Environment International 199 (2025) 109489

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

A comprehensive assessment of non-indigenous species requires the combination of multi-marker eDNA metabarcoding with classical taxonomic identification

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ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Environmental DNA
Alien species
Marine biodiversity
molecular-based NIS identification
Morphological identification
Mediterranean Sea

ABSTRACT

In marine environment, non-indigenous species (NIS) can alter natural habitats and cause biodiversity loss with important consequences for ecosystems and socio-economic activities. With more than 1000 NIS introduced over the last century, the Mediterranean Sea is one of the most threatened regions worldwide, requiring an early identification of newly entered alien species for a proper environmental management. Here, we carried out environmental-DNA (eDNA) metabarcoding analyses, using multiple molecular markers (i.e., *18S rRNA*, *COI*, and *rbcL*) and different genetic databases (i.e., NCBI, PR2, SILVA, MIDORI2, MGZDB, and BOLD), on seawater and sediment samples collected on a seasonal basis in three Mediterranean ports located in the North Adriatic, Ionian and Tyrrhenian Sea to identify marine species, and particularly NIS. The use of the multi-marker eDNA metabarcoding allowed the identification of a higher number of species compared to the morphological analyses (1484 vs. 752 species), with a minor portion of species shared by both approaches. Overall, only 4 NIS were consistently identified by both morphological and molecular approaches, whereas 27 and 17 NIS were exclusively detected by using eDNA metabarcoding and classical taxonomic analyses, respectively. The eDNA metabarcoding allowed also identifying the genetic signatures of 5 NIS never reported in the Italian waters. We conclude that eDNA metabarcoding can represent a highly sensitive tool for the early identification of NIS, but a comprehensive census of the NIS requires the combination of molecular and morphological approaches.

1. Introduction

Our oceans are increasingly threatened by anthropic activities, among these, the globalization of maritime transportation, which favors the spread of non-indigenous species (NIS) (Molnar et al., 2008; Occhipinti-Ambrogi, 2007), some of which can become invasive (Katsanevakis et al., 2014; Ricciardi et al., 2017). The introduction and establishment of NIS into new environments can be favoured also by global climate change and anthropogenic stressors (e.g. habitat damage/alteration, pollution, overexploitation of biological resources) (Albano et al., 2021; Beca-Carretero et al., 2024; Gissi et al., 2021). NIS can profoundly alter biodiversity by replacing native species (e.g., through efficient competition for food sources and space), and modify the structure and functioning of the marine food web, with cascade effects on the provisioning of ecosystem's goods and services (Occhipinti-Ambrogi, 2007; Ojaveer et al., 2023).

The Mediterranean Sea is one of the most invaded marine basins worldwide (Edelist et al., 2013; Molnar et al., 2008; Tempesti et al.,

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https://doi.org/10.1016/j.envint.2025.109489
Received 5 September 2024; Received in revised form 16 April 2025; Accepted 18 April 2025

2020) with more than 1000 NIS recorded (Galanidi et al., 2023) out of an overall estimate of 17,000 marine species described, mostly belonging to animals and photosynthetic organisms (Coll et al., 2010), and a rising trend of reported NIS over time, which has increased by 40 % over the last two decades (Galanidi and Zenetos, 2022; Zenetos et al., 2022). The main vectors of NIS introduction in the Mediterranean Sea include: i) the opening (and recent enlargement) of the Suez Canal, ii) aquaculture activities and aquarium trade, and iii) maritime transportation (Bailey et al., 2020; Gewing and Shenkar, 2017; Hulme, 2015; Molnar et al., 2008). Currently, approximately 90 % of the global trades are based on shipping (United Nations Conference on Trade and Development; UNCTAD, 2023, 2021), which implies the potential transfer of NIS through ballast waters and biofouling (Fernandes et al., 2016; Slišković et al., 2021). For this reason, commercial and touristic ports are the first terminals for the introduction of NIS in marine environments and their spreading in the surrounding areas (Galanidi et al., 2023; Tempesti et al., 2022, 2020; Ulman et al., 2017). Ports are impacted systems characterized by low diversity, where opportunistic NIS can exploit available ecological niches and outcompete native species (Ferrario et al., 2017; Sempere-Valverde et al., 2024; Ulman et al., 2017). The establishment, diffusion, and impacts of NIS can vary depending on specific environmental conditions, invader characteristics, resource availability, and community structure within the invaded ecosystem (Geburzi and McCarthy, 2018).

To date, prevention remains the most effective strategy for minimizing the NIS impact, considering their early detection as a key management priority in controlling their spread (Trebitz et al., 2017). To this aim, different national and international, policies and guidelines have been implemented (Katsanevakis et al., 2023). For European countries such issues are defined in the Marine Strategy Framework Directive (MSFD—2008/56/EC) Descriptor 2, according to which member countries develop standardized samplings and methodological procedures for evaluating new NIS introduction and trends in abundances and spatial distribution of established NIS (Magliozzi et al., 2024).

The presence of larval stages or juveniles of NIS is sometimes difficult to be recognized by using traditional morphological approaches and can remain unnoticed, until these organisms reach the maturity stage and a high population size (Pochon et al., 2013; Zaiko et al., 2015, 2016). This limits the timely detection of NIS especially during their initial phases of introduction (Bax et al., 2003), since once established the chances of their eradication are very low (Geburzi and McCarthy, 2018; Olenin et al., 2011).

Environmental DNA (eDNA) metabarcoding is widely used for marine biodiversity identification of both planktonic and benthic organisms (Pawlowski et al., 2022; Yang and Zhang, 2020) and can also be used to track the presence of NIS (Duarte et al., 2021; Knudsen et al., 2022; Maggio et al., 2023), in samples collected from hard natural and artificial substrates (e.g. settlement plates) and of seawater in ports, marinas, and estuaries (Duarte et al. 2021). NIS identification in soft sediments have received less attention (Gollasch et al., 2019) even though can provide an early detection for a wide range of taxa particularly resting stages of planktonic organisms, which can settle to sea floor after ballast water discharge (Holman et al., 2019; Shaw et al., 2019). Thus, the integration of metabarcoding analysis of eDNA from seawater and sediment samples may provide a more comprehensive view of the NIS, including benthic species that have planktonic larval stages (Couton et al. 2019). However, there are still uncertainties related to the reliability and accuracy of the eDNA approach for NIS detection which still requires additional investigations, including multiple-based marker approaches (Aglieri et al., 2023; Azevedo et al., 2020; Couton et al., 2022; Westfall et al., 2020).

Here we used eDNA metabarcoding targeting multiple molecular markers in combination with different genetic databases to improve NIS identification in seawater and sediment samples collected on a seasonal basis in three Italian ports located in different regions of the Mediterranean Sea (North Adriatic, Ionian and Tyrrhenian Sea). Such sampling areas were selected because they represent major sites of introduction of NIS through maritime traffic and their potential spreading and as such are surveyed by the Italian Environmental Protection Agencies under the European Marine Strategy Framework Directive (MSFD). We compared eDNA results with those obtained by classical morphological approaches to assess their effectiveness for NIS detection.

2. Materials and methods

2.1. Study sites and environmental characteristics

Three Italian port systems (i.e., Civitavecchia, Trieste, and Taranto ports) of international socio-economic relevance have been investigated since they are located in different Mediterranean sectors with special attention to high-priority areas with increased likelihood of NIS introductions. The port of Civitavecchia is located in the Tyrrhenian Sea and it is one the largest ports of the Western Mediterranean Sea (Lorenčič et al., 2020), with regular ferry links to other Mediterranean ports and important traffic of cruise ships, cargo ferries, and carrier ships (Gobbi et al., 2020). The port of Taranto is located in the Ionian Sea and it is one of the most important commercial hubs of the Central Mediterranean Sea. This area is influenced by different anthropogenic pressures including urbanization, discharge of industrial wastewaters, commercial fishing, and aquaculture. It is included in a highly contaminated area (Site of National Interest, Cardellicchio et al., 2016) and is considered a hotspot of NIS (Tempesti et al., 2020). Finally, Trieste's port is situated in the North Adriatic Sea and it represents a core for diverse maritime pathways and transportation routes (Caramuta et al., 2021).

2.2. Sampling strategy

A pre-survey was conducted in Trieste port in July 2021 to test the different genetic markers and to set up the most appropriate methodology (e.g., filter type, volume). Then, sampling campaigns were conducted in late winter (March), summer (July), and autumn (October) 2022 in all ports.

In each port sampling sites were located in areas subject to the influence of commercial and/or touristic maritime traffic or close to areas designated for ballast water discharge. Such sampling strategy was selected as the most suitable for detecting NIS introduction in the three selected ports following protocols used by Italian Environmental Protection Agencies under the MSFD and can be also adopted in other ports worldwide (Hewit and Martin, 1996; Hewitt and Martin, 2001; Campbell et al., 2007).

In the selected ports, we collected water and sediment samples at two stations (hereafter defined as Station 1 and Station 2) for eDNA analyses and morphological identifications of species (Fig. 1). In the Trieste port, water samples at each station were collected contextually for eDNA analysis and morphological identification of planktonic organisms. In Civitavecchia and Taranto ports, seawater samplings for morphological and molecular analyses were carried out within approximately one week. Such sampling strategy allows to conciliate the logistic needs with preservation of the genetic signatures of NIS in eDNA (Scriver et al., 2024a; Wood et al., 2020).

Sediment samples for eDNA analyses were collected at the same time of seawater samples. For the morphological identification of organisms present in the sediment (i.e. soft substrates), samples were collected in the same season within two weeks of those of eDNA analysis. Organisms of artificial hard substrates (e.g., piers and other artificial structures) as well vagile epi-megabenthos were collected in the same season in an area close to the stations where soft sediments and seawater samples were taken to identify genetic signatures of organisms in the water column, such as larvae, cells, or excretion material, transported by currents and settling on the seafloor through sedimentation processes. The collection of eDNA samples was carried out, before those for

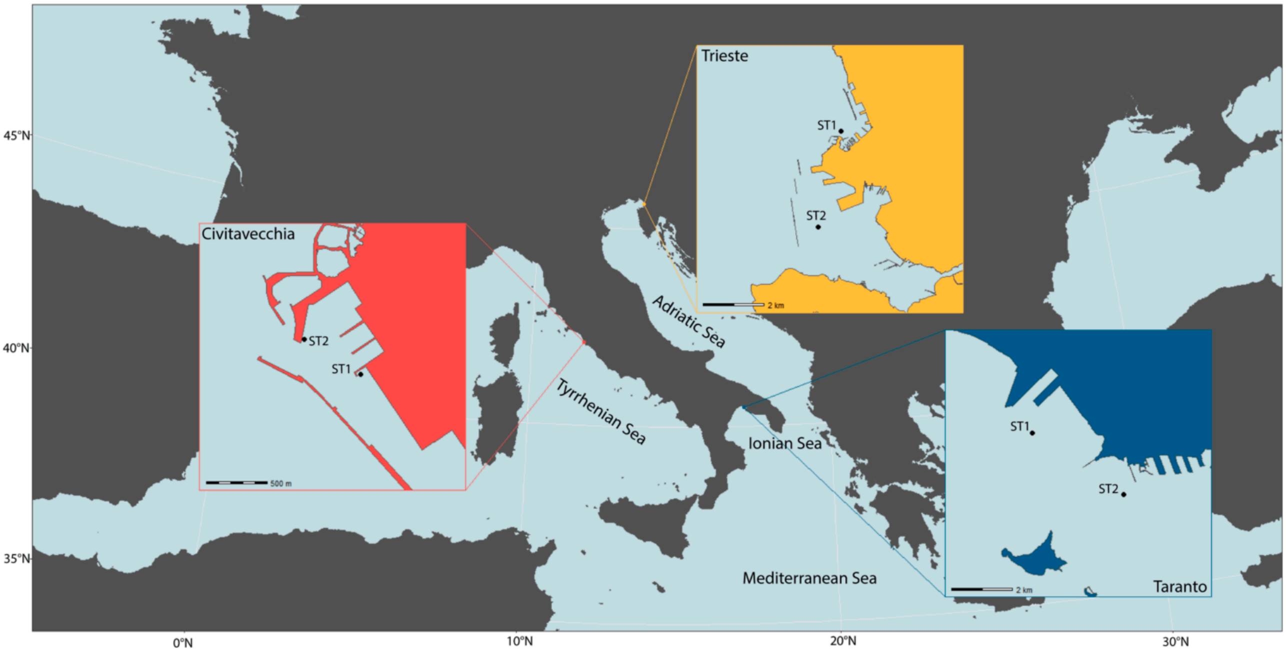

Fig. 1. Sampling locations at three major ports in the Mediterranean Sea: the port of Trieste in the Adriatic Sea (yellow colored), Taranto port located in the Ionian Sea (blue colored) and Civitavecchia port in the Tyrrhenian Sea (red colored). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

morphological identification since this strategy can allow the detection of organisms in the early stages potentially released by ballast waters and ship hulls before their settlement, colonization and growth of soft and hard substrate. For eDNA analyses, a total of 20 L of seawater for each station was collected through two independent deployments of 5 L Niskin bottles carried out at the surface (at ca. 1 m depth) and bottom (ranging from 12 to 20 m depth) of the water column. Aliquots of 2 L of seawater were vacuum-filtered with sterile apparatus using mixed cellulose ester membranes (MCE, 0.45 µm pore size, 47 mm diameter, Merck KGaA, Darmstadt, Germany) and stored at –20 °C until DNA extraction.

Water samples for the taxonomic identification of phytoplankton based on morphological approach were collected by plankton net (with a mesh size of 20 µm) and Niskin bottle, whereas large organisms were identified by visual census. Nets were rinsed with seawater and subsamples of 250 ml were collected. Samples from nets and Niskin bottles were transferred into dark glass bottles and stored at 4 °C and fixed with Lugol's solution. Identification and counting of phytoplankton were carried out using an inverted microscope, equipped with phase contrast, following the Utermöhl method (Hasle 1978).

For zooplankton analysis, samples were collected with a WP2 plankton net with a mouth area of 0.25 m^2 and a mesh aperture of 200 µm, each equipped with a 500 ml plexiglass filtering code-end. The net was towed vertically from the surface down to 1 m above the seafloor. The filtered water volume was calculated using a mechanical flowmeter. Upon collection, zooplankton samples were preserved in 70 % ethanol for taxonomic identification which was carried out by a stereomicroscope.

For eDNA analyses sediment samples were collected at each site by 3 independent deployments of a Van Veen grab. Aliquots of sediment obtained from the surface layer were kept at −20 °C until DNA extraction.

For morphological identifications of macrobenthos, sediments (soft substrates) were collected using a Van Veen grab (N = 6 replicates) and sorted with a sieve of a mesh of 500 µm whereas those of hard substrates were collected by scuba divers through scraping of an area equal to 0.1 m^2. All samples were fixed onboard with an aqueous solution of formaldehyde buffered with sodium tetraborate in a final concentration of 4 %. Vagile epi-megabenthos was collected by means of baited traps kept in selected sites for at least 12 h. Samples taken for taxonomic identification were stored in 70 % ethanol (modified from HELCOM and OSPAR, 2013).

Samples collected from hard and soft substrates were analyzed under a stereomicroscope for sorting organisms and light microscopy for taxonomic identification at the species level using the standard dichotomic analysis. All taxonomic identifications have been carried out by Italian Environmental Protection Agencies.

At each site and sampling period, temperature and salinity values of the surface and bottom waters were acquired by conductivity, temperature, depth (CTD) casts, whereas water transparency data were measured through the Secchi disk. The sampling effort (as the number of samples analyzed) and environmental conditions at the sampling locations are reported in (Tables S1-S2).

2.3. *Environmental DNA extraction, amplification and sequencing*

Environmental DNA (eDNA) was extracted from MCE filters using DNeasy PowerWater kit (QIAGEN©, Düsseldorf, Germany) and from 10 g of sediments (wet weight) using DNeasy PowerMax Soil kit (QIAGEN©, Düsseldorf, Germany). Two independent replicates of filters were analyzed for each station and depth, whereas three independent replicates were analyzed for each station. The DNA concentration was determined by absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using NanoDrop spectrophotometer (ND-1000 UV–vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA).

To identify a large number of marine species belonging to different phyla, five different genetic markers were selected for metabarcoding: 3 different regions of the ribosomal *18S rRNA* gene (V4, V9, and V7), a region of the mitochondrial Cytochrome Oxidase subunit 1 (*COI*) gene and the Ribulose Bisphosphate Carboxylase gene (*rbcL*). For the amplification of the *COI* gene two primer sets were used: mlCOIintF – jgHCO2198 for eDNA extracted from filters, whereas mlCOIintF – dgHCO2198 for eDNA extracted from sediments (Leray et al., 2013), for the amplification of V9 region of *18S rRNA* (Amaral-Zettler et al., 2009), for the amplification of V4 region (Piredda et al., 2018), for the V7 region (Guardiola et al., 2015) and for *rbcL* (Little, 2014) (Table S3).

Three aliquots of extracted eDNA from each sample (ranging from 5 to 20 ng) were subjected to 1st-round PCR amplification using specific conditions for each genetic marker primer set (Table S4A) with overhang Illumina adapters, as well as different enzymatic reaction conditions (Table S4B). The PCR products were checked on 1.5 % agarose gel and cleaned from free primers and primer dimers using the CleanNGS beads (CleanNA, The Netherlands) following the manufacturer's instructions. After pooling PCR replicates, a 2nd-round PCR was carried out to apply dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina) by 8 cycles PCR (16S Metagenomic Sequencing Library Preparation, Illumina). After purification by the CleanNGS beads (CleanNA, The Netherlands), the libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) by the Synergy^{TM} 2 microplate reader (Biotek). The libraries were pooled in equimolar quantities in a final amplicon library and analyzed on a 4150 TapeStation platform (Agilent Technologies, Santa Clara, CA, USA). The final library was sequenced on an Illumina® MiSeq (PE300) platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54) at the Sequencing Platform of Edmund Mach Foundation.

2.4. *Mock communities*

We used two mock communities as a positive control to validate the taxonomic identification of eDNA by bioinformatics, one for phytoplankton and one including benthic metazoan taxa. A total of 9 previously identified taxa covering a range of taxonomic groups plus 3 algal species were selected. DNA was isolated from species using DNeasy Blood & Tissue Kit (QIAGEN©, Düsseldorf, Germany). DNA from algal cultures was extracted with DNeasy PowerSoil Pro (QIAGEN©, Düsseldorf, Germany). The DNA of *Saccharomyces cerevisiae* was purchased from (Merck KGaA, Darmstadt, Germany). DNA isolated from different taxa was pooled in equal amounts in each sample before library preparation. Mock DNA was amplified with different primer pairs and sequenced along with DNA extracted from sediment and seawater samples.

2.5. *Bioinformatic analyses*

Raw sequences resulting from the metabarcoding with *18S rRNA V4, V7, V9*, *COI* and *rbcL* markers were processed through QIIME2 (Bolyen et al., 2019). Primer sequences were removed using Cutadapt v4.5 with a maximum error rate of 0.1 % (Martin, 2011). Sequences were then merged with a minimum overlap of 12 nucleotides, and errors were corrected with DADA2 v1.20 to obtain amplicon sequence variants (ASVs), excluding singletons (Callahan et al., 2016). Taxonomic assignments were performed using VSEARCH v2.21.2 (Rognes et al., 2016) and the Basic Local Alignment Search Tool (BLAST; Camacho et al., 2009). *COI* sequences were aligned against four different databases: National Center for Biotechnology Information (NCBI; Sayers et al., 2022), MIDORI2 (Leray et al., 2022), Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007), MetaZooGene Atlas and Database (MGZDB; Bucklin et al., 2021), whereas 18S rRNA sequences were aligned against Protist Ribosomal Reference (PR2; Guillou et al., 2013) and SILVA (Quast et al., 2012), and *rbcL* sequences were classified using NCBI.

For the taxonomic assignment of ASVs five different percentage values of sequence similarity were used (ranging from 80 % to 99 %) since optimal thresholds can vary depending on the marker used (Bonin et al., 2023; Hleap et al., 2021). The most accurate percentage values of sequence similarity of each genetic marker were chosen calculating the best metrics for benchmarking (Hleap et al., 2021) resulting from sequences of mock communities clustered at different similarity levels (see figures S1-S2 and Tables S5A-E; Ershova et al., 2023). For each genetic marker, the combination of taxonomic databases, identity percentages, software for taxonomic assignment of ASVs selected is reported in Table S6.

To account for contamination in the field and laboratory, a small number of ASVs present in the negative controls from all subsequent datasets were excluded. Sequences belonging to terrestrial species were removed through the package WoRMS (Ahyong et al., 2024) in R studio (Team, 2021) retaining only the sequences of species present in the World Register of Marine Species database. Pandas and NumPy libraries in a Python script were used to categorize all obtained species as native or non-indigenous and to perform cross-checks between the list of identified species and databases (McKinney, 2012). The list of Mediterranean NIS was retrieved from (Galanidi et al., 2023) and from the Global Invasive Species Database (GISD) of the International Union for Conservation of Nature (IUCN) for identifying non-indigenous invasive species.

3. Results

3.1. *Sequencing results and taxonomic assignment*

Mock community analyses by metabarcoding using various molecular markers and databases revealed differences in taxonomic resolution, with an average of 46 % of reads being classified at the species level (Fig. S3). The combination of different markers and genetic databases allowed us to successfully identify 9 out of the 12 taxa in the mock community samples (Table 1).

The eDNA metabarcoding conducted in the pre-survey at the Trieste port using the different molecular markers allowed us to define the most appropriate combination of filter type and water volume for detecting the highest number of species and NIS (Fig. 2A-B, Fig. S4). Analyses carried out with 2 L of seawater samples concentrated on MCE identified 274 genetic signatures classified at species level, of which 9 were identified as NIS. No species was identified in water and sediment samples by using all molecular markers and from 5 to 156 species were exclusively detected using *rbcL* and *COI* markers, respectively (Fig. S5).

MiSeq runs carried out on eDNA from seawater and sediment samples collected in the three ports in the three sampling periods over 2022 resulted in a total of ~ 90 million raw paired reads, reduced to ~ 72 million after removal of the low-quality sequences, aligning paired ends for all markers (Table S7). After bioinformatic processing of raw reads, the highest number of ASVs was observed through *18S V4 rRNA* sequencing (48,666 ASVs), while the lowest number was observed with the *rbcL* marker (4,843 ASVs). Rarefaction curves indicate that the sequencing depth was sufficient to capture the ASV diversity across all three ports over the three sampling periods (Supplementary Figs. S6-S8).

Only 1.3 % of the total number of ASVs identified through eDNA metabarcoding were assigned at the species level. The highest number of species was obtained using 18S rRNA V9 (686 species), followed by *18S rRNA V4* (568), 18S rRNA V7 (482), *COI* (397) and *rbcL* (15). Exclusive genetic signatures belonging to different species were identified using the different molecular markers (*COI*, three regions of *18S rRNA,* and *rbcL*) and only one ASV classified at the species level was consistently identified by all genetic markers (Fig. 3A). Clustering ASVs with different genetic databases greatly improved the power of detection of eDNA metabarcoding. Each database allowed the identification of unique species, and particularly, when MIDORI2 was used, the highest number of exclusive species (108) was detected compared to the other *COI* databases (NCBI, MGZDB and BOLD, Fig. 3B). For *18S V4*, *V7*, and *V9*, ASVs classified at species level were compared between the two data sets based on PR2 and SILVA database (Fig. 3C-E). On average, only 21 % of the identified species by the different 18S rRNA markers was shared between the two databases.

3.2. *Comparison between eDNA metabarcoding and classical taxonomic approach for species identification*

The eDNA metabarcoding analysis carried out on seawater and

Table 1
Mock community composition at lowest taxonomic level analyzed through DNA metabarcoding. Black circles indicate taxa identified at molecular level.

Kingdom	Phylum	Class	Order	Family	Genera	Species	Mol Id
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Chamelea*	*gallina*	•
Animalia	Cnidaria	Hexacorallia	Actiniaria	Actiniidae	*Anemonia*		
Animalia	Annelida	Polychaeta		Chaetopteridae	*Chaetopterus*		•
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae			•
Animalia	Chordata	Teleostei	Clupeiformes	Clupeidae	*Clupea*	*harengus*	•
Animalia	Annelida	Polychaeta	Sabellida	Oweniidae	*Oweniia*	*fusiformis*	•
Plantae	Chlorophyta	Chlorodendrophyceae	Chlorodendrales	Chlorodendraceae	*Tetraselmis*	*suecica*	•
Plantae	Chlorophyta	Chlorophyceae	Chlamydomonadales	Dunaliellaceae	*Dunaliella*	*salina*	•
Chromista	Heterokontophyta	Bacillariophyceae	Naviculales	Phaeodactylaceae	*Phaeodactylum*	*tricornutum*	•
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*cereviesiae*	•
Animalia	Arthropoda	Malacostraca	Amphipoda	Caprellidae			
Animalia	Arthropoda	Malacostraca	Tanaidacea				

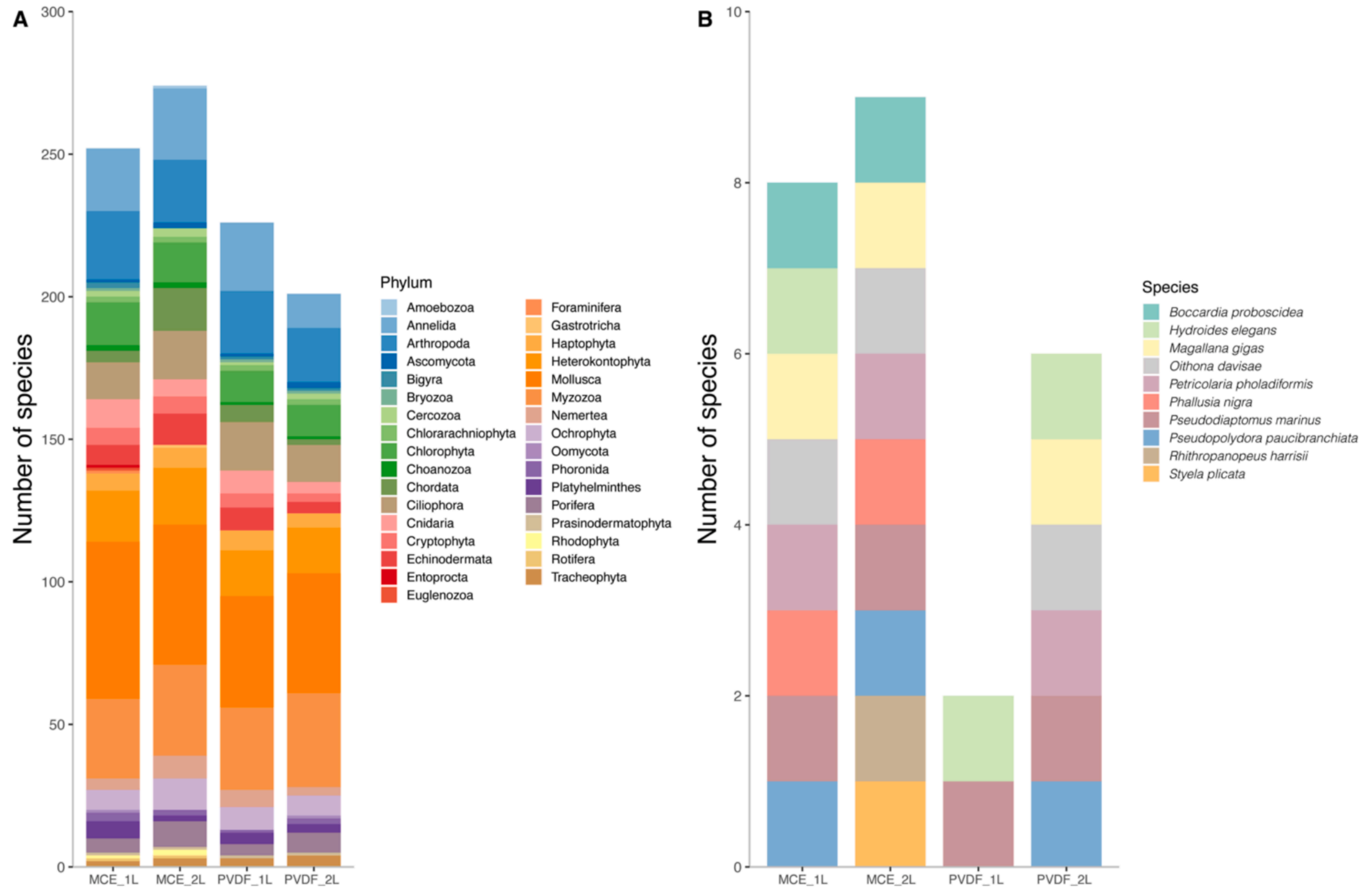

Fig. 2. Number of species belonging to the different phyla identified through eDNA metabarcoding with different genetic markers carried out in Trieste during the pre-survey with different filters (mixed cellulose ester (MCE) and polyvinylidene difluoride (PVDF)), and seawater volumes (1 L and 2 L) (A). NIS detected using different combinations of filters and seawater volumes (B).

sediment samples collected in the three ports over the three sampling periods of 2022 allowed the identification of 1484 species belonging to 48 phyla (Fig. 4). The greatest number of species of the kingdom Animalia belong to the phyla Annelida (154 species), Mollusca (130), and Arthropoda (113). Regarding the chromists, the most abundant species found belong to the phylum Myzozoa (160 species), followed by Bacillariophyta (153) and Ciliophora (121). The kingdom Plantae is mainly represented by species belonging to the phyla Rhodophyta (56 species) and Chlorophyta (52 species).

The analysis conducted using the classical taxonomic approach on the soft and hard bottom and seawater samples collected in the three ports during the three sampling periods allowed the identification, of 752 species, belonging to 23 phyla (Table S8). A total of 560 species were identified only through morphological approaches, of which 253 species were not found in reference sequence databases. In all sampling periods and ports, the number of species identified by eDNA metabarcoding was higher than those determined by classical taxonomic approach (Fig. 5A-B). Overall, 1292 species of unicellular and multicellular organisms belonging to 47 phyla were exclusively identified through eDNA metabarcoding. Only 192 species belonging to 15 phyla were detected by both molecular and morphological approaches.

The eDNA metabarcoding analysis based on the use of *COI* and *18S rRNA* allowed the identification of 22 and 17 NIS, respectively (Table 2). No NIS were detected with the *rbcL* marker. Overall, 25 NIS were identified in seawater and 14 in sediment samples by the molecular approach. 14 NIS were exclusively detected using the *COI* marker, and

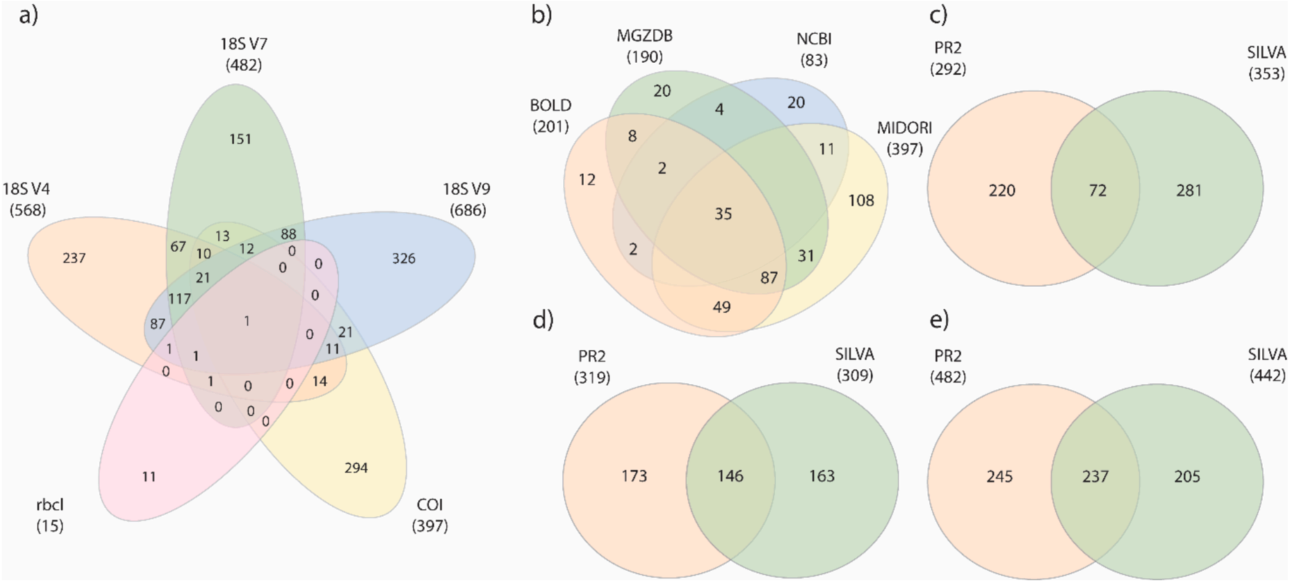

Fig. 3. Venn diagrams of the species identified with different genetic markers (A), species identified with *COI* databases (MGZDB, MIDORI2, NCBI, and BOLD) (B), and species identified with 18S databases (PR2 in orange and SILVA in green) for *18S V4* (C), *18S V7* (D), *18S V9 rRNA* (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

some of them were only specifically found in one port and sampling time. Nine NIS were identified only by the *18S rRNA* gene marker, including *Aidanosagitta neglecta*, *Herdmania momus*, *Petricolaria pholadiformis*, and *Phallusia nigra* which have never been detected in the Italian coastal environments. Among NIS, *Aidanosagitta neglecta*, *Hydroides elegans*, *Oithona davisae*, *Petricolaria pholadiformis*, and *Pseudodiaptomus marinus* were present in all ports investigated, although not always in the same sampling period. The eDNA metabarcoding analysis allowed us also to identify species listed in the Global Invasive Species Database (GISD) such as *Rhithropanopeus harrisii*, *Mnemiopsis leidyi* recorded in the Trieste port and *Styela plicata* both in Trieste and Taranto ports. The molecular approach allowed the identification of 31 NIS genetic signatures, which mainly belong to the Phyla: Rhodophyta (7 species), Chordata (5) and Mollusca (5) (Fig. 6A). With the morphology-based approach, 21 NIS mainly belonging to Arthropoda (8), and Annelida (7) were detected (Fig. 6B). Taxonomic analyses allowed the identification of 5 NIS, which have not been found in all genetic databases used (Table S9). Overall, only 4 NIS were detected with both molecular and morphological approaches.

Combining molecular and morphological approaches, the total NIS number detected in the Port of Civitavecchia and Trieste was 24, whereas 22 NIS were detected in the port of Taranto (Fig. 6C). The highest number of identified NIS through eDNA metabarcoding was observed in Civitavecchia port (17 species), followed by Trieste (14), and Taranto (13).

4. Discussion

4.1. Assessing the marine biodiversity through molecular and morphological approaches

We carried out a massive sequencing using multiple markers on eDNA from water and sediment samples collected on a seasonal basis in three ports located in different Mediterranean regions in comparison with a classical taxonomic approach to comprehend whether eDNA can be used for the identification of NIS and facilitate the early identification and eventually the prevention of biological invasions as previously recommended (Roy et al., 2023).

The key issues for an adequate representation of biodiversity and species identification, including NIS, are the collection of appropriate volumes/surface of samples (i.e., definition of sample size) and *a priori* definition of the environments to be investigated (e.g. seawater vs sediment) (Pawlowski et al., 2022; Takahashi et al., 2023). These issues have been taken into account in this study, which also allowed the identification of the most appropriate combination of filter types and seawater volumes to be used for samples concentration. The integrated analyses of eDNA metabarcoding of water and sediment samples conducted in this study allowed us to provide a wide overview of marine biodiversity, including NIS in different developmental stages, which are difficult to be morphologically identified.

In recent years, multi-marker metabarcoding approaches are spreading to improve the assessment of the diversity of marine species (including alien species) based on a single molecular marker (Duarte et al., 2021). We report a limited overlap of number of species identified using different genetic markers (range 0.7 %–22 %), indicating that the use of multi-markers allows to obtain a more representative picture of the local biodiversity. The present study highlights the usefulness of applying the multi-marker metabarcoding approach not only for the detection of NIS but also for identifying a greater number of taxa and phyla compared to classical taxonomic identifications (Keck et al., 2022; von Ammon et al., 2018). On the other hand, our data highlighted also that multi-marker-based eDNA metabarcoding did not allow the detection of all species that were identified through the taxonomic approach, and only a minor portion (5.5 %) was shared between the two approaches. The limited overlap between the two approaches could also be due to temporal variations due to the sampling strategy adopted (up to ca. one week between different samplings), despite discrepancies remain also when samples for eDNA analysis and morphological identification contextually collected (as in the Trieste port) were compared. At the same time, although bloom-forming species might have considerable quantitative changes even within 7 days, changes in the census of species present are unlikely to be significant in 7 days (Kenitz et al., 2023; Suthers et al., 2009; Zingone et al., 2023).

These results further reinforce previous findings on the differences between biodiversity assessment based on eDNA metabarcoding analysis and morphological approach (Fediajevaite et al., 2021; Keck et al., 2022; Nistal-García et al., 2021). The differences between the two approaches can be due to difficulties in identifying morphologically taxa to the species level especially in the early stages (Thiele et al., 2021), the lack of specialized taxonomists for certain biological components, and

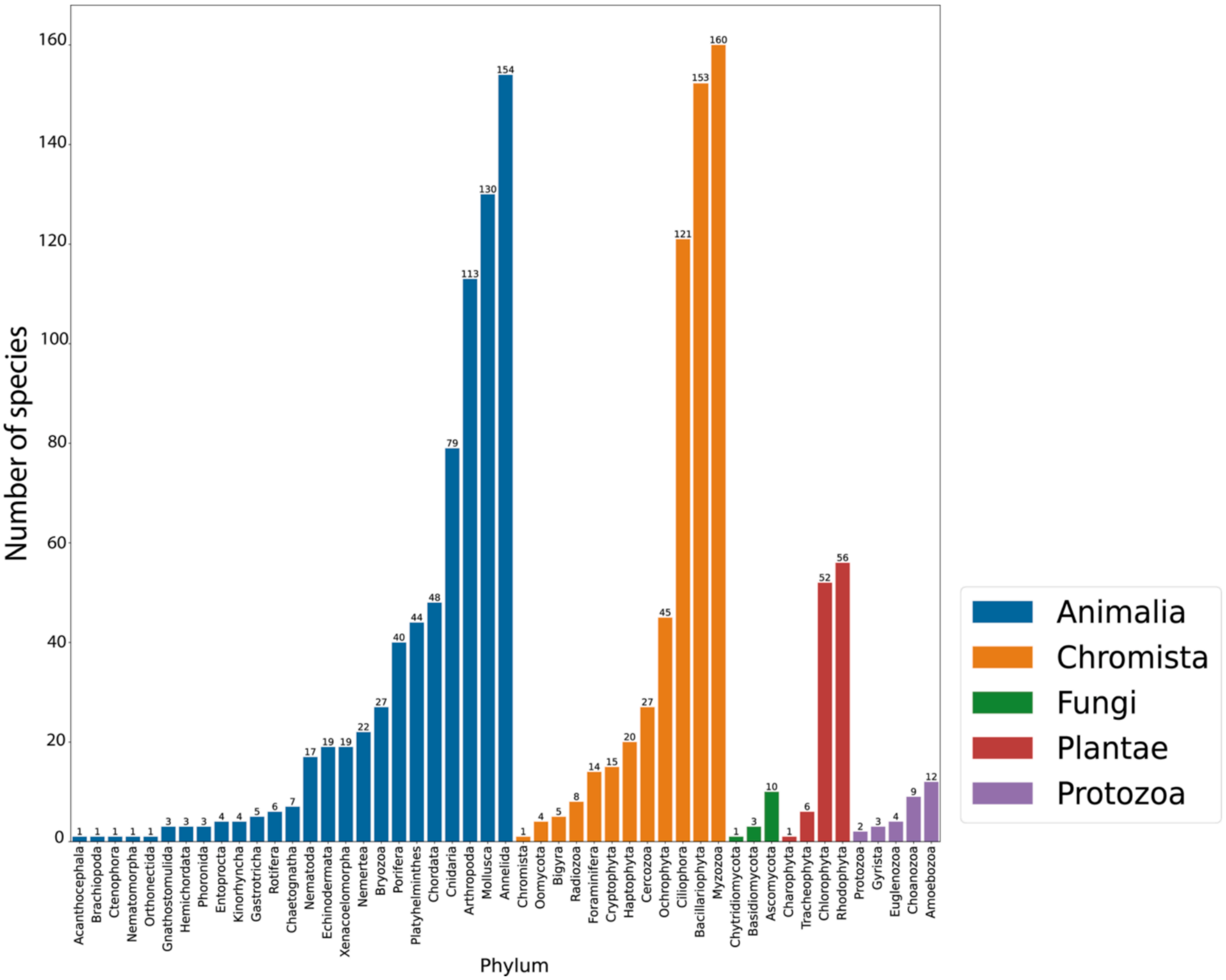

Fig. 4. Number of species belonging to the different phyla identified through eDNA metabarcoding with different genetic markers in different ports over the three sampling periods (spring, summer, and autumn 2022).

the presence of undescribed or cryptic species, but also to the limits associated with eDNA metabarcoding (van der Loos and Nijland, 2021). In particular, despite the advantage of using eDNA metabarcoding for the identification of several species, some important caveats remain, especially with regard to the incorrect identification of some species.

Primer bias and variations in resolution among markers can be solved using different molecular markers. An optimized portfolio of primer sets can mitigate biases in amplification efficiency and taxonomic resolution, but it can also lead to a higher number of false positive results due to non-target amplifications of template sequences (Baetscher et al., 2023). Furthermore, PCR amplification inefficiencies may lead to the non-detection of certain taxa (van der Loos and Nijland, 2021), as also suggested by our metabarcoding analyses of the mock community. These limitations become more pronounced when identification at species level is required for biodiversity assessment, and particularly for NIS detection. Bioinformatic pipelines play a key role when using molecular methodologies (Brandt et al., 2021; Hakimzadeh et al., 2023). In this regard, for NIS identification, the sequence clustering could lead to a taxonomic assignment of NIS that can be incorrectly grouped with other congeneric species (Brown et al., 2016; Pearman et al., 2021). Amplicon sequence variants (ASVs) have been employed since they are characterized by a combination of infraspecific and taxonomic diversity, offering a more accurate resolution than previously used operational taxonomic units (OTUs) (Callahan et al., 2017; Pearman et al., 2021). The latter method involved clustering reads at a specific similarity threshold, typically ranging from 97 to 99 % (Porter and Hajibabaei, 2020). Another important constraint of metabarcoding analysis for the simultaneous detection of multiple species of interest is the incompleteness of comprehensive and curated reference databases specific to each genetic marker employed for taxonomic assignments (Blackman et al., 2023; Carugati et al., 2015; Keck et al., 2023). As shown in this study, to date this limitation can be partially overcome by combining results of sequences clustered against different genetic databases until the building of a unique, public, curated, and screened world genetic bank (Ruppert et al., 2019), which would improve the reliability of species identification. For rigorous species identifications through eDNA metabarcoding, filling the gaps in reference libraries is essential and thus future efforts must be devoted to improve barcode records of marine species and particularly with those with few deposited sequences (e.g. marine mollusks, ascidians; Duarte 2020; Weigand et al., 2019).

4.2. eDNA metabarcoding vs classical taxonomic approach for detecting NIS

Overall, 31 NIS have been identified in the present study by high-

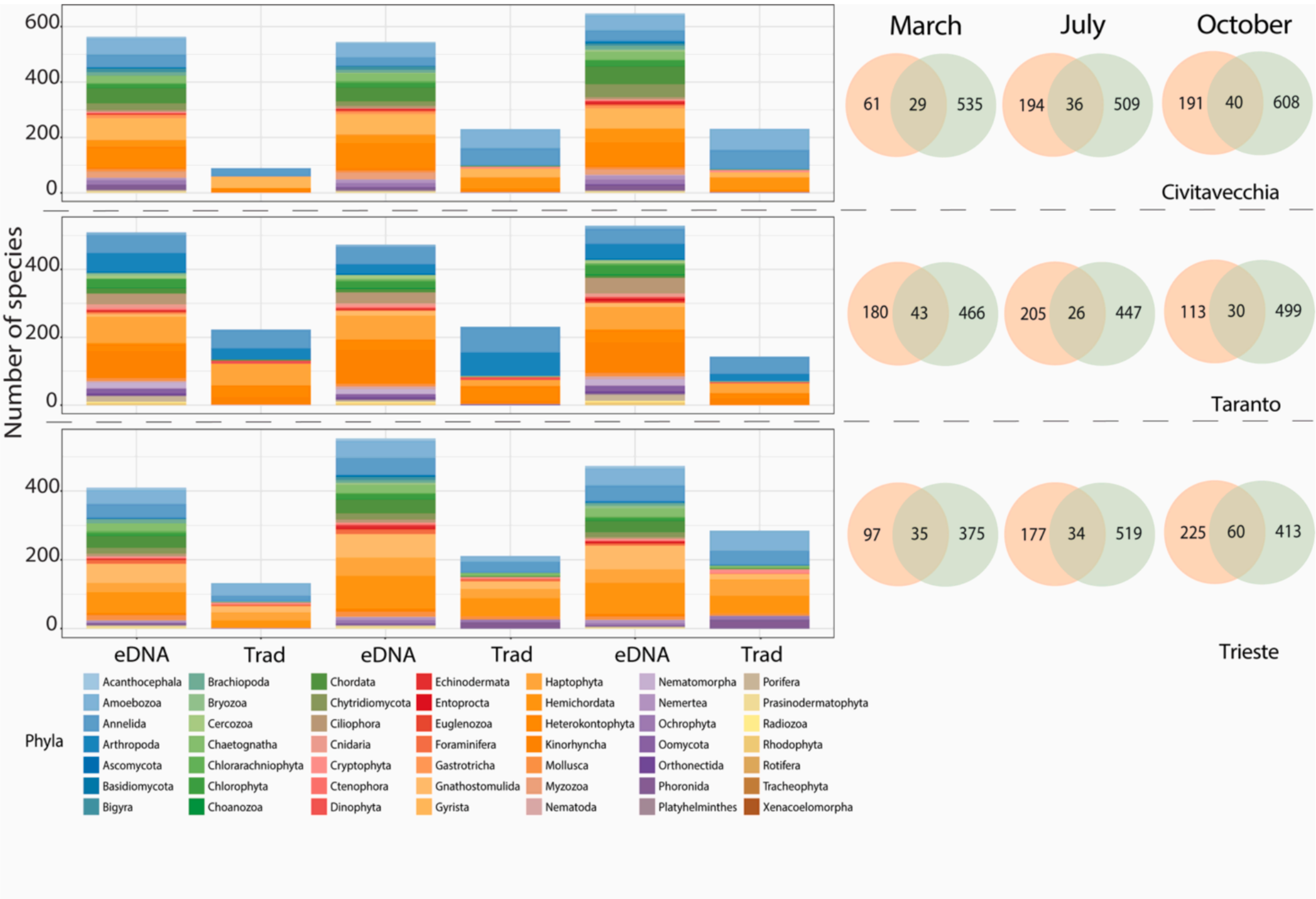

Fig. 5. Comparison between species number obtained by eDNA metabarcoding and morphological analysis. Illustrated are: (left panel) number of species grouped by Phylum identified through molecular (eDNA) and morphological approaches (Trad.) in the three ports; (right panel) Venn Diagrams showing the numbers of shared and exclusive species found with eDNA metabarcoding (green circle) and traditional approaches (orange circle) in March, July and October 2022 in three ports. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

throughput sequencing analysis of eDNA extracted from sediment and seawater samples. Among these, based on available information, the chaetognath *Aidanosagitta neglecta*, the Atlantic polychaete *Boccardia proboscidea*, the bivalve *Petricolaria pholadiformis*, the tropical tunicate *Herdmania momus*, and the solitary tunicate *Phallusia nigra* were detected for the first time in Italian waters. *A. neglecta* and *P. pholadiformis* were identified by molecular analyses in all investigated ports along with the copepods *Pseudodiaptomus marinus* and *Oithona davisae,* which were only detected in some locations by morphological analyses. Previous investigations, based on both eDNA metabarcoding (*18S V4 rRNA*; Lin et al., 2022) and morphological approaches (Pansera et al., 2021; Uttieri et al., 2023) identified these copepods in different Mediterranean areas, besides ports, suggesting a high capacity to adapt to a variety of environmental conditions, and possibly replacing the autochthonous congeneric species (Pansera et al., 2021; Sabia et al., 2014). The discrepancy between molecular tools and classical taxonomic approaches that we found on samples contextually collected suggests that planktonic NIS detection through morphological analyses can require a larger sampling effort (i.e. number of stations, sample volumes, number of replicates) compared to eDNA metabarcoding.

Data reported here also provide evidence of the presence in seawater of genetic signatures of NIS of both hard and soft substrates. The benthic bivalve *P. pholadiformis* and the tunicate *P. nigra*, for instance, have been identified through eDNA metabarcoding from seawater samples, but they were not observed through the morphological approach. On the other hand, we identified in all ports and different seasons the genetic signatures of the serpulid *Hydroides elegans* in the water column, whose presence has been also documented by morphological analyses carried out on artificial hard substrates located inside the investigated ports. The detection of this species by eDNA metabarcoding in the water column of ports located in different marine basins is of relevance for a better understanding of its spatial distribution and spreading in the Mediterranean Sea, being recognized as an invasive species (Streftaris and Zenetos, 2006). Since the genetic imprints of all these NIS were identified from seawater samples, we report here the possibility of detecting the presence of the meroplanktonic larvae of these species. The same applies to the bivalve *Magallana gigas* identified by eDNA metabarcoding in seawater of the Trieste port in July and October 2022, corresponding to periods in which temperature regimes of the North Adriatic Sea can favor spawning events (Ezgeta Balić et al., 2020), but not found in the adult stage by morphological approach. The eDNA metabarcoding of seawater samples allowed also the detection of the American crab, *Rhithropanopeus harrisii*, which has been documented in the Mediterranean Sea since 1996 (Mizzan and Zanella, 1996) and was identified for the first time in the Trieste port. This species has been included in the list of the Global Invasive species Database (GISD; Pagad et al., 2015) and can cause clogging of pipes and damage fishing nets (Roche and Torchin, 2007). These results indicate that eDNA metabarcoding can detect the genetic signatures of organisms in the early life stage (i.e., the meroplanktonic larvae of benthic species), which escape the classical taxonomic identification methods (Rishan et al., 2023). In light of this evidence, eDNA metabarcoding of water samples can

Table 2
Comparison between NIS identified by eDNA metabarcoding with different genetic markers in the present study and those morphologically detected in the ports of Trieste (TR), Taranto (TA) and Civitavecchia (CI) and reported in Italian marine waters (IT). The different sampling periods and environmental matrices (W = water and S = sediment) along with species recorded in the Global Invasive Species Database (GISD) are shown. Black circles indicate where species were identified. Data derived from sampling carried out in Trieste during the pre-survey are indicated with asterisks on TR.

Species	Genetic marker					Taxonomic approach				Season			Matrix		GISD
	COI	*18S V4*	*18S V7*	*18S V9*	*rbcl*	CI	TA	TR	IT	March	July	October	W	S	
Acanthosiphonia echinata				•					•		TA	TA		•	
Aglaothamnion feldmanniae		•	•						•			CI		•	
Aidanosagitta neglecta				•							TA	CI/TA/TR	•		
Amathia verticillata	•		•						•		TR		•		
Asparagopsis taxiformis	•								•		CI	CI	•	•	
Aurelia solida	•								•		TR			•	
Balanus trigonus	•					•		•	•		CI	TR	•		
Boccardia proboscidea	•	•									TR*		•	•	
Botrylloides niger	•								•			CI	•		
Clytia linearis	•	•							•			CI	•		
Dasysiphonia japonica	•								•		CI			•	
Herdmania momus				•							TA		•		
Hydroides elegans	•	•	•			•	•	•	•	CI/TA/TR	CI/TR/TR*	CI/TA/TR	•		
Kapraunia schneideri		•							•	TA		CI	•	•	
Magallana gigas	•		•						•		TR/TR*	TR	•	•	
Melibe viridis	•								•			TA	•		
Mercenaria mercenaria				•					•	TA	TA	CI/TA	•	•	
Mnemiopsis leidyi	•							•	•	TR		TR	•		•
Oithona davisae	•	•	•	•					•	CI/TA	TA/TR*	CI/TA/TR	•	•	
Petricolaria pholadiformis		•		•							CI/TA/TR*	CI	•		
Phallusia nigra			•	•							CI/TR/TR*	CI/TR	•	•	
Polyandrocarpa zorritensis	•								•	TA		TA	•	•	
Polydora cornuta	•								•		TR			•	
Pseudodiaptomus marinus	•	•	•			•		•	•	TR	CI/TR/TR*	CI/TA/TR	•		
Pseudopolydora paucibranchiata	•	•	•						•		TR*		•		
Pyropia koreana	•								•	TA			•		
Rhithropanopeus harrisii	•								•		TR*		•		•
Saccharina japonica				•					•	CI			•		
Spermothamnion cymosum	•								•		CI			•	
Styela plicata	•							•	•	TA	TR/TR*	TR	•		•
Thecacera pennigera	•								•			CI	•		

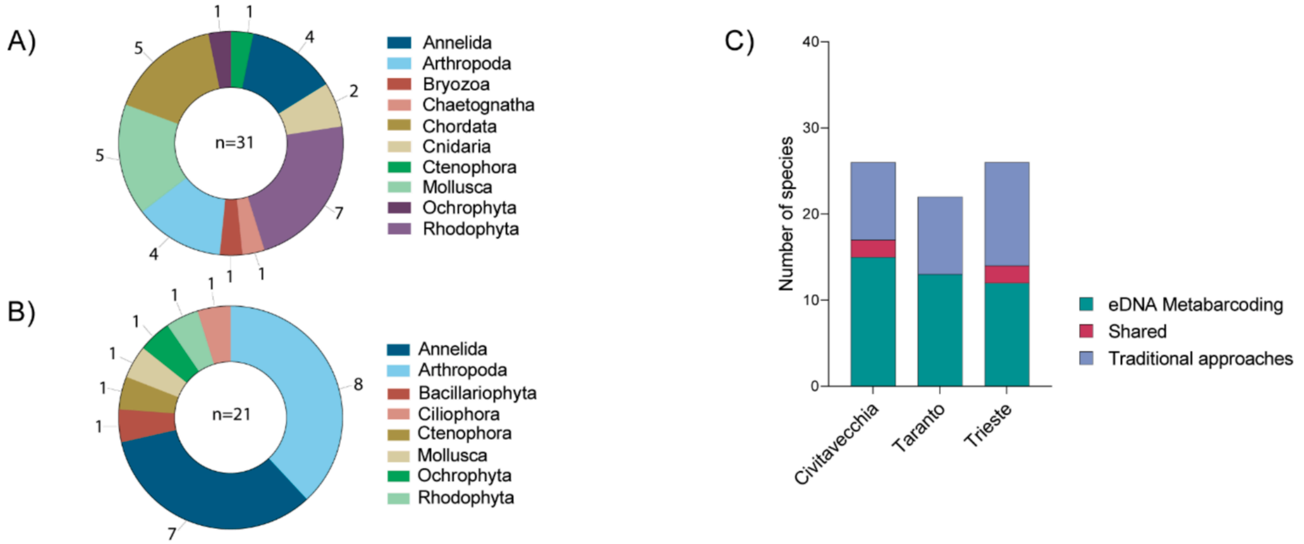

Fig. 6. Donut plot of NIS grouped at phylum level identified by eDNA metabarcoding (A) and morphological approaches (B). The number of NIS belonging to each phylum is reported. The total number of NIS is reported within donuts plot. Number of exclusive and shared NIS recorded in three ports and in the three sampling periods of 2022 through eDNA metabarcoding and morphological approaches (C).

provide valuable information not only on the overall biodiversity (Rees et al., 2014; Takahashi et al., 2023) but also it can improve the reliability of detection of NIS from different environments (Borrell et al., 2017). However, as previously reported (Couton et al., 2022), also in our study water eDNA metabarcoding did not allow us detecting the genetic signatures of all NIS colonizers of hard substrates (e.g. *Cutleria multifida* and *Ianiropsis serricaudis*). Thus, we cannot rule out that molecular analyses of samples collected also from hard substrates (e.g., hulls, piers) could further increase our ability to detect the NIS exploiting these habitats.

Marine sediments are the repository of genetic information present in the environment, including planktonic species that can be transported on the seafloor and/or with benthic life-cycle stages. For instance, we detected in the sediments of the port of Trieste the genetic signature of the invasive jellyfish *Aurelia solida,* which can cause detrimental consequences on the local zooplanktonic communities (Malej and Ramsak 2012; Scorrano et al., 2016). These data support previous findings on the sensitivity of metabarcoding on sediment eDNA for early detection of NIS (Holman et al., 2019; Pearman et al., 2021), which is crucial for enhancing the chances of successful control and management of invasive species.

The *18S rRNA* and *COI* metabarcoding allowed us to identify eight macroalgal NIS, including *Acanthosiphonia echinata* and *Pyropia koreana*, whose presence has been also reported by previous morphological and molecular investigations carried out in the Adriatic Sea (Sfriso et al., 2020; Wolf et al., 2020). Among the non-indigenous macroalgae identified in this study, *Dasysiphonia japonica,* a harmful algal bloom species, can cause mortality in the early life stage of fish and bivalves (Young et al., 2022), whereas the invasive red macroalgae *Asparagopsis taxiformis* is replacing indigenous macroalgal species leading to biodiversity and ecosystem functioning loss (Mancuso et al., 2023; Navarro-Barranco et al., 2018). Additional evidence of the presence of invasive alien species was provided by the *COI* metabarcoding, which allowed to detect the ctenophore *Mnemiopsis leidyi* (one of the 100 most invasive species in the world, threatening biological diversity (Putelli et al., 2024). This species is spreading from the Black Sea into the Mediterranean Sea (Malej et al., 2017; Shiganova, 1998) and can potentially provoke fishery declines and impact marine ecosystem food webs (Fuentes et al., 2010; Piccardi et al., 2024). This species was reported for the first time in the Adriatic Sea only in 2005 (Shiganova and Malej, 2008), and it is now invading the region, reaching up to 100 individuals/m^3 (Malej et al., 2017; Paliaga et al., 2021). Our results revealed a high sensitivity of eDNA metabarcoding to detect the genetic signatures of NIS even when their abundance morphologically determined is relatively low such as in the case of *M. leidyi* in the Trieste port in 2022 (0.67 individuals/m^3). We also report, by the *COI* metabarcoding, the presence of the alien tunicate *Styela plicata*, which is another invasive species frequently detected in Mediterranean ports (Tempesti et al., 2020), outcompeting native species for the colonization of artificial and natural hard substrates (Platin and Shenkar, 2023).

In this investigation, consistent with what has been previously reported (von Ammon et al., 2018; Zaiko et al., 2016), the use of a classical taxonomic approach resulted in a lower number of NIS when compared to eDNA metabarcoding. Among the 21 NIS morphologically identified, only 4 were shared with those detected by the eDNA metabarcoding approach. Summing up the number of species identified using both approaches the overall number of NIS rises to 48. For example, we report the case of the Mediterranean invader crustacean *Stenothoe georgiana* (Martínez-Laiz et al., 2020), which has been identified through morphological approaches and not by eDNA metabarcoding due to the lack of sequences deposited in genetic databases up to now.

These findings indicate that NIS identified through eDNA metabarcoding, although valuable, fast, and cost-effective cannot be used as a unique tool for marine NIS identification (Duarte et al., 2021). Therefore, we argue that multi-marker-based eDNA metabarcoding can be useful to complement and expand NIS lists obtained using morphological identification. In this context, the data obtained with eDNA metabarcoding can represent a precious alert for environmental protection agencies and/or institutional entities.

At the same time, the molecular approach did not identify the same species in all ports, nor every single port in all seasons, suggesting that a complete assessment of NIS requires a sampling strategy designed to capture the variability of these species over time and in space. Discrepancies between species detected through eDNA analyses and those identified via classical morphological approach present great challenges for the current regulatory decision-making. Integrating expert-driven guidelines will facilitate the complementary use of both approaches, enhancing the accuracy and reliability of biodiversity assessments, including NIS detection.

Marine NIS are considered a major threat worldwide and encompass a very diverse group of organisms. This study highlights that the number of NIS can be much higher than those recorded in the Mediterranean Sea and as such requires extensive investigations for improving the knowledge on their abundance, distribution, and extent of impacts on marine environments. The advent of new technologies (e.g. molecular techniques, remote systems) will provide the opportunity to gain more detailed knowledge on marine species as well as on NIS introductions and spreading into new environments.

5. Conclusions

The use of an eDNA-based approach to analyze biodiversity has been repeatedly recommended due to the increasing threat posed by alien species to marine ecosystems and this study provides clear indications in this perspective. At the same time, we do not exclude that the use of environmental RNA-derived amplicons can represent a further step forward for the molecular identification of NIS (Scriver et al., 2024b). In addition, we think that future studies will likely use an increasing number of DNA markers to detect a larger number of species and NIS as this has been the trend in similar studies, starting with a single or a pair of markers and now using multiple markers (as in the present study). Based on the results obtained in the present study, and the genetic signatures belonging to ca. 1500 species identified in only three ports we confirm that eDNA metabarcoding of both water and sediment samples has a high potential to capture a large portion of biodiversity, but it is insufficient to identify the whole number of species and particularly the total number of NIS. In this work, these aspects have been addressed to provide a reliable pipeline that could be used in the future as a complementary approach to traditional monitoring tools for easily and timely detection of NIS and large-scale mapping the patterns of species invasion fronts within the Descriptor 2 of the Marine Strategy Framework Directive (MSFD). The integration of eDNA metabarcoding with traditional approaches increases the chances of early NIS detection, aiding more effective prevention plans and minimizing ecological impacts.

CRediT authorship contribution statement

Stefano Varrella: Writing – original draft, Visualization, Supervision, Formal analysis, Data curation. **Silvia Livi:** Writing – review & editing, Data curation, Conceptualization. **Cinzia Corinaldesi:** Writing – review & editing, Funding acquisition, Conceptualization. **Luca Castriota:** Methodology. **Teresa Maggio:** Methodology. **Pietro Vivona:** Data curation. **Massimo Pindo:** Methodology, Formal analysis. **Sebastiano Fava:** Data curation. **Roberto Danovaro:** Writing – review & editing, Funding acquisition. **Antonio Dell'Anno:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgments

This study was funded by the Italian Ministry of Environment and Energy Security (MASE) under the Operating Agreement with the Italian Institute for Environmental Protection and Research (ISPRA), implementing art. 11 “Monitoring Programs” of Legislative Decree n. 190/2010 transposing Marine Strategy Framework Directive 2008/56/EC. This work was carried out in the framework of the research convention between the Italian Institute for Environmental Protection and Research (ISPRA) and CoNISMa – research unit Polytechnic University of Marche and in the framework of the National Recovery and Resilience Plan

(NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n° 3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union - NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree n° 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, Project title "National Biodiversity Future Center - NBFC". This study was also supported by the Project DiverSea (HORIZON-CL6–2022-BIODIV-01; Grant agreement 101082004). Authors thank the Italian Environmental Protection Agency of Friuli Venezia Giulia, Puglia and Lazio for their support during sampling activities, Dr. Marco Lo Martire, Dr. Andrea Sagrati, Dr. Agnese Fumanti, Dr. Anna Salvatori, Dr. Julian Ivaldi and Dr. Luigi Maria Cusano for supporting sample processing and laboratory analyses and Dr. Nico Salmaso for methodological suggestions and data analysis. We would like to thank the six reviewers of the present work for their useful comments and suggestions.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109489.

Data availability

Data will be made available on request.

All sequences of *18S rRNA V4, V7, V9*, *COI* and *rbcL* markers have been deposited in the NCBI's Sequence Read Archive under BioProject ID PRJNA1055974.

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