oceans

MDPI

Article

The Advantages of Combining Morphological and Molecular Methods to Characterise Zooplankton Communities: A Case Study of the UNESCO Biosphere Reserve of the Berlengas Archipelago, Portugal

Marco Simões [1,2,3], Sónia Cotrim Marques [4], Cátia Costa [1,5,6], Maria da Luz Calado [1], Jorge Lobo-Arteaga [7,8], Cátia Bartilotti [7,8], Maria Jorge Campos [4], Sérgio Miguel Leandro [4] and Agostinho Antunes [2,3,*]

1 MARE—Marine and Environmental Sciences Centre/ARNET—Aquatic Research Network Associate Laboratory, Polytechnic Institute of Leiria, 2520-630 Peniche, Portugal; marco.a.simoes@ipleiria.pt (M.S.); catia.g.costa@ipleiria.pt (C.C.); maria.m.calado@ipleiria.pt (M.d.L.C.)
2 CIIMAR/CIMAR—Interdisciplinary Centre of Marine and Environmental Research, University of Porto, 4450-208 Porto, Portugal
3 Department of Biology, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal
4 MARE—Marine and Environmental Sciences Centre/ARNET—Aquatic Research Network Associate Laboratory, School of Tourism and Maritime Technology, Polytechnic Institute of Leiria, 2520–614 Peniche, Portugal; sonia.cotrim@ipleiria.pt (S.C.M.); mcampos@ipleiria.pt (M.J.C.); sergio.leandro@ipleiria.pt (S.M.L.)
5 Associate Laboratory TERRA, Department of Life Sciences, Centre for Functional Ecology, Faculty of Sciences and Technology, University of Coimbra, 3000-456 Coimbra, Portugal
6 Centre for Environmental and Marine Studies (CESAM) and Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
7 IPMA—Portuguese Institute for Sea and Atmosphere I.P., 1495-165 Lisbon, Portugal; jorge.arteaga@ipma.pt (J.L.-A.); cbartilotti@ipma.pt (C.B.)
8 MARE—Marine and Environmental Sciences Centre/ARNET—Aquatic Research Network Associate Laboratory, NOVA School of Science and Technology, NOVA University Lisbon, 2829-516 Caparica, Portugal
* Correspondence: aantunes@ciimar.up.pt

Abstract: Understanding the ecological dynamics of zooplankton communities is crucial to precisely assessing the health of marine ecosystems and their management. Metabarcoding has contributed to a better understanding of biodiversity in marine environments. However, this methodology still requires protocol optimisation. Here, we used a complementary approach combining molecular and morphological identification methods to identify the zooplankton community inhabiting the Berlengas Archipelago, Portugal. The presence of non-indigenous species was also assessed. The results showed that the metabarcoding approach outperformed the classical morphological identification method, detecting more species with higher resolution. Nevertheless, the classical method was able to identify species that were not detected by the molecular approaches, probably due to a lack of reference data in the databases. The comparison between different molecular approaches showed that COI and bulk DNA gave better results than 18S rRNA and eDNA by detecting higher species diversity. However, complementarities were observed between them. Molecular tools also proved effective in identifying several potential non-indigenous species, identifying, for the first time, several potentially unreported NIS inhabiting the Portuguese marine ecosystems. Overall, our results confirmed the importance of combining both classical and molecular methods to obtain a more refined assessment of the zooplanktonic communities in marine environments.

Keywords: community assessment; complementarity; metabarcoding; eDNA; community DNA; non-indigenous species

check for updates

Citation: Simões, M.; Cotrim Marques, S.; Costa, C.; da Luz Calado, M.; Lobo-Arteaga, J.; Bartilotti, C.; Jorge Campos, M.; Leandro, S.M.; Antunes, A. The Advantages of Combining Morphological and Molecular Methods to Characterise Zooplankton Communities: A Case Study of the UNESCO Biosphere Reserve of the Berlengas Archipelago, Portugal. *Oceans* **2024**, *5*, 805–824. https://doi.org/10.3390/oceans5040046

Academic Editor: Santiago Hernández-León

Received: 4 September 2024
Revised: 8 October 2024
Accepted: 17 October 2024
Published: 26 October 2024

1. Introduction

Marine zooplankton, comprising a wide variety of species, plays a central role in the maintenance of marine ecosystems [1,2]. Acting as both regulators of the biological

pump and contributors to global biogeochemical cycles, these organisms exert a profound influence on aquatic food webs and, consequently, on fisheries dynamics [1–6].

Beyond their ecological importance, the sensitivity of zooplankton to environmental variables [7–12] and their ubiquitous distribution, have made them valuable bioindicators for assessing the health of marine ecosystems [13–15]. This recognition positions zooplankton communities as valuable components among the Biological Quality Elements (BQE), in accordance with the European Water Framework Directive (WFD) [16], as well as the European Marine Strategy Framework Directive (EU-MSFD) [17].

Despite their ecological importance, the diversity of zooplankton species in the marine environment is not fully known. It has been estimated that approximately 1600 holoplanktonic species remain to be discovered or described [18,19]. This estimate increases to 28,000 when meroplankton is considered [18]. Therefore, the study and characterisation of zooplankton using classical taxonomy methods, as well as molecular tools, is of paramount importance in unravelling the intricacies of these undiscovered communities [20,21].

Morphological identification has been the main approach used to characterise zooplankton [16,21,22]. Typically, these identifications are made by ecologically oriented biologists, but their often-limited taxonomic expertise can make it difficult to achieve the required accuracy and consistency in species assignment [23]. In addition, the complexity of zooplankton diversity, the presence of cryptic and sibling species, and the larval stages and phases of many species make their study even more difficult, jeopardising biodiversity assessments and our understanding of zooplankton community dynamics [24–27].

DNA metabarcoding, a combination of DNA barcoding and Next-Generation Sequencing (NGS) technology, has proven highly effective in the identification of species and characterisation of communities, including zooplankton [28–31]. The advantages of DNA metabarcoding, such as increased sample processing capacity at a sustainable cost and reduced time, together with the ability to identify cryptic and sibling species—difficult to distinguish using morphological characteristics alone—and to identify species regardless of their life cycle stage or phase [21], have provided a comprehensive understanding of zooplankton biodiversity [32–36].

Although several mitochondrial and nuclear genes have been used in zooplankton metabarcoding studies [6,13,24], cytochrome c oxidase subunit I (COI) and 18S ribosomal RNA (18S rRNA) are the most frequently used [13,24], presenting different advantages. As a mitochondrial gene, COI is the standard metazoan barcode gene due to its high copy number, conserved regions, and rapid evolutionary rate, allowing high-resolution discrimination of closely related species [29]. In turn, the nuclear gene 18S rRNA, characterised by hypervariable regions bounded by conserved primer-binding regions, shows divergence patterns suitable for distinguishing invertebrate and vertebrate taxa, excelling in phyla for which COI is not an effective marker, such as Porifera, Ctenophora, Appendicularia, and Thaliacea [29,35,37–39].

Metabarcoding sequencing can be applied to DNA extracted from either bulk community environmental DNA (CeDNA) [40–42] or environmental DNA (eDNA) [23,43,44]. CeDNA, the first template in metabarcoding, has played a crucial role in exploring ecosystem diversity by revealing the "hidden" species that remained unidentified by traditional morphological methods [40,42,44]. In turn, eDNA has several advantages over CeDNA. It is easy and quick to collect and is a non-invasive method that detects species in low abundances [45–47], which are unlikely to be caught in net tows or detected by morphological techniques [45–49].

However, the use of these genetic methods for ecological status assessment needs improvement, due to a lack of standardisation and harmonisation. Progress is also needed in the determination of species abundance and the availability of more accurate reference barcode databases [16,44]. Furthermore, unlike morphological methods, molecular tools are not able to provide information on the life stage or phase and sex of zooplankton species [40,42,44], which could be relevant information to include in certain management plans.

As maritime traffic intensifies, holoplanktonic species, or species with a planktonic phase or stage, are more likely to be transported out of their native habitat by ballast water or biofouling, which can alter their distribution and cause a range of ecological and socio-economic problems [50–52]. The capabilities of molecular tools, such as their sensitivity to detect low-abundance species, have improved the detection of non-indigenous species (NIS), thereby aiding their management [53–55].

The main objectives of this study were as follows: (i) to assess the diversity of the zooplanktonic community using an integrative approach including eDNA and bulk-sample metabarcoding, along with classical morphological identification; (ii) to compare species recovery rates between the genetic markers mitochondrial COI and 18 rRNA and morphology; and (iii) to reveal hidden species diversity, including NIS. Our primary finding indicates that COI and CeDNA emerged as the most effective approaches, detecting a higher number of species. However, remarkable complementarity between classical (morphological) and molecular approaches was also observed, highlighting the value of integrating different methods for a more comprehensive understanding of zooplankton communities.

2. Materials and Methods

2.1. Sampling Methodology

Zooplankton sampling was carried out twice a year at the same station (39°25′ N, 9°31′ W) within the Berlengas Biosphere Reserve (UNESCO), Portugal, on 16 June (B04) and 23 September 2020 (B05), and on 26 June (B09) and 11 August 2021 (B10; Figure 1). The four bulk zooplankton samples were used for morphological identification and metabarcoding analysis.

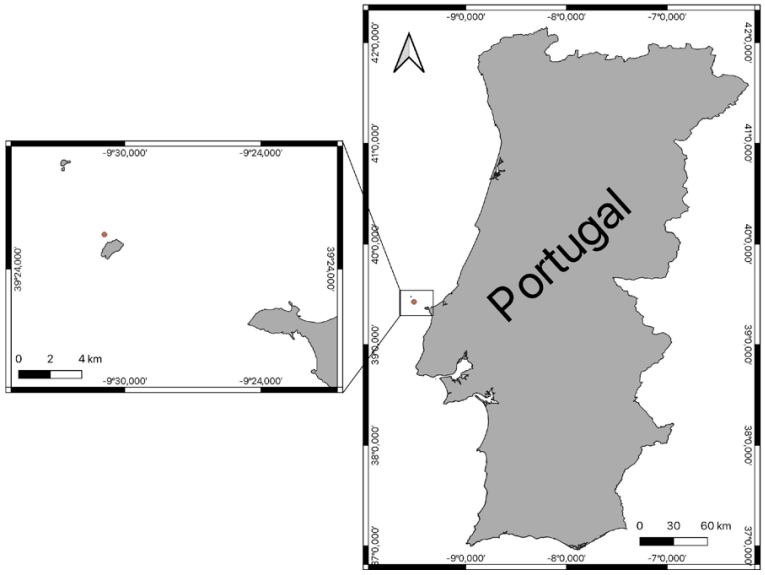

Figure 1. Geographical location of the sampling station in the Berlengas Biosphere Reserve (UNESCO). Figure created in QGIS (version: 3.28.3-Firenze). Adapted from [56].

At the same time, two litres of seawater were collected from the surface using sterile bottles for subsequent eDNA metabarcoding. At the laboratory, the collected seawater was filtered through a 0.2 µm membrane filter (Whatman, Maidstone, UK) using a vacuum pump and a sterilised filter cup. One litre of water was filtered in each filter, and the filters were stored at −80 °C until DNA extraction was carried out. A litre of sterilised water was also filtered and used as a negative control (NC).

2.2. Morphological Identification of Zooplankton Community

Zooplankton samples were collected vertically with a 57 cm diameter WP2 net (mesh size: 200 µm) from near the bottom, approximately 40 m depth, to the surface at each

station during the day. The net was retrieved at a speed of~0.4 m s^{-1} and fitted with a flowmeter (General Oceanics, model 2030 RC, Miami, FL, USA), which was used to measure the volume of water filtered (average of 20 m^3). Two net hauls were taken at each of the four sampling events. One sample was preserved in 4% neutral formalin prepared with seawater for morphological identification, and the second was immediately preserved in 96% ethanol and stored at −20 °C until DNA extraction.

In the laboratory, large (>1000 µm) and rare organisms were separated from the formalin-preserved samples, morphologically identified, and counted. The remaining sample was then subsampled using a Folsom Plankton Splitter until 300–500 individuals were obtained per fraction. All specimens in this subsample were morphologically identified to the lowest taxonomic level possible and counted under a stereomicroscope (Leica, S8APO, Wetzlar, Germany). Zooplankton abundance (ind m^{-3}) was calculated from the volume of water filtered.

2.3. DNA Extraction from Zooplankton Communities (CeDNA)

Each bulk sample was collected using nets according to the method described in Section 2.2. All handling materials were previously sterilised with 1% bleach and washed with Milli-Q water to avoid contamination. Total DNA was then extracted from each sample through the following steps: absolute ethanol was added to each sample up to a volume of 200 mL (including ethanol used for preservation), and the solution was blended and divided into four Falcon tubes. Each subsample was centrifuged (Eppendorf, Germany) at 4 °C for 10 min at 10,000 rpm, and 1 mL of the pellet was collected and centrifuged and the supernatant discarded. This pellet was dried at 50 °C for 5–10 min to remove excess ethanol. The dried pellets were then manually crushed using liquid nitrogen, and the tissue DNA extraction protocol of the Blood and Tissue Qiagen Kit (Qiagen, Hilden, Germany) was followed according to the manufacturer's instructions.

2.4. Environmental DNA Extraction from Water Samples (eDNA)

DNA extraction was performed using an adapted protocol from the manufacturer of the Qiagen Blood and Tissue kit. Using a sterilised scalpel blade, the filter was cut into thin strips 1 mm wide and 1 cm long, which were then placed in a Falcon tube. Then, 960 µL ATL (Qiagen kit buffer, Hilden, Germany) and 40 µL proteinase K were added, and the solution was vortexed and incubated at 56 °C for 2 h. The solution was manually homogenised every 10 min. Then, 1 mL absolute ethanol and 1 mL AL (Qiagen kit buffer) were added, and the solution was homogenised and transferred to the columns. The next steps were performed according to the original protocol.

2.5. Metabarcoding Procedure

The integrity of the extracted CeDNA and eDNA was then assessed by agarose gel electrophoresis, and their concentrations quantified using a Qubit fluorometer (Thermofisher Scientific, Waltham, MA, USA). Prior to sequencing, PCR was performed to verify the amplification of the selected fragments of the COI and 18S rRNA genes, using the following sets of primers: for COI, the forward primer mlCOIintF [57] and the reverse primer HCO2198 (313 base pair bp) [58], and for 18S rRNA, the forward primer 18S-F04 and the reverse primer 18S-R22 (V1–V2 region, 365 bp) [59,60]. eDNA extracted from NC was also tested for each primer pair.

After confirmed the presence of the respective DNA fragments, the extracted CeDNA and eDNA samples were sent to STABVIDA to be sequenced (Costa da Caparica, Portugal). The generated DNA fragments (DNA libraries) were sequenced with MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) on the Illumina MiSeq platform, using 2 × 300 bp paired-end sequencing reads. The standard protocol 16S Metagenomic Sequencing Library Preparation of Illumina was applied, with the following adapted conditions on the first PCR: for COI, 95 °C for 3 min, 10 cycles of 95 °C for 30 s, 57 °C–47 °C for 30 s, 72 °C for 40 s,

29 cycles of 95 °C for 30 s, 47 °C for 30 s, 72 °C for 40 s, and 72 °C for 4 min; for 18S rRNA, 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min.

2.6. Bioinformatic Analysis of Metabarcoding Data

The raw NGS data were processed and analysed through the QIIME 2 platform (version 2022.2), following the guide of the "QIIME2docs" official website [61,62]. The imported raw data were denoised (with a minimum quality of ≥30) and merged using the DADA2 plugin, excluding singletons. Taxonomic assignment was performed using the "classify-consensus-blast" function of QIIME2.

For taxonomic assignment, different percentages of identification were used due to the level of confidence offered by each gene at the species level, and different databases were used as references (summary on Table 1). The databases used for the analysis of COI data were the National Center for Biotechnology Information (NCBI), the Barcode of Life Data System (BOLD), and BOLDigger (a Python package from BOLD [63]), with a percentage of identity of ≥ 97% at the species level. The NCBI and BOLD reference databases were downloaded for use in QIIME's blast. The COI reference database from NCBI was downloaded using the Entrez Direct tool from NCBI, using as part of the script "-query "COI" efilter -query "NOT Bacteria NOT environmental NOT viruses NOT unclassified"", and the taxonomy file was obtained using the script "Entrez_qiime_py" [64], while the COI reference database of BOLD was downloaded following the R software script "COIdatabases" available on the GitHub platform, as described by Devon O'rourke and Benjamin Kaehler [65].

Table 1. A summary of the methodology used for the taxonomic assignment of COI and 18S rRNA metabarcoding ASVs, from the databases used and the corresponding download to the taxonomic classification.

	COI	**18S rRNA**
Percentage of identification (species level)	≥97%	≥99%
Databases used for taxonomic classification	NCBI BOLD BOLDigger (software)	NCBI SILVA
Database download	NCBI (downloaded for use in QIIME's blast): - Entrez Direct tool from NCBI ➔ "-query "COI" efilter -query "NOT Bacteria NOT environmental NOT viruses NOT unclassified"" ➔ Taxonomy file was obtained using the script "Entrez_qiime_py"	NCBI (downloaded for use in QIIME's blast): - Entrez Direct tool from NCBI ➔ "txid6340[ORGN] AND (18S rRNA OR 18S ribosomal RNA OR small subunit ribosomal RNA) NOT environmental sample [Title] NOT environmental samples [Title] NOT environmental [Title] NOT uncultured [Title] NOT unclassified [Title] NOT unidentified [Title] NOT unverified [Title]" ➔ Taxonomy file was obtained using the script "Entrez_qiime_py"
	BOLD (downloaded for use in QIIME's blast) ➔ Downloaded following the R software script "COIdatabases" available on the GitHub platform	SILVA (downloaded for use in QIIME's blast) ➔ Downloaded from SILVA database website

The sequences of 18S rRNA were compared with those from the NCBI and SILVA databases, with a percentage of identity of ≥99% at the species level. The NCBI 18S rRNA reference database was downloaded using the Entrez Direct tool. However, due

to sequence length, the download process was performed for different taxon groups. An example of the script used for the download was "txid6340[ORGN] AND (18S rRNA OR 18S ribosomal RNA OR small subunit ribosomal RNA) NOT environmental sample [Title] NOT environmental samples [Title] NOT environmental [Title] NOT uncultured [Title] NOT unclassified [Title] NOT unidentified [Title] NOT unverified [Title]". The generated fasta files were merged and the taxonomy file was obtained using the script "Entrez_qiime_py" [64].

Whenever the taxonomic classification of the databases did not agree at the species level, both proposals were accepted, but separated by a backslash, or verified using the Basic Local Alignment Search Tool (BLAST) in NCBI. For 18S rRNA, careful taxonomic assignment analysis was performed using BLAST in NCBI, especially when certain Amplicon Sequence Variant(s) (ASV(s)) showed multiple assignments at 99% similarity. In such cases, BLAST parameters such as Sequence Coverage, Q-score, and Total Score were used together with the biogeography of the assigned species. In order to use 18S rRNA for NIS detection, it was essential to employ a combination of BLAST parameters, in conjunction with biogeography, to restrict and refine the numerous assignments at 99% similarity. The aforementioned methodology proved invaluable in achieving the most plausible classification, given that in some ASVs, there were upwards of five potential classifications.

To check for species overlap, Venn diagrams were constructed using the R package "ggVenDiagram" ((version 1.2.3) [66]). For a correct and comparable analysis, organisms were only included if they corresponded at each taxonomy level (e.g., organisms classified only at the class level were only included in the analysis up to the class level and excluded at lower levels). At the species level, species of unclassified organisms ("sp.") were not included. After taxonomic assignment, organisms belonging to fungi, phytoplankton, non-marine organisms, and unassigned ASVs were systematically removed.

2.7. Statistical Analysis

For biodiversity and statistical analysis, both ASV tables obtained from QIIME 2 were rarefied (random subsampling) separately through the "rarefy" function of the "vegan" R package (version 2.6-4; [67]), and merged manually at the species level. Alpha-diversity was determined by the Shannon and species richness indices, using the "diversity" function of the "vegan" R package (version 2.6-4, [67]). A Tukey test was used to assess the statistical significance of alpha-diversity using the IPSUR plugin for R Commander (version 0.2-1.1, [68]). The Bray–Curtis dissimilarity index and NMDS were applied to assess beta-diversity using the "vegdist" (to generate a dissimilarity matrix) and "metaMDS" (to perform Nonmetric Multidimensional Scaling—NMDS) functions of the "vegan" R package (version 2.6-4, [67]), respectively. The "adonis2" and "betadisper" functions of the vegan R package were used to assess statistical significance between groups of samples (COI vs. 8S rRNA and eDNA vs. CeDNA) and between samples within each group, respectively (version 2.6-4, [67]). As a complement to beta-diversity, an Indicator Species Analysis statistical test was conducted using the R package "indicspecies" (version 1.7.12, [69]) to indicate the species responsible for the differences between the COI and 18S rRNA communities. Only the most significant species are shown.

2.8. Non-Indigenous Species (NIS) Detection

For NIS detection, two comparative approaches were used. The first approach was checking the species identified in this study using molecular and morphological approaches in AquaNIS—the online information system on aquatic non-indigenous species [70]—to assess which NIS reported for the Portuguese coast and surrounding coastlines were detected in this study. The second approach was based on consulting the following databases to check the species' known distribution: the World Register of Marine Species [71], the Ocean Biodiversity Information System (OBIS), and Biodiversity of Marine Planktonic Copepods (BMPC) [72]. In this case, the goal was to assess unreported NIS probably present on the Portuguese coast.

3. Results

3.1. Zooplankton Species Identification

The approaches used in this study showed different efficiencies in species detection (Table 1). Morphological methods identified 38 species, whereas molecular approaches identified 267 species. Of these 267 species, 234 were identified by COI, 97 by 18S rRNA, 105 by eDNA, and 271 by CeDNA, with many species only detected by one approach (Table 2; Supplementary Table S1).

Table 2. Number of reads and ASVs throughout bioinformatic filtering, and number of species identified by genetic markers, DNA source, and morphological method.

	Marker Genes		DNA Sources		Morphological
Totals	**COI**	**18S rRNA**	**eDNA**	**CeDNA**	
Total input reads	1,012,606	1,049,204	1,100,781	961,029	---
Total non-chimeric reads	749,150	648,605	796,070	601,685	---
Number of total ASVs	2392	1585	3021	1211	---
Total reads (filtered taxa)	316,428	368,787	324,183	361,032	---
Number of ASVs (filtered taxa)	536	166	154	590	---
Number of classes identified (filtered taxa)	20	20	22	26	14
Number of genera identified (filtered taxa)	170	85	68	193	30
Number of species identified (filtered taxa)	234	97	105	271	38
Species only retrieved by the approach	172	56	26	181	8

Regarding the NGS data, the number of reads was similar for both studied genes (Table 2), although 18S rRNA showed a slightly higher number of input reads (1,049,204) compared to COI (1,012,606). However, after sequence filtering, this trend was reversed, resulting in more COI ASVs (2392 versus 1585 from 18S rRNA). The same trend persisted for the number of ASVs after taxa filtering, despite higher total 18S rRNA reads. This difference in the number of ASVs, together with the number of taxa identified, underlines the greater ability of COI to discriminate species compared to 18S rRNA, resulting in higher number of species detected by COI. Since the PCRs of NC for COI and 18S rRNA were negative, they were not sequenced.

A comparison of the results provided by the two DNA sources revealed a notable disparity in the total input reads (Table 2), with eDNA input reads (1,100,781) showing a higher number than CeDNA (961,029). This difference persisted through the filtering process to taxa filtering. A greater number of reads, ASVs, and species were identified for CeDNA (361,032, 590, and 271, respectively). This difference in the number of reads was due to the fact that a significant number of the eDNA reads corresponded to phytoplanktonic organisms.

Despite the lower number of species identified by 18S rRNA and eDNA, both were able to detect and identify species that COI and CeDNA could not (Figure 2(B1,B2); Supplementary Table S1). Focusing on the class level (Figure 2(A1,A2)), the results provided by the two marker genes and DNA sources were also different. Specifically, the classes Tentaculata, Scyphozoa, Calcarea, and Anthozoa were detected exclusively by the 18S rRNA gene, whereas Sipuncula, Ophiuroidea, Gymnolaemata, and Cephalopoda (Annelida, Echinodermata, Bryozoa, and Mollusca phyla, respectively) were detected exclusively by the COI gene (Figure 2(A1); Supplementary Table S1). In terms of DNA sources (Figure 2(A2)), the classes Scyphozoa and Cephalopoda were detected only by eDNA, whereas Tentaculata, Sipuncula, Gymnolaemata, and Calcarea were detected only by CeDNA.

The morphological analysis identified the lowest number of classes (Figure 2(A1,A2)) and species (Figure 2(B1,B2)) compared to the molecular approaches. Nevertheless, eight species were only detected by the morphological approach (Figure 2(B1,B2); Supplementary Table S1), demonstrating that currently, not all zooplankton species can be detected by molecular approaches, due to several reasons, such as the incompleteness of databases.

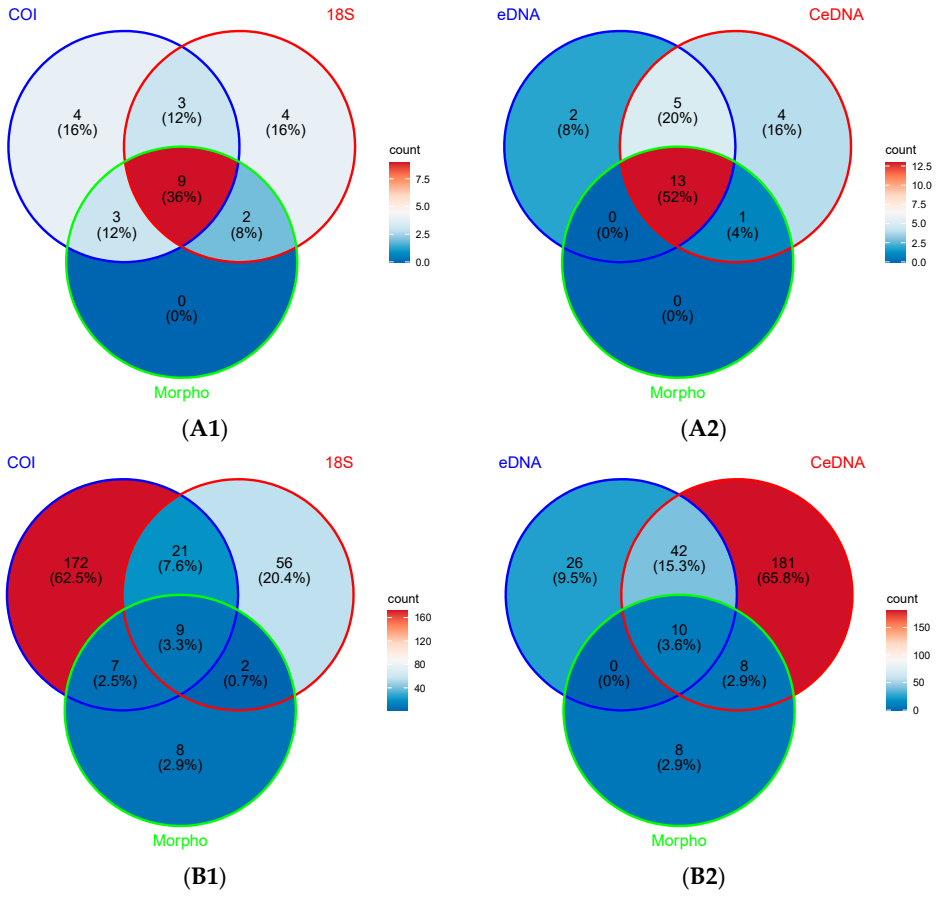

Figure 2. These Venn diagrams illustrate the number of classes (plots As) and species (plots Bs) that were detected simultaneously or exclusively by the various approaches analysed. Two comparisons were conducted: genes (COI and 18S rRNA) with morphology at the class level (**A1**) and species level (**B1**), and DNA sources (eDNA and CeDNA) with morphology at the class level (**A2**) and species level (**B2**). This figure was created using the R package "ggVenDiagram". Adapted from [65].

The bar plots in Figure 3 show that COI and CeDNA identified a greater number of classes/species than 18S rRNA and eDNA (larger grey area on COI and CeDNA bars). The most abundant species detected by both genes differed, with only 2 out of 24 species, *Paracalanus parvus* (Claus, 1863) and *Eualus cranchii* (Leach, 1817), being recovered by both genes. Copepods were one of the most represented taxa in these results, with only *P. parvus* being recovered by both genes. Another difference between the two genes was a higher ability of 18S rRNA to detect the class Hydrozoa.

Some classes and species were detected differently depending on the DNA source. For example, Ascidiacea (*Ascidia ahodori* (Oka, 1927)), Hydrozoa (*Liriope tetraphylla* (Chamisso & Eysenhardt, 1821)), and unclassified classes of the Porifera phylum were detected by eDNA, whereas Malacostraca (*E. cranchii* (Leach, 1817)), Bivalvia, and Gastropoda were detected by CeDNA.

In the morphological data, classes detected by this method were generally detected by the molecular approaches (Figure 2(A1) and Figure 3(A3)). At the species level, some taxa were classified only at the genus level, indicating difficulties in classifying some taxa (Figure 3(B3)). Despite these difficulties, this method was able to identify species not detected by the molecular approaches, such as *Centropages chierchiae* (Giesbrecht, 1889) (Figure 3(B3)).

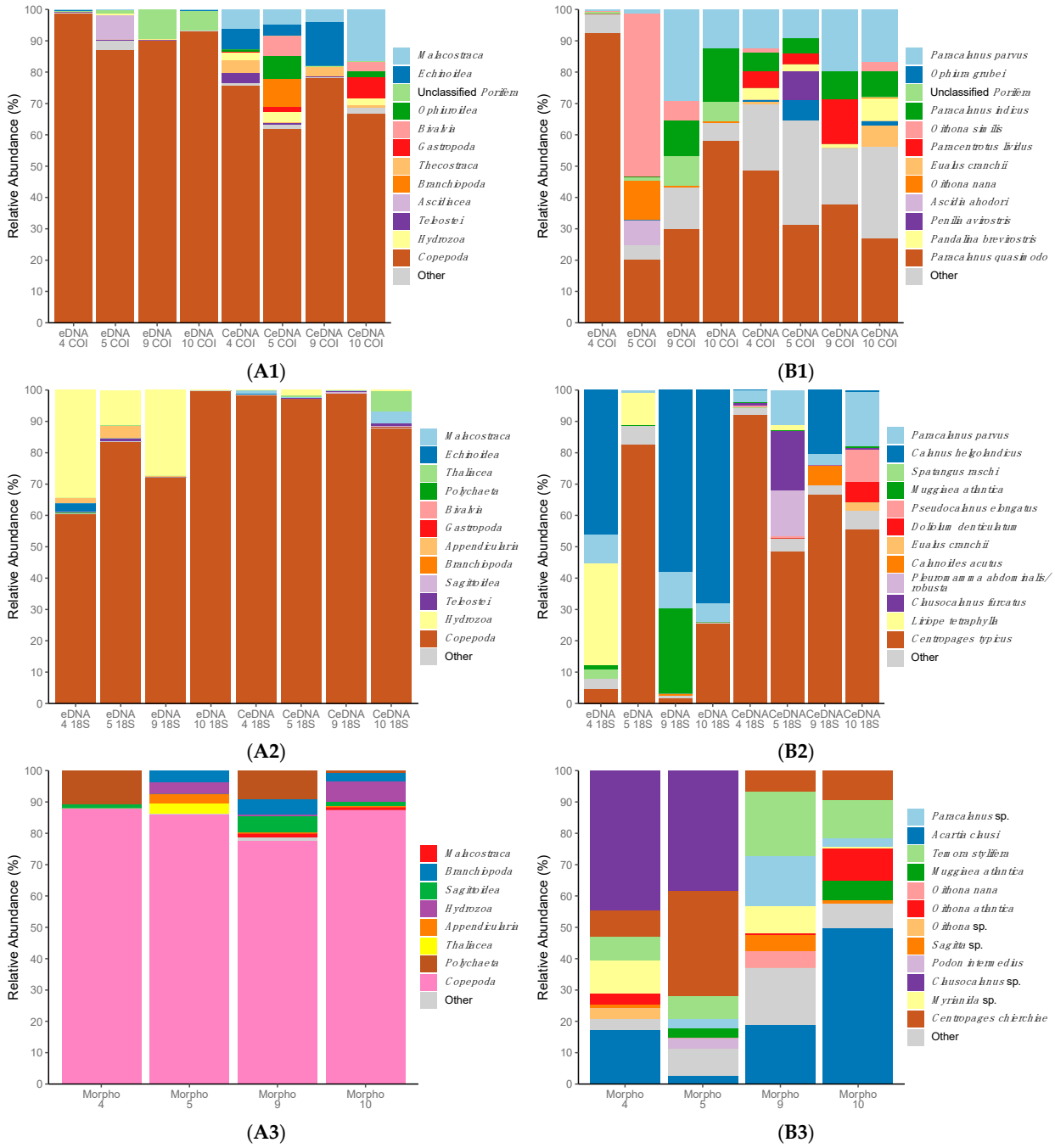

Figure 3. The relative abundance (%) of the twelve most abundant taxa identified through the various approaches (eDNA, CeDNA, and morphology), presented at the taxonomic levels of class (plots As) and species (plots Bs). The relative abundance of these taxa is also shown by the genes COI (**A1**,**B1**) and 18S rRNA (**A2**,**B2**), as well as by morphology (**A3**,**B3**). In (**A3**), only eight classes are present, as no further classes were identified. Figures created in R studio (2023.09.0+463). Adapted from [73].

3.2. Alpha- and Beta-Diversity

Prior to any ecological analysis, the data were rarefied to a depth of 13,219 and 29,183 sequences for the COI and 18S rRNA genes, respectively. Only three 18S rRNA samples were analysed for alpha- and beta-diversity due to the low sequencing depth of the BE09 sample, in order to include as many ASVs as possible in the remaining samples.

The alpha-diversity metrics showed that the combination of CeDNA-COI allowed the detection of the greatest number of species (Figure 4A), while CeDNA-18S had a higher median value than eDNA-COI and eDNA-18S samples, indicating a higher number of

species. CeDNA-COI had the highest Shannon index (Figure 4B), showing a higher species diversity and evenness of species abundance compared to the CeDNA-18S, eDNA-COI, and eDNA-18S samples. Although not statistically significant, eDNA-COI had a higher median value than eDNA-18S rRNA and CeDNA-18S rRNA, indicating higher species richness (Figure 4B). Therefore, COI and CeDNA were the molecular approaches with the highest alpha-diversity indexes.

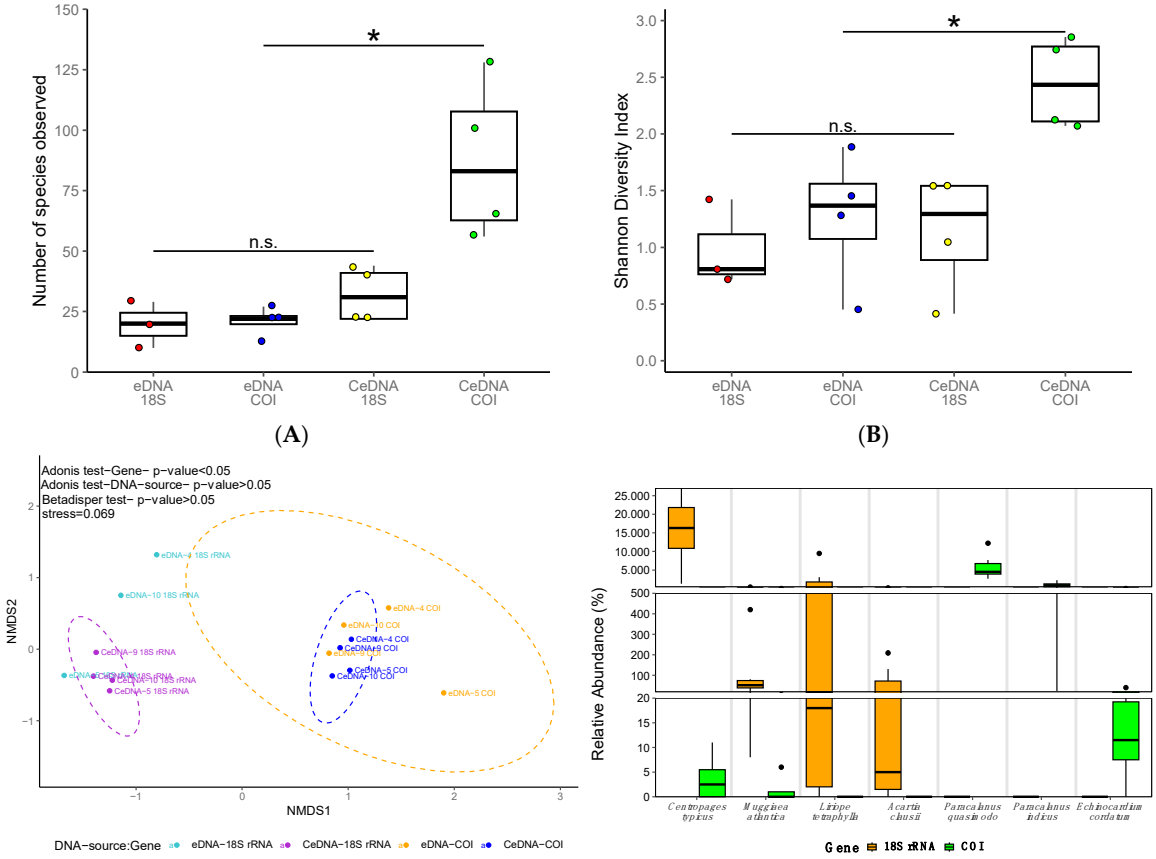

Figure 4. Ecological indexes to assess the richness (number of species observed, plot (**A**)) and diversity (Shannon index, Plot (**B**)) as alpha-diversity metrics, and the dissimilarity of the zooplankton communities (Bray–Curtis, Plot (**C**)), detected by the different molecular approaches for DNA sources and genes. To complement the Bray–Curtis analysis, the species responsible for such dissimilarity between genes were determined using the R package "indicspecies". (**A**,**B**) Tukey test, * $p < 0.05$; (**C**) adonis and betadisper statistical tests; (**D**) indicspecies, * $p < 0.05$ (only species with higher significance). These figures were created in R studio. Adapted from [73].

Beta-diversity analysis using the Bray–Curtis index showed that species diversity was similar between eDNA-COI and CeDNA-COI, and the same pattern was observed for eDNA-18S and CeDNA-18S (Figure 4C). This shows that species diversity did not differ significantly between DNA sources. However, when the molecular markers were compared, each gene formed a distinct cluster, meaning that species diversity detected by 18S rRNA was statistically different from COI. This difference was explained by an indicspecies test (Figure 4D, which showed that *Centropages typicus* (Krøyer, 1849) and *Paracalanus quasimodo* (Bowman, 1971) were two of the main contributors (Supplementary Table S2), represented as the most abundant (number of reads) by one of the genes, 18S rRNA and COI, respectively (Figure 3(B1,B2)).

3.3. Non-Indigenous Species (NIS) Detection

Based on cross-referencing the species obtained in this study with various lists from the AquaNIS database, 10 of the taxa detected in this work can be classified as NIS. Four out of the ten have already been reported to occur on the continental coast of Portugal, including Acartia (Acanthacartia) tonsa (Dana, 1849) (Copepoda), Amphibalanus improvisus (Darwin, 1854) (Cirripedia), *Austrominius modestus* (Darwin, 1854) (Amphipoda), and *Balanus trigonus* (Darwin, 1854) (Cirripedia) (Table 3). However, six species classified as NIS on the continental coast of Spain (Mediterranean and Atlantic coasts), in the Macaronesia region, in the Northeast Atlantic Ocean (from the Gulf of Biscay in France to Russia), in the Mediterranean, and in the Black Sea which have not been reported so far on the Portuguese mainland coast, were detected in this study, suggesting that they may have arrived in Portugal (Table 3). Of these 10 species, only *Oithona plumifera* (Baird, 1843) was identified through the morphological approach, while the remaining species were only detected by the molecular approach.

Table 3. Crossing of NIS detected by molecular and classical approaches with AquaNIS lists from continental Portugal, continental Spain, Macaronesia (Maca), Northeast Atlantic (excluding Portugal, Spain, and Mediterranean and Black Seas), and Mediterranean and Black Seas (excluding Spain). R-species reported in AquaNIS.

Taxa	AquaNIS Portugal	AquaNIS Spain	AquaNIS Maca	AquaNIS NE Atlantic	AquaNIS Medi-Blac Seas	Native Region
Copepoda						
Acartia (Acanthacartia) tonsa (Dana, 1849)	R	R	-	R	R	Indian and Pacific Oceans
Oithona plumifera (Baird, 1843)	-	-	-	R	R	Pacific Ocean
Clausocalanus arcuicornis arcuicornis (Dana, 1849)	-	-	-	-	R	Circum-global tropical and subtropical
Cirripedia						
Amphibalanus improvisus (Darwin, 1854)	R	R	R	R	R	NW Atlantic
Austrominius modestus (Darwin, 1854)	R	R	R	R	R	Pacific Ocean
Balanus trigonus (Darwin, 1854)	R	R	R	R	R	Indo-Pacific Oceans
Branchiopoda						
Penilia avirostris (Dana, 1849)	-	-	-	R	R	Asia, New Zealand
Ascidiacea						
Ecteinascidia turbinata (Herdman, 1880)	-	-	-	R	R	NW Atlantic Ocean
Perophora japonica (Oka, 1927)	-	R	-	R	-	NW Pacific Ocean
Hydrozoa						
Ectopleura crocea (Agassiz, 1862)	-	-	R	-	-	Atlantic coast of North America

In addition to the species classified as NIS using the AquaNIS lists (Table 3), 35 other potential NIS were detected after cross-checking their known native distribution on several databases, as described in the Materials and Methods, and scientific publications (Table 4 and Supplementary Table S3). Of these potential NIS, detected by molecular methods, the

classes with a greater number of species were Copepoda (13 species, 37%) and Gastropoda (7 species, 20%), while the classes with more reads were Copepoda (156,059) and Polychaeta (4897) (Supplementary Table S3). The copepod species with higher number of reads was *Paracalanus quasimodo*, which, according to the WoRMS, OBIS, and BMPC databases, does not occur along the Portuguese coast.

Table 4. A list of the 10 most abundant potential NIS, verified based on their known native distribution. The remaining potential species are listed in Supplementary Table S3.

List of 10 Most Abundant Potential NIS	Total Number of Reads	Gene	Native Region
Paracalanus quasimodo (Bowman, 1971)	119669	COI	Gulf of Mexico
Paracalanus indicus (Wolfenden, 1905)	22447	COI	Tropical and subtropical waters
Oncaea waldemari (Bersano & Boxshall, 1996)	6054	COI	Southern Brazil
Calanoides acutus (Giesbrecht, 1902)	4217	18S rRNA	Antarctic Ocean
Oncaea scottodicarloi (Heron & Bradford-Grieve, 1995)	1284	COI	Pacific Ocean
Ascidia ahodori (Oka, 1927)	1273	COI	Northwest Pacific
Subeucalanus subtenuis (Giesbrecht, 1888)	1241	18S rRNA	Circum-global tropical and subtropical waters
Ectopleura dumortierii (Van Beneden, 1844)	1132	COI	Mediterranean Sea, northeast (France and UK) and northwest Atlantic
Nyctiphanes simplex (Hansen, 1911)	1051	18S rRNA	North and equatorial Pacific
Temora stylifera (Dana, 1849)	518	COI	Western Pacific and Western Central Atlantic

4. Discussion

4.1. Databases' Influence and Complementarity Between COI and 18S rRNA

Among the genes studied, COI showed better results than 18S rRNA, detecting more ASVs (536 for COI, 166 for 18S rRNA) and identifying a greater number of species (234 for COI, 97 for 18S rRNA) (Table 2). This performance is further evidenced by the Venn diagrams (Figure 2(B2)) as well as the higher alpha-diversity indices observed for COI compared to 18S rRNA (Figure 4A,B). One possible explanation for the observed differences is that mitochondrial DNA is more abundant within cell organisms than nuclear DNA [29], making it more detectable in the marine environment. This high detectability increases the likelihood of capturing a wider range of species, including those that are less abundant or considered rare.

The variable number of sequences of each gene published in databases could also explain the results obtained. As COI is often successful in discriminating genetic differentiation in terms of both intra- and interspecific divergence [74], and presents conserved regions that allow us to design conserved primers for a large spectrum of metazoan phyla [58] despite some re-designing for specific groups [75,76], it has become a gold standard gene marker [77]. Thus, the amount of metazoan data available in public databases is significantly higher for COI than for 18S rRNA [29,35], increasing the probability of matching metabarcoding sequences. Currently, the NCBI and BOLD databases contain approximately 3,000,000 and 700,000 available COI sequences, respectively, while the NCBI and SILVA databases contain approximately 150,000 and 50,000 18S rRNA available sequences, respectively. To improve species recovery, this study used two databases for each gene, rather than a single database per gene, as is common in this type of study [23,35,39,40,43,78,79].

In addition to the number of sequences available, the completeness of the databases and the morphological similarity of the species can also influence the taxonomic classi-

fication of ASVs. For example, *C. typicus* was only detected by molecular methods, and according to the OBIS, WoRMS, and BMPC databases, this species occurs on the continental coast of Portugal. However, instead of *C. typicus*, the species has always been morphologically identified as *Centropages chierchiae* in this region. Despite the lack of existence of 18S rRNA sequences for *C. chierchiae*, the observed incongruence could not be a consequence of the incompleteness of the databases, because both genes matched *C. typicus* with 99% similarity or higher. Similar situations have occurred in previous studies for other organisms [23,33–35,80], demonstrating the importance of continuously updating and ensuring the accuracy of public databases in collaboration with taxonomists to overcome this lack of data [23,33,35,80–82], or constructing specific geographic sequence databases [21,29,42]. Therefore, the most probable explanation for this incongruence can be the morphological misidentification of the two species, due to their similarity [83], suggesting a cryptic species complex for *C. typicus* and *C. chierchiae*, requiring further clarification studies.

The genes selected and their affinity to the organisms studied can also influence the results. A significant number of ASVs were excluded from the 18S rRNA classification because the matches were phytoplanktonic organisms (~44%), a result consistent with several authors (e.g., [38,84,85]). Another difficulty was the lack of species-level resolution of 18S rRNA, so a higher BLAST similarity percentage was used, although some taxa were still undifferentiated, such as *Pleuromamma abdominalis* and *Pleuromamma robusta*. While on average, the 18S rRNA gene was less effective than COI, it complemented the data by detecting taxa not depicted by the COI [33,38,39], including some copepods such as the genus *Pleuromamma* and the species *Calanoides acutus*. In addition to copepod species, the 18S rRNA gene was also very useful to complement the lack of compatibility of COI with some phyla, detecting taxa from Ctenophora, Cnidaria, and Porifera. As generally accepted, the COI gene is unable to detect or identify the lowest taxonomic levels of some of these taxa [29,33,35,39]. This complementarity between genes demonstrated the advantage of using two gene markers [23,34,38,39] to characterise zooplankton communities. The use of a third gene has also been proposed [23,80], such as 12S rRNA for the selective detection of vertebrates [86,87], 16S rRNA for a better resolution of Cnidaria and Copepoda [88–90], and internal transcribed spacer (ITS) for Ctenophora [91]. It could therefore contribute to more comprehensive characterisation of zooplanktonic communities.

4.2. Complementarity Between DNA Sources and Optimisation of the eDNA Approach

Over the last decade, eDNA has emerged as another source of DNA that can provide information on the diversity of marine communities, as it is a simple and non-invasive sampling method. In this study, the use of eDNA accounted for the detection of only one-third of the species when compared to the use of CeDNA (Table 1). To our knowledge, only two studies have focused on the comparison between bulk and eDNA metabarcoding of zooplankton samples [23,79], and the results obtained in these studies are not consistent. While Djurhuus et al. (2018) [23] observed higher species richness in eDNA than in CeDNA samples, Suter et al. (2021) observed higher richness in CeDNA than in eDNA. A possible reason for the observed differences could be the volume of the samples used in the two studies, and consequently, the eDNA concentration. The authors of Djurhuus et al. (2018) [23] used 1 L of seawater in triplicate, while Suter et al. (2021) considered half of the membrane to filter 2 L of seawater (technically 1 L), the same as in this study. In addition, Suter et al. (2021) also found that the eDNA metabarcoding sequencing of larger volumes of filtered seawater (~2200 L) detected approximately the same species richness as bulk metabarcoding samples, highlighting the importance of analysing higher volumes of water to obtain a more reliable representation of zooplankton communities. Given the fact that higher volumes of water are logistically more difficult to process, Suter et al. (2021) recommended the use of multiple small volumes.

Despite the small number of species identified in the eDNA samples, these results were essential in complementing those from the CeDNA samples. Indeed, eDNA demonstrated

its effectiveness in detecting planktonic or larval stages of benthic organisms such as *Ascidia ahodori* and Porifera (3451 COI reads), which were detected in much lower abundance by CeDNA (264 COI reads). This difference may be due to the source of the DNA molecules. On the one hand, the higher abundance detected by eDNA could be due to the sum of two sources of DNA, digestive remains from fish faeces that feed on these species [92–94], and the organisms living in the water column in the planktonic phase or stage, as well as on benthos. On the other hand, CeDNA only has the living organisms in the water column as a source of DNA. Furthermore, this difference may also be related to the short planktonic period of these organisms [95,96], which makes them more difficult to detect using CeDNA than eDNA.

In addition, eDNA successfully identified pelagic organisms, specifically *Oithona similis*, *Oithona nana*, and *Muggiaea atlantica*, that were not detected by CeDNA. In the case of the genus Oithona, this could be due to the vertical distribution of the organisms [97,98], while in the case of *M. atlantica*, it could be due to the fragility of the organisms during net collection [99]. This highlights the nuanced and complementary nature of eDNA and CeDNA in capturing different facets of the marine environment, and demonstrates their distinct strengths in revealing the presence of specific taxa across different ecological niches and life stages. One potential solution to the challenge of detecting species with diel migration through eDNA is the collection of sea water samples at different depths, which could help to circumvent this bias.

4.3. Benefits of Combining Classical and Molecular Approaches

Although the molecular approach offers clear advantages, reliance on morphology-based identification remains essential. The use of both approaches provides a synergistic framework that allows data to be cross-checked. This not only facilitates database refinement, but also serves as a mechanism for correcting taxonomic classification using the classical method, thereby increasing the robustness of both techniques. Despite the limitations of the morphological approach, such as its reduced ability to detect cryptic species complexes and organism life stages or phases, especially in the case of meroplankton, and a comparatively lower number of species recovered by the morphological method, our results highlight the importance of an integrative approach [34,35,80]. In addition, the morphological method provides valuable supplementary information, including details of the life stages [23,35,80,100] and abundance of organisms. Therefore, a comprehensive understanding of zooplankton communities requires the simultaneous application of both approaches [23,35,100], which not only increases the accuracy of taxonomic identification, but also provides a more holistic perspective that enriches ecological studies of zooplankton communities [34,43].

4.4. Detection of Non-Indigenous Species Through Metabarcoding Analysis

The identification and tracking of NIS are fundamental aspects of marine ecology and conservation. The arrival and spread of NIS, enabled by shipping, particularly ballast waters [101], poses a significant threat to marine ecosystems worldwide [50,102,103]. Recognising the ecological and economic impacts of NIS introductions [50,51], researchers are increasingly relying on advanced molecular techniques such as metabarcoding for their efficient early detection [35,104]. In this study, molecular methods played a key role in the identification of NIS on the continental coast of Portugal. Known NIS inhabiting the Portuguese mainland were successfully detected, allowing a thorough assessment of their presence (Table 3). Also, NIS that have not been reported on the Portuguese mainland coast were detected through molecular methods (Tables 3 and 4), suggesting their presence, warning that further studies are needed for confirmation.

One of the unreported species (Table 4), among other potential examples, was *P. quasimodo*, native to the Gulf of Mexico. In the present study, it had the highest number of reads using COI. The authors of Stefanni et al. (2018) [35] detected this species for the first time in the Adriatic Sea using metabarcoding. However, according to the authors, it is

likely that erroneous morphological and genetic identification with its congeners *P. parvus* or *P. indicus* prevented the detection of *P. quasimodo* in the western Mediterranean and Black Sea years earlier [35,72,105,106]. Therefore, it can be hypothesised that *P. quasimodo* and *P. indicus* are currently inhabiting the Portuguese mainland coast, although morphological identification is required to confirm the presence of both species. This finding highlights the effectiveness of metabarcoding in not only verifying the presence of established NIS, but also shedding light on new and potentially invasive species that might otherwise have gone unnoticed.

5. Conclusions

In conclusion, the present study clearly shows that an integrative approach, by combining metabarcoding with morphological identification, CeDNA with eDNA, and at least two genetic markers, provides very useful complementarity, improving the evaluation of ecological analyses that can be applied to the management of marine ecosystems and marine fisheries, as well as to the detection of NIS. These findings highlight the multiple benefits of integrating different approaches, ultimately establishing metabarcoding as a powerful tool in the wider field of marine ecology and conservation. These results serve to improve our understanding of the diversity and dynamics of marine species, while providing vital information for informed decision making and proactive management strategies in the marine environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/oceans5040046/s1, Supplementary Table S1—Excel file representing species list obtained from Venn diagram results, with taxa identified only by each gene, DNA source, morphology, and taxa in common between these approaches. Supplementary Table S2—Excel file presenting all significant species using indicspecies test, with all taxa responsible for the divergence in beta-diversity. Supplementary Table S3—Excel file with a list of all potential NISacording to their native distribution, checked on several databases.

Author Contributions: M.S. performed the research, analysed the data, and wrote the original draft; S.C.M. collected the data, performed the research, analysed the data, and reviewed and edited the manuscript; C.C. performed the research and reviewed and edited the manuscript; M.d.L.C. performed the research and reviewed and edited the manuscript; J.L.-A. contributed to valuable discussions of the obtained results and reviewed and edited the manuscript; C.B. contributed to valuable discussions of the obtained results and reviewed and edited the manuscript; M.J.C. designed the research, supervised the research, and reviewed and edited the manuscript; S.M.L. designed the research, collected the data, supervised the research, and reviewed and edited the manuscript; A.A. designed research, supervised the research, and reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The present work was supported by the Portugal 2020 program, through the project e-Fishing (MAR20-01-77P3-FEAMP-000006), the Strategic Project granted to MARE—Marine and Environmental Sciences Centre (UIDB/04292/2020 https://doi.org/10.54499/UIDB/04292/2020, and UIDP/04292/2020, https://doi.org/10.54499/UIDP/04292/2020), the project granted to the Associate Laboratory ARNET (LA/P/0069/2020, https://doi.org/10.54499/LA/P/0069/2020), and the FCT grant awarded to Marco Simões (2020.07688.BD). Cátia Costa and Maria da Luz Calado were each supported by a grant from the e-Fishing Project. Agostinho Antunes was partially supported by Strategic Funding, UIDB/04423/2020 and UIDP/04423/2020, through national funds provided by FCT and the European Regional Development Fund (ERDF) in the framework of the program PT2020, and by the European Structural and Investment Funds (ESIF) through the Competitiveness and Internationalization Operational Program—COMPETE 2020.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw sequences are available on National Center for Biotechnology Information (NCBI) database under GenBank accession no. PRJNA956157.

Acknowledgments: We would like to thank the anonymous reviewers for their time and constructive suggestions.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Lampert, W. Zooplankton research: The contribution of limnology to general ecological paradigms. *Aquat. Ecol.* **1997**, *31*, 19–27. [CrossRef]
2. Stemmann, L.; Boss, E. Plankton and Particle Size and Packaging: From Determining Optical Properties to Driving the Biological Pump. *Annu. Rev. Mar. Sci.* **2012**, *4*, 263–290. [CrossRef] [PubMed]
3. Daewel, U.; Hjollo, S.S.; Huret, M.; Ji, R.B.; Maar, M.; Niiranen, S.; Travers-Trolet, M.; Peck, M.A.; van de Wolfshaar, K.E. Predation control of zooplankton dynamics: A review of observations and models. *ICES J. Mar. Sci.* **2014**, *71*, 254–271. [CrossRef]
4. Jeong, H.J.; Kim, J.S.; Lee, K.H.; Seong, K.A.; Du Yoo, Y.; Kang, N.S.; Kim, T.H.; Song, J.Y.; Kwon, J.E. Differential interactions between the nematocyst-bearing mixotrophic dinoflagellate *Paragymnodinium shiwhaense* and common heterotrophic protists and copepods: Killer or prey. *Harmful Algae* **2017**, *62*, 37–51. [CrossRef]
5. Morabito, G.; Mazzocchi, M.G.; Salmaso, N.; Zingone, A.; Bergami, C.; Flaim, G.; Accoroni, S.; Basset, A.; Bastianini, M.; Belmonte, G.; et al. Plankton dynamics across the freshwater, transitional and marine research sites of the LTER-Italy Network. Patterns, fluctuations, drivers. *Sci. Total Environ.* **2018**, *627*, 373–387. [CrossRef]
6. Schroeder, A.; Pallavicini, A.; Edomi, P.; Pansera, M.; Camatti, E. Suitability of a dual COI marker for marine zooplankton DNA metabarcoding. *Mar. Environ. Res.* **2021**, *170*, 105444. [CrossRef]
7. Beaugrand, G. Monitoring pelagic ecosystems using plankton indicators. *ICES J. Mar. Sci.* **2005**, *62*, 333–338. [CrossRef]
8. Beaugrand, G.; Edwards, M.; Legendre, L. Marine biodiversity, ecosystem functioning, and carbon cycles. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 10120–10124. [CrossRef]
9. Hay, S. Marine ecology: Gelatinous bells may ring change in marine ecosystems. *Curr. Biol.* **2006**, *16*, R679–R682. [CrossRef]
10. Polyakov, I.V.; Alkire, M.B.; Bluhm, B.A.; Brown, K.A.; Carmack, E.C.; Chierici, M.; Danielson, S.L.; Ellingsen, I.; Ershova, E.A.; Gardfeldt, K.; et al. Borealization of the Arctic Ocean in Response to Anomalous Advection from Sub-Arctic Seas. *Front. Mar. Sci.* **2020**, *7*, 491. [CrossRef]
11. Weydmann, A.; Carstensen, J.; Goszczko, I.; Dmoch, K.; Olszewska, A.; Kwasniewski, S. Shift towards the dominance of boreal species in the Arctic: Inter-annual and spatial zooplankton variability in the West Spitsbergen Current. *Mar. Ecol. Prog. Ser.* **2014**, *501*, 41–52. [CrossRef]
12. Weydmann, A.; Walczowski, W.; Carstensen, J.; Kwasniewski, S. Warming of Subarctic waters accelerates development of a key marine zooplankton Calanus finmarchicus. *Glob. Chang. Biol.* **2018**, *24*, 172–183. [CrossRef] [PubMed]
13. Bucklin, A.; Peijnenburg, K.; Kosobokova, K.N.; O'Brien, T.D.; Blanco-Bercial, L.; Cornils, A.; Falkenhaug, T.; Hopcroft, R.R.; Hosia, A.; Laakmann, S.; et al. Toward a global reference database of COI barcodes for marine zooplankton. *Mar. Biol.* **2021**, *168*, 1–26. [CrossRef]
14. Gannon, J.E.; Stemberger, R.S. Zooplankton (Especially Crustaceans and Rotifers) As Indicators of Water-Quality. *Trans. Am. Microsc. Soc.* **1978**, *97*, 16–35. [CrossRef]
15. Ferdous, Z.; Muktadir, A. A Review: Potentiality of Zooplankton as Bioindicator. *Am. J. Appl. Sci.* **2009**, *6*, 1815–1819. [CrossRef]
16. Hering, D.; Borja, A.; Jones, J.I.; Pont, D.; Boets, P.; Bouchez, A.; Bruce, K.; Drakare, S.; Hanfling, B.; Kahlert, M.; et al. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water Res.* **2018**, *138*, 192–205. [CrossRef]
17. Ndah, A.B.; Meunier, C.L.; Kirstein, I.V.; Göbel, J.; Rönn, L.; Boersma, M. A systematic study of zooplankton-based indices of marine ecological change and water quality: Application to the European marine strategy framework Directive (MSFD). *Ecol. Indic.* **2022**, *135*, 108587. [CrossRef]
18. Lenz, J. 1—Introduction. *ICES Zooplankton Methodol. Man.* **2000**, *11*, 215–226. [CrossRef]
19. Wiebe, P.H.; Bucklin, A.; Madin, L.; Angel, M.V.; Sutton, T.; Pages, F.; Hopcroft, R.R.; Lindsay, D. Deep-sea sampling on CMarZ cruises in the Atlantic Ocean—An Introduction. *Deep Sea Res. Part II Top. Stud. Oceanogr.* **2010**, *57*, 2157–2166. [CrossRef]
20. Bucklin, A.; Batta-Lona, P.G.; Questel, J.M.; Wiebe, P.H.; Richardson, D.E.; Copley, N.J.; O'Brien, T.D. COI Metabarcoding of Zooplankton Species Diversity for Time-Series Monitoring of the NW Atlantic Continental Shelf. *Front. Mar. Sci.* **2022**, *9*, 867893. [CrossRef]
21. Matos, A.; Ledoux, J.-B.; Domínguez Pérez, D.; Almeida, D.; Antunes, A. *Omics Advances in the Study of Zooplankton*; CRC Press: Boca Raton, FL, USA, 2020; pp. 264–277. [CrossRef]
22. Bucklin, A.; Ortman, B.D.; Jennings, R.M.; Nigro, L.M.; Sweetman, C.J.; Copley, N.J.; Sutton, T.; Wiebe, P.H. A "Rosetta Stone" for metazoan zooplankton: DNA barcode analysis of species diversity of the Sargasso Sea (Northwest Atlantic Ocean). *Deep Sea Res. Part II Top. Stud. Oceanogr.* **2010**, *57*, 2234–2247. [CrossRef]

23. Djurhuus, A.; Pitz, K.; Sawaya, N.A.; Rojas-Marquez, J.; Michaud, B.; Montes, E.; Muller-Karger, F.; Breitbart, M. Evaluation of marine zooplankton community structure through environmental DNA metabarcoding. *Limnol. Oceanogr. Methods* **2018**, *16*, 209–221. [CrossRef]
24. Bucklin, A.; Lindeque, P.K.; Rodriguez-Ezpeleta, N.; Albaina, A.; Lehtiniemi, M. Metabarcoding of marine zooplankton: Prospects, progress and pitfalls. *J. Plankton Res.* **2016**, *38*, 393–400. [CrossRef]
25. Choquet, M.; Kosobokova, K.; Kwasniewski, S.; Hatlebakk, M.; Dhanasiri, A.K.S.; Melle, W.; Daase, M.; Svensen, C.; Soreide, J.E.; Hoarau, G. Can morphology reliably distinguish between the copepods Calanus finmarchicus and C-glacialis, or is DNA the only way? *Limnol. Oceanogr. Methods* **2018**, *16*, 237–252. [CrossRef]
26. Knowlton, N. Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia* **2000**, *420*, 73–90. [CrossRef]
27. Lindsay, D.J.; Grossmann, M.M.; Bentlage, B.; Collins, A.G.; Minemizu, R.; Hopcroft, R.R.; Miyake, H.; Hidaka-Umetsu, M.; Nishikawa, J. The perils of online biogeographic databases: A case study with the 'monospecific' genus Aegina (Cnidaria, Hydrozoa, Narcomedusae). *Mar. Biol. Res.* **2017**, *13*, 494–512. [CrossRef]
28. Brannock, P.M.; Waits, D.S.; Sharma, J.; Halanych, K.M. High-Throughput Sequencing Characterizes Intertidal Meiofaunal Communities in Northern Gulf of Mexico (Dauphin Island and Mobile Bay, Alabama). *Biol. Bull.* **2014**, *227*, 161–174. [CrossRef]
29. Bucklin, A.; Steinke, D.; Blanco-Bercial, L. DNA Barcoding of Marine Metazoa. *Annu. Rev. Mar. Sci.* **2011**, *3*, 471–508. [CrossRef]
30. Coissac, E.; Riaz, T.; Puillandre, N. Bioinformatic challenges for DNA metabarcoding of plants and animals. *Mol. Ecol.* **2012**, *21*, 1834–1847. [CrossRef]
31. Taberlet, P.; Coissac, E.; Pompanon, F.; Brochmann, C.; Willerslev, E. Towards next-generation biodiversity assessment using DNA metabarcoding. *Mol. Ecol.* **2012**, *21*, 2045–2050. [CrossRef]
32. Carroll, E.L.; Gallego, R.; Sewell, M.A.; Zeldis, J.; Ranjard, L.; Ross, H.A.; Tooman, L.K.; O'Rorke, R.; Newcomb, R.D.; Constantine, R. Multi-locus DNA metabarcoding of zooplankton communities and scat reveal trophic interactions of a generalist predator. *Sci. Rep.* **2019**, *9*, 281. [CrossRef]
33. Cicala, F.; Arteaga, M.C.; Herzka, S.Z.; Hereu, C.M.; Jimenez-Rosenberg, S.P.A.; Saavedra-Flores, A.; Robles-Flores, J.; Gomez, R.; Batta-Lona, P.G.; Galindo-Sanchez, C.E. Environmental conditions drive zooplankton community structure in the epipelagic oceanic water of the southern Gulf of Mexico: A molecular approach. *Mol. Ecol.* **2022**, *31*, 546–561. [CrossRef]
34. Clarke, L.J.; Beard, J.M.; Swadling, K.M.; Deagle, B.E. Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecol. Evol.* **2017**, *7*, 873–883. [CrossRef]
35. Stefanni, S.; Stankovic, D.; Borme, D.; de Olazabal, A.; Juretic, T.; Pallavicini, A.; Tirelli, V. Multi-marker metabarcoding approach to study mesozooplankton at basin scale. *Sci. Rep.* **2018**, *8*, 12085. [CrossRef]
36. Zhang, G.K.; Chain, F.J.J.; Abbott, C.L.; Cristescu, M.E. Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. *Evol. Appl.* **2018**, *11*, 1901–1914. [CrossRef]
37. Giebner, H.; Langen, K.; Bourlat, S.J.; Kukowka, S.; Mayer, C.; Astrin, J.J.; Misof, B.; Fonseca, V.G. Comparing diversity levels in environmental samples: DNA sequence capture and metabarcoding approaches using 18S and COI genes. *Mol. Ecol. Resour.* **2020**, *20*, 1333–1345. [CrossRef]
38. Wangensteen, O.S.; Palacin, C.; Guardiola, M.; Turon, X. DNA metabarcoding of littoral hard-bottom communities: High diversity and database gaps revealed by two molecular markers. *PeerJ* **2018**, *6*, e4705. [CrossRef]
39. Zhao, L.N.; Zhang, X.; Xu, M.Y.; Mao, Y.; Huang, Y. DNA metabarcoding of zooplankton communities: Species diversity and seasonal variation revealed by 18S rRNA and COI. *PeerJ* **2021**, *9*, e11057. [CrossRef]
40. Lindeque, P.K.; Parry, H.E.; Harmer, R.A.; Somerfield, P.J.; Atkinson, A. Next Generation Sequencing Reveals the Hidden Diversity of Zooplankton Assemblages. *PLoS ONE* **2013**, *8*, e81327. [CrossRef]
41. Lobo, J.; Shokralla, S.; Costa, M.H.; Hajibabaei, M.; Costa, F.O. DNA metabarcoding for high-throughput monitoring of estuarine macrobenthic communities. *Sci. Rep.* **2017**, *7*, 15618. [CrossRef]
42. Yang, J.H.; Zhang, X.W.; Xie, Y.W.; Song, C.; Zhang, Y.; Yu, H.X.; Burton, G.A. Zooplankton Community Profiling in a Eutrophic Freshwater Ecosystem-Lake Tai Basin by DNA Metabarcoding. *Sci. Rep.* **2017**, *7*, 1773. [CrossRef]
43. Garcia-Vazquez, E.; Georges, O.; Fernandez, S.; Ardura, A. eDNA metabarcoding of small plankton samples to detect fish larvae and their preys from Atlantic and Pacific waters. *Sci. Rep.* **2021**, *11*, 7224. [CrossRef] [PubMed]
44. Yang, J.H.; Zhang, X.W. eDNA metabarcoding in zooplankton improves the ecological status assessment of aquatic ecosystems. *Environ. Int.* **2020**, *134*, 105230. [CrossRef]
45. Davy, C.M.; Kidd, A.G.; Wilson, C.C. Development and Validation of Environmental DNA (eDNA) Markers for Detection of Freshwater Turtles. *PLoS ONE* **2015**, *10*, e0130965. [CrossRef]
46. Flynn, J.M.; Brown, E.A.; Chain, F.J.J.; MacIsaac, H.J.; Cristescu, M.E. Toward accurate molecular identification of species in complex environmental samples: Testing the performance of sequence filtering and clustering methods. *Ecol. Evol.* **2015**, *5*, 2252–2266. [CrossRef]
47. Harvey, J.B.J.; Johnson, S.B.; Fisher, J.L.; Peterson, W.T.; Vrijenhoek, R.C. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. *J. Exp. Mar. Biol. Ecol.* **2017**, *487*, 113–126. [CrossRef]
48. Kelly, R.P.; O'Donnell, J.L.; Lowell, N.C.; Shelton, A.O.; Samhouri, J.F.; Hennessey, S.M.; Feist, B.E.; Williams, G.D. Genetic signatures of ecological diversity along an urbanization gradient. *PeerJ* **2016**, *4*, e2444. [CrossRef]
49. Olds, B.P.; Jerde, C.L.; Renshaw, M.A.; Li, Y.Y.; Evans, N.T.; Turner, C.R.; Deiner, K.; Mahon, A.R.; Brueseke, M.A.; Shirey, P.D.; et al. Estimating species richness using environmental DNA. *Ecol. Evol.* **2016**, *6*, 4214–4226. [CrossRef]

50. Sala, E.; Knowlton, N. Global marine biodiversity trends. *Annu. Rev. Environ. Resour.* **2006**, *31*, 93–122. [CrossRef]
51. Bailey, S.A.; Brown, L.; Campbell, M.L.; Canning-Clode, J.; Carlton, J.T.; Castro, N.; Chainho, P.; Chan, F.T.; Creed, J.C.; Curd, A.; et al. Trends in the detection of aquatic non-indigenous species across global marine, estuarine and freshwater ecosystems: A 50-year perspective. *Divers. Distrib.* **2020**, *26*, 1780–1797. [CrossRef]
52. Bailey, S.A.; Brydges, T.; Casas-Monroy, O.; Kydd, J.; Linley, R.D.; Rozon, R.M.; Darling, J.A. First evaluation of ballast water management systems on operational ships for minimizing introductions of nonindigenous zooplankton. *Mar. Pollut. Bull.* **2022**, *182*, 113947. [CrossRef] [PubMed]
53. Ardura, A.; Borrell, Y.J.; Fernandez, S.; Arenales, M.G.; Martinez, J.L.; Garcia-Vazquez, E. Nuisance Algae in Ballast Water Facing International Conventions. Insights DNA Metabarcoding Ships Arriving Bay Biscay. *Water* **2020**, *12*, 2168. [CrossRef]
54. Ardura, A.; Zaiko, A.; Martinez, J.L.; Samulioviene, A.; Semenova, A.; Garcia-Vazquez, E. eDNA and specific primers for early detection of invasive species—A case study on the bivalve Rangia cuneata, currently spreading in Europe. *Mar. Environ. Res.* **2015**, *112*, 48–55. [CrossRef] [PubMed]
55. Borrell, Y.J.; Miralles, L.; Do Huu, H.; Mohammed-Geba, K.; Garcia-Vazquez, E. DNA in a bottle-Rapid metabarcoding survey for early alerts of invasive species in ports. *PLoS ONE* **2017**, *12*, e0183347. [CrossRef]
56. QGIS. *QGIS Geographic Information System. Open Source Geospatial Foundation Project*; Version: 3.28.3; QGIS Development Team: Firenze, Italy, 2024.
57. Leray, M.; Yang, J.Y.; Meyer, C.P.; Mills, S.C.; Agudelo, N.; Ranwez, V.; Boehm, J.T.; Machida, R.J. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. Frontiers in Zoology. *Front Zool* **2013**, *10*, 34. [CrossRef]
58. Folmer, O.; Black, M.; Wr, H.; Lutz, R.; Vrijenhoek, R. DNA primers for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **1994**, *3*, 294–299.
59. Pitz, K.J.; Guo, J.C.; Johnson, S.B.; Campbell, T.L.; Zhang, H.B.; Vrijenhoek, R.C.; Chavez, F.P.; Geller, J. Zooplankton biogeographic boundaries in the California Current System as determined from metabarcoding. *PLoS ONE* **2020**, *15*, e0235159. [CrossRef]
60. Sommer, S.A.; Van Woudenberg, L.; Lenz, P.H.; Cepeda, G.; Goetze, E. Vertical gradients in species richness and community composition across the twilight zone in the North Pacific Subtropical Gyre. *Mol Ecol.* **2017**, *26*, 6136–6156. [CrossRef]
61. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
62. Estaki, M.; Jiang, L.; Bokulich, N.A.; McDonald, D.; González, A.; Kosciolek, T.; Martino, C.; Zhu, Q.; Birmingham, A.; Vázquez-Baeza, Y.; et al. QIIME 2 enables comprehensive end-to-end analysis of diverse microbiome data and comparative studies with publicly available data. *Curr. Protoc. Bioinform.* **2020**, *70*, e100. [CrossRef]
63. Buchner, D.; Leese, F. BOLDigger – a Python package to identify and organise sequences with the Barcode of Life Data systems. *Metabarcoding Metagenomics* **2020**, *4*, e53535. [CrossRef]
64. Baker, C.B. A utility to generate input files for taxonomy assignment in QIIME from the NCBI database. Available online: https://github.com/bakerccm/entrez_qiime (accessed on 1 September 2024).
65. Robeson, M.S.; O'Rourke, D.R.; Kaehler, B.D.; Ziemski, M.; Dillon, M.R.; Foster, J.T.; Bokulich, N.A. RESCRIPt: Reproducible sequence taxonomy reference database management for the masses. *PLoS Comput. Biol.* **2021**, *17*, e1009581. [CrossRef]
66. Gao, C.H.; Yu, G.C.; Cai, P. ggVennDiagram: An Intuitive, Easy-to-Use, and Highly Customizable R Package to Generate Venn Diagram. *Front. Genet.* **2021**, *12*, 706907. [CrossRef] [PubMed]
67. vegan: Community Ecology Package. Available online: https://cran.r-project.org/web/packages/vegan/vegan.pdf (accessed on 1 September 2024).
68. Kerns, G.J.; Chang, G.A. RcmdrPlugin.IPSUR: An IPSUR Plugin for the R Commander. Available online: https://ipsur.r-forge.r-project.org/rcmdrplugin/installation.php (accessed on 1 September 2024).
69. De Caceres, M.; Legendre, P. Associations between species and groups of sites: Indices and statistical inference. *Ecology* **2009**, *90*, 3566–3574. [CrossRef] [PubMed]
70. AquaNIS. Information system on Aquatic Non-Indigenous and Cryptogenic Species. Available online: http://www.corpi.ku.lt/databases/index.php/aquanis/ (accessed on 1 September 2024).
71. WoRMS EB. World Register of Marine Species. Available online: https://www.marinespecies.org/ (accessed on 1 September 2024).
72. Razouls, C.D.N.; Kouwenberg, J.; de Bovée, F. Biodiversity of Marine Planktonic Copepods (Morphology, Geographical Distribution and Biological Data). Available online: http://copepodes.obs-banyuls.fr/en (accessed on 20 January 2024).
73. RStudio Team. *RStudio: Integrated Development for R*; RStudio, PBC: Boston, MA, USA, 2020. Available online: http://www.rstudio.com/ (accessed on 20 January 2024).
74. Hebert, P.D.N.; Ratnasingham, S.; deWaard, J.R. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. B-Biol. Sci.* **2003**, *270*, S96–S99. [CrossRef]
75. Bucklin, A.; Hopcroft, R.R.; Kosobokova, K.N.; Nigro, L.M.; Ortman, B.D.; Jennings, R.M.; Sweetman, C.J. DNA barcoding of Arctic Ocean holozooplankton for species identification and recognition. *Deep Sea Res. Part II Top. Stud. Oceanogr.* **2010**, *57*, 40–48. [CrossRef]

76. Geller, J.; Meyer, C.; Parker, M.; Hawk, H. Redesign of PCR primers for mitochondrial cytochrome *c* oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Mol. Ecol. Resour.* **2013**, *13*, 851–861. [CrossRef]
77. Stoeckle, M.Y.; Hebert, P.D.N. Barcode of life. *Sci. Am.* **2008**, *299*, 82. [CrossRef]
78. Bik, H.M.; Porazinska, D.L.; Creer, S.; Caporaso, J.G.; Knight, R.; Thomas, W.K. Sequencing our way towards understanding global eukaryotic biodiversity. *Trends Ecol. Evol.* **2012**, *27*, 233–243. [CrossRef]
79. Suter, L.; Polanowski, A.M.; Clarke, L.J.; Kitchener, J.A.; Deagle, B.E. Capturing open ocean biodiversity: Comparing environmental DNA metabarcoding to the continuous plankton recorder. *Mol. Ecol.* **2021**, *30*, 3140–3157. [CrossRef]
80. Sawaya, N.A.; Djurhuus, A.; Closek, C.J.; Hepner, M.; Olesin, E.; Visser, L.; Kelble, C.; Hubbard, K.; Breitbart, M. Assessing eukaryotic biodiversity in the Florida Keys National Marine Sanctuary through environmental DNA metabarcoding. *Ecol. Evol.* **2019**, *9*, 1029–1040. [CrossRef] [PubMed]
81. Ershova, E.A.; Descoteaux, R.; Wangensteen, O.S.; Iken, K.; Hoperoft, R.R.; Smoot, C.; Grebmeier, J.M.; Bluhm, B.A. Diversity and Distribution of Meroplanktonic Larvae in the Pacific Arctic and Connectivity with Adult Benthic Invertebrate Communities. *Front. Mar. Sci.* **2019**, *6*, 490. [CrossRef]
82. Miralles, A.; Bruy, T.; Wolcott, K.; Scherz, M.D.; Begerow, D.; Beszteri, B.; Bonkowski, M.; Felden, J.; Gemeinholzer, B.; Glaw, F.; et al. Repositories for Taxonomic Data: Where We Are and What is Missing. *Syst. Biol.* **2020**, *69*, 1231–1253. [CrossRef] [PubMed]
83. Blanco-Bercial, L.; Cornils, A.; Copley, N.; Bucklin, A. DNA barcoding of marine copepods: Assessment of analytical approaches to species identification. *PLoS Curr.* **2014**, *6*, 6. [CrossRef]
84. Abad, D.; Albaina, A.; Aguirre, M.; Estonba, A. 18S V9 metabarcoding correctly depicts plankton estuarine community drivers. *Mar. Ecol. Prog. Ser.* **2017**, *584*, 31–43. [CrossRef]
85. Albaina, A.; Aguirre, M.; Abad, D.; Santos, M.; Estonba, A. 18S rRNA V9 metabarcoding for diet characterization: A critical evaluation with two sympatric zooplanktivorous fish species. *Ecol. Evol.* **2016**, *6*, 1809–1824. [CrossRef] [PubMed]
86. Andruszkiewicz, E.A.; Sassoubre, L.M.; Boehm, A.B. Persistence of marine fish environmental DNA and the influence of sunlight. *PLoS ONE* **2017**, *12*, e0185043. [CrossRef]
87. Port, J.A.; O'Donnell, J.L.; Romero-Maraccini, O.C.; Leary, P.R.; Litvin, S.Y.; Nickols, K.J.; Yamahara, K.M.; Kelly, R.P. Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. *Mol. Ecol.* **2016**, *25*, 527–541. [CrossRef]
88. Goetze, E. Species discovery in marine planktonic invertebrates through global molecular screening. *Mol. Ecol.* **2010**, *19*, 952–967. [CrossRef]
89. Lindeque, P.K.; Harris, R.P.; Jones, M.B.; Smerdon, G.R. Simple molecular method to distinguish the identity of Calanus species (Copepoda: Calanoida) at any developmental stage. *Mar. Biol.* **1999**, *133*, 91–96. [CrossRef]
90. Lindsay, D.; Grossmann, M.; Nishikawa, J.; Bentlage, B.; Collins, A. DNA barcoding of pelagic cnidarians: Current status and future prospects. *Bull. Plankton Soc. Jpn.* **2015**, *62*, 39–43.
91. Johansson, M.L.; Shiganova, T.A.; Ringvold, H.; Stupnikova, A.N.; Heath, D.D.; MacIsaac, H.J. Molecular Insights Into the Ctenophore Genus Beroe in Europe: New Species, Spreading Invaders. *J. Hered.* **2018**, *109*, 520–529. [CrossRef]
92. Bowman, R.E. Food of Northwest Atlantic Fishes and Two Common Species of Squid. In *NOAA Technical Memorandum NMFS-NE-155*; Northeast Fisheries Science Center (U.S.): Woods Hole, MA, USA, 2000.
93. CJ, P.V.; Carballo, J.L.; Camacho, M.L. A qualitative assessment of sponge-feeding organisms from the Mexican Pacific coast. *Open Mar. Biol. J.* **2010**, *4*, 39–46.
94. Lorders, F.L.; Miranda, R.J.; Nunes, J.; Barros, F. Spongivory by Fishes on Southwestern Atlantic Coral Reefs: No Evidence of Top-Down Control on Sponge Assemblages. *Front. Mar. Sci.* **2018**, *5*, 256. [CrossRef]
95. Holland, L.Z. Tunicates. *Curr. Biol.* **2016**, *26*, R146–R152. [CrossRef]
96. Sadro, S. *Porifera: The Sponges*; 2008. Available online: https://api.semanticscholar.org/CorpusID:5650532 (accessed on 1 September 2024).
97. Cornwell, L.E.; Fileman, E.S.; Bruun, J.T.; Hirst, A.G.; Tarran, G.A.; Findlay, H.S.; Lewis, C.; Smyth, T.J.; McEvoy, A.J.; Atkinson, A. Resilience of the Copepod *Oithona similis* to Climatic Variability: Egg Production, Mortality, and Vertical Habitat Partitioning. *Front. Mar. Sci.* **2020**, *7*, 29. [CrossRef]
98. Hure, M.; Batistic, M.; Garic, R. Copepod Diel Vertical Distribution in the Open Southern Adriatic Sea (NE Mediterranean) under Two Different Environmental Conditions. *Water* **2022**, *14*, 1901. [CrossRef]
99. Bucklin, A.; Yeh, H.D.; Questel, J.M.; Richardson, D.E.; Reese, B.; Copley, N.J.; Wiebe, P.H. Time-series metabarcoding analysis of zooplankton diversity of the NW Atlantic continental shelf. *ICES J. Mar. Sci.* **2019**, *76*, 1162–1176. [CrossRef]
100. Kelly, R.P.; Closek, C.J.; O'Donnell, J.L.; Kralj, J.E.; Shelton, A.O.; Samhouri, J.F. Genetic and Manual Survey Methods Yield Different and Complementary Views of an Ecosystem. *Front. Mar. Sci.* **2017**, *3*, 283. [CrossRef]
101. Chan, F.T.; Briski, E.; Bailey, S.A.; MacIsaac, H.J. Richness-abundance relationships for zooplankton in ballast water: Temperate versus Arctic comparisons. *ICES J. Mar. Sci.* **2014**, *71*, 1876–1884. [CrossRef]
102. Cariton, J.T.; Geller, J.B. Ecological Roulette: The Global Transport of Nonindigenous Marine Organisms. *Science* **1993**, *261*, 78–82. [CrossRef]
103. Sardain, A.; Sardain, E.; Leung, B. Global forecasts of shipping traffic and biological invasions to 2050. *Nat. Sustain.* **2019**, *2*, 274–282. [CrossRef]

104. von Ammon, U.; Wood, S.A.; Laroche, O.; Zaiko, A.; Tait, L.; Lavery, S.; Inglis, G.J.; Pochon, X. Combining morpho-taxonomy and metabarcoding enhances the detection of non-indigenous marine pests in biofouling communities. *Sci. Rep.* **2018**, *8*, 16290. [CrossRef] [PubMed]
105. Cornils, A.; Held, C. Evidence of cryptic and pseudocryptic speciation in the Paracalanus parvus species complex (Crustacea, Copepoda, Calanoida). *Front. Zool.* **2014**, *11*, 19. [CrossRef]
106. Ounissi, M.; Khelifi-Touhami, M. Le Zooplancton du plateau continental d'El-Kala (Méditerranée sud-occidentale): Composition et abondance en mai 1996. *J. Rech. Océanogr.* **1999**, *24*, 5–11.