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eDNA Metabarcoding Analysis as Tool to Assess the Presence of Non-Indigenous Species (NIS): A Case Study in the Bilge Water

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Abstract: One of the most important causes of biodiversity loss are non-indigenous species (NIS), in particular invasive ones. The dispersion of NIS mainly depends on anthropogenic activities such as maritime traffic, which account for almost half of the total NIS introduction in the European seas, as reported by the European Environmental Agency. For this reason, NIS management measures are mainly focused on commercial ports (i.e., ballast water management and Marine Strategy Framework Directive monitoring), underestimating the role of marinas and tourist harbors; these host small vessels (<20 m), such as recreational, fishery, and sail ones without ballast waters, but are also responsible for NIS arrival and spread through the bilge water as well as from hull fouling. With the aim of paying attention to marinas and tourist harbors and validating an innovative molecular methodology for NIS surveillance and monitoring, in the present work, eDNA metabarcoding of cytochrome oxidase subunit I (COI) was applied to both bilge waters and adjacent ones to assess species composition and particularly NIS presence. A total of 140 OTUs/species with extra-Mediterranean distribution were found in the bilge samples; several of these are most likely ascribed to food contamination (e.g., *Salmo salar*). Excluding food contamination species, twelve of these found in the bilge waters were already known as NIS in the Mediterranean Sea, belonging to algae, mollusks, crustaceans, annelids, echinoderms, and fishes. Nine of these species are new to Italian waters. The results obtained in the present work support the importance of NIS monitoring in marinas and small harbors, particularly in the bilge waters, through eDNA metabarcoding, having detected several potential NIS that otherwise would not have been discovered.

Keywords: alien species surveillance; biodiversity; alien species spread; marinas; recreational boating

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1. Introduction

Non-indigenous species (NIS), and in particular invasive alien species (IAS), are considered one of the most important causes of biodiversity loss as well as having socio-economic and health impacts. The effects of NIS/IAS on biodiversity include displacement of native species, habitat modifications, changes in ecosystem functioning, introduction of new diseases and parasites, and genetic modifications, such as hybridization with the native taxa [1]. Furthermore, IAS pressure may even act on human health in the case of toxic species or the spread of pathogens [2], and in some cases, it might affect the economy in sectors such as fisheries and tourism [3,4]. In the Mediterranean Sea, new species can enter from the Atlantic Ocean through the Strait of Gibraltar and from the Red Sea by the

Suez Canal; in the first case, the new species are called range-expanding species because their entrance is mediated by natural areal expansion. On the other hand, species entering the Mediterranean through the Suez Canal are considered NIS according to the most recent literature because of their human-mediated dispersion, and they are considered in management efforts/policy initiatives. The European Environmental Agency (EEA) reported that anthropogenic dispersion of NIS mainly depends on vessel movements (maritime traffic), accounting for almost half of the total NIS introduction in the European seas. NIS transport occurs by moving live organisms attached to ships in the hull fouling or present in ballast water and sediments, as demonstrated by many authors [5,6]. Once a NIS has become established, its eradication is an expensive and often unsuccessful measure. Prevention is, by far, the best approach to limiting the impact of NIS, and detection at the early stages of incursion is fundamental. For this reason, monitoring programs that allow early detection of NIS are recommended as a priority strategy for management efforts. Monitoring programs are mainly focused on commercial ports since they are NIS hot spots of introduction and subsequent spread. This is the case of the monitoring activities carried out by nine European States, including Italy, within the Marine Strategy Framework Directive (MSFD—2008/56/EC) for the Descriptor 2 non-indigenous species [7]. Furthermore, great importance is given to the early detection of NIS in Regulation (EU) n. 1143/2014, which requires states to establish a surveillance system of the IAS of Union concern for their early detection [8]. The International Maritime Organization (IMO), recognizing the problem of invasive species in ships' ballast water, adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments [9]. The Convention requires all ships to carry ballast water management procedures to minimize the transfer of harmful aquatic organisms and pathogens from one port to another. More recently, attention has been given to the hulls as NIS vectors of introduction, but no international regulations or 'best practices' currently exist that specifically address NIS transported attached to the hull of vessels, except for the hull fouling management guidelines for international merchant vessels developed by IMO (MEPC.207(62), 2011). These guidelines define biofouling as the undesirable accumulation of microorganisms, plants, algae, and animals on submerged structures (especially ships' hulls), which can include NIS. Boat hulls were demonstrated to be vectors of the introduction of some macroalgae, such as *Undaria pinnatifida* in British coastal waters [10] and *Codium fragile* in European waters [11], as well as some mussel species, including *Mytilopsis sallei* in northern Australia [12] and the invasive *Dreissena polymorpha* [13].

To achieve the objectives of alien species policies, it is necessary to have a wide knowledge of their distribution and introduction pathways. With this aim in mind, several authors reviewed the recorded alien species in Italian waters [14–16]. The number of NIS in Italian seas tended to increase rapidly in the last decades, reaching a number of 282 (updated to 2020). Most of the introduced species are native to the Indian/Indo-Pacific/Red Sea, followed by those of North Pacific and Tropical Atlantic origin; as well as the other European States, maritime traffic is the most relevant pathway of introduction, accounting for 52%.

As stated above, NIS management measures only concern commercial ports and ballast waters, although the issue of invasive species spread is widely acknowledged, and recreational boating is increasingly recognized as responsible for the spread of marine NIS. NIS transport in fact can occur through hull fouling and bilge waters of moving small vessels (<20 m) such as recreational, fishery, and sail boats hosted in the small harbors and marinas; small boats moving from one harbor to another can bring NIS into the hull fouling as well as into the bilge water and carry them around, favoring their spread in a region. These artificial systems are mainly dominated by highly competitive species, in particular NIS, that show greater tolerance to variable environmental conditions and are often thought to be more resilient to disturbance than native communities. A study of the benthic community of some selected Mediterranean marinas and of the associated boat fouling led Ulman et al. [17] to demonstrate the pivotal role of recreational boating for both first

introduction events to a given country and secondary spread. Moreover, Ferrario et al. [18] showed that marinas can host as many NIS as larger commercial ports and recommended the importance of considering this vector in the future planning of sustainable development of maritime tourism in Europe. In particular, NIS transport among marinas is mediated by boats' hulls in the fouling, as confirmed by many researchers [19–22].

Likewise, but perhaps less studied, the bilge water can also act in the transport of the NIS. Bilge water is any seawater that is stored within the hull of a vessel, such as in the engine room, as well as the uncontained water on the deck area of a vessel. Bilge water can often contain marine debris, oil, dirt, terrestrial vegetation, and detergents; it is pumped for discharge to the sea without any treatment. As the hull fouls, it can host organisms and NIS as adults or propagules that are released into the sea when the bilge waters are dumped. Its role as a potential transporter was already demonstrated for several invasive algae [23,24], and for the invasive ascidian *Didemnum vexillum* [25]. To date, studies on the transport of NIS through bilge waters are very few, although their role as NIS vectors is widely recognized [26,27]. These authors, investigating the taxonomic diversity in bilge water samples with different techniques (morphological and molecular), identified the presence of NIS and demonstrated the important contribution of such studies to a better understanding of the potential spread of marine organisms.

As set out above about the role of bilge water in NIS transport, it should be useful and interesting to carry out monitoring activities in the bilge water to early detect the NIS presence, but to date no monitoring programs on bilge waters exist. This type of activity shows some difficulties in the taxonomic identification of species present as larvae or spores. In the last decade, the development of molecular techniques applied to monitoring activities for species identification has greatly improved thanks to eDNA analysis [28–30]. This methodology, first applied in freshwater, has developed in a few years and allows to identify the presence of target species as well as assess biodiversity with increased speed and lower cost than traditional monitoring activities [31,32]. eDNA allows for the study of the whole diversity of a community as well as the detection of the presence of rare species [30,33] and early identification of the presence of NIS due to its features [33,34]. Biodiversity assessment using eDNA analysis involves metabarcoding, defined as the amplification of environmental DNA samples (extracted from water or sediment) by polymerase chain reaction (PCR) with general or universal primers, followed by high-throughput next-generation sequencing (NGS) to generate thousands to millions of reads. From these reads, the species presence is identified, and subsequently, biodiversity is assessed. As with all novel methodologies, eDNA metabarcoding suffers from some bias, e.g., the PCR primer bias, that is, the different primer efficiencies in the PCR for a broad set of species [35]. On the other hand, it was demonstrated to be an interesting tool to complement biodiversity assessment. Its application to bilge water species composition seems to be a promising tool in NIS identification, as highlighted by [27,36]. Fletcher et al. [27] reported that morphological assessments of bilge water samples provided less taxonomic resolution compared to metabarcoding, and 70% of the identified taxa were putative NIS, supporting the usefulness of the method. On the other hand, Ponchon et al. [36], focusing on the biodiversity patterns of dead/alive organisms in bilge water by metabarcoding analysis of co-extracted eDNA and eRNA, concluded that the presence of an OTU from a NIS in the eDNA-only group may assist in early detection, although they recommend further research to improve understanding of the persistence of RNA in the environment.

In the present work, eDNA metabarcoding was used to screen and study species composition in bilge water and adjacent seawater samples, looking for NIS presence by using different primer pairs to reduce primer bias. The validation of this innovative methodology for NIS surveillance and monitoring in bilge water as well as in small harbors not included in the institutional monitoring activities was an objective of the present study. Two different small harbors, one touristic and the other dedicated to fishing, were taken into account to assess by eDNA metabarcoding the differences in NIS occurrence between the two sites.

2. Materials and Methods

2.1. Water Sampling, Samples Preparation and DNA Extraction

The sampling was carried out in February 2021 in two small harbors in the south Tyrrhenian Sea: Cala Harbor (CA) (N 38°07′21.08″–E 013°22′13.76″) in Palermo and Isola delle Femmine Harbor (IF) (N 38°11′59.96″–E 013°14′52.96″) (Figure 1). The first is a leisure harbor mainly hosting sail and recreational boats, while IF is mainly a fishery harbor, even though it also hosts recreational boats in the summertime. In both sites' sea bottoms, which are predominantly clay and/or rocky and subject to rearrangement due to shipping and possible inputs from land, biotic assemblages are characterized by species tolerating pollution and human disturbance [37] (author's observation). In both harbors, a total of ten samples were collected from six bilges and four adjacent seawaters; for each sample, two replicates were done. In CA, bilge water came from two sailboats and one fishboat, and in IF, bilge water only came from fishboats. The sampled boats had carried out some exits the previous day. All water samples (seawater and bilge) were collected in replicates of two-liter bottles and subsequently stored at −20 °C. For each replicate sample, after thawing, water was filtered using a Mixed Cellulose Esters (MCE) 0.45 µm filter using an autoclaved filtration unit and sterilized forceps and scissors to avoid contamination among filters; a water blank control was used for the subsequent analyses. The filters were thereafter preserved with 96% ethanol for the future bulk DNA extraction.

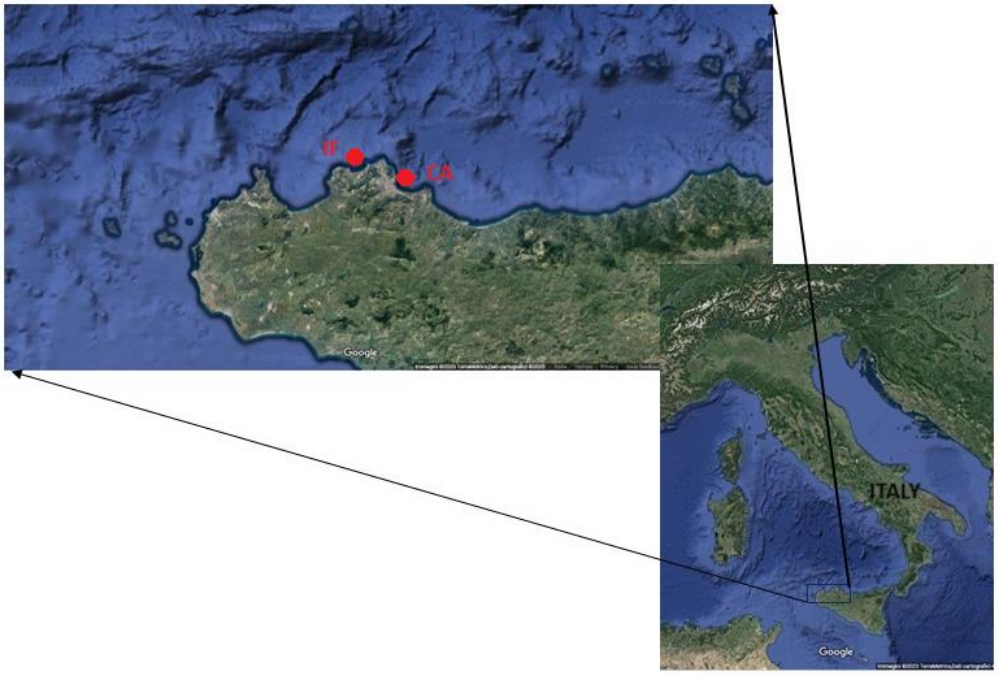

Figure 1. Sampling sites: CA Cala harbor and IF Isola delle Femmine harbor. From each site, water was sampled from sea and from bilge of selected boats. ©2023 Google, GeoBasis-DE/BKG (©2009), Inst. Geogr. Nacional 200 km.

DNA was extracted in a dedicated area from the MCE 0.45 µm filters by using the DNeasy PowerWater kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quantity of extracted DNA samples was assessed using fluorimeter analysis (Qbit, Tokyo, Japan); the mean yield of DNA was 9 ng/µL.

2.2. Amplicon Sequencing (Metabarcoding Analysis)

Metabarcoding analysis was carried out by using three different primer pairs in order to address the potential PCR primer bias and to recover as many species as possible. PCR amplifications of Cytochrome Oxidase Subunit I (COI) were undertaken in a total volume

of 25 µL using Platinum Multiplex PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA), and the concentration of each primer used (Microsynth, Balgach, Switzerland) is reported in Table 1. Reaction cycling conditions were the same as reported in each reference. After PCR, the amplicons were purified using the AMPure XP beads and checked on a 2% agarose gel to verify DNA amplification.

Table 1. Primer pairs used in the metabarcoding analyses; conc is primer concentration in the PCR reaction; ref is the reference of the primer pair.

PCR	Primer Name	Conc	Primer Sequence	Amplicon Lenght	Ref.
Mini COI	mlCOIintF jgHCO2198	0.2 µM	GGWACWGGWTGAACWGTWTAYCCYCC TAIACYTCIGGRTGICCRAARAAYCA	350 bp	[38]
COI-2	jgLCO1490 jgHCO2198	0.2 µM	TITCIACIAAYCAYAARGAYATTGG TAIACYTCIGGRTGICCRAARAAYCA	600 bp	[39]
Ca	CasF CasR	1 µM	GGTTCTTCTCCACCAACCACAARGYATHGG ATTTCTATCHGTTARYAACATTGTRAT	600 bp	[40]

Primers used in PCR were modified by adding Illumina overhang adapter sequences to locus-specific sequences: forward overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′ and reverse overhang: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′.

After the purification of amplicons, index PCR was performed using Nextera XT index kit v2 (Illumina, San Diego, CA, USA) and KAPA HiFi Hot Start ready mix (Roche, Basel, Switzerland) as described in the Illumina metagenomics sequencing library preparation guide. Libraries qualities were checked by an Agilent 2100 Bioanalyzer with a DNA 1000 kit; library size profiles with an average fragment size of 500–550 bp were obtained. Library quantification was performed by QuBit using the QuBit dsDNA BR kit.

Illumina library preparations of amplicons obtained by PCRs were performed with the Illumina DNA prep kit (Illumina) according to the manufacturer's protocol; Illumina Nextera CD DNA indices were used. Amplicons obtained from PCR libraries were checked by an Agilent 2100 Bioanalyzer with a high-sensitivity DNA kit. library size profiles with an average fragment size of 450 bp were obtained. Library quantification was performed by QuBit using the QuBit dsDNA BR kit. 30% PhiX was spiked in to improve the sequence quality. The quality of the sequence data was high (average % >Q30 higher than 90), and after primer trimming, more than 350,000 reads per sample were obtained.

2.3. Bioinformatics and Statistical Analyses

Quality testing and trimming were done by Trimmomatic (version 0.39) considering a minimum read length of 100 and a minimum Phred quality score of 25. The selected reads for each sample were classified by a proprietary algorithm called GAIA (https://metagenomics.sequentiabiotech.com/ (accessed on 31 May 2023)) against a database of COI sequences. This database is a custom database created with COI sequences extracted from the National Center for Biotechnology Information (NCBI) with a length ranging from 400 to 800 bp; the query used was: COI | cytochrome oxidase subunit 1 | (COI) | cytochrome c oxidase subunit 1 as title AND 400:800 as Slen. An assignment-first strategy approach was used (https://doi.org/10.1101/804690 (accessed on 31 May 2023)). In the first step, the reads were aligned against the custom COI database, and then, using the last common ancestor (LCA) algorithm, the reads were classified according to the percentages of identity obtained (domain (<75%), phylum (>78%), class (>85%), order (>89%), family (>91%), genus (>93%), and species (>95%). The methods are fully described in [41,42].

Absolute and relative read counts per OTU (Operational Taxonomic Unit) at different taxonomic levels were calculated. Taxonomy was assigned based on the best BLAST hit to a sequence with taxonomic information available. The best hits for metazoans with a minimum of 370 bp and a 97% sequence were considered. We assumed that the recovery of

operational taxonomic units (OTUs) at the species level corresponds to species presence in the analyzed sample. With the aim of comparing both ports and, in particular, the differences between bilge and port samples, biodiversity was assessed through richness, alpha diversity (the total number of observed OTUs/species), and the Shannon index, measuring both the number of OTUs/species and the inequality between OTUs/species abundances. OTUs corresponding to organisms of terrestrial origin were intentionally kept in the datasets as they may be representative of legacy DNA from non-living biodiversity.

Furthermore, in order to quantify compositional dissimilarity between different samples (bilge and port water), we calculated the Bray–Curtis dissimilarity among samples, and we used them for the creation of a Multidimensional Scaling (MDS) plot looking for differences between port and bilge samples. These analyses were performed using the normalized total number of reads per sample (sample size normalization); they used the standard methodology suggested by McMurdie and Holmes [43].

Starting from the list of OTUs identified at species level by metabarcoding, the marine ones were selected according to *The World Register of Marine Species* (WoRMS), considered an authoritative and comprehensive list of names of marine organisms, the AlgaeBase (http://www.algaebase.org/ (accessed on 31 May 2023)) and Encyclopedia of Life (http://eol.org/ (accessed on 31 May 2023)) databases. The curated sequence dataset, including all OTUSs/species, was employed for the subsequent analyses. For the detection of putative NIS, a check on species distribution, looking for species with an extra Mediterranean distribution, was done. In particular, it was compared with the Mediterranean NIS database [16] to verify their occurrence and with the Italian NIS database [44] and subsequent updates with the final aim of detecting a new NIS for the Mediterranean Sea and for Italian seas; subsequently, these comparisons were done for bilge and port samples separately. Only for the OTUs corresponding to NIS already present in the Mediterranean Sea, we checked the sequence similarity of the OTUs with the congeneric available sequences to exclude a possible bias in the species assignment.

3. Results

DNA extractions from all samples yielded good-quality DNA, as indicated by the nanophotometer absorbance ratios 260/280. The concentration of DNA extracted ranged between 1.3 and 22.4 ng/µL (Figure S1). A total of 2,893,451 reads for samples were obtained, and the filtering of sequences with ambiguous bases, barcode errors, or mismatches has resulted in the removal of 18% of reads. The remaining sequences were clustered into the number of OTUs reported in Table 2 using a 97% sequence similarity threshold.

Table 2. Total number of retrieved OTUs in the three analyses and percentage of specie-level match.

Target	OTUs Retrieved	% Species Level Match
miniCOI	329	29.8
COI-2	3730	28.9
Cas	2595	28.2

The total number of OTUs retrieved by the three primer pairs (miniCOI, COI-2, and Cas) ranged from 329 to 3730, where COI-2 and Cas primers yielded a higher number of OTUs than miniCOi. However, the percentage of aligned reads at the species level was similar in the three analyses (Table 2).

The species with more assigned OTUs for the three primer pairs (as percentage of total reads) was the green alga *Micromonas pusilla*, followed by the fish *Mullus surmuletus* for mini COI, by the fish *Merluccius merluccius* for COI-2, and by the sea urchin *Arbacia lixula* for Cas (Figure 2). In the barplot of the taxonomic composition of each sample, it is evident that miniCOI and COI-2 showed a higher number of OTUs assigned at species level.

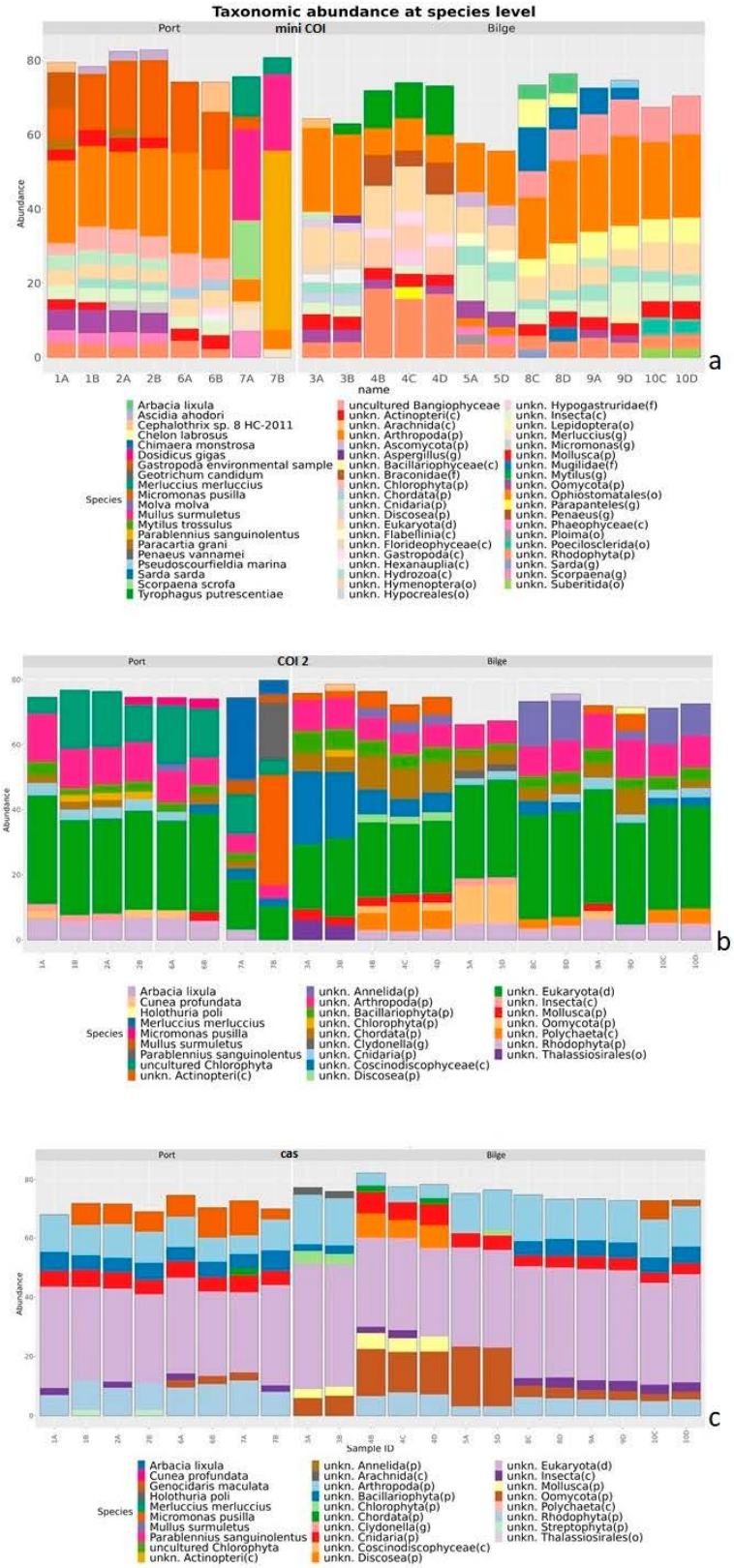

Figure 2. Taxonomic composition of samples in bilge and port for miniCOI (**a**), COI-2 (**b**), and Cas (**c**). Samples from 1 to 5 are from CA, and samples from 6 to 10 are from IF. Abundance is the percentage of reads classified for a specific OUT/taxon. Each bar represents the taxonomic composition of the samples, assigning a color to each taxon. Taxa with a percentage of less than 1% of the total reads classified in each sample are not represented in this bar plot.

In the MiniCOI metabarcoding analysis, a large number of species within the phyla Arthropoda, Rhodophyta, and Oomycota were found in all samples. However, the presence of species such as the green alga *Micromonas pusilla* and the tunicate *Ascidia ahodori* stood out in the samples from the water extracted from the port samples. Species belonging to the classes Bangiophyceae and Bacillariophyceae were the most identifiable taxa in the taxonomic profiles of the samples extracted from the IF bilge water (8, 9, and 10), in addition to the fish *Sarda sarda* in the bilge samples (8 and 9). Finally, the mite *Tyrophagus putrescentiae* appeared in 10% of the port samples (4), while one IF port sample (7) showed a different species composition for the presence of fish such as *Parablennius sanguinolentus*, *Mullus surmuletus*, and *Merluccius merluccius*. Regarding the COI-2 and Cas analyses, despite finding some similarities, such as the high frequency of the phyla Artropoda and Rhodophyta, there were differences in the overall taxonomic composition. In the Cas group, there were species within the classes Bacillariophyta and Coscinodiscophyceae and the phylum Cnidaria. The presence of the fish *Parablennius sanguinolentus* in an IF port sample (7) with ~35% of the total classified reads is also noteworthy.

Alpha diversity calculated considering the total number of species in the three analyses showed different results between bilge and port water (Figure 3); it was higher in bilge water for Cas primer at IF and lower for COI-2 at IF. The Shannon index was always higher in bilge water than in port water (Figure 3).

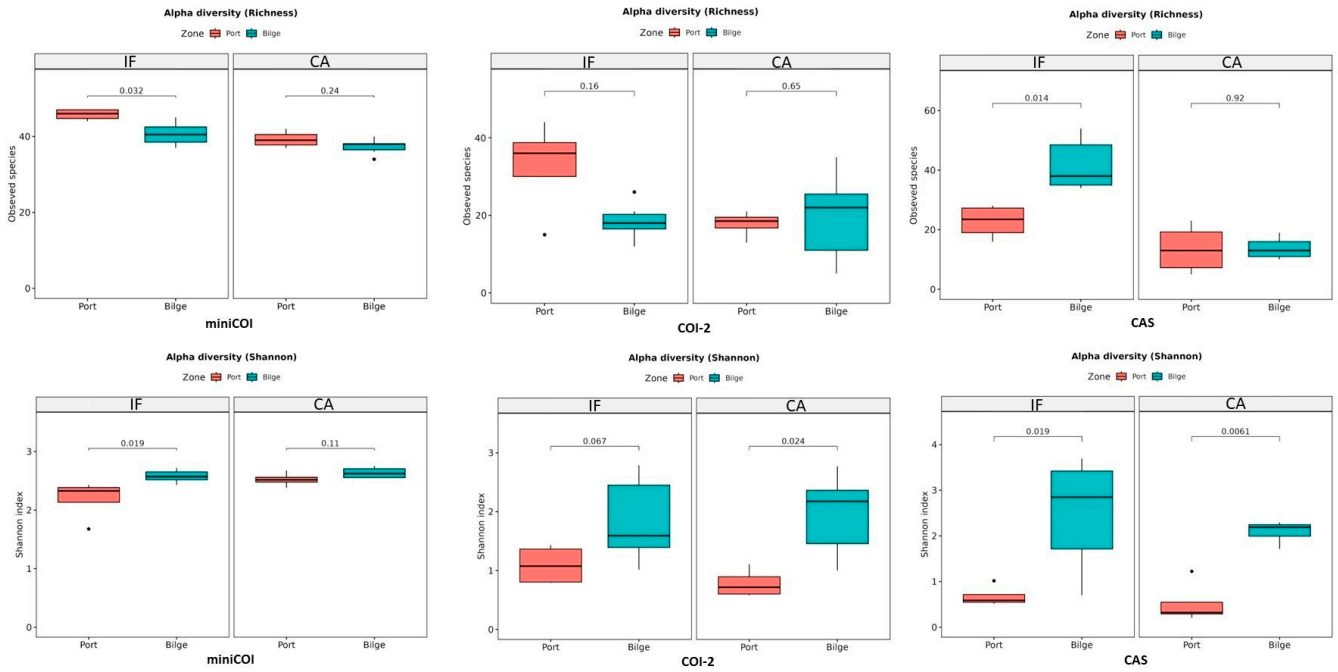

Figure 3. Diversity metrics. On the top, boxplots represent alpha diversity for observed species retrieved with miniCOI, COI-2, and Cas primers in port and bilge. On the bottom, boxplots representing Shannon index for the same primers and locations. Black dot indicates outliers values that are far from the majority of the others.

Multidimensional scaling (MDS) of the Bray–Curtis beta diversity metric shows that groups are clustered according to the extraction zone of each sample (bilge or port) in the case of MiniCOI, COI-2, and Cas (Figure 4).

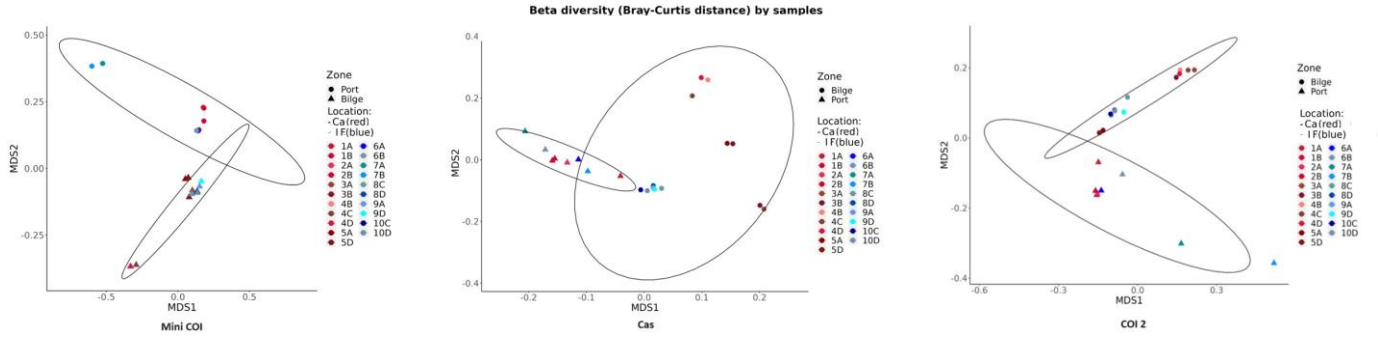

Figure 4. Multidimensional scaling (MDS) plot based on Bray–Curtis dissimilarity matrix for miniCOI, COI-2, and Cas primer sets. In blue, samples from IF, and in red the ones from CA. Different point shapes identified the zone extraction: bilge (circle) and port (triangle).

The total number of OTUs identified at species level was reported in Table 3 for the three primer pairs and combined bilge and port samples, together with the number of marine species. The number of OTUs with species-level matches was higher for COI-2 and lower for MiniCOI. A number of terrestrial organisms (e.g., plants, ants, flies, and spiders) were detected in the samples through the metabarcoding approaches, and it was not possible to determine the source of these organisms. Differences in the number of retrieved OTUs were shown in comparisons among the three primer pairs, even though the gene marker was the same. For marine species, information on their geographical distribution was collected. Looking for non-indigenous species (NIS), the number of marine species not distributed in the Mediterranean Sea was calculated; among these species, only a small number (30) were already known as NIS in the Mediterranean Sea according to [16,44] and the following literature update. These NIS belong to Algae (6), Cnidarians (1), Molluscs (4), Crustaceans (5), Annelids (3), Echinoderms (1), Tunicates (1), and Fishes (9) (Tables 3 and 4). Some of the 30 NIS likely belong to food contamination, so they were excluded from the counts. The check carried out with the co-generic sequences available in the database reported that the highest value of sequence similarity was with the species previously assigned for all 30 OTUs. Among these NIS, 13 were not present in the Italian NIS database.

Table 3. Total number of identified species; number of marine species according to WORMS, AlgaeBase, and Encyclopedia of Life databases; number of species with extra Mediterranean distribution according to references information, number of extra Mediterranean species already found in Mediterranean as non-indigenous species (NIS); number of new non-indigenous species (NIS) for Italian seas.

	N of Species	N of Marine Species	Extra Med Species	Extra Med Species Already in MED (NIS)	NIS Not Yet Recorded in Italy
Mini COI	90	81	29	7	2
COI-2	654	306	180	18	8
Cas	470	168	126	10	6

Furthermore, some of the identified marine species were found only in the bilge samples from CA or IF, and some were common to bilge from the two zones and were not present in the port water samples (Figure 5). In the bilge water, NIS was found in a percentage ranging from 15% to 80%, and the Cas primer set identified the highest percentage of NIS.

Table 4. List of the Non-ndigenous Species (NIS) detected with three primer pairs and already present in the database of Mediterranean NIS; in bold, NIS found in the bilge water. NIS new for the Italian seas, and NIS considered food contamination are reported.

	Non-Indigenous Species	Mini COI	COI 2	Cas	New NIS for Italy	Food Contamination
Algae	*Asparagopsis taxiformis*		x			
Algae	***Bonnemaisonia hamifera***	x	x			
Algae	***Grateloupia subpectinata***		x		x	
Algae	*Herposiphonia parca*		x		x	
Algae	***Kapraunia schneideri***	x				
Algae	***Sphaerotrichia divaricata***		x		x	
Cnidaria	*Cassiopea ornata*		x	x		
Mollusca	***Dosidicus gigas***				x	
Mollusca	***Mytilus edulis***	x	x	x		x
Mollusca	***Mytilus trossulus***	x				x
Mollusca	*Sepia pharaonis*		x		x	
Annelids	*Branchiomma boholense*		x			
Annelids	***Lysidice collaris***		x			
Annelids	***Polydora cornuta***		x			
Crustacea	*Penaeus brasiliensis*			x		x
Crustacea	*Penaeus monodon*	x	x			x
Crustacea	*Penaeus subtilis*			x		x
Crustacea	*Penaeus vannamei*	x	x	x		x
Crustacea	***Solenocera crassicornis***		x		x	
Echinoderm	***Acanthaster planci***				x	
Tunicate	*Styela plicata*		x			
Pisces	***Abudefduf bengalensis***		x		x	
Pisces	*Carassius auratus*			x		
Pisces	*Clupea harengus*			x		x
Pisces	*Cyclopterus lumpus*			x	x	
Pisces	*Gadus morhua*			x		x
Pisces	***Lagocephalus guentheri***		x		x	
Pisces	*Merluccius gayi*		x			x
Pisces	*Rhabdosargus haffara*		x		x	
Pisces	*Salmo salar*	x	x	x		x
Pisces	***Scorpaena neglecta***	x			x	

The taxonomic groups better represented within the number of identified marine species are algae (11, 64, and 62) and fish (34, 84, and 30) with all the analysis (Table 5).

Table 5. Number of marine species identified for each taxonomic group for the three primer sets.

Taxonomic Group	miniCOI	COI 2	Cas
Algae	11	64	62
Porifera	6	15	5
Cnidaria	4	30	21
Mollusca	5	28	12
Annelids	8	22	3
Crustacea	7	18	24
Tunicate	1	4	0
Echinoderms	4	20	8
Pisces	34	84	30

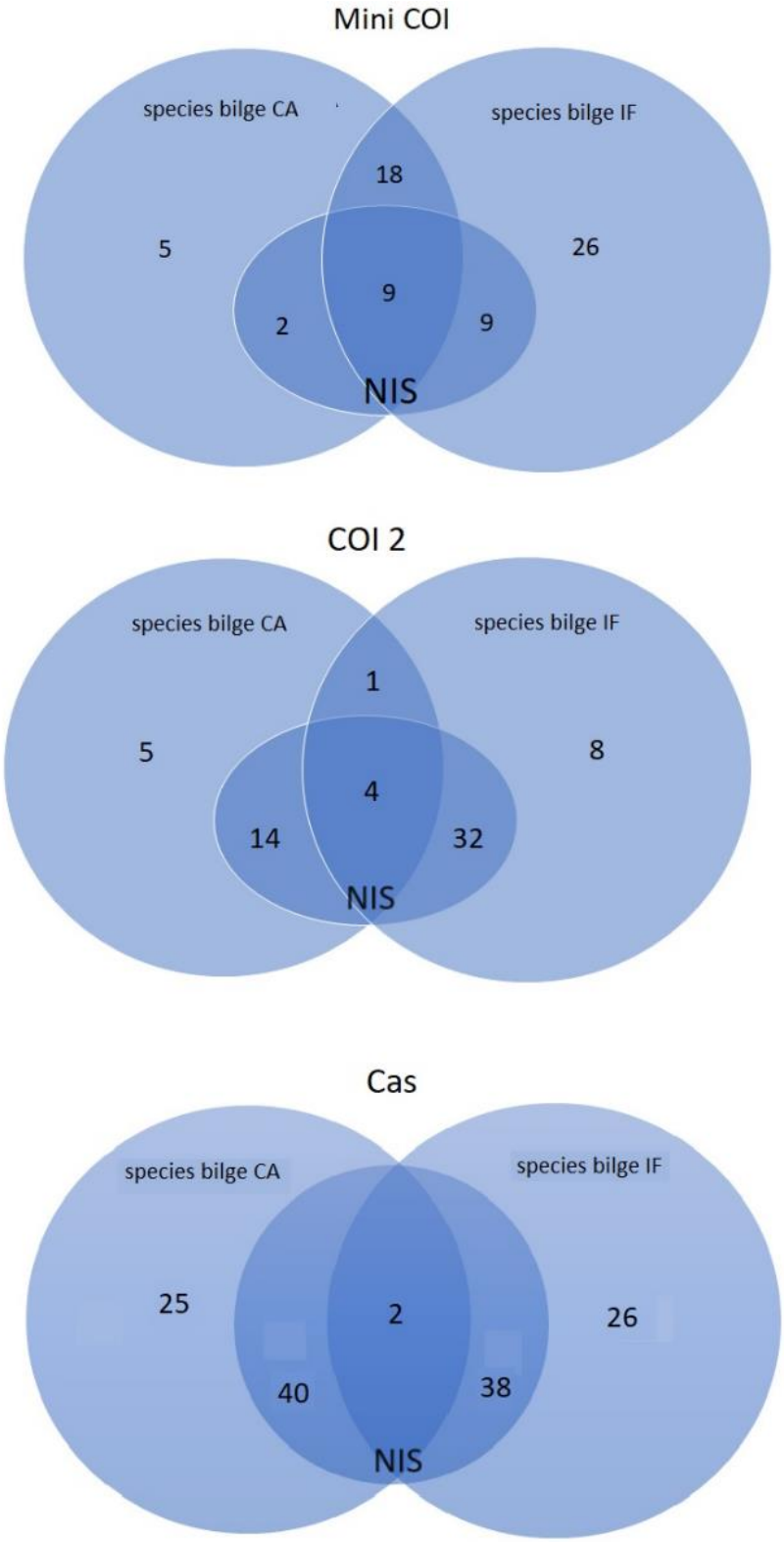

Figure 5. Venn diagram illustrating the number of species found only in bilge samples from Cala (CA) and from Isola delle Femmine (IF). Number of extra Mediterranean species (NIS) found only in bilge waters for the three primer sets.

4. Discussion

The use of eDNA metabarcoding has been reported as a promising tool for biodiversity monitoring, given the continuous improvement in molecular biological technologies. It can

be considered a rapid and cost-effective method to identify the taxonomic composition of many samples [30,45]. Such information provides a more complete picture of the organisms inhabiting the different local communities and then integrates traditional information with species invisible to classical monitoring. eDNA metabarcoding proves to be even more useful when the biodiversity of a specific place is historically monitored, like in the Mediterranean Sea, since it highlights relevant changes in the community composition that are deepening in this direction. During the last century, the Mediterranean Sea has been affected by the increasing introduction of non-indigenous species (NIS) from the whole globe through anthropogenic activities, mainly maritime traffic and aquaculture [42], causing evident changes in community composition and sometimes resulting in real environmental and economic disasters such as those produced by the alga killer *Caulerpa taxifolia* or the Manila clam *Ruditapes philippinarum* [46–49].

In the last few years, the classical monitoring of biodiversity has been supported by the innovative eDNA metabarcoding tool also valid for the detection of NIS, which is fundamental in the management of bio invasion [50,51]. Considering also that recent research proved that NIS may be consistently found in marinas and small harbors [52] and that these areas are not usually involved in institutional monitoring, the use of eDNA metabarcoding can be considered a valid support in NIS surveillance and monitoring; in particular, it has been demonstrated to be a powerful method in the detection of NIS even at low abundances [5], and it can be considered a crucial tool in the implementation of early warning strategies [53].

The results obtained in the present work support the importance of NIS monitoring in marinas and small harbors through eDNA metabarcoding, having screened several NIS that otherwise would not have been detected. The percentage of marine NIS detected by using the three different primer pairs was high, ranging from 37 to 75%, with COI-2 and Cas being the primer pairs that detected the highest number of species. The use of more primer pairs will allow for the identification of different species arrays, which together provide a more complete picture of biodiversity and provide a promising NIS surveillance tool within an early detection and rapid response system. Furthermore, the highlighted differences between bilge and adjacent seawater support the role of bilge in the transport of different species, including the NIS.

Regarding most identified species having an extra Mediterranean distribution in both the port and the bilge water, the main questions that arise are how these new species, or better, their DNA, entered the Mediterranean and if they can be considered a new arrival. There are some main issues to discuss on this. The first is that the presence of species-specific DNA does not mean that the species is present and alive in that sample. Several studies have demonstrated that extracellular eDNA molecules can persist in water for several days to weeks [31,54], even if degraded by the action of environmental factors [55], so Ponchon et al. [36] underlined the need for further research to improve knowledge on eDNA persistence. However, the presence of NIS as DNA in most of the analyzed samples lets us hypothesize their occurrence as living organisms.

The other point worthy of note is that the high number of reads corresponding to species not yet detected in the Mediterranean may depend on the incompleteness of the available databases; some reads could not be assigned to a species due to incomplete databases. This is the case, e.g., of *Ascidia ahodori*: the obtained reads showed a very high match (99.8%) with this north Pacific species, but the Genbank nucleotide database does not contain any sequences of Mediterranean *Ascidia* species. This stresses the importance of further improvements to the database in order to advance the performance of metabarcoding in monitoring studies and suggests considering as valid only those species whose taxonomic groups are well studied. Many authors reported that, albeit the importance of biodiversity assessment is well known and the metabarcoding was demonstrated to be a useful tool, reference databases for many marine taxa are still very incomplete [56–58]. One of the challenges of metabarcoding studies should, therefore, be the production of exhaustive databases in order to avoid a high proportion of missing taxa. Furthermore,

a valid database should be taxonomically diverse, cover each target group, and be free from mislabeled sequences [59]. Implementation of databases does not mean a mere increase in the number of different sequences; it is necessary to have taxonomic expertise for species validation, especially for those groups with difficulties in classification, such as those including cryptic species or species complexes.

Given all the above, the identification of DNA from extra-Mediterranean species surely needs further investigation; the development and testing of targeted metabarcoding could validate the occurrence of several selected species. This hybrid methodology is based on the use of metabarcoding and the subsequent design of primer sets to amplify mitochondrial DNA regions of selected species with higher PCR efficiency, so it combines the positive aspects of qPCR and metabarcoding. It was demonstrated to be a potentially valuable tool in invasive species monitoring and detection in aquatic systems [60]. The analyses of eDNA metabarcoding carried out in the bilge waters were able to detect the DNA presence of marine species and specifically of NIS, although they contained concentrated debris that could have complicated laboratory procedures and consequently affected the results. In general, the analysis returned a heterogeneous set of species, including terrestrial/aerial (mainly insects), freshwater, and marine species, some of which derive from food contamination (e.g., Atlantic salmon and giant tiger prawn; see Table 4) and from other kinds of contamination, such as terrestrial organisms' faeces. Furthermore, differences in DNA persistence in the environment have been demonstrated to have a direct impact on DNA transfer by boats and consequently on results. After the OTUs validation, taking into account the abovementioned bias sources, marine species accounted for 36–47% of the total identified OTUs. In terms of species richness and evenness, bilge and port samples resulted in differences, with bilge samples being generally more heterogeneous than port samples, possibly depending on the different boat movements. In terms of species composition, bilge waters contained a different species array compared to that found in the ports, with some species present only in one or the other compartment. Although it is only a hypothesis to be tested with a targeted approach, such as active surveillance with digital drop PCR [61], it can be affirmed that the analyzed bilge waters could be vectors of species spread among visited ports by boats or in the environment. In the last decades, several authors have studied the role of bilge water as a vector of species spread, analyzing the species composition. In particular, Darbyson et al. [26] conducted a biosecurity assessment of bilge water, looking for the presence of the invasive species *Styela clava* and *Carcinus maenas*, confirming the important role of bilge water in the spread of these species. Zaiko et al. [62] demonstrated that invertebrates can survive in bilge and ballast water for at least 16 days, supporting the idea that bilge can carry propagules of them from one site to another. Among the surviving species, there might also be non-indigenous species and/or marine pests, as demonstrated by Fletcher et al. [27] in a study carried out in New Zealand marinas where five different non-indigenous species were identified in bilge water samples. In the present work, a total of 140 species with extra-Mediterranean distribution were found in the bilge samples, 12 of which were already reported as non-indigenous species in the Mediterranean Sea and nine new for the Italian waters. With all the necessary precautions regarding the potential bias of the methodology, we can say that bilge could be the vector of introduction for this species. The high number of NIS found in the bilge water highlights the importance of considering bilge waters as a "sampling matrix" to be monitored in NIS management. Early detection of NIS, when their population is small and still confined in the arrival area, is demonstrated to be one of the most reliable management measures, maximizing the probability of containment and control of the species [63]. eDNA metabarcoding can thus be considered a complement to conventional approaches, and as stated above, it has to be followed by active surveillance, including visual confirmation of the species [28,50,64]. Furthermore, up to date, only commercial ports and sometimes ballast waters were monitored, being considered the primary hub of NIS introduction, leaving aside the role of bilge waters as a vector of NIS spread. The dense network of recreational and fishing boat routes, together with the lack of NIS surveillance on these,

would increase the chances of spreading around little harbors and marinas. Bilge waters have been little investigated for NIS detection, and even less through eDNA analysis that has been applied only by Fletcher et al. [27] and Ponchon et al. [36]. They compared species detection in bilge water by traditional methods and eDNA metabarcoding and found that traditional assessment provided less taxonomic resolution compared to eDNA metabarcoding. This novel approach proved to be a promising tool for NIS surveillance and subsequent management actions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15111117/s1, Figure S1: Concentration of extracted DNA from port and bilge water in Isola delle Femmine harbor (IF) and Cala harbor (CA).

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