

**SPECIAL ISSUE** Environmental DNA: New Horizons in Aquatic Sciences, Terrestrial Ecology, and Beyond



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# Multi-Seasonal eDNA Metabarcoding Highlights a Resurgence in Fish Diversity Across a Severely Impacted Estuarine Ecosystem

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## ABSTRACT

Aquatic ecosystems have been in an alarming state of decline for decades. In particular, estuarine ecosystems have experienced long-term declines in fish diversity due to factors such as habitat degradation, pollution and altered hydrology. Monitoring these systems is often limited by the difficulty and cost of conventional survey methods. In this study, we applied environmental DNA (eDNA) metabarcoding to assess fish diversity in the Mersey Estuary (UK), a historically severely impacted system. Monthly water samples were collected over a year (2023–2024) across saline, brackish, and freshwater zones. Overall, 69 species were detected, surpassing both historical (46 species) and recent (39 species) records. Richness was highest in the upper freshwater zones, and several species were recorded returning to the estuary for the first time since pre-industrial times (~1850s). Peak species richness occurred during winter (December–February). Species composition varied monthly and spatially, though not consistently by season. Approximately 15% of detected species were diadromous, with the endangered Atlantic salmon (*Salmo salar*) being frequently detected during its key spawning period (October–December), for example. The results presented here indicate a resurgence in estuarine fish diversity in the Mersey and highlight eDNA metabarcoding as a rapid, sensitive tool for monitoring both contemporary and historically absent species, supporting conservation and restoration efforts.

## 1 | Introduction

The world's aquatic ecosystems are in a perpetual state of pressure from anthropogenic stressors (Dubois et al. 2018), with freshwater and estuarine systems being heavily impacted (Kennish 2002; Mahoney and Bishop 2017; Dudgeon and Strayer 2025). The deterioration of these habitats is driven by factors such as pollution, overfishing, the introduction of invasive species, habitat loss, and is augmented by climate change (Cai et al. 2021). The heterogeneous chemistry of estuarine waters, formed

through the mixing of freshwater and terrestrial loads from rivers with coastal saltwater, creates a unique range of habitats that support a diverse array of species (Ray 2005). These conditions are especially beneficial for the early life stages of many marine organisms (Tagliapietra et al. 2009). Beyond supporting early life stages, estuaries are also crucial for the migratory behaviour of fishes (Potter et al. 2015). Migratory patterns, along with other behaviours such as spawning and foraging, are integral for understanding ecosystem health, as they provide insights into how species interact with and adapt to their changing

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environment (Franco et al. 2008; Whitfield et al. 2023). For many diadromous species, coastal ecosystems serve as key stopover points where individuals rest, feed and prepare for migration, playing an integral role in their life cycle (Chalifour et al. 2019). Between 1970 and 2020, populations of migratory freshwater fish have plummeted by an average of 76%, with the most pronounced losses observed in Europe (Deinet et al. 2024).

Monitoring fish in complex ecosystems, such as estuaries and rivers, can be challenging, expensive and potentially yield inconsistent results depending on the method used (Fullerton et al. 2010; Elliott et al. 2022). Environmental DNA (eDNA) metabarcoding has emerged as an established and effective technique for monitoring fish biodiversity globally (Garlapati et al. 2019; Pawlowski et al. 2020; Schenekar 2023), with the advantages of being non-invasive and providing either detections comparable to, or superseding, more traditional fish monitoring methods (McDevitt et al. 2019; McElroy et al. 2020). There is a plethora of eDNA studies demonstrating the ability and potential of eDNA metabarcoding for the effective monitoring of fishes across both marine and freshwater ecosystems (e.g., Stat et al. 2017; Bessey et al. 2020), and increasingly in estuarine ecosystems also (Jackman et al. 2025; Cunnington et al. 2024). However, few studies have attempted to capture full-season cycles of fish diversity using eDNA metabarcoding in estuarine systems (Stoeckle et al. 2017; Gibson et al. 2024; Cunnington et al. 2024). Seasonality, driven by factors such as temperature, rainfall, and day length, plays a crucial role in estuarine ecosystems. These periodic shifts can influence breeding cycles, food availability and habitat conditions (Arevalo et al. 2023). The dynamic nature of estuaries further shapes these processes: salinity fluctuates hourly with tides, episodically with rainfall and seasonally with evaporation and heating (Carter et al. 1979). Such variability makes migration an essential but challenging behavioural adaptation for many species.

This study investigates fish biodiversity in the Mersey Estuary (UK; Figure 1), an example of an estuarine ecosystem that has suffered from long-term and lingering anthropogenic degradation since the Industrial Revolution (Lallias et al. 2015). Rapid industrialisation and urbanisation within the catchment led to substantial alterations in water quality, habitat structure and hydrodynamics, including severe organic and chemical pollution, loss of intertidal habitats, channel modification and changes to freshwater inflows (Moore 1978; Wilson et al. 2005; Hartnett et al. 2006). It is estimated that before the Industrial Revolution, the Mersey was home to a wide variety of fish species, with the estuary playing an important role in supporting both freshwater and marine species, including migratory species like Atlantic salmon *Salmo salar*, European smelt *Osmerus eperlanus*, and European eel *Anguilla anguilla* (Mersey Rivers Trust 2021). Post-Industrial Revolution, however, the Mersey was considered biologically dead up until ~50 years ago (Jones 2000, 2006). However, the implementation of new water quality legislation in the 1980s through the efforts of the Mersey Basin Campaign (Alexander and Harper 1989) aimed to improve the water quality and environmental conditions of the Mersey and its surrounding area. The effort focused on reducing industrial pollution, improving sewage treatment and wastewater management, restoring river habitats, enhancing biodiversity, and raising public awareness about environmental issues. As a result of these efforts, significant

improvements in the overall health of the Mersey estuary were achieved.

Before widespread industrialisation throughout the system, which is estimated to span from the 1760s to 1860s (Mokyr 1988; Stokey 2001; Clark 2005), around 46 species were estimated to occur in the Mersey, and between 2015 and 2020, 39 species were documented as having returned (see Table A3 for full species lists; Mersey Rivers Trust 2021). Among the 46 pre-industrial species, several are diadromous (~17%), relying on the estuary as a crucial migratory route. In the more recent 2015–2020 survey (39 species), around ~25% of the returning species were diadromous, indicating some recovery of ecological connectivity within the estuary. However, the estimated abundances of these migratory species are likely still far from pre-industrial levels, especially considering their disrupted migration routes and altered environmental conditions. While their return is promising, the continued fragility of these populations underscores the ongoing challenges in restoring full ecological health to the system. This means that monitoring programmes would need to take this into account and limit invasive approaches and general handling of the specimens. For this reason, eDNA metabarcoding offers the potential for applying a non-invasive method to obtain estimates of biodiversity in the system.

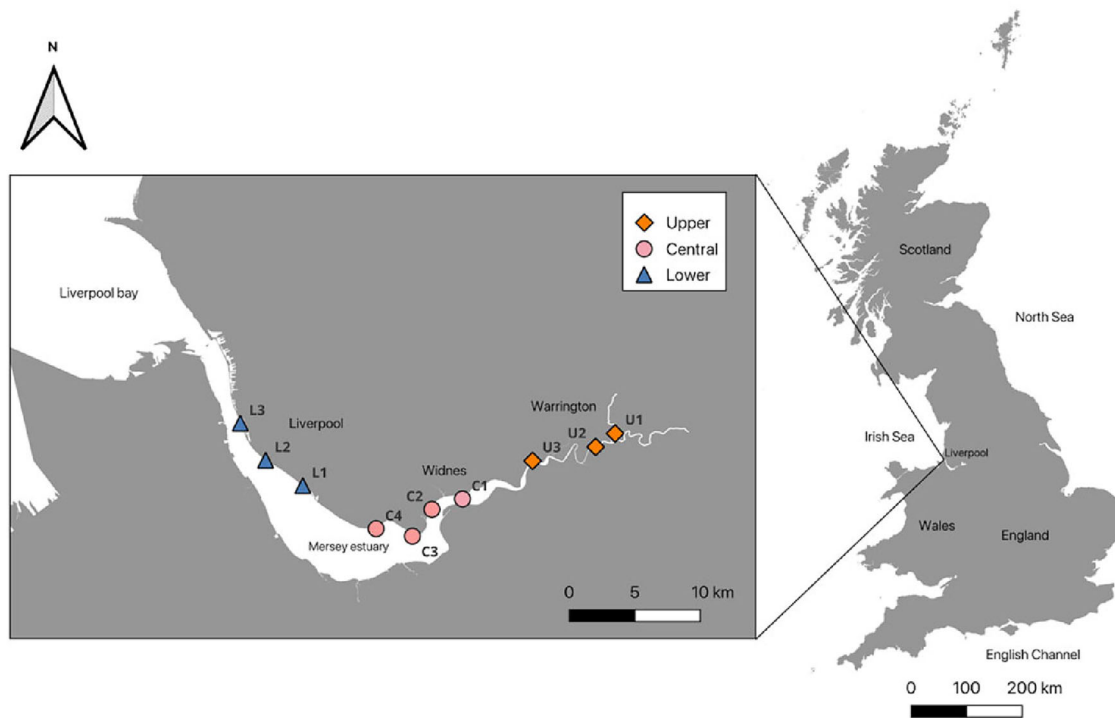
This study aims to apply eDNA metabarcoding as a fish monitoring method across large spatial and temporal scales in the Mersey Estuary across a marine to freshwater gradient, collecting samples during a 12-month period using an optimised eDNA protocol (Jackman et al. 2025). In doing so, we aim to detect broad-scale fish diversity, compare seasonal differences in richness estimates, and highlight the presence/absence of key diadromous species (UK Biodiversity Action Plan [BAP] species; Joint Nature Conservation Committee 2007). The findings will reinforce the prospect of eDNA metabarcoding as a routine biomonitoring method in similar systems worldwide and help inform the many organisations currently working to preserve and increase fish biodiversity within this ecosystem.

## 2 | Methods

For this study, 10 sampling locations were selected along the northern bank of the Mersey Estuary (53.314474° N, –2.899608° W; Figure 1; Jackman et al. 2025). Three sites were sampled in the upper freshwater zone (U1, U2 and U3) and three in the lower marine zone (L1, L2 and L3). As the central zone is the largest area of the entire system (~5 km in width) and features added complexity due to the convergence of freshwater and marine water, four sites (C1, C2, C3 and C4) were selected here.

### 2.1 | Sample Collection

Samples were collected during 12 consecutive months from September 2023 to August 2024. Samples were collected during three subsequent days in each month (Table A1), always during high tide due to multiple sampling locations being frequently dry at low tide and to harmonise the hydrodynamic conditions at the time of sampling, as much as possible (Jackman et al. 2025). Five



**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).

water sample replicates were taken at each of the 10 sites using 0.45 µm Sterivex filters (Merck Millipore), following an optimised protocol comparing different filtering methods (Jackman et al. 2025). Water samples were collected in individual 1 L sterilised buckets from surface water at each site, using one bucket per replicate, and filtered using the Sterivex 0.45 µm filters. A single 1 L field blank water replicate was taken at the beginning of each sampling day using sealed bottled water. Each filter 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 filter replicate became clogged, volume filtered was recorded (Table A2), and the filter was sealed in an individual sterile air-tight bag and stored at  $-20^{\circ}\text{C}$  in the laboratory until processed. In total, 636 samples were collected across the study, including 600 eDNA samples (10 sites  $\times$  12 months  $\times$  5 replicates) and 36 field negative controls (3 days  $\times$  12 months).

## 2.2 | DNA Extractions, PCR Amplification and Sequencing

All DNA extractions were performed in a dedicated eDNA clean room at the University of Salford, decontaminated via UV for at least 6 h before extractions; all personnel wore full PPE. The DNA extractions followed the Mu-DNA protocol (Sellers et al. 2018) tailored for water samples (see Jackman et al. 2025 for further details). During each extraction session, an extraction negative control (extraction reagents excluding the DNA template) was included to account for possible contamination.

DNA was PCR amplified using the fish-specific Tele02 primers (forward: 5'-AAACTCGTGCCAGCCACC-3'; reverse: 5'-

GGGTATCTAATCCCAGTTTG-3'; Taberlet et al. 2018) according to the protocol described in Jackman et al. (2025). Both positive (*Hoplias malabaricus*, a neotropical freshwater species absent in the United Kingdom) and negative (nuclease-free water) PCR controls were included to account for possible tag jumping and contamination. Due to a high number of samples (600 eDNA samples, 36 field blanks, 24 extraction blanks, 16 PCR blanks and 16 positive controls), PCRs were prepared across eight libraries as follows: Libraries 1–4 each contained 75 eDNA samples, four field blanks, three extraction blanks, two positive controls and two PCR negative controls (86 samples in total). Libraries 5–8 each contained 75 eDNA samples, five field blanks, three extraction blanks, two positive controls and two PCR negative controls (87 samples in total). PCRs were performed in triplicate for each sample. Each sample was amplified using 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 minimise bias in individual reactions. The PCR reaction consisted of a total volume of 20 µL, including 10 µL AmpliTaq Gold 360 Master Mix (1X; Applied Biosystems), 0.16 µL of BSA (20 mg/mL), 1 µL of each of the two primers (5 µM), 5.84 µL of ultra-pure water and 2 µL of eDNA template. All PCR amplifications of libraries were 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 visualised 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 1:1.1 ratio for a left-sided size selection. The purified libraries were visualised 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 (1:0.8 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/ $\mu$ L at a volume of 50  $\mu$ 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). All prepared libraries were sequenced using the Illumina NovaSeq X Plus PE150 (Novogene, UK) with a minimum of 6 GB of raw data generated per library.

### 2.3 | Bioinformatic Analysis

The bioinformatics analysis was completed using the OBITools metabarcoding software 1.2.11 (Boyer et al. 2016). Read quality was checked with *fastqc*, and low-quality ends were trimmed for downstream analysis. We used *illumina-paired-end* to merge all paired reads showing 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 chimerae 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 United Kingdom, 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). Due to limitations in the reference database, the final dataset had to be manually curated using the NCBI nucleotide database (Altschul et al. 1990) to improve taxonomic assignments by checking MOTUs identified at the genus level (see Jackman et al. 2025 for further details). All subsequent analyses were performed in Rv4.3.1 (R Core Team 2023).

MOTUs/reads originating from sequencing errors or contamination were removed, as well as non-fish and non-target species (e.g., human and domestic species reads). To address MOTUs that contained potential contaminants, the maximum number of reads recorded in the controls (field collection blanks, DNA extraction blanks and PCR blanks) was removed from all samples. Finally, all MOTUs with < 5 reads were removed from the final dataset. 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). After all filtering stages, all libraries were separated into their respective sampling months (September 2023–August 2024).

### 2.4 | Data Analysis

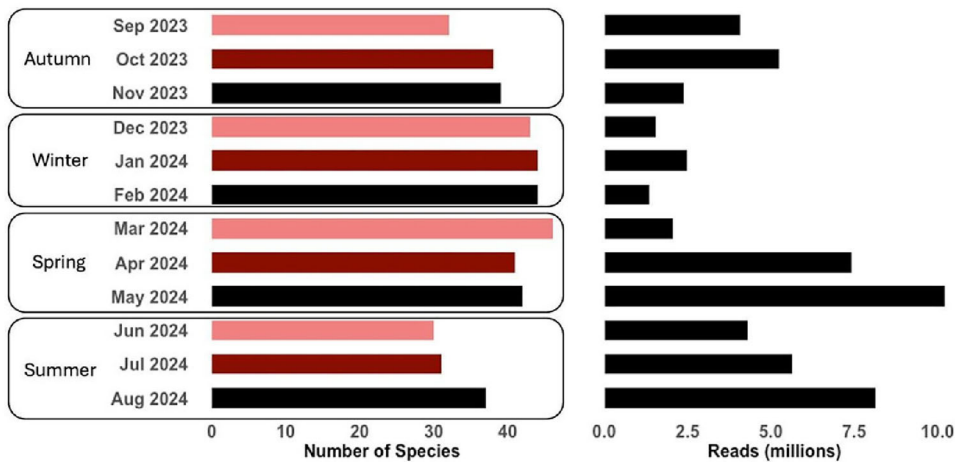
Seasonality of the data was determined based on the typical seasonal cycles in the United Kingdom, following the classification by the UK Met Office: autumn (September, October and November), winter (December, January and February), spring (March, April and May) and summer (June, July and August) (UK Met Office 2024). These periods are also aligned with key migratory patterns of estuarine fish species, such as autumn migrations to overwintering sites (Graham and Harrod 2009) and spring migrations associated with spawning, as observed in several species across UK estuaries (Ibbotson et al. 2013).

To evaluate the relationship between sequencing depth and biodiversity, we conducted a Kendall's rank correlation test (Kendall's tau) between the total read count (sequencing depth) and total observed species richness across all samples. This non-parametric correlation analysis was performed using the *cor.test()* function v4.3.1 (stats package; R Core Team 2023), chosen due to the non-normality of both variables as confirmed by Shapiro–Wilk tests (total reads:  $W = 0.69266$ ,  $p < 0.001$ ; total richness:  $W = 0.94934$ ,  $p < 0.001$ ). The analysis included all 12 sample months to assess the global relationship independent of temporal or spatial factors.

The number of species detected through the eDNA survey was compared with the species listed in the Mersey Rivers Trust (2021) report, which details species estimated to inhabit the Mersey pre-Industrial Revolution and species detected during a 5-year survey between 2015 and 2020. This comparison was performed using a Venn diagram (VennDiagram v1.7.3; Chen and Boutros 2011). The brook lamprey *Lampetra planeri*, European river lamprey *Lampetra fluviatilis* and mullet species, the thick-lipped grey mullet *Chelon labrosus* and thin-lipped mullet *Chelon ramada*, were excluded from this comparison because each species pair is indistinguishable using the target 12S rRNA region. Since we could not determine whether all or only one species from each genus was detected, we could not conclusively state whether they remain exclusive to the pre-industrial list or were also present in the eDNA list.

Species richness across the 10 sampled sites and seasons was visualised using boxplots (generated with ggplot2 v3.1.5; Wickham et al. 2016). Differences in richness between seasons were assessed using the Kruskal–Wallis test, followed by Dunn's post hoc test for pairwise comparisons. Each species from the eDNA survey and the 2021 report has been classified to a physiological saline tolerance (F = freshwater, M = marine and B = brackish) using classifications as described per species in FishBase (Froese and Pauly 2025; Table A3). In addition, the number of species detections in each season was assessed using a heatmap, using the ggplot2 package.

To assess the overlap in species detected between sampling months and seasons, UpSet plots were created using the 'UpSetR' package v1.4.0 (Conway et al. 2017). UpSet plots provide an efficient visualisation of intersections (number of species) between sets (sample sizes), making them particularly useful for identifying shared and unique species across different sampling periods. To assess temporal changes in species composition, analyses were first conducted at the monthly scale before examining broader



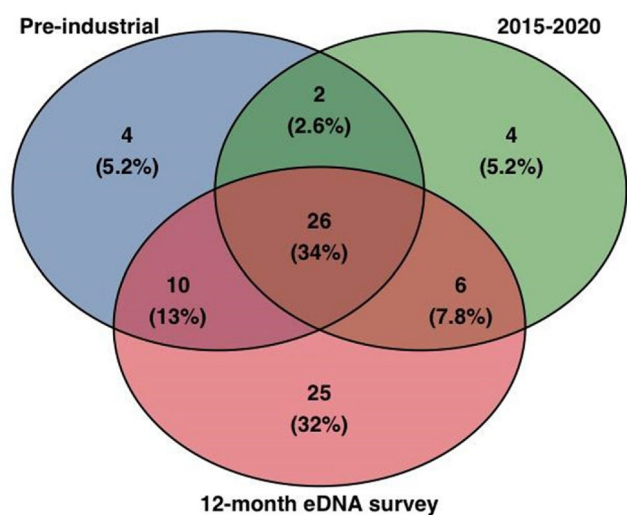
**FIGURE 2** | Total number of species and reads (x-axis) recovered by each of the 12 sampling months by season (y-axis).

patterns at the seasonal level using non-metric multidimensional scaling (NMDS). A PERMANOVA was performed using the Jaccard dissimilarity index to test for statistical differences in species composition across the 12 sampling months and the four seasons, and was applied to test for spatial variation between zones within each month. This approach first allowed for an assessment of fine-scale temporal variation, capturing potential short-term shifts in community structure which may not be visible on a broader, seasonal scale. NMDS ordinations were generated using the ‘vegan’ package v2.6.8 (Oksanen et al. 2007) to visualise patterns of similarity and dissimilarity across months and seasons. This is followed up by a pairwise comparison, conducted using the pairwiseAdonis function to assess the differences in species composition between each pair of months and seasons.

Finally, notable migratory species (e.g., protected UK BAP fish species), the season and site they were detected have been visualised using an alluvial plot, using the package ‘ggalluvial’ (Brunson 2023). For this comparison, *Lampetra* spp. was included in the analysis as *L. fluviatilis* is a key migratory species (but with the caveat that this could be *L. planeri*; see above). This was also performed for all detected species classified as freshwater/brackish or marine/brackish (Table A3), showcasing their physiological saline tolerance and the estuarine habitat zone in which they were detected.

### 3 | Results

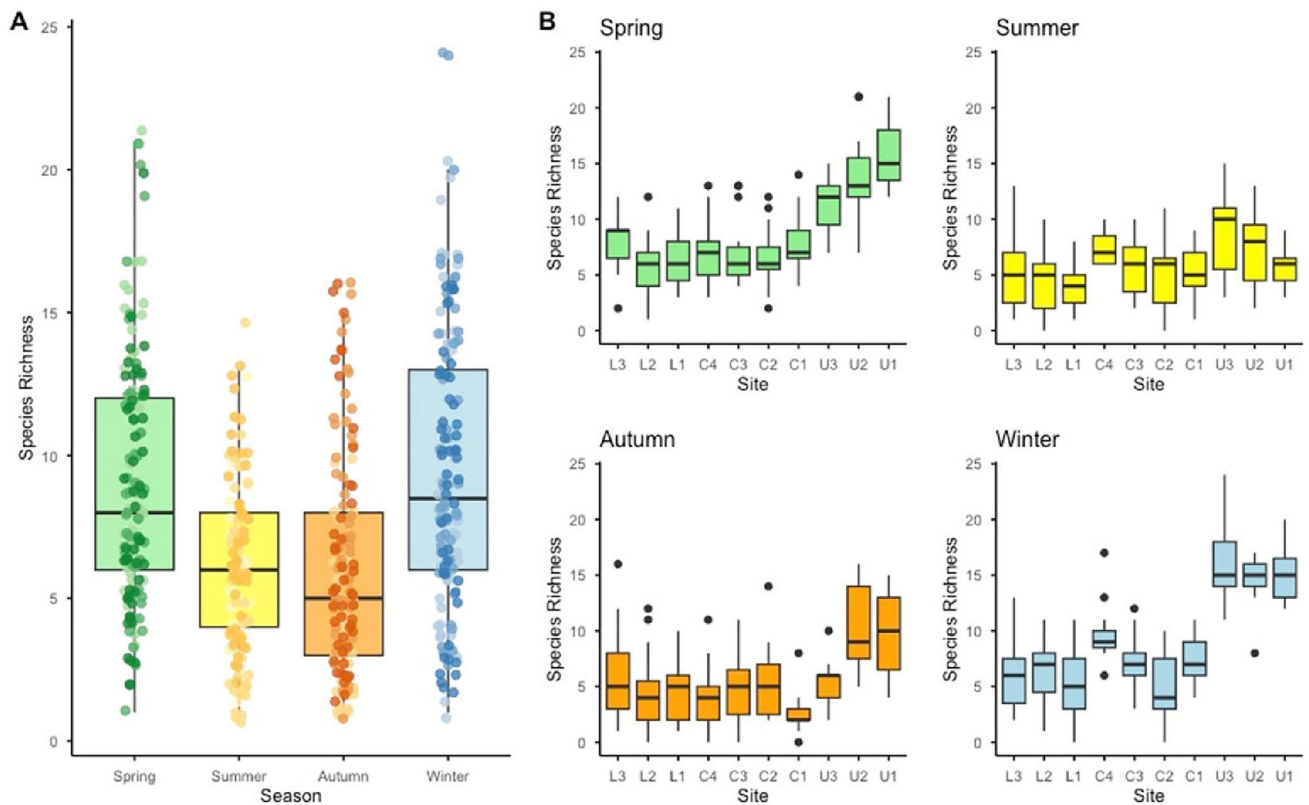
The eDNA dataset obtained after quality-checking and filtering allowed for the detection of at least 69 unique fish species in total (Table A3). *Lampetra* spp. and *Chelon* spp. were retained at the genus level for subsequent analyses (see Section 2). The highest number of species was detected in March 2024 ( $n = 46$ ) and the lowest in June 2024 ( $n = 30$ ; Figure 2). The Kendall’s tau correlation test revealed a weak but statistically significant positive correlation between total read count and species richness ( $\tau = 0.34$ ,  $p < 0.001$ ; Figure A1). However, four of the months with generally the lowest read counts returned the highest species richness values (Figure 2).



**FIGURE 3** | Venn diagram illustrating the number of species shared between, and uniquely detected in, each of the compared species’ lists. The pre-industrial period extends up to the ~1850s, while the 12-month period spans September 2023 to August 2024.

The species lists from the Mersey Rivers Trust (2021) report, alongside the species detected in the eDNA survey for this study, document a total of 81 unique species across three species lists (Table A3). A Venn diagram (Figure 3) visualises the species overlap and exclusivity across these datasets. Of the total species, 26 species are shared among all three lists (~34%). In addition, 25 species were exclusively detected through the eDNA survey (32%), while 4 species were unique to the pre-industrial estimates (5.2%) and 4 species were exclusive to the 2015–2020 survey data (5.2%).

The four species that are exclusive to the pre-industrial list are the burbot *Lota lota* (now extinct within the United Kingdom; NatureServe and Freyhof 2024), torpedo ray *Torpedo* sp., shore rockling *Gaidropsarus mediterraneus*, and the European sea sturgeon *Acipenser sturio* (deemed ‘possibly extinct’ in this region; Gessner et al. 2022). The four species that are exclusive to the 2015–2020 survey are the butterflyfish *Pholis gunnellus*, sea lamprey



**FIGURE 4** | Overall species richness between seasons across the sampling year, with jitter points representing the variation in richness between months within seasons (A). Panel species richness detected at each sample site for each season of the year (spring, summer, autumn and winter; B).

*Petromyzon marinus*, poor cod *Trisopterus minutus* and the tub gurnard *Chelidonichthys lucernus*.

Species richness variations across the sampled seasons, sites and months are evident (Figure 4A,B). Across all seasons, species richness is consistently highest in the spring and in the winter (Figure 4A). Richness at the sample sites across the seasons shows a higher richness in the upper zone (U1–U3) compared to the central (C1–C4) and lower (L1–L3) zones (Figure 4B). In spring and summer, richness gradually increases from the lower to the upper sites, with the highest values observed between U1 and U3. Autumn follows a similar trend, although there is more variability in richness at the central sites. Winter shows the most pronounced separation, with the upper sites maintaining the highest richness, while richness at lower and central sites remains more constrained, with lower median values and fewer outliers.

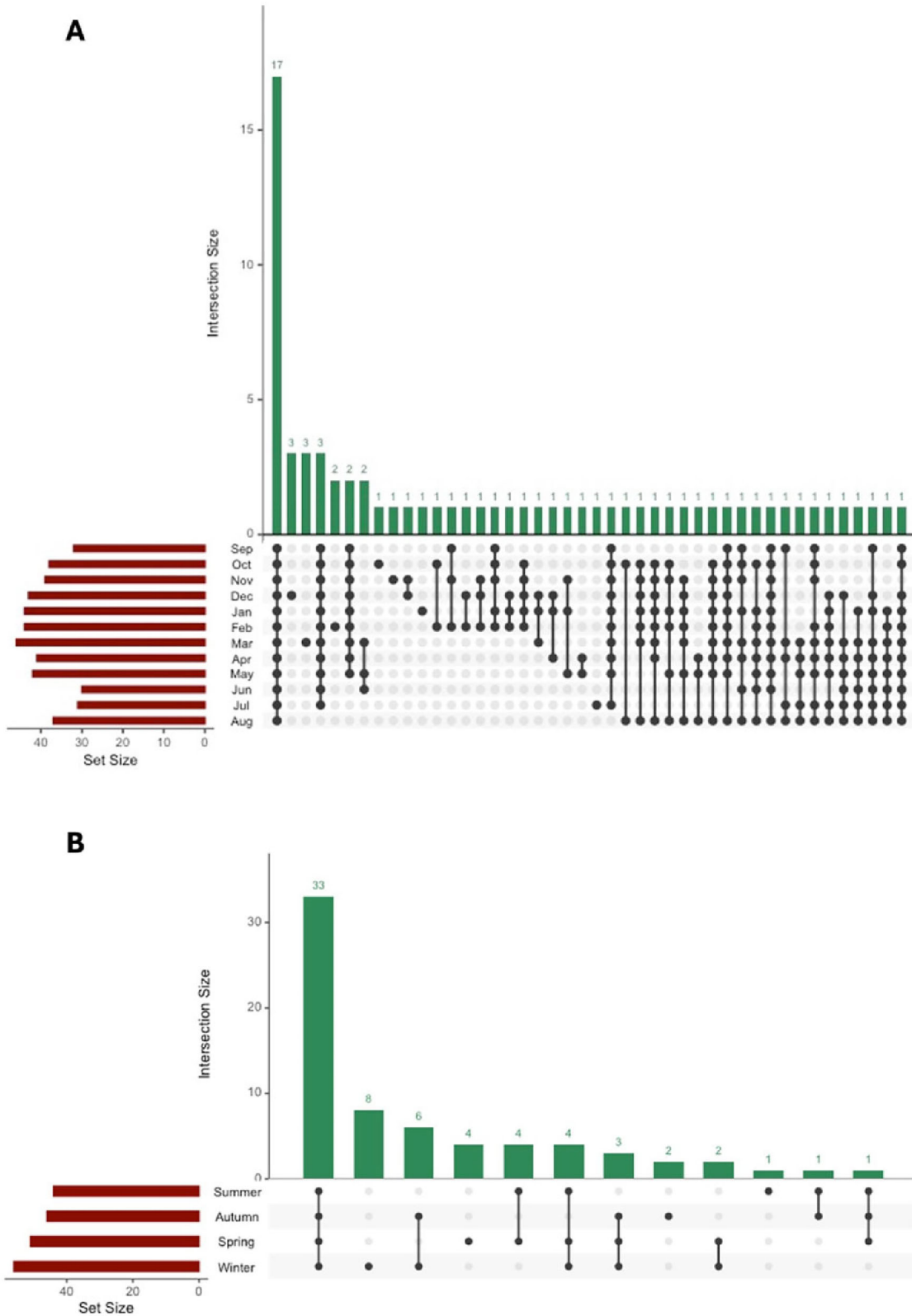
Overall, species richness varied significantly across the four seasons ( $\chi^2 = 83.415$ ,  $df = 3$ ,  $p < 0.001$ ; Figure 4A). Dunn's post hoc pairwise comparisons indicate that species richness differs significantly between multiple comparisons, except for spring versus winter and summer versus autumn (see Table A4 for all comparisons). The lack of significant differences between spring and winter, as well as summer and autumn, suggests more stable richness levels within these seasonal pairs, whereas transitions between other seasons show stronger shifts in species richness.

Species detection varied across both individual sampling months and broader seasonal scales (Figure 5A,B). In total, 12 species

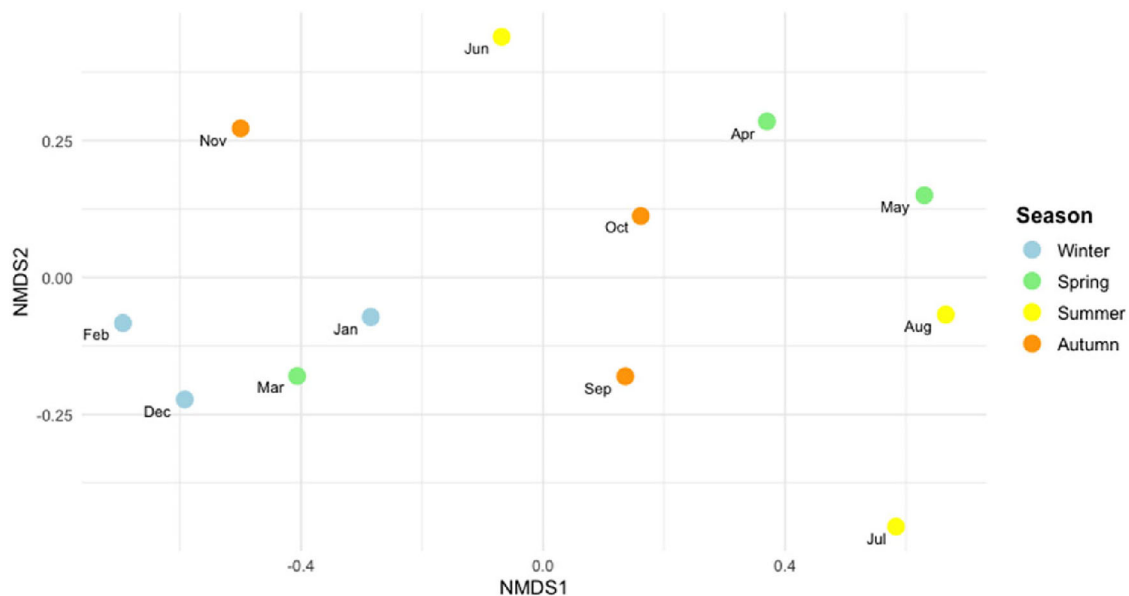
were detected exclusively within individual months (Figure 5A), representing approximately 17.3% of the total richness. When considering seasonal patterns (Figure 5B), the highest number of exclusive detections occurred in winter, with eight species (~12% of the total richness). Spring had four exclusively detected species (~5.8%), autumn had two (~3%), and summer had the lowest, with only one species (~1.5%) (Table A3).

The eight species exclusively detected in winter were European hake *Merluccius merluccius*, pollack *Pollachius pollachius*, longspined bullhead *Taurulus bubalis*, tench *Tinca tinca*, Atlantic horse mackerel *Trachurus trachurus*, corkwing wrasse *Symphodus melops*, turbot *Scophthalmus maximus* and lesser pipefish *Syngnathus rostellatus*. The four in spring were Crucian carp *Carassius carassius*, crystal goby *Crystallogobius linearis*, common seasnail *Liparis liparis* and European anchovy *Engraulis encrasicolus*. The two in autumn were big-scale sand smelt *Atherina boyeri* and striped red mullet *Mullus surmuletus*. The single exclusive detection in summer was the starry smoothhound *Mustelus asterias*.

When assessing temporal species composition changes, a PERMANOVA analysis revealed that species composition varied significantly across the 12 sampling months ( $F = 5522.8$ ;  $p = 0.001$ ; Figure A3) and significantly between the sampled zones within each month (Table A5). This result suggests that species assemblages shift meaningfully over time, indicating strong temporal community structuring. All post hoc pairwise comparisons between months can be seen in Table A6. When fish community composition was compared across the four seasons, it was found



**FIGURE 5** | Upset plots of the number of species detected by month (set size) and exclusively between months (intersection size) (A) and the number of species detected by season (set size) and exclusively between seasons (intersection size) (B).



**FIGURE 6** | A non-metric multidimensional scaling (NMDS) analysis was performed using Jaccard dissimilarity (stress = 0.071). The plots show all eDNA samples aggregated by sampling month and coloured by different seasons.

that differences were not significant (PERMANOVA  $F = 1.5172$ ;  $p = 0.159$ ; Figure 6). A heatmap summarising species detections across seasons further illustrates this, showing that the majority of species are frequently detected year-round (Figure A3).

Across the dataset, several migratory species, which are also listed on the UK BAP list, were detected. The European eel *A. anguilla* was present across all sampling months and seasons and at all sampling sites, and the brown trout *S. trutta* was detected in all seasons and sites (the month of July being the only exception). Atlantic salmon *S. salar* was found in winter (December, January and February) at sites U1, U2, U3, L1, and L2, and in autumn (October and November) at sites U1, U2 and U3. Detections of *Lampetra* spp. were recorded in spring (April) at site L1 and in winter (December–January) at sites U3 and C1. European smelt *O. eperlanus* occurred across all seasons at sites U1, U3, C2, C3, C4 and L3 (Figure 7).

Of the 69 detected species (Table A3), 20 are classified as freshwater/brackish tolerant and 37 are classified as marine/brackish tolerant. Of the 20 freshwater/brackish species, 31.2% were detected in the central zone and 27.1% were detected in the lower zone. Of the 37 marine/brackish species, 34.7% were detected in the central zone and 24% were detected in the upper zone (Figure A4).

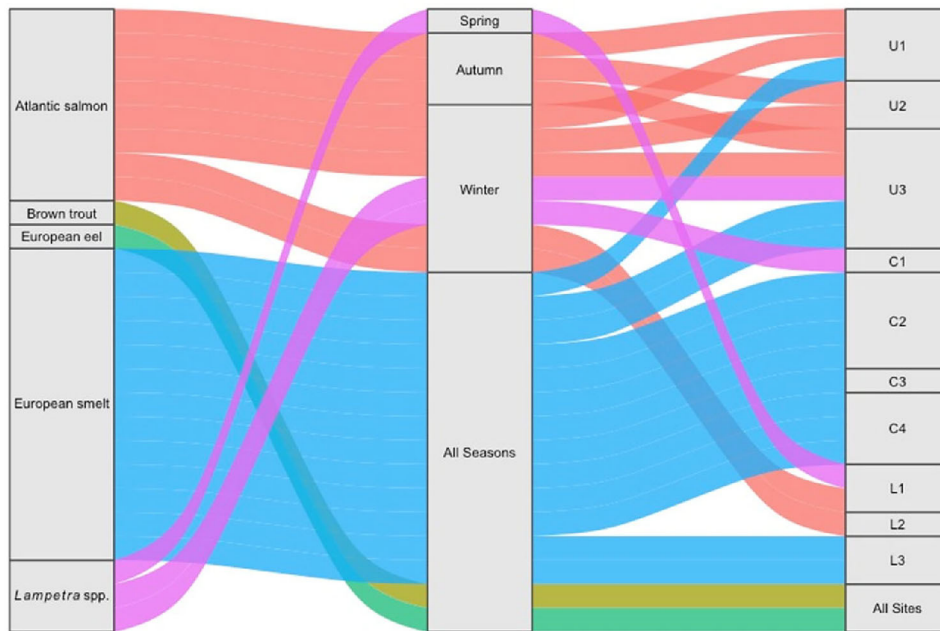
## 4 | Discussion

With climate change intensifying, estuarine ecosystems face mounting pressures from rising sea levels (projected to increase by +1 m this century), more frequent storm surges, and ocean warming (with temperatures potentially rising by +4°C by 2100; IPCC 2021). These shifts exacerbate existing anthropogenic stressors, including altered river flows, increased nutrient/sediment loads and pollution from industrial and urban sources, legacies of the Industrial Revolution that persist in many systems (Clausen

and York 2008; Arthington et al. 2016). Such cumulative impacts have led to widespread declines in coastal and estuarine fish populations (Kennish 2002; Whitfield et al. 2018), underscoring the need for efficient, scalable monitoring tools like eDNA metabarcoding. In this study, we assess fish biodiversity in a heavily modified post-industrial estuary. Despite historical degradation, our data reveal a rich assemblage of species across seasons, many of which have reappeared after decades of absence, a likely result of the restoration initiatives implemented in the 1980s (Kim and Batey 2021).

### 4.1 | Species Richness and Fish Diversity in the Mersey

In total, at least 69 distinct species of fish were identified across 12 months of sampling. The number of species detected here exceeded all previous estimates, with all current documented species lists recovered through more traditional methods featuring a maximum of ~46 species recorded (Mersey Rivers Trust 2021). All species detected through eDNA analysis were cross-examined against expected species for the geographic location and are consistent with the anticipated composition for these environments (Baldock and Dipper 2023). At least five UK BAP priority fish species were detected: the European eel, the brown trout, the Atlantic salmon, the European smelt and *Lampetra* spp. (Joint Nature Conservation Committee 2007), representing an increase in species detected in comparison to previous eDNA work conducted during November 2022–January 2023 (Jackman et al. 2025). The consistent identification of UK BAP priority species further underscores the power of eDNA to detect species of conservation concern, many of which may exist in low populations or occupy complex habitats (Beng and Corlett 2020). This is further substantiated by the detection of thick-lipped grey mullet *C. labrosus* or thin-lipped mullet *C. ramada* (designated here as *Chelon* spp. due to indistinguishable 12S rRNA gene sequences; Cunningham et al. 2024) and the transparent goby *Aphia minuta*,



**FIGURE 7** | Alluvial plot illustrating detections of returning migratory species of conservation concern, as listed under the UK Biodiversity Action Plan, and their associated sampling site(s).

a set of species/genera previously assumed to be absent due to insufficient habitat quality (Mersey Rivers Trust 2021). The Mersey Estuary's ability to support more habitable conditions for these fish species underscores its ecological significance and highlights clear signs of ecological recovery over the past four decades (Hawkins et al. 2020).

We see a generally higher observed richness within the upper estuary sampled zones, in which the water chemistry is predominantly freshwater. This trend is present over the four seasons (Figure 4). In highly dynamic and turbid environments such as tidally energetic estuaries, the suspended particles within a water sample can quickly obstruct the filter and inhibit the filtration process (Barnes et al. 2021; Hallam et al. 2021). However, water volume filtered is likely not the sole contributing factor toward the increased richness estimates (Jackman et al. 2025). Ecological factors, such as habitat structure, salinity gradients, and connectivity between saline gradient zones, can significantly influence species richness (Leibold et al. 2004; Lin et al. 2024). Furthermore, only a weak correlation between species richness and sequencing depth was observed (Figure A1), suggesting that other biological and ecological factors may drive richness variation, and the balanced library preparation ensures that differences in sequencing depth are unlikely to bias richness estimates between the sampled zones. In addition, the classification of species (Table A3) to either freshwater or marine indicates more marine species present in the data, and the alluvial plot, showcases prevalent abiotic influence of potential eDNA transport from the marine zone to the upper (25% of marine species detected in the upper freshwater zone; Figure A4) contributing to the elevated richness observed in this zone.

The observed richness is notably higher during the winter and spring seasons, which may provide valuable insights into the ecological dynamics influencing eDNA persistence in the water

column. While some winter-exclusively detected species (e.g., European hake, pollack and turbot) are typically associated with offshore movements in winter (Casey and Pereiro 1995; Imsland et al. 1996; Gonse et al. 2025), their detection in the system during this season contrasts with migratory behavioural expectations. Similarly, year-round residents like tench and long-spined bullhead were unexpectedly detected only in winter, further complicating the identification of clear behavioural patterns underlying these observations. The spring season is a critical time for fish spawning (Wright and Trippel 2009), which could further explain the elevated richness and DNA reads observed during this period. Spawning represents a biologically intense phase for many fish species, during which large quantities of genetic material, such as gametes, are released into the water (Alix et al. 2020). Our analysis of seasonal reads reveals a higher abundance of eDNA reads in the spring (Figure 2), suggesting that the heightened eDNA concentrations and observed richness during spring may be driven by the reproductive behaviours of fish, supporting studies showing that spawning events contribute to the higher genetic material present in the water (Schreck et al. 2001; Tillotson et al. 2018; Alix et al. 2020; Collins et al. 2022). However, it is also worth noting that eDNA follows a state of exponential decay upon release (Sassoubre et al. 2016), so one potential explanation for this seasonal increase in observed richness in winter and spring may be the conditions associated with these seasons (less UV exposure and more temperate water conditions) contributing to the extended survival of DNA fragments in the water (Barnes et al. 2014; Strickler et al. 2015; McCartin et al. 2022), resulting in a larger window for capturing species' DNA between shedding and filtration.

In estuarine systems, tidal patterns and the flow of water directly influence the transport of eDNA particles (Andruszkiewicz et al. 2019; Pont 2024), which, in turn, should be considered when interpreting the results. For example, here, there are frequent

instances of species detected outside of their physiological tolerances (Table A3; Figure A4). Common marine/brackish species such as Atlantic herring *Clupea harengus*, and European sprat *Sprattus sprattus* have been detected across multiple freshwater sites and sampling months. Furthermore, we also see the opposite pattern, with freshwater/brackish species such as the common roach *Rutilus rutilus* and common bream *Abramis brama* frequently detected within the lower marine zones of the system. The strong tidal dynamics of the Mersey Estuary, with its pronounced tidal asymmetry throughout its length (flood tides fill the upper estuary in as little as  $\sim 2$  h with ebb tides retreating for up to  $\sim 10$  h), extensive tidal excursion range (particularly at the estuary mouth as it flows into Liverpool Bay) and complex hydrology (The Mersey Gateway Project 2021), can facilitate long-distance transport of eDNA. It has been documented that eDNA can be transported over large spatial scales (Fremier et al. 2019; Wood et al. 2021), with DNA from freshwater species being transported through estuarine regions to saltwater zones, and vice versa. Therefore, it is important to note that we are detecting the species' DNA at a particular location and not necessarily the species itself. While this might be less important for monitoring an overall system in terms of species diversity/composition, as we have undertaken here, it could be critical for identifying important habitats for species of concern within these systems. This emphasises an urgent need for more integrative approaches, such as coupling eDNA surveys with hydrodynamic modelling and particle tracking methods to simulate hydrological patterns and the subsequent dispersal of eDNA (Fukaya et al. 2021; Pastor Rollan et al. 2024). Such approaches would help clarify how tidal regimes and estuarine hydrodynamics influence eDNA transport, and how these factors relate to the presence of a particular species both spatially and temporally.

## 4.2 | Seasonal Compositions and Diadromous Species Detections

Analysing the data on a finer monthly scale revealed significant fluctuations in species compositions (Figure A2), indicating that short-term temporal dynamics play a crucial role in shaping the observed fish biodiversity within the Mersey. These findings reinforce the importance of implementing a robust temporal sampling strategy to accurately capture broad-scale biodiversity estimates (Beentjes et al. 2019; Seymour et al. 2021). The monthly variability observed here may be driven by factors such as seasonal migrations, reproductive cycles, hydrological conditions and environmental fluctuations that influence species presence and detectability (De Souza et al. 2016; Buxton et al. 2017). However, when the data is aggregated into broader seasonal periods, species compositions exhibit relatively non-significant variation across seasons (Figure 6). Although several eDNA studies in estuaries have identified seasonal differences in fish community compositions (e.g., Stoeckle et al. 2017; Cunnington et al. 2024), other studies have found this not to be the case (e.g., Hallam et al. 2021; Gibson et al. 2023). Here, this consistency across seasons suggests that while some species exhibit strong temporal shifts at a more fine-scale monthly level, the overall assemblage remains relatively stable when viewed on a seasonal scale. A key factor contributing to this stability could be the presence of approximately half of all recorded species detected through all seasons (Figures 5B and A3). Consequently, while

seasonal sampling can provide a general overview of biodiversity trends, higher-resolution monthly data (or, if feasible, including more fine-scale sampling to account for tidal variations and their influence on eDNA transport) is important for detecting fine-scale temporal changes and ensuring comprehensive assessments of community dynamics.

The species detected year-round may represent resident populations or species that are less affected by short-term environmental fluctuations. Patterns of fish diversity within the estuary are likely influenced by a combination of environmental stability, habitat connectivity, and anthropogenic modification. Artificial barriers, such as weirs, docks, and shipping canals, are prevalent throughout the length of the estuary (Cunnington et al. 2024) and can alter species distributions by impeding the natural migratory routes of certain fish species (Brönmark et al. 2014; Rahel and McLaughlin 2018), potentially leading to unintended permanent residency and shifts in traditional migratory behaviour (Chapman et al. 2012). Such processes may influence local diversity patterns by favouring resident or disturbance-tolerant species while reducing the contribution of migratory fishes. The year-round detection of typically migratory species in the eDNA data, such as the European flounder *Platichthys flesus* (which migrates to deeper waters during winter; Tesch and Thorpe 2003), represents an example of this process, although alternative explanations related to eDNA ecology must also be considered. Furthermore, DNA shed by migrating individuals may persist in sediments and later be resuspended into the water column, resulting in detections even in the absence of live fish (Jerde et al. 2016; Ellegaard et al. 2020). While this study used eDNA metabarcoding, future work incorporating eRNA assays, which degrade rapidly and may better indicate the presence of live organisms, could help distinguish active populations from residual genetic material (Littlefair et al. 2022; Janik-Superson et al. 2025). Such approaches would clarify whether observed diversity patterns reflect true behavioural shifts (e.g., halted migration or trapping) or methodological limitations associated with eDNA-based monitoring in dynamic estuarine ecosystems.

Overall, around  $\sim 15\%$  of the species detected with eDNA here are diadromous, highlighting the estuary's role as a crucial corridor and habitat for migratory species. Atlantic salmon, for instance, rely on clean, well-oxygenated freshwater habitats for spawning, making their presence a valuable indicator of improving water quality (Thorstad et al. 2008; Smialek et al. 2021). Interestingly, the detections of salmon align with their key migratory period in late autumn to winter (Figure 7), reinforcing the idea that conditions are becoming more suitable for their life cycle after a lengthy absence (Jones 2006). Similarly, European smelt also depend on clean, fast-flowing water for spawning (Aarts and Nienhuis 2003). While their presence is more sporadic, any detection suggests that improving habitat conditions may be supporting their return. The detection of *Lampetra* spp. also aligns with the spawning season of the migratory *L. fluviatilis* between April and May (Silva et al. 2015), and they also rely on clean freshwater for spawning. It is important to reiterate, however, that the 12S sequence fragment used here cannot distinguish between the migratory *L. fluviatilis* and the non-migratory *L. planeri*, so a more targeted species-specific eDNA approach would be warranted for the future detection and monitoring of these two species. European eel *A. anguilla*, a critically endangered species (Freyhof 2024), was

frequently detected, corroborating their known presence in the Mersey over the past decade (e.g., BBC News 2023; Springwatch 2016). Their persistence highlights the estuary's role in supporting at-risk diadromous species, with eDNA emerging as a promising tool for monitoring their recovery (Hillsdon 2023; Rodriguez 2023). Collectively, these findings signal ecological restoration in this previously degraded estuarine ecosystem, particularly for species with stringent habitat requirements (Hawkins et al. 2020).

It is also worth noting that non-target eDNA detections are present within the dataset. For this study, we used the Tele02 primer set, which is designed for the detection of teleost fishes (Taberlet et al. 2018), but we also detected two elasmobranch species in the estuary, the starry smooth-hound *Mustelus asterias* (class Elasmobranchii, infraclass Selachii) and the thornback ray *Raja clavata* (class Elasmobranchii, infraclass Batoidea). This highlights a major benefit to the eDNA as a monitoring method overall, demonstrating that non-target species can be detected as 'molecular bycatch' (Mariani et al. 2021). These infrequent non-target detections could be improved by re-analysing the samples using different primer sets designed to target elasmobranch species (e.g., Elas02; Taberlet et al. 2018).

## 5 | Conclusions

This study demonstrates the utility of eDNA metabarcoding as a powerful tool for monitoring coastal and freshwater fishes within a complex and heavily urbanised ecosystem. The resulting data represent the most comprehensive fish species inventory compiled to date for the Mersey Estuary. The ecological importance of this system is underscored not only by the remarkable diversity of species detected but also by the presence of returning migratory species that depend on this ecosystem for critical life cycle stages.

In addition, detecting species of conservation concern listed in the UK BAP (Joint Nature Conservation Committee 2007) provides invaluable insights to support targeted conservation efforts for these vulnerable populations. However, the study also highlights a recurring pattern of unexpected species detections, such as freshwater species detected at marine sampling sites. This recurring phenomenon in tidal and lotic ecosystems underscores the complexity of distinguishing between species presence and eDNA particle distributions, as well as highlighting complex sampling dynamics in such environments. Looking ahead, integrating advanced techniques like hydrodynamic modelling and particle tracking to examine these anomalous detections will enhance data interpretation and improve the precision of species monitoring using eDNA for effective management and conservation strategies.

### Author Contributions

A.D.M., I.C., J.M.J. and P.E.R. conceived and acquired funding for the study. J.M.J. carried out the eDNA sampling, laboratory work and bioinformatic analyses. J.M.J., N.G.S., C.B., I.C. and A.D.M. analysed the data. J.M.J. and A.D.M. prepared the original draft of the manuscript, with input from I.C., N.G.S., C.B. and P.E.R. All other authors (A.D. and A.W.) contributed to editing and discussing the manuscript.

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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 on figshare at: <https://doi.org/10.6084/m9.figshare.31242334>.

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**Appendix A: Additional Data and Results That Support the Findings in the Main Text.**

**TABLE A1** | The month, year, date, tidal regime (high/low tide), tidal phase (spring/neap tide) and daily average mean flow (m<sup>3</sup>/s) on each of the three sampled days during the 12 sampled months.

<b>Month/year</b>	<b>Dates</b>	<b>Tidal regime (high/low)</b>	<b>Tidal phase (spring/neap)</b>	<b>Daily mean flow (m<sup>3</sup>/s)</b>
September 2023	25	High	Neap	33.91
	26	High	Neap	28.24
	27	High	Neap	28.32
October 2023	23	High	Neap	59.71
	24	High	Neap	57.932
	25	High	Neap	45.78
November 2023	22	High	Neap	54.145
	23	High	Neap	55.579
	24	High	Neap	55.423
December 2023	19	High	Spring	59.235
	20	High	Spring	60.219
	21	High	Spring	61.81
January 2024	29	High	Spring	50.959
	30	High	Spring	58.325
	31	High	Spring	57.348
February 2024	27	High	Spring	52.091
	28	High	Spring	56.203
	29	High	Spring	53.733
March 2024	25	High	Spring	52.746
	26	High	Spring	51.238
	27	High	Spring	55.981
April 2024	21	High	Neap	40.487
	22	High	Neap	39.184
	23	High	Neap	36.731
May 2024	29	High	Spring/neap	52.786
	30	High	Spring/neap	41.315
	31	High	Spring/neap	35.616
June 2024	26	High	Spring	19.076
	27	High	Spring	17.022
	28	High	Spring	15.97
July 2024	24	High	Spring	16.794
	25	High	Spring	16.579
	26	High	Spring	14.379
August 2024	21	High	Spring	14.095
	22	High	Spring	20.232
	23	High	Spring	30.698

**TABLE A2** | The filter (0.45 µm) sample replicates collected at each of the 10 sample locations for all 12 months and their associated dates and volumes (mL).

Replicate	Month/dates	U1	U2	U3	C1	C2	C3	C4	L1	L2	L3
25–27 (2023)											
1	September	720	680	540	360	300	290	220	320	300	320
2	September	680	600	520	380	360	240	210	360	320	310
3	September	650	590	500	340	330	220	260	340	380	380
4	September	670	610	570	360	340	230	260	380	360	350
5	September	610	570	510	330	360	260	220	360	310	340
23–25 (2023)											
1	October	700	745	615	395	255	225	165	275	255	275
2	October	785	645	545	415	385	205	155	375	335	325
3	October	675	615	525	305	295	185	225	315	355	355
4	October	705	655	615	325	305	195	225	345	325	315
5	October	635	595	535	285	315	215	175	325	275	305
22–24 (2023)											
1	November	755	715	575	345	285	265	195	305	285	305
2	November	665	585	505	365	345	225	195	345	305	295
3	November	635	575	485	325	320	205	245	325	365	365
4	November	655	595	555	335	315	215	245	365	345	335
5	November	595	555	495	315	345	245	205	345	295	325
19–21 (2023)											
1	December	680	700	560	330	270	250	180	290	270	290
2	December	715	600	520	350	330	210	180	330	290	280
3	December	650	590	500	310	300	190	230	310	350	350
4	December	670	610	540	320	300	200	230	350	330	320
5	December	610	570	510	300	330	230	190	330	280	310
29–31 (2024)											
1	January	850	790	650	440	380	360	280	400	380	400
2	January	720	640	560	400	380	260	230	380	340	330
3	January	690	630	540	380	370	260	300	380	420	420
4	January	710	650	610	390	370	270	300	420	400	390
5	January	650	610	550	370	400	280	240	400	350	380
27–29 (2024)											
1	February	540	500	420	330	280	260	220	290	270	290
2	February	480	430	380	350	320	250	230	320	300	290
3	February	460	420	360	310	300	240	270	310	350	350
4	February	490	450	410	330	310	260	270	350	330	320
5	February	430	410	370	300	330	260	230	330	290	320
25–27 (2024)											
1	March	520	480	390	310	260	240	200	270	250	270
2	March	460	410	360	330	300	230	210	300	280	270
3	March	440	400	340	290	280	220	250	290	330	330
4	March	470	430	390	310	290	240	250	330	310	300
5	March	410	390	350	280	310	2460	210	310	270	300

(Continues)

TABLE A2 | (Continued)

Replicate	Month/dates	U1	U2	U3	C1	C2	C3	C4	L1	L2	L3
21–23 (2024)											
1	April	360	330	280	190	160	150	140	170	150	170
2	April	320	290	260	200	180	150	140	180	170	160
3	April	310	280	240	180	170	140	160	180	210	210
4	April	330	300	270	190	170	150	160	210	190	180
5	April	290	270	250	170	190	150	140	190	160	190
29–31 (2024)											
1	May	460	430	350	240	200	190	150	210	190	210
2	May	420	380	330	260	240	180	160	240	220	225
3	May	400	370	310	230	220	170	200	230	260	260
4	May	410	380	350	240	220	180	200	260	240	230
5	May	380	360	320	220	240	190	160	240	210	230
26–28 (2024)											
1	June	840	770	630	490	420	400	320	440	420	440
2	June	760	680	600	510	490	370	340	490	450	415
3	June	730	670	580	490	480	370	410	490	530	530
4	June	750	690	650	500	480	380	410	530	510	500
5	June	690	650	590	480	510	390	350	510	460	490
24–26 (2024)											
1	July	880	810	670	520	450	430	350	470	450	410
2	July	780	700	620	540	520	400	370	520	480	470
3	July	750	690	600	520	510	400	440	520	560	560
4	July	770	710	670	530	515	410	440	560	540	530
5	July	710	670	610	510	540	420	380	540	490	520
21–23 (2024)											
1	August	700	860	720	580	510	490	390	530	510	530
2	August	740	740	660	600	580	460	430	580	540	530
3	August	790	730	640	580	570	460	500	580	620	620
4	August	810	750	710	590	570	470	500	620	600	590
5	August	750	710	650	570	600	480	440	600	550	580

**TABLE A3** | Complete list of estimated pre-industrialisation species by the Mersey Rivers Trust (2021) report, the number of species identified during a 5-year survey from 2015 to 2020, the total species eDNA detections from this study and the estuarine zones in which the eDNA for each species was detected. Each species has been classified according to its physiological saline tolerance (F = freshwater, M = marine and B = brackish).

Species	Common name	Tolerance	Pre-industrial presence	Fish survey 2015–2020	12-Month eDNA survey (2023–2024)	eDNA Zone detection
<i>Abramis brama</i>	Common bream	F, B	×		×	Upper, central, lower
<i>Acipenser sturio</i>	Sturgeon	M, B, F	×			
<i>Agonus cataphractus</i>	Pogge	M	×	×	×	Central, lower
<i>Ammodytes marinus</i>	Raitt's sand eel	M, B		×	×	Upper, central, lower
<i>Anguilla anguilla</i>	European eel	M, B, F	×	×	×	Upper, central, lower
<i>Aphia minuta</i>	Transparent goby	M, B			×	Upper, central, lower
<i>Arnoglossus laterna</i>	Mediterranean scaldfish	M			×	Upper, central, lower
<i>Atherina boyeri</i>	Sand smelt	M, B, F			×	Central
<i>Barbatula barbatula</i>	Stone loach	F	×	×	×	Upper, central, lower
<i>Barbus barbus</i>	Common barbel	F			×	Central, upper
<i>Blicca bjoerkna</i>	White bream	F, B	×		×	Upper, central, lower
<i>Buglossidium luteum</i>	Solenette	M		×	×	Upper, central, lower
<i>Carassius carassius</i>	Crucian carp	F, B	×		×	Upper
<i>Chelidonichthys cuculus</i>	Red gurnard	M			×	Upper, lower
<i>Chelidonichthys lucernus</i>	Tub gurnard	M		×		
<i>Chelon labrosus</i>	Thicklip mullet	M, B, F	×		×	Upper, central, lower
<i>Chelon ramada</i>	Thinlip mullet	M, B, F	×		×	Upper, central, lower
<i>Ciliata mustela</i>	Fivebeard rockling	M	×		×	Central, lower
<i>Clupea harengus</i>	Atlantic herring	M, B	×	×	×	Upper, central, lower
<i>Conger conger</i>	Conger eel	M			×	Central, lower
<i>Cottus gobio</i>	European bullhead	F, B	×	×	×	Upper, central, lower
<i>Crystalllogobius linearis</i>	Crystal goby	M			×	Central
<i>Cyprinus carpio</i>	Common carp	F, B	×	×	×	Upper
<i>Dicentrarchus labrax</i>	European seabass	M, B, F	×	×	×	Upper, central, lower
<i>Echichthys vipera</i>	Lesser weaver	M	×	×	×	Central, lower
<i>Engraulis encrasicolus</i>	European anchovy	M, B			×	Central, lower

(Continues)

TABLE A3 | (Continued)

Species	Common name	Tolerance	Pre-industrial presence	Fish survey 2015–2020	12-Month eDNA survey (2023–2024)	eDNA Zone detection
<i>Esox lucius</i>	Northern pike	F, B	×	×	×	Upper, central, lower
<i>Gadus morhua</i>	Atlantic cod	M, B	×	×	×	Central, lower
<i>Gaidropsarus mediterraneus</i>	Shore rockling	M, B	×			
<i>Gasterosteus aculeatus</i>	Three-spined stickleback	M, B, F	×	×	×	Upper, central, lower
<i>Gobio gobio</i>	Gudgeon	F, B	×		×	Upper, central, lower
<i>Gymnammodytes semisquamatus</i>	Smooth sandeel	M			×	Upper, central
<i>Gymnocephalus cernua</i>	Eurasian ruffe	F, B	×	×	×	Upper, central
<i>Lampetra fluviatilis</i>	River lamprey	M, B, F	×	×	×	Upper, lower
<i>Lampetra planeri</i>	Brook lamprey	F	×		×	Upper, lower
<i>Leuciscus idius</i>	Ides	F, B			×	Upper
<i>Leuciscus leuciscus</i>	Dace	F, B	×		×	Upper, central, lower
<i>Limanda limanda</i>	Common dab	M	×	×	×	Upper, Central, Lower
<i>Liparis liparis</i>	Common seasnail	M			×	Lower
<i>Lipophrys pholis</i>	Shanny	M			×	Upper, central, lower
<i>Lota lota</i> (extinct in the UK)	Burbot	F, B	×			
<i>Melanogrammus aeglefinus</i>	Haddock	M			×	Central, lower
<i>Merlangius merlangus</i>	Whiting	M, B	×	×	×	Central, lower

(Continues)

TABLE A3 | (Continued)

Species	Common name	Tolerance	Pre-industrial presence	Fish survey 2015–2020	12-Month eDNA survey (2023–2024)	eDNA Zone detection
<i>Merluccius merluccius</i>	European hake	M			×	Central
<i>Mullus surmuletus</i>	Striped red mullet	M			×	Upper
<i>Mustelus asterias</i>	Starry smooth-hound	M			×	Lower
<i>Oncorhynchus mykiss</i>	Rainbow trout	M, B, F			×	Central, lower
<i>Osmerus eperlanus</i>	European smelt	M, B, F	×	×	×	Central, lower
<i>Perca fluviatilis</i>	European perch	F, B	×		×	Upper, central, lower
<i>Petromyzon marinus</i>	Sea lamprey	M, B, F		×		
<i>Pholis gunnellus</i>	Butterfish	M, B		×		
<i>Phoxinus phoxinus</i>	Common minnow	F, B		×	×	Upper, central, lower
<i>Platichthys flesus</i>	European flounder	M, B, F	×	×	×	Upper, central, lower
<i>Pleuronectes platessa</i>	European plaice	M, B	×	×	×	Upper, central, lower
<i>Pollachius pollachius</i>	Pollack	M			×	Lower
<i>Pomatoschistus microps</i>	Common goby	M, B, F		×	×	Upper, central, lower
<i>Pomatoschistus minutus</i>	Sand goby	M, B	×	×	×	Upper, central, lower
<i>Pungitius pungitius</i>	Ninespine stickleback	M, B, F			×	Upper, central, lower
<i>Raja clavata</i>	Thornback ray	M		×	×	Lower
<i>Rutilus rutilus</i>	Common roach	F, B	×	×	×	Upper, central, lower
<i>Salmo salar</i>	Atlantic salmon	M, B, F	×	×	×	Upper, central, lower
<i>Salmo trutta</i>	Brown trout	M, B, F	×	×	×	Upper, central, lower
<i>Sardina pilchardus</i>	Pilchard	M, B, F	×	×		
<i>Scardinius erythrophthalmus</i>	Rudd	F, B	×		×	Upper, central
<i>Scomber scombrus</i>	Atlantic mackerel	M, B			×	Upper, central, lower
<i>Scophthalmus maximus</i>	Turbot	M, B			×	Lower

(Continues)

TABLE A3 | (Continued)

Species	Common name	Tolerance	Pre-industrial presence	Fish survey 2015–2020	12-Month eDNA survey (2023–2024)	eDNA Zone detection
<i>Scophthalmus rhombus</i>	Brill	M			×	Upper, central
<i>Scyliorhinus canicula</i>	Small-spotted catshark	M	×	×		
<i>Solea solea</i>	Common sole	M, B	×	×	×	Upper, central, lower
<i>Sparus aurata</i>	Gilt-head bream	M, B			×	Upper, lower
<i>Sprattus sprattus</i>	European sprat	M, B	×	×	×	Upper, central, lower
<i>Squalius cephalus</i>	Common chub	F, B	×		×	Upper, central, lower
<i>Symphodus melops</i>	Corkwing wrasse	M			×	Central
<i>Syngnathus rostellatus</i>	Lesser pipefish	M, B	×	×	×	Lower
<i>Taurulus bubalis</i>	Longspined bullhead	M, B	×		×	Lower
<i>Thymallus thymallus</i>	European grayling	F, B	×	×	×	Upper, central, lower
<i>Tinca tinca</i>	Tench	F, B	×		×	Upper
<i>Torpedo sp.</i>	Torpedo ray sp.	M	×			
<i>Trachurus trachurus</i>	Atlantic horse mackerel	M			×	Lower
<i>Trisopterus luscus</i>	Whiting-pout	M, B	×	×	×	Upper, central, lower
<i>Trisopterus minutus</i>	Poor cod	M		×		
	Total		46	39	69	

Note: Species detected with eDNA featuring \* could not be assigned to a species level.

**TABLE A4** | All Dunn's post hoc comparisons of the differences in species richness between seasons.

Pairs	Z	Unadjusted p	Adjusted p
Spring vs. summer	5.91	3.44e-09	5.16e-09 <sup>a</sup>
Spring vs. autumn	-6.48	9.43e-11	2.83e-10 <sup>a</sup>
Spring vs. winter	-0.51	0.607	0.607
Summer vs. autumn	-0.60	0.552	0.662
Summer vs. winter	-6.40	1.54e-10	3.08e-10 <sup>a</sup>
Autumn vs. winter	-6.96	3.31e-12	1.98e-11 <sup>a</sup>

<sup>a</sup>Significant difference.**TABLE A5** | All post hoc pairwise comparisons of species composition changes between sampled zones within each of the 12 months.

Month	Zones	df	Sums of Sqs	F model	R <sup>2</sup>	p	p adjusted	Sig
September	U vs. C	1	1.65675286	5.7350397	0.15611906	0.001	0.003	*
	U vs. L	1	1.97284422	6.63964802	0.19737567	0.001	0.003	*
	C vs. L	1	0.37490888	0.93619001	0.03026197	0.524	1	
October	U vs. C	1	1.71738151	9.0970035	0.21609622	0.001	0.003	*
	U vs. L	1	2.39577592	15.791474	0.36060613	0.001	0.003	*
	C vs. L	1	0.80566548	3.93192705	0.10646417	0.002	0.006	*
November	U vs. C	1	2.19077165	10.148758	0.24663583	0.001	0.003	*
	U vs. L	1	3.94094917	25.9752734	0.48124394	0.001	0.003	*
	C vs. L	1	0.73670306	2.65358744	0.07885006	0.013	0.039	.
December	U vs. C	1	3.03254091	22.8169963	0.40878223	0.001	0.003	*
	U vs. L	1	4.27034186	36.4068716	0.57417864	0.001	0.003	*
	C vs. L	1	1.10872114	4.96580007	0.13433498	0.001	0.003	*
January	U vs. C	1	2.40786813	20.8372063	0.39436616	0.001	0.003	*
	U vs. L	1	5.4740909	77.9199243	0.73564936	0.001	0.003	*
	C vs. L	1	1.66273065	10.9393087	0.25476211	0.001	0.003	*
February	U vs. C	1	0.60264824	4.83785785	0.1278576	0.001	0.003	*
	U vs. L	1	3.74254915	24.387694	0.46552333	0.001	0.003	*
	C vs. L	1	2.66900281	13.2915901	0.28712753	0.001	0.003	*
March	U vs. C	1	1.73046179	12.1463521	0.26904393	0.001	0.003	*
	U vs. L	1	2.82888036	22.6249802	0.44691336	0.001	0.003	*
	C vs. L	1	0.98920574	4.60337217	0.12241913	0.001	0.003	*
April	U vs. C	1	1.87867947	16.967036	0.33956459	0.001	0.003	*
	U vs. L	1	2.66810385	18.5014422	0.39786814	0.001	0.003	*
	C vs. L	1	0.96138948	5.20161953	0.13616228	0.001	0.003	*
May	U vs. C	1	2.29652745	22.5405163	0.40583916	0.001	0.003	*
	U vs. L	1	2.0777841	11.8021264	0.29652	0.001	0.003	*
	C vs. L	1	0.81467615	4.35377119	0.11655506	0.001	0.003	*
June	U vs. C	1	2.35447627	16.0367319	0.32703509	0.001	0.003	*
	U vs. L	1	1.63107235	8.49351408	0.23274037	0.001	0.003	*
	C vs. L	1	0.77417728	3.76225983	0.10234028	0.001	0.003	*
July	U vs. C	1	1.48839175	5.89720123	0.15561047	0.001	0.003	*
	U vs. L	1	2.15534262	9.29555698	0.24924033	0.001	0.003	*
	C vs. L	1	0.29677638	0.85928381	0.02615041	0.54	1	
August	U vs. C	1	1.29007957	8.6838875	0.2083272	0.001	0.003	*
	U vs. L	1	0.81154339	4.62251237	0.1461779	0.001	0.003	*
	C vs. L	1	0.58450753	3.04287146	0.08683282	0.015	0.045	

Note: Zones are classified as: U = upper, C = central and L = lower.

**TABLE A6** | All post hoc pairwise comparisons of monthly species composition changes for each of the 12 months.

<b>Pairs</b>	<b>df</b>	<b>Sums of Sqs</b>	<b>F model</b>	<b>R<sup>2</sup></b>	<b>p</b>	<b>Sig</b>
January vs. February	1	20.2141683	11.5027478	0.11007125	0.011	*
January vs. March	1	151.300168	57.1319345	0.38054485	0.003	*
January vs. April	1	3436.24522	140.758793	0.602154	0.002	*
January vs. May	1	149.356388	33.1376431	0.26271018	0.002	*
January vs. June	1	-685.75464	-37.760755	-0.6835856	1	
January vs. July	1	14.834781	10.2738749	0.09948184	0.003	*
January vs. August	1	19.0959101	10.1328751	0.09825068	0.006	*
January vs. September	1	2860.02585	124.082362	0.57159117	0.006	*
January vs. October	1	447.942793	12.9192232	0.12197241	0.055	
January vs. November	1	-5448.7535	-43.130476	-0.8648664	1	
January vs. December	1	164.393104	17.0768476	0.15513569	0.03	*
February vs. March	1	614.757343	235.993764	0.70658135	0.004	*
February vs. April	1	197.671003	8.49883537	0.07980214	0.109	
February vs. May	1	739.928728	169.357899	0.63345014	0.001	*
February vs. June	1	2349.12616	135.58556	0.58045352	0.003	*
February vs. July	1	14.2176155	9.72416363	0.0902691	0.008	*
February vs. August	1	34.4802711	18.3383413	0.15762939	0.002	*
February vs. September	1	-105.2676	-4.7924602	-0.0514171	0.887	
February vs. October	1	943.629971	28.5988348	0.22590125	0.004	*
February vs. November	1	59809522.6	498503.28	0.99980345	0.002	*
February vs. December	1	-232.89535	-25.23971	-0.3468885	0.998	
March vs. April	1	-157.26638	-6.5244744	-0.0713248	0.942	
March vs. May	1	796.878208	152.819856	0.60928133	0.001	*
March vs. June	1	-504.65346	-27.772077	-0.3954563	1	
March vs. July	1	550.455583	238.5443	0.70880505	0.002	*
March vs. August	1	1450.22433	532.056061	0.84445829	0.003	*
March vs. September	1	402.575513	17.6485203	0.15260481	0.023	*
March vs. October	1	2442.90911	72.1881315	0.42416666	0.001	*

(Continues)

TABLE A6 | (Continued)

Pairs	df	Sums of Sq	F model	R <sup>2</sup>	p	Sig
March vs. November	1	-5767.9851	-47.73887	-0.9498169	1	
March vs. December	1	229382.643	22772.4621	0.995715	0.001	*
April vs. May	1	193.682944	7.48732198	0.07097841	0.107	
April vs. June	1	-1019.7295	-26.264845	-0.3661363	1	
April vs. July	1	-742.18785	-32.323583	-0.4921642	1	
April vs. August	1	13420.3285	574.025503	0.8541722	0.002	*
April vs. September	1	151816.134	3492.88868	0.97270871	0.001	*
April vs. October	1	2546.13218	46.7227408	0.32284312	0.002	*
April vs. November	1	-6023.6722	-42.576955	-0.7682176	1	
April vs. December	1	1949.06034	63.4326886	0.39293584	0.001	*
May vs. June	1	-620.01593	-31.101401	-0.4649036	0.998	
May vs. July	1	575.530468	141.351977	0.59056114	0.003	*
May vs. August	1	-9.5125928	-2.1187357	-0.0220975	0.904	
May vs. September	1	260.474352	10.5992571	0.09759972	0.051	
May vs. October	1	29330.7265	823.782956	0.8936843	0.004	*
May vs. November	1	-5466.3446	-44.591292	-0.8349068	1	
May vs. December	1	109164.142	9222.39074	0.98948542	0.001	*
June vs. July	1	4935.8129	289.858168	0.74733032	0.003	*
June vs. August	1	-615.84603	-35.299096	-0.5629759	1	
June vs. September	1	-719.20819	-19.162771	-0.2430675	0.994	
June vs. October	1	-1648.5898	-33.948368	-0.5300157	1	
June vs. November	1	-6280.2861	-46.333768	-0.8967901	1	
June vs. December	1	-991.41562	-39.986724	-0.6892685	1	
July vs. August	1	9.21394298	5.82123121	0.05606976	0.047	*
July vs. September	1	-117.82111	-5.4376026	-0.0587453	0.893	
July vs. October	1	15713.9043	480.577106	0.83061895	0.006	*
July vs. November	1	-5725.1297	-47.836667	-0.9536182	1	
July vs. December	1	-251.8012	-28.197454	-0.4039603	0.999	
August vs. September	1	42.6068633	1.92913628	0.01930504	0.421	
August vs. October	1	214855.888	6487.95381	0.98511985	0.004	*

(Continues)

TABLE A6 | (Continued)

Pairs	df	Sums of Sq	F model	R <sup>2</sup>	p	Sig
August vs. November	1	-5164.905	-43.005424	-0.7819939	0.998	
August vs. December	1	99.1719097	10.6088182	0.09767916	0.107	
September vs. October	1	-725.04101	-13.628296	-0.1615269	0.996	
September vs. November	1	-2835.4329	-20.226517	-0.2600696	0.987	
September vs. December	1	-455.80104	-15.486008	-0.1876774	0.998	
October vs. November	1	-6237.386	-41.248709	-0.726833	1	
October vs. December	1	1166920.49	28839.0313	0.99661334	0.001	*
November vs. December	1	-6041.4033	-47.403622	-0.9368975	1	

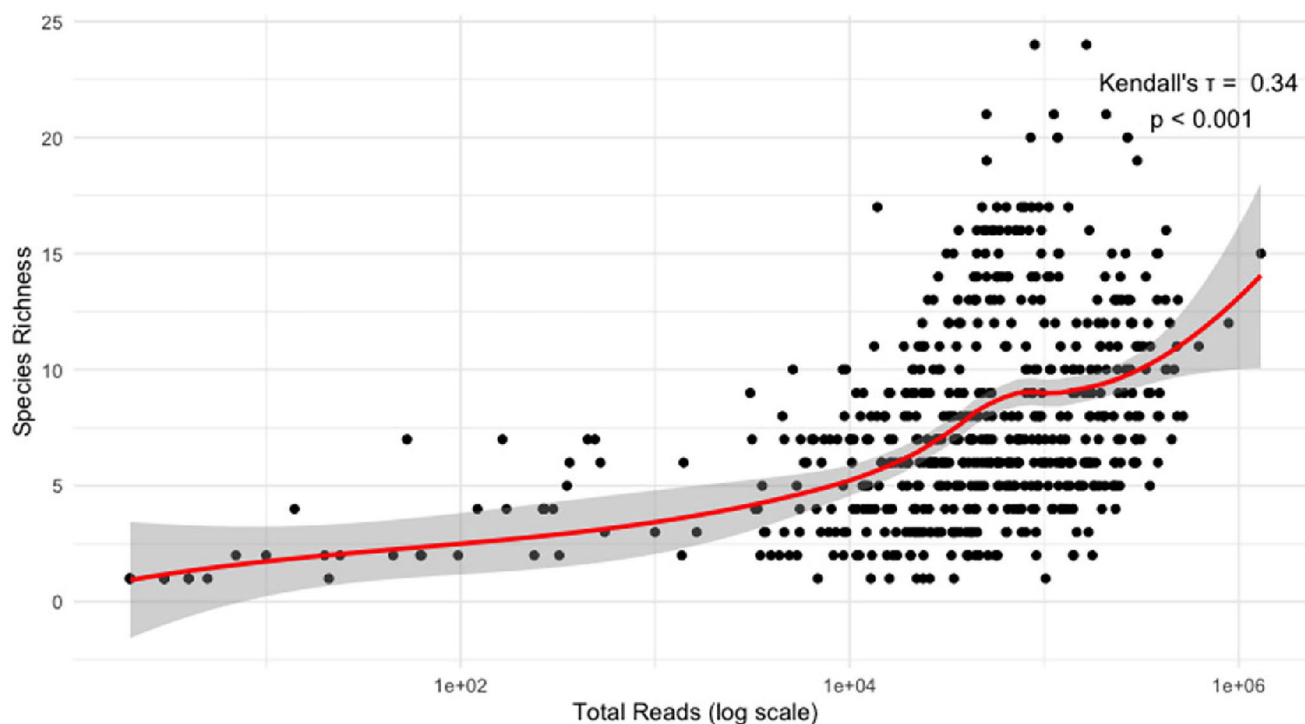
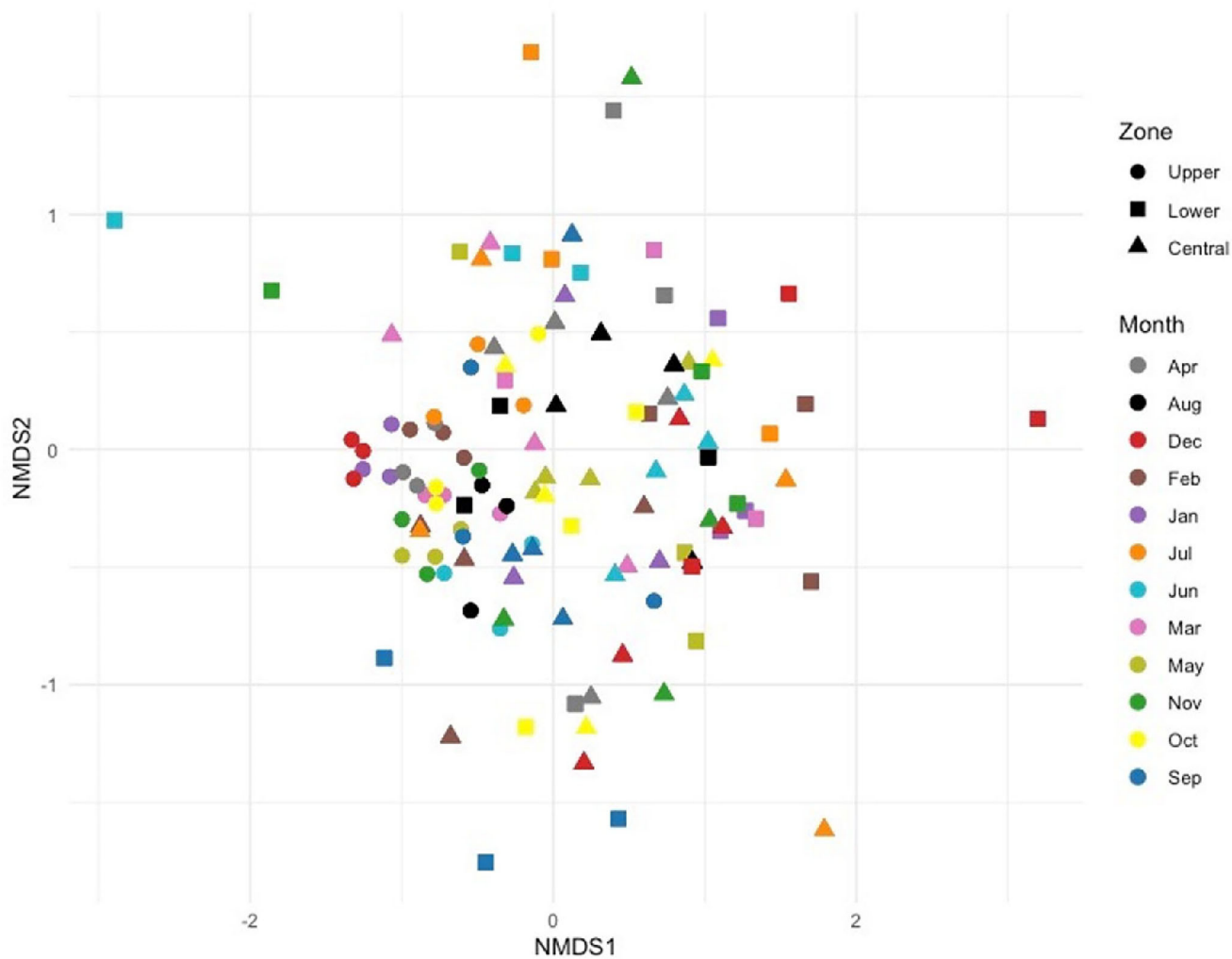
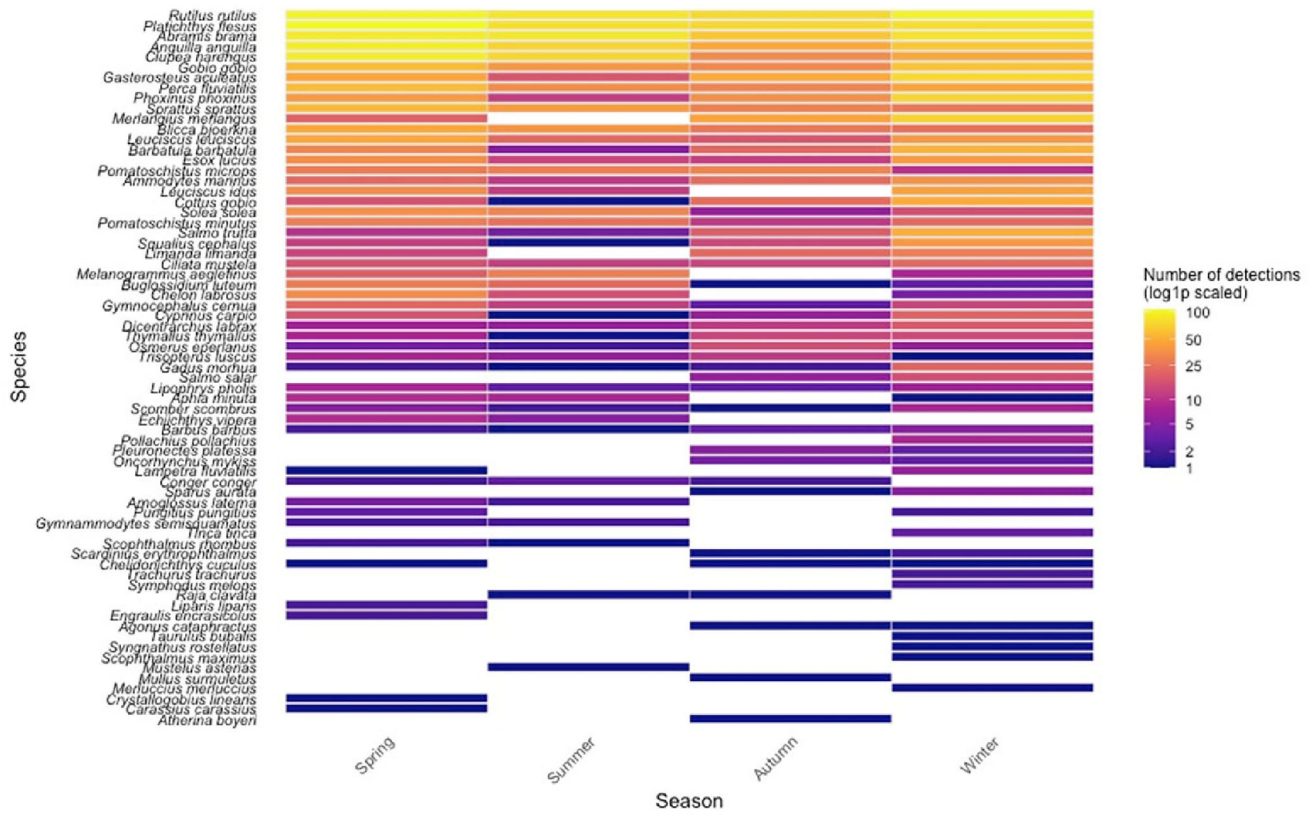


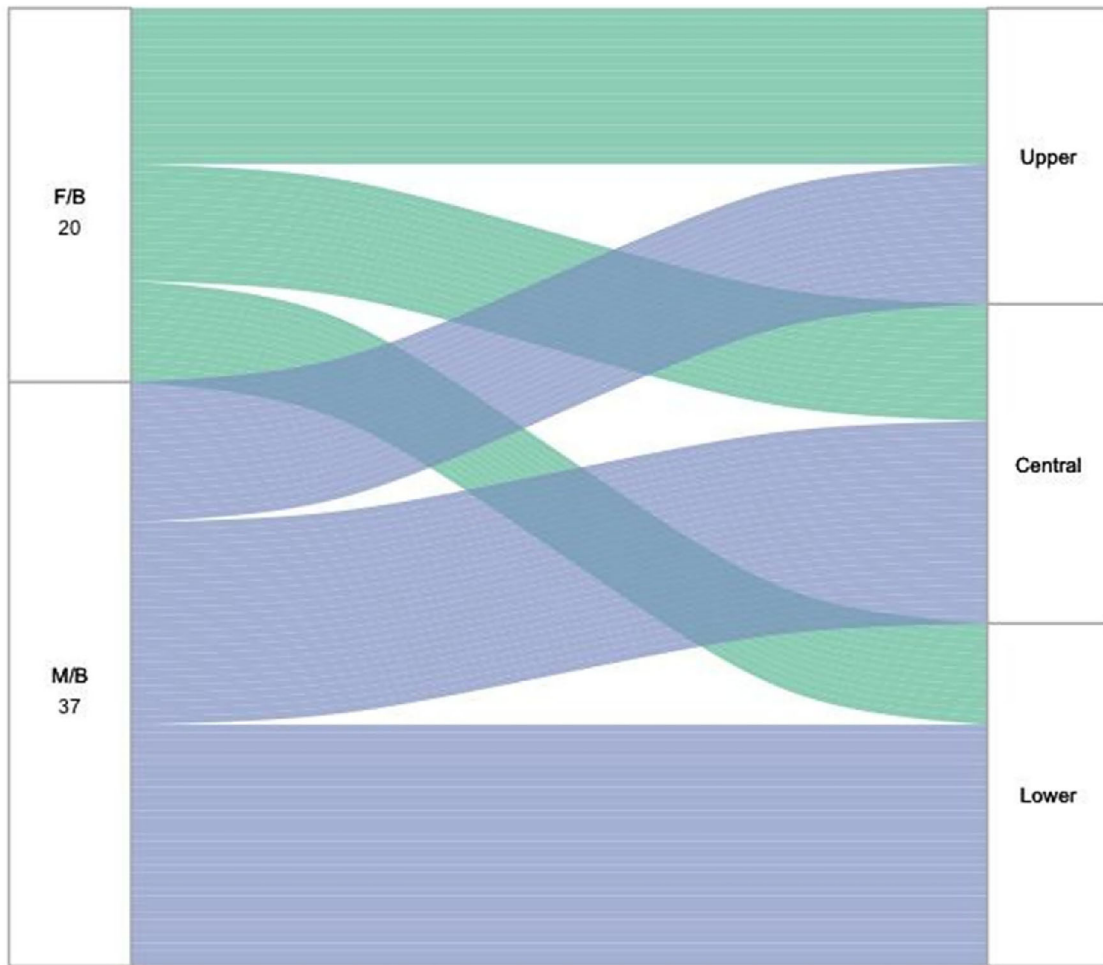
FIGURE A1 | Kendall's tau correlation between total sequencing reads and species richness. A weak but statistically significant positive correlation ( $\tau = 0.34$ ,  $p < 0.001$ ).



**FIGURE A2** | A non-metric multidimensional scaling (NMDS) analysis using Jaccard dissimilarity (stress = 0.161). The points in the plot represent the 10 sampling sites for each of the 12 months sampled (10 × 12 sites = 120 points). Each month's 10 points are colour-coded to differentiate between months.



**FIGURE A3** | Seasonal variation in fish species detections. Each row represents a species, and each column represents a season. Colour intensity indicates the number of samples in which each species was detected,  $\log(1 + x)$  transformed for improved visibility. This heatmap allows comparisons of species detections across seasons while highlighting both abundant and rare taxa.



**FIGURE A4** | Alluvial plot showing detections of freshwater/brackish (green) and marine/brackish (blue) species (left) and different habitat zones (right) in which the eDNA signal was detected. Species classifications are based on known physiological tolerances (see Table A3), with detections outside their expected zones indicating potential eDNA transport via tidal dynamics.