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Environmental DNA as a complementary tool for monitoring fish assemblages in coastal lagoons: Insights for conservation

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ABSTRACT

For their scarcity, invasive species in early invasion stages and endangered species are often difficult to detect compromising both conservation and ecosystem protection. Here environmental DNA (eDNA) was compared with beach seining for monitoring fish diversity in the protected Natural Park Ria Formosa lagoon (Portugal). Surface water for eDNA extraction was collected before sampling a mean of 1400m^2 from ten diverse shallow water habitats with a 25 m beach seine. Fragments of the 12S rRNA and COI mitochondrial genes were PCR-amplified and taxonomy was assigned to amplicon sequence variants. Sampling with the beach seine resulted in the identification of 33 species and 4 genera while 28 species were identified based on eDNA, with 18 taxa in common. Greater taxonomic resolution at a species level was possible with eDNA. While the majority of species detected with both methods were of least concern according to IUCN criteria, eDNA detected one invasive (the weakfish *Cynoscion regalis* with 12S marker) and two critically endangered species (the ray *Aetomylaeus bovinus* with 12S and the eel *Anguilla anguilla* with COI marker), none of which were caught by beach seining. *C. regalis* is a threat in Portuguese waters and should be surveyed in Ria Formosa. The results highlight the usefulness of eDNA as a cost-effective complementary method to traditional monitoring, especially for rare species. Based on these results, we recommend the use of eDNA with multiple markers in surface and bottom water samples in long-term monitoring programmes, to enhance the detection of rare, elusive species in coastal lagoons.

1. Introduction

Coastal lagoons and estuarine saltmarshes are highly productive ecosystems that act as efficient carbon sinks (Santos et al., 2004; Cunha et al., 2024), thus having enormous ecological value under current conditions of climate change. In the interface of freshwater and marine habitats, they provide numerous ecosystem services that extend beyond the lagoon area (Rodrigues-Filho et al., 2023). Among these, they shelter rich communities and diverse fish assemblages that are the basis of important coastal fisheries (e.g., Cavraro et al., 2017; Scapin et al., 2018, 2022; Erzini et al., 2022). Moreover, they are important shelters of vulnerable and endangered species, being key conservation areas (Newton et al., 2018; Facca et al., 2020; Mateos-Molina et al., 2024). For these reasons, long-term monitoring by member states of the fish diversity and abundance in transitional waters such as lagoons is required under the Water Framework Directive (WFD) in Europe (European Parliament and Council, 2000; Coates et al., 2007; Cabral et al., 2012).

Currently, eDNA is not integrated in the assessment of ecological status of transitional waters for the WFD, mainly because fish-based indices, such as the AZTI Fish Index for estuaries require fish composition and absolute abundance data obtained from bottom trawl tows (Borja et al., 2004; Uriarte and Borja, 2009). However, it has been recognized that there is considerable potential for DNA-based identification of fish to be used for assessment procedures to fulfil the requirements of the WFD, with ongoing research to replace absolute by relative abundance in fish-based metrics (Hering et al., 2018; Pont et al., 2021).

Here we have focused on the Ria Formosa (Algarve, South Portugal). This coastal lagoon has high fish diversity and is an important nursery for many commercial fish species, with an important role in terms of fish provisioning services (Monteiro, 1989; Erzini et al., 2002; Adão et al., 2022; Erzini et al., 2022, 2024). More than 120 species of fish have been

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recorded in studies since the 1980s (Monteiro, 1989; Erzini et al., 2002). Effective monitoring of the ichthyofauna of the Ria Formosa is essential, given the impact of climate change, loss of seagrass habitat and the numerous activities and anthropogenic threats that are threatening the lagoon (Ribeiro et al., 2008; Correia, 2022). These include pollution, tourism, fishing, harvesting of invertebrates at low tide, aquaculture, and boating.

WFD sampling guidelines specify the use of standardized beam trawl tows for monitoring of transitional waters. However, Adão et al. (2022) compared beam trawl catches with those of a 25 m beach seine, a 50 m beach seine and a Riley push net based on monthly sampling carried out in the Ria Formosa lagoon from September 2000 to April 2002 (Erzini et al., 2002) and found significant differences in diversity, assemblage structure and relative abundance between the sampling gear, concluding that a combination of complementary sampling methods is required for effective monitoring of the ichthyofauna.

Environmental DNA (eDNA) has been chosen in estuary monitoring (Valentini et al., 2016; Van Driessche et al., 2024) to detect vulnerable species because it has multiple advantages in the assessment of these populations (Ahn et al., 2020; Kume et al., 2021). Fishing gear can damage seagrass habitats and large numbers of fish, especially juveniles, are killed since it is not possible to record all the data in the field and release all the fish alive. The use of eDNA avoids the disturbance as it is a non-invasive sampling technique (Maruyama et al., 2018; Banerjee et al., 2022). Moreover, while sampling with nets is costly, requiring a small research vessel, a team of researchers and considerable time in the laboratory for processing the samples (sorting, identifying, measuring and weighing all the fish), using eDNA can be more cost-effective (Thomsen and Willerslev, 2015; Piggott et al., 2021). However, traditional sampling methods such as beach seine, beam trawl and others allow to measure fish abundances and sizes which are crucial to analyse population trends (Adão et al., 2022).

Since 2002, annual fish assemblage monitoring has been carried out in July with a 25 m beach seine, at eleven sampling locations in the Ria Formosa lagoon (Fig. 1). The objective of this study is to compare fish diversity obtained from beach seine sampling in the 2024 monitoring survey with eDNA results from water samples taken at the same time and sampling locations. We evaluate the eDNA potential as a complementary or perhaps an alternative method to sampling with nets for monitoring the fish assemblages in coastal lagoons, with a focus on rare species (e.g., Jerde et al., 2011; Fukumoto et al., 2015; Budd et al., 2021; Piggott et al., 2021).

2. Material and methods

2.1. Study area and sampling methodology

The Ria Formosa is a mesotidal coastal lagoon with six inlets that extends for 55 km along the south coast of Portugal (Fig. 1). It consists of salt marshes, subtidal channels, sand banks, tidal creeks and tidal flats covering a surface area of approximately 170 km^2 (Ribeiro et al., 2006; Erzini et al., 2002). The semi-diurnal tidal averages 2 m, varying from 1.5 to 3.5 m on neap and spring tides, with the area covered by water during spring tides ranging from 14.1 to 63.1 km^2 (Águas, 1986; Jacob and Cravo, 2019).

Unvegetated soft substrate accounts for 15.9 km^2 of the subtidal zone, while vegetated habitat covers 3.1 km^2 (Erzini et al., 2022). Three species of seagrasses are found in the lagoon, with *Zostera marina* and *Cymodocea nodosa* occupying the shallow subtidal zones and *Zostera noltei* the exposed intertidal areas. In recent years the green algae *Caulerpa prolifera* has expanded its distribution within the lagoon, at the expense of *Z. marina* and *C. nodosa*, and is now found in large areas, especially in the deeper subtidal areas (Parreira et al., 2021).

Beach seine sampling was authorized by the Instituto da Conservação da Natureza e das Florestas, I.P. (RUBUS@ICNF), under permit reference n° 679/2024/CAPT. Eleven points within Ria Formosa were sampled on July 22nd and 23rd, 2024 as part of the annual monitoring program of Ria Formosa lagoon ichthyofauna (Fig. 1). The geographical coordinates, tide, tide phase, type of habitat (% vegetation cover or unvegetated bottom, maximum depth, location in relation to the nearest inlet, area sampled, and temperature at the time of sampling) are given in Supplementary table 1. Based on distance to the nearest inlet, the sampling locations are classified as outer (within 2 km of an inlet), middle (between 2 and 6 km from an inlet) or inner lagoon (greater than

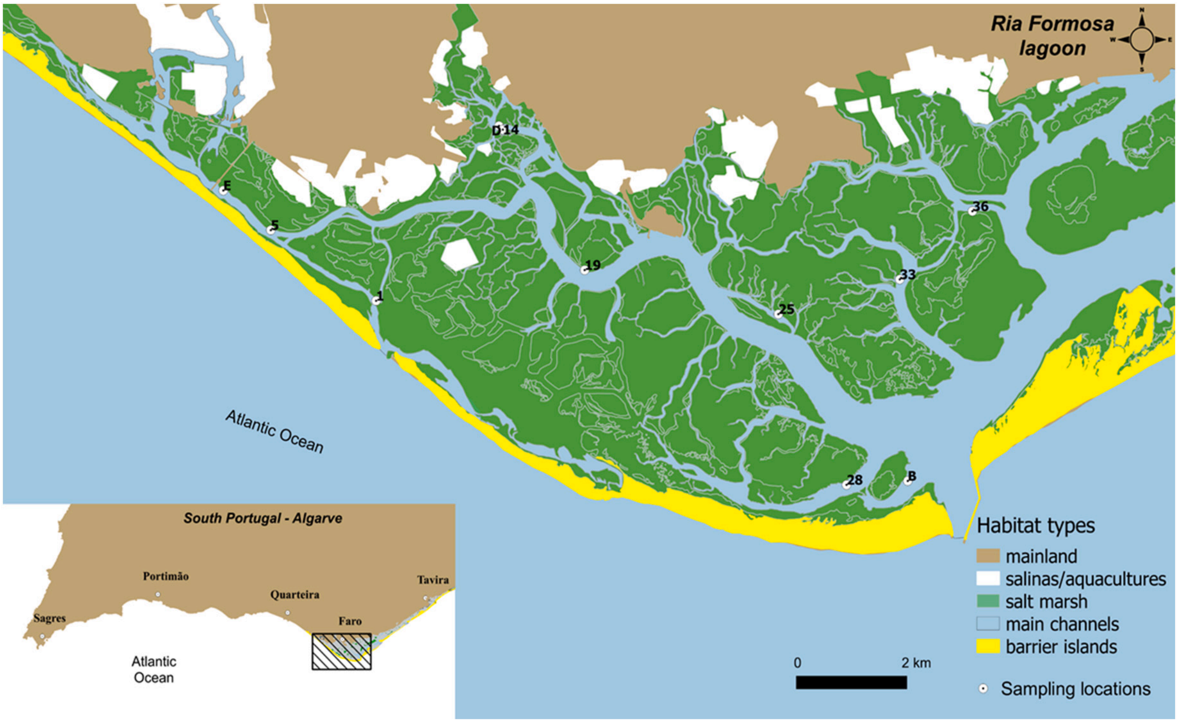

Fig. 1. Sampling sites within the Ria Formosa lagoon.

6 km from an inlet).

Fish were sampled using a 25 m beach seine, 3.5 m high in the middle with a codend and a stretched mesh size of 9 mm. Sampling took place on two days when the amplitude of the tide was less than 2 m, within a window of 1 h and 45 min before and after low tide, with the majority of the sampling taking place less than an hour before or after the low tide (Supplementary Table 1). The net was deployed and towed from a boat with two people on shore holding one end. GPS measurements were used to estimate the area sampled by the net. Sea horses and pipefish (Syngnathidae) were measured and released alive. Large individuals of other fish species were also released. The remainder of the catches, consisting largely of small species and juveniles, mostly less than 7–8 cm in total length, were too numerous to measure and/or difficult to identify. They were placed in labelled bags in a cooler with ice pads for a maximum of three to four hours before arrival at the laboratory where they were stored in freezers. One sample per day was thawed in the following weeks and all the fish were identified, measured (total length in cm) and weighed (g).

For environmental DNA, six 2 L replicates of surface water were taken using sterilized bottles from each sampling point except one (site #14) that is a few meters in front of site #D and water eDNA would be expectedly mixed. The water samples were filtered in situ through 0.2 μm filters using manual pumps. The filters were immediately stored in 100 % ethanol. In one sample of difficult filtration –perhaps due to suspended solids- two filters were employed. Three blanks of sterile water were opened in the field on the first and second sampling days, filtered in situ as well, and included in the pipeline to monitor for contamination across the sampling. Researchers wore gloves all the time during sampling, and cleaned all the materials with 10 % bleach before sampling and between sampling points.

2.2. DNA methodology

DNA analysis followed the methodology described in Douard et al. (2024). Briefly, eDNA was extracted from filters with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), incorporating an initial ten minutes bead beating step with Power Water DNA Bead Tubes (Qiagen, Hilden, Germany) and the mixer mill MM 400 homogenizer by Retsch (Haan, Germany), in tubes with 720 μL of ATL Buffer and 80 μL of Proteinase K (2 mg/L); then incubation at 56 °C for 1 h, and quantification with Qubit 1× High Sensitivity dsDNA Assay (Thermo Fisher Scientific). Extraction negative controls were included at each extraction round and treated with the rest of the samples to check for extraction cross-contamination.

Possible inhibitors in eDNA extracts were tested with an internal positive control added to a qPCR run using Applied Biosystems TaqMan® Exogenous Internal Positive Control Reagents (Thermo Fisher Scientific, Waltham, MA, USA). Ct values were compared among samples amplified under the same conditions used for library preparation using 1× Platinum II Hot-Start PCR Master Mix (Invitrogen) in a final volume of 20 μL.

DNA metabarcoding library preparation and sequencing were carried out by AllGenetics & Biology SL (www.allgenetics.eu). Two mitochondrial markers were chosen; one of the most broadly used for metazoans within the 12S rRNA gene, and a second one targeting a fragment of cytochrome oxidase I (COI), which has shown to provide a good identification rate (Wangensteen et al., 2018). The 12S fragment, of 220 bp, was PCR-amplified using the following primers: Forward - MiFish-U-F (5’ GTCGGTAAAACTCGTGCCAGC 3′) and Reverse - MiFish-U-R (5’ CATAGTGGGGTATCTAATCCCAGTTTG 3′) (Miya et al., 2015). A COI fragment of 365 bp was amplified using the primers: Forward - mlCOIintF-XT (5’ GGWACWRGWTGRACWITITAYCCYCC 3′) (Leray et al., 2013, as modified by Wangensteen et al., 2018) and Reverse - jgHCO2198 (5’ TANACYTCNGGRTGNCCRAARAAYCA 3′) (Yu et al., 2012). These primers also included the Illumina sequencing adaptors attached to their 5′ ends.

PCRs were carried out with 4 μL of template DNA, 0.5 μM of the primers, 10 μL of the master mix cited above, and ultrapure water up to 20 μL. The cycles were: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 53 °C for 1 min and 68 °C for 30 s. Three PCR replicates per sample were performed independently and pooled before the next steps. For COI primers the PCRs were optimised as explained in Douard et al. (2024), with 6 μL of template DNA, 0.5 μM of the primers, 6.25 μL of the master mix, and ultrapure water up to 12.5 μL. Incubation was: initial denaturation step at 94 °C for 2 min, followed by 37 cycles of 94 °C for 15 s, 52 °C for 1 min and 68 °C for 30 s.

The library preparation process followed Vierna et al. (2017), attaching oligonucleotide indices in a second amplification step with 2.5 μL of pooled PCR product from the first amplification step, 1 μM of the dual-indexed primers, 6.5 μL of the PCR master mix, and ultrapure water up to 25 μL. The reaction mixture was incubated as follows: an initial denaturation at 94 °C for 5 min, followed by 5 cycles of 94 °C for 30 s, 60 °C for 45 s, 68 °C for 45 s, and a final extension step at 68 °C for 7 min. A negative control without DNA was included to check for contamination during library preparation. Library size was verified under UV light on 3 % agarose gels stained with GreenSafe (NZYTech). Libraries were purified with Mag-Bind RXNPure Plus magnetic beads (Omega Bio-tek), then pooled in equimolar amounts (Douard et al., 2024). The pool was sequenced with a NovaSeq PE250 flow cell (Illumina) and a total output of two gigabases.

2.3. Bioinformatic analysis

Illumina paired-end raw data consist of forward (R1) and reverse (R2) reads stored in separate files together with the reads' quality scores. Traces of adapter dimers were removed using Cutadapt v3.5 (Martin, 2011). The quality of the FASTQ files was assessed with the software FastQC (Andrews, 2010) and the output was summarised using MultiQC (Ewels et al., 2016).

Qiime2 2024.4 (Bolyen et al., 2019) was employed for further analysis. Primers were trimmed with q2-cutadapt plugin (Martin, 2011) and denoising was done with DADA2 (Callahan et al., 2016) (via q2-dada2). The output of the sequence quality filters can be found in Supplementary Table 2. Denoised sequences were then assigned against a local database of nucleotide sequences downloaded from NCBI using the electronic search query “12S NOT unverified NOT virus NOT environmental NOT unclassified NOT genome” for 12S and “COI NOT environmental NOT unclassified NOT viruses NOT archaea” for COI in November 2024. Finally, taxonomy was assigned to ASV (amplicon sequence variants) using the q2-feature-classifier (Bokulich et al., 2018) with 97 % identity and an e-value of 10^{-50} as thresholds. A table of ASV of fish species was constructed containing the corresponding sequences (Supplementary table 3) and the number of reads of each assigned species. The ASV table was expert-checked for taxonomy and confirmation of the pipeline assignment using manual BLAST against GenBank database with the following thresholds: identity 99 % (12S) or 98 % (COI), *E*-value 0.000, coverage 98 %. Singletons were not considered in the ASV table and downstream analysis. The taxonomic reference was the World Register of Marine Species (WoRMS Editorial Board, 2025).

2.4. Statistical analysis

The relationships between variables were tested using Pearson's correlation coefficient (r). Differences in the proportion of species detected from one, two or no genes across ecological types were assessed using Chi-square contingency analysis and confirmed using Fisher's exact test. Fish diversity in the sampling sites was measured from Shannon-Wiener index, using 0/1 as absence/presence for eDNA because the number of individuals per species cannot be estimated.

3. Results

No evidence of inhibition was found with Ct variation being 1 cycle maximum. The dataset of sequences (raw FASTQ files) can be found in NCBI Gen (https://www.ncbi.nlm.nih.gov/gene) under BioProject accession number PRJNA1271295.

3.1. Fish diversity detected in ria Formosa from net sampling and eDNA

A total of 48 fish classified down to a genus (4) or species level (44) were identified. From them, 33 species and 4 genera (1392 individuals) were detected visually, 20 species from COI (4306 quality sequences) and 14 species from 12S eDNA (702,004 sequences) (Table 1), with 28 species in total for the molecular analysis. Sequences of the two eDNA markers were obtained for six species, five of them also sampled by netting: *Sardina pilchardus*, *Dicentrarchus labrax (*Near Threatened -NT- and decreasing in Ria Formosa), *Pagrus pagrus*, *Chelon auratus* (NT and decreasing) and *Gobius niger*. By eDNA it was possible to recognise there were two species of sand smelt (*Atherina* sp. from morphology): *A. boyeri* and *A. presbyter* (Table 1).

Sequences of eDNA were not found for all the fish visually identified. The results point out at some effect of the species abundance, as estimated from net sampling, on eDNA detectability, because the proportion of species detected from eDNA increased significantly with species

Table 1

Fish species identified from eDNA (number of sequences of COI, cytochrome oxidase and 12S, 12S rRNA) and visually (number of individuals sampled). Species/genera identified in previous surveys (all surveys since 2001) are also identified (Previous). 1 A: Invasive, endangered and data deficient species; 1B, rest of species. Ecology as the main habitat of adults as in FishBase (2025). Conservation status as from IUCN (2025): Critically endangered CR, Least concern LC, EN endangered, NT Near threatened, DD data deficient. Population trend in Ria Formosa as from IUCN records is indicated when available.

A)

Species/Genus	COI	12S	Visual	Previous	Ecology	Conservation
Aetomylaeus bovinus	5	0	0	0	Benthopelagic	CR
Anguilla anguilla	0	233	0	1	Benthic	CR
Chelon auratus	154	90,037	78	0	Pelagic-neritic	NT / Decreasing
Chelon labrosus	113	0	9	1	Benthopelagic	NT / Decreasing
Chelon saliens	60	0	0	0	Benthopelagic	NT / Decreasing
Cynoscion regalis	0	53	0	1	Benthopelagic	Invasive in Ria Formosa - EN in native NW Atlantic coast
Dicentrarchus labrax	3	12,764	2	1	Benthopelagic	NT / Decreasing
Hippocampus guttulatus	0	0	21	1	Demersal	DD / Decreasing in Ria Formosa
Hippocampus hippocampus	0	0	1	1	Demersal	DD / Decreasing in Ria Formosa

B)

Species/Genus	COI	12S	Visual	Previous	Ecology	Conservation
Arnoglossus thori	0	0	8	1	Demersal	LC
Atherina boyeri	0	61,358	0	0	Pelagic-neritic	LC / Decreasing
Atherina presbyter	963	0	0	0	Pelagic-neritic	LC
Atherina sp.	0	0	241	1	Pelagic-neritic	
Belone belone	0	0	1	1	Pelagic-oceanic	LC
Chelidonichthys lastoviza	0	0	1	1	Demersal	LC
Chelidonichthys lucerna	0	0	1	0	Demersal	LC
Chelon sp.	0	0	1	–		
Coris julis	0	419	65	1	Demersal	LC / Stable
Diplodus vulgaris	12	0	327	1	Benthopelagic	LC
Diplodus annularis	3	0	6	1	Benthopelagic	LC / Stable
Diplodus bellottii	3	0	11	1	Benthopelagic	LC
Diplodus puntazzo	0	29,022	12	1	Benthopelagic	LC
Diplodus sargus	0	0	79	1	Demersal	LC / Stable
Gobius couchi	3	0	0	1	Demersal	LC
Gobius niger	11	2648	34	1	Demersal	LC / Stable
Gobius paganellus	0	0	3	1	Demersal	LC / Stable
Halobatrachus didactylus	0	0	1	1	Demersal	LC
Lipophrys pholis	6	0	0	0	Demersal	LC
Microchirus azevia	0	0	1	1	Demersal	LC / Stable
Monochirus hispidus	0	0	1	1	Demersal	LC
Mugil cephalus	0	160	0	0	Benthopelagic	LC / Decreasing
Mullus surmuletus	0	0	14	1	Demersal	LC
Pagrus pagrus	7	34,095	1	1	Benthopelagic	LC
Pomatoschistus microps	0	6144	1	1	Demersal	LC
Pomatoschistus sp.	0	0	6	–	Demersal	
Sardina pilchardus	2131	464,456	79	1	Pelagic-neritic	LC
Scomber colias	9	55	0	0	Pelagic-neritic	LC
Scorpaena porcus	0	0	18	1	Demersal	LC
Serranus hepatus	0	0	1	1	Demersal	LC
Sparus aurata	182	0	32	1	Demersal	LC / Stable
Spondyliosoma cantharus	595	0	269	1	Benthopelagic	LC
Symphodus cinereus	5	613	20	1	Demersal	LC / Stable
Symphodus bailloni	0	0	36	1	Demersal	LC
Symphodus roissali	0	0	7	1	Demersal	LC / Stable
Symphodus sp.	0	0	1	–		
Syngnathus acus	0	0	2	1	Demersal	LC
Syngnathus typhle	17	0	1	1	Demersal	LC
Trachinus draco	27	0	0	1	Demersal	LC / Stable

abundance (individuals caught from nets) (Table 2; $r = 0.96$, $p = 0.001$). The direct correlation between the sample size of a species (standardized by the sampled area of the sites where the species appeared) and the detection of species' DNA (as 1 presence, 0 absence) was positive and statistically significant ($r = 0.334$, $p = 0.04$).

The ecological traits of fish species may also be important, considering that water samples were collected near the surface. This effect can be seen in Fig. 2: the majority of species not detected from eDNA were demersal (only 37 % of the demersal species were found in eDNA), while eDNA sequences were missing only for one pelagic (the needlefish *Belone belone*, $n = 1$ individual trapped). The difference between the two types of fish ecology was significant ($\chi^2 = 14.96$, 2 d.f., $p = 0.001$, and Fisher's exact test with $p = 0.0002$).

3.2. DNA of species with conservation issues

Regarding conservation status, the majority of species were according to IUCN criteria (Table 1). We found several species with conservation issues (Table 1 A): two classified as critically endangered (CR), the duckbill eagle ray *Aetomylaeus bovinus* (Jabado et al., 2021) and the European eel *Anguilla anguilla* (Pike et al., 2020); four near threatened (NT), the European seabass *Dicentrarchus labrax* and the golden grey mullet *Chelon auratus* mentioned above, plus the leaping mullet *C. saliens* and the thicklip grey mullet *C. labrosus,* all of them declining; and one species that is endangered in its native NE America but is considered invasive in the Ria Formosa (Morais et al., 2017): the weakfish *Cynoscion regalis*.

These seven species were detected by eDNA, but only three were also caught by the beach seine (*D. labrax*, *C. auratus* and *C. labrosus*), making an important 57.1 % of the species with conservation issues (three endangered and one invasive) detected only from eDNA (Table 1). These species not found in nets belong to four different families (Anguillidae, Mugilidae, Myliobatidae, Sciaenidae), two were detected by 12S (*A. anguilla, C. regalis*) and two by COI marker (*A. bovinus, C. saliens*).

Environmental DNA also detected other species with declining population status: *Atherina boyeri* and *Mugil cephalus* (Table 1 A). However, DNA of two Syngnathidae demersal species with national conservation status (Law DL n°38/2021) found in nets that are declining in the Ria Formosa (*Hippocampus guttulatus* and *H. hippocampus*) were not detected by the eDNA. Moreover, molecular analysis did not find two of the most important commercial species of Least Concern (LC) that use the coastal lagoon as a nursery: the white seabream (*Diplodus sargus*) and the red mullet (*Mullus surmuletus*) (Table 1B). This could be due to the demersal way of life of these species as explained above, without excluding a primer bias, relative scarcity of sequences in the databases, or even the spatial heterogeneity of eDNA distribution (Troth et al., 2021). This will be commented with more detail later.

3.3. Fish diversity detected at small scale

Considering each sampling point individually, we found in total (regardless of the sampling method) between 8 species in sampling point #36 and 23 species in sampling point #1 (Table 3). The diversities calculated from the results obtained by the two sampling methods (Table 3) were positively but not significantly correlated ($r = 0.354$, $p = 0.316$, n.s.).

Table 2
Variation of the proportion of species detected from eDNA along increasing number of individuals per species caught in nets. Standardized by the area sampled.

	Minimum number of individuals caught per species						
	1	5	10	15	20	25	30
Number of species	37	23	20	16	15	12	11
% of species detected from eDNA	54.1	60.9	60	68.8	66.7	75	81.8

At the site level, the overlap between net and eDNA results was lower than at the lagoon scale, something that could be explained from the dispersion of eDNA in a mesotidal system (Xiong et al., 2025). The proportion of shared species between two methods ranged between 11.8 % and 37.5 % (Fig. 3).

In sampling points #19, #25, #28, #36, and #E, the proportion of species detected exclusively by eDNA was higher than that detected only by nets. In contrast, the remaining five points showed a higher proportion of species detected only by nets. The proportion of species found only from eDNA and those found only from nets were negatively and significantly correlated ($r = -0.81$, $p = 0.005$). The points where >40 % of the species were missed by eDNA (#B, #33, #D, #1) were located in very different zones regarding the main channels, #B and #1 being on more open waters and #D and #33 in smaller channels, with no apparent relation with the distance to inlets (outer, middle, inner; Fig. 1, Supplementary table 1) nor with physic-chemical factors (Supplementary table 1). The number of individuals was significantly correlated with the area (m^2) sampled by the net ($r = 0.73$, $p = 0.016$), and with the tide in m ($r = 0.71$, $p = 0.022$), but the proportion of netted species found (or missing) from eDNA was not significantly correlated with this factor (data not shown). Considering the tidal phase (Fig. 3), in general the number of species detected was higher during floods than in ebbs or lows, with the exception of site #5 that was located in a middle position and relatively shallow (Supplementary table 1). That site was the smallest in sampling area and exhibited the largest number of species per unit sampling effort (Table 3). Excluding #5, the difference between the points sampled during floods and those sampled in ebbs and lows for the species detected from any method (Fig. 3) was significant (PERMANOVA with F = 4.49, $p = 0.03$). A higher number of species was detected during flood phase than when tides were washing eDNA away. However, during the floods eDNA missed a mean of 13.25 species caught in nets (SE 2.98), while in other phases eDNA missed fewer netted species (mean 8.6 with SE 3.58), although the difference was not significant ($t = 2.12$, $p = 0.07$).

4. Discussion

4.1. Implications for conservation

Here, environmental DNA (eDNA) has been shown to be a useful tool for fish monitoring coupled with netting or trawling, as reported by other authors (e.g., Van Driessche et al., 2024; Kasmi et al., 2025). In this case the results were especially important because it was the only methodology that detected some species of conservation importance, two of them critically endangered (*Aetomylaeus bovinus* and *Anguilla anguilla*) and one invasive (*Cynoscion regalis*).

The occurrence of weakfish *C. regalis* eDNA points at a biodiversity threat in Ria Formosa: a biological invasion, suggested by Morais et al. (2017). This Northeast American native species appeared in Europe in 2009 and colonized rapidly Atlantic European waters (Bañón et al., 2018). Its introduction in multiple locations of Europe has likely happened independently through ballast water, as for other fish (see Morais et al., 2017, and references therein). The species was detected in Ria Formosa by local fishers and the first report of its presence was published in 2016 (Morais and Teodósio, 2016). While its lack of appearance in net surveys in our study suggests its density is still low in the lagoon, at least in the survey points, there is a serious concern about weakfish in Portugal, where its fishing for consumption is being explored to minimize its impact (Cerveira et al., 2022). A close surveillance of its evolution in Ria Formosa and other European lagoons where it may be undetected yet would be recommended.

Opposite to weakfish, the viviparous duckbill eagle ray *A. bovinus* and the catadromous European eel *A. anguilla* are of special concern for their level of threat at a global level. *A. bovinus* had not been found in Ria Formosa in the previous 22 years of fish surveys with nets, although it

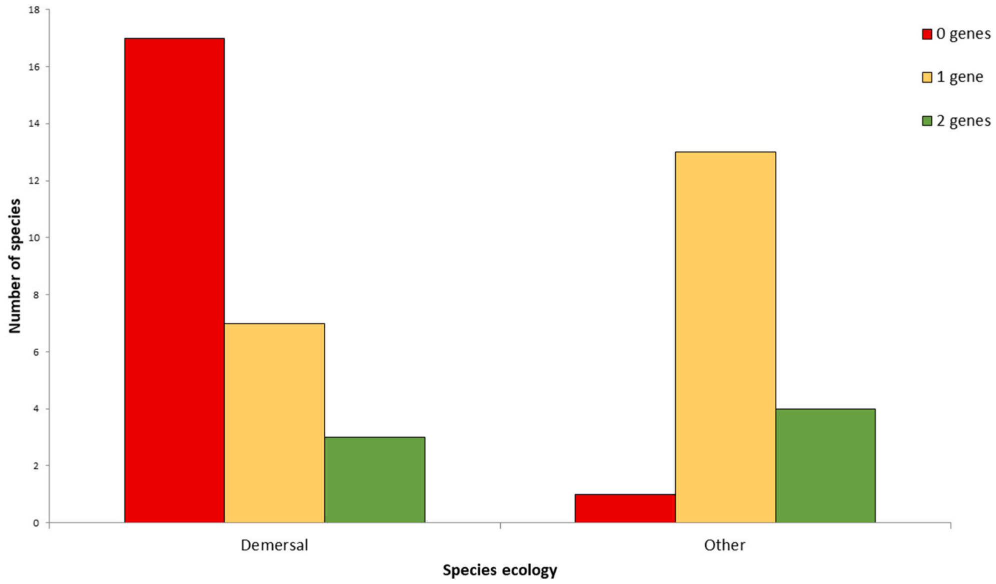

Fig. 2. Number of eDNA markers (genes) amplified from water samples for demersal versus other fish ecology.

Table 3
Fish diversity measured from the two sampling methods employed in the eleven sampling points.

Sampling point	Number of species per unit sampling effort (1000 m^2)	Species detected from nets	Total number of species detected	Individuals sampled	Diversity from nets (Shannon index)	Diversity from eDNA (Shannon index)
#1	9.6	14	23	186	1.5	2.485
#19	7.3	8	15	185	1.38	2.485
#25	7.9	10	19	191	1.66	2.565
#28	4.5	6	12	131	1.003	2.197
#33	17.4	14	19	223	2.18	2.079
#36	6.3	5	8	75	1.13	1.609
#5	21.4	14	21	53	2.25	2.565
#B	12.6	17	21	273	2.07	2.079
#D	15.9	10	17	55	1.73	2.079
#E	20.1	12	21	20	2.29	2.565

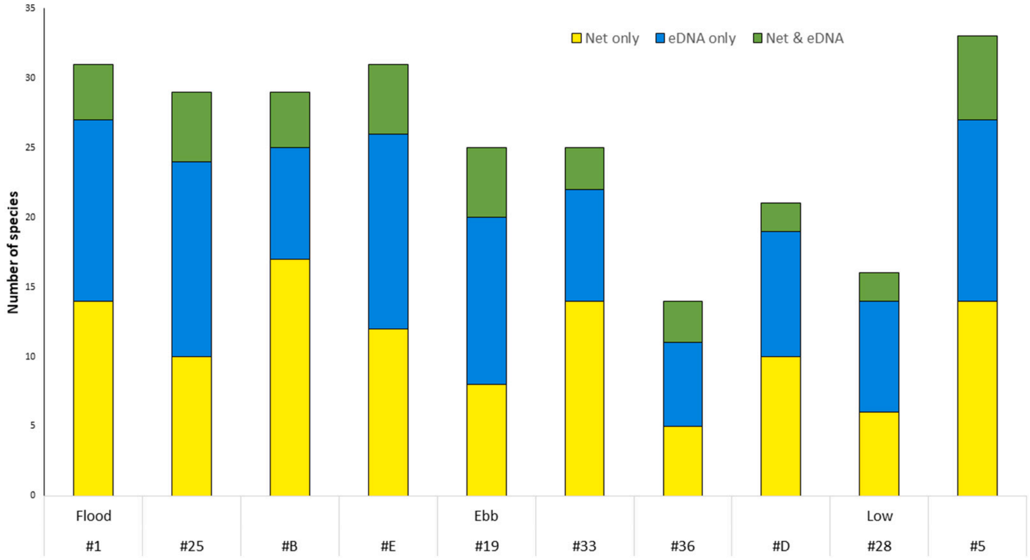

Fig. 3. Number of species detected by each method from each sampling point. The tidal phase is indicated.

can enter estuaries and lagoons from the continental shelf (Last et al., 2016). Some species occur unnoticed for a long time, like scalloped hammerhead sharks (*Sphyrna lewini*) in Micronesia, where eDNA helped to find the species after 25 years of unsuccessful visual monitoring (Budd et al., 2021). No longer commercialized in the Mediterranean Sea where it used to be fished, *A. bovinus* is an important protein source in West

Africa (Walker et al., 2005). The population has been reduced by >80 % over the last three generations (51 years), and because of overfishing and by-catch it was classified as CR in 2021 (Jabado et al., 2021). Its presence in the Ria Formosa confirmed here by eDNA emphasizes the value of this natural park as a shelter for fish.

On the other hand, *A. anguilla* had been found in previous surveys in Ria Formosa, although it failed to be caught in 2024. Its strong global population decline explains the difficulty of detecting it in regular surveys. It has been classified as CR by IUCN since 2008, with the last assessment maintaining the status in 2018 (Pike et al., 2020). This migratory, panmictic species exhibited a reduction of 89 % in catch rate of leptocephali larvae in the Sargasso Sea, the spawning site, in 2011 (Hanel et al., 2014). The decline of maturing silver eels, those that are migrating from continental waters to the sea, ranged between 50 % and 80 % over three generation lengths −39 years (Pike et al., 2020). Multiple factors like drastic habitat loss, contamination and parasites/pathogens account for the species decline (Podda et al., 2021), as well as fishing pressure including illegal fishing (Hutchinson et al., 2024). As in the case of *A. bovinus*, the occurrence of *A. anguilla* in Ria Formosa indicates the need of further efforts in the protection of the whole area as a refuge for endangered fish. This is emphasized by the presence of other four NT species (individuals and/or eDNA).

4.2. Implications for coastal lagoon management

From the technical point of view, differences in species detection between eDNA and net surveys, especially at a small spatial scale, support the inclusion of various methodologies for monitoring fish. Fifteen species and three genera overlapped between netting and molecular approaches, although the proportion of species found simultaneously from the two methods in each sampling site (small scale) ranged between 11.8 % and 37.5 %. The use of a combination of sampling gear, namely beach seine, beam trawl and gill nets would undoubtedly increase the number of species caught and improve the monitoring of the fish assemblage of the Ria Formosa lagoon, but would be prohibitive in terms of effort and cost (Adão et al., 2022).

The advantages of the use of DNA include an increase in taxonomic resolution (Behrens-Chapuis et al., 2021), as was here the case for *Atherina* genus sequences that were assigned to species level while the visual identification only reached genus due to difficult and time-consuming morphological identification. Also, the fact that sampling with nets could have impacts on the seagrass meadows and requires large numbers of fish to be killed for identification and quantification. However, in this moment the eDNA techniques and protocols still need improvements and, although they are recommended as a complement, they cannot replace current physical monitoring now. As applied in this study they have several drawbacks that will be commented next.

First, it is important to highlight the need of using more than one molecular marker in eDNA metabarcoding, because we have found an overlapping of just six species (22.2 %) between COI and 12S markers. Regarding primers choice, it is important to consider the group targeted. In this study we found COI marker, amplified from Wangensteen et al. (2018) primers, to be more efficient than the 12S marker (primers by Miya et al., 2015) because a higher number of species was detected. Without the COI marker we would have missed the CR duckbill eagle ray *A. bovinus* in our study. Differences between primers in taxonomic resolution, i.e. the lack of truly universal primers (global or for a target taxon) together with the differences in databases completeness (Xiong et al., 2022), are drawbacks of eDNA-based techniques for biodiversity inventories and could explain the differences between 12S and COI performance found in this study. Thus, employing several primer pairs in diversity inventories, and an effort in new primers design as long as the number of genome sequences in databases grows, are strongly recommended.

Another drawback of eDNA is the existence of false negatives, such as the commercial species *D. sargus* and *M. surmuletus* that were captured but not detected using eDNA in our study. These two species are demersal, as most of the species missed here by molecular approaches. This shows how the sampling conducted based on surface water collection encompasses an inherent bias against demersal and benthic species; surface water expectedly contains more eDNA from planktonic fish. In future studies it is important to consider collecting water samples from the whole water column, including near the bottom, to be able to capture all the fish diversity (Fernandez et al., 2021).

On the other hand, some differences in the efficiency of eDNA were found between tidal phases. More species were found during floods but also a higher proportion of visually detected fish was missing from eDNA. This could be interpreted from the highly dynamic nature of coastal eDNA (Sevellec et al., 2024); floating DNA would be more diluted during the floods while at the same time the majority of fish would be physically present in situ and trapped in nets. However, it is important to note that sampling did not take place at the same state of flood and ebb. Therefore these results should be considered preliminary as the state of the tide affects the area and volume sampled (i.e. dilution effect). Another consideration is the dispersion of eDNA in a mesotidal system where some DNA detected can be originated beyond the sampling area; this could also explain the differences with fish captures (Xiong et al., 2025).

As a final remark, eDNA approach should be included as a complement in monitoring programs as it enhances conservation efforts by enabling non-invasive, efficient monitoring in habitats that are critical yet challenging to study.

5. Conclusions

The use of eDNA to monitor the fish diversity of the Ria Formosa allowed to detect rare, endangered and invasive species that were not caught by sampling with beach seines in the same locations, or even in all the sampling carried out annually since 2001. While beach seining is an appropriate methodology for monitoring the most abundant species, focusing on the juveniles of commercially important marine migratory species that use the Ria Formosa as a nursery, it is costly and time consuming, with a maximum of 5 or 6 sampling locations possible per low tide period and requiring a boat, a skipper and a team of at least three or four researchers, who also need to process the samples in the laboratory. It also has a lower taxonomic resolution due to difficulties in identifying morphologically very similar species, and is invasive and destructive.

In light of the combined effects of climate change and anthropogenic impacts, it is recommended to use a combination of complementary methods to monitor the evolution of the fish assemblages and diversity of the ichthyofauna of essential fish habitats such as the Ria Formosa, to provide a sound scientific basis for management and conservation. In this context, the use of eDNA could complement nets sampling as it is non-destructive, cost-effective, provides taxonomic resolution, and is highly efficient in detecting rare species like non-native invaders in early invasion stages. It could be employed for surveillance of the spread of *C. regalis* over Ria Formosa and other European lagoons.

Better results can be obtained by improving the sampling design, including more sampling locations within the Ria Formosa and sampling water close to the bottom in order to increase the detection of demersal species. Better taxonomic resolution can also be obtained by using more molecular markers in eDNA metabarcoding.

CRediT authorship contribution statement

Sara Fernandez: Writing – original draft, Validation, Software, Methodology, Investigation. **Pedro Monteiro:** Writing – review & editing, Validation. **Jorge M.S. Gonçalves:** Writing – review & editing, Validation. **Eva Garcia-Vazquez:** Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation. **Karim Erzini:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.118951.

Data availability

All the raw data are available. Sequences are in https://www.ncbi.nlm.nih.gov/gene under BioProject accession number PRJNA1271295, and sampling locations and data are provided in the Supplementary materials and article tables.

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