printing of the probes. This may not be an easily accessible option for all end users. The gauze material, once it has been used to capture eDNA, also requires a storage solution, typically ethanol (Maiello et al. 2022, 2024; Neave et al. 2023). Currently, there is no data available comparing storage solutions for alternative approaches to ethanol, which again may cause issues in the accessibility of ethanol for purchase, transport, and use in the field. This needs to be carefully planned and considered before use.

Finally, the interpretation of the data collected from this dynamic system should be underpinned by the fact that the richness recovered here is based on eDNA presence and not necessarily the presence of a physical organism (Bohmann et al. 2014). The high-tide sampling in this strongly tidal system (Bowden 1963) could facilitate cross-zone eDNA transport via hydrological movement (Deiner and Altermatt 2014; Ruppert et al. 2019; Laporte et al. 2022; Van Driessche et al. 2024). This may lead to false-positive species detections in certain zones (e.g., freshwater species detected in marine habitats or vice versa; Burian et al. 2021). However, studies on eDNA transport dynamics show variability in spatial and temporal estimates of eDNA transport

ranges and degradation rates (Marshall et al. 2021; Jo and Yamanaka 2022). Therefore, further investigations are needed to establish conclusive estimates. This emphasizes the need for integrative approaches that include hydrodynamic modeling and particle tracking methods to simulate hydrological patterns and the subsequent dispersal of eDNA (Andruszkiewicz et al. 2019; Pont 2024). This would allow for more accurate interpretation of eDNA results, better identification of actual species presence at a particular location, and contribute towards building more effective monitoring strategies.

5 | Conclusions

The four filtration methods detected a high number of species, ranging from 39 (0.45 μm Sterivex filter) to 33 (5 μm KX Nylon filter) over three months of sampling. Overall, the 0.45 μm filter emerged as the optimal choice, delivering the most consistent results across the salinity gradient for all 10 sampling sites. It yielded the highest species richness, detected the most UK BAP species, and exhibited the lowest variability in species detection probabilities. Nevertheless, all four methods were effective for eDNA monitoring of fish biodiversity. We also demonstrated that if passive filtration is the desired method for logistical and/or budgetary reasons, the metaprobe is an effective method that can be deployed in estuarine and freshwater systems.

Author Contributions

A.D.M. and I.C. acquired funding for the study. J.M.J. carried out the eDNA sampling. J.M.J. performed eDNA laboratory work. J.M.J. carried out the bioinformatic analyses. J.M.J., N.G.S., C.B., I.C., and A.D.M. analyzed the data. J.M.J., N.G.S., C.B., I.C., and A.D.M. wrote the manuscript, with all other authors (A.D., A.W., and P.E.R.) contributing to editing and discussions.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in JmJackman27/Mersey_eDNA_data and at https://github.com/JmJackman27/Mersey_eDNA_data.git.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** edn370206-sup-0001-AppendixS1.docx.