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Unveiling the UNESCO Biosphere Reserve of the Berlengas Archipelago in Portugal as a Hotspot of Fish Species Using eDNA Metabarcoding and the Collaboration of Fishing Crews

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Abstract: Managing fishery resources is crucial to ensure the marine environment continues to provide diverse goods and services. To overcome difficulties of classical methods used for fish stock management, molecular tools have shown potential to address this issue assessing both targeted and non-targeted species. This study aims to evaluate the spatiotemporal diversity of fish using 12S rRNA gene eDNA metabarcoding sequencing in the Berlengas archipelago and compare two seawater eDNA sampling sources: samples collected by fishermen during their activities and those collected by our research team. The results indicated that autumn presented the highest diversity and that the area around Berlenga Island was the richest area, increasing biodiversity across the region. Fisher-collected samples were generally less diverse than those by the research team but detected species typical of deeper and open-ocean habitats, validating this sampling method. Our study also highlighted eDNA's role in monitoring fish species by detecting unexpected species for the region, such as Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*), while cautioning against false positives like orange clownfish (*Amphiprion percula*) and blue tilapia (*Oreochromis aureus*). Future optimisation of our eDNA sampling methodology could better refine marine ecosystem dynamics around the UNESCO Biosphere Reserve of the Berlengas Archipelago, Portugal.

Keywords: environmental DNA; genetics; fish diversity; fisheries management; Northeast Atlantic; Portugal

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1. Introduction

The Portuguese mainland coast is a transitional zone with flora and fauna adapted to both cold and warm water [1–3], for which the Iberian upwelling is a key factor to keep the species richness characteristic from the Portuguese coast, as well as significant recruitment rates for fisheries (abundance) [4–6]. Fisheries play a crucial role in the functioning of the Portuguese society, being socially, culturally and economically important for many coastal

Portuguese communities [7,8]. However, if not properly managed, fisheries can negatively impact the well-functioning of marine ecosystems, threaten their provisioning, regulation and cultural services [9], including fisheries sustainability. In Portugal, there have been fish landings decreasing [10] that may be related to overfishing, as it was considered for the case of the European sardines *Sardina pilchardus* (Walbaum 1792) [11]. The main purpose of fisheries management is therefore to ensure that fisheries operate in an appropriate manner, providing benefits without damaging the structure of the ecosystem, fish stocks or diversity [9].

The European fisheries management is governed by a number of directives, such as the European Common Fisheries Policy (CFP), the Integrated Maritime Policy (IMP) and the Marine Strategy Framework Directive (MSFD) [12]. In general, these guidelines focus on long-term sustainability and require information on the state of the marine environment, including indicators of fishing pressure on target and non-target species [13,14]. However, European fisheries management has traditionally relied on information provided by (1) the fishing industry, such as landings and effort data (fishery-dependent) [15], and (2) fisheries management agencies, which conduct scientific surveys, such as capture or underwater visual census (UVC) of the species of interest (fishery-independent) [15,16]. Both methods are essential to assess the status of resources and provide data to formulate the best possible scientific advice for a sustainable exploration [16,17]. However, these methods suffer from data deficiencies [16,18,19], which can be up to 50% off [20], providing low accuracy and reliability. As a result, it can lead to uncertainty about the real state of the fisheries stocks and, consequently, misleading perceptions about the availability of fish resources, increasing the likelihood of overfishing [21]. Furthermore, those methods mainly focus on commercial fish species, disregarding surveys of non-commercial species [17], as clearly advocated by the European policies [12]. Such constrains highlight the urgent need to develop more effective approaches assisting the fisheries management plan.

A new era of fisheries management relying on the use of molecular tools is currently emerging [22], with great promise placed on the environmental DNA (eDNA) surveys. The eDNA represents the DNA shed by organisms, through body cells or metabolic waste into the water, which can be collected by filtration [23]. The eDNA retained in the filter may then be extracted and subjected to barcoding and next-generation sequencing (NGS). These techniques allow the identification of multiple species and the characterisation of large complex communities [22] by amplifying and sequencing one or more target genes from multiple species in multiple samples simultaneously [24]. Therefore, eDNA metabarcoding allows a rapid and accurate taxonomic identification of the organisms down to the species level, which is a challenging task for most traditional methods [22]. The ease and non-invasiveness of the sample collection and the greater spatial and temporal sampling capacity also improve the assessment of fish community status [25–30]. Such advantages make eDNA metabarcoding a potential tool for improving fisheries resource management.

In comparative studies between traditional and molecular methods used for monitoring species composition in the marine aquatic environment, eDNA metabarcoding detected more species than trawling [31] or UVC [32,33]. The latter, which requires trained observers and little post-survey processing [34,35], is the most widely used technique to assess the abundance, biomass and composition of fish communities [36,37]. However, this technique has several limitations, such as depth, visibility limitations and underestimation of diversity [38–40]. eDNA metabarcoding has shown a high species overlap, detecting 75–100% of the species caught by trawling [17,31,41]. However, eDNA metabarcoding lacks efficiency for detecting less abundant species in the samples (one individual in a sample) [17,31]. This situation represents only <0.1% of reads detected [31]. At different depths, eDNA metabarcoding also showed good performance, with no significant differences in species

detected between surface and bottom samples, in contrast to trawling, where fish diversity was high near shore and progressively decreased at deeper strata [31].

There is also a positive correlation between the number of reads and species abundance or biomass [17,21,22,41], which is important for efficient fish stock management. Nevertheless, further studies are needed to investigate the influence of eDNA production and degradation rates, transport by currents and biotic and abiotic factors in this correlation [42,43].

Despite its potential, eDNA metabarcoding has rarely been used to assess fish communities in Portugal, including in the Berlengas archipelago, which presents a great lack of studies on biodiversity, making its application of extreme importance. Classified as UNESCO Biosphere Reserve since 2011, Berlengas Natural Reserve is made up of a small number of Islands (the large island of Berlenga, the Islands of Estela and Farilhões-Forcado) and the city of Peniche [44], with a total area of 18,502.00 ha (726.00 ha of terrestrial area and 17,777.00 ha of marine area) [45]. Ecologically, and due to its geographical location under Mediterranean and Atlantic climatic influences, the Berlengas Reserve is very important, because it is a very nutrient-rich area due to its proximity to the Nazaré Canyon, which, with upwelling events, makes it a productive area [46,47]. These characteristics make it a relevant area for the conservation of several species, namely dusky grouper *Epinephelus marginatus* (Lowe 1834) and common dolphin *Delphinus delphis* (Linnaeus 1758) [48], as well as for fisheries. Another ecosystem service is the observation of fish biodiversity through scuba diving, as the Berlengas Reserve is considered a biodiversity hotspot due to its endemic species richness [49–51].

Therefore, the objectives of this study included (1) the characterisation of fish species diversity around the Berlengas archipelago in different seasons and distances from the coast using eDNA metabarcoding and (2) the evaluation of the fish diversity assessed with two different sampling sources, fishers and research team collection. The main findings are thtat (1) autumn was the richest and most diverse season, (2) the Berlengas region showed the highest diversity values, contributing to the enrichment of the area, (3) both sampling sources showed complementarity, and (4) eDNA metabarcoding detected rare and unexpected species for the study area, such as Atlantic cod and the orange clownfish, respectively.

2. Materials and Methods

2.1. Sampling Methodology and eDNA Extraction

The eDNA metabarcoding analyses were performed on 35 seawater samples collected in the vicinity of the Peniche coastline and Berlengas Natural Reserve during the years of 2020 and 2021 (Figure 1). Two litres of seawater were collected from the surface by the research team (RT, representing B samples) and by the fishing crew (FC) of two purse seine vessels of the National Association of Fishing Producers' Organisations of Peniche (Portugal), namely Guerreiro-do-Mar (GM) and Rio-Minho (RM), while hauling the seine net during fishing operation. Later, information on the collected species and quantities was provided by the fishers, which can be found in Table 1.

Samples were transported to the Cetemares Research Centre (Peniche, Portugal) and filtered within a few hours after collection or stored at 4 °C and filtered within a maximum of 12 h of collection. The time between collection and filtration varied according to the collection approach, approximately 3 h for RT samples. For FC samples, this interval varied between 5 and 12 h, depending on the species caught and the fishing time. Two litres of collected seawater were filtered using a vacuum pump and a filter cup (previously bleached (1%) and sterilised), with a membrane filter of 0.2 µm (Whatman, Dassel, Germany) for

each litre. One additional litre of Mili-Q water was filtered as a negative control in the laboratory. The filters were stored at −80 °C until eDNA extraction.

A modified version of the DNeasy Blood and Tissues Kit (Qiagen, Hilden, Germany) protocol was used to extract the filtered DNA. This method required the fragmentation of the filter into thin strips of 1 mm wide and 1 cm long using a sterilised scalpel blade and placed into a falcon. Then, 960 µL of ATL buffer and 40 µl of proteinase K were added, vortexed and incubated for 2 h at 56 °C. The solution was manually homogenised every 10 min. Then, 1 mL of ethanol and 1 mL of AL buffer were added and homogenised. The entire mixture was passed through the columns provided by the kit. The next steps followed the original protocol.

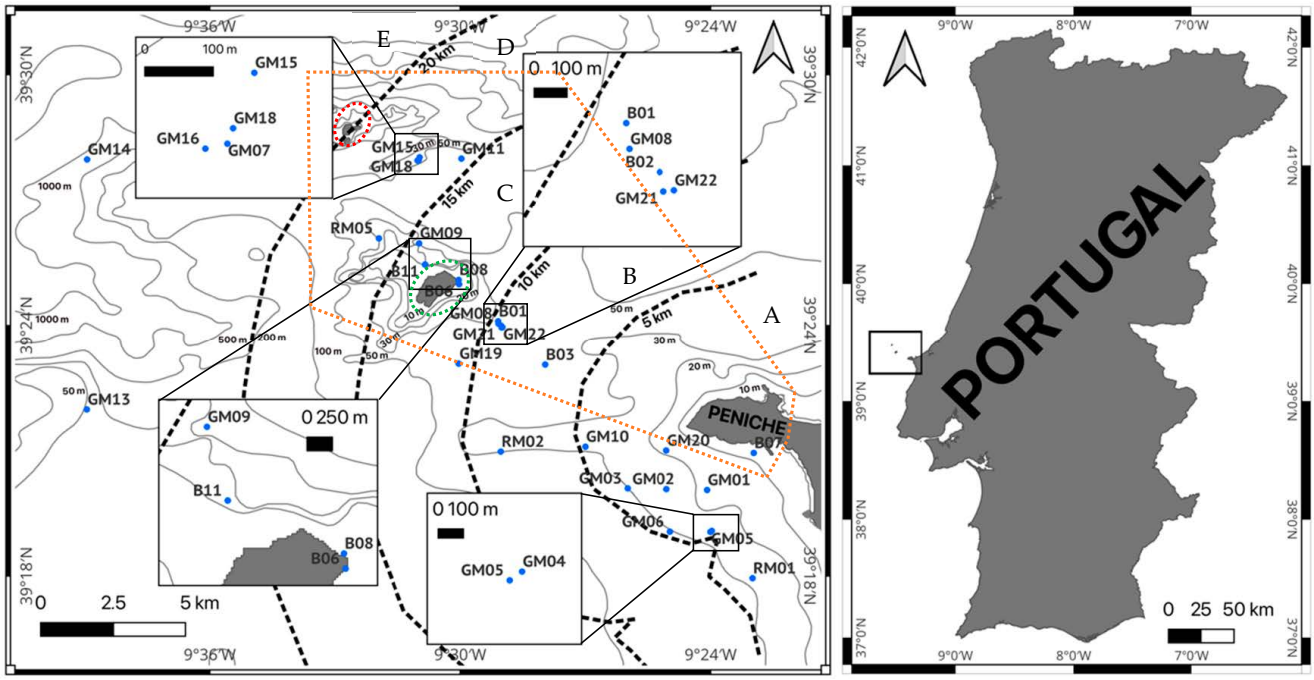

Figure 1. Geographical location of eDNA samples (blue dots) collected by fishermen (RM—Rio-Minho, GM—Guerreiro-do-Mar) and by the research team (B). The black dotted lines represent the distance from the coastline to which the sample belongs (5 km—map region A, 10 km—map region B, 15 km—map region C (includes the large Berlenga Island), 20 km—map region D and >20 km—map region E). The continuous grey line represents depth in metres (m). The orange dotted line represents the area of UNESCO Biosphere Reserve of the Berlengas archipelago. The green dotted circle represents the large Berlenga Island in region C. The red dotted circle represents Farilhões Island. Samples B00, B04, B05, B09, B10 and B11 were collected at the same geographical location.

Table 1. Data from eDNA samples collected by fishermen (RM—Rio-Minho, GM—Guerreiro-do-Mar) and the research team (B—Berlengas).

Date	Samples	Species Caught	Amount	Season	Map Region
30/January/20	B00	-	-	Winter	C
02/June/20	B01	-	-	Spring	B
02/June/20	B02	-	-	Spring	B
02/June/20	B03	-	-	Spring	B
17/June/20	B04	-	-	Spring	C
09/July/20	GM01	Sardine	2700 kg	Summer	A
10/July/20	GM02	Sardine	2700 kg	Summer	A

Table 1. *Cont.*

Date	Samples	Species Caught	Amount	Season	Map Region
15/July/20	GM03	Sardine	2700 kg	Summer	A
15/July/20	RM01	Sardine	2700 kg	Summer	A
16/July/20	RM02	Sardine	2700 kg	Summer	B
20/July/20	GM04	Sardine	2700 kg	Summer	A
31/July/20	GM05	Sardine	2700 kg	Summer	A
10/July/20	GM06	Sardine	2700 kg	Summer	A
11/August/20	GM07	Sardine	2700 kg	Summer	D
17/August/20	GM08	Sardine	2700 kg	Summer	B
18/August/20	GM09	Sardine	2700 kg	Summer	C
20/August/20	RM05	Mackerel	-	Summer	D
11/September/20	GM10	Sardine	2700 kg	Summer	A
23/September/20	B05	-	-	Autumn	C
30/September/20	GM11	Mackerel	7000 kg	Autumn	D
17/October/20	B06	-	-	Autumn	C
22/April/21	GM13	Horse mackerel	500 kg	Spring	E
27/April/21	GM14	Horse mackerel	3500 kg	Spring	E
06/May/21	GM15	Horse mackerel	8000 kg	Spring	D
18/May/21	GM16	Horse mackerel	6000 kg	Spring	D
01/June/21	GM18	Mackerel	1900 kg	Spring	D
01/June/21	B07	-	-	Spring	A
01/June/21	B08	-	-	Spring	C
16/June/21	GM19	Sardine	900 kg	Spring	C
16/June/21	GM20	Sardine	2000 kg	Spring	A
26/June/21	B09	-	-	Summer	C
06/July/21	GM21	Sardine	60 kg	Summer	B
05/August/21	GM22	Sardine	570 kg	Summer	B
11/August/21	B10	-	-	Summer	C
12/October/21	B11	-	-	Autumn	C

2.2. Library Preparation for Metabarcoding Analysis

DNA quality and integrity of the extracted DNA was evaluated by agarose gel electrophoresis. The eDNA concentration was measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR was pe rformed on both replicates of each sample to ensure the presence of the fish 12S rRNA gene using the primer pair FW–GTCGGTAAAACTCGTGCC and Rev–CATAGTGGGGTATCTAATCCCAGTTTG (170 base pairs (bp)) [52], as well as on the negative control.

Library preparation was performed in the laboratory. Two PCR reactions were performed for each replicate of each eDNA sample using the same pair of primers with the tags of the Illumina system, according to the manufacturer's instructions for the Taq polymerase used in the reactions: 1x PCR buffer (NZYTaq II 2x Green Master Mix) (Nzytech, Lisbon, Portugal), 0.5 µM of each primer and 1 µL of DNA template. A Bio-Rad T100 thermal cycler (Bio-Rad, Hercules, CA, USA) was used with the following PCR conditions: 3 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 20 s at 72 °C, and a final extension of 5 min at 72 °C. Negative controls were also performed in the same conditions, not

amplifying any product. PCR products were purified using the NZYGelpure kit (NZYTech, Lisbon, Portugal). Subsequently, the STAB VIDA Company (Costa da Caparica, Portugal) sequenced both replicates of each sample according to the MiSeq Reagent Kit v3 protocol on the lllumina MiSeq platform, using 2 × 300 bp paired-end sequencing reads.

Some data from cytochrome oxidase subunit I (COI) gene metabarcoding sequencing, from another study performed by our team, were added to the 12S rRNA dataset due to the detection of fish species, specifically sharks. The methodology about COI library preparation and sequencing is in Supplementary Information S2.

2.3. Bioinformatic Analysis of Metabarcoding Data

Raw metabarcoding data were processed using "Quantitative Insights Into Microbial Ecology 2" (QIIME 2) platform (version 2022.2) [53]. The DADA2 plugin was used to denoise (minimum quality of ≥30), merge raw data and exclude singletons. Taxonomic classification of Amplicon Sequencing Variants (ASVs) was performed by the Basic Local Alignment Search Tool (BLAST) in QIIME 2, with 97% of similarity at the species level. A second BLAST was performed on unassigned ASVs, with 90% of similarity classifying to the genus level. The remaining unassigned ASVs and non-fish species were excluded.

Two databases were used for taxonomic classification: the National Center for Biotechnology Information (NCBI), downloaded using the Entrez Direct tool from NCBI, with taxonomic information obtained through the script "Entrez_qiime_py" [54], and the Mitochondrial Genome Database of Fish MitoFish, available on its respective online platform [55–57]. Both taxonomic classifications were crossed, and ASVs with different classifications were verified on NCBI using BLAST.

After taxonomic classification, all ASVs were checked for species and genus distribution by consulting the World Register of Marine Species (WoRMS) [58], Ocean Biodiversity Information System (OBIS) [59] and FishBase [60].

2.4. Plots and Statistical Analysis

For ease of interpretation, reads from both replicates of each sample were pooled for all plots and statistical analyses. Venn diagrams were performed using the R package "ggVenDiagram" (version 1.2.3) [61]. Alpha and beta diversity analyses were performed using the "vegan" package (version 2.6-4) [62]. Reads rarefaction was applied to a sequencing depth of 7177 reads.

For alpha diversity analysis, the number of species and Brillouin diversity indices were applied, through "diversity" vegan's function and tabula package (version 3.0) [63,64], respectively. Normality test and Homogeneity of Variance were tested before any statistical analysis using the IPSUR plugin of R commander (version 0.2-1.1) [65]. Welch and Tukey statistical analyses with 95% significance were performed through the IPSUR plugin of R commander (version 0.2-1.1) [65] for collection analyses with the season and map region, respectively. For beta diversity, the Jaccard index was used to assess the species dissimilarity of the species with respect to each variable using the "vegdist" and "metaNMDS" functions. The statistical analyses "Adonis2" and "betadisper" were performed through the R package "vegan", with 95% of significance. The "pairwiseAdonis" package was used to identify which pairwise presented statistical differences, with 95% of significance [66]. The "indicspecies" R package (version 1.7.12) [67] was used to identify the species responsible for the dissimilarity detected in beta-diversity, with statistical significance of 95%.

3. Results

3.1. Bioinformatic Analysis, Unexpected Species and Chondrichthyans

Raw data were deposited in the NCBI database with the accession number PRJNA1110393. After processing the raw data of the 35 samples, 3,210,977 reads were obtained. The lowest number of reads was 7177 (B09) and the highest was 134,643 (GM04), with an average of 91,742 reads per sample. Overall, 1585 ASVs were detected: 1007 were classified as unassigned (63%) and 578 were classified as fish (36%), corresponding to 80 different species.

Out of the 80 species identified, four were unexpectedly detected. Atlantic salmon *Salmo salar* (Linnaeus 1758) with three ASVs was detected in three samples: GM09, B05 and B04, with a total of 501 reads. Atlantic cod *Gadus morhua* (Linnaeus 1758) presented three ASVs distributed by four samples: B00, B06, B09 and B10, with a total of 2915 reads. Orange clownfish *Amphiprion percula* (Lacepède 1802) had one ASV in sample B00 with 23,438 reads, and blue tilapia *Oreochromis aureus* (Steindachner 1864) had three ASVs detected in three samples: GM01, GM08 and RM05, with 75 reads in total. Although excluded from the analysis due to their detection in unlikely environments, *A. percula* and *O. aureus* are included in the discussion to warn of the cautious analysis that eDNA metabarcoding may require.

For cartilaginous fish, only one species was detected, small-spotted catshark *Scyliorhinus canicula* (Linnaeus 1758), with 13 reads in the B10 sample. In a parallel study developed by our team to characterise zooplankton communities, the cytochrome oxidase subunit I (COI) gene was sequenced by metabarcoding for the same samples analysed in this work, and blue shark *Prionace glauca* (Linnaeus 1758) and shortfin mako shark *Isurus oxyrinchus* (Rafinesque 1810) were detected by COI metabarcoding with two and eight reads in the B00 and GM18 samples, respectively (Methodology in Supplementary Information S2). Considering both datasets of 12S rRNA and COI, three shark species were detected.

3.2. Species Abundance and the Influence of Samples Collector

Focusing on the twelve most abundant species (Figure 2a,b), RT samples generally presented a greater number of species compared to FC samples, which were clearly dominated by the species caught by fishermen during sampling, such as European pilchard, Atlantic horse mackerel *Trachurus trachurus* (Linnaeus 1758) and Atlantic chub mackerel *Scomber colias* (Gmelin 1789) (Figure 2a, Table 1). Despite the general agreement between the dominance of these three fish species (Figure 2a) and the species caught by fishermen (Table 1), there were some samples where this tendency was not observed, such as GM05, GM09, GM11, GM13 and GM18.

The RT samples generally showed a balanced abundance of species, showing the higher abundance of fish species occurring around the Berlengas archipelago. The exceptions were samples B00, B03, B05 and B07, where *S. pilchardus* or Common pandora *Pagellus erythrinus* (Linnaeus 1758) were detected as the dominant species.

The differences in species richness between RT and FC samples were confirmed by alpha-diversity analysis (Figure 3a), although without statistically significant differences (Welch test, Df = 1, F = 2.25, *p*-value = 0.15). However, using the Brillouin diversity index, RT samples showed higher species richness and evenness statistically significant when compared to FC samples (Welch test, DF = 1, F = 6.27, *p*-value = 0.02; Figure 3b), which confirm the balanced abundances observed in Figure 2b.

When assessing the presence/absence of species, the beta diversity Jaccard index (Figure 3c) showed a significant species dissimilarity between RT and FC samples (Adonis test, R^2 = 0.08, F = 3.12, *p*-value = 0.001; Betadisper test, DF = 1, F = 8.22, *p*-value = 0.007).

This is further illustrated in the Venn diagram (Figure 3d), where 16 species were exclusive to FC samples and 22 were exclusive to RT samples.

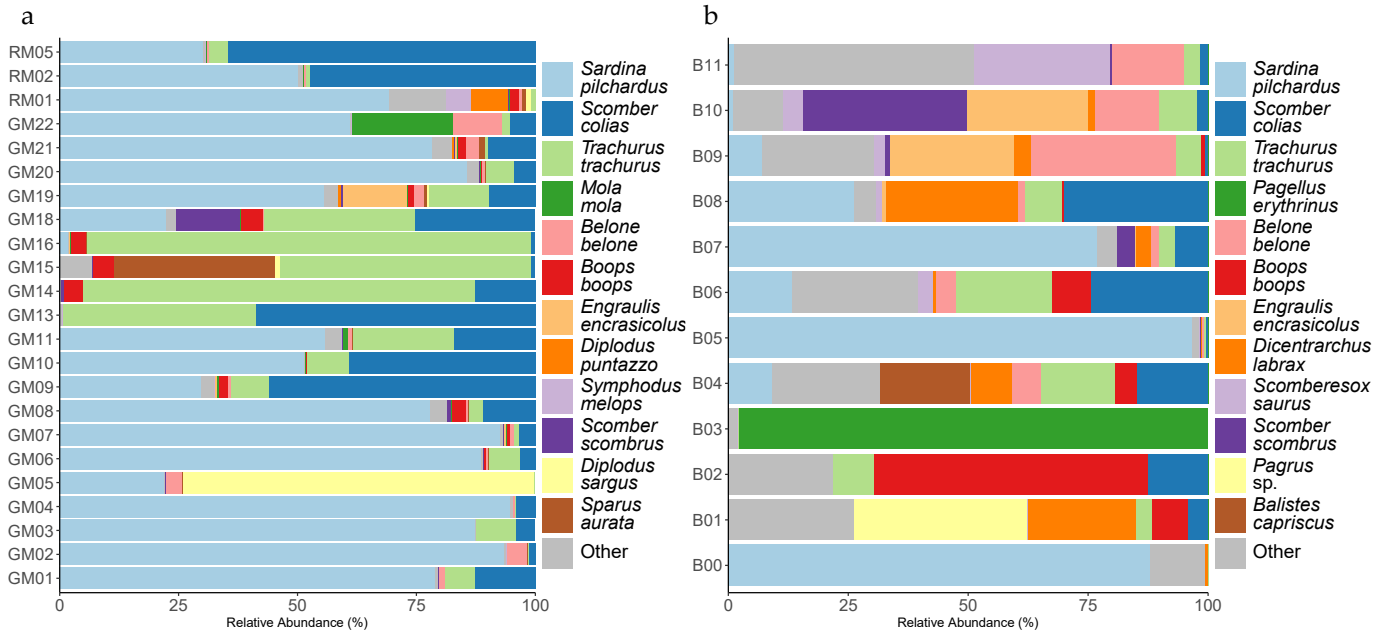

Figure 2. Relative abundance of the twelve most abundant fish species, detected by eDNA metabarcoding analyses. (**a**) Fishing crew (FC) samples. (**b**) Research team (RT) samples.

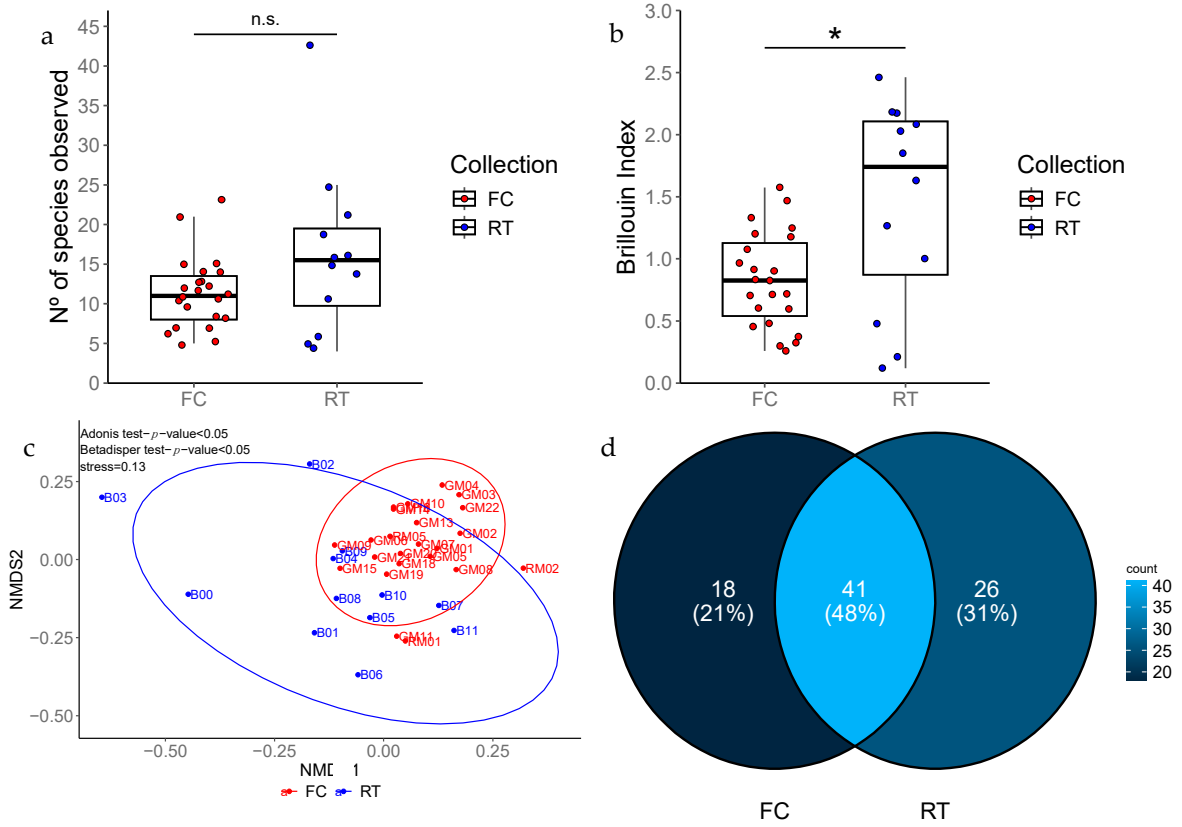

Figure 3. Fish species diversity regarding sample sources FC and RT. (**a**,**b**) The alpha-diversity index for number of species detected and alpha-diversity index Brillouin, respectively, for FC samples (red box and points) and RT samples (blue box and points). (**c**) The beta-diversity Jaccard index. (**d**) Venn diagram. Welch statistical analysis (**a**,**b**) and Adonis, pairwiseAdonis and Betadisper test (**c**). *—statistically significant; n.s.—not significant.

Despite the significant dissimilarity, the sample sources did not form distinct clusters, indicating that many species were shared (41; Figure 3d; Supplementary Information S1). Additionally, the RT samples showed a higher dispersion than the FC samples (Figure 3c), highlighting how variable species detection can be (B00, B02 and B03 samples), meaning a high variability in species detection.

3.3. Effect of Distance from the Coast and Season on Species Richness

The comparison of species richness in different map regions (Figure 4a) did not reveal statistically significant differences (Tukey test, DF = 3, F = 3.01, p-value = 0.05), although a higher number of species was detected in map region C (Figure 4a), with 21 out of 78 species detected only in this region (Figure 4d; Supplementary Information S1). The remaining regions showed similar species richness values.

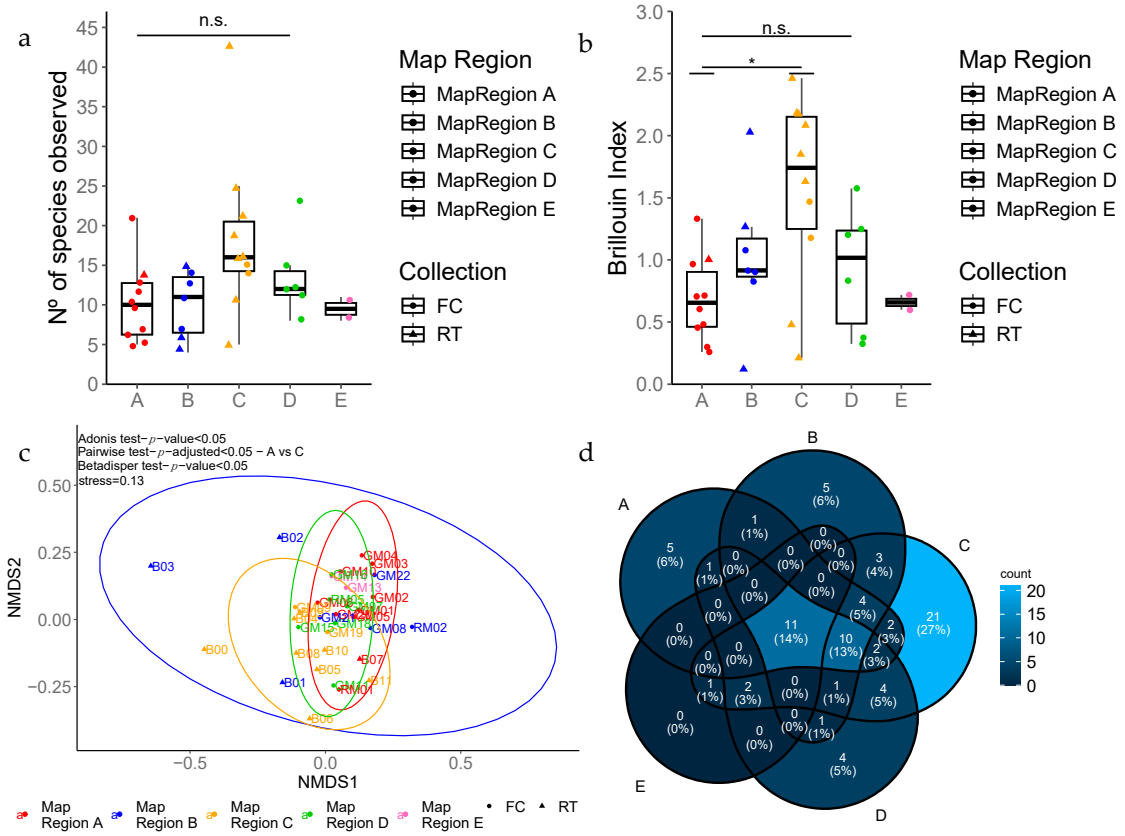

Figure 4. Fish species diversity regarding distance from the coastline. (**a**,**b**) The alpha-diversity index for number of species detected and alpha-diversity index Brillouin, respectively, for map regions A (red), B (blue), C (yellow), D (green) and E (pink). (**c**) The beta-diversity Jaccard index. (**d**) A Venn diagram with the overlapping species among the 5 regions. Tukey statistical analysis (**a**,**b**) and Adonis, pairwiseAdonis and Betadisper test (**c**). *—statistically significant; n.s.—not significant. Dots represent FC samples, triangles represent RT samples (**a**–**c**).

The results provided by the Brillouin index showed the same tendency, with a pattern of increasing species richness and diversity until map region C, followed by a decline (Tukey test, DF = 3, F = 4.31, p-value = 0.01; Figure 4b). However, in this case, there were significant differences (Tukey statistical analysis, p-value < 0.05) between map regions A and C, which means that the samples collected in region C, close to the large Berlenga Island, had a high species diversity and evenness.

The significant differences between map regions A and C were also observed on the Jaccard index (Adonis test, R^2 = 0.16, F = 1.49, p-value = 0.006; Betadisper test, DF = 4,

F = 5.50, *p*-value = 0.001; Figure 4c), reinforcing the dissimilarity of the species between regions A and C, indicating the detection of different species on each region.

Regarding seasonality, the results revealed a significantly higher number of species (Tukey test, DF = 2, F = 15.83, *p*-value = 0.00001) in autumn than in spring and summer (Figure 5a). Similar numbers of species were detected in spring and summer, with a median value of 11 and 12 species, respectively. In contrast, the Brillouin diversity index did not show significant differences (Tukey test, DF = 2, F = 1.77, *p*-value = 0.18; Figure 5b). Nevertheless, a similar trend was observed, with a higher fish species diversity detected in autumn than in spring and summer. Although not significant, summer showed lower diversity.

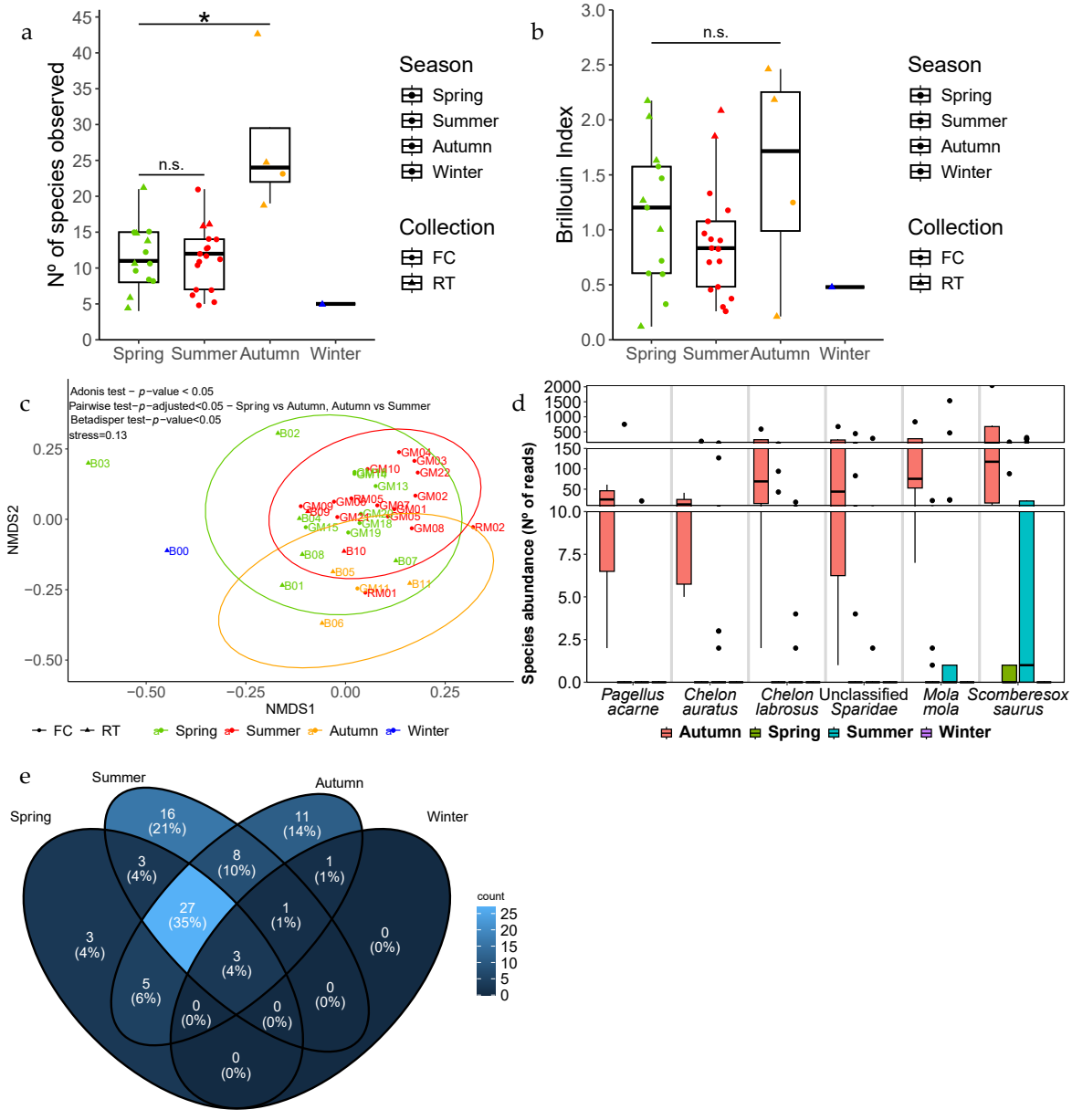

Figure 5. Fish species diversity regarding seasons. (**a**,**b**) The alpha-diversity index for number of species detected and alpha-diversity index Brillouin, respectively, for spring (green), summer (red), autumn (yellow) and winter (blue). (**c**) The beta-diversity Jaccard index. (**d**) The species responsible for the dissimilarity observed on the Jaccard index. (**e**) A Venn diagram with the overlapping species among seasons. Tukey test (**a**,**b**) and Adonis, pairwiseAdonis and Betadisper test (**c**). *—statistically significant; n.s.—not significant. Dots represent FC samples, triangles represent RT samples (**a**–**c**).

The Jaccard index also showed significant differences between autumn–spring and autumn–summer (Adonis test, R^2 = 0.18, F = 2.28, *p*-value = 0.001; Betadisper test, Df = 3, F = 7.50, *p*-value = 0.0006; Figure 5c), indicating dissimilarity in the species identified in autumn. This dissimilarity was explained by six species detected in higher abundance in autumn: axillary seabream *Pagellus acarne* (Risso 1827), golden grey mullet *Chelon auratus* (Risso 1810), thicklip grey mullet *Chelon labrosus* (Risso 1827), unclassified Sparidae, ocean sunfish *Mola mola* (Linnaeus 1758) and Atlantic saury *Scomberesox saurus* (Walbaum 1792; Figure 5d).

Despite this seasonal variability, 27 species overlapped during spring, summer and autumn and three more species that were also detected during winter (Supplementary Information S1).

4. Discussion

4.1. Not Everything That Comes into the Net Is Fish—Careful Analysis Is Needed

In this study, orange clownfish and blue tilapia were unexpectedly detected considering their native habitat conditions when compared to the Northeast Atlantic Ocean. Orange clownfish inhabits warm water, 26–30 °C [68], so its detection suggests some kind of environmental contamination and a false-positive site detection [69,70]. One of the possible explanations is most likely to come from an aquarium hobbyist, either from a boat or from one of the fishers' houses in the main island of the archipelago.

As for blue tilapia, and despite its great adaptability, tolerating moderate salinity and living in tropical and subtropical regions [71,72], it is not likely that it tolerates the salinity level of seawater [73] in the long term, nor the low water temperature of the Portuguese coast [72]. Therefore, its detection could have been due to an contamination and a false-positive site detection that may have had an origin in (1) its use as bait in fishing or in the diet of some crews, (2) the existence of aquaponic companies in Portugal that can use this species, although this is highly unlikely, or (3) its presence in freshwater systems, as this species is classified as a potential invasive species in Iberian aquatic environments [74], thus rejecting the hypothesis of the presence of blue tilapia on the Portuguese coast.

In the case of Atlantic salmon and Atlantic cod, despite their probable occurrence in Portuguese waters according to the FishBase database [60], sightings and catches are not common. However, Atlantic salmon was previously reported in Portuguese rivers by studies carried out in Northern Portugal [75], including the Lima and Minho Rivers [76,77] and the Mondego River [78,79], where monitoring programs have occurred. On marine waters, a fishing vessel has caught salmon specimens in July 2018 and September 2021 (Otávio Farto, personal communication) around Peniche on more than one occasion, confirming their presence in the Central Portuguese coastal waters.

The detection of Atlantic cod, although unexpected, is not entirely surprising. Even though it is recognised that this species is distributed in the northern areas of the North Atlantic [80–82], FishBase mentions its possible presence in Portugal. Moreover, there are popular rumours from decades ago about the existence of Atlantic cod in the Tagus River. Also, two specimens were caught off Peniche on two separate occasions about 10 years ago (Nuno Vasco-Rodrigues, personal comment). An error in classification due to a lack of information in databases could be considered, although it is unlikely. On NCBI, 100% of similarity with Atlantic cod was obtained in the BLAST, and there are several 12S rRNA gene sequences of other Gadiformes or Gadidae taxa inhabiting the Portuguese coast, such as *Merluccius merluccius* (Linnaeus 1758) or *Pollachius pollachius* (Linnaeus 1758), which match with lower similarity. The detection of *G. morhua* could be due to eDNA transport by currents from the north; however, this is rather unlikely due to the dilution rates resulting from long transport distances [83–85]. Its identification could be by transported roe instead

of eDNA. However, the known spawning areas in the North Sea and Baltic Sea [86,87], the Gulf of Maine [88] and Northern Canada [89] are far from Portugal, and horizontal transport of Atlantic cod roe is reduced (up to 20 km) [90]. The use of cod as bait by fishermen or even its disposal in some way by the small population living on Berlenga Island seems to be a reasonable explanation for this detection, resulting from environmental contamination. Although further studies are needed to clarify this detection, the results suggest the possible presence of Atlantic cod on the Portuguese coast and demonstrates the usefulness and relevance of the application of eDNA for the detection of rare or less abundant species and for fisheries management methods due to its high sensitivity.

These results confirm eDNA metabarcoding as a promising molecular tool for fisheries management and the detection of rare/uncommon species such as Atlantic salmon and Atlantic cod. However, it must be used cautiously with regard to the species identified due to false-positive site detection, i.e., erroneous occurrence at an unoccupied site [69,70], as happened with orange clown fish and blue tilapia. Critical analysis of the results is essential in order to avoid erroneous conclusions.

4.2. 12S rRNA Gene Affinity to Bony and Cartilaginous Fish

Over the last decades, tonnes of elasmobranchs landed in Portuguese ports, distributed among 58 taxa and including sharks, rays and skates [91]. The number of sharks, rays and skates species detected in this study was very low, though. Small-spotted catshark was the only species of shark detected with the 12S rRNA gene, while blue shark and shortfin mako shark were detected with COI.

In total, the number of reads detected was very low, 23 for the three species (small-spotted catshark, blue shark and shortfin mako shark). On the one hand, this suggests a low abundance of these species around Berlengas, which is in line with popular reports that many hound sharks (Family Triakidae) came to Berlengas to spawn decades ago but are now rare.

On the other hand, it can suggest a low affinity of the primers used for cartilaginous species. The 12S rRNA is the most used in fish surveys, but the region sequenced can influence the results. Kumar et al. (2022) compared three different primer sets for 12S rRNA: MiFish-12S [92], Riaz-12S [93] and Valentini-12S [94], concluding that the Riaz-12S primers were the most efficient, detecting more elasmobranch species and higher species diversity scores. The use of other markers together (multi-marker approach) or separately, such as 12S rRNA, 16S rRNA and COI, has shown advantages [95–97]. In fact, the use of a specific primer pair for the elasmobranch COI gene sequence has also been proposed [97].

Therefore, as (1) the primers used in our study focused on the same region as the MiFish primers, (2) as the Riaz-12S primer pair showed potentially better results and (3) as another work of ours with the COI gene also detected other elasmobranch species, the results of our study suggest that the low detection of elasmobranch species may not only be due to the low abundance of these species but also to the chosen primer pair.

This reinforces the need for new comparative studies using Riaz-12S/16S rRNA/COI primers and/or the use of multiple markers to confirm this trend and to understand the best primer or combination of primers that can be used to simultaneously obtain more complete data on elasmobranchs and bony fishes.

4.3. Benefits of Cooperation Between Fishers and Scientific Community

The comparison of the results provided by the two sampling sources revealed that dominant species were mostly surveyed in the FC samples (Figure 2a,b), indicating the presence of a large school of the target species of the fisheries. However, this tendency was not observed in all samples. In the samples GM05, GM09, GM11, GM13 and GM18

(Figure 2a), other species were detected in high abundance, although the same amount of the target species was caught (Table 1). As eDNA metabarcoding has shown its potential to estimate fish abundance [17,98,99], this result suggests the presence or the passage of a larger school of an untargeted species, demonstrating the sensitivity of the technique. The same was observed for some RT samples (Figure 2b).

Two main reasons may be pointed out to explain the species dominance. While, in the FC samples, it was due to the fishing activity, in the RT samples, it was due to the natural presence or passage of a species. Regardless of the origin, it affected the evenness and diversity of the species detected. Nevertheless, samples collected by fishermen can be useful for a more complete diversity assessment by detecting species not detected by the RT samples (Supplementary Information S1). In contrast to the RT samples, FC samples were generally collected in regions further away from shore, in deeper regions (open ocean). This increased the probability of detecting species compatible with such environments, such as angler *Lophius piscatorius* (Linnaeus 1758) and *Lepidotrigla* sp. (Günther 1860), which can live in greater depths [60,100,101], and longfin yellowtail *Seriola rivoliana* (Valenciennes 1833), Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus 1758) and shortfin mako shark in oceanic regions [60,102,103].

FC samples can also be useful to study correlations between specific species. In general, garfish *Belone belone* (Linnaeus 1760) was detected in samples where sardines were the most abundant species. This correlation was confirmed by fishermen who reported the presence of garfish as a by-catch when fishing for sardines, suggesting a potential interspecific relationship. According to [104], such species overlap can be explained by a predator–prey relationship, as juveniles of sardines were found in the guts of garfish caught in the Adriatic Sea.

These results showed that, despite the need to optimise the methodology, samples collected by fishers can be useful and relevant as they are in the field every day, facilitating the access to samples from a wide and variable area, and, no less importantly, they have empirical knowledge that can help to further understand some of the results. As such, increasing the volume of seawater can be an approach of optimisation, since the number of species is as great as the volume of water used [105], showing the possibility to be Vessels of Opportunity (VOO). Another approach could be to perform sample collection prior to fishing activity.

4.4. Berlengas Archipelago, an Area Rich in Fish Biodiversity

The indices of diversity determined in each geographical region might be interpreted in two ways. By excluding region C, which includes the large Berlenga Island, the results showed that, in general, species richness and diversity did not vary significantly, suggesting that fish species richness was stable up to 30 km from the coast. However, when region C is included in the analysis, there is a clear highlight of this region by the highest richness and diversity values (Figure 4a,b), as well as the most dissimilar region compared to the other regions (Figure 4c), demonstrating the high fish diversity around the large Berlenga Island. A similar trend was found by Vasco-Rodrigues et al. 2011 [106], with UVC results showing a greater number and dissimilarity of species around the large Berlenga Island compared to other sampling points of the archipelago.

In this study, the richness around the large Berlenga Island (region C) could be explained by the unequal number of RT and FC samples collected: eight and two, respectively, with the former showing higher biodiversity values. However, as Figure 4a, 4b show, the two lowest values belong to RT samples, demonstrating that Berlengas richness is not exclusively due to the greater number of species detected on the RT samples. In addition,

the second-richest regions, B and D, were mainly composed of FC samples, confirming their ability to detect diversity values as significant as RT samples.

In addition, it seemed that the high number of species occurring in region C might explain the diversity found on closer regions B and D enriched. Vasco-Rodrigues et al. 2011 [106] observed the existence of a coast-to-ocean gradient of species richness from the large Berlenga Island to the other archipelago islands of Estelas and Farilhões, with the Nazaré Canyon having a relevant influence in this gradient by its great depths [107], conferring oceanic characteristics to the most distant region of the archipelago [106]. This suggests that the geographical characteristics (shallow and deep waters) make it a biodiversity hotspot [51] with a large spectrum of species distributed in the different habitats around it, new studies being of great importance to assess the whole diversity, as Rodrigues 2012 [108] demonstrated for the area around the large Berlenga Island. The results also showed that the species richness analysis could be biased in relation to the distance from the coast, as the farthest regions, D and E, from the coast did not show lower species richness values.

Comparing our results with other studies, the same tendency of enrichment of the surrounding areas was observed in the UVC results of the Sagres Marine Protected Area (MPA) in Portugal [109] and the Scorpion State Marine Reserve (SSMR) on the island of Santa Cruz [110]. However, this trend was not observed in the eDNA metabarcoding results of the SSMR, where less biodiversity was detected outside the MPA. This may be related to the composition of the fauna and flora, biasing the true species richness [110]. Despite the possible biases observed in these studies and the unclear benefits of these areas for fisheries management [109], MPAs and the UNESCO Biosphere Reserve (UBR) suggest that they may be important for species conservation, increasing biodiversity and maintaining sustainable fisheries. Further studies are needed to clarify their role in fisheries management [109].

4.5. Seasonal Variability

There are not many studies characterising fish communities using eDNA metabarcoding, and those available show differences in collection time. Despite this, it has been shown that fish communities vary along the seasons [111–113], and in some cases, such changes have been linked to fish migration [114], spawning [115] or seasonal events [116].

In general, the results of this study showed a stable species diversity throughout the year (Figure 5b,e), with 30 species detected along all seasons (Supplementary Information S1). This suggests that they can be considered the core species that are present at the Berlengas Archipelago area throughout seasons, regardless of abundance variations, although a higher species richness was observed in the autumn (Figure 5a). The differences in the indices of diversity determined in the autumn and the spring/summer (Figure 5c) can be explained by the presence of species that have their spawning period in autumn, such as axillary seabream (from May to September (males) or November (females) [117]), golden grey mullet (between August and November [118]) and ocean sunfish (from August to October [119]). In the case of thicklip grey mullet, it has been suggested that this species spawns during the spring [120,121], which is not consistent with the obtained results. However, Arruda et al. (1991) also referred to the need for further studies to better clarify the spawning behaviour of this species. The spawning of Atlantic saury occurs during different seasons depends on the region of the Atlantic Ocean. While, in the Northeast Atlantic, it occurs during winter–spring [122,123], in Southern Africa, it occurs during summer [124]. Considering the Northeast Atlantic data, there are no agreements with our data. However, as Atlantic saury is a migratory species, it is possible that such an increase in autumn is due to its passage during migration [123] and not due to its spawning season.

5. Conclusions

Our study illustrates the potential of molecular methods, specifically the eDNA metabarcoding approach, for non-invasive monitoring and assessment of fish communities. A direct correlation between the number of individuals and collected eDNA samples was also demonstrated, one of the advantages of eDNA metabarcoding. This first study of fish communities using eDNA metabarcoding for the Berlenga Natural Reserve area revealed that autumn and the waters closer to Berlenga Island were the richest season and area, respectively. Thus, the Berlengas Reserve could act as a migration corridor for certain species, namely the migratory Atlantic saury.

Moreover, this study highlights the benefits of combining both sampling approaches (fishers and researchers) to obtain a better understanding of the dynamics of the marine environment, including protected areas, which can be important to protect biodiversity and to enrich the surrounding areas, thus contributing to a more resilient fish community and sustainable fisheries. The cooperation of fishers can be extremely valuable, not only to obtain samples by their use as VOO but also to acquire empirical knowledge assisting a better analysis of the marine ecosystem. Nevertheless, our methodology could still be further optimised in order to more consistently and accurately improve the reliability of future obtained results (e.g., the lower diversity detected by FC samples due to the bias caused by the fishing activity). Further studies are needed to better understand how the proximity of the Nazaré Canyon and the recurring upwelling events influence the fish communities of the UNESCO Biosphere Reserve of the Berlengas Archipelago in Portugal.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jmse13010060/s1: Supplementary Information S1—Excel file representing the Venn diagram results, with shared and non-shared taxa identified between the several analysis approaches. Supplementary Information S2—Methodology of metabarcoding of the COI gene (References [125,126] are cited in the Supplementary Materials).

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