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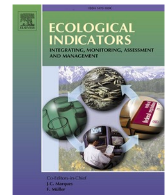
Ecological Indicators 165 (2024) 112215

Contents lists available at ScienceDirect

Ecological Indicators

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



Original Articles

Environmental DNA reveals ecologically relevant spatial and temporal variation in fish assemblages between estuaries and seasons

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ARTICLE INFO

Keywords:
Biomonitoring
Coastal Habitats
Ecosystem Management
eDNA
Fishes
Metabarcoding
Estuaries

ABSTRACT

Estuarine ecosystems are threatened by numerous anthropogenic pressures. Fish assemblages are a dominant component of estuarine macrofauna and serve as indicators for the health of these transitional water ecosystems. Environmental DNA (eDNA) metabarcoding is increasingly used to assess the biodiversity of fishes in estuaries. However, there is a need to further establish how effective eDNA metabarcoding can be relative to conventional fish sampling methods across multiple estuaries and seasons. This study compared fish assemblages detected via eDNA metabarcoding of surface water samples to contemporary sampling with conventional fishing gears in three temperate estuaries (UK), during early summer and autumn. Most species caught by fishing were detected by eDNA. Species richness estimates from eDNA were two to ten times higher than estimates based on fishing, and included taxa of conservation importance and a non-native species. The eDNA assemblage composition was significantly different to the assemblage detected by seine nets. Importantly, eDNA methods could effectively discriminate between fish assemblages of different estuaries and seasons. Fish assemblages in estuaries are often not monitored due to resource constraints. The dynamic nature of estuaries may make fishing gear deployment difficult and inconsistent. The findings indicate that eDNA metabarcoding is suited to gathering large amounts of information on fish biodiversity, at a relatively low sampling effort, compared to established fishing methods. Therefore, eDNA shows promise as an assessment tool for fish assemblage structure and ecosystem health in estuarine environments, with application to statutory monitoring.

1. Introduction

Estuaries are coastal ecosystems of substantial ecological and economic value (Costa et al., 2002; Costanza et al., 1997; Nixon, 1988), but are threatened by significant anthropogenic pressures (Kennish, 2002) and require protection. Within the European Union (EU) and the United Kingdom (UK) protective legislation includes, or is derived from, the Water Framework Directive (WFD; 2000/60/EC; EC, 2000; UK Parliament, 2017a) and the Habitats Directive (92/43/EEC; EC, 1992; UK Parliament, 2017b). Fish assemblages are important indicators of the ecosystem health of estuaries and other transitional waters (Whitfield, 2002) and must be monitored under the WFD. Multi-metric indices of fish assemblage structure and function (Coates et al., 2007), show clear relationships with anthropogenic disturbance (Lepage et al., 2016; Teichert et al., 2016). Regardless, fishes in the vast majority (74 %) of transitional waters in the EU and the UK are not monitored (EEA, 2018a,

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https://doi.org/10.1016/j.ecolind.2024.112215
Received 20 February 2024; Received in revised form 31 May 2024; Accepted 3 June 2024
Available online 9 June 2024
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2018b). This is due to the resource intensive nature of fish sampling in estuaries, which often uses multiple fishing gear types to gain a comprehensive sample of the assemblage (Coates et al., 2007; Harrison and Kelly, 2013). The dynamic nature of these environments also means consistent fishing gear deployment – within and across estuaries – can be difficult (Waugh et al., 2019). Species richness trends from WFD monitoring in England and Wales showed only one netting method, seine nets, out of the three used in monitoring, had a consistent sampling effort for comparison between estuaries. Therefore, in an assessment context, use of data from the other two methods: fyke nets and beam trawls, is likely to reduce comparability of estimates of fish assemblage health between estuaries (Waugh et al., 2019). Therefore, there is a requirement to develop methods that can be consistently applied to complement and enhance existing fish sampling designs to allow accurate assessment of fish assemblages and ecosystem health.

Environmental DNA (eDNA) is trace DNA isolated from an environmental sample rather than directly from individual organisms (Taberlet et al., 2012). An increasing number of studies have examined the potential of eDNA metabarcoding to assess fish biodiversity within individual estuaries (Ahn et al., 2020; Cole et al., 2022; DiBattista et al., 2022; García-Machado et al., 2022; Gibson et al., 2023; Hallam et al., 2021; Saenz-Agudelo et al., 2022; Stoeckle et al., 2017; Zou et al., 2020). They show that, firstly, eDNA detects a greater species richness and a different assemblage composition to conventional fish sampling. Secondly, eDNA detects spatial and temporal changes in assemblage composition within estuaries. Thirdly, variation in assemblage composition may be correlated with physicochemical variables (Gibson et al., 2023). In addition, eDNA can be used to assess variation in the structure of fish assemblages among multiple estuaries and coastal areas at the regional scale in relation to geographic and anthropogenic factors (Kume et al., 2021). However, at present, there have been no comparative studies across multiple estuaries, over multiple seasons using both eDNA metabarcoding and fishing. Temporal studies on estuarine habitats tend to focus on one system (DiBattista et al., 2022) and the studies that compare multiple estuaries have used eDNA metabarcoding data alone (Ahn et al., 2020; Kume et al., 2021). Comparative studies are essential to provide comprehensive assessments of the fish assemblage using eDNA analysis and fishing, thereby facilitating further integration of eDNA into the wider set of methods used to study and monitor fishes in estuaries and coastal ecosystems.

The estuaries of North East England and Yorkshire (UK) are good test ecosystems for studying the application of eDNA as a fish monitoring method. The fish assemblages within these estuaries have been monitored for many years and are well characterised (Waugh et al., 2019). This allows the contextualisation of eDNA detections using prior historical records in addition to comparison with contemporary fishing. Differences in the fish assemblages of three estuaries in this region have also been observed: the species richness of the Tees and Esk is estimated as being lower than the Tweed (Waugh et al., 2019). The differences between the Esk and Tweed may be due to the Tweed being a larger estuary with a wider mouth (ABPmer and HR Wallingford, 2007), which typically promotes higher fish species richness (Nicolas et al., 2010b; Waugh et al., 2019). The Tees, Esk and Tweed estuaries also differ in the distribution of habitat types (Environment Agency, 2016), which can influence the composition of fish assemblages (Teichert et al., 2018a). These estuaries also show variation in the concentration of chemical pollutants. Both the Esk (Environment Agency, 2022a) and Tees (Environment Agency, 2022b) frequently fail assessments of chemical pollution and hazardous substances and the Tees has a history of industrial pollution (Law et al., 1997; Woodhead et al., 1999). Comparatively, at present the Tweed has lower levels of chemical pollutants and hazardous substances (Environment Agency, 2022c). Heavy metals and organic contaminants negatively affect fish densities, occurrence, and species richness (Courrat et al., 2009). Therefore, given the differences in estimated species richness and variation in environmental and anthropogenic factors which may influence fish assemblage structure, these estuaries are good test ecosystems for determining if eDNA can detect differences in assemblage composition between estuaries. In addition, given that seasonal changes in the fish assemblage structure in temperate estuaries are well documented (Henderson and Bird, 2010; Maes et al., 2005; Selleslagh et al., 2012), these estuaries will also be useful for determining if eDNA can detect seasonal variation in fish assemblage composition.

This study aims to compare the fish assemblages detected via eDNA metabarcoding of surface water samples to contemporary sampling with conventional fishing gears in three estuaries, over two seasons and years: early summer and autumn of 2016-2017. A qualitative comparison was made between eDNA metabarcoding and a wider dataset of species detected with fishing gears in the estuaries from 2007 to 2017. We also tested the following hypotheses. Hypothesis 1: eDNA metabarcoding will detect more species in each estuary overall and will detect a different assemblage composition to fishing gears. Hypothesis 2: Differences in assemblage composition will be detected by both eDNA and fishing between estuaries, seasons and different salinity zones.

2. Methods

2.1. Fish sampling

Study ecosystems were the estuaries of the Tweed, Tees and Esk (England, UK, Fig. 1). Fish monitoring was conducted by the Environment Agency (EA) in early meteorological summer (late May/June) and autumn (September/October), as part of statutory monitoring. During each survey, double fyke nets, seine nets and 1.5 m beam trawls were deployed at multiple stations (Colclough et al., 2002). Fishing gear was typically deployed 2 and 4 hrs after low tide on a neap tide. Surface water physicochemical parameters: dissolved oxygen (%), salinity (practical salinity units), temperature (°C) and pH, were recorded at each station (Pro-Plus YSI Inc., Yellow Springs, OH, USA). In the Esk and Tweed, seine nets were deployed at three stations in the lower, middle, and upper regions of each estuary. In the Tees only one seine net station was present in the lower estuary. In the Tweed and Tees, beam trawling occurred at one seine netting station in the lower region of each estuary. In the Esk and Tweed, fyke nets were set at one independent station in the lower region of each estuary (Fig. 1). Sampling numbers for each gear type over the study period are given in Table S3. This sampling has occurred consistently since 2011, although data from 2007 to 2017 was used to contextualise eDNA detections (SI methods 1.1).

2.2. eDNA sampling

Water samples were collected alongside fishing at almost all fishing stations, or within a day of it at the same tidal state on seven sampling events (Table S1). The exception was summer 2017 Tweed seine net station 1 (Station Code: TW.S1), on this event this fishing station was not sampled with eDNA. Therefore, it was not used in direct comparison of assemblage composition between eDNA and seine nets, only in general comparisons of species richness and composition at the estuary level. An additional eDNA station was also sampled in the upper Tees estuary where no fishing was conducted. The Esk and Tees were sampled in autumn 2016 and 2017 and summer 2017. The Tweed was only sampled in summer and autumn 2017 (Table S1). Four Esk stations were sampled for eDNA: one fyke net station and three seine net stations. Four Tweed stations were sampled for eDNA: one fyke net station, one beam trawl station and two seine net stations. The upstream station on the Tweed was not sampled in summer 2017 (Table S2). In autumn 2016 and summer 2017 two stations were sampled for eDNA on the Tees: a seine net station, and the independent eDNA station where fishing did not occur. During comparisons of beam trawl and eDNA data, eDNA samples from the seine net station were compared to the nearby beam trawl station. In autumn 2017, three Tees stations were sampled for eDNA, the seine net station, a beam trawl station and the independent eDNA

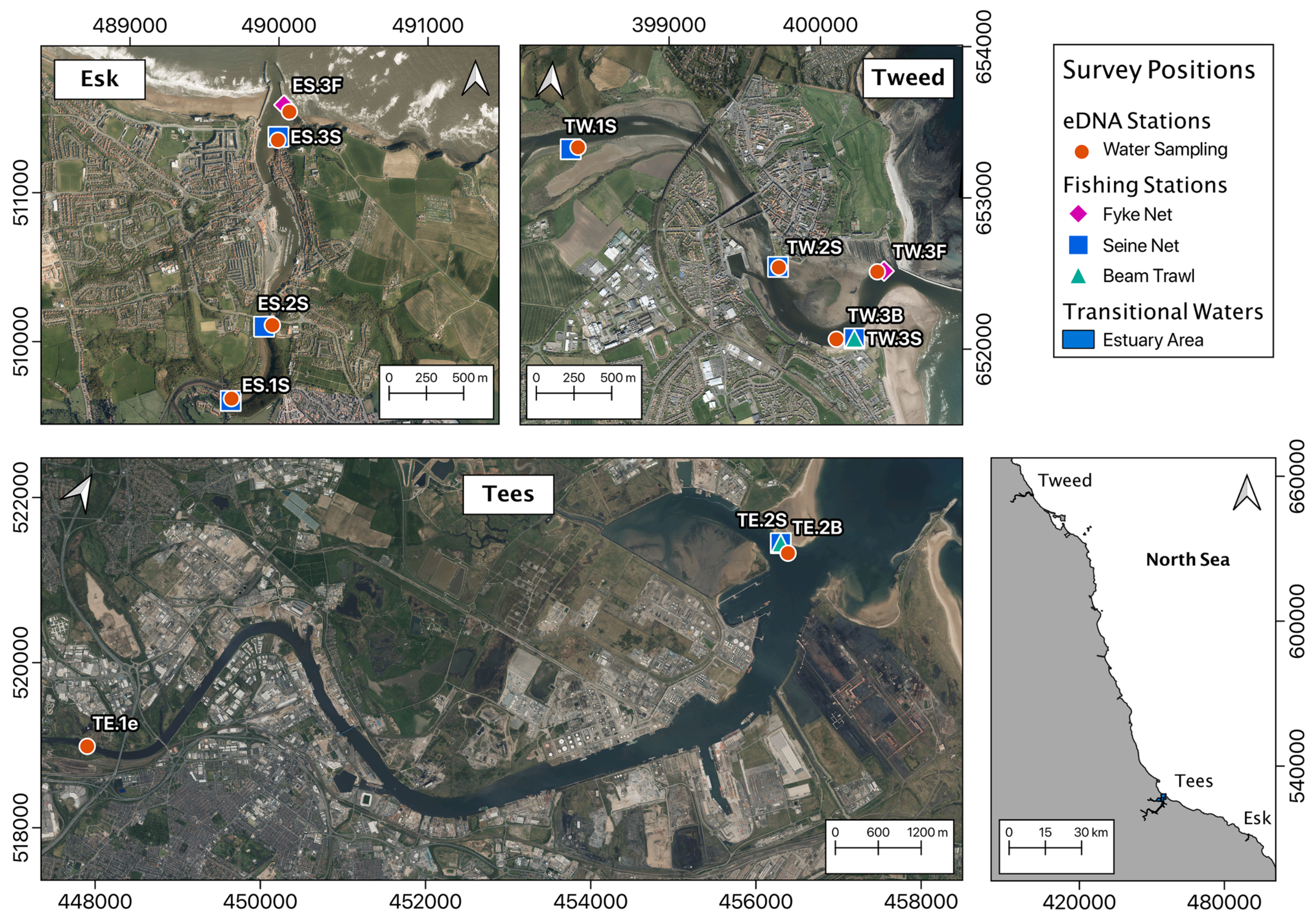

Fig. 1. Locations of the Esk, Tees and Tweed estuaries and the distribution of eDNA and fish sampling stations in Autumn 2017. Stations are labelled with codes for estuary (ES = Esk, TW = Tweed, TE = Tees), a site number and sampling method (S = seine nets, F = fyke nets, B = beam trawl and e = eDNA only). Coordinate System: British National Grid (EPSG:27700) axis in eastings/northings (m). British Coastline (Wessel and Smith, 1996), Satellite Photography (Getmapping, 2014), Estuary position and extent (Environment Agency, 2021). British National Grid is a projected coordinate system which provides constant lengths, angles and areas for 2D maps of the region of interest, the United Kingdom.

station. At each sampling station, immediately before fish sampling, triplicate 2 L surface water samples (0–1 m depth) were collected in HDPE bottles each covered with a 250-µm nylon mesh (previously cleaned with 10 % bleach and triple rinsed with deionised water). On the Esk, 36 samples were collected, while 21 were collected from both the Tees and Tweed respectively.

Samples were packed in sterile plastic bags and transported to the laboratory on ice. Field blanks (commercial bottled water) were taken into the field for the Esk sampling. The blanks were opened in the field, then sealed, and transported, filtered, stored, and analysed in the same way as samples. Within 5 hrs of collection, at the laboratory, water samples were filtered, individually, using PES 0.22 µm Sterivex filters (Merck Millipore, Burlington, MA, USA) using a 100 mL polypropylene syringe until the filter blocked. The mean filtered volume per sample was 400 mL (sd: 200 mL). Following filtration, the filters were stored at − 20 °C.

2.3. Laboratory methods

DNA extraction and pre-PCR preparations were carried out in a pre-PCR laboratory, in separate rooms. Equipment and surfaces were cleaned using 10 % commercial bleach solution and subjected to UV irradiation following work. Total eDNA was extracted from filters using DNeasy PowerSoil kits (Qiagen, Hilden, Germany, see Collins et al., 2019). Field and extraction blank controls were processed in parallel. Following extraction, a ~167 bp fragment of the 12S rRNA region was amplified using the fish-targeting Tele02 PCR primers (SI methods 1.2, Miya et al., 2015; Taberlet et al., 2018). Each primer possessed a unique 8-bp index tag for identification of each individual samples after sequencing (the forward and reverse primer had the same tag). PCR reactions for each DNA template were conducted in triplicate along with PCR blanks using water instead of template. The total reaction volume was 26 µl containing: 16 µl Amplitaq Gold 360 Master Mix (Applied Biosystems, Waltham, MA, USA), 0.16 µl of Bovine Serum Albumin, 2 µl of DNA template, 1 µl of each forward and reverse primer (5 µM) and 5.84 µl H_2O. The thermocycler profile was as follows: 95 °C for 10 mins, followed by 40 cycles of 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 mins. Following amplification, sample amplicons and field blanks (n = 4), extraction blanks (n = 4), PCR blanks (n = 2) and well blanks (unused tag combinations containing just water, n = 10), were combined by equal volume into three separate pools. Primer dimers were removed using a HighPrep PCR clean-up (MagBio Genomics, Gaithersburg, MD, USA), with 1X paramagnetic beads to pool ratio. For each pool (three in total), PCR-free dual-indexed libraries were prepared using the KAPA Hyper Prep Kit (Roche, Basel, Switzerland). This followed the manufacturer instructions but with a prolonged adaptor ligation step of 90 mins and at a lowered temperature of 37 °C. Libraries were then quantified using qPCR, pooled in equimolar concentrations and loaded onto an Illumina MiSeq platform at a concentration of 8 pM, with 1 % PhiX, and sequenced using V2 chemistry (2

x 150 bp paired-end; Illumina, San Diego, CA, USA). The samples from this study were sequenced in libraries alongside other fish eDNA samples collected off the south and southwest coast of England (Collins et al., 2022), two sequencing runs were conducted in total.

2.4. Bioinformatics

Bioinformatic processing followed Collins et al. (2019). This entailed sample demultiplexing using cutadapt v2.10 (Martin, 2011), sequence denoising and dereplication using dada2 v1.18.0 (Callahan et al., 2016), homology filtering of ASVs (amplicon sequence variants) using hidden Markov models with hmmer v3.1 (Eddy, 2011). Reference library sequences were used as priors during dada2 denoising to avoid erroneously discarding rare sequences. Following initial processing, approximate taxonomic assignment was conducted using SINTAX (Edgar, 2016) and NCBI RefSeq v205 (O'Leary et al., 2016) to exclude non-fish ASVs. Final consensus taxonomic assignment of taxa was carried out using a curated reference library for fishes of the British Isles (Collins et al., 2021) using phylogenetic placement with epa-ng v0.3.7 (Barbera et al., 2019) and sequence similarity blastn v2.10.1 (Camacho et al., 2009) see Collins et al. (2019). To control for potential laboratory cross-contamination between different libraries, an exclusion list of sequences generated from other lab projects sequencing fishes was used. For closely related species which shared haplotypes, species identification was not possible and a higher-level taxonomic assignment was used, except in cases were the presence of one of those species was considerably more likely than the other (SI methods 1.3). To account for contamination detected in blanks, a per species read threshold cut-off was calculated using an adaption of the approach in Yamamoto et al. (2017). Species which contributed equal to or less than 0.08 % of the total target fish reads in a sample were considered absent (SI methods 1.4).

Samples were checked for extreme outliers in taxonomic composition using species accumulation curves vs read depth and nMDS (SI methods 1.5). Species were assigned to estuarine-use functional guilds, which describe the ecological use of an estuary (Elliott et al., 2007). The guilds were Marine Stragglers (MS), Marine Migrants (MM), Estuarine Species (ES), Anadromous Species (A), Catadromous Species (C), Freshwater Species (F) (Franco et al., 2008) or Unassigned (UN) (Gibson et al., 2023).

2.5. Statistical analysis

Each fishing gear station was paired with their nearest neighbour eDNA station (SI methods: 1.6). Exact physiochemical readings were not used given eDNA sampling was not always concurrent with fishing. Instead, the salinity at each station was classified: Euhaline (>30), Polyhaline (18–30), Mesohaline (5–18), Oligohaline (0.5 to 5.0) and Limnetic (<0.5) (McLusky, 1993). Species detected with eDNA were checked against species lists from fishing surveys for each estuary from 2007 to 2017. Species lists were also generated for each estuary for eDNA and fishing, for each season, using all the data for 2016 and 2017. The taxonomic composition of these lists was compared using UpSet plots (R-packages: 'ComplexUpset' v1.3.5, Krassowski, 2020). For these qualitative comparisons, where species were identified to a lower taxonomic resolution by fishing than eDNA, a single match was counted between the methods. This represented the diversity of species caught by fishing gears but accounted for the ability to identify higher level taxa to species level using expert judgement, if prior knowledge of a species presence was available.

Overall, species richness estimates were summarised for eDNA samples and fishing stations using rarefaction and extrapolation (R/E) sampling curves (R-package: 'iNEXT' v2.0.20; (Chao et al., 2014; Hsieh et al., 2016). R/E curves were calculated with 95 % confidence intervals (CI) and standard errors calculated using 1000 bootstrap replicates. To compare species richness from eDNA metabarcoding and conventional fishing methods (Hypothesis 1), asymptotic species richness and richness estimates per eight samples were compared (along with the 95 % CI) for each estuary, per season for eDNA and the combined fishing data. To compare species richness among estuaries (Hypothesis 2), the procedure was repeated for each estuary using only the 2017 data (when all estuaries were sampled) and pooled across seasons.

To compare assemblage composition (presence/absence) resolved by eDNA metabarcoding and fishing (Hypothesis 1), samples for each method, for each sampling event at a station were aggregated together and treated as an independent replicate. Aggregation accounted for the spatio-temporal non-independence of eDNA samples within each sampling event at a station (Hurlbert, 1984). This was not required for the previous species richness analysis as the aim was to make general comparisons of richness at the estuary scale, rather than explicitly test hypotheses with a spatial component. Fishing data was aligned to the taxonomic resolution of the eDNA data as exact differences in species richness were less important here than differences in composition. Assemblage composition was compared using ordination with generalised linear latent variable models (GLLVM; binomial distribution, probit link; R Package: gllvm; v1.3.1, Niku et al., 2019; SI methods 1.7). GLLVM model selection showed read depth had no effect on eDNA assemblage composition (SI methods 1.7). Since the spatial deployment of fishing gears was not consistent within and between estuaries (Fig. 1), eDNA and fishing data were subdivided and analysed separately. Each group consisted of stations sampled with each gear type and nearest neighbour eDNA stations.

Comparisons were made using ordination and differences in assemblage composition were tested using multivariate GLMs (binomial distribution, logit link; R Package: mvabund v4.1.12, Wang et al. (2012); SI methods 1.7). For the comparison of eDNA and seine nets, the fixed effects of sampling method (Hypothesis 1), estuary, season, salinity zone and year (Hypothesis 2) on assemblage composition were tested. In addition, the interaction between method and each environmental variable was tested to determine environmental effects were consistent across methodologies (Hypothesis 2). The analysis was repeated omitting the 2016 data ensuring differential sampling of estuaries across years had not influenced the overall conclusions. For comparisons with fyke nets and beam trawls no interaction terms were included and only the fixed effects of method, estuary, season and salinity zone were included in the initial model due to small sample sizes. Model selection was applied using backwards selection and assessing the AIC (Zuur et al., 2007).

To further address Hypothesis 2, assemblage composition across estuaries and seasons was analysed using sample level eDNA data from 2017 using ordination and a multivariate GLM. A preliminary multivariate GLM model contained a main effect of estuary, season, and salinity zone, with an interaction term between estuary and season. However, salinity was dropped from this preliminary model using model selection (SI methods 1.7). This allowed incorporation of additional data from a station in the upper Tees which lacked salinity classification. Therefore, the initial model contained only the main effects of estuary and season and an interaction term between estuary and season. This model was also subject to model selection. To account for spatio-temporal non-independence between samples collected together at a station (Hurlbert, 1984), sampling event was used as a blocking factor (Wang et al., 2012).

3. Results

3.1. Qualitative comparison of eDNA with fish surveys

A total of 20,322,880 reads were returned in total by the two sequencing runs. After read processing, filtering, and taxonomic assignment 1,786,739 target fish reads were obtained for the samples used in this study, with 95 species detected overall. In the blanks 12,128 target fish reads were detected from 57 species. After applying the 0.08

Table 1
24 species detected by eDNA with the highest read abundance across estuaries (see Table S4. for other species).

Species	Common Name	Guild	Esk			Tees			Tweed		
			Detections	Reads	Fishing 2007–2017	Detections	Reads	Fishing 2007–2017	Detections	Reads	Fishing 2007–2017
Salmo trutta †	Brown trout	A	36	242,351	●	16	18,237	●	17	22,044	●
Clupea harengus †	Atlantic herring	MM	36	95,931	●	20	71,537	●	16	37,702	●
Pleuronectes platessa †	European plaice	MM	29	65,728	●	19	64,142	●	12	43,663	●
Phoxinus phoxinus †	Eurasian minnow	F	36	52,109	○	12	1073	○	21	112,728	●
Sprattus sprattus †	European sprat	MM	35	29,967	●	21	56,058	●	14	13,472	●
Gadus morhua †	Atlantic cod	MM	35	82,717	●	15	4212	●	9	1632	●
Platichthys flesus †	European flounder	MM	34	27,738	●	21	39,655	●	19	11,549	●
Salmo salar †	Atlantic salmon	A	35	53,759	●	13	2400	○	19	10,228	●
Ammodytidae †	Sand lances	UA	28	10,767	■	18	47,060	■	10	4112	■
Sardina pilchardus †	European pilchard	MM	25	23,969	○	15	16,150	○	12	19,729	○
Limanda limanda †	Dab	MM	10	2742	●	13	54,678	●	2	12	○
Gasterosteus aculeatus †	Three-spined stickleback	A	27	3614	●	8	532	●	21	49,512	●
Scomber scombrus †	Atlantic mackerel	MS	33	29,322	○	13	10,268	○	10	10,685	○
Barbatula barbatula †	Stone loach	F	33	23,391	○	9	258	○	21	25,459	○
Merlangius merlangus †	Whiting	MM	27	5348	●	21	34,215	●	9	1844	●
Zeugopterus punctatus †	Topknot	MS	21	12,262	○	13	8555	○	8	8872	○
Trachurus trachurus †	Atlantic horse mackerel	MS	24	12,585	○	14	7036	○	9	7703	○
Pomatoschistus microps †	Common goby	ES	22	11,720	●	11	5837	●	12	8875	●
Anguilla anguilla †	European eel	C	25	5780	●	6	2838	●	19	9877	●
Labrus bergylta †	Ballan wrasse	MS	20	5676	○	13	4509	○	8	4370	○
Dicentrarchus labrax †	European seabass	MM	24	3144	○	15	8702	●	9	2394	○
Molva molva †	Ling	MS	30	10,733	○	10	758	○	9	927	○
Atherina presbyter †	Sand smelt	MM	20	5787	●	11	2934	●	8	3600	○
Gobio gobio	Gudgeon	F	13	7014	○	0	0	○	5	184	○

Detections: Total presence/absence per estuary. **Fishing 2007 to 2017:** species present in fishing data**. Presence Cat.:** Exact Species Present: ●; Species present within clade: ■; No Species Present: ○. †: indicates detected in blanks.

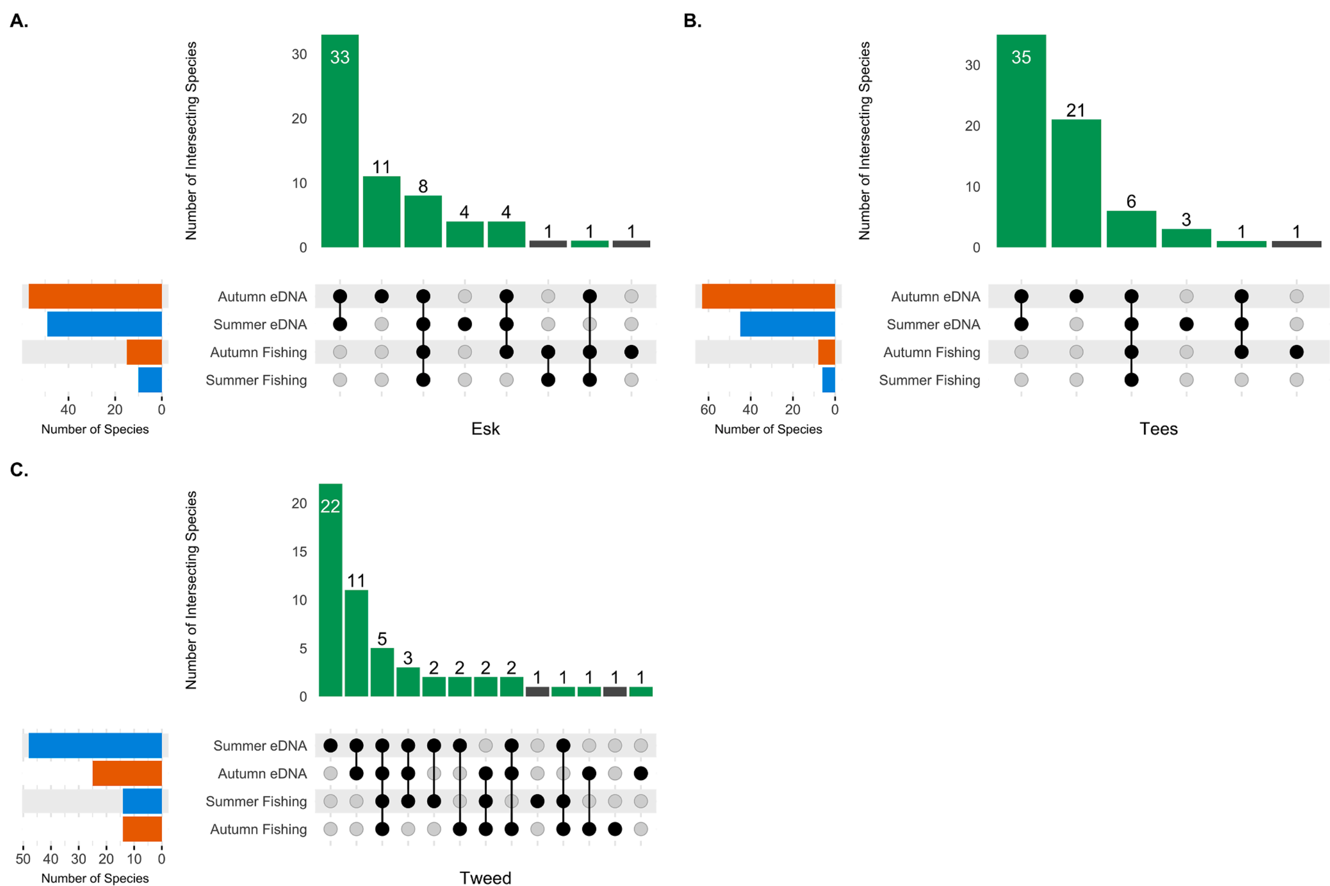

Fig. 2. UpSet plots showing shared (intersecting) species between species lists from eDNA and fishing per seasons and estuary (2016 and 2017). In each plot, bottom panels indicate species lists and intersections; single black dots indicate species not shared between multiple lists; black dots connected by lines indicate which lists shared species; top bar graphs gives the number of species shared between each list in each intersection; green bars indicate intersections which contained species detected via eDNA, grey bars indicate intersections where only fishing contributed; lower left-hand bar charts show the total number of species within each species list (blue bars indicate summer and orange autumn). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

% per sample cut-off threshold, 76 species were detected by eDNA, across all estuaries. Comparably, a total of 24 species were detected by contemporary fishing across all estuaries in 2016 and 2017. All the 76 species detected by eDNA had the potential to occur in UK estuaries and 70 of the 76 species could be assigned to estuarine use guilds (Table 1 and Table S4). Interestingly 36 of the 76 species detected by eDNA had never been detected in any of the estuaries by fishing surveys (2007 to 2017). Most of the previously undetected 36 species were from the Marine Straggler (17), Freshwater (8) and Marine Migrant guilds (6). Most species detected by contemporary fishing in 2016 and 2017 in each estuary were detected with eDNA. Specifically, in the Esk, Tees and Tweed, 13 of 15, 7 of 8 and 18 of 20 species detected by fishing were detected by eDNA in each estuary respectively (Fig. 2). Most species detected by fishing surveys from 2007 to 2017 in each estuary were detected by the eDNA sampling surveys in 2016 and 2017. Specifically, in the Esk, Tees and Tweed, eDNA detected 23 of 30, 32 of 40 and 23 of 32 species that had been detected over a decade.

3.2. Overall species richness comparisons

Species richness rarefaction/extrapolation (R/E) curves calculated using all data for each estuary, per season, showed eDNA detected a four to ten times higher asymptotic species richness compared to fishing on the Esk in summer and autumn, and the Tees in autumn (95 % CI none-overlapping, Fig. 3; Table S5). There was no difference in asymptotic species richness between eDNA and fishing on the Tweed in either season or on the Tees in summer (95 % CI overlapping; Fig. 3; Table S5). Comparably, species richness estimates per eight samples showed the eDNA data consistently detected on average four times as many species compared to fishing, except for the Tweed in Autumn (Fig. 3; Table S6). On the Esk and Tweed there was no difference in asymptotic species richness between seasons for eDNA data. In the Tees, asymptotic species richness was higher in autumn than early summer for the eDNA data. There were no differences in asymptotic species richness between seasons, per estuary for fishing gears (data combined across gears; Table S5).

Species richness R/E curves calculated by pooling the data across both seasons from 2017 showed eDNA detected no difference in asymptotic species richness between estuaries. Fishing data showed the Tweed had a higher asymptotic species richness than the Esk and Tees. Environmental DNA detected a five to seven times higher species richness than fishing in the Esk (eDNA species richness: 72, fishing: 14) and Tees (eDNA: 60, fishing: 9) respectively, but not in the Tweed (CI interval overlapping; Fig. 4, Table S7).

3.3. Station level assemblage composition between methods and ecological variables

Ordination of sampling stations, across both years, suggested a difference in assemblage composition (presence/absence) between eDNA and fishing gears (Fig. 5; model residuals: Fig. S6). Overall, the assemblage composition detected by fishing methods was relatively similar

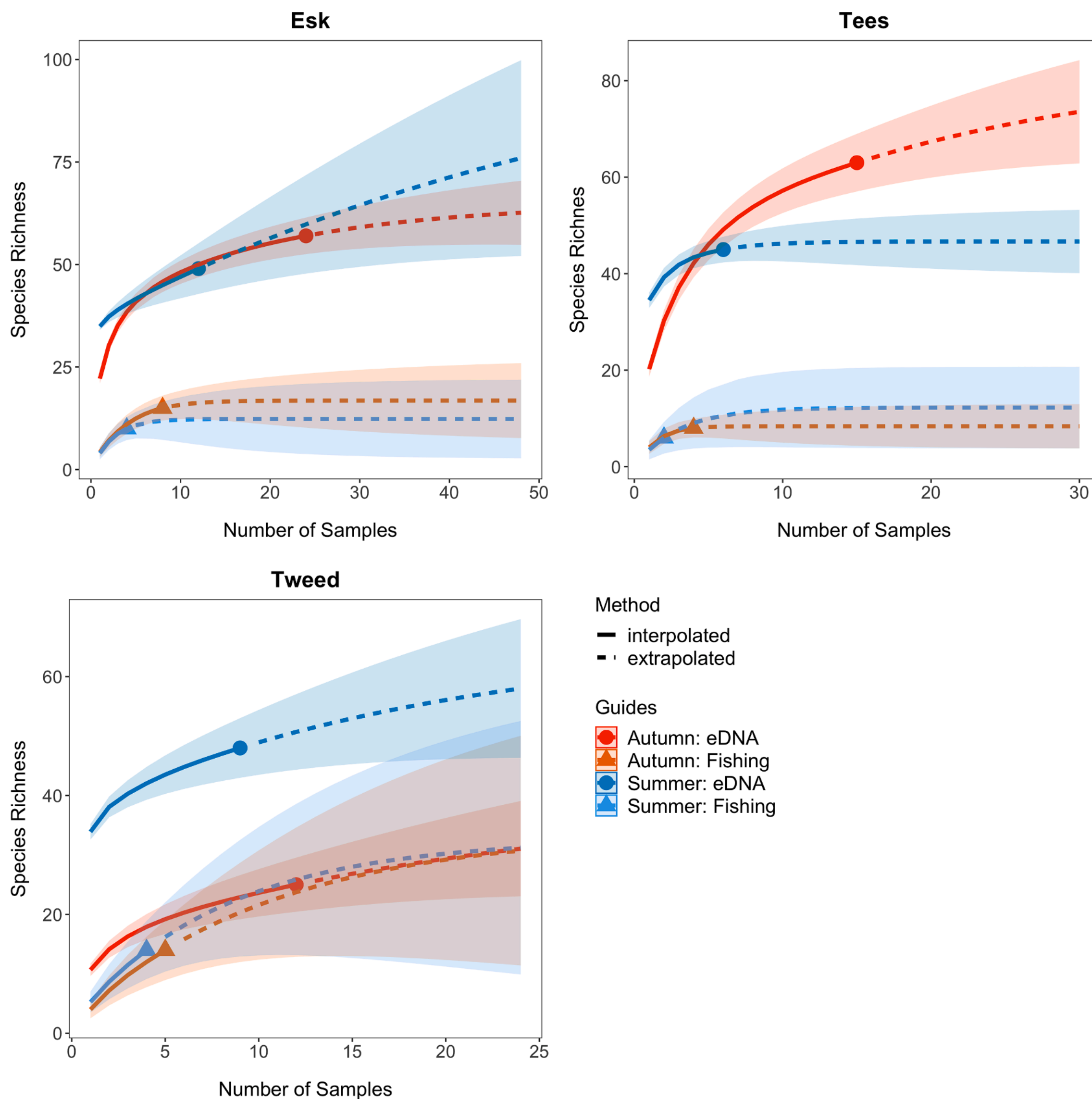

Fig. 3. Species richness Rarefaction and Estimation (R/E) curves and with the standard error (shaded area) calculated on species presence/absence data for eDNA-derived data (filtered by species detected in fishing 2007 to 2017), and fishing gears (combined across methods) for each estuary, per season. All available data was used for each estuary.

across stations, seasons, and estuaries with a subtle gradient present in assemblage composition between the Tweed, Esk and Tees. Comparably, eDNA data pooled at the level of sampling stations showed a greater variability in assemblage composition (Fig. 5A). There appeared to be a separation in composition between eDNA stations in autumn and summer. In autumn, differences in assemblage composition appeared greater between the three estuaries, than in summer (Fig. 5B). Variation in eDNA composition between estuaries appeared to mirror the subtle change in composition detected by fishing (Fig. 5A).

For comparisons of assemblage composition between eDNA and seine netting stations using multivariate GLMs, the best fitting model contained the explanatory variables: method, season, estuary and year. The effect of salinity zone was dropped, along with interaction terms, during model selection (initial model AIC: 2345). There was a statistically significant effect of method on assemblage composition, indicating a difference between the community composition of eDNA and seine nets. Comparably, year, season and estuary had no statistically significant effect on the community composition of eDNA or seine netting stations (Table 2 model 1; residuals: Fig. S7), regardless of the initial patterns for eDNA shown in the ordinations (Fig. 5A and B). There was no change in the statistical significance of the effects of method, season and estuary in an alternative model using only the 2017 data (Table S9). Differences in assemblage composition between methods was confirmed by GLLVM ordination (Fig. S8). Six species were detected significantly more frequently in eDNA than in seine nets: Atlantic Salmon (*Salmo salar*; Anadromous), three spined stickleback (*Gasterosteus aculeatus*; Anadromous), European plaice (*Pleuronectes platessa*; Marine Migrant), common and sand gobies (*Pomatoschistus microps* and *Pomatoschistus minutus*; Estuarine Species) and the marine taxon *Pollachius* sp. (Table S8). Comparably there was no statistically significant difference in composition between eDNA and fyke nets and beam trawls (Table S10 and S11; Fig. S9 and 10).

3.4. Sample level assemblage composition between seasons and estuaries

Given that no statistically significant differences in assemblage composition were found between different estuaries and seasons for eDNA and seine net data pooled at the station level, further investigation of eDNA data at the sample level was conducted. Ordination of the eDNA samples from 2017 alone showed a clear difference in assemblage

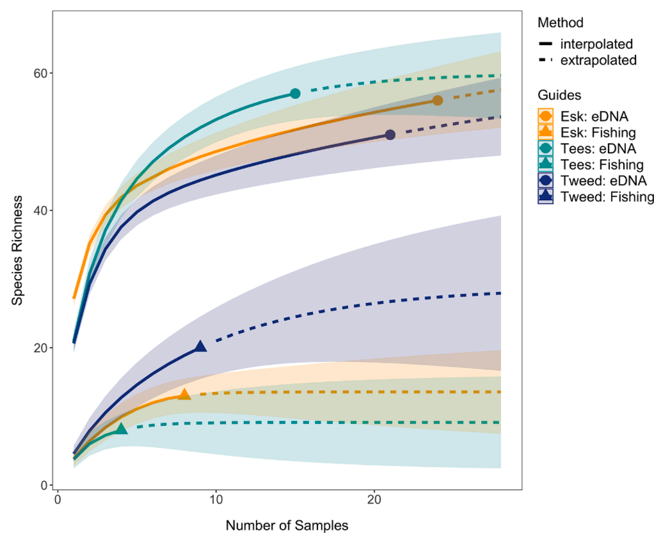

Fig. 4. Species richness Rarefaction and Estimation (R/E) curves and with the standard error (shaded area) calculated on species presence/absence data for eDNA and fishing gears (combined across methods) for each estuary for 2017.

composition between the different estuaries and between summer and autumn in each estuary. Differences in assemblage composition between estuaries appeared marginally greater in autumn compared to summer (Fig. 5C and D; residuals: Fig. S11). But for the multivariate GLM, the interaction term between season and estuary did not improve model fit (initial model AIC: 2215), regardless of the patterns in the ordination. Both season and estuary showed a strong statistically significant effect on assemblage composition (Table 2 model 2, residuals: Fig. S12), supporting the evidence from the ordination (Fig. 5C and D). In addition, seven species show a significantly higher incidence in summer compared to autumn. This included Estuarine Species: sand smelt (*Atherina presbyter*), two goby species (*P. microps* and *P. minutus*), Marine Stragglers: poor cod (*Trisopterus minutus*), Topknot (*Zeugopterus punctatus*) and Ballan Wrasse (*Labrus bergylta*) and a mullet (*Chelon ramada* or *Chelon labrosus;* unclassified). Four other species, common dab (*Limanda limanda*, Marine Migrant), common ling (*Molva molva*, Marine Straggler), European eel (*Anguilla anguilla*, Catadromous) and Atlantic mackerel (*Scomber scombrus*, Marine Straggler) showed statistically significant differences in incidence across estuaries (Table S12), with the exact relationship depending on the species (Fig. S13). For example, *A. anguilla* was detected more frequently in the Esk and Tweed than the Tees (Fig. S13).

4. Discussion

This is one of the first studies to compare the fish assemblages detected via eDNA metabarcoding and conventional fishing gears, across multiple estuaries and seasons. Analysis of eDNA detected 76 species, half had never been previously recorded in these estuaries. Five species of conservation interest were detected by eDNA: European eel (*A. anguilla*), European smelt (*Osmerus eperlanus*), shad (*Alosa* sp.), lamprey (*Lampetra* sp.) and Atlantic salmon (*S. salar*). Out of these five species shad had not previously been detected by fishing from 2007 to 2017. The non-native pink salmon (*Oncorhynchus gorbuscha*) was also detected in the Tees. This invasive species has been extensively sited in Scotland and has also been reported from northern England, but has never previously been detected in the Tees to our knowledge (GB Non-Native Species Secretariat, 2019). Therefore, eDNA metabarcoding is an effective way of detecting species of conservation interest and non-natives in estuaries, as concluded by Kume et al. (2021). Overall, eDNA detected 87 to 90 % of the species caught by fishing, depending on the estuary, between 2016 and 2017, a higher coverage than comparable studies (~70 %, Gibson et al., 2023; Hallam et al., 2021). Therefore, although a combination of eDNA and fishing detects the most species, only a few species were missed by eDNA metabarcoding.

Metabarcoding generally detected a higher asymptotic species richness than fishing gears in different seasons in 2016 and 2017, and for both seasons in 2017. Calculating the expected species richness for a given sample size showed eDNA was often more efficient at detecting species than fishing. This supports Hypothesis 1 that eDNA would detect more species in an estuary overall, as found by other studies (Gibson et al., 2023; Hallam et al., 2021). However, eDNA did not detect a higher asymptotic species richness than fishing in autumn in the Tweed. It is not known why this occurred. It potentially could have been due to a random issue with sample preservation or extraction, causing eDNA degradation. Alternatively, it may have been due to a seasonal increase in the concentration of inhibitors in the Tweed in autumn, given that sample inhibition can be high in temperate rivers in autumn (Jane et al., 2015). Additionally, the Tweed was sampled with three fishing gears and therefore a larger component of the fish assemblage was captured by fishing (Fig. 4, and see Hallam et al., 2021). There was a clear difference in assemblage composition of stations sampled with eDNA and seine nets, supporting Hypothesis 1, as demonstrated in other studies (Gibson et al., 2023; Hallam et al., 2021). However, differences between eDNA and fyke and beam trawl compositions were not statistically significant. This is probably primarily because of the lower total number of fyke and beam trawl stations relative to seine netting stations. Despite the none statistically significant result, the ordinations comparing fyke nets and beam trawls to eDNA did show a difference in composition (Fig. S8). This suggests greater replication would allow a statistically significant difference in species composition between these two gear types and eDNA to be detected. Many of the species detected more frequently using metabarcoding were also detected in seine nets in 2016 and 2017. Specifically, these were three-spined stickleback (*Gasterosteus aculeatus*), European plaice (*Pleuronectes platessa*), common goby (*Pomatoschistus microps*), sand goby (*Pomatoschistus minutus*), saithe/pollock (*Pollachius* sp.) and Atlantic salmon (*S. salar*). All these species, except *S. salar*, are known to have been detected at least once in every estuary from 2007 to 2017. More frequent detection of these species maybe due to a range of factors. For example, active gear avoidance by more mobile species (plaice, saithe/pollock and salmon), difficulty in identifying small specimens of closely related goby species in fishing hauls and eDNA transport causing a wider range of habitats to be sampled by eDNA than those that could be accessed with a seine net. Overall, this demonstrates that eDNA metabarcoding is a reliable method for detecting species commonly found in estuaries. However, sources of uncertainty around eDNA detections in our study may have contributed to high numbers of previously undetected species. Specifically, these include sources of *in situ* contamination such as wastewater effluent and transport of eDNA from the adjacent river and the sea. Several studies have inferred eDNA transport may influence species detection in aquatic ecosystems (Deiner and Altermatt, 2014; Gibson et al., 2023; Yamamoto et al., 2017). It is estimated that eDNA maybe detected for around 48 h in an inshore environment (Collins et al., 2018), over which time it will be transported in an open system such as an estuary. However, only with detailed hydrological modelling and studies of eDNA decay and transport within estuaries will the influence of these factors on species detection be established.

Initial analyses using eDNA and fishing data aggregated at the level of the sampling station were unable to detect assemblage differences between estuaries and seasons. When the 2017 data were analysed independently and samples were not aggregated over stations, eDNA data revealed a clear difference in assemblage composition between estuaries and seasons. This supported the hypothesis that eDNA would show differences in composition between seasons and estuaries. This suggests, with the current sampling design, that eDNA would outperform a fishing surveys ability to detect ecologically relevant differences. There was a consistent shift in assemblage composition between early

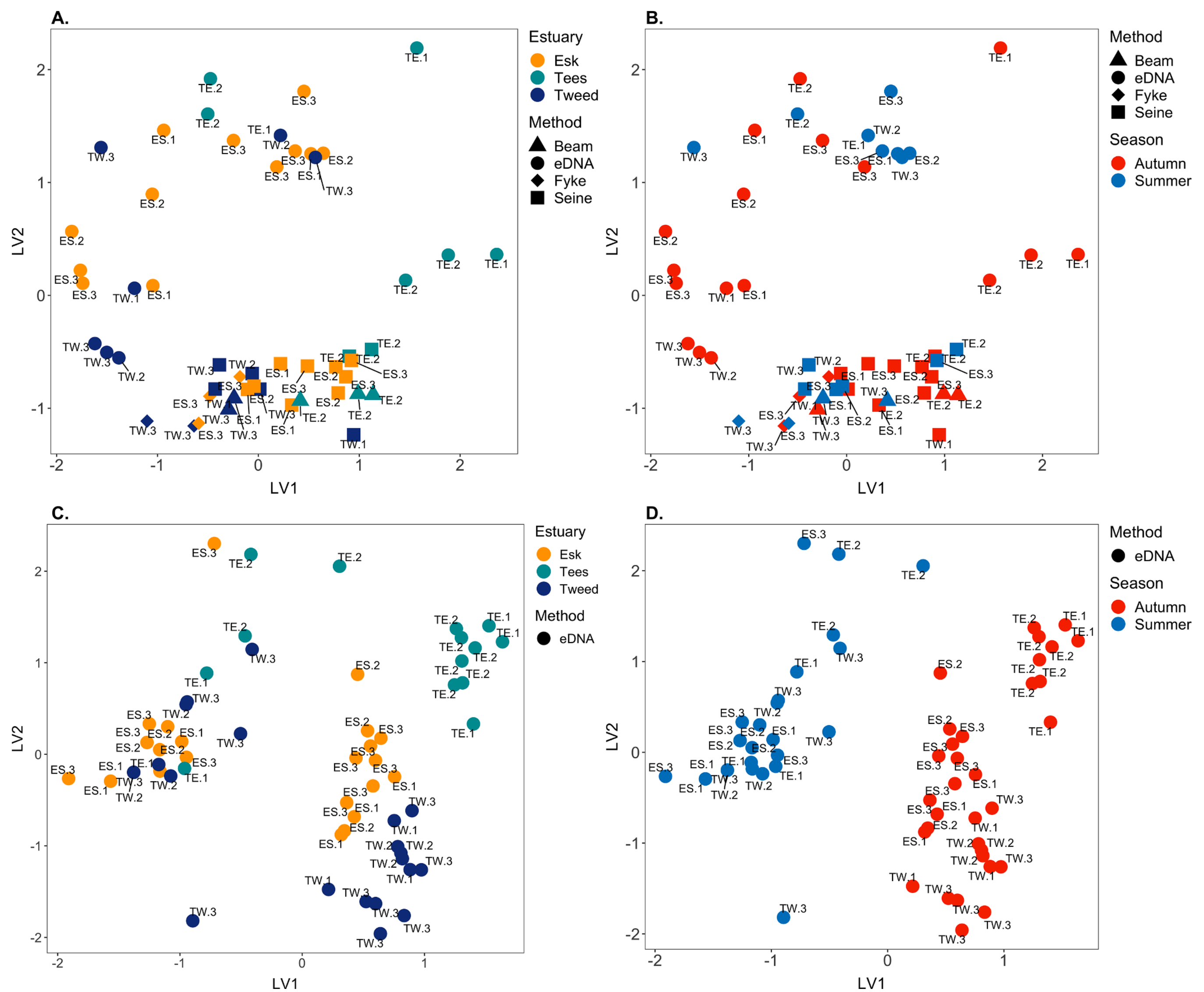

Fig. 5. Presence/absence of fish species modelled using a binomial GLLVM (probit link). Panel A and B shows composition per station for eDNA and all fishing methods. Panel C and D shows composition per sample for eDNA in 2017. Point labels show estuary and general sampling site within which stations were situated.

summer (late May/June) and autumn (October/September) across estuaries. This is comparable to eDNA metabarcoding studies which have detected seasonal changes in the fish assemblage composition within estuaries (DiBattista et al., 2022; Hallam et al., 2023; Stoeckle et al., 2017; Zou et al., 2020) and in coastal ecosystems (Sigsgaard et al., 2017). Seasonal changes in the assemblage structure of temperate estuaries are caused by sequential immigration and emigration of fishes controlled by spawning times, and recruitment of juveniles and larvae into estuaries (Maes et al., 2005; Teichert et al., 2018b). Spawning and changes in abundance across various life history stages, depending on species, may have driven the seasonal differences in eDNA assemblage composition (Collins et al., 2022).

Regarding seasonal changes in assemblage composition at the species level, seven species occurred more frequently in early summer than in autumn. Two of the species, the Marine Stragglers: topknot (*Z. punctatus*) and ballan wrasse (*L. bergylta*), were novel detections. In addition, a species of mullet *C. labrosus* or *C. ramada* was also detected. *C. ramada* has previously been caught by fishing on the Esk. It is notable that for five of the seven species detected more frequently in autumn, their spawning times in the waters around Britain overlap with the sampling period in early summer (Table S13). It is possible that spawning within, or outside of the estuaries, depending on the ecological guild, caused greater detection in early summer. For species such as *P. minutus*, and other *Pomatoschistus* spp., adult abundance is generally higher in autumn, rather than early summer (Maes et al., 2005). This supports the assumption that it was spawning rather than adult abundance driving this pattern. The exception to this was Poor cod (*T. minutus*) which spawns from February to March (and can be more abundant in autumn, Henderson and Bird, 2010) and *C. labrosus* which spawns from July to August (Table S13). However, in the case of *C. labrosus* it is notable that juveniles move into estuaries in April-June (Kottelat and Freyhof, 2007) whereas *C. ramada* does spawn in June (Maitland and Campbell, 1992). Therefore, changes in abundance across various life history stages, depending on species, probably drove the differences in assemblage composition in eDNA rather than spawning alone.

In comparison to seasonal variation in community composition, differences in asymptotic species richness between seasons were harder to identify. Only the Tees showed a greater species richness in autumn. This is surprising, as a higher species richness is expected in autumn compared to early summer (Henderson and Bird, 2010) and eDNA metabarcoding using MiFish_U primers has been able to detect seasonal changes in fish species richness at an intertidal site in the Thames estuary (Hallam et al., 2023). There was a clear shift in the eDNA

Table 2
ANOVA results for multivariate GLM model 1 and 2.

eDNA vs. Seine Nets	**Model 1:** Species Presence/Absence ~ Method + Season + Estuary + Year			
	AIC: 1301			
	Sampling Years Included: 2016 and 2017			
	Explanatory Variable	**Residual DF**	**Wald-Test**	**P-Value**
	Intercept	31		
	Method	30	8.008	$< 2 \times 10^{-16}$
	Season	29	6.766	0.082
	Estuary	27	6.226	0.447
	Year	26	3.213	0.513
eDNA Only Seasons and Estuaries	**Model 2: (Block — Station):** Species Presence/Absence ~ Season + Estuary			
	AIC: 2012			
	Sampling Years Included: 2017			
	Explanatory Variable	**Residual DF**	**Wald-Test**	**P-Value**
	Intercept	59		
	Season	58	12.66	$< 2 \times 10^{-16}$
	Estuary	56	11.31	0.001

assemblage composition between estuaries, over both seasons, probably because of differences in the fish assemblages between estuaries due to differences in environmental factors e.g. habitat type (Teichert et al., 2018a), levels of pollution (Courrat et al., 2009) and estuary size (Nicolas et al., 2010a, 2010b; Waugh et al., 2019). Comparably, salinity was not required to explain variation in assemblage composition, despite ecological expectations (Ahn et al., 2020; Gibson et al., 2023; Nicolas et al., 2010a; Selleslagh et al., 2009). It may be the use of salinity zone classifications rather than exact salinity measurements which produced this effect, as the latter is considered a better measure when studying the effect of salinity on the nekton (Greenwood, 2007). Future studies should collect physicochemical parameters simultaneously to collecting eDNA samples (Gibson et al., 2023). In addition, because the eDNA sampling mirrored the fish biomonitoring surveys it was not possible to sample all estuaries in early summer 2016 or both seasons in this year. While the spatial distribution of sampling within the Tees was also affected and therefore may have influenced the observation of differences in assemblage composition between this estuary and the Esk and Tweed. Given that eDNA has less deployment conditions than fishing, more spatially and temporally consistent designs should be implemented in future.

Despite the present advances, further research into fish eDNA in estuaries is required: focusing on calculations of fish assemblage health metrics, establishing links between metrics and anthropogenic impacts (Gibson et al., 2023), abundance estimation (Bleijswijk et al., 2020) and developing occupancy models accounting for uncertainty in eDNA detections (Burian et al., 2021). Regardless, eDNA metabarcoding could contribute to biomonitoring at present, in several ways. Firstly, fishing sampling efforts in estuaries are often low and inconsistent gear deployment can bias comparisons between estuaries (Waugh et al., 2019), but see Harrison and Kelly (2013). Future surveys could instead combine high effort with a single gear type (e.g. seine nets) to obtain quantitative data on a subset of species, with data on the wider fish community generated through eDNA metabarcoding. Secondly, eDNA could increase the temporal resolution of fish monitoring. Budget restraints currently means sampling is now triennial rather than bi-annual in some areas of the UK. Less frequent fishing could be used to provide a 'ground-truthed' datasets to estimate rates of false positive and negative detection for species in eDNA data collected more frequently (Burian et al., 2021). Thirdly, eDNA could be used to sample waterbodies not currently assessed. In the UK, routine phytoplankton or chemical monitoring occurs in a greater number of estuaries at a higher temporal frequency than fishing (Environment Agency, 2022d; Natural Resources Wales, 2019). Therefore, the concurrent collection of eDNA samples may be an efficient way to collect data on fishes and other biological groups (Mariani et al., 2021). Overall, in combination with the result that eDNA is suited to the collection of large amounts of data on fish biodiversity, these avenues for application suggest eDNA has the potential to be a core assessment tool for monitoring estuarine fish assemblages and the health of these ecosystems.

Ethics Statement

The routine fish monitoring surveys conducted by the EA were conducted in accordance with the relevant legislation relating to the protection of fish stocks and the environment. Water sampling for eDNA by Salford and Bristol University was conducted with the consent of the EA in accordance with local regulations.

CRediT authorship contribution statement

Thomas I. Gibson: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Charles Baillie:** Methodology, Investigation. **Rupert A. Collins:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Owen S. Wangensteen:** Methodology, Investigation, Conceptualization. **Laura Corrigan:** Conceptualization. **Amy Ellison:** Writing – review & editing, Supervision. **Morton Heddell-Cowie:** Writing – review & editing, Investigation. **Hannah Westoby:** Writing – review & editing, Investigation, Data curation. **Barry Byatt:** Writing – review & editing, Investigation. **Lori Lawson-Handley:** Writing – review & editing, Supervision. **Ana Z. Soto:** Investigation. **Simon Creer:** Writing – review & editing, Supervision. **Martin J. Genner:** Writing – review & editing, Funding acquisition, Conceptualization. **Stefano Mariani:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare no conflict of interest. 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 (available at: https://research.bangor.ac.uk/portal/files/49682869/T_I_Gibson_Thesis_2022.pdf).

Data availability

The raw sequence data was deposited in the NCBI BioProject database, accession number PRJNA725897 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA725897). The data used in the ecological analysis is available at: https://zenodo.org/records/10478979. The exclusion list of species generated from other concurrent laboratory projects is available at: https://doi.org/10.5281/zenodo.6858158.

Acknowledgments

This work is dedicated to the memory of Dr Laura Corrigan, who sadly passed away in 2020. Laura was critical to the initial collaboration and coordination of this project between the 'SeaDNA' team and the Environment Agency. We acknowledge Naiara Sales, Riccardo Lollobrigidi, Sandra Garces-Pastor, and also the Environment Agency staff for their sampling efforts. This study has been funded by Grant NE/N005759/1 from the UKRI Natural Environment Research Council. TG's PhD was funded by a KESS2 Knowledge Exchange Scholarship (https://kess2.ac.uk), Grant Number: BUK2147. 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.ecolind.2024.112215.

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