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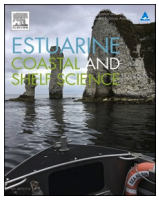

Environmental DNA enhances comprehension of the spatial and temporal dynamics of fish diversity in a coastal lagoon

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ABSTRACT

Transitional environments have great ecological value and high productivity, and many species can benefit from their sheltered conditions and food resources. In this study, we performed for the first time a fish-targeted eDNA metabarcoding of the 12S rRNA gene at 16 sites broadly covering the different water bodies of the Marano and Grado Lagoon (northern Adriatic Sea) in two seasons. The eDNA was collected at the same time as the beach seine net, allowing a direct comparison of the two approaches.

With eDNA we detected 34 species, covering all the functional guilds occurring in the lagoon. Species of regional interest, that uses the area as a nursery and feeding ground, and diadromous species, highlighting the ecological connectivity between freshwater and marine habitats, were found. While some species were constantly present (e.g. *Atherina boyeri, Sparus aurata*), others (*Squalius cephalus, Platichthys flesus*) were influenced by salinity (higher in Grado and lower in Marano), which was confirmed as the main ecological driver in this environment. The comparison with traditional methods, which identified 18 species (11 of which were detected with both approaches), showed that eDNA is very sensitive in detecting most of the biodiversity in the lagoon with a limited sampling effort. Few relevant species (*Chelon saliens*, *Knipowitschia panizzae*) lacked reference sequences, which need to be implemented in the databases. Our study represents a significant advance in the understanding of lagoon fish biodiversity and ecological dynamics and contributes to the improvement of management strategies in these ecologically sensitive habitats.

1. Introduction

Environmental DNA (eDNA), the genetic material found in an environment (e.g. water, soil) without its biological source being obviously present (Thomsen and Willerslev, 2015), can come from a variety of sources, including epidermis, exoskeleton, mucus, faeces, excretions, and gametes (Bohmann et al., 2014) and can persist and eventually accumulate in the environment (Bairoliya et al., 2022). The eDNA metabarcoding approach, which combines eDNA detection and high-throughput sequencing (HTS), is one of the fastest growing, most efficient, and robust methods for the non-invasive study of biological populations (Yao et al., 2022). Compared to traditional techniques involving captures, visual or acoustic surveys, eDNA metabarcoding has been shown to be comparable and often superior in terms of representativeness of biological communities (Mirimin et al., 2021; Cole et al., 2022). In this context, fish represent the most common target of eDNA studies in both freshwater and marine environments, and the number of related publications has steadily increased since the first study in 2011 (Dejean et al., 2011; Tsuji et al., 2019; Yao et al., 2022; Gibson et al., 2023). Recently, Yao et al. (2022) provided an overview of eDNA-based studies on fish, highlighting how this approach can greatly improve the monitoring, conservation, and management of these organisms. Fish eDNA-based surveys can address a wide range of research questions ranging from monitoring species distribution, population dynamics, reproduction to biodiversity assessment, prey-predator interactions, and diet estimation. When comparing the performance of eDNA to conventional fish survey methods, metabarcoding allows for higher spatial and temporal resolution without disturbing the target organisms and their habitats (Ruppert et al., 2019; Zou et al., 2020), making eDNA a cost-effective tool for monitoring fish communities (Carvalho et al.,

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2024). The use of eDNA has been shown to be effective in identifying spawning migrations (Yatsuyanagi and Araki, 2020) and range expansions (Nardi et al., 2019) with particular attention paid to species of conservation concern (Simpfendorfer et al., 2016). In addition, it enables the tracking of cryptic, rare, and endangered species, including one of the world's most threatened marine fish taxa (Bonfil et al., 2024). Nonetheless, eDNA testing has proven its worth in the early detection of non-native and invasive species, helping to assess the overall health of an ecosystem (Ota et al., 2020). It is worth mentioning that while some species are detected by both eDNA and conventional methods, not all species are reliably detected by eDNA (Zou et al., 2020; Hallam et al., 2021; Cole et al., 2022) suggesting that eDNA provides extremely useful information when combined with other monitoring methods.

The potential of eDNA is particularly relevant in transitional environments, due to their high ecological value (Hering et al., 2018; Nagarajan et al., 2022). Coastal lagoons, as well as estuarine environments, are highly productive ecosystems intermittently subjected to variation in salinity and other parameters (Ahn et al., 2020). If trophic transfer is effective, the high productivity of these environments can be of great benefit to biodiversity conservation (de Wit, 2011). Many animal species frequently experience stress and limitation as a result of the varying salinity levels. On the other hand, the high productivity and generally calm conditions in the lagoons are advantageous. For these reasons, many fish and invertebrate species have developed a life cycle that includes spawning in the open sea, where salinity is more constant, and the migration of juveniles to the lagoons, where they can develop and benefit from the diversity of habitats and food resources. When they reach adulthood, they return to the sea. As a result, lagoons serve as nurseries for a variety of fish and invertebrate species, and coastal fisheries undoubtedly benefit from this nursery function. Apart from their importance, lagoons have been less studied with eDNA compared to other aquatic environments (Cananzi et al., 2022), and further efforts are needed to exploit the potential of this methodology in these transitional ecosystems as well. When applying this molecular approach, it is important to consider that the detection of eDNA of a fish does not necessarily imply its presence in that environment (West et al., 2020); in lagoons, as in other transitional waters, such genetic material could be transferred from freshwater or marine species via rivers or via flood tides (Yamamoto et al., 2017).

The Marano and Grado Lagoon (MGL; northern Adriatic Sea) is a shallow transitional system of migrations (Cananzi et al., 2022) which is considered one of the best-preserved wetlands in the whole Mediterranean (Bettoso et al., 2010, 2013). The lagoon has been designated as a Natura 2000 site, i.e. the European Union network of sites prioritized for their naturalistic value and the protection of biodiversity itself. According to the Habitats Directive 92/43/EEC, this lagoon is a Special Area of Conservation (SAC - IT3320037) for the protection of habitats and important species of flora and fauna at European level, and according to the Birds Directive 2009/147/EC it is a Special Protection Area (SPA - IT3320037) for the protection of wild bird species and their habitats. It also includes two Regional nature reserves established by the Regional Law No. 42/96: the "Valle Canal Novo" (121 ha) and the "Foci dello Stella" (1377 ha). Due to the close interaction between natural processes and human activities, this lagoon is an example of a conflict between the needs of nature conservation and human use, as this basin also plays an important role for fishing and fish and shellfish farming (Bettoso et al., 2013).

The first comprehensive characterization of the fish community in the Marano and Grado Lagoon began in 2010, when annual monitoring was carried out using fyke nets as sampling method, as this is the traditional fishing method employed in the lagoons of the northern Adriatic (Bettoso et al., 2013). Since 2018, sampling with the beach seine net has replaced sampling with fyke nets and is now the official method adopted in Italian transitional waters to estimate the ecological quality status of fish fauna according to the Water Framework Directive (WFD/2000/60/EC) (Catalano et al., 2017). As this sampling is carried out every 3 years in the MGL, we used the 2021 monitoring to test the eDNA method to detect fish species for the first time in this lagoon. The objectives of the study were: *i*) to test the effectiveness of eDNA metabarcoding in describing seasonal fish diversity in the Marano and Grado Lagoon, *ii*) to evaluate the performance of the eDNA approach compared to the fishing with seine net in terms of resolution and sensitivity, highlighting both strengths and potential biases, and *iii*) to produce a DNA-based list of fish occurrences.

2. Materials & methods

2.1. Study area

The Marano and Grado Lagoon (MGL, Fig. 1) is one of the most important coastal wetlands in Italy and it is located between the low coastal plain of Friuli Venezia Giulia and the northern Adriatic Sea. It stretches from the Tagliamento River in the West to the Isonzo River in the East and covers an area of about 160 km^2, 32 km long and 5 km wide. A series of barrier islands separated by six tidal inlets surround the lagoon (Fontolan et al., 2012). The MGL is divided into two halves by a historical-administrative designation: the Marano Lagoon to the West and the Grado Lagoon to the East, split by the old border between Italy and the Austro-Hungarian Empire, which runs along the line connecting the mouth of the Aussa-Corno River to the Buso inlet. The Marano Lagoon is the deepest lagoon basin, with many marshes and channels that receive the water of many tributaries, including Stella, Turgnano, and Cormor. The Grado Lagoon is shallower (average depth <1 m), has a series of relict morphological reliefs (islands) and marshes and is only slightly characterised by estuaries due to the relatively low freshwater contribution of the Natissa River. As a result, the MGL exhibits a clear West-East salinity gradient (Ferrarin et al., 2010, Fig. 1B and C), with average salinity values lower (~20) in the western part of the lagoon (referred to as the "Marano Lagoon") and higher (~34) in the eastern part (referred to as the "Grado Lagoon").

According to the Water Framework Directive (WFD), the MGL is divided into 16 water bodies (Fig. 1), based on surface salinity and other characteristics such as nutrient and organic matter enrichment, presence of priority substances, aquaculture activities, geomorphology, and tides. Water bodies with a salinity of 30–40 are classified as euhaline (TEU), 20 to 30 as polyhaline (TPO) and 5 to 20 as mesohaline (TME) (Bettoso et al., 2010). Some water bodies are classified as heavily modified (FM) (Fig. 1), due to the presence of fish farms or the bridge between Grado and Aquileia, which severely constrain the hydrological regime (Bettoso et al., 2020).

2.2. eDNA sampling

Environmental DNA (eDNA) surveys were carried out at the 16 water bodies (Fig. 1) in Spring and Autumn 2021 (Table 1): at each station, 5 L precleaned tanks were used to collect surface (~25 cm) water immediately before the seine net sampling, for a total of 32 water samples (16 for each season). Once in the laboratory, the water samples were prefiltered through 50 μm mesh, before passing through 1.2 μm PES membrane filters (PALL Laboratory) until clogging (1–1.6 L per filter) in duplicates, for a total of 64 filters (two for each station). Before filtration, all filtration equipment and surfaces were cleaned with 10% bleach. After each cleaning, pure water (MilliRo) was allowed to circulate in the system and then 1 L was filtered as a "filtration blank". All filters were stored at −80 °C until further processing.

2.3. eDNA extraction and sequencing

A clean environment with regular decontamination was maintained in the laboratory, in a facility dedicated exclusively to molecular procedures, with separate rooms for DNA extraction and PCR preparation (performed under a laminar flow hood prior to bleach and UV

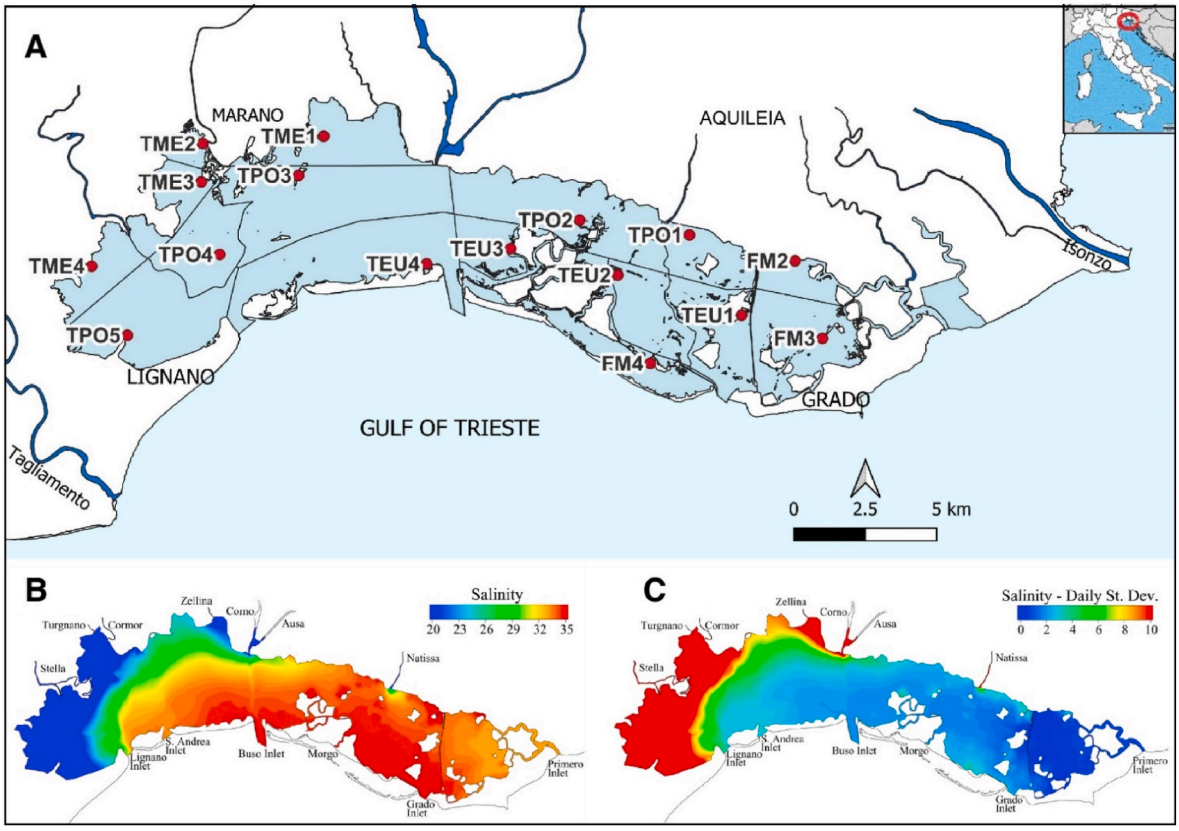

Fig. 1. Map of the Marano and Grado Lagoon (Italy). Fishing and sampling sites (red dots) at each water body (A); average annual salinity distribution (B) and average daily standard deviation of salinity (C) as computed by the numerical model of Ferrarin et al., (2010) (Figures B and C modified after Ferrarin et al., 2010). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Stations, coordinates, and sampling dates (2021) in the Marano and Grado Lagoon.

Station	Latitude	Longitude	Spring	Autumn	Water body	Lagoon
FM2	45.724717	13.402167	14-May	24-Sep	TPO	Grado
FM3	45.700200	13.415117	14-May	24-Sep	TEU	Grado
FM4	45.691133	13.338017	10-Jun	26-Oct	TEU	Grado
TEU1	45.707050	13.378600	10-Jun	28-Sep	TEU	Grado
TEU2	45.719017	13.322600	28-May	29-Sep	TEU	Grado
TEU3	45.726750	13.274133	10-Jun	28-Sep	TEU	Grado
TEU4	45.721333	13.236567	11-Jun	28-Sep	TEU	Grado
TME1	45.761217	13.189000	11-Jun	28-Sep	TME	Marano
TME2	45.757983	13.134550	03-Jun	27-Sep	TME	Marano
TME3	45.745750	13.134333	28-Apr	27-Sep	TME	Marano
TME4	45.718150	13.085950	28-Apr	29-Oct	TME	Marano
TPO1	45.732300	13.354433	28-May	01-Oct	TPO	Grado
TPO2	45.736283	13.304933	28-May	28-Sep	TPO	Grado
TPO3	45.748500	13.178000	11-Jun	26-Oct	TPO	Marano
TPO4	45.722850	13.143400	03-Jun	27-Sep	TPO	Marano
TPO5	45.696400	13.102750	28-Apr	29-Oct	TPO	Marano

decontamination) and PCR post-processing, using only filtered tips. DNA was extracted from membrane filters using the DNeasy PowerWater Kit (Qiagen) according to the manufacturer's instructions. For each sample, two filters were extracted independently, and the eluted DNAs were pooled. To assess possible contamination at each step of the workflow, DNA extraction was performed on different blank samples ("filtration blank", an untreated membrane filter, and an "extraction blank" without any filter). The amount of extracted DNA was determined using the Qubit fluorimeter (Thermo Fisher Scientific), with all blanks below the detection limit (<0.005 ng/μL). For fish eDNA metabarcoding, the mitochondrial 12S rRNA gene was amplified using the primers teleo_f/L1848 and teleo_r/H1913 (Valentini et al., 2016) in combination with the primer teleo_blk to prevent amplification of human DNA (Valentini et al., 2016). PCR amplifications were performed in duplicates for each sample, in a total volume of 50 μl with 1 U Hiproof HF Master Mix (Bio-Rad), 0.5 μM F and R primers, 10 μM blocking primer, and 5 μl DNA. The thermal cycling profile started with 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 7 min. All blanks were amplified at the same conditions of the samples, and no template controls (NTCs) were added for each reaction. Samples, blanks and NTCs were run on an electrophoresis gel (1.8 %) to verify presence of absence of amplification. The absence of any amplicons in the negative controls were considered index of absence of contamination, and blanks were not further processed. PCR amplicons from the samples were purified with 1:2 diluted Thermolable Exonuclease I (New England Biolabs) and amplified following the Nextera XT Index protocol (Illumina), with indexes incorporated by PCR. The amplicons were then normalized by the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific) and multiplexed. The pool was purified with 1 × Magnetic Beads Agencourt XP (Beckman Coulter), loaded on an Illumina MiSeq System and sequenced following the V2 – 150PE strategy with approximately 20% PhiX at BMR Genomics S.r.l., Padua, Italy (www.bmr-genomics.it).

2.4. Bioinformatic analyses

Bioinformatic analyses were performed with QIIME2 (v. 2023.5; Bolyen et al., 2019). Given the short length of the amplified region and the possible readthrough, primers were removed with *Cutadapt* (Martin, 2011) and sequences were then denoised with DADA2 (Callahan et al., 2016).

For the taxonomic classification of the fish, the list of Mediterranean species was retrieved from Fishbase (https://www.fishbase.se/tropheiceco/FishEcoList.php?ve_code=13; March 1, 2023). The 758 species, (plus *Knipowitschia panizzae* and *Pomatoschistus canestrinii,* found with seine net but not included in Fishbase) were used as Entrez query from NCBI on March 1, 2023. The query was "12S[All Fields] OR mitochondrion[All Fields] OR mithocondria[All Fields]) AND (("*Species*"[Organism] OR *Species*[All Fields]) NOT ("predicted" [All Fields]) NOT ("unverified"[All Fields])) AND ("80"[SLEN]: "25,000"[SLEN])". The query returned 6,803 sequences, that, after a 100% similarity dereplication using cd-hit-est v. 4.8.1 (Fu et al., 2012), were reduced to 4, 494, corresponding to 720 species. RESCRIPt (v. 2021.11.0; Robeson et al., 2021) was used to construct a QIIME2-formatted database, starting from the dereplicated accession list retrieved from the NCBI query. Taxonomic assignment of the amplicon sequence variants (ASVs) was performed using *classify-consensus-blast* in QIIME2 (v. 2023.5; Bolyen et al., 2019) with decreasing identity percentages (1, 0.99, and 0.97), and the assignment was manually curated. As an additional identity check, ASVs were also aligned against the NCBI nucleotide collection using BLASTN 2.12.0+ (Altschul et al., 1997) and against Complete + Partial mtDNA MiFish Database (Zhu et al., 2023). The ASVs which could not be assigned at the species level were considered "unassigned" and removed from the dataset. To each of the identified species, the IUCN status (following Rondinini et al., 2022), functional guild (following Franco et al., 2008) and other relevant features, such as trophic level and habitat (https://www.fishbase.se/), were assigned.

2.5. Fish sampling and environmental parameters

Fish was sampled using a beach seine net (10 m length x 2 m height; mesh size 2 mm)(Fig. S1). At each sampling station, two net tows were carried out in parallel to the shore or the tidal flat, for a total sampling area of 280 m^2 and on all available habitats (vegetated, unvegetated) (Franco et al., 2012; Cavraro et al., 2017). The sampling depth ranged from 40 to 80 cm. The fish samples were stored at −20 °C until the identification at species level in the laboratory. Water temperature and salinity were measured at the surface (first 50 cm) at each station using a Hydrolab MS5 probe, and significant differences among samples were calculated by Kruskal-Wallis H test. Presence of vegetation was also assessed by direct observation during sampling.

2.6. Data analysis

For the eDNA metabarcoding data, multivariate analyses were performed using R environment v. 4.0.3 (R Core Team, 2021) and Plymouth Routines in Multivariate Ecological Research (PRIMER) software v. 7.0 (Clarke et al., 2014). We checked the role of read depth in fish species detection using the *manyglm* function in the *mvabund* R package (Wang et al., 2012) and verifying the AIC values with or without this factor (Gibson et al., 2023). Then, to assess the differences in fish community structure, an agglomerative hierarchical cluster analysis (group average linkage) based on the Bray-Curtis similarity matrix of the presence/absence data was performed. A non-metric multidimensional scaling (nMDS), based on the same matrix, was used to better visualize the distribution patterns of the studied faunal groups. Statistically significant variation in taxa composition was calculated by the Similarity Profile (SIMPROF) tests (Clarke et al., 2014). A similarity percentage (SIMPER) was performed to test which species in the fish community were responsible for statistical differences between samples. The relationships between the environmental variables and the fish fauna were investigated using a Distance-based Linear Model (DISTLIM) and Distance-based Redundancy Analysis (dbRDA).

Species detected with eDNA and seine nets were assessed and compared in terms of occurrence, functional groups and persistence. Estuarine Use Functional Group (EUFG) categories were assigned to each species according to Franco et al. (2008): Estuarine Species (ES), Marine Migrants (MM), Marine Stragglers (MS), Diadromous (D), represented by Catadromous and Anadromous Species, and Freshwater (F). The persistence of fish species, i.e. the frequency with which the taxon was found in the total samples, was assessed using a constancy index (C) (Félix et al., 2013) as follows: $Cij=(nij/nj)x100$ where *nij* is the number of occurrences of taxon *i* in group *j* and *nj* is the number of samples in group *j.* Thus, each species was considered as Permanent (C = 100%), Constant (100% > C ≥ 50%), Frequent (50% > C ≥ 25%), Temporary (C < 25%) or Absent (C = 0%).

3. Results

3.1. Environmental parameters

Salinity ranged from 0.32 to 31.32, and 10.62 and 33.62 in Spring and Autumn respectively. Lower salinities were found in Marano Lagoon ($p < 0.05$): the average salinity in the Marano Lagoon was 16.8 ± 7.3, the one of the Grado Lagoon was 28.7 ± 5.6. Temperature, on the other hand, did not differ significantly between Spring (20.7 ± 4.2 °C) and Autumn (21.6 ± 4.8 °C). Vegetation was observed at all sites in the Grado Lagoon and at the TPO sites in the Marano Lagoon.

3.2. eDNA fish detection

A total of 2,628,001 raw sequences were generated for the 32 samples. After the trimming/denoising procedure, 2,073,439 reads were retained with an average of 64,795 ± 16,402 per sample. Overall, the total number of ASVs was 1,399, with an average of 98 ± 50 per sample. The AVSs belonging to fishes of the classes of Actinopterygii and Chondrichthyes were 98 (20 ± 5), representing the 72% (1,494,680 reads) of the original dataset (Fig. S2). The total number of fish species detected was 34 (Table 2).

The *manyglm* test considering the numerical variables temperature, salinity and read depth indicated a not significant role of the latter factor in the fish species detection and assemblages composition, with AIC decreasing from 1,580 to 1,522 after its removal.

Hierarchical clustering (Fig. 2) highlighted two sample clusters, grouped mostly according to the distribution of sampling sites in the Marano (lower salinities) and Grado (higher salinities) lagoons, with the exception of the sample collected at station TEU4 in Autumn, which appeared isolated from both groups (Fig. 2). Representation on a non-metric multidimensional scaling (nMDS), showing the significant groups highlighted by SIMPROF, is presented in Fig. S3.

The SIMPER analysis carried out on the Marano and Grado lagoons, showed that the dissimilarity between fish faunas was mainly related to the higher occurrence of the species *S. cephalus, P. flesus, S. trutta, S. solea, M. cephalus* in the Marano Lagoon (Table S1). The presence of *P. flesus, S. cephalus* and *M. cephalus* in unvegetated sites contributed to the differentiation between vegetated and unvegetated stations (Table S2) Only a few species (mainly *S. pilchardus* and *S. solea*) contributed to the distinction between the seasons, as they were more frequently detected in Spring (Table S3).

Salinity, temperature, and the presence of vegetation were significantly related to the distribution of fish presence in the DISTLIM marginal tests (Table 3), while salinity emerged as the only significant variable in sequential tests (Table 3). The role of salinity in the composition of the fish community is also demonstrated by the dbRDA analysis (Fig. 3).

Of the total of 34 species, 4 were classified as permanent, 9 as

Table 2
List of fish species detected by eDNA metabarcoding in the Marano and Grado Lagoon reported together with relevant taxonomic, ecological, and functional features (https://www.fishbase.se/). Med = Mediterranean. For IUCN status (Rondinini et al., 2022): CR = critically endangered, DD = data deficient, EN = endangered, LC = least concern, NA = not available, NT = near threatened, VU = vulnerable. For the EUFG (Estuarine Use Functional Group; Franco et al., 2008): D = diadromous, ES = Estuarine species, F = freshwater, MM = marine migrants, MS = marine stragglers.

Family	Species	Common name	Med Status	EUFG	IUCN	Habitat	Trophic level
Anguillidae	*Anguilla anguilla*	European eel	Native	D	CR	Demersal	3.5
Aphaniidae	*Aphanius fasciatus*	Mediterranean banded killifish	Native	ES	LC	Benthopelagic	2.7
Atherinidae	*Atherina boyeri*	Big-scale sand smelt	Native	ES	LC	Demersal	3.2
Bothidae	*Arnoglossus laterna*	Mediterranean scaldfish	Native	MS	LC	Demersal	3.6
Carangidae	*Trachurus* sp	Atlantic horse mackerel	Native	MS	LC	Pelagic-Neritic	3.7
Clupeidae	*Alosa fallax*	Twaite shad	Native	D	VU	Pelagic-Neritic	4
	Sardina pilchardus	European pilchard	Native	MM	LC	Pelagic-Neritic	3.1
	Sprattus sprattus	European sprat	Native	MM	LC	Pelagic-Neritic	3
Cyprinidae	*Squalius cephalus*	European chub	Native	F	NA	Benthopelagic	3.3
Engraulidae	*Engraulis encrasicolus*	European anchovy	Native	MM	LC	Pelagic-Neritic	3.1
Gobiidae	*Gobius niger*	Black goby	Native	ES	LC	Demersal	3.3
	Pomatoschistus marmoratus	Marbled goby	Native	ES	LC	Demersal	3.3
	Pomatoschistus minutus	Sand goby	Native	MM	DD	Demersal	3.4
	Zosterisessor ophiocephalus	Grass goby	Native	ES	LC	Demersal	3.2
Labridae	*Symphodus cinereus*	Grey wrasse	Native	MS	LC	Demersal	3.5
Moronidae	*Dicentrarchus labrax*	European seabass	Native	MM	LC	Demersal	3.5
Mugilidae	*Chelon auratus*	Golden grey mullet	Native	MM	LC	Pelagic-Neritic	2.8
	Chelon labrosus	Thicklip grey mullet	Native	MM	LC	Demersal	2.6
	Chelon ramada	Thinlip grey mullet	Native	D	LC	Pelagic-Neritic	2.3
	Mugil cephalus	Flathead grey mullet	Native	D	LC	Benthopelagic	2.5
Pleuronectidae	*Platichthys flesus*	European flounder	Native	MM	LC	Demersal	3.3
Pomatomidae	*Pomatomus saltatrix*	Bluefish	Native	MS	LC	Pelagic-Oceanic	4.5
Salmonidae	*Oncorhynchus mykiss*	Rainbow trout	Non native	F	NA	Benthopelagic	4.1
	Salmo trutta	Sea trout	Native	D	NA	Pelagic-Neritic	3.4
Scombridae	*Scomber scombrus*	Atlantic mackerel	Native	MS	VU	Pelagic-Neritic	3.6
Soleidae	*Solea solea*	Common sole	Native	MM	LC	Demersal	3.2
Sparidae	*Diplodus puntazzo*	Sharpsnout seabream	Native	MS	NA	Benthopelagic	3.1
	Sparus aurata	Gilthead seabream	Native	MM	LC	Demersal	3.7
Squalidae	*Squalus acanthias*	Picked dogfish	Native	MS	CR	Benthopelagic	4.4
Syngnathidae	*Hippocampus hippocampus*	Short snouted seahorse	Native	ES	NT	Demersal	3.2
	Syngnathus taenionotus	Darkflank pipefish	Endemic	ES	DD	Demersal	3.4
	Syngnathus typhle	Broadnosed pipefish	Native	ES	DD	Demersal	4.3
Torpedinidae	*Torpedo marmorata*	Marbled electric ray	Native	MS	LC	Reef-Associated	4.6
Triakidae	*Mustelus mustelus*	Smooth-hound	Native	MS	EN	Demersal	4.3

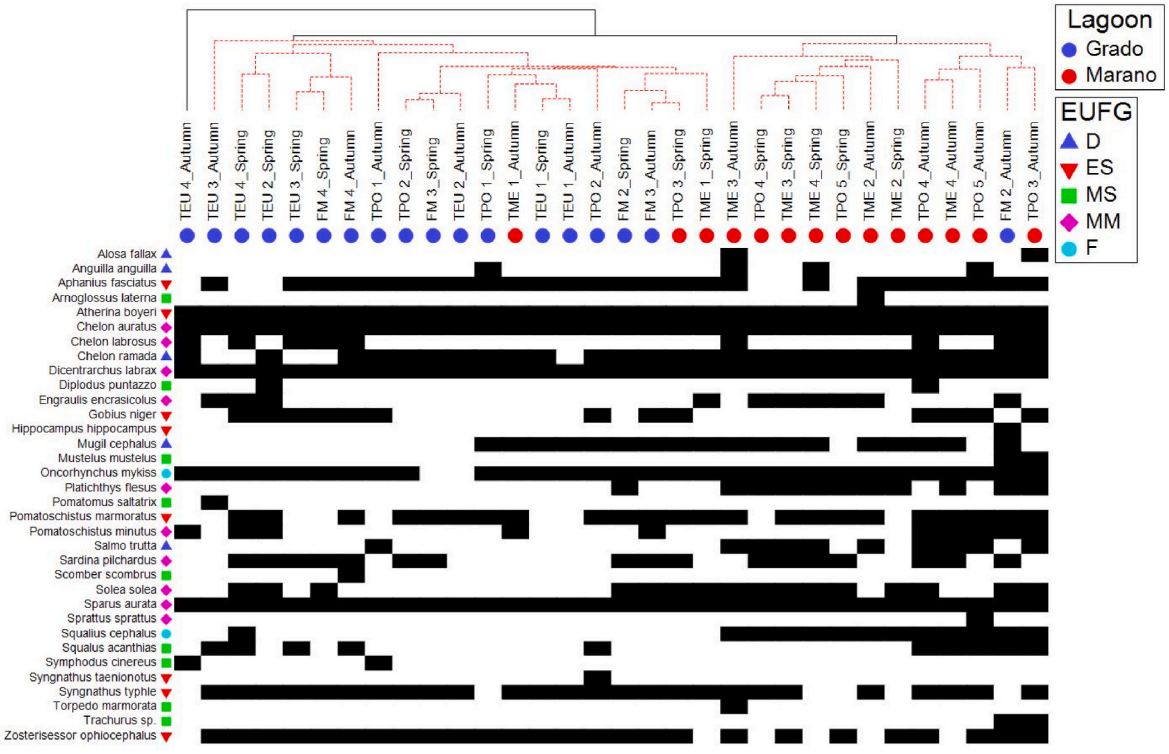

Fig. 2. Shade plot based on presence/absence of fish species detected with eDNA metabarcoding. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
DISTLIM results on the relationship between environmental variables and the presence of fish by eDNA in the Marano and Grado Lagoon.

Environmental parameter	Pseudo-F	p	% Explained variance
Marginal tests			
Salinity	62,085	0.0003	17.147
Temperature	27,463	0.0245	8.3865
Vegetation	47,416	0.0012	13.648
Sequential tests			
Salinity	62,085	0.0002	17.147
Temperature	0.952	0.4771	2.6346
Vegetation	1661	0.1596	4.4922
Total variance explained			24.27

constant, 8 as frequent and 14 as temporary (Table S4). According to the EUFG, 10 species were marine migrants (MM), 9 marine stragglers (MS), 8 estuarine (ES), 5 diadromous (D) and 2 freshwater (F) species (Table 2). The permanent species were *Atherina boyeri* (ES), *Chelon auratus* (MM), *Dicentrarchus labrax* (MM) and *Sparus aurata* (MM). Although we relied on presence/absence data in our study, it is noteworthy that these permanent taxa accounted for almost half (1,007,076) of the reads in the entire dataset, with an average of 31,440 ± 18,323 and 31.2 ± 12.5 % respectively.

The ES species *A. boyeri*, *Aphanius fasciatus*, *Syngnathus typhle*, *Pomatoschistus marmoratus* and *Zosterisessor ophiocephalus* were constantly found in the MGL, in all water bodies and in both seasons; *Z. ophiocephalus* in particular was detected in all samples from the Grado Lagoon and prevailed in vegetated sampling stations as did *S. typhle*. *Gobius niger* was frequent in MGL, but constant in TEU, TPO on both seasons and in vegetated areas, whereas was temporary in TME. *Hippocampus hippocampus* and *Syngnathus taenionotus* were temporary species in vegetated habitats.

The MM *Sardina pilchardus* and *Solea solea* were constant in the MGL, while *S. pilchardus* prevailed in TEU and TPO during Spring, while *S. solea* was constantly detected always in Spring, but in TPO and TME of the Marano Lagoon. Also worth mentioning are the *Platichthys flesus* and *Engraulis encrasicolus*, both of which were frequently detected in MGL but constantly only in TME.

Most MS species were temporary in MGL and prevalent in Autumn, while *Squalus acanthias* was frequent in TEU and TPO. Among the diadromous species (D), *Chelon ramada* and *Mugil cephalus* were constantly detected, especially *C. ramada* proved to be permanent in TPO, TME and in Spring, while *M. cephalus* was permanent in TME. *Anguilla anguilla* and *Alosa fallax* were recorded as temporary species. Finally, the freshwater species (F) were constantly represented by *Oncorhynchus mykiss* throughout the MGL, but were permanent in the Marano Lagoon, TPO, TME and during Spring; *Squalius cephalus* and *Salmo trutta* were both constant in the TME water types of Marano.

3.3. Fish survey

Fish fauna monitoring using the seine net method detected 18 species (Table S5): 10 species were ES, 7 MM (all temporary) and 1 D; MS and F species were not caught. Of the ES species, *A. boyeri*, *Knipowitschia panizzae* and *A. fasciatus* were constantly recorded, *Syngnathus abaster* and *P. marmoratus* frequently and other species only temporarily. Finally, *C. ramada* was the only D species caught with the seine net and was frequent only in TPO, TME and in Spring.

3.4. Comparison between eDNA metabarcoding and seine net

The two methods eDNA metabarcoding and seine net detected 34 and 18 fish species respectively. Of these, 11 (Fig. 4A) were common to both methods, while 23 were detected only with the former and 7 only with the latter (Fig. 4A and B).

4. Discussion

This study is the first fish-targeted eDNA survey in the Marano and Grado Lagoon and presents valuable insights into the composition and ecological dynamics of the fish fauna in this transitional environment. By employing eDNA metabarcoding alongside traditional methodologies such as beach seine net sampling, we achieved a comprehensive comparison between molecular and conventional approaches, shedding light on the strengths and limitations of each method. The findings of the

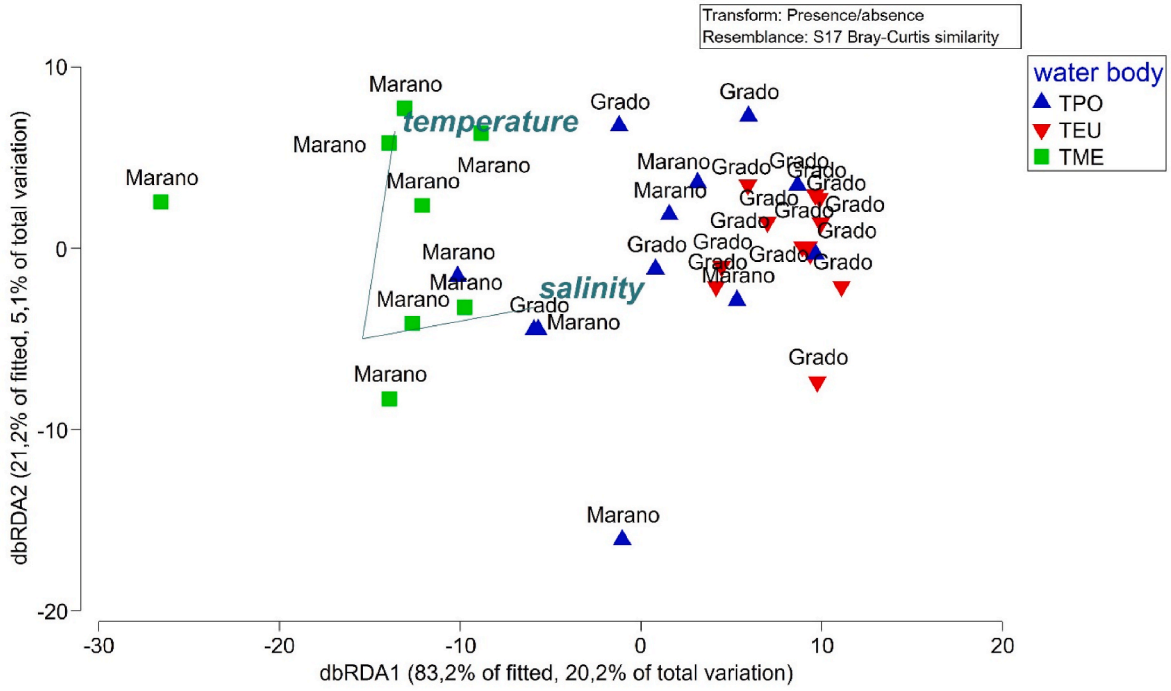

Fig. 3. DbRDA plot of fish fauna detected by eDNA in the Marano and Grado Lagoon (Italy).

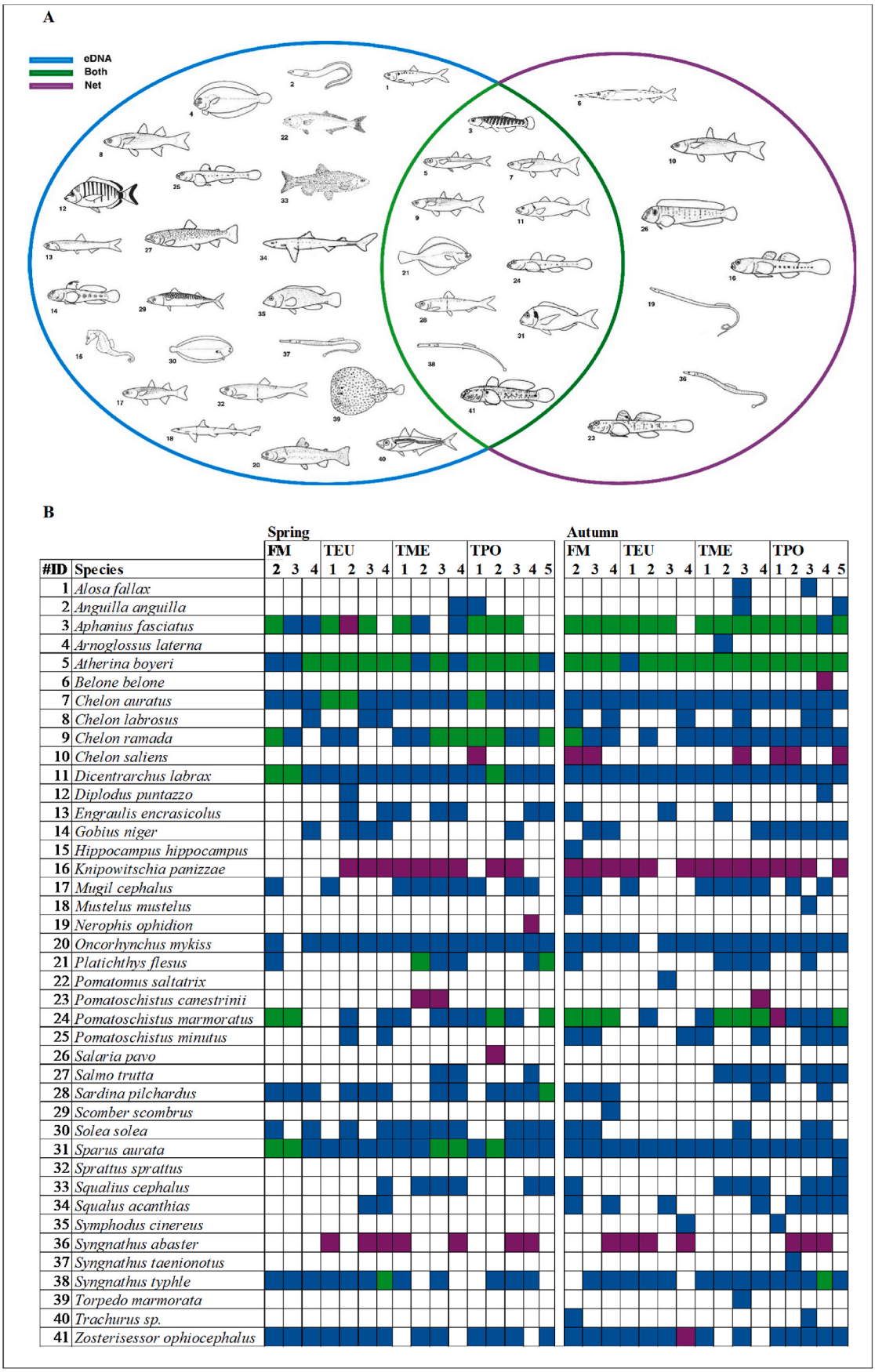

Fig. 4. A. Fish species detected with eDNA metabarcoding and/or seine net at each sampling station in the Marano and Grado Lagoon. B. Venn diagram showing the species detected with eDNA metabarcoding and/or seine net in the Marano and Grado Lagoon.

current study using eDNA revealed a diverse fish community, with 34 species occurring according to seasonal distribution and habitat preference (Bettoso et al., 2013). The ability to discern species distributions, based on EUFG and habitat preference, underscores the utility of eDNA metabarcoding in elucidating complex ecological relationships within transitional ecosystems (Blabolil et al., 2021; Cananzi et al., 2022; Zainal Abidin et al., 2022). Moving forward, the versatility of the use of eDNA to integrate other methods for monitoring aquatic species is welcome in wider applications (Mirimin et al., 2021; Aguzzi et al., 2022; Tibone et al., 2022) as well as for the screening of biodiversity in remote environments (Aguzzi et al., 2024; Stefanni et al., 2022).

Overall, the species richness of the fish fauna detected in the MGL using eDNA metabarcoding was broadly comparable to that reported by Bettoso et al. (2013) and referred to species caught by fike nets. The eDNA approach has demonstrated its effectiveness in detecting species belonging to different functional guilds, including estuarine residents (ES), marine migrants (MM), diadromous (D), and freshwater (F) species, as well as their relationships with the major environmental drivers (salinity, vegetation, seasonality). In this study, the reliability of eDNA metabarcoding was supported by the detection of important ES species (e.g. *Atherina boyeri*) and in capturing ubiquitous and abundant taxa, essential for artisanal fisheries and conservation efforts. Similarly, the identification of species of regional interest (e.g. *Aphanius fasciatus*) pointed out the conservation significance of transitional environments like the MGL. The detection of MM (e.g. Dicentrarchus labrax, *Sparus aurata*) across both seasons confirmed the importance of lagoon as nursery and feeding grounds for these species. Additionally, the presence of D species (e.g. *Allosa fallax*, *Anguilla anguilla)* highlighted the ecological connectivity between freshwater and marine habitats.

Among ES species, the sand smelt *A. boyeri* was detected in all analyzed water samples, and it was also constantly caught with the beach seine net. *A. boyeri* is indeed a target species for artisanal fisheries in the lagoon, as it is ubiquitous and abundant in every water body, both in Spring and Autumn (Franco et al., 2006; Bettoso et al., 2013). The group of ES species includes 3 species of Community Interest whose conservation requires the designation of Special Protection Areas, as indicated in the Annex II of the Habitat Directive 92/43/EEC (EEC, 1992), such as the Mediterranean banded killifish *A. fasciatus*, the Adriatic dwarf goby *Knipowitschia panizzae* and the Canestrini's goby *Ninnigobius canestrinii*. *A. fasciatus* is a typical euryhaline species inhabiting the saltmarsh creeks of the brackish environment, lagoons, estuaries and salt flats (Franco et al., 2006; Lipej et al., 2006) and was constantly detected in both eDNA and seine nets samples. *K. panizzae* and *P. canestrinii* are typical gobiid species of the northern Adriatic lagoons. In particular, the Canestrini's goby is endemic to the Adriatic Sea (Miller, 1986) and is regularly found in the TME water bodies of the MGL (Bettoso et al., 2013), where the salinity ranges between 5 and 20 and there is a sedimentary habitat without vegetation (Franco et al., 2005). Among the ES species, the grass goby *Zosterisessor ophiocephalus* stands out, a fundamental component of the typical lagoon resident fish fauna in the MGL (Bettoso et al., 2013), which is very common in seagrass beds and sparsely vegetated habitats of the northern Adriatic lagoons (Franco et al., 2006); as it was confirmed by the eDNA, which constantly detected this species in vegetated sampling stations and, less frequently, in the non-vegetated ones. Among MM species, the seabream *Sparus aurata*, the sea bass *D. labrax*, the grey mullets *Chelon* spp., the common sole *Solea solea* and the flounder *Platichthys flesus* are particularly noteworthy. The eDNA detected *S. aurata*, *D. labrax* and *C. auratus* in all samples in both Spring and Autumn, while only their juvenile stages could be caught with the seine net in Spring. Among the flatfish, the common sole was constantly detected by eDNA in the inner water bodies of the Marano Lagoon in Spring, probably due to the presence of juveniles, which are particularly abundant in the mesohaline water bodies (TME) during this season (Bettoso et al., 2013); however, it was not found in the seine net samples. In contrast, the flounder was found in Spring in some seine net samples in the inner water bodies of the Marano Lagoon. Among MM it is also important to mention the detection of small pelagic marine species, migrating into the lagoon environment (Franco et al., 2006). These species are the anchovy *Engraulis encrasicolus*, the sprat *Sprattus sprattus* and the sardine *Sardina pilchardus*. Their juveniles are very abundant in the coastal marine areas off the transitional waters in the northern Adriatic Sea, entering the lagoon in Spring months and migrating seawards a few months later. In this case, the lagoon environment seems to be only a part of the juveniles habitat, without playing a special role as a nursery (Franzoi et al., 2010). Most of the MS were temporarily detected by eDNA in the MGL. These are stenohaline species that occur irregularly and sporadically in transitional environments (Elliott et al., 2007), because they are not dependent on these systems for any of their life stages (Franzoi et al., 2010). In fact, these species do not show a clear seasonality of occurrence in the lagoon. They are found occasionally and with few individuals in the zones more influenced by the sea (e.g. near sea inlets of the lagoon). The D species included both anadromous and catadromous species. In the transitional waters of the northern Adriatic, the category of the anadromous species includes sturgeons (Fam. Acipenseridae, e.g. *Acipenser naccarii*) and the twaite shad *A. fallax*, the latter detected in a few samples from the TPO and TME water bodies of the Marano Lagoon. Among catadromous species, the European eel *A. anguilla* was frequent only in the innermost part of the Marano Lagoon (TME) and it was detected only by eDNA in both seasons. *A. anguilla* enters the lagoon at elver stage and it can spend a large part of its life there, reaching a considerable size. This fish, which was once very common and abundant, represents a traditional and highly prized species for the artisanal fisheries in MGL. The dramatic global decline of its stock led to the adoption of specific management plans and catch quotas, according to the Council Regulation (EC) n. 1100/2007, establishing measures for the recovery of European eel stocks. The thin lip grey mullet *Chelon ramada* and the flathead grey mullet *Mugil cephalus* were constant species in eDNA samples and permanent in the TME water bodies; these species are in fact also categorised as catadromous, as they can occur in freshwaters, far from estuaries (Elliott and Hemingway, 2002; Franzoi et al., 2010). Finally, F species occasionally occur in transitional waters, but they are found in the oligohaline zone of coastal lagoons, usually close to estuaries (Franzoi et al., 2010). In this case, only 3 species were detected by eDNA in the MGL, of which the rainbow trout *Oncorhynchus mykiss* was permanently present in the Marano Lagoon, probably as a consequence of trout farming and river inputs in areas along the coasts of this lagoon.

Seven taxa were found with the WFD surveys but not by eDNA. These species, although present in the reference database, were not detected due to two main reasons: because the 12S target region present in NCBI was not in its full length, so that an assignment was not possible (*B. belone*, *C. saliens*, *K. panizzae*, *N. ophidion*, *P. canestrinii*, *S. pavo*); or because the primer site contained a mismatch, that prevented amplification (*S. abaster*). With regard to the implementation of site-specific eDNA monitoring of the MGL, these biases could be at least partially prevented by a combined strategy: *i*) the DNA of these species could be extracted directly from the lagoon specimens, sequenced by Sanger, and included in the reference database to increase its coverage and resolution; *ii*) primers with degenerate nucleotides, or a mixture with different primers showing specific polymorphisms could be tested to increase the number of species detected in a study tailored to the MGL. Moreover, other fish-specific systems could be applied alone or together with the system used by Valentini et al. (2016), that we used as well, such as the one on the 16 S rRNA gene (16SF/D/16S2R-degenerate; Deagle et al., 2007; Berry et al., 2017) or the 12S rDNA (MiFish-U-F/MiFish-U-r; Miya et al., 2015). These approaches target longer DNA regions (160–400 bp and ~170 bp, respectively), in contrast to Valentini et al. (2016), which covers 70–80 bp only. In eDNA surveys the use of so-called "mini-barcodes" facilitates the detection of such genetic material due to the possible degradation of DNA (Meusnier et al., 2008), although the short length of the reads may not be functional to discriminate among close related species.

Both eDNA metabarcoding and traditional approaches, including seine net sampling, have advantages and drawbacks. The number of species of fish caught by the seine net was lower than the ones detected by eDNA, although catches allow to estimate information on fish abundance and biomass essential for the application of the multimetric indices required by the WFD Directive (Pérez-Domínguez et al., 2012; Zucchetta et al., 2021). It is known that this fishing gear catches mainly the ES species of small sizes, as well as the early juvenile stage of MM and D, as adults manage to escape, therefore it is necessary to take into consideration the bias introduced by this monitoring method at different seasons. Furthermore, MS and F species are known to enter the lagoon (Franzoi et al., 2010) but are only transient and spend little time in this environment, reducing the likelihood of their capture (also due to their high mobility). In contrast, eDNA methods appeared to have the ability to trace the presence of a higher number of fish species, providing a more complete assessment of the composition and richness of the studied area (Ruppert et al., 2019). It is worth noting the high sensitivity for the detection of exogenous DNA, i.e. genetic material transported into the sampling area, although the actual species is not present. This was probably the case of *Salmo salar* detected at TME1 Spring and attributed to contamination also by Cananzi et al. (2022) in the Venice Lagoon. As we cannot exclude this possibility as well as other options (such as genetic material coming from nearby farming), this taxon should require further investigations, and the relative sequences were excluded from the analysis also in the present study. Another important difference of the two approaches regards the sampling effort. When sampling with the beach seine net, 140 m^2 of lagoon were searched from the water surface to the bottom in each tow, which corresponded to a total area of 280 m^2 per sampling station. The molecular approach needed the filtration of a low volume of water (only ~2 L per filter due to the high concentration of organic and inorganic particulate, typical of transitional environments).

Other studies comparing the fish richness recognized through eDNA and conventional methods in such environments have shown that the former method detected a greater number of taxa, highlighting the sensitivity of this approach in assessing fish biodiversity. Cole et al. (2022) found a higher number of genera (107 vs 29) in an Australian estuary using molecular or remotely sensed underwater bait video. eDNA was also more powerful in resolving alpha diversity of fish communities associated with oyster reefs as well as the estuarine gamma diversity. When comparing eDNA with bottom trawling in coastal wetland (China) (Zou et al., 2020), the number of genera detected was higher (60 vs 26), and the former approach was better suited to monitor a wide range of taxa and assess seasonal fluctuations in fish diversity. In relation to seine netting, the same traditional method to which we compared eDNA metabarcoding (detecting 18 and 34 fish species, respectively), Gibson et al. (2023) found that the molecular approach detected more species than the seine net (38 vs 14) in a macrotidal estuary (United Kingdom) and that the former provided a more complete picture of biodiversity and thus better management and ecological inferences. Overall, the standardization and integration of the two approaches can significantly increase the resolution and efficiency of biodiversity monitoring and improve the protection and management of this important and complex transitional environments.

The use of eDNA metabarcoding in well-studied and monitored areas such as the MGL allows the selection of the most appropriate taxonomic assignment, as we were able to take into account previous data at the same sites as well as the ecology of the species, thus maximizing the efforts of the different approaches. In our study, this information helped to choose between different options with the same similarity parameters in several cases. The first case was *Platichthys flesus/Pleuronectes platessa.* Sequences of *P. platessa* detected by Cananzi et al. (2022) in the Venice Lagoon were attributed to possible contamination. However, blasting our ASVs on NCBI (on December 1, 2023), *P. flesus* and *P. platessa* (5 and 4 sequences for our target gene region available respectively) had the same similarity thresholds (98.81% with 100% coverage) and, as pointed out by Lleonart and Farrugio (2012), the morphologically similarity of the two species can lead to misidentification, and this can be a source of biases also in the reference databases. Moreover, *P. platessa* has not yet been identified in the Mediterranean Sea so far (Lleonart and Farrugio, 2012; https://fishbase.se/summary/Pleuronectes-platessa.html). A confirmation of this approach comes for the seine net sampling, which actually detected *P. flesus* in the lagoon. For these reasons, we decided to select *P. flesus* as the correct assignment in our dataset. Another case regarded the choice of *Symphodus cinereus* among others *Symphodus* spp. (*S. cinereus, S. mediterraneus*, and *S. roissali*) which had the same similarity thresholds (100% with 100% coverage, with 3, 1, 3 sequences respectively) but is the only species commonly found the MGL (Bettoso et al., 2013). A third case worth mentioning is *Trachurus* sp.: different species (*T. lathami, T. trachurus and T. japonicus*) had the same similarity thresholds (100% with 100% coverage) but the one which is more commonly found in MGL, *T. mediterraneus* (although *T. trachurus* is also present in the northern Adriatic), is present in NCBI with only one sequence which do not cover our 12S region. Therefore, even if we decided to remain at the genus level to be more cautious, we think the *T. mediterraneus* is the most likely option.

The lack of completeness of the reference database is one of the main methodological limitations of DNA metabarcoding (Stefanni et al., 2018), as the missing sequences of a particular taxon prevent its detection by molecular methods. On the other hand, the presence of close taxa together with the target species (e.g. other species of the same genus) could lead to a "false positive" assignment. We applied a conservative approach by testing different similarity thresholds for taxonomic assignment and preferring to lose resolution (as in *Trachurus* sp.) rather than reliability. Overall, the general trend was the absence (or only partial presence) of reference sequences of species commonly found in transitional environments (e.g. *B. belone*, *C. saliens*, *K. panizzae*, *N. ophidion*, *P. canestrinii*, *S. pavo*), highlighting the need for greater efforts to fill the gaps in local reference databases. In our study, we emphasized the importance of informed taxonomic assignment in eDNA analyses, leveraging ecological knowledge and reference databases to mitigate potential biases and inaccuracies. By carefully considering species similarities and ecological context, the study demonstrates the value of integrating molecular and ecological expertise in optimizing eDNA-based assessments.

In the northern Adriatic Sea, Cananzi et al. (2022) conducted a first fish-targeted eDNA survey in two sampling sites of the Venice Lagoon. The outcomes of this study highlighted the relevance of this approach for biodiversity assessment and monitoring, also with regard to the isolation of the Venice Lagoon by mobile dams designed to prevent the flooding of the city during extreme high tides (Zonta et al., 2018). The study reported that the local fish community reflected both the influence of marine and freshwater sources as well as the seasonal trends. As this is, at the best of our knowledge, the only study on fish eDNA in the North Adriatic Lagoon, a comparison with our results is of interest. Out of the 54 species detected in the two sampled stations (Torcello and Chioggia, the latter more influenced by marine water), 20 (*A. fallax, A. anguilla, A. boyeri, C. auratus, D. labrax, E. encrasicolus, M. cephalus, M. mustelus, O. mykiss, P. saltatrix, P. minutus, S. salar, S. pilchardus, S. scombrus, S. aurata, S. sprattus, S. cephalus, S. acanthias, S. typhle, Z. ophiocephalus*) were also detected in our study. When comparing the two studies, it must be taken into account the spatial coverage of the lagoons (only 2 vs 16 sampling sites) and the frequency of sampling (12 months vs 2 seasons) which may influence the detection of fishes based on their distribution and ecological features. Another discrepancy could be due to the different portion and length of the 12S barcode region used (~167 bp vs ~80 bp, respectively), which can lead to different taxonomic resolution. This comparison highlights the broader applicability of eDNA metabarcoding in different transitional environments.

In 2016, the MGL was one of the first sites in the Adriatic where the presence of the non-indigenous species (NIS) *Mnemiopsis leidyi* was detected and it has since been monitored after its bloom in summer as in

the entire northern Adriatic Sea (Malej et al., 2017). This species is considered one of the 100 most dangerous aquatic invasive species (Lowe et al., 2000) due to its significant negative impacts on the functioning of the ecosystem and on fisheries (e.g. Piccardi et al., 2024). And more recently, in 2023, MGL experienced the invasion of the blue crab (*Callinectes sapidus*) (unpublished data), another NIS whose impact on local fisheries has not yet been quantified. The impact of NIS on the local fish fauna requires an additional monitoring program and eDNA protocols, which have proven effective in detecting resident and visiting species in this type of environment.

This study represents a significant step forward in understanding the fish biodiversity and ecological dynamics of transitional environments. By leveraging the strengths of eDNA metabarcoding alongside traditional methodologies, the research provides valuable insights into the complexities of fish communities, contributing to informed conservation and management strategies in these ecologically sensitive habitats.

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CRediT authorship contribution statement

Elisa Banchi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Nicola Bettoso:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Diego Borme:** Writing – review & editing, Writing – original draft. **Sergio Stefanni:** Writing – review & editing, Writing – original draft, Methodology. **Valentina Tirelli:** Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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.

Data availability

The sequences generated for this study are available at the Sequence Reads Archive (SRA) at NCBI under the accession numbers PRJNA1049655.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2024.108824.

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