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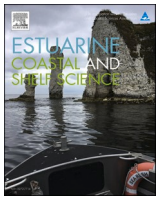
Estuarine, Coastal and Shelf Science 294 (2023) 108522

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss



# Environmental DNA metabarcoding for fish diversity assessment in a macrotidal estuary: A comparison with established fish survey methods

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ARTICLE INFO

*Keywords:*
Estuaries
Fishes
Environmental DNA
Water framework directive
Metabarcoding
Biomonitoring

ABSTRACT

Fishes are a dominant component of the macrofauna in estuaries and are important for assessing the health of these threatened ecosystems. Several studies have applied environmental DNA (eDNA) metabarcoding to assess the biodiversity of fishes in estuaries. However, none have combined measurement of physicochemical variables with a spatially extensive sampling design across the full salinity gradient. This study aimed to compare spatial fish assemblage composition detected via eDNA metabarcoding of surface water samples with conventional fishing gear surveys in a macrotidal estuary (river Dee, North Wales, UK). In addition, eDNA assemblage composition across seasons was investigated. In autumn 2018, triplicate eDNA samples were taken at 13 stations in a spatially systematic design alongside seine, fyke and beam trawl sampling. In summer 2019, eDNA samples from eight of the 13 original stations were collected again in the upper and lower estuary. DNA was extracted from samples and subjected to metabarcoding analysis using an established assay targeting teleost fishes. The key findings were that in autumn, eDNA detected 17 of the 26 (71%) species caught by fishing gears, which included the most abundant species. Overall, eDNA detected a greater species richness, per 30 samples, than seine or fyke nets (but not beam trawling). Additionally, there was a clear correlation between salinity and assemblage composition, which was consistent across seasons. Overall, the study indicates that eDNA metabarcoding could enhance existing fish sampling methods, by generating a more comprehensive picture of estuarine fish biodiversity and providing additional information for ecological inference and management actions.

## 1. Introduction

Estuaries play a crucial role in the ecology of numerous fish species, providing an environment for truly estuarine fishes, nursery habitat for many marine species and a migratory route for diadromous fishes (McLusky and Elliott, 2004). Within estuaries, numerous abiotic and biotic environmental factors may influence the spatio-temporal distribution of fish directly and via interactions between environmental variables (reviewed in Martino and Able, 2003). Generally, in temperate regions, salinity on a spatial scale (Selleslagh et al., 2009; Whitfield et al., 2012) and temperature on a temporal scale are the best predictors for the abundance and assemblage structure of fishes in estuaries (Selleslagh et al., 2009). In temperate estuaries, the species richness of the fish assemblage declines from its maximum in the euhaline marine environment through to the oligohaline river (Martino and Able, 2003; Selleslagh and Amara, 2008; Whitfield et al., 2012). Furthermore, fish assemblages in estuaries show strong spatial changes in taxonomic composition (Nicolas et al., 2010; Teichert et al., 2018). Freshwater and diadromous species dominate in the lower salinity reaches of the upper estuary, while marine and estuarine species dominate in the higher

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https://doi.org/10.1016/j.ecss.2023.108522
Received 6 April 2023; Received in revised form 5 September 2023; Accepted 1 October 2023
Available online 10 October 2023

salinity zones towards the sea (Nicolas et al., 2010).

An understanding of fish assemblages in estuaries has important practical applications. Fishes are a dominant component of the macrofauna in estuaries (Martino and Able, 2003), and fishes are good indicators of the health of estuarine ecosystems (Teichert et al., 2016; Whitfield, 2002). In the European Union, monitoring of fish assemblages is specifically required to assess the ecological status of estuaries (and other transitional waters) under the Water Framework Directive (WFD; EC, 2000). Legislation equivalent to the WFD requiring fish monitoring is currently present within the UK (UK Parliament, 2017). Monitoring allows the calculation of multi-metric indices of fish assemblage health (Coates et al., 2007; Delpech et al., 2010; Harrison and Kelly, 2013). Indices currently require data from capture-based methods using a variety of fishing gear types (e.g. beam and otter trawls, seine and fyke nets; Coates et al., 2007; Colclough et al., 2002; Delpech et al., 2010; Harrison and Kelly, 2013). However, established capture based techniques have disadvantages, including known sampling biases, and requiring the use of multiple fishing gear types to gain a comprehensive assessment of the assemblage (Coates et al., 2007; Elliot and Hemingway, 2002a). Furthermore, capture-based assessments can result in mortality of captured fish, damage to habitats (Kubečka et al., 2012), and low detection probabilities (Evans and Lamberti, 2018).

In addition to innovation in conventional fish sampling methods (Becker et al., 2010; Warry et al., 2013; Samedy et al., 2015; Harrison et al., 2017), there have been substantial advances in the analysis of environmental DNA (eDNA) for the detection of fish species. Environmental DNA is defined as DNA isolated from an environmental sample without capturing the organism (Taberlet et al., 2012). The application of eDNA metabarcoding (Deiner et al., 2017; Lawson Handley, 2015) is potentially well suited to biodiversity assessment of fish, including those in estuaries (Hering et al., 2018). The main conclusions of these studies are as follows. Firstly, in estuaries, fish eDNA metabarcoding generally detects greater species richness than other conventional methods, such as beam trawls (Zou et al., 2020), baited remote underwater videos (BRUVs; Cole et al., 2022) and combined multi-method netting techniques (Hallam et al., 2021). However, while some species are detected by both eDNA and conventional methods, not all species are reliably detected by eDNA (Cole et al., 2022; Hallam et al., 2021; Zou et al., 2020). In addition, eDNA may detect a different assemblage composition than conventional methods (Cole et al., 2022; Hallam et al., 2021). Secondly, eDNA metabarcoding can detect changes in fish assemblage composition within estuaries, and between estuaries and adjacent environments at various spatial scales: 100s of kilometres (García--Machado et al., 2022), 10s of kilometres (Hallam et al., 2021; Saenz-Agudelo et al., 2022) and even at distances of less than 1 km (Cole et al., 2022; DiBattista et al., 2022). Finally, eDNA can detect seasonal changes in the composition of fish assemblages in estuaries (DiBattista et al., 2022; Stoeckle et al., 2017; Zou et al., 2020), but not in all studies (Hallam et al., 2021).

Despite the advances made in the study of fish eDNA in estuaries, further research is required, particularly given the wide diversity of estuarine environments globally (Nagarajan et al., 2022). Few eDNA studies have measured physicochemical variables (but see Ahn et al., 2020). Without the collection of physicochemical data, it may be difficult to contextualise fish eDNA data if assemblage composition is correlated with parameters such as salinity. In addition, many studies have generally focused on specific sections of the estuary (Ahn et al., 2020; Cole et al., 2022; Hallam et al., 2021) or amalgamated several surveys from different seasons (García-Machado et al., 2022). Therefore, more spatially comprehensive studies, which not only compare eDNA metabarcoding with fishing gears, but directly correlate changes in fish assemblage composition with physicochemical variables are required. In addition, given that estuaries are often open systems, species eDNA detections within estuaries will likely be affected by eDNA transport from outside the ecosystem, a widespread phenomenon in aquatic ecosystems (Deiner et al., 2017; Shaw et al., 2016; Yamamoto et al., 2017). Allochthonous eDNA could be transported in from the river, the sea and potentially wastewater outflows (Nakagawa et al., 2018) contributing noise to ecological interpretations (Yamamoto et al., 2017). Further to this, the ability of eDNA to detect species from transported eDNA is of concern to environmental managers as spatially specific data is often required in ecological assessment. Therefore, there is a clear requirement to support eDNA detections using the wealth of previous fish survey data available for UK estuaries (Waugh et al., 2019), to give environmental managers confidence that results are relevant for ecological assessment.

### 1.1. Aims and Objectives

This study aimed to compare the fish assemblage detected via eDNA metabarcoding of surface water samples to conventional fishing gears in a macrotidal estuary (river Dee, Wales, UK), and determine if eDNA could detect ecologically relevant spatial and seasonal patterns in assemblage composition. Ecological inference was supported by assigning species to estuarine-use guilds, which describe the overall ecological use of an estuary by a species and its links between the estuary and marine and freshwater areas (Elliott et al., 2007). Objective 1 was to compare eDNA data collected in autumn (October 2018) with data from fishing gears (seine, beams and trawls) collected concurrently. It was hypothesised that eDNA would detect more species in the estuary overall and would show a different assemblage composition (species presence/absence) to fishing gears. In addition, it was hypothesised that the assemblage composition would be correlated with salinity, across methods. Objective 2 was to determine if seasonal changes could be detected between autumn and summer using eDNA. It was hypothesised that there would be a greater species richness in the estuary overall in autumn than in summer, and that assemblage composition would differ between seasons. In addition, it was hypothesised that there would be a consistent correlation between eDNA assemblage composition and salinity between each season. Objective 3 was to directly determine if clear changes in eDNA assemblage composition could be detected longitudinally along the estuary in both autumn and summer (June 2019).

## 2. Methods

### 2.1. Study location

The river Dee (on the Welsh-English border, UK) ends in a macrotidal estuary, with a mean spring tidal range of ~10 m and high peak river flows (~300 $m^3$ $s^{-1}$). The water column is generally well-mixed, although stratification may occur in deeper channels at the mouth (Bolaños et al., 2013). There is substantial urban development and industrial activity around the Dee and the upper reaches are heavily canalised. Previously, WFD fish surveys were conducted from 2002 to 2017 in spring/summer (May to July) and, or autumn (September to November) by NRW (Natural Resources Wales; see SI Methods 1.1).

### 2.2. Autumn 2018 Fish Survey

In October 2018 a new fish survey design was implemented by NRW with assistance from the Department of Agriculture, Environment and Rural Affairs (Northern Ireland, UK). The survey was designed to provide data for the Estuarine Multi-metric Fish Index (EMFI; Harrison and Kelly, 2013). The survey was conducted from October 15, 2018 to October 18, 2018, approximately at low tide, around a neap tide. Sampling stations were placed in a spatially systematic manner (Rozas and Minello, 1997), from the head to the mouth of the estuary. Exact station placement accounted for deployment suitability for fishing gears. Fifteen stations were sampled using fyke nets and beam trawls. Thirty stations were sampled using seine nets, 15 of which were located near to the fyke and beam trawl stations (Fig. 1). Generally, a single sample per

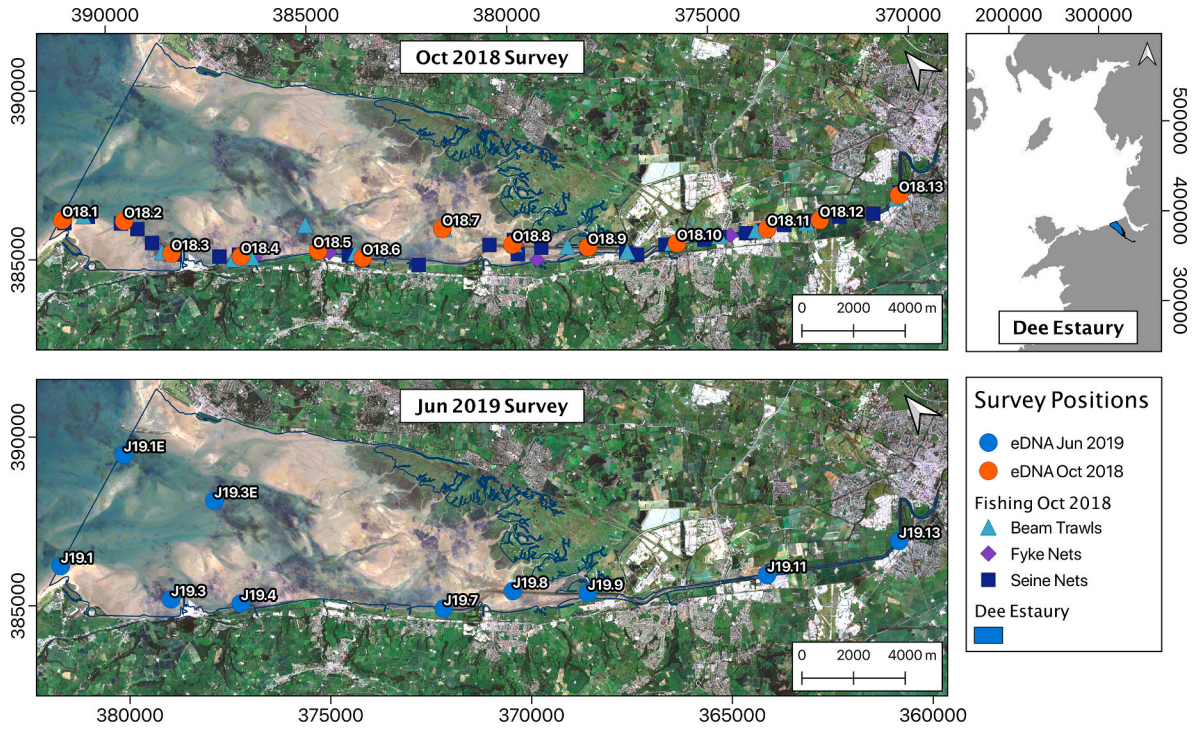


**Fig. 1.** Map of the Dee estuary, giving its geographic location within Britain and the distribution of eDNA sampling stations in October 2018 relative to fishing gear sampling stations and distribution of eDNA stations in June 2019. Coordinate System: British National Grid (EPSG:27,700) axis in eastings/northings (m). British Coastline (Wessel and Smith, 1996, 2017). Dee Satellite Photography (Copernicus, 2019), Dee Estuary Extent (Natural Resources Wales, 2019).

gear was taken at each station. For gear specification and operation see SI Methods 1.2.

### 2.3. eDNA sampling

In autumn 2018 (15–17 October) eDNA was sampled in the vicinity of 13 of the 30 seine stations, in a spatially systematic design around low tide. At each station three 1 L water surface samples were taken (total samples: 39). In summer (10–11 June 2019) eight of the original 13 eDNA stations were re-sampled in the upper and lower estuary at low tide, around a neap tide, with two additional stations added at the request of NRW (total samples: 33; Fig. 1). No fish sampling was conducted in summer 2019. Sampling equipment was prepared, and sampling conducted using procedures aiming to limit DNA contamination from external sources (Goldberg et al., 2016, SI Methods 1.3). A range of physicochemical variables were measured but only temperature (°C), salinity (recorded as practical salinity units) and dissolved oxygen (%) were recorded in both surveys. Physicochemical measurements were taken at the surface (~1 m depth), at every sampling station, using a Pro Plus Quatro multiprobe (YSI Inc.), and station location was recorded using a GPSMAP 64s (Garmin Ltd.). At the end of each day a 1 L dd$H_2O$ field blank was opened on board the vessel, sealed and placed inside the box with the other samples (3 field blanks per survey). Samples were immediately transported back to the laboratory on ice. At the laboratory samples were stored on ice or in a cold room (4 °C) until filtration at the end of each day. Samples were filtered through an encapsulated 0.8 μm PES filter with an integrated 5.0 μm glass fibre pre-filter (Nature Metrics Ltd.) using a Geopump™ peristaltic pump (Geotech Environmental Equipment, Inc.). The pre-filter reduces blocking of the underlying 0.8 μm filter allowing a relatively large volume of turbid water to be filtered. After filtration, filters were capped, bagged in sterile Whirl-Pak bags and frozen at – 20 °C. Field blanks were processed in an identical manner.

### 2.4. DNA extraction

Total DNA was extracted from each filter capsule using DNeasy Blood and Tissue Kits (QIAGEN) following a modification of the Spens et al. (2017) protocol. Briefly, 720 μl of Buffer ATL (QIAGEN) and 80 μl Proteinase K were added into each filter capsule and incubated overnight at 56 °C to allow sample lysis. Turbid estuarine waters have high concentrations of humic compounds which may inhibit PCR (Petit et al., 1999). Therefore to remove PCR inhibitors, 300 μl of flocculant solution (Sellers et al., 2018) was added to ~ 1 ml of sample lysate, vortexed and incubated for ~ 1 h at 4 °C in the refrigerator (G. Sellers and R. Donnely *pers. comm.*). Each sample was then centrifuged at 10,000×*g* for 2 min and 1200 μl of the supernatant removed (Sellers et al., 2018). The remaining DNA extraction followed Spens and Evans et al. (2017), and 70 μl of AE buffer (QIAGEN) was used for the final elution. Extraction blanks, consisting only of Buffer ATL and Proteinase K, were added at the sample lysis step and treated identically to samples. DNA extracts were stored at – 20 °C in DNA LoBind tubes (Eppendorf) in the pre-PCR lab. In total 35 samples were successfully extracted for October-2018; four samples from four separate stations were lost from the analysis due to a laboratory error. In June 2019, all samples were successfully extracted. For details of laboratory anti-contamination procedures and technical notes see SI Methods 1.4.

### 2.5. Library preparation & sequencing

A variable ~167 bp region of the mitochondrial 12S rRNA was PCR amplified using Tele02 primers (Taberlet et al., 2018), a modification of the MiFish_U primers (Miya et al., 2015), that generate an amplicon coincident with an extensive reference database for UK fish species (Collins et al., 2021). The Tele02 primers were chosen instead of MiFish_U primers as they have a higher primer universality *in silico*, the consistency with which primers bind here to fish species found in the UK (Collins et al., 2019). Total volume for each PCR reaction was 25 μl containing 12.5 μl 2x QIAGEN Multiplex PCR Master Mix (QIAGEN),

1.25 μl of eDNA template (or control), 0.5 μl of forward and reverse Tele02 primer at 10 μM concentration and 10.25 μl of RNase-Free Water (QIAGEN). The thermal cycle profile was: 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 60 °C for 1 min 30 s, 72 °C for 20 s and finally 72 °C for 10 min. QIAGEN Multiplex Master Mix was used because it consistently amplified these samples, which contained PCR inhibitors (Gibson, 2022). For each triplicate, 20 μl from each reaction was pooled to reduce effects of single-tube PCR bias. From each pool, 15 μl of product was cleaned of DNA fragments under 200 bp using ProNex (Promega) size-selective paramagnetic beads (bead ratio: 1.75X). Each cleaned PCR product was indexed with its own unique i5/i7 dual index combination (Integrated DNA Technologies) via a second-round of PCR (after Brennan et al., 2019). Total reaction volumes for each PCR were 25 μl, containing 12.5 μl 2x QIAGEN Multiplex PCR Master Mix, 3.0 μl of template, 1.0 μl of premixed i5/i7 indexes at 10 μM concentration (Integrated DNA Technologies) and 8.5 μl of RNase-Free Water (QIAGEN). This thermal cycling profile was: 95 °C for 15 min; 15 cycles of 94 °C for 30 s, 60 °C for 1 min 30 s, 72 °C for 20 s; 72 °C for 10 min. Following PCR, reaction concentrations were quantified using a Qubit dsDNA Broad Range Assay Kit (ThermoFisher Scientific). All PCR products were then pooled in equimolar quantities. This final pool was loaded into a 1.5% agarose gel, run for 30 min at 90V, and the target amplicon manually excised to remove primer dimer and high molecular weight material from the PCR product. The gel slice was purified using a QIAEX II Gel Extraction Kit (QIAGEN) and quantified using a Qubit dsDNA HS Assay Kit (Invitrogen). Finally, the purified pool was diluted to a concentration of 12 pM, with a 3% PhiX spike and sequenced on an Illumina MiSeq instrument using the Illumina MiSeq Reagent Kit v2 (500 cycles).

### 2.6. Bioinformatics

Primers were removed from reads using Cutadapt v2.9 (Martin, 2011), reads were trimmed using fastp v0.20.1 (Chen et al., 2018, see SI Methods 1.5) and denoised, dereplicated, merged and cleaned of chimaeras in dada2 v1.14.1 (Callahan et al., 2016). Taxonomic assignment followed Collins et al. (2019). Here, Amplicon Sequence Variants (ASVs) were assigned an approximate taxonomic classification by BLAST v2.9.0 searching (evalue: $1 \times 10^{-4}$; (Camacho et al., 2009) against the full NCBI nucleotide 'nt' database (NCBI, 2021). A specific classification was then assigned by BLAST (evalue: $1 \times 10^{-5}$) against the curated Meta-Fish-Lib database of UK fish species (Collins et al., 2021). All ASVs identified as fishes were then assigned a taxonomy using the phylogenetic Evolutionary Placement Algorithm v0.3.6 (Barbera et al., 2019; Czech et al., 2020; Czech and Stamatakis, 2019) to verify BLAST assignments. ASVs were assigned to species based on rules adapted from Collins et al. (2019). Rule 1: Species level taxonomy was assigned if both the best scoring BLAST hit and species level EPA result were identical and sequence identity was ≥97%. Rule 2: Species level taxonomy was assigned if both the BLAST hit and the species level EPA results were identical, BLAST identity was ≥95% and EPA probability ≥90%. Rule 3: Species level taxonomy was assigned if the BLAST identity alone was 100% and no species had tied on BLAST score, even if EPA had not given a species level result. For flounder (*Platichthys flesus*) and plaice (*Pleuronectes platessa*) Rule 3 was relaxed to 99% identity, as the EPA algorithm struggled to place these species and the reference library was complete for close relatives.

The majority of ASVs were identified to species level. Any ASVs not assigned to species level were assigned a higher-level phylogenetic classification, as determined from EPA. Collectively, taxa are therefore generally referred to as species. Species were assigned to estuarine-use functional guilds using the classification system for European estuaries (Franco et al., 2008). Single guild classifications in Elliot and Hemingway (2002b) and Elliott and Dewailly (1995) were used when multiple guild assignments were present in Franco et al. (2008) for single species. Briefly, these guilds are Marine Stragglers (MS), Marine Migrants (MM), Estuarine Species (ES), Anadromous Species (A), Catadromous Species (C) and Freshwater Species (F; Franco et al., 2008). It is acknowledged that single estuarine-use guild classification may be an oversimplification for certain species, e.g. three-spined stickleback (*Gasterosteus aculeatus,* Franco et al., 2008). Where no guild could be assigned, species were counted as unassigned (UA). The *Melanogrammus aeglefinus/Merlangius merlangus* taxon was considered as whiting (*M. merlangus*), because haddock (*M. aeglefinus*) were absent from catches from 2002 to 2018.

To account for contamination, a conservative, per species read threshold cut-off was calculated using an adaption of the approach in Yamamoto et al. (2017; SI Methods 1.6). This method resulted in species that contributed equal to or less than 0.55% of the total target fish reads in a sample being considered absent. Following cleaning, any samples which had sequenced poorly were removed (SI Methods 1.7; SI Figs. 1, 2, 3 and 4.).

### 2.7. Statistical analysis

Statistical analysis was conducted using R v3.6.3 (R Core Team, 2021). Firstly, for Objective 1 and 2 species lists were compared, in terms of which species did and did not intersect, using Upset plots (Conway et al., 2017). For Objective 1 species lists from eDNA sampling in October 2018 was compared to lists from the contemporary 2018 fish survey and the prior fishing surveys from 2002 to 2017. For Objective 2 the species lists from eDNA in October 2018 and June 2019 were compared along with the species lists for prior and contemporary fishing from 2002 to 2018.

#### 2.7.1. Objective 1 and 2: Species richness

Sample-size-based rarefaction and extrapolation (R/E) sampling curves were used to compare species richness estimates for the estuary overall (R-package: 'iNEXT' v2.0.20; Chao et al., 2014; Hsieh et al., 2016a). For Objective 1 curves were calculated for each gear type and the eDNA data for October 2018, using per sample presence/absences. For the eDNA data three curves were generated, one using all species, a second using only species detected at least once by prior or contemporary fishing (2002–2018), and a third using species only detected by contemporary fishing in 2018. These reduced datasets containing only species which had been detected in a specific fishing dataset were described as 'filtered'. R/E curves were generated for twice the sample size of each gear type and 95% confidence intervals (CI) and standard errors calculated using 1000 bootstrap replicates (Hsieh et al., 2016). Asymptotic species richness and a richness estimate per 30 samples were compared. Comparison of an estimate for 30 samples, the number of seine net samples, allowed a clearer comparison of the species richness captured by each method for a given sample size. For Objective 2, separate R/E curves and asymptotic species richness were compared for the eDNA data for October 2018 and June 2019 and these data filtered by all fish detections (2002–2018).

#### 2.7.2. Objective 1 and 2: Comparisons of assemblage composition

The reads of samples at each eDNA sampling station were summed and treated as an independent sample to account for non-independence of samples in spatial comparisons (Hurlbert, 1984). Only species detected in the estuary from prior or contemporary fish surveys (2002–2018) were included in the following analyses. This reduced the potential for spurious detections from eDNA transport influencing results to increase the studies relevance to environmental managers.

For Objective 1, to determine if eDNA showed a different assemblage composition (species presence/absence) to fishing, composition at all 13 eDNA stations was compared to nearby seine net stations (SI Methods 1.8; SI Table 1). Not all eDNA stations had a full complement of gear deployments nearby, therefore only a subset of nine stations was compared against all three fishing gear types. Assemblage composition was compared using ordination from generalised linear latent variable models (GLLVM) using the binomial distribution (probit link; R Package:

gllvm v1.3.1; Niku et al., 2019; SI Methods 1.9). Temperature and dissolved oxygen were collinear with salinity (Pearson correlation >0.8; SI Fig. 5); both were removed, and salinity retained, as it is the primary environmental driver of fish assemblage structure in estuaries (Whitfield et al., 2012). Direct comparisons of the fixed effects of sampling method, salinity and the interaction between method and salinity, on assemblage composition and individual species presence/absence was made using multivariate GLMs (R package: mvabund v4.1.12; Wang et al., 2012). *P*-values were calculated using 5000 bootstrap replicates (SI Methods 1.9) and backwards model selection was applied by assessing the AIC (Zuur et al., 2007).

For Objective 2, to determine seasonal changes in assemblage composition, the subset of stations that were sampled in both October and June were retained. For GLLVM ordinations, all the stations in summer were used, but for formal analysis using multivariate GLMs the additional two stations in the lower estuary were removed as they had not been sampled in both seasons (eight stations per season). Temperature, salinity and dissolved oxygen were checked for collinearity, and salinity and dissolved oxygen were retained in the analysis as temperature was colinear with salinity (SI Fig. 6). Direct comparisons of the fixed effects of season, salinity, dissolved oxygen, and the interaction between season and salinity, on fish assemblage composition was made using multivariate GLMs as above.

#### 2.7.3. Objective 3: Spatial variation in eDNA assemblage composition

For Objective 3, longitudinal spatial variation in eDNA assemblage composition was determined separately for October and June and analysed using GLLVM ordination as above. SIMPROF (Clarke et al., 2008; Whitaker and Christman, 2015) confirmed if groupings of stations apparent from ordination had the same assemblage composition (Bray-Curtis distances; *p*-value <0.001). Indicator species for each SIMPROF grouping were identified using the Indicator (IndVal) index (Cáceres and Legendre, 2009; Dufrene and Legendre, 1997; R-package: indicspecies v1.7.9, Cáceres and Legendre, 2009). *P*-values were calculated using 10,000 bootstrap iterations and adjusted for multiple testing using "fdr" (Benjamini and Hochberg, 1995).

## 3. Results

### 3.1. Sequencing and sample quality

In total 13,409,393 paired reads were sequenced on the MiSeq and 828,499 reads (6.2%) were assigned to fishes present in the Meta-FishLib database (SI Table 2 and SI Fig. 7). Two samples from October 2018 and two from June 2019 were removed due to extremely low read depth. A total of 6415 reads, from 12 fish species, were detected in negative controls, 98% from one field and one extraction blank. Following application of the 0.55% read contribution per sample cut off, the total number of species detected by eDNA was 39 in October, and 38 in June (SI Results 2.1). Species accumulation curves showed the number of taxa in most samples from both seasons were approaching saturation (SI Figs. 1 and 2). The volume of sample filtered varied between surveys due to variation in turbidity. In October the mean volume was 513 ml (SD: 23 ml), in June it was 907 ml (SD: 67 ml).

### 3.2. Physicochemical environment

In October 2018 salinity increased from 0.06 in the upper estuary to 30.04 in the lower estuary. Surface water temperature increased from 12.6 °C in the upper estuary to 13.4 °C in the lower estuary. Dissolved oxygen saturation increased from ≥84.5 to ≤92.1% in the upper estuary to 99.5% in the lower estuary (SI Fig. 8). In June 2019, salinities increased from 0.07 in the upper estuary to 30.23–27.65 in the lower estuary. Surface water temperatures ranged from 12.2 °C in the upper estuary, increasing to 14.1–14.3 °C in the lower estuary. Dissolved oxygen saturation increasing from 95% in the lower estuary to 101.3–101.2% in the upper estuary (SI Fig. 9).

### 3.3. Objective 1: Comparison of eDNA and fishing (Autumn, 2018)

#### 3.3.1. Species list comparison

Comparison of species lists detected by eDNA and fishing gears in October 2018, and the species list from 2002 to 2017, showed a high overlap between methods (Fig. 2 and Table 1). In October 2018, 24 species were detected by a combination of fyke nets, seine nets and beam trawls. All these species had been previously caught in the estuary (2002–2017). Sixteen of these species were detected by eDNA. In addition, detection of *Chelon* sp. by eDNA was likely *Chelon ramada,* detected by fishing. These 17 species included the 11 most abundant species detected by fishing which accounted for 98.8% of the total catch abundance. In October 2018, eDNA detected an additional 22 species not detected by fishing, eight of which had been detected by fishing in previous years in autumn. Overall, 71.8% of the reads in the October eDNA data came from fish species detected concurrently or previously in the estuary. Of the 14 taxa never detected by fishing, two could not be identified to species level (assignments: Gadidae and Cottidae) and therefore were not compared with species level records from fishing. Of the remaining 12 species, 11 belong to the Freshwater guild, including *Coregonus* sp. Which was likely from a freshwater upstream population. The tropical freshwater guppy (*Poecilia* reticulata), which did not receive a formal guild classification, was also detected. These previously undetected taxa represented 27.7% of the reads in October.

#### 3.3.2. Species richness comparison

Comparison of species rarefaction/estimation curves at the estuary level in October 2018 for the eDNA data, eDNA data filtered by all fishing species detections (2002–2018) and eDNA data filtered by species caught by fishing in October 2018, compared to fishing gears showed the unfiltered eDNA data generally had the highest asymptotic species richness. Although when the richness estimate for beam trawls was extrapolated to the asymptote it overlapped with the estimated asymptotic species richness for eDNA (95% CI overlapping, SI Table 3). The two filtered eDNA datasets showed more rapid increases in species richness at lower sampling levels than fishing gears (Fig. 3). Comparisons of asymptotic species richness between the filtered eDNA datasets and fishing gears showed that only eDNA data filtered by prior or contemporary fish detection, detected a higher asymptotic species richness than fyke nets (SI Table 3). Comparably, when species richness was estimated for a standardised sample size of 30 (number of seine nets) it was shown that eDNA data filtered by prior or contemporary detections also had a higher estimated species richness than seine nets, per 30 samples (SI Table 4). The eDNA data filtered by contemporary detections alone also had a higher estimated species richness than the fyke nets, per 30 samples (SI Table 4).

#### 3.3.3. Assemblage composition comparison (eDNA vs. seine)

After omitting species detected by eDNA not detected by prior or contemporary fishing, ordination of 13 seine net and eDNA stations showed a difference in assemblage composition (species presence/absences) between methods. There was a clear spatial trend in composition along the estuary detected by eDNA, with a comparable but less variable spatial shift in composition detected by seine nets (Fig. 4; residuals: SI Fig. 10). Sample read depth was also not required to describe variation in assemblage composition (SI results 2.2). The best fitting multivariate GLM showed statistically significant differences in assemblage composition between seine nets and eDNA and an effect of salinity (Table 2: model 1.; residuals: SI Fig. 11). A consistent effect of salinity on species presence/absence, between methods, was present as the salinity-method interaction term was dropped by model selection. Regarding species level patterns, two Freshwater guild species, dace (*Leuciscus leuciscus*), perch (*Perca fluviatilis*) and the potentially Anadromous three-spined stickleback (*G. aculeatus*) were detected less frequently at seine net

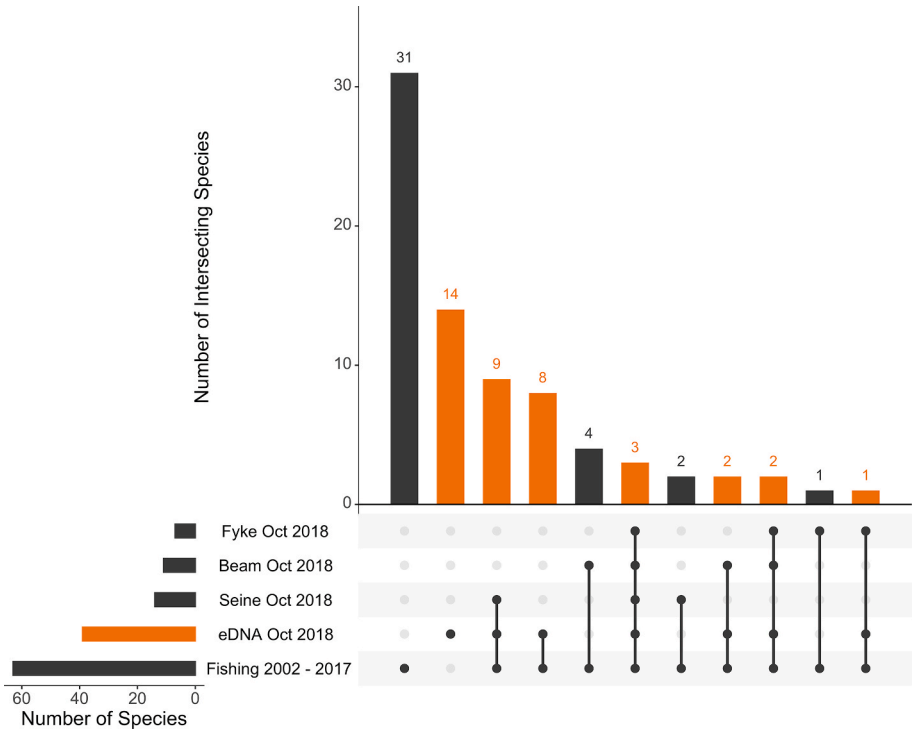


**Fig. 2.** UpSet plot (Conway et al., 2017) showing the number of intersecting species (i.e. shared groups of species) between each species list generated for each dataset. Species lists were generated for October 2018 fyke, beam and seine catch datasets, the cleaned eDNA October 2018 dataset and the prior fish survey data (2002–2017). The bottom table shows each dataset and which intersections between species lists they contributed to. Single black dots indicate no intersections between species lists. Black dots connected by lines indicate which lists shared species. The top bar graph gives the number of species shared between each species list in each intersection, intersections including species detected in the eDNA data are indicated in orange. The left-hand bar chat shows the total number of species within each species list (eDNA species list indicated in orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

than at eDNA stations. In addition, four Marine Migrant and one Estuarine species showed positive relationships with salinity (SI Table 5).

#### 3.3.4. Assemblage composition comparison (eDNA vs. all fishing gears)

Ordination of nine stations sampled with eDNA and all fishing gears showed some differences in assemblage composition between eDNA and fishing. Spatial variation in assemblage composition at eDNA stations along the estuary was still apparent with a more limited spatial trend present in fishing gears (Fig. 4; residuals: SI Fig. 13). Following model selection, a multivariate GLM showed no statistically significant difference in assemblage composition between methods, but there was a statistically significant effect of salinity on assemblage composition (Table 2 model 2; residuals: SI Fig. 14). At the species level two Marine Migrant species, whiting (*Merlangius merlangus*) and plaice (*Pleuronectes platessa*), showed positive associations with salinity, as they had in the eDNA vs. seine comparison (SI Table 6).

### 3.4. Objective 2: Seasonal comparisons

#### 3.4.1. Seasonal species list comparison

Comparison of the species lists between the unfiltered 2018 and 2019 eDNA data and all the fish monitoring data (2002–2018) showed a high degree of overlap (Fig. 5 and Table 1). Of the 38 species detected by eDNA in June 2019, 32 had been detected in October 2018 by eDNA. Of the 38 species detected in June, 22 had been detected at least once from 2002 to 2018 by fishing in summer and a further 5 detected in autumn. These previously detected species accounted for 69.7% of the reads in June 2019, comparable to October 2018 (71.8%). A further 11 Freshwater guild species had never previously detected by fishing in June. Similarly, to October (27.7%), these species accounted for 30.3% of the total reads. In addition, 9 of these species previously undetected by fishing had been detected in October by eDNA.

#### 3.4.2. Seasonal species richness comparison

Rarefaction/extrapolation curves calculated for eDNA data for October and June, using all samples collected in each survey, showed no differences in asymptotic species richness between seasons, regardless of whether the data was the full eDNA dataset or filtered by prior or contemporary fish detections (2002–2018; Fig. 6; SI Table 7).

#### 3.4.3. Seasonal assemblage composition comparison

For seasonal comparisons between the eDNA data, filtered by all fish detections (2002–2018), in October and June a subset of eight stations in the upper and lower estuary were compared. However, for ordination, the two additional stations sampled in June 2019 were retained in the analysis. Ordination showed an overlap in assemblage composition between seasons and both seasons showed a shift in assemblage composition along the estuary (Fig. 7; model residuals: SI Fig. 14). Sample read depth in June was also not required to describe variation in assemblage composition as in October (SI Results 2.2). Following model selection, only salinity, which had a statistically significant effect on assemblage composition, was retained in the multivariate GLM (Table 3; model residuals: SI Fig. 15). Given that the interaction between season and salinity were dropped from the model, this suggests the effect of salinity was consistent across seasons. In addition, salinity had a positive effect on the incidence of sand goby (*Pomatoschistus minutus*) an Estuarine species, and a negative effect on the incidence of the Freshwater guild species chub (*Squalius cephalus*; SI Table 8).

### 3.5. Objective three: spatial variation in eDNA assemblage

Ordination and SIMPROF analysis showed clear spatial changes in

**Table 1**
Taxonomic and guild assignments for each species and comparison to contemporary and prior fishing.

| Taxa | Common Name | Guild | Oct-18 | | | | Jun-19 | | Detected in Fishing (2002–18) |
|---|---|---|---|---|---|---|---|---|---|
| | | | eDNA | | Fishing | | eDNA | | |
| | | | Total Reads | Incidence | Total Catch | Incidence | Total Reads | Incidence | |
| *Platichthys flesus* † | European flounder | MM | 78,091 | 31 | 201 | 41 | 77,903 | 26 | ● |
| *Merlangius merlangus* † | Whiting | MM | 38,163 | 13 | 31 | 2 | 673 | 2 | ● |
| *Phoxinus phoxinus* † | Eurasian minnow | F | 34,113 | 27 | – | – | 79,402 | 20 | ○ |
| *Cottus gobio* † | Bullhead | F | 24,414 | 28 | – | – | 36,344 | 22 | ○ |
| *Salmo trutta* | Brown Trout | A | 18,426 | 25 | – | – | 16,633 | 19 | ● |
| *Anguilla Anguilla* † | European eel | C | 13,107 | 23 | 24 | 5 | 18,644 | 19 | ● |
| *Pleuronectes platessa* † | European plaice | MM | 11,947 | 11 | 106 | 19 | 14,941 | 14 | ● |
| *Salmo salar* † | Atlantic salmon | A | 9667 | 20 | – | – | 6050 | 16 | ● |
| *Oncorhynchus mykiss* † | Rainbow trout | F | 8185 | 24 | – | – | 6960 | 18 | ○ |
| *Barbatula barbatula* | Stone loach | F | 7020 | 22 | – | – | 12,310 | 19 | ○ |
| *Pomatoschistus microps* | Common goby | ES | 6145 | 13 | 1707 | 29 | 4318 | 11 | ● |
| *Leuciscus leuciscus* † | Common dace | F | 5853 | 17 | 16 | 8 | 9842 | 16 | ● |
| *Rutilus rutilus* | Roach | F | 5456 | 19 | – | – | 10,503 | 18 | ● |
| *Gasterosteus aculeatus* † | Three-spined stickleback | A | 4633 | 15 | 8 | 5 | 31,095 | 21 | ● |
| *Thymallus thymallus* | Grayling | F | 4068 | 18 | – | – | 8233 | 14 | ○ |
| *Dicentrarchus labrax* † | European seabass | MM | 3461 | 12 | – | – | 11,734 | 11 | ● |
| *Squalius cephalus* | Chub | F | 2914 | 13 | – | – | 7733 | 17 | ● |
| *Gymnocephalus cernua* | Ruffe | F | 2708 | 12 | – | – | 10,227 | 16 | ○ |
| *Perca fluviatilis* | European perch | F | 2539 | 15 | 1 | 1 | 7679 | 17 | ● |
| *Pomatoschistus minutus* | Sand goby | ES | 2473 | 10 | 61 | 15 | 8560 | 9 | ● |
| *Gobio gobio* | Gudgeon | F | 2431 | 11 | 1 | 1 | 5373 | 16 | ● |
| *Clupea harengus* | Atlantic herring | MM | 2028 | 10 | 4 | 3 | 4401 | 6 | ● |
| *Sprattus sprattus* | European sprat | MM | 2002 | 5 | – | – | 57,473 | 13 | ● |
| Ammodytidae | Sand Lances | UA | 1702 | 6 | – | – | 45,673 | 10 | ■ |
| *Solea solea* | Common sole | MM | 1401 | 2 | 1 | 1 | 4173 | 9 | ● |
| *Barbus barbus* | Barbel | F | 1351 | 5 | – | – | 186 | 1 | ○ |
| *Limanda limanda* | Dab | MM | 1324 | 3 | 21 | 1 | 5554 | 6 | ● |
| Gadidae | Cods | UA | 1009 | 1 | – | – | – | – | – |
| *Esox lucius* | Northern Pike | F | 976 | 9 | – | – | 948 | 5 | ● |
| *Poecilia reticulata* † | Guppy | UA | 944 | 2 | – | – | – | – | ○ |
| *Osmerus eperlanus* | European smelt | A | 920 | 5 | 2 | 2 | 3072 | 8 | ● |
| *Chelon* sp.* | Mullet Genus | UA | 794 | 2 | 6 | 4 | 1011 | 3 | ■ |
| Cottidae | Sculpins | UA | 759 | 1 | – | – | – | – | – |
| *Gadus morhua* | Atlantic Cod | MM | 636 | 4 | 9 | 2 | – | – | ● |
| *Coregonus* sp. | Whitefish | F | 394 | 5 | – | – | – | – | ○ |
| *Abramis brama* | Freshwater Bream | F | 266 | 2 | – | – | 1116 | 5 | ○ |
| *Pungitius pungitius* | Ninespine stickleback | F | 161 | 2 | – | – | 213 | 2 | ○ |
| *Salvelinus fontinalis* | Brook trout | UA | 83 | 1 | – | – | – | – | ○ |
| *Scophthalmus rhombus* | Brill | MS | 64 | 1 | 2 | 1 | – | – | ● |
| *Buglossidium luteum* | Solenette | MS | – | – | – | – | 1795 | 5 | ● |
| *Carassius auratus* | Goldfish | F | – | – | – | – | 101 | 1 | ○ |
| *Cyprinus carpio* | Common carp | F | – | – | – | – | 368 | 1 | ○ |
| *Echiichthys vipera* | Lesser weever | MS | – | – | 1 | 1 | 144 | 3 | ● |
| *Myoxocephalus scorpius* | Shorthorn sculpin | ES | – | – | – | – | 1147 | 2 | ● |
| *Scardinius erythrophthalmus* | Rudd | F | – | – | – | – | 126 | 1 | ● |

**Incidence:** Total presence/absence in samples. **Detected in Fishing:** Exact Species Present: ●, No Species or Clade Present: ○, Identified Undifferentiated Taxa Present: ■, Taxa where comparison not possible indicated with a dash (–). **Chelon* sp. Refers to *Chelon ramada* in contemporary fishing data. † Indicates species detected in blanks.

assemblage composition across stations in October and June for the eDNA data filtered by prior or contemporary fishing detections (Fig. 8; SI Figs. 16 and 17). In June three groupings of stations in assemblage composition (groups: A - C) along the estuary were obvious from the ordination (Fig. 8; SI Fig. 16) and SIMPROF analysis, which identified an additional outlier station (group D; $p = <0.001$; SI Fig. 17). Indicator species analysis showed the Marine Migrant species whiting (*Merlangius merlangus*) and sea bass (*Dicentrachus labrax*) were associated with group A and B and the Freshwater common roach (*Rutilus rutilus*) was associated with group B and C (SI Table 9). This suggests the presence of two, overlapping eDNA assemblages characterised by Marine Migrant and Freshwater species in the lower and upper estuary, respectively. In June a comparable split was seen in assemblage composition in the ordination and SIMPROF analysis ($p = <0.001$; SI Figs. 16 and 17), between stations in the lower estuary (A) and those stations in the upper estuary (B, Fig. 8). Indicator species analysis showed that six Marine Migrant and Estuarine species and the Ammodytidae were associated with the group A grouping, B was not characterised by indicator species. June 2019 A group overlapped spatially with October 2018 A + B grouping and shared an indicator species, *D. labrax* (SI Table 9; Fig. 8).

## 4. Discussion

### 4.1. Overview

This study has provided a comprehensive comparison of the teleost fish assemblage detected via eDNA metabarcoding of surface water samples and conventional fishing gears in a temperate macrotidal estuary. The analysis focused on species which had been detected by prior or contemporary fish surveys, thus giving confidence the species

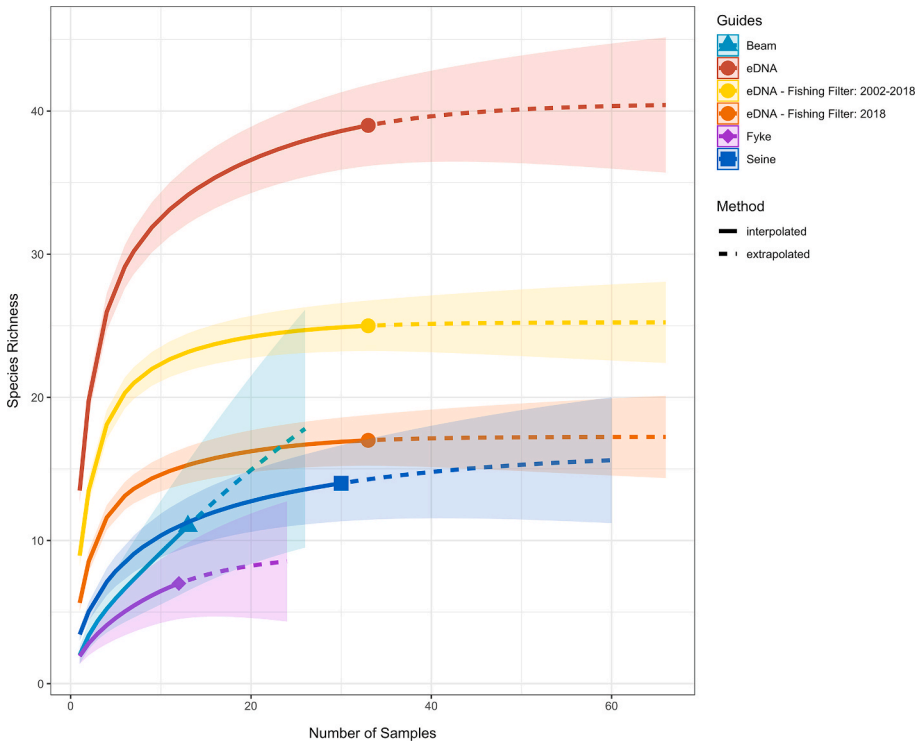


**Fig. 3.** Species Rarefaction and Estimation (R/E) curves and 95% confidence intervals calculated on species (including some higher-level taxa) presence/absence data for eDNA and catches from October 2018. R/E curves were calculated individually for each gear type (Beam, Fyke and Seine). For the 2018 eDNA data, R/E curves were calculated including all species (eDNA), species only detected by prior or contemporary fishing (eDNA - Fishing, 2002–2018 Filter) and species only detected in the October 2018 catch data (eDNA - Fishing, 2018 Filter). R/E curves were calculated, to double the observed sample size, using the iNEXT software (Hsieh et al., 2016), confidence intervals were calculated using 1000 bootstrap iterations.

detected with eDNA had the potential to occur in the estuary, rather than being the result of eDNA transport alone. Higher species richness was detected by eDNA in the estuary overall and a different assemblage composition was detected relative to certain fishing gear types, even when the data was filtered by species detected by prior and contemporary fishing. These results partially support the initial hypothesis (Objective 1). Additionally, there was a clear correlation between salinity (and its colinear variables) and assemblage composition, consistent between eDNA and fishing gears (Objective 1), which was also consistent over seasons (Objective 2) as hypothesised. Conversely, it was not possible to detect changes in assemblage composition across seasons as initially hypothesised (Objective 2). Finally, clear spatial changes in eDNA assemblage composition were obvious along the estuary, in both seasons, as was hypothesised (Objective 3).

### 4.2. Detection and composition between eDNA and fishing gears

Overall, 17 of the 26 (71%) species detected in fishing gears in autumn were detected by eDNA, including the 11 most abundant. This is comparable to coverage in the tidal Thames, where 13 out of 18 species (72%) detected by fishing gears were detected by eDNA metabarcoding using a 12S rRNA and a CO1 marker (Hallam et al., 2021). At present, the greatest coverage of fish species would be provided by a combination of eDNA and sampling using multiple fishing gears, as with previous studies in estuaries (Cole et al., 2022; Hallam et al., 2021; Zou et al., 2020). Species coverage in the present study would have risen without the specific contamination threshold used, e.g. Nilsson's pipefish (*Syngnathus rostellatus*) was removed (SI Results 2.1). The presence of contamination necessitated the use of a threshold cut off, which is a standard approach in eDNA metabarcoding (Sepulveda et al., 2020). Field contamination in October 2018 was probably due to working alongside a fish survey, whereas lab contamination was sample to sample contamination from operator error. Therefore, no species detected in the blanks were removed *a priori* as their eDNA was most likely present in the estuary. In future more nuanced filtering methodologies may allow some of the rare species which were lost by filtering to be retained. In addition, two species detected by fishing gears not detected in October 2018 were detected in June 2019: lesser weever (*Echiichthys vipera*) and common dragonet (*Callionymus lyra*; prior to data cleaning; SI Results 2.1). Although *C. lyra* has a very poor fit with the primers used, resulting in poor amplification and efficiency (Collins et al., 2022). Failure to detect these species in autumn could have been due to variation in sample storage period prior to extraction (12 months in autumn, 6 months in summer), stochastic factors or primer bias. In addition, a further species not detected by eDNA in October 2018 was pogge (*Agonus cataphractus*), absent from the reference database at the time of analysis. It is possible that species detections would rise with ongoing development of the reference database. Further to this, although no elasmobranchs were detected by fishing in 2018, the addition of primers targeting this taxonomic group e.g. MiFish_E/Elas02 (Miya et al., 2015; Taberlet et al., 2018) would allow their detection. It should also be acknowledged that the yield of fish reads in this study from target species was low (6.2% of total reads), compared to other such studies using Tele02 primers (Aglieri et al., 2021; Zhang et al., 2020). This is largely due to the primers amplifying other vertebrate groups present within the samples e.g. mammals and birds (SI Fig. 7). Future re-analyses of these samples using MiFish_U primers (Miya et al., 2015) may be useful to determine if they reduce non-target amplification. However, fish sequence read depth had a limited effect on assemblage composition as shown by species accumulation curves and model selection of GLLVM ordinations.

Metabarcoding of eDNA detected more species (filtered by fishing detections from 2002 to 2018) in the estuary overall, than seine and fyke nets, per 30 samples. This was also the case for eDNA compared to fyke nets when only species caught in autumn 2018 were retained in the eDNA data. Comparisons between asymptotic species richness showed

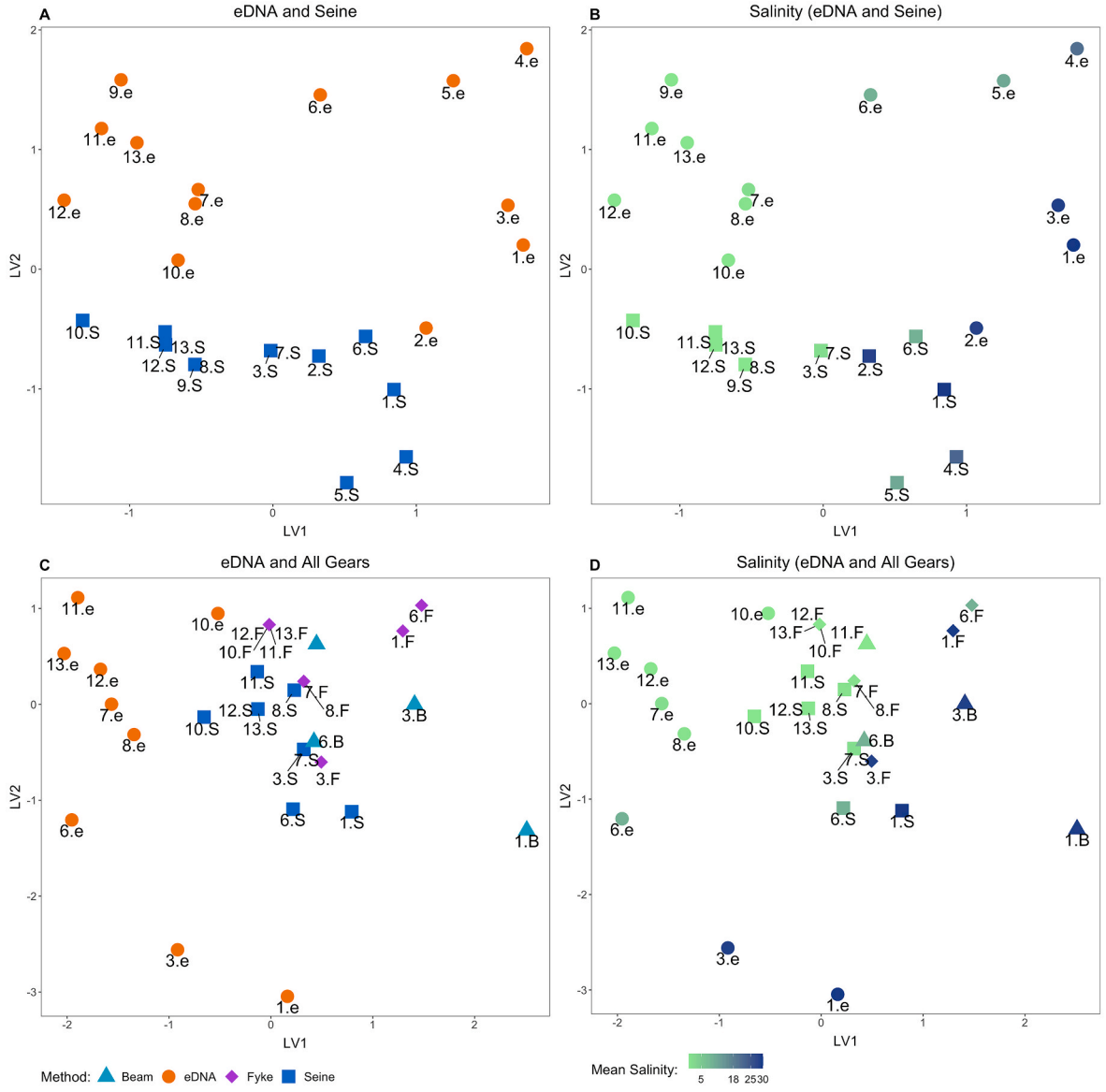


**Fig. 4.** Presence/absence of fish species per station in October 2018, modelled using a binomial GLLVM (log link; two latent variables; 50 iterations) for eDNA (filtered by prior or contemporary fish detections, 2002–2018) and fishing stations. Panel A shows eDNA station composition relative to seine net stations. Panel C shows eDNA station composition relative to beam trawl, seine net and fyke net stations. Panel B and D shows salinity, in practical salinity units, at each station for eDNA and seine, and eDNA and all gears, respectively.

more limited differences, probably due to the high uncertainty associated with extrapolating to the asymptote (Hsieh et al., 2016). However, eDNA metabarcoding is clearly a more sensitive method for detecting differences in species richness at the estuary level than seine, and particularly fyke nets. Therefore, there is broad support for the hypothesis that eDNA will detect more species in the estuary overall than each gear type, except for beam trawls. In addition, eDNA richness estimates approached an asymptote. Therefore, eDNA may be useful for calculating assessment metrics of fishes in estuaries that rely on species richness estimates, which are particularly sensitive to sampling effort (Gamito et al., 2012). The demonstrated sensitivity to species richness is comparable to other studies in estuaries where eDNA generally detects greater species richness, per site, than conventional methods (Cole et al., 2022; Hallam et al., 2021; Zou et al., 2020). Although, in the tidal Thames no differences in eDNA and fishing gears in species richness estimates at the level of the estuary were detected (95% confidence intervals overlapping). This was probably because data from the multimethod technique was aggregated rather than each gear type being compared individually (Hallam et al., 2021). It is arguable that in the present study, replicate eDNA samples should also have been aggregated for each station when compared to the fishing gears. However, this was not required as the aim of this particular analysis was to compare different methodologies at the estuary scale, on a sample-per-sample basis, rather than explicitly test hypotheses with a spatial component.

Comparing assemblage spatial composition with fishing gears, metabarcoding of eDNA showed a different composition to the assemblages derived from the seine nets. Although, no difference was detected in the less well replicated comparison with all gear types, probably due to a reduced sample size. Therefore, there is partial support for the initial hypothesis that eDNA would show a different assemblage composition compared to fishing gears. Differences in assemblage composition between eDNA and seine nets are comparable to studies that have shown different compositions between eDNA and a multimethod netting technique (Hallam et al., 2021) and BRUVs (Cole et al., 2022) in estuaries. The species which were detected less frequently with the seine netting method: three-spined stickleback (*G. aculeatus*), common dace

**Table 2**
ANOVA for multivariate GLMs – Species Presence/Absence.

| | | | | |
|---|---|---|---|---|
| **Model 1 eDNA + Seine** | **Model 1:** Species Presence/Absence ~ Salinity + Method | | | |
| | **AIC:** 477 | | | |
| | **Explanatory Variable** | **Residual DF** | **Wald-Test** | **P-Value** |
| | **Intercept** | 25 | | |
| | **Salinity** | 24 | 6.883 | $<2 \times 10^{-16}$ *** |
| | **Method (Seine vs. eDNA)** | 23 | 4.944 | 0.035 * |
| **Model 2 eDNA + All Fishing Gears** | **Model 2:** Species Presence/Absence ~ Salinity + Method | | | |
| | **AIC:** 523 | | | |
| | **Explanatory Variable** | **Residual DF** | **Wald-Test** | **P-Value** |
| | **Intercept** | 35 | | |
| | **Salinity** | 34 | 6.515 | $<2 \times 10^{-16}$ *** |
| | **Method (Seine vs. Fishing)** | 31 | 4.755 | 0.487 |

Significance codes: *** <0.001 ** <0.01 * <0.05.

(*Leuciscus leuciscus*) and European perch (*Perca fluviatilis*), were also caught by seine nets. The latter suggests that the greater detection of these species by eDNA was driven by the greater detection probability of eDNA and potential transport within the estuary, rather than potentially spurious detections in the eDNA alone from transport into the estuary.

### 4.3. Assemblage composition and salinity

Exploration of spatial changes in assemblage composition showed that in both seasons, regardless of differences in design, a clear spatial shift in composition along the estuary was present. This is remarkable given the river was in flood during both surveys (SI Figs. 18 and 19). In October the three sub-assemblages that showed a change in assemblage composition over space, also exhibited overlapping indicator species, with a similar pattern in June. Two Marine Migrant species, *M. merlangus* and *D. labrax,* were associated with the assemblages in the lower estuary. Whereas common roach (*R. rutilus*) was associated with the assemblage in the upper estuary and one of the assemblages in the lower estuary. This supports the growing body of evidence that despite the impact of eDNA persistence and transport, localised patterns in fish assemblages can be detected within estuaries using eDNA (Cole et al., 2022; DiBattista et al., 2022; García-Machado et al., 2022; Hallam et al., 2021; Saenz-Agudelo et al., 2022). This complements the evidence for spatial patterns from marine assemblages more generally (Jeunen et al., 2019; Port et al., 2016; Yamamoto et al., 2017).

It is likely that eDNA transport influenced the results to some degree given its prevalence in aquatic systems (Deiner and Altermatt, 2014; Shaw et al., 2016; Yamamoto et al., 2017). Fundamentally, eDNA data possesses inherent detection uncertainty and it is not possible to prove definitively that a fish was present when the sample was taken (Jerde, 2021). This study assumed that eDNA of species not detected by prior, or contemporary fishing were likely to have been transported into the estuary, either from upstream or from wastewater sources, and omitted them from much of the analysis. This appears to be a valid, if simplistic, assumption, given that all the previously undetected taxa identified by eDNA to species level were freshwater species rather than being a random assortment across different estuarine use guilds. The guppy (*P. reticulata*), a common freshwater tropical aquaria species which tolerates brackish conditions (Froese and Pauly, 2023; Rodriguez, 1997), was also removed by prior detection filtering. This species was probably introduced by wastewater effluent; however, guppies can establish themselves in the warm cooling water expelled from power stations (Wheeler and Maitland, 1973). Guppies were only detected downstream (station 4 and 8) of a power station which is a thermal pollution source (NE and CCW, 2010). It is plausible guppies were established at the power station. Comparably, Atlantic Salmon (*S. salar*)

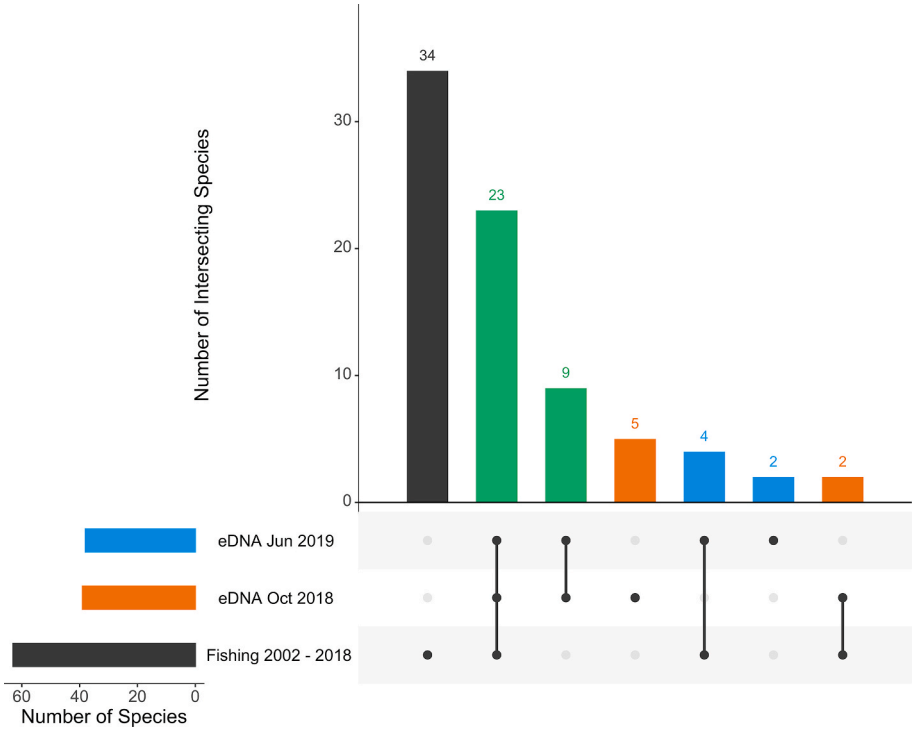


**Fig. 5.** UpSet plot (Conway et al., 2017) showing the number of intersecting species (i.e. shared groups of species) between each species list generated for each dataset. Species lists were generated for the cleaned eDNA October 2018 and June 2019 datasets and the combined prior and contemporary fish data (2002–2018). The species lists for June are shown in blue and the species lists for October are shown in orange in the table. Intersections which only overlapped with October are shown in orange, intersections which only overlapped with June are shown in blue. Intersections which overlapped with June and October are shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

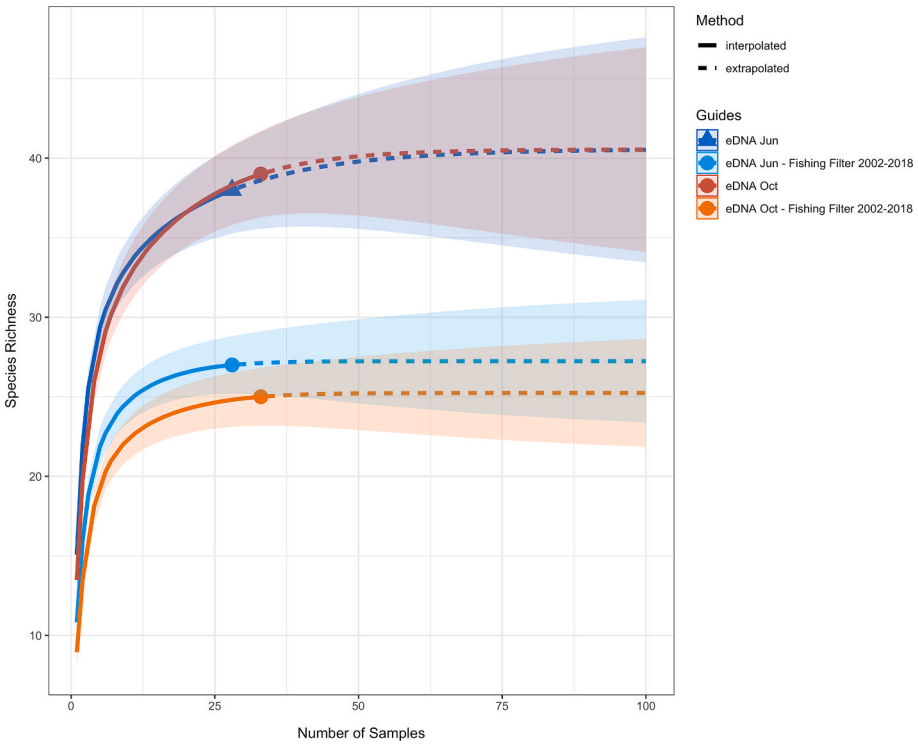


**Fig. 6.** Species Rarefaction and Estimation (R/E) Curves and 95% confidence intervals calculated on species (including some higher-level taxa) presence/absence data for eDNA from October 2018 and June 2019 (all samples and sites). For the 2018 and 2019 eDNA data, curves were calculated including all species and species only previously detected in prior or contemporary fish surveys from 2002 to 2018. R/E curves were calculated, to double the observed sample size, using the iNEXT software (Hsieh et al., 2016), confidence intervals were calculated using 1000 bootstrap iterations.

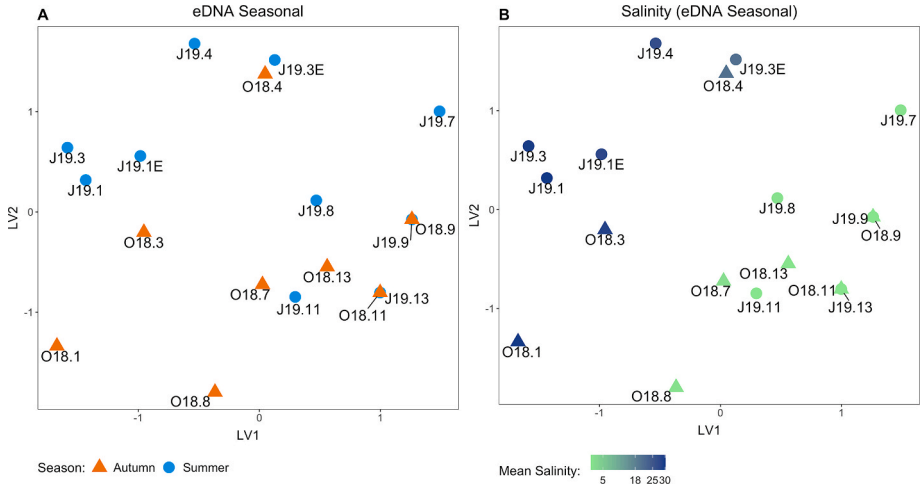


**Fig. 7.** Presence/absence of fish species per station as modelled using a binomial GLLVM (log link; two latent variables; 50 iteration) for eDNA (filtered by fish detections 2002–2018) in October 2018 and June 2019. Panel A shows season, panel B shows salinity at each station.

**Table 3**
ANOVA for multivariate GLM – Species Presence/Absence.

| | | | |
|---|---|---|---|
| **AIC:** 297 | | | |
| **Final Model:** Species Presence/Absence ~ Salinity | | | |
| **Explanatory Variable** | **Residual DF** | **Wald-Test** | **P-Value** |
| **Intercept** | 15 | | |
| **Salinity** | 14 | 6.683 | $<2 \times 10^{-16}$ *** |

Significance codes: *** <0.001 ** <0.01 * <0.05

has been detected by prior fishing and was retained in the analysis, however it is widely eaten and its eDNA could have been introduced to the estuary via wastewater. These cases illustrate some of the difficulties with inferring detections based on eDNA. The method of filtering eDNA detections using fishing data does not however consider the higher detection probability of eDNA (Jerde, 2021) and more sophisticated occupancy modelling methods should be used in future (Burian et al., 2021). In addition, certain novel detections may be useful in other contexts, such as the study of changes in species distributions due to global climate change.

In addition, comparisons between the assemblage composition of eDNA and fishing gears showed a consistent correlation with salinity

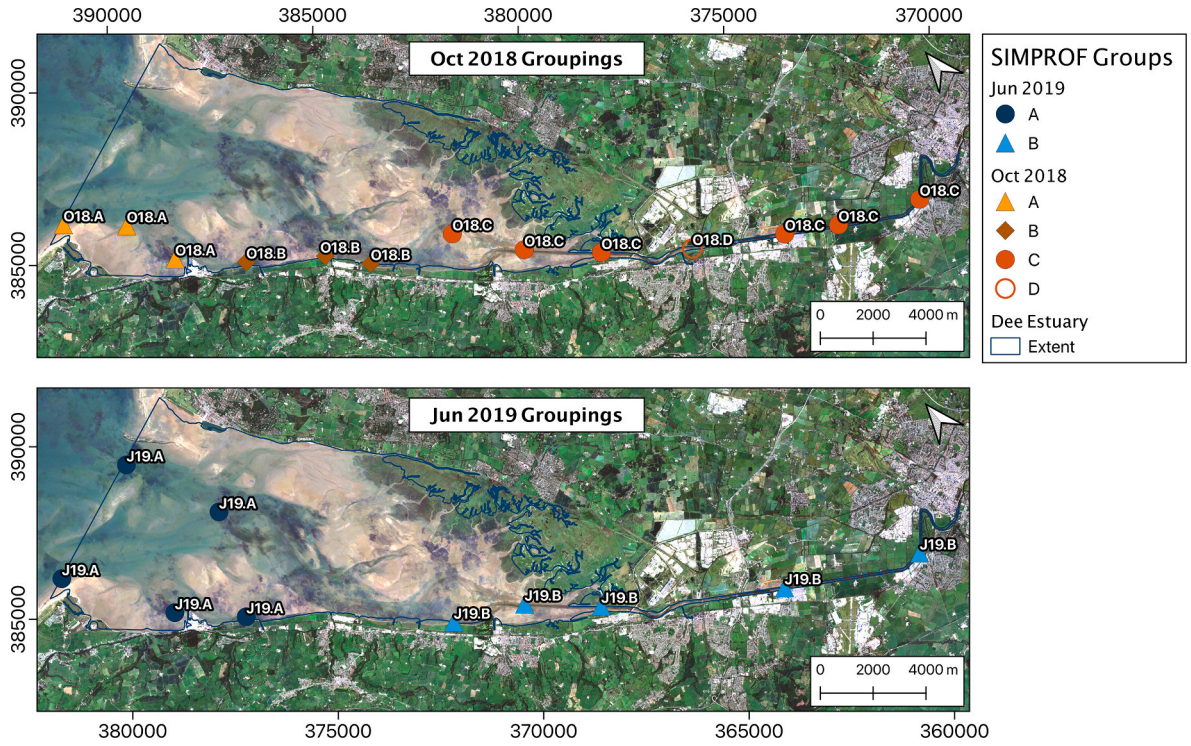


**Fig. 8.** The SIMPROF assemblage groupings mapped onto their sampling locations. Colours and symbols indicate the SIMPROF groupings for each season ($p = < 0.001$) calculated using Bray-Curtis dissimilarities calculated using species presence/absences (SI Fig. 17). Coordinate System: British National Grid (EPSG:27,700) axis in eastings/northings (m; see Fig. 1 for map data references). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(and its collinear variables temperature and dissolved oxygen), regardless of the methodology. Focusing on the comparison between seine nets and eDNA, four Marine Migrant and one Estuarine Species had a statistically significant positive association with salinity. This included *M. merlangus* and *D. labrax*, also identified as the indicator species for the eDNA assemblages in the lower estuary. All five species were absent in the upper estuary, where salinity was below 1. These results support those reported from Japanese estuaries where the proportion of marine species detected increased, compared to freshwater and brackish species, with increasing salinity (Ahn et al., 2020). Salinity influences the distribution of fishes and the structure of the assemblage through their physiological salinity tolerance (Marshall and Elliott, 1998; Selleslagh et al., 2009; Whitfield et al., 2012). Although a variety of other environmental factors, including temperature and dissolved oxygen, can influence fish assemblage structure (Martino and Able, 2003). In the context of eDNA detections, the correlation with salinity could also be due in part to changes in the relative quantities of transported eDNA from the marine environment and the river. It is notable that no freshwater species showed a species level correlation with salinity, potentially due to downstream transport obscuring these associations. Vertical gradients in eDNA assemblage composition were not considered in this study although they can occur in highly stratified Fjords, for example (Jeunen et al., 2020). The water column of the Dee is generally well-mixed (Bolaños et al., 2013). However, vertical eDNA gradients in well-mixed systems should be investigated further to determine if they occur and influence the detection of species.

### 4.4. Comparison between Seasons

Compared to spatial changes in assemblage composition, a direct comparison between the assemblage composition of eight stations in autumn and summer showed no difference in assemblage composition, as was also found by Hallam et al. (2021). This contrasts with other eDNA studies which have been able to detect seasonal changes in the assemblage composition within estuaries (DiBattista et al., 2022; Stoeckle et al., 2017) and in coastal fish assemblages more generally (Sigsgaard et al., 2017). There was additionally no difference in the estimated asymptotic species richness at the estuary level between either season, comparable to the observation that there were no statistically significant differences in species richness between winter and summer in the tidal Thames (Hallam et al., 2021). Seasonal changes in the structure of the fish fauna in temperate estuaries are a well-established phenomenon (Henderson and Bird, 2010; Maes et al., 2005; Selleslagh et al., 2012). Consistent seasonal changes in the fish fauna are caused by sequential immigration and emigration of marine, freshwater, estuarine and diadromous species, probably controlled by the spawning times and the time needed for larval and juvenile stages to recruit into the estuary (Maes et al., 2005). However, given that much of these seasonal changes are related to changes in abundance it maybe that a presence/absence analysis using a relatively sensitive method is not the best way to assess seasonal variation. Generating proxies of abundance and biomass from eDNA read counts is challenging as any relationship between these is affected by numerous biological, environmental, and methodological factors (Hansen et al., 2018; Lamb et al., 2019; Rourke et al., 2022). An assessment of the quantitative relationship between fish abundance and read count is outside the scope of this publication. However, given the encouraging results of other studies (Rourke et al., 2022), this should be addressed in future. Other factors may have prevented detection of seasonal trends, the threshold cut-off to remove contamination applied across species may have smoothed out any differences in assemblage composition between the two seasons. In addition, approximately twice the volume of water was filtered per sample in summer compared to autumn, due to variation in turbidity. It is unknown how this influenced the results, however it seems most likley this would create artificial differences rather than a lack of difference in assemblage composition (see Sigsgaard et al., 2017). Further study and method development is required in this area. The most likely explanation however is the design employed in the current study was also not

optimal for assessing seasonal changes in assemblage composition. Given that there were effectively only two temporal replicates in the analysis (two seasons) a design with monthly sampling would have been more appropriate (DiBattista et al., 2022; Stoeckle et al., 2017).

### 4.5. Implications for management

This study has focused on providing direct comparisons of species richness and assemblage composition between eDNA and fishing gears. Further research should build on these findings to devise ways to calculate eDNA-based metrics for indices of fish assemblage health (Coates et al., 2007; Delpech et al., 2010; Harrison and Kelly, 2013). For example, eight of the 14 metrics in the Estuarine Multi-metric Fish Index use varying measures of species richness (Harrison and Kelly, 2013) and therefore should be straightforward to calculate using eDNA data. In addition, further surveys should be conducted on other estuaries which are known to show variation in anthropogenetic impacts, and the health of the fish assemblage, to assess if eDNA reflects this variance. However, the current research does have some important implications for survey design in an applied setting. Firstly, eDNA sampling in estuaries should have a spatially replicated design to take account of spatial variation in the fish assemblage. Secondly, future surveys should record salinity, and other physicochemical parameters, at each sampling station to contextualise the results and provide valid comparisons, as is best practice in fishing surveys (Elliot and Hemingway, 2002a). Thirdly, any eDNA detections should be scrutinised to determine if they are likely to occur in the ecosystem before species are included in bioassessments of estuarine ecosystem health. More sophisticated occupancy modelling methods which combine both data from eDNA and from fishing should be used in future (Burian et al., 2021).

## Ethics statement

The routine monitoring fish surveys conducted by NRW were exempt by the Welsh Government from the relevant legislation relating to the protection of fish stocks and the environment (Exemption Reference: DISP068). Water sampling by Bangor University on the Dee estuary, a protected area, was conducted with the consent of NRW, the competent conservation authority, in accordance with local regulations.

## CRediT authorship contribution statement

**Thomas I. Gibson:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gary Carvalho:** Methodology, Funding acquisition, Conceptualization. **Amy Ellison:** Writing – review & editing, Methodology, Formal analysis. **Enrica Gargiulo:** Investigation. **Tristan Hatton-Ellis:** Writing – review & editing, Funding acquisition, Conceptualization. **Lori Lawson-Handley:** Writing – review & editing, Methodology, Conceptualization. **Stefano Mariani:** Writing – review & editing, Methodology, Conceptualization. **Rupert A. Collins:** Writing – review & editing, Formal analysis. **Graham Sellers:** Methodology. **Marco Antonio Distaso:** Investigation. **Carlo Zampieri:** Investigation. **Simon Creer:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare no competing interests. All authors have approved this manuscript. This material is original and has not been published elsewhere in any format except for the lead author's PhD thesis (Chapter 2; Gibson, 2022; available at: https://research.bangor.ac.uk/portal/files/49682869/T_I_Gibson_Thesis_2022.pdf).

## Data availability

The raw sequence data was deposited under BioProject accession number PRJNA930409 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/). Processed ASV, species level and other supporting data is attached as supplementary files.

## Acknowledgements

T. Gibson would like to thank the following individuals for their help on this project. Firstly, Mark Kyriacou, Adam Cooper, Adam Lyshon and the rest of the NRW survey team. Trevor Harrison and the rest of the DAERA survey team. Ian Marland of Marland Marine Services Ltd. All these individuals planned and executed the fish survey and contributed to other fieldwork. Molly Czachur for providing initial bioinformatic practice data. Robert Donnelly, Charles Baillie, Georgina Brennan, Kirthana Pillay and Wendy Grail for their excellent advice on laboratory matters. Simon Creer additionally acknowledges initial contributions to project planning from Mathew Seymour. This work was funded by a KESS2 Knowledge Exchange Scholarship (https://kess2.ac.uk), Grant Number: BUK2147 with additional direct funding from NRW. The completion of this manuscript would not have been possible without the exemplary support of CEFAS and Veronique Creach.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2023.108522.

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