Title:

Estuarine molecular bycatch as a landscape-wide biomonitoring tool

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Author Contributions

SM and MG conceived the study, OSW and MHC participated in sampling, OSW and CB carried out lab work, LRH and RAC analysed the data, and SM and LRH drafted the manuscript. All authors contributed to data interpretation.

Abstract

Environmental DNA analysis is rapidly transforming biodiversity monitoring and bolstering conservation applications worldwide. This approach has been assisted by the development of metabarcoding PCR primers that are suited for detection of a wide range of taxa. However, little effort has gone into exploring the value of the non-target DNA sequences that are generated in every survey, but subsequently discarded. Here we demonstrate that fish-targeted markers widely employed in aquatic biomonitoring can also detect birds and mammals present in the surrounding habitats. We showcase this feature in three temperate estuaries over multiple seasons, where dozens of bird and mammal species offer valuable insights into spatial and temporal faunal variation. Our results indicate that existing metabarcode sequence data sets are suitable for mining and exploration of this 'molecular by-catch', and that any future eDNA-based surveys can be designed to accommodate this enhanced property of this widely applicable tool.

Keywords:

biomonitoring, birds, coastal, conservation, environmental DNA, estuaries, mammals, metabarcoding

63 **Introduction**

64

65 The speed with which environmental DNA (eDNA) analysis has broadly permeated
66 biomonitoring studies worldwide is arguably unprecedented in the history of DNA-based
67 applications to environmental science (Hering et al. 2018; Tsuji et al. 2019). As is typically
68 the case with novel approaches, limitations and pitfalls have led to eDNA-based methods
69 facing much scrutiny, and technological developments remain the ongoing focus of a
70 thriving technical literature (Deiner et al. 2017; Harper et al. 2019a; Kelly et al. 2019;
71 Loeza-Quintana et al. 2020). However, for every caveat raised, elegant solutions are
72 proposed, and new advantages of the methods realised (Thomas et al. 2019; Salter et al.
73 2019; Russo et al. 2020).

74 Aquatic environments have been the main beneficiaries of this 'eDNA revolution',
75 largely owing to the utility of eDNA-based methods for exploring inherently poorly
76 accessible realms, and the relative ease of collecting water, within which DNA naturally
77 disperses, thus facilitating species detection. The utility of the methods ranges from the
78 relatively straightforward recovery of rare (Boussarie et al. 2018) and invasive (Imamura
79 et al. 2020) species, to more sophisticated inference on habitat gradients (Sigsgaard et al.
80 2019), productivity dynamics (Kelly et al. 2016; Djurhuus et al. 2020) and ecosystem
81 structure (Aglieri et al. 2020; Harper et al. 2020). It is now possible consider aquatic eDNA
82 as a useful tool for tackling some of the most pressing biodiversity conservation
83 challenges in a swift, affordable and standardised way, particularly given growing interest
84 in the generation and curation of reference DNA sequence databases. Moreover, the
85 utility of aquatic eDNA may stretch into biomonitoring of associated terrestrial habitats.
86 Recently, it has been shown that DNA retrieved from smaller water bodies can be used to
87 map the distribution of terrestrial mammals that are active in proximity of the aquatic
88 source (Harper et al. 2019b; Sales et al. 2020), suggesting water masses can act as
89 natural biodiversity 'collectors'.

Fundamental to the success of multi-species eDNA investigations is the choice of the genetic marker, which should be 'universal' across the whole taxonomic group of interest, and 'specific' enough to minimise the amplification of DNA from non-target taxa (Collins et al. 2019; Leese et al. 2020). As the most abundant and speciose vertebrate class on Earth, bony fishes (Osteichthyes) have played a major role in the development and consolidation of eDNA applications in marine and freshwater systems (McElroy et al. 2020), and there are now a widely recognised set of procedures that have proven successful globally (Miya et al. 2020). Interestingly, even the most efficient 'fish' primers tend to also amplify some DNA from other vertebrates, and whilst such components typically amount to rather pervasive biological material shed by humans and farmed animals (e.g. cattle, pig, chicken), they may sometimes unveil taxonomic records of substantial ecological and conservation value (Mariani et al. 2019).

Here we explored the concept that eDNA in estuarine areas, at the interface between land and sea, would originate from across the river drainage basin. We therefore examined samples from three UK estuaries flowing into the North Sea, collected as part of the routine monitoring operations of the UK Environment Agency, using a metabarcoding workflow designed for teleosts. Results confirm the versatility of the assay, which, beyond the 93 fish species identified as part of the primary survey, was also able to detect at least 32 birds and eight mammals, including marine, freshwater and terrestrial taxa as well as endangered and exotic species. Spatial and temporal analyses also showed significant variation in richness and community structure, which reflected the known landscape features and seasonality of the studied region. We conclude that future eDNA monitoring programmes along the coastal zone could harness this 'molecular by-catch' gathered by estuaries as a valuable catchment-wide biodiversity assessment tool without incurring any additional costs.

Methods

Data Collection

Sample locations included estuarine segments of the Rivers Tweed, Tees and Esk, situated along the North Sea coast of Britain, between 55º46′N, 1º59′W and 54º29′N, 0º36′W. Sites mirrored those targeted by the regular TraC survey (Environment Agency 2020), which included three netting sites each in the Tweed and Esk estuaries, and two in the Tees estuary. The Esk and Tees were surveyed in October 2016, May 2017 and October 2017, whereas the Tweed was only sampled in May and October 2017. Three 2 L water samples per site were collected immediately ahead of netting operations. Each sample was filtered through a 0.22 µm Sterivex-GP PES filter (Merck Millipore) using a 100 mL polypropylene syringe, and the filters were stored at −20°C.

We extracted DNA from filters following the mu-DNA tissue protocol (Sellers et al. 2018) and PCR-amplified an approximately 167-bp fragment of the mitochondrial 12S rRNA region using the fish-specific MiFish (Miya et al. 2015) and Teleo02 primers (Taberlet et al. 2018). Each primer pair was designed with a unique 8-bp tag to facilitate sample identification after sequencing. We then prepared three PCR-free, dual-indexed libraries using the KAPA Hyper Prep Kit, which were quantified using qPCR, pooled in equimolar concentrations, and loaded onto an Illumina MiSeq at 8pM concentration for 2x150-bp paired-end sequencing. Further details on laboratory procedures are in the Supporting Information.

Raw reads were filtered for PCR primers and demultiplexed (tag required on both ends of the amplicon, no mismatches allowed) into sample replicates using cutadapt v2.10 (Martin, 2011), followed by correction of Illumina sequencing errors (denoising) and quality filtering (default settings), using dada2 v1.16 (Callahan et al. 2016), and removal of non-homologous reads, using hmmer v3.1b2 (Eddy, 1998); further details can be found in Collins et al. (2019). Taxonomic identification followed a two-step procedure: (1) we obtained the NCBI RefSeq mitochondrion database v201 (https://www.ncbi.nlm.nih.gov/refseq/) and used the sintax algorithm in vsearch v2.15.0 (Edgar, 2016; Rognes et al. 2016) to assign a rough taxonomy; (2) we then removed reads assigned to fishes and used BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to more

accurately identify the remaining reads based on conditions outlined in the Supporting Information.

Data analysis

All downstream analyses were performed in R v.3.6.3 (R Core Team, 2020). The raw data were summarised as the number of taxa and number of reads belonging to each vertebrate groups across seasons within each estuary (Fig. S1). Subsequent refinement of non-fish data, including removal of spurious taxa, correction of misassignments, false positive removal (see Fig. S2), and noise mitigation using a sequence threshold, is fully described in the Supporting Information. All fish assignments and corresponding reads were then omitted for downstream analyses.

Sequence data for PCR replicates were then pooled across biological replicates from each sampling location (Fig. S3), within each estuary. Sequence data for biological replicates taken at each sampling location were then pooled, and a bubble plot summarising eDNA detections in each estuary across different seasons produced (Fig. 1). For comparison, this bubble plot was reproduced to include species whose tissue had been sequenced in laboratories concurrently with this project and whose sequences were removed from the present data set (Fig. S4; see also Appendix 2). The pooled sequence data were converted to presence/absence using the *decostand* function in vegan v2.5-6 (Oksanen et al. 2019) for downstream analyses.

We investigated spatial variation in α- and β-diversity between estuaries, followed by temporal variation in α- and β-diversity within each estuary, using the packages vegan v2.5-6, stats v3.6.3, FSA v0.8.30 (Ogle et al. 2020), iNEXT v2.0.20 (Hsieh et al. 2016), and betapart v1.5.1 (Baselga & Orme 2012). We define α-diversity as taxon richness of individual sampling locations, and β-diversity as the difference between communities present at each sampling location whilst accounting for taxon identity (Baselga & Orme 2012). β-diversity (Jaccard dissimilarity) was partitioned by community dissimilarity due to taxon replacement (i.e. 'turnover') or taxon subsets (i.e. 'nestedness-resultant'). Details of

174 α- and β-diversity analyses are provided in the Supporting Information.

175

176 **Results**

177

178 Alongside 93 fishe species, teleost eDNA metabarcoding recovered two amphibian, 51
179 bird, 51 mammal, and 13 invertebrate species from 78 water samples (Fig. S1a). Most
180 reads belonged to fishes, followed by mammals and birds (Table 1; Fig. S1b). After
181 dataset refinement, 32 birds (21 aquatic, 11 terrrestrial) and eight mammals (three
182 aquatic, five terrestrial) remained in 69 (88.5%) water samples. This included 18 birds and
183 two mammals of conservation concern within Europe (Fig. 1).

184 The 69 remaining samples included 33, 16, and 20 water samples from the Esk, Tees,
185 and Tweed estuaries respectively were analysed. Alpha diversity differed across estuaries
186 ($H = 7.95$, $p = 0.018$), where taxon richness was lower in the Tees than the Esk ($Z = 2.263$, $p = 0.036$) or Tweed ($Z = -2.715$, $p = 0.020$) (Fig. 2a). Taxon richness in the Esk
188 and Tweed did not significantly differ ($Z = -0.781$, $p = 0.435$). Rarefaction and
189 extrapolation curves indicated that lower taxon richness of the Tees may be due to
190 differences in sample size between estuaries (Fig. 2b).

191 Beta diversity in each estuary was driven by turnover as opposed to nestedness-
192 resultant (Table 2). MVDISP was present between estuaries for all β-diversity components
193 (Table 2). Estuary had a moderate positive influence on turnover (Fig. 2bi) and total β-
194 diversity (Fig. 2biii) of communities, but not nestedness-resultant (Fig. 2bii; Table 2),
195 generally indicating that a substantial proportion of taxa at a given estuary appear to be
196 replaced by different taxa at other estuaries.

197 Alpha diversity differed across seasons within the Esk estuary ($H = 20.635$, $p < 0.001$)
198 but not the Tees ($H = 1.298$, $p = 0.523$) or Tweed ($H = 1.364$, $p = 0.243$) estuaries (Fig.
199 3a). Taxon richness was higher in autumn (2016: $Z = 2.621$, $p = 0.013$; 2017: $Z = 4.537$, p
200 < 0.001) than spring in the Esk (Fig. 3a). Furthermore, taxon richness was comparable
201 between autumn 2016 and autumn 2017 in the Esk ($Z = -1.910$, $p = 0.056$).

Beta diversity of estuarine communities across seasons was also largely driven by turnover, but nestedness-resultant played a greater role in some seasons. MVDISP was absent between seasons for total β-diversity (Esk), turnover and total β-diversity (Tees), and nestedness-resultant (Tweed) (Table S2). Season had a strong positive influence on all β-diversity components for the Esk (Figs. 3bi, S5ai-iii), and on turnover (Figs. S5bi, S5ci) and total β-diversity (Figs. 3bii-iii, S5biii, S5ciii) but not nestedness-resultant (Figs. S5aii, S5bii, S5cii) for the Tees and Tweed (Table S2). Therefore, taxa detected in a given season appear to be replaced by different taxa in other seasons within each estuary.

Discussion

Since the inception of eDNA-based biodiversity assessment, there has been an emphasis on comparing detection performance with well-established biomonitoring approaches that use capture, visual or acoustic identification (Jerde et al. 2011; Foote et al. 2012; Thomsen et al. 2012; Yamamoto et al. 2016). The popularity of eDNA-based analysis today owes much to the realisation that, in many important contexts, the new tool offered significant advantages over conventional sampling methods, either through sheer improvement of detection efficacy (Boussarie et al. 2018; McElroy et al. 2020), through the discovery of its unique complementarity (Aglieri et al. 2020; Harper et al. 2020), or by simply being less resource-intensive (Bálint et al. 2018; Aglieri et al. 2020). On the other hand, little effort has gone into evaluating the intrinsically serendipitous nature of high-throughput sequencing, which, irrespective of the metabarcoding markers chosen, consistently yields substantial amounts of non-target sequences. Here we offer a demonstration that non-target sequences from metabarcoding assays contain valuable biodiversity information that can be harnessed, at no extra cost, from existing studies and ongoing surveys, dramatically expanding the reach and value of eDNA-derived data for conservation science.

We were able to conduct a multi-seasonal, parallel biodiversity survey from samples collected and analysed in three estuaries for an unrelated purpose. From a data set originally generated for monitoring coastal fish (Collins et al 2019; Siegenthaler et al 2019; Table S1), we extracted a faunal list including 32 birds and eight mammals. Of these, 52.5% were taxa that are typical of coastal marine areas, such as oystercatcher (*Haematopus ostralegus*), guillemot (*Uria algae*), common seal (*Phoca vitulina*), grey seal (*Halichoerus grypus*) and harbour porpoise (*Phocoena phocoena*). These species are directly associated with the sampled habitat, but their presence at the time of sample collection would not have been monitored by a fish-surveying team. Furthermore, some of the detected species (e.g. whimbrel (*Numenius phaeopus*), white-fronted goose (*Anser albifrons*), lapwing (*Vanellus vanellus*), redshank (*Tringa totanus*), dunlin (*Calidris alpina*), harbour porpoise) are currently listed as species of conservation concern (IUCN 2010; Eaton et al. 2015), making these DNA signatures a useful permanent record of these organisms' presence at a certain time and space, which can serve as a baseline for future surveys, and required no financial investment to obtain.

Perhaps more surprisingly, 47.5% of the detected non-target species were not strictly associated with coastal marine areas, but rather more typical of the rural landscape, demonstrating the role of estuaries as physical collectors of eDNA transported through the drainage basin. We found ducks, passerines, waders, grouse and partridges amongst the birds, and European rabbit (*Oryctolagus cuniculus*) and Daubenton's bat (*Myotis daubentonii*) amongst the mammals. Prior to data set refinement, a number of rodents and mustelids were also detected. Although most of these species would be expected in rural Britain, we also recovered data from species of high conservation relevance, such as the occurrence of spoonbill (*Platalea leucorodia*) in the Esk catchment, a bird that has only started breeding again in Britain in the last decade. The detection of water buffalo (*Bubalus bubalis*) as well as western and eastern kangaroo (*Macropus fuliginosus* and *M. giganteus*) in the Esk and Tweed is more puzzling. This could reflect drainage/sewage processes from nearby wildlife parks or farms: it is worth mentioning that an exotic meat

257 company purveying both kangaroo and buffalo meat is located in the Tweed drainage,
258 only a few miles upstream of the monitoring sites.

259 The utility of this 'molecular by-catch' in the context of landscape-wide biomonitoring is
260 further corroborated by the marked spatial and temporal patterns identified. Taxon
261 richness was shown to significantly vary among estuaries, and this was also reflected in
262 the overall β-diversity configuration: the least taxon-rich estuary, the Tees, also supported
263 a more divergent community from the other two. This can be explained by the
264 characteristics of the catchment. Both the Tweed and the Esk run through rural
265 landscapes, with little urbanisation, meeting the North Sea by the picturesque coastal
266 towns of Berwick and Whitby, respectively. In contrast, the Tees flows through more
267 urbanised areas, including the large post-industrial towns of Darlington, Middlesbrough
268 and Hartlepool, which may arguably result in greater environmental impact on the
269 catchment. However, rarefaction and extrapolation analyses indicated that sample
270 coverage may have also influenced lower diversity of the Tees. With greater sample
271 coverage, future studies may consider modelling eDNA-based results against land-use
272 and satellite data to examine potential urbanisation and environmental gradients
273 influencing biodiversity at landscape-scale.

274 The faunal records from eDNA also delineated clear temporal changes in the studied
275 systems, with autumn samples significantly more taxon-rich than and divergent from
276 spring samples, although this was less evident in the less diverse Tees estuary. The Esk
277 and Tweed estuaries both supported more bird species than the Tees, including moult
278 migrants (e.g. shelduck, *Tadorna tadorna*), winter migrants (e.g. Canada goose, *Branta*
279 *canadensis*; whooper swan, *Cygnus cygnus*), passage migrants (e.g. dunlin), and partial
280 migrants (e.g. common starling, *Sturnus vulgaris*). Additionally, more mammals were
281 detected in the Esk and Tweed during autumn which coincides with moulting, breeding
282 and dispersal in some species (e.g. harbour seal, *Phoca vitulina*). Autumnal influxes of
283 birds and mammals to the Esk and Tweed may drive increased richness and community
284 divergence, compared to less diversity in the Tees and spring generally.

The bird and mammal biodiversity 'bonus' showcased in this work will represent an underestimation of the actual bird and mammal eDNA diversity in the studied estuaries, and more exhaustive faunal inventories are likely to be obtained by employing taxon-specific markers for birds (Ushio et al. 2018) or mammals (Sales et al. 2020), or possibly less specific markers for vertebrates (Harper et al. 2019b). Nevertheless, the volume of information retrieved allows for educated inference on spatial and temporal variation between and within catchments, and inform and propel further focussed research activity leading to conservation actions.

In the midst of a global biodiversity crisis, rapid, powerful and affordable methods are crucial for assessing and monitoring biotas. Environmental DNA metabarcoding projects typically generate an extraordinary amount of biological information, which often exceeds the scope of the original investigation (Hupało et al. 2020). Data are routinely stored in publicly available repositories, and sequencing and computational power costs continue to drop. With this in mind, researchers and environmental managers only need to be aware of the potential of this 'molecular by-catch' and start designing aquatic surveys accordingly. Meanwhile, after a decade of high-throughput sequencing in natural habitats, we have already accumulated a vast amount of environmental barcodes, which remain partly untapped. We only have to start sieving through these data sets with renewed endeavour.

Data Accessibility

Code and data to be archived in public repositories upon article acceptance.

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484 https://doi.org/10.1371/journal.pone.0153291
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487 **Table 1.** Summary of raw sequence output using teleost eDNA metabarcoding of
488 estuarine water samples.

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Group	Number of taxa	Read counts	Reads (%)
Fishes	93	2,379,539	81.563
Amphibians	2	88	0.003
Birds	51	178,465	6.117
Mammals	51	342,979	11.756
Invertebrates	13	16,354	0.561
Total	**210**	**2,917,425**	**100.000**

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Table 2. Summary of analyses statistically comparing homogeneity of multivariate dispersions between communities at sampling locations in each estuary (ANOVA), and variation in community composition of sampling locations in each estuary (PERMANOVA). Relative contributions of taxon turnover and nestedness-resultant to total β-diversity (Jaccard dissimilarity) for each estuary are given in brackets.

	Homogeneity of multivariate dispersions (ANOVA)				**Community similarity (PERMANOVA)**			
	Mean distance to centroid ± SE	**df**	***F***	***P***	**df**	***F***	***R^2***	***P***
Turnover		2	2.822	0.067	2	6.014	0.156	**0.001**
Esk (95.16%)	0.404 ± 0.042							
Tees (97.27%)	0.543 ± 0.029							
Tweed (94.21%)	0.415 ± 0.037							
Nestedness-resultant		2	0.242	0.786	2	0.264	0.008	0.673
Esk (4.84%)	0.189 ± 0.026							
Tees (2.73%)	0.155 ± 0.017							
Tweed (5.79%)	0.173 ± 0.033							
Total β-diversity		2	1.839	0.167	2	4.203	0.115	**0.001**
Esk (100%)	0.522 ± 0.013							
Tees (100%)	0.589 ± 0.013							
Tweed (100%)	0.543 ± 0.011							

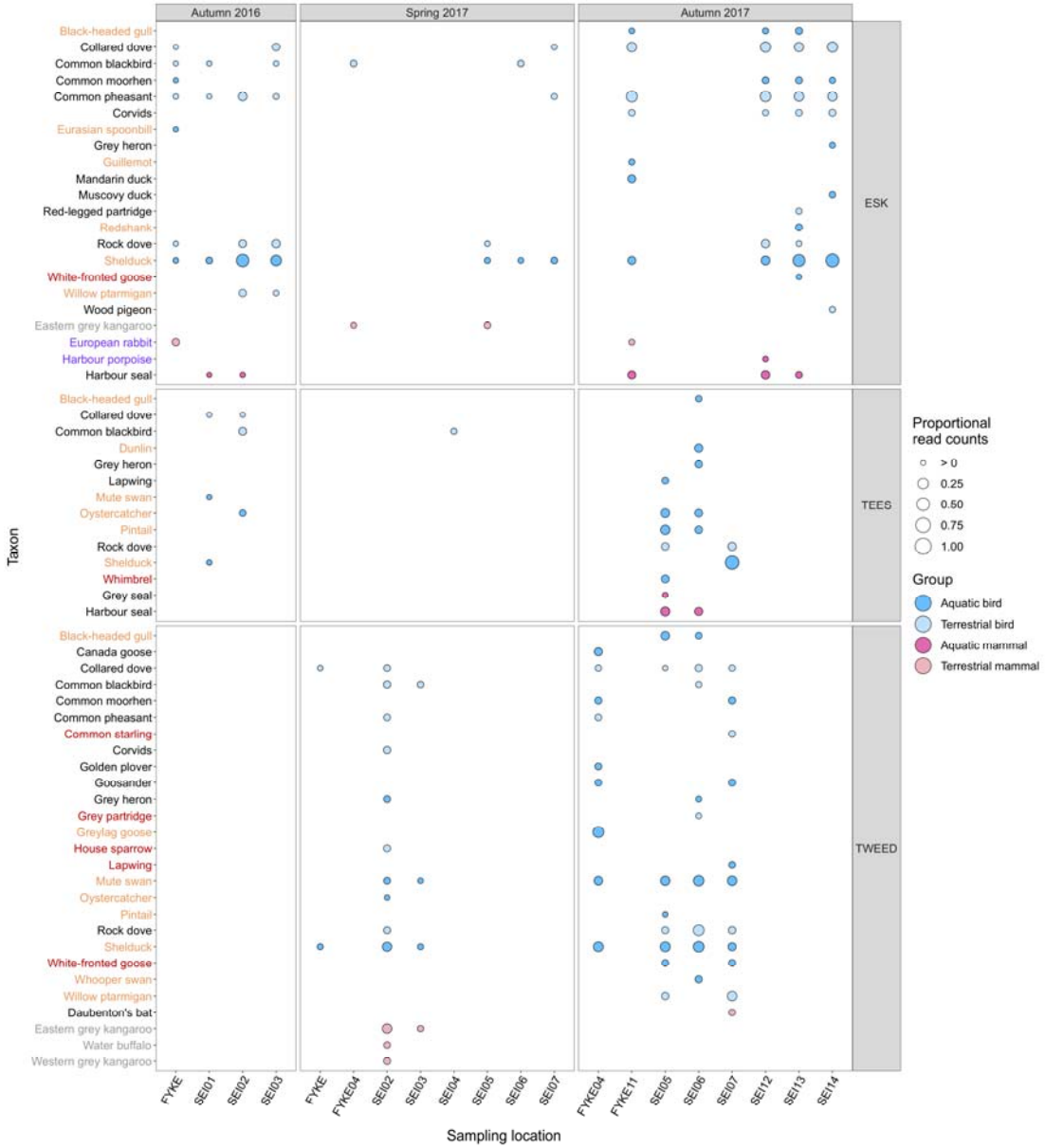

Figure 1. A bubble graph showing proportional read counts for taxa detected in water samples from different sampling locations within each estuary. Bubbles are coloured according to vertebrate group, and whether taxa have aquatic or terrestrial life histories. Names of birds on the Birds of Conservation Concern 4 red and amber lists (Eaton et al. 2015) are coloured red and orange respectively. Names of endangered mammals on the European Red List (IUCN 2010) are coloured purple. Names of taxa found in captivity are coloured grey.

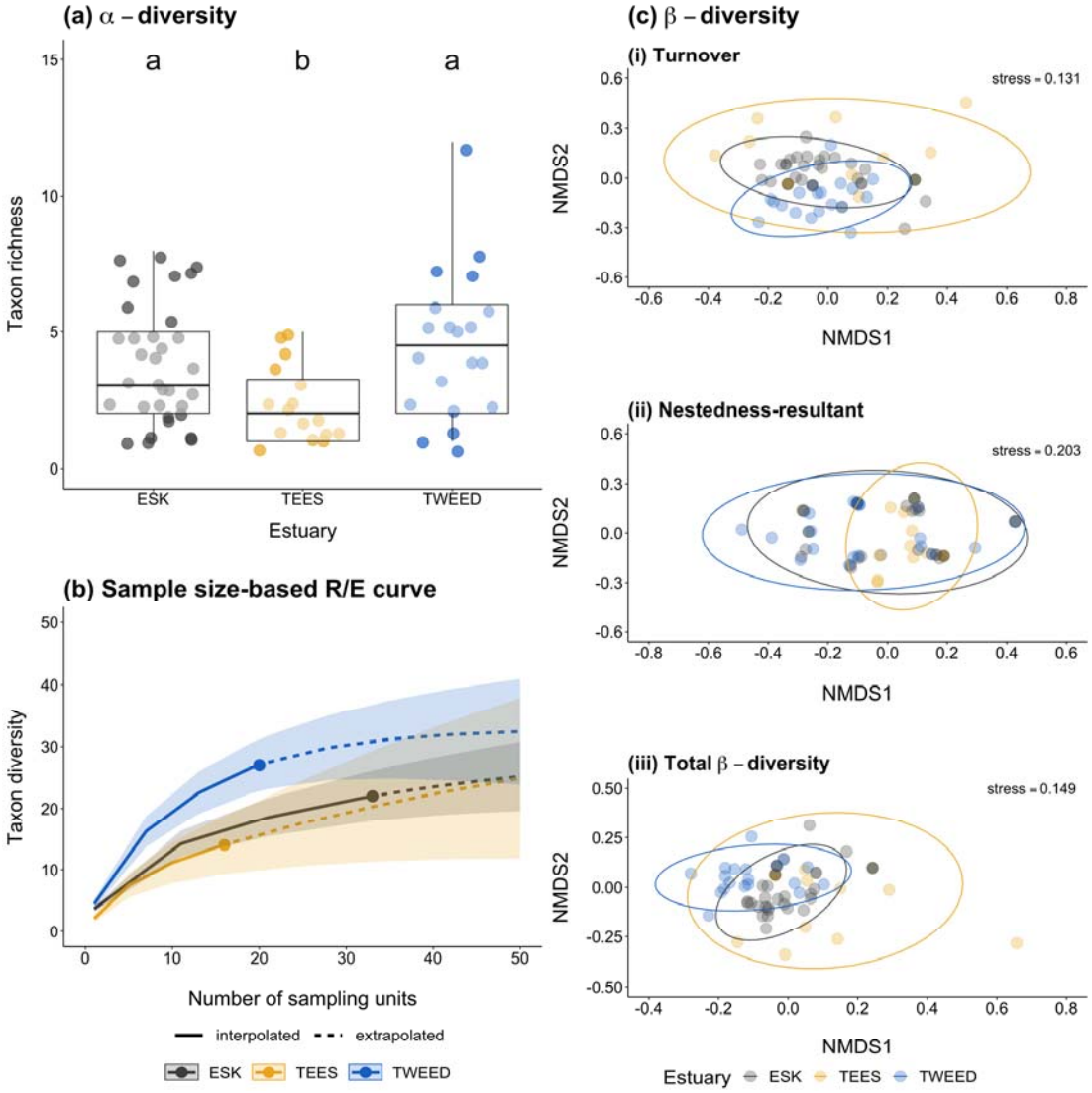

Figure 2. Summaries of α- and β-diversity comparisons made between sampling locations in the Esk (grey points/lines/ellipses), Tees (yellow points/lines/ellipses), and Tweed (blue points/lines/ellipses) estuaries: **(a)** boxplot showing the number of taxa detected at each estuarine sampling location, **(b)** sample size-based rarefaction/extrapolation (R/E) for each estuary, and **(c)** non-metric multidimensional scaling (NMDS) plots of estuarine communities for each β-diversity component. Letters denote significance, where different letters indicate a statistically significant difference in taxon richness derived from Dunn's test. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles.

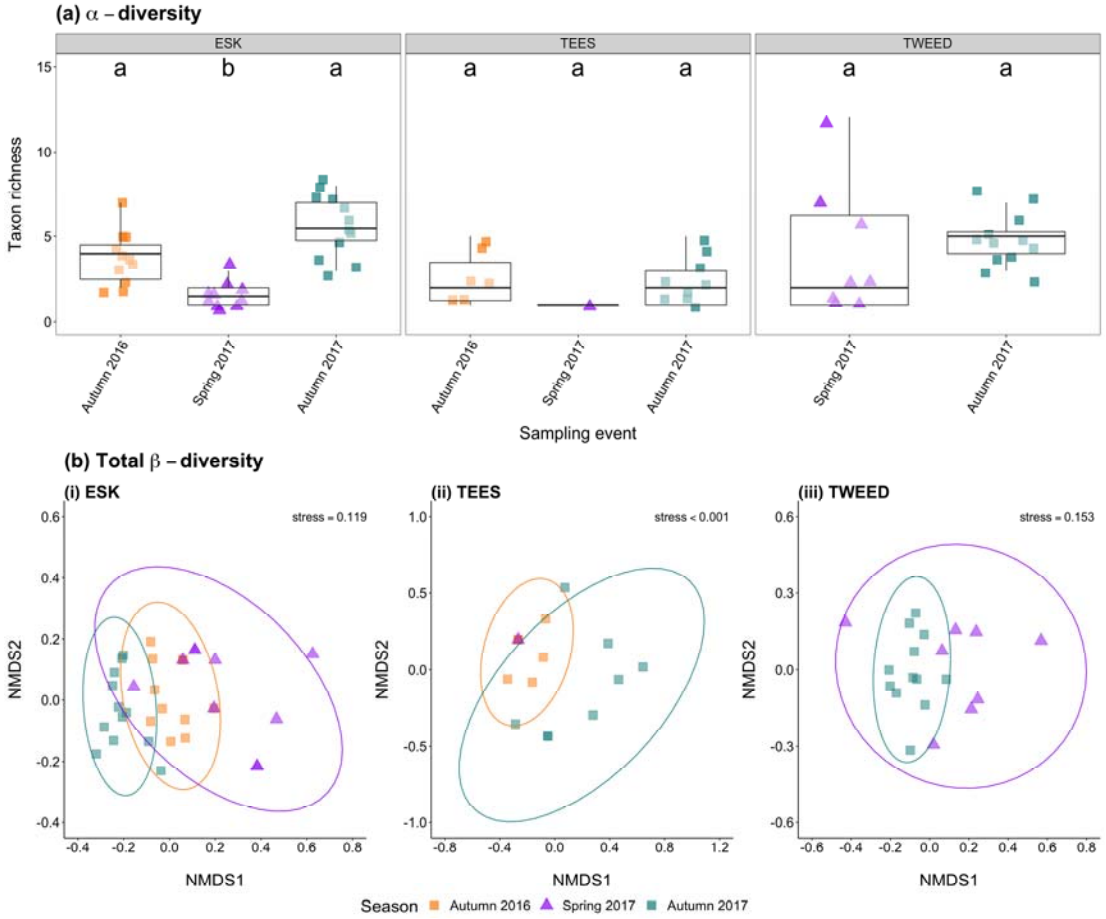

Figure 3. Summaries of α- and β-diversity comparisons made between sampling locations in each estuary during different seasons, including autumn 2016 (orange squares/ellipses), spring 2017 (purple triangles/ellipses), and autumn 2017 (green squares/ellipses): **(a)** boxplot showing the number of taxa detected at estuarine sampling locations across seasons, and **(b)** non-metric multidimensional scaling (NMDS) plots of communities in each estuary across seasons for each β-diversity component. Letters denote significance, where different letters indicate a statistically significant difference in taxon richness derived from Dunn's test. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles.