scientific reports

OPEN

Integrative approach to monitoring metazoan diversity and distribution in two Mediterranean coastal sites through morphology and organismal eDNA

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Marine and coastal ecosystems respond to climate change in various ways, such as the type of ecosystem, the species composition, interactions, and distribution, and the effect of local stressors. Metazoan organisms, particularly zooplankton, are important indicators for monitoring the effects climate-driven warming in marine coastal ecosystems over the long term. In this study, the diversity and distribution of zooplankton communities in the Mediterranean Sea (Canyon Dohrn and LTER-MareChiara, Gulf of Naples), a known biodiversity and climate changes hotspot, have been assessed using the integration of morphological-based identification and organismal eDNA. Our findings showed that the multi-locus strategy including the mitochondrial cytochrome c oxidase I (COI) gene and the hypervariable region V9 of the 18S rDNA (18S V9) as targets, improved the taxonomic overview, with the COI gene being more effective than the 18S V9 region for metazoans at the species level. However, appendicularians were detected only with the 18S V9 region. Overall, organismal eDNA is a powerful approach for revealing hidden biodiversity, especially for gelatinous and meroplankton components, and provided new insights into biodiversity patterns. The ecological importance of calanoid copepods in coastal ecosystems has been confirmed. In contrast, the discovery of 13 new metazoan records in the Mediterranean Sea, including two non-indigenous copepod species, suggested that local stressors affect zooplankton community structure and resilience, highlighting the importance of biomonitoring and protecting marine coastal ecosystems.

Marine metazoan zooplankton is a group of highly diverse multicellular organisms that either spend their entire life cycle in the water column (holoplankton) or temporarily live as microscopic planktonic life stages, occupying as adults the benthic or nekton environment (meroplankton). Zooplanktonic organisms perform critical ecological functions in marine ecosystems, linking primary producers (phytoplankton) to higher trophic levels (fish, seabirds, marine mammals) and regulating, directly and indirectly, ecosystem services such as carbon cycling and fish stocks[1,2]. Several studies have explored and modeled the zooplankton assemblages' responses to climate-driven warming scenarios in marine ecosystems[3–6]. In this context, ecological data collected at long-term sites provide meaningful temporal scales baselines of biodiversity assessment, supporting the early identification of community changes, the plan and track of measures management, and the prediction and modeling of future relapses on the composition or function of marine ecosystems[7–10].

The zooplankton taxonomic complexity, with ca. 2×10^5 species distributed in 11 different phyla[11], has been traditionally identified and monitored by a microscopy-based approach, a time-consuming process requiring a high level of taxonomic expertise and almost impossible in the case of eggs, early larval live stages and

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gelatinous taxa with fragile bodies. Considering climate changes, it is essential to monitor the distribution and to identify possible shifts in the zooplankton communities in a relatively fast, cheap, and large-scale way. Over the past few decades, environmental DNA metabarcoding (eDNA) from the bulk samples (e.g., organisms collected using plankton nets or organisms isolated from sediment collected with sediment cores or grabs) or from the environmental matrix (e.g., water or sediment samples) has been reported to improve the limitation of morphology-based species identification and to be successful to uncover the marine metazoan diversity and to speed up environmental monitoring[12–14]. The non-invasive nature, cost-effectiveness, rapid turnaround, and high yield of data generated have made eDNA metabarcoding a very successful tool for assessing the diversity of marine zooplankton at spatial[15,16] and/or temporal scales[10,17–19]. Such an approach has also been used to evaluate changes in vertical migrations of species and ecosystem responses to climatic events[20] and to discover cryptic, non-indigenous, and rare species[21,22]. Despite its many advantages, there are several biases connected to the crucial choices needed for a successful application of eDNA. Among them, based on target taxa and the field of application, careful consideration is necessary for the selection of the substrate used as a source of the DNA sour (bulk samples or environmental matrix), the choice of primer pair (universal or specific), and the marker region, determining the further availability of reference sequences and the power discrimination at species level[23–25].

This study aims to assess the diversity and distribution of the marine zooplankton metazoan community in the Gulf of Naples (Central Tyrrhenian Sea, Western Mediterranean Sea). A microscopy-based approach has been integrated with organismal eDNA (bulk samples) using two genetic markers: the mitochondrial cytochrome oxidase I (COI) gene and the hypervariable region V9 of the 18S ribosomal DNA (18S V9). The Mediterranean Basin is a global hotspot for biodiversity[26] and climate change hotspot, with warming of this sea is expected to exceed global rates by 25%[27,28]. The Gulf of Naples, which spans roughly 195 km of coastline, is also highly affected by multiple anthropogenic pressures such as high population density, industrial and commercial activities, and tourism. In this study, we selected two different sites: the coastal site, LTER MareChiara (LTER-MC), and the Canyon Dohrn, located off-shore and about 12 nautical miles away. LTER MareChiara represents one of the most extended plankton time series available for the Mediterranean basin (https://www.st.nmfs.noaa.gov/copepod/time-series/it-30101/). The second site, Canyon Dohrn, is an active geological site is characterized by upwelling waters that provide crucial ecological benefits and serve as a breeding ground for various marine species along the coastline.

Results
General description
The data analyzed in this study were collected in two different sampling sites located in the Gulf of Naples. The first site is the coastal long-term ecological research site LTER MareChiara (LTER-MC), and the second site, Canyon Dorhn (C), is located in the area offshore of the Naples Bay (Fig. 1).

After the preprocessing steps, taxonomic filtering (90% threshold) and normalization, 8,562,606 reads representing 8322 ASVs were retained for the COI gene (Supplementary file COI gene). Output of the BLAST searches highlighted that 97% of the sequences (ASVs) found matches with reference sequences with values of similarity of 98–100% that were considered assigned at species level (the full path of taxonomic ranks of reference sequence has been considered valid); 1% of the sequences found matches with reference sequences in the range of 95–97%, and in this case the assignment was considered reliable at the genus level (the path of taxonomic ranks of reference sequence was truncated at genus level); finally, 2% of the sequences found matches with reference sequences with values of similarity lower than 95% (the path of taxonomic ranks was truncated at family or order level). After this step, we applied the ASV clustering (the abundances of ASVs with the same taxonomic path were summed), resulted in a list of 206 taxa (Supplementary file COI).

For the 18S V9, after the preprocessing steps, taxonomic filtering (90% threshold) and normalization, the dataset consisted of 10,045,999 reads, corresponding to 1640 ASVs (Supplementary file 18S V9 gene).The output of the BLAST searches highlighted that 69% of the sequences found a match with reference sequences with values of similarity of 99–100% and were considered assigned at the species level (the full path of taxonomic ranks of reference sequence has been considered valid); about 20% of the sequences found matches with reference sequences in the range of 95–98% and in this case the assignment was considered reliable at genus level (the path of taxonomic ranks was truncated at genus level); 10% of the sequences found matches with reference sequences with values of similarity lower than 95% (the path of taxonomic ranks was truncated at family or order level). After this step, we applied the ASV clustering (the abundances of ASVs with the same taxonomic path were summed), producing a list of 139 taxa (Supplementary file 18S V9).

Using morphological identification, we reported that the zooplankton community constituted of 105 taxa identified at different taxonomic levels (Supplementary file Morpho) (Table 1).

Overall, alpha diversity indexes did not show specific trends. The values, calculated using the post-clustering ASVs tables normalized, highlighted higher species richness values (observed taxa) in the COI gene than in the 18S V9 region and morphological dataset, but quite similar ranges of values were found for the Shannon index in all three datasets (Supplementary Fig. S1a–c).

Taxonomic overview of the three datasets (COI vs V9 vs Morphology)
Overall, in the case of the COI gene 11 holoplanktonic and nine meroplanktonic taxa were found, including ichthyoplankton assemblage (Fig. 2). For the 18S V9 region, eight holoplanktonic and 12 meroplanktonic were found (Table 1). Using morphological identifications, 11 holoplanktonic and 6 meroplanktonic taxa were reported (Table 1). Both molecular and morphological approaches showed that holoplanktonic organisms were more abundant than meroplanktonic ones. Copepoda was the dominant taxon, representing an average of 76% of the total reads in the COI gene, 48% in the18S V9 region, and 78% in microscopy identification. The other
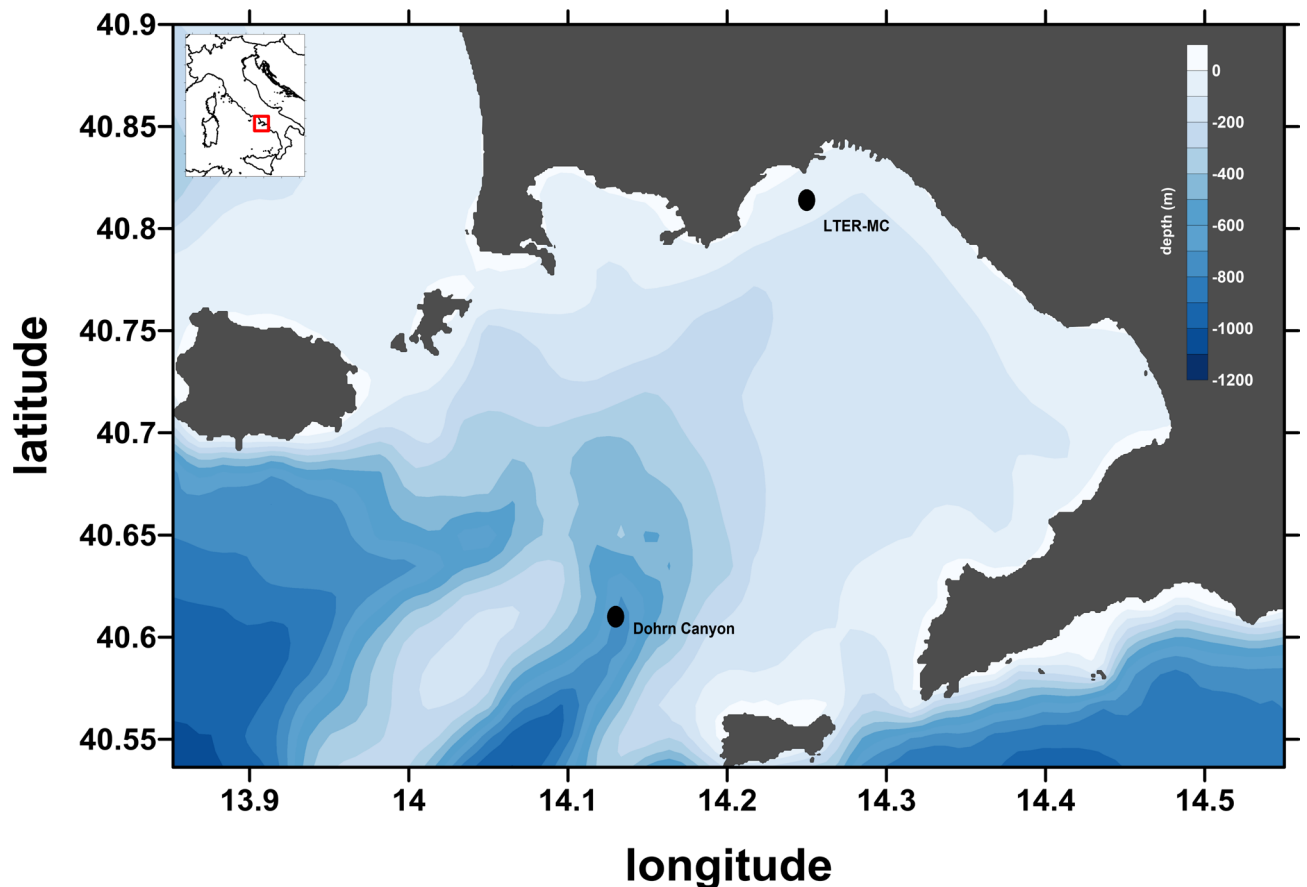
Figure 1. The sampling site (black dot), Canyon Dohrn and the LTER-MC site in the Gulf of Naples (Central Tyrrhenian Sea, Mediterranean Sea). Created by I. Di Capua with Surfer Ver. 10.7.972 (64-bit); www.goldensoft ware.com. Coastline data: NOAA National Geophysical Data Center, Coastline extracted: WLC (World Coast Line), Date Retrieved: 20 March, 2024, http://www.ngdc.noaa.gov/mgg/shorelines/shorelines.html.

crustacean taxa (Amphipoda, Diplostraca, Euphausiacea, Ostracoda) represented an average of 10% in the COI gene, 12% in 18S V9 region, and 14% in microscopy dataset. Lower percentages were found for the holoplankton taxa, including Chaetognatha, Hydromedusae, Pelagiidae, Siphonophorae, Thaliacea, and Appendicularia, were included with lower percentages.

The 18 V9 region generally detected more meroplanktonic larvae than the COI gene dataset. Meroplanktonic larvae (Anthozoa, Bryozoa, Cirripedia, Decapoda, Echinodermata, Hemichordata, Malacostraca, Mollusca, Polychaeta, Others) represented an average of 7% of the total reads in the COI gene and 30% in 18S V9 region. Microscopy counts for the meroplanktonic taxa allowed the identification of fewer taxa at higher taxonomic levels (Mollusca, Polychaeta, Decapoda, and Echinodermata) with a total average abundance of 3% (Table 1). The ichthyoplankton assemblage was mainly assigned to Teleostea (on average, 2% in COI gene and 0.2% in 18S V9 region). In contrast, only a few unidentified fish eggs (2.4 ind. m^{-3}) were counted with microscopy only at LTER-MC (Table 1).

Metazoan distributions across the two stations (Canyon vs LTER-MC)

The distribution of taxa at the two stations showed that in the COI gene, the crustacean dominated all samples, accounting for a total average of 92%. This comprises 93% of samples in Canyon and 90% in LTER-MC. A similar dominance of crustaceans was observed also for the 18S V9 region, with an average value of 79%. Crustacean accounted for 73% in the Canyon, while at LTER-MC it was 85% (Table 1). The second most abundant taxa differed between the molecular markers. In the COI gene dataset, cnidarians (Anthozoa, Hydromedusae, Pelagiidae, and Siphonophorae) account for an average of 3% of reads (Table 1, Fig. 4a). In contrast, in the 18S V9 region dataset, chaetognaths (Sagittoidea) represented the second most abundant phylum with an average of read abundance of 8% (Table 1, Fig. 4b). The analysis of the COI gene and morphology analyses revealed that micro-crustacean copepods were the dominant taxa at both stations (Fig. 3a,c). However, in the 18S V9 region, the dominance of the copepods was most evident in the Canyon. At the same time, at LTER-MC, the group only dominated in the spring sample (MC1_April) with 68.5% (Fig. 3b, Supplementary file 18S V9).

Focusing on non-copepod holoplankton components, it was found that the COI gene and morphological data showed similar distributions. The Ostracoda taxa, mainly belonging to the Order Halocyprida, with *Porroecia spinirostris*, was more abundant in the Canyon based on COI gene and morphology, but this presence

Taxa	Molecular								Morphology			
	COI				V 9				total abundance (ind. m⁻³)		Percentage (%)	
	No of total reads		Percentage (%)		No of total reads		Percentage (%)					
	Canyon	LTER-MC	Canyon	LTER-MC	Canyon	LTER-MC	Canyon	LTER-MC	Canyon	LTER-MC	Canyon	LTER-MC
Holoplankton												
Amphipoda	4212		0.1		849	0	0.4	0.0	0.48		0.0	
Appendicularia					6858	608	2.9	0.3	57.83	54.97	2.7	0.5
Chaetognata	87,405	37,391	2.0	0.9	21,560	14,033	9.1	6.0	12.53	74.99	1.0	0.7
Copepoda	3,466,556	3,004,170	81.0	70.2	145,109	81,608	61.4	35.0	1897.98	7937.79	83.7	72.3
Diplostraca (Cladocera)	139,916	229,572	3.3	5.4					165.71	1962.76	5.8	17.9
Euphausiacea	103,743	157,320	2.4	3.7	3160	29,470	1.3	12.7	4.60	98.15	0.2	0.9
Hydromedusae	9828	14,826	0.2	0.3	1450	29	0.7	0.0	0.96	5.65	0.2	0.1
Ostracoda	195,002	5570	4.6	0.1	23,361	137	9.9	0.1	82.51	1.52	3.5	0.0
Pelagiidae	2994	1928	0.1	0.0						0.96		0.0
Pteropoda	82,478	8464	1.9	0.2								
Siphonophorae	77,769	110,336	1.8	2.6					4.08		0.1	
Thaliacea	19	60	0.0	0.0	7003	328	3.0	0.1	7.12	583.48	0.3	5.3
Meroplankton												
Anthozoa	25,430	1	0.6	0.0	548	10	0.2	0.0				
Bryozoa		5	0.0	0.0	0	0	0.0	0.0				
Cirripedia	393	248,715	0.0	5.8	0	0	0.0	0.0		76.09		0.7
Decapoda	1451	198,907	0.0	4.6					0.51	76.28	0.0	0.7
Echinodermata	28,809	18,994	0.7	0.4	0	0	0.0	0.0	8.19	9.12	0.3	0.1
Hemichordata		2085	0.0	0.0	0	0	0.0	0.0				
Malacostraca					15	87,166	0.0	37.4				
Mollusca_Bivalvia	1510	768	0.0	0.0	1213	840	0.5	0.4	48.04	101.72	2.0	0.9
Mollusca_Gastropoda	0	0	0.0	0.0	3015	1642	1.3	0.7				
Pisces	2712	180,875	0.1	4.2	702	57	0.3	0.0		2.40		0.0
Polychaeta	39,978	13,330	0.9	0.3	5381	832	2.3	0.4	1.01	0.12	0.0	0.0
Others					15,893	15,818	6.7	6.8				

Table 1. Taxonomic overview and comparison of metazoan taxa identified with molecular (COI gene and 18S V9 region) and morphological data. For molecular data is reported the number of total reads and the percentage (normalised dataset); for morphology the total abundance (ind. m⁻³) and the percentage. The Others taxa reported in the list of 18S V9 region includes 4 phyla (Crustacea, Mollusca, Phoronida and Platyhelminthes, referred to the taxa_31, 73, 128, 129, 135 in the Supplementary file 18S V4).

was partially lost in the 18S V9 region (Fig. 4a,b). Similarly, the Diplostraca taxa were more abundant at station LTER-MC based on COI gene and morphology (Fig. 4a,c), but cladocerans (Diplostraca) were absent in the 18S V9 region dataset (Fig. 4b).

Meroplankton was more abundant at the coastal station LTER-MC than at Canyon on the COI gene, with the Cirripedia and Decapoda as the main taxa (Table 1). At the Canyon, meroplankton was mainly represented by Echinodermata (Echinoidea, Ophiuroidea, Holothuroidea) and Polychaeta, with the presence of the species *Magelona lusitanica* being detected in the layer 0–200 m in spring using the COI gene.

The two genetic markers showed different accuracies in identifying fish at the species level. The 18S V9 region had lower resolution power compared to the COI gene. Using the COI gene, we were able to identify several fish species at LTER-MC, including *Mullus barbatus*, *Engraulis encrasicolus*, *Trachurus mediterraneus*, *Apogon imberbis*, *Serranus hepatus*, *Anguilla rostrata*, *Auxis rochei*, *Megalops atlanticus*, and *Sarpa salpa*. Additionally, at the Canyon, we found *Boops boops* in the layer 0–50 m and the deep-sea fish *Ceratoscopelus maderensis* in the layer 0–200 m.

Some taxonomic groups were detected using only one of the two markers: the 18 V9 region allowed for the detection of Appendicularia (Fig. 4b), but this was lost with the COI gene (Fig. 4a). Within appendicularians, we reported *Oikopleura dioica* and *Oikopleura* sp., as well as the Thaliacea, which include *Doliolum denticulatum* and *D. nationalis* (Supplementary file 18S V9). On the other hand, the 18 V9 region failed to detect the Amphipoda, Diplostraca, Pelagiidae, and Siphonophorae (Fig. 4b,a, Table 1).

Overall, the COI dataset shared a core of 23 species, whereas only 10 were shared among the 18S V9 region samples. However, both datasets highlighted that each sample showed a low number of exclusive taxa except for the sample C1_200 at Canyon, which had 39 exclusive species with the COI region and 14 with V9 (Supplementary Fig. S2a–c).
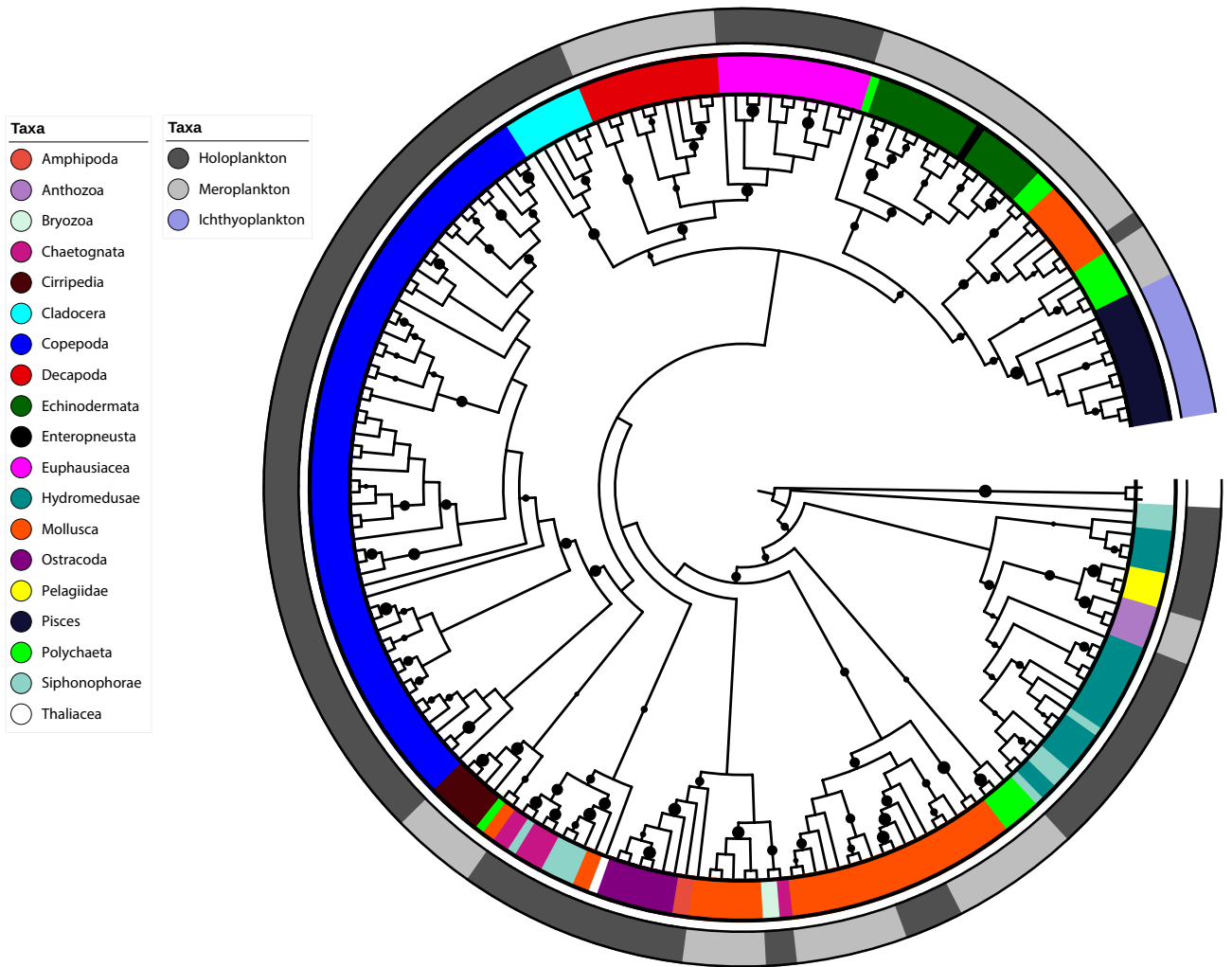
Figure 2. Maximum Likelihood tree generated for COI dataset. Taxa are colored based on both plankton type and their affiliation to the main 19 taxonomic groups. Dots on the tree branches represent a bootstrap value from 0.8 to 1.
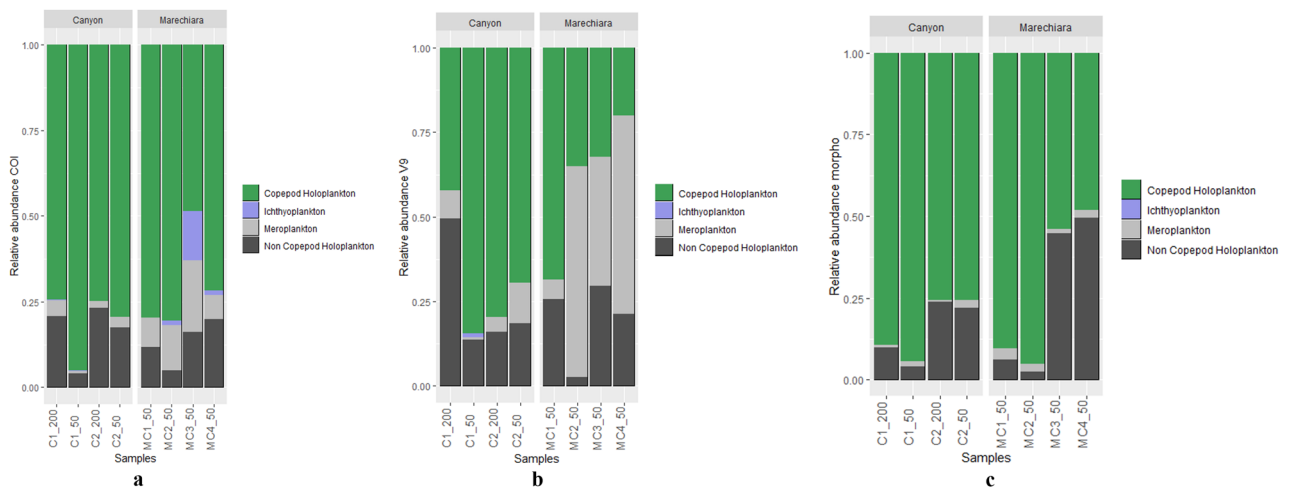

Figure 3. Barplots of relative abundances of the taxonomic groups colored based on four plankton type in the three datasets: COI gene (**a**), 18S V9 region (**b**) and morphology (**c**).
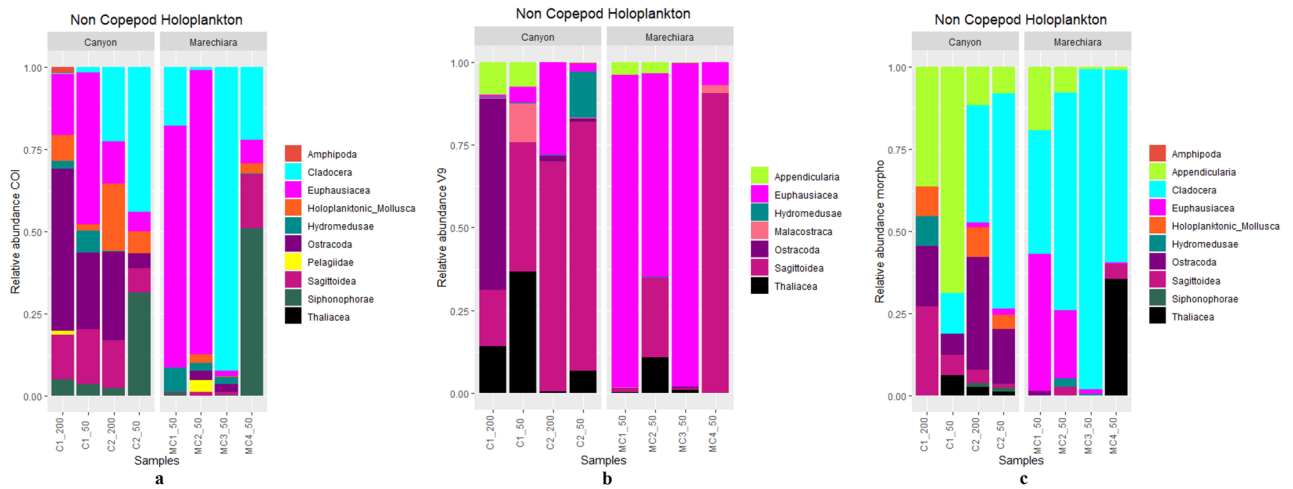
Figure 4. Barplots of relative abundances of the taxonomic groups assigned to non-Copepod Holoplankton in the three datasets: COI gene (**a**), 18S V9 region (**b**) and morphology (**c**).

Focus on dominant taxa (COI vs morphology)

Similar results were observed for the identification of Copepoda using the COI gene and morphology-based identification, with respectively 63 species and 72 species identified. Thirty four copepod species from 14 families were identified at both stations, LTER-MC and Canyon, using both approaches, with 31 being calanoids (Fig. 5a,b, Supplementary Table S1). The distribution details of these 34 species showed recurrent presence in the COI data set (Fig. 5a, Supplementary Table S1).

The dominance of the calanoids *Acartia clausi* and *Temora stylifera* was confirmed by both the COI gene and morphological approaches. Moreover, other calanoids, eight species of *Clausocalanus* and four of *Calocalanus* were identified (Fig. 5a,b). As expected, the COI gene distinguished the three calanoid species *Paracalanus quasimodo*, *P. parvus*, and *P. indicus*. In contrast, the microscopic approach identified them as a unique species complex (*P. parvus* complex), with a total abundance of 1273 ind. m^{-3}. The non-indigenous calanoid species *Pseudodiaptomus marinus* was detected using the COI gene only at LTER-MC in the June sample (Supplementary Table S1). Additionally, several meso-bathypelagic calanoid species (*Haloptilus longicoris*, *Heterorhabdus papilliger*, *Lucicutia flavicornis*, *Pleuromamma abdominalis*, *Neocalanus gracilis*) were exclusively detected and identified at the Canyon (Fig. 5a,b).

The COI gene and morphology showed different results for cyclopoid copepod species, specifically within the *Oithona* and *Coryaceus* genera (Supplementary file COI and 18S V9). The COI gene indicated only the presence of *O. davisae*, while morphological identification revealed the presence of eight different species, excluding *O. davisae* (Supplementary File Morpho) and any corycaeids species were detected. Both approaches detected *Oncaea* (Fig. 5a,b) and *Triconia* at species level (Supplementary Table S1).

The larvae of Euphausiacea, including calyptopis and eggs, were reported in morphological dataset with a very low abundance (1.1 ind. m^{-3}), but the COI gene successfully identified 11 Euphasiacea species across five genera. Specifically, at the Canyon, nine species from four genera were detected, with five of them exclusively found at the deeper layer of the Canyon (*Meganyctiphanes norvegica*, *Nematoscelis atlantica*, *N. megalops*, *N. difficilis*, *Thysanoessa raschii*) (Fig. 6a). *Euphausia krohni*, which was abundant in all Canyon samples, was only present in two samples at the LTER-MC station with very low abundance. *Nyctiphanes simplex*, another commonly represented euphausiid, showed an opposite distribution pattern, dominating at the LTER-MC rather than the Canyon samples (Fig. 6a).

Finally, the morphological approach to identifying cnidarians was ineffective, but only the COI gene revealed the presence of 19 gelatinous taxa (Fig. 6b). These included Hydromedusae, six species of Siphonophorae, and one species of Pelagidae (*Pelagia noctiluca*) (Fig. 6b).

Discussion

Understanding the dynamics of the zooplankton community in the Mediterranean basin is essential to properly address local anthropogenic pressures and global climate change challenges on marine ecosystems. At the LTER-MC, morphological data of phytoplankton and zooplankton components have been collected weekly since 1984, also linking them with oceanographic, chemical, and physical parameters[29]. However, the standard procedures for monitoring marine communities in time series monitoring and field research must integrate advanced molecular approaches (DNA barcoding and metabarcoding) to expand knowledge and expedite routine actions and studies[30]. The implementation of environmental DNA metabarcoding requires understanding its limits and potentialities in terms of ecological inferences, as well as harmonizing protocols with the establishment of best practices to apply to different marine ecosystem. This study is the second using eDNA to analyze metazoan communities from the Gulf of Naples[11], and are currently undergoing various studies to enhance and supplement traditional monitoring methods with DNA-based approaches such as metabarcoding, metagenomics, and metatranscriptomics in the framework of the Naples Ecological Research augmented observatory Project (NEREA) (https://www.nerea-observatory.org/nereasampling).
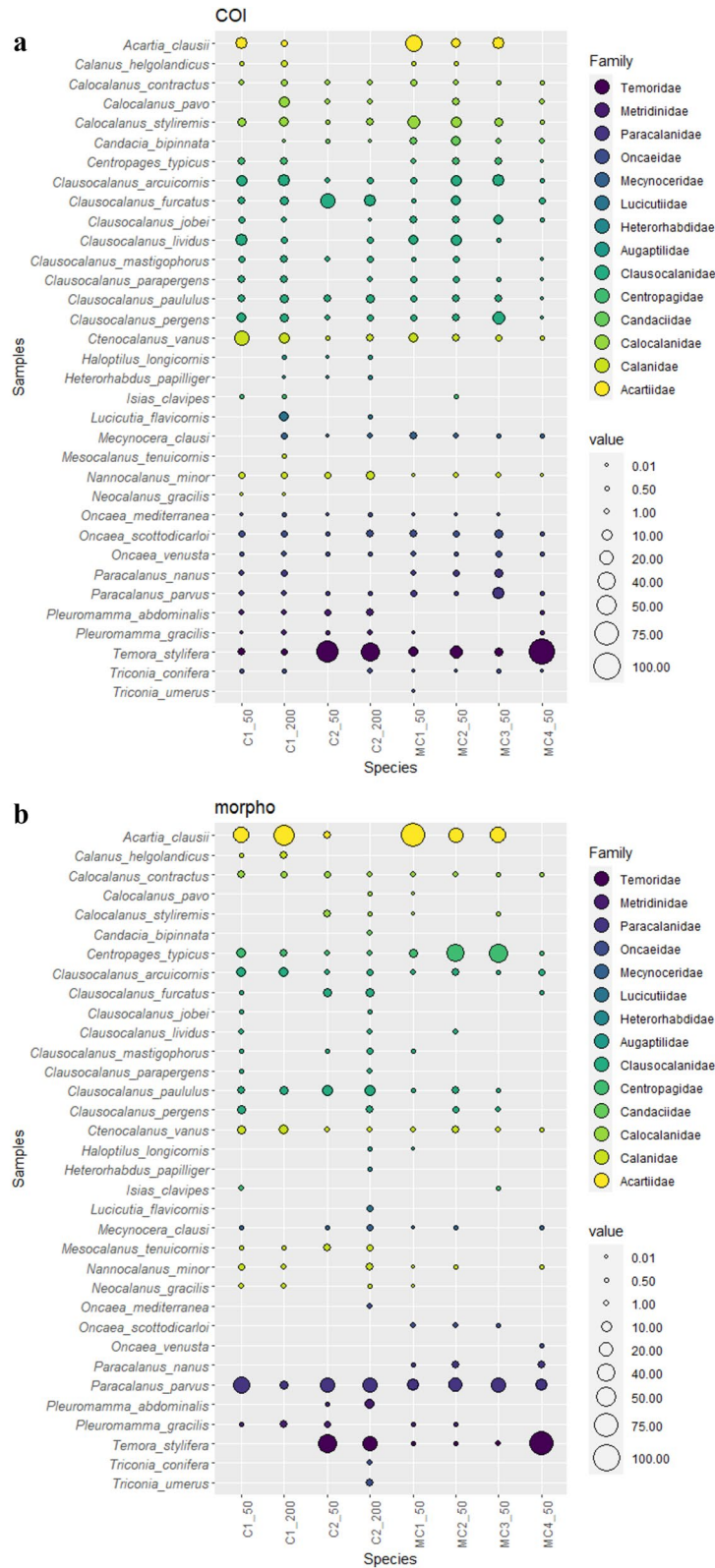
Figure 5. Bubble plot showing the distribution (relative abundance) obtained for Copepoda species between COI gene (**a**) and morphology (**b**).
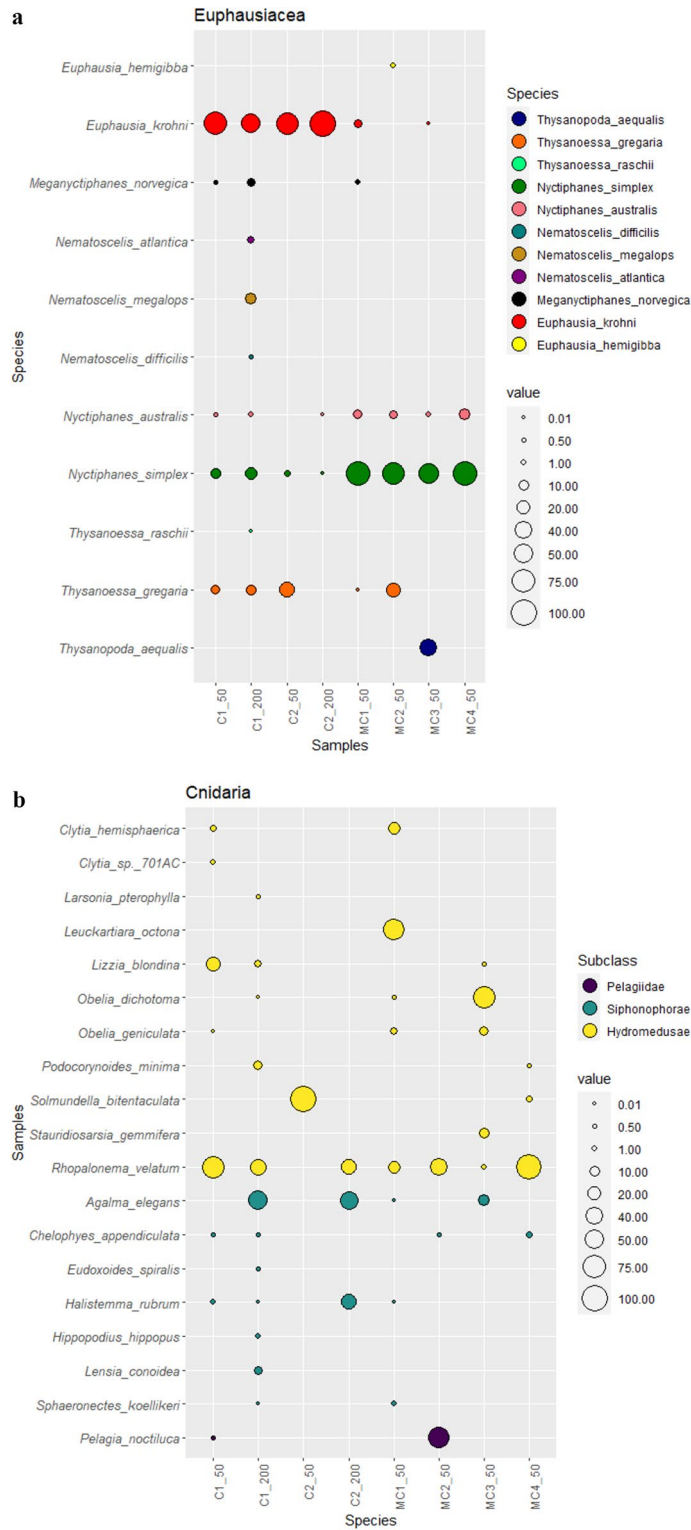
Figure 6. Bubble plot showing the distribution (relative abundance) obtained with COI gene for Euphausiacea (**a**) and Cnidaria (**b**) species.

COI gene vs 18S V9 region

The combined use of the molecular markers COI and 18S is a common practice in marine zooplankton metabarcoding-based assessments[10]. The COI gene, in particular, is known to reveal a greater number of metazoan species and provide better taxonomic resolution compared to the 18S regions, which have highly conserved primer sites,

allowing for amplification of a wide range of metazoan and protists[17,31]. Our results further demonstrate the value of this approach, showing that using both markers identifies more metazoan zooplanktonic taxa than using a single molecular marker. This leads to higher taxonomic resolution and better assignment quality, enhancing the accuracy and reliability of our findings.

The COI gene detected more taxa (206) than the 18S V9 region (139). It also provided more accurate assignments, with 97% of COI sequences being assigned at the species level, compared to 69% for the 18S V9 region. At a higher taxonomic level, the two markers performed similarly, with the main differences being the detection of Diplostraca and Siphonophorae only with the COI gene and Appendicularia only with the 18S V9 region. Difficulties detecting Diplostraca with an 18S marker have been reported previously, including when using the 18S V4 region[10,11]. Unlike what we observed in the 18S V9 region, Siphonophorae can also be detected in the 18S V4 region[11]. The absence of Appendicularia in the taxonomic profile from the COI gene was expected, as reference COI barcodes exist for only 8% of their described diversity[32], and PCR primers for the COI barcodes have been reported to fail in several cases[32,33].

Our study confirmed that using two molecular markers in organismal eDNA assessments improves species detection. This helps to reduce biases related to different PCR primer amplification affinities among organisms and the varying taxonomic coverage of reference sequences in databases. However, our results also suggest that caution is needed when choosing primer pairs. Universal primers that amplify alternative regions within the same molecular marker, such as the V4 and V9 regions in the 18S marker, can detect different phyla.

However, the success of taxonomic assignments in eDNA assessments depends on the quality and quantity of reference barcodes in the databases. Several initiatives are being undertaken to create a comprehensive and curated COI reference database for marine zooplankton[32]. On the other hand, the resources available for 18S rRNA mainly focus on protists[34] or are less curated[35]. In our case, having COI barcode references previously generated for zooplankton specimens collected in the studied region[36,37], and reference therein] could have improved the taxonomic assignments of COI gene at the species level compared to the 18S V9 region. This would also have provided a taxonomic profile more consistent with the morphological observations.

Organismal eDNA vs morphology

Our results show that organismal eDNA data closely matches the findings from morphological identification, confirming previous studies of zooplankton communities at different spatial and temporal scales[32,33,38]. Overall, there is strong agreement between morphological and molecular results for holoplanktonic organisms. However, morphology-based results are slightly more accurate, especially for the important and abundant group of micro-crustacean copepods, which reported a higher total number of species (72). On the other hand, the COI gene consistently shows the presence of copepods in all samples, likely due to the inclusion of nauplii and/or copepodid stages in the molecular results, which were not identified and/or were lost using the traditional morphological approach. Out of 34 species shared between the molecular identification with the COI gene and the morphological list, 31 were belonged to the calanoid order (Supplementary Material, Table S1). The morphological list includes only nine species not detected by the COI gene. Most of these species belong to the cyclopoid order of the *Oithona* and *Corycaeus* genera, that do not yet have available barcodes. Despite this, the COI gene provides correct identification in the case of the cryptic species *Paracalanus quasimodo*, *P. parvus*, and *P. indicus*[37].

The combination of molecular and morphological approaches improves the understanding gained solely from morphological studies, especially when studying the meroplanktonic component. It was expected that eDNA would outperform morphology-based surveys for screening meroplankton, as demonstrated in previous studies[39,40]. Molecular identification is particularly useful for identifying larval stages of benthic species, which can be challenging or impossible using morphology-based methods. Previous knowledge of the diversity of meroplanktonic metazoans in the Gulf of Naples study areas was limited, mainly including morphological identification of decapod larvae in spring and larvae of cirripedes and echinoderms in winter[37]. However, this study revealed a greater diversity, confirming and expanding our previous eDNA results obtained using the 18S V4 region[11], with overall the presence of 13 phyla.

Biodiversity and ecological patterns

Our study provides for the first overview of zooplankton metazoan communities and diversity in the Canyon Dohrn site in two different depth layers. With a length of approximately 25 km and a width of nearly 2 km, the Canyon Dohrn represents a crucial bathymetric feature of the Gulf of Naples[41,42]. The head of the Canyon is steep, and gets very close to the coastline, consequently supporting the exchanges between the deep sea and the shelf area[35,43], and reference therein]. A recent study investigation, performed by visual identification of ichthyoplankton, reported the presence of larvae belonging to 11 families and of eggs belonging to five families[44]. In addition, the deep-sea benthic community was also explored based on an ROV survey by[45] et al., highlighting the Dohrn Canyon's high biodiversity and evidence of severe anthropogenic impacts. The authors also reported the co-existence of living specimens of the bivalves with the cold-water corals, which has never been previously reported in the Mediterranean Basin. In contrast, no planktonic surveys exist, but our preliminary findings revealed differences between the two sampling sites (Canyon and LTER-MC), highlighting in the submarine Canyon Dohrn the presence of deeper-living zooplankton species such as meso-bathypelagic copepod species, euphausiids, and hydrozoan-groups. These species, often challenging to identify or poorly characterized through morphological methods, were successfully identified at the species level using the COI gene[46]. Nevertheless, euphausiids are hard to identify using only morphology beyond the genus level, particularly at larval stages[36]. Of note, among the euphasiids, we recently obtained the barcodes for the *Euphausia krohnii*, *Nematoscelis megalops*, and the genus *Nyctiphanes* in this study area[36].

Morphological features are often insufficient for accurate identification beyond the family level. It is difficult, and therefore impossible, to morphologically distinguish the larval stages of many benthic species and within groups of gelatinous holoplankton, especially when using samples fixed with ethanol (Di Capua, personal communication). In these cases, the use of eDNA organismal can help to improve our understanding of the presence and distribution of these taxa, especially in coastal areas, such as the LTER-MC site, and also better to understand the relationship between benthic and pelagic coupling.

Molecular data, however, have revealed the presence of 20 cnidarian species belonging to Anthozoa, Hydromedusae, Siphonophorae, and Pelagiidae. Notably, COI gene analysis has detected medusae *P. noctiluca* in the 200–0 m of the Canyon in April and at LTER-MC in May, where they are expected to feed on spring zooplankton and ichthyoplankton[47]. These findings confirm the significant role of submarine canyons in regulating vertical matter fluxes and facilitating exchanges between deeper layers and the surface[44]. The connection between the offshore submarine Canyon station and the coastal station LTER-MC depends on specific surface and deep circulation patterns, which may vary depending on the seasonal forcing scheme[48]. Furthermore, the *P. noctiluca* ephyrae in both sites aligns with the hypothesis of seasonal vertical migration of this species in the Mediterranean Sea[49].

Overall, the fish in the area belong to 14 species across nine families, including commercially important ones like *Engraulis encrasicolus* and *Mullus barbatus*. Recently, five of these families (Engraulidae, Gobiidae, Myctophidae, Serranidae, Carangidae) have been identified in the sampling area through morphological-based identification[44].

The calanoid copepods, such as *Acartia clausi* and *Temora stylifera* have been confirmed as the most abundant species using COI gene and morphology, confirming the ecological significance of these two species in this neritic ecosystem[48–52]. Additionally, our data showed a recurrent copepod community structure in both sites in the depth layer 50–0 m, probably related to the water mixing process in the surface mixed layer at 30–50 m depth[44,48].

Surveys of non-indigenous species (NIS) are especially important in LTER sites, where knowledge of species composition can aid in the early detection of NIS and their invasive potential. Organismal eDNA has proven effective in tracing rare species and larval stages, with several studies demonstrating its accuracy in detecting NIS in marine environments[11,53,54]. In our survey, the COI gene revealed 12 new records of metazoans in the Mediterranean Sea, including holoplankton taxa such as *Larsonia pterophylla*, *Euchaeta rimana*, *Oithona davisae*, *Nyctiphanes australis*, *Nyctiphanes simplex*, *Proceroecia procera*, and meroplankton taxa including *Magelona lusitanica*, *Balanus trigonus*, *Psammechinus miliaris*, *Glossobalanus marginatus*, *Aplysia Juliana*, and *Philine indistincta*. Identifying these previously undocumented species needs to be confirmed in future assessments, but raises concerns about the impact of climate change and other anthropogenic pressures on biodiversity in the Mediterranean Sea. Notably, the polychaete *M. lusitanica* has already been molecularly characterized in nearby Atlantic waters[47], suggesting it may be a new record for the Mediterranean Sea, as recently observed for other non-indigenous polychaetes along the coasts of Italy[55]. Additionally, our results confirmed the presence of the non-indigenous copepod *Pseudodiaptomus marinus* at the long-term coastal station[56], and the COI gene detected a new non-indigenous species, *Oithona davisae* for the first time.

Our research confirms that using organismal environmental DNA (eDNA) is an effective molecular approach for studying the diversity and distribution of metazoan communities, and it is an improvement over traditional morphology-based approaches. Using a multi-loci strategy, we could cover a wide range of metazoan phyla. However, the choice of primer pairs can significantly impact the taxonomic results. We found that the COI gene identified more holo- and meroplanktonic organisms than the 18S V9 region and provided more accurate classifications in according to morphological identification. Specifically, 97% of COI sequences were matched to specific species, compared to 69% for the 18S V9 region. Both markers performed similarly when identifying groups of organisms at high taxonomic levels (es. subclass, order), but they complemented each other. Despite some limitations, organismal eDNA data provided valuable insights into hidden biodiversity, especially for the gelatinous and meroplankton components, including juvenile or larval stages that cannot be identified through morphology-based approaches. Additionally, the use of local barcode references obtained from COI analysis of zooplankton specimens probably contributed to the accuracy of this marker in producing a taxonomic profile that closely matches morphological observations. This highlights the importance of enhancing the reference databases globally and locally, emphasizing their crucial role in ensuring reliable ecological inferences derived from metabarcoding data across various sample types. Furthermore, it identified signs of potential ecosystem deterioration by detecting new entries and non-indigenous species (NIS). Overall, applying the organismal eDNA approach at LTER sites increases its potential impact, as it quickly identifies changes in zooplanktonic community biodiversity and guides the optimization of biomonitoring approaches.

Materials and methods
Study area
The site LTER-MC (40° 48.5′ N; Long. 14° 15′ E) is a coastal station (depth ca. 75 m), located two nautical miles from the city of Naples, is influenced by the eutrophic coastal zone and also the oligotrophic waters of the mid Tyrrhenian Sea[40]. LTER-MC has long been sampled for plankton and other parameters since 1984, and the dataset represents one of the few plankton time series available in the Mediterranean Sea (https://www.st.nmfs.noaa.gov/copepod/time-series/it-30101/). All these observations highlight a strong seasonal signature of the biodiversity pattern of the area, and zooplankton communities are diversified and stable throughout the year[52]. The Canyon site (C) (40° 36.232′ N; 14° 08.012′ E) located approximately 12 nautical miles offshore from the Naples metropolitan area, was originated by ancient eruptive events involving the area offshore the Naples Bay[57]. From a geological point of view, the Canyon was explored, integrating stratigraphic study of geophysical and geological to provide information for evaluating and mitigating natural hazards (i.e., volcanism, seafloor stability)

in managing densely populated coastal areas[57]. Biodiversity and ecology knowledge of Canyons are limited and focused on deep-sea benthic communities. Nevertheless, based on the ROV survey, Taviani et al. (2019) highlighted that, despite evidence of severe anthropogenic impacts, the Dohrn Canyon was a highly diverse habitat, also reporting the co-existence of living specimens of the bivalves with the cold-water corals, which has never been previously reported in the Mediterranean Basin.

Sampling strategy and sample treatment

Sampling of communities was performed during 2019: four for the Canyon station (C1_50; C1_200; C2_50; C2_200) and four for the LTER-MC station (MC1_50; MC2_50; MC3_50; MC4_50) (Fig. 1). The sampling covered three different seasons; in particular, the LTER-MC station covered three different seasons (autumn, spring, winter), while two seasons (autumn, spring) for the Canyon (C) station. Details regarding station label and surface environmental data are reported in Supplementary File final, Supplementary File sampling site. Zooplankton samples were collected with a double 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 cod-end. This double WP2 plankton net allowed two simultaneous samples for each tow for morphology-based and molecular analyses. The net was towed vertically from 50 m to the surface at LTER-MC, from 200 m to the surface, and from 50 to the surface at Canyon. The filtered water volume was calculated using a mechanical flowmeter (Hydrobios). Upon collection, zooplankton samples were immediately preserved in 95% ethanol for taxonomic identification. For metabarcoding, samples were prefiltered on a 2000 μm filter mesh to remove larger organisms, then the samples were filtered on a 200-μm filter mesh; the resulting pellets of bulk zooplankton were collected and placed into 2 mL cryogenic vials, immediately flash-frozen in liquid nitrogen and then stored at − 80 °C until later DNA extraction.

Morphology-based approach

In the laboratory, the ethanol-fixed samples were concentrated and re-suspended in ethanol 200 mL volume. Aliquots ranging from 1/4 to 1/32 according to sample density, were taken initially by using the Huntsman beaker subsampling technique and successively using a large bore graduated pipette after careful mixing[43]. These aliquots were transferred in a mini-Bogorov chamber (10 mL) and examined under a stereomicroscope (Leica M165C). For all copepods, adult females and males were separately identified at species level. Depending on the species, copepodites (CII-CV) were identified at the species level in most cases or grouped at genus or family level (e.g. *Calocalanus*, *Oithona*, Oncaeidae, Corycaeidae). Copepod species abundances (ind. m^{-3}) reported here correspond to the whole population (adult females and males, and copepodites), unless otherwise indicated. Among other zooplankters, some groups were identified at the species level (e.g. cladocerans, chaetognaths, siphonophors), while other holoplankton (e.g. amphipods, mysids, ostracods, euphasids, pteropods, hydromedusea) and meroplankton taxa (decapods, cirripedes, echinoderms, bivalvs, molluscs, gastropods) were identified at higher taxonomic levels. All zooplankters were identified and counted, then the rest of the sample was checked for the presence of rare species. The calculation of abundance (ind. m^{-3}) needs to consider the number of individuals counted in the subsample (n), which fraction of the original sample does the counted sub-sample represent (k), and the water volume filtered by the sampling net (V, m^3), according to the formula: ind. m^{-3} = (n × k)/V m^3.

Molecular approach

All samples were shipped on dry ice to Genoscope Sequencing Laboratory (France) where DNA extractions and sequencing were performed. DNA extraction from each zooplankton pellet was performed using Cryogrinding of two different pellets separately, followed by Nucleospin RNA/DNA co-extraction (Macherey Nagel). For each sample, PCR amplifications were performed in triplicates (5–10 ng DNA per amplification) that were then merged. Two different metabarcoding markers were amplified: mitochondrial cytochrome c oxidase gene (COI) and the V9 region of the nuclear 18S rRNA (V9). For COI gene we amplified a fragment of ca. 313 bp using a primer pair described in Leray et al. (2013) (forward m1COIint 5′-GGWACWGGWTGAACWGTWTAYCCY CC-3′ and reverse jgHCO2198 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′. For V9 region of 18S rRNA were used a primer pair described in[31] (forward 1389F 5′-TTGTACACACCGCCC1510R-3 and reverse 1510R 5′-CCT TCYGCAGGTTCACCTAC-3′) amplifying a ca. 136 bp fragment. Illumina HiSeq sequencing paired-end were performed by Genoscope as described in[58] using (2 × 250 bp) for the COI gene and (2 × 150 bp) for the V9 18S region.

Data preprocessing and taxonomic assignment

Illumina paired-end raw reads were processed to generate ASVs using the DADA2 R package[59], with some modifications for the COI gene and the V9 18S markers.

COI: Quality check was performed with the vsearch –fastq_stats command (Rognes et al. 2016). Primers were trimmed with cutadapt[60]. After primer removal, forward and reverse reads were truncated based on the Quality score plots (forward: 210 nucleotides; reverse: 200 bp respectively) and then used to train the error model using machine learning approach. Forward and reverse reads were then separately reduced in sequence variants using error rates calculated in the previous step for forward and reverse applying pseudo pooling option. Finally, forward, and reverse reads were merged to obtain the full denoised sequences. The ASV table was further filtered based on expected amplicons length (309–317 bp) and checked for presence of chimera (option method = *consensus* in the removeBimeraDenovo function in DADA2). ASVs were taxonomically assigned using standalone Blast 2.8.1 in the blast + suite[61,62] against a custom database integrating MetaCOXI and MetaZooGene reference database[63,64]. In addition, we added a selection of 13,507 protist and macroalgae sequences (organisms not included in MetaCOXI and MetaZooGene) obtained from GenBank (full list is available in the Supplementary File final) and 23 copepod sequences from the Gulf of Naples (GenBank accessions MZ710171 to MZ710194).

Assignments with similarity lower than 90% and query coverage lower than 250 bp were excluded from Blast results. Finally, ASVs assigned to non-marine taxa (Arachnidae, Collembola, Insecta, Amphibia, Craniata and terrestrial Mammalia and protists) were removed from the analyses.

18 V9: Quality check was performed with the vsearch –fastq_stats command[60]. Primers were trimmed with cutadapt[65]. After primer removal, forward and reverse reads were truncated based on the Quality score plots (forward: 120 nucleotides; reverse: 130 bp respectively). Denoising steps were performed with the same procedure described for the COI gene. The ASV table was filtered based on expected amplicons length (125–150 bp) and checked for presence of chimera as performed for COI gene. Taxonomic assignment of ASVs was performed using standalone Blast 2.8.1 in the blast + suite[61,62] against a custom version of PR2_4.14.3 reference Database[34]. This Database was integrated with 330 sequences of Protists from the Gulf of Naples (Supplementary File PR2) and 30 other environmental marine Fungi sequences[66]. Assignments with lower than 90% similarity and query coverage lower than 90 bp were excluded from Blast results. ASVs assigned to non-marine taxa (Arachnidae, Collembola, Insecta, Amphibia, Craniata and terrestrial Mammalia and protists) were removed from the analyses.

Post ASV clustering

In metazoan diversity assessments, especially in comparison with counts from morphological data, the assumption that each ASV is a biological entity (species) could produce a strong overestimation. Nevertheless, biological variations (intra genomic, intra individual, intra species variations) remain in the ASVs table. After the taxonomic assignment with BLAST, we applied a taxonomy-guided clustering of ASVs that share the same taxonomic path to overcome this pattern. We first ranked the assignments of ASVs into three levels based on the similarity with the reference from the BLAST output.

In the case of COI gene: (i) assignments showing similarity with the reference sequence in the range 98–100% were considered assigned at species level (meaning that the full path of taxonomic ranks of reference sequence has been considered valid); (ii) assignments showing similarity with the reference sequence in the range 95–97% were considered reliable at genus level (meaning that the path of taxonomy of reference sequence was truncated at genus level); (iii) assignments showing similarity with the reference sequences lower than 95% were considered reliable at higher levels (the path of taxonomy was truncated at family or order level). After that, the ASVs with the same taxonomic path were collapsed together. In the case of several ASVs with taxonomic path truncated at the same genus (or family/order), they have been collapsed together.

The same approach but different thresholds were applied for the 18S V9 region: (i) assignments showing similarity with the reference sequence in the range 99–100% were considered assigned at species level (meaning that the full path of taxonomic ranks has been considered valid); (ii) assignments showing similarity with the reference sequence in the range 95–98% were considered reliable at genus level (meaning that the path of taxonomy was truncated at genus level); (iii) assignments showing similarity with the reference sequences lower than 95% were considered reliable at higher levels (the path of taxonomy was truncated at higher level). After that, the ASVs with the same taxonomic path were collapsed together. In the case of several ASVs with taxonomic path truncated at the same genus (or family/order), they have been collapsed together.

Data analysis

Normalization of the datasets was applied to counterbalance the potential biases in the analyses related to the different number of reads obtained in the sequencing of the samples. Thus, after the post ASVs clustering step, the COI gene and 18S V9 tables were subsampled to the lowest number of reads (1,070,326 for the COI and 58,252 for the 18S V9) using the function *rrarefy* in vegan R package[67]. The phyloseq[68] and ggplot2[69] R packages were used to generate alpha diversity descriptors (Richness (number of observed taxa) and Shannon entropy index) and the plots of the results. After logarithmic transformation, cluster analyses of molecular and morphological datasets were performed based on a Bray–Curtis dissimilarity matrix with vegan package. Part of the analyses (the comparison with morphological data and the generation of a phylogenetic tree) were performed only using the COI dataset representing the most frequently used and powerful barcode region, also providing a large number of curated reference sequences for a successful species-level identification of marine zooplankton[32]. The phylogenetic tree was built (only for COI dataset) using a ASV representative sequence and two protist sequences detected in our dataset as outgroups (*Hematodinium sp.* and *Azadinium dalianens*). The alignment was performed with online MAFFT version 7[69] (https://mafft.cbrc.jp/alignment/server/index.html) then visualized in SeaView[70]. The maximum-likelihood tree was built in FastTree[71] with generalized time-reversible models and visualized in iTOL (https://itol.embl.de/) [72]. In addition, the molecular and morphological taxa lists were annotated as holo or meroplankton.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Files (Sampling sites, COI gene, 18 S V9 region, Morpho). The metabarcoding data generated in this study have been deposited in the ENA (European Nucleotide Archive, http://www.ebi.ac.uk/ena) under the umbrella project permanent identifier PRJEB74649 'The NEREA Augmented Observatory: a holistic approach to ecological long-term research'.

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Acknowledgements

Author contributions

IDC conceptualization and design; IDC, FL acquisition of data; IDC, FL GZ, RP analysis and interpretation of data; IDC, RP, FL, VR, YC drafting the manuscript. All authors significantly participated in critical revision of the manuscript draft.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-69520-2.

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