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Contribution to the Themed Section: '*Patterns of biodiversity of marine zooplankton based on molecular analysis*'

Original Article

Metazoan diversity and seasonality through eDNA metabarcoding at a Mediterranean long-term ecological research site

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Metabarcoding of environmental DNA has provided striking insights into marine microbial diversity. With this approach, we assessed the diversity of metazoan assemblages and their temporal variations at the Long-Term Ecological Research site MareChiara (LTER-MC) in the Gulf of Naples (Mediterranean Sea). The Illumina sequencing of the V4-18S rRNA fragment from 48 surface samples collected from 2011 to 2013 produced a total of 5 011 047 marine metazoan reads. The normalized dataset was generally dominated by copepods (60.3%), followed by annelids (34.7%) mostly represented by the invasive benthic polychaete *Hydroides elegans*. Non-copepod holoplankton was mainly represented by siphonophores, rotifers, and appendicularians, with occasional mass occurrences of jellyfish. The rest of meroplankton (mainly molluscs, annelids, and anthozoans) showed a high diversity, covering all 11 zooplankton phyla, from Porifera to Craniata. A high number of copepod genera were identified, with seasonal recurrence matching patterns observed in 30 years of studies in the Gulf of Naples. Despite limitations related to the molecular marker resolution and reference dataset, the study provided valuable insights into diversity and seasonal patterns of the whole metazoan assemblage, expanding the knowledge on rare or hardly identifiable taxa and confirming DNA metabarcoding as a powerful approach to be integrated at LTER sites.

Keywords: biodiversity, copepod, eDNA, LTER-MC, Mediterranean Sea, metabarcoding, V4-18S rRNA, zooplankton

Introduction

Plankton diversity ensures the functionality of marine biota through the occupation of numerous ecological niches and the establishment of various interactions that give rise to complex trophic webs. Despite the growing interest, plankton diversity is still one of the less known aspects of the pelagic realm (e.g. Chust *et al.*, 2016), while concern about its threats from anthropogenic impacts and global climate change is increasing (e.g. Staudinger *et al.*, 2012; Beaugrand and Kirby, 2018).

We have estimated that ~38% of the 2×10^5 species distributed in 11 phyla of metazoans living on Earth occur in marine zooplankton, with an increasing number of new species discovered over the last two decades (http://www.marinespecies.org, at VLIZ-Flanders Marine Institute, Belgium, accessed on 12

November 2020). Our knowledge of zooplankton diversity is still limited owing to the poor exploration of some marine habitats and the extreme variety of its components. Small size, complex morphology, and numerous and different developmental stages further contribute to blur our view of extant zooplankton diversity even in accessible and widely investigated coastal areas and shelf seas. Some holoplanktonic organisms, like the gelatinous ones, have fragile bodies that break easily during the sampling procedures making their identification very difficult or impossible. The classification of meroplanktonic larvae, which are ubiquitous and abundant especially in coastal waters, is also challenging. Numerous are therefore the reasons why zooplankton diversity still needs to be further and deeper explored.

Molecular approaches applied to zooplankton diversity studies have revealed enormous potential to overcome some limitations of morphological analyses, which are costly, time consuming and require a wide taxonomic expertise. In fact, the molecular and morphological approaches effectively complement each other in the new discipline of integrative taxonomy (Padial *et al.*, 2010; Gómez Daglio and Dawson, 2019). Metabarcoding, i.e. the taxonomic identification of a natural organism ensemble through the analysis of DNA regions, has become a widely used approach to appraise zooplankton diversity rapidly and with a higher taxonomic resolution than morphological approaches (Mohrbeck *et al.*, 2015). Indeed metabarcoding has revealed temporal and spatial patterns of zooplankton distribution similar to those detected through morphology-based species identification (Abad *et al.*, 2017; Bucklin *et al.*, 2019; Schroeder *et al.*, 2020) and has been used to shed light on variability at different time scales, as well as on vertical migrations and ecosystem responses to climatic events (Pearman and Irigoien, 2015; Berry *et al.*, 2019; Carroll *et al.*, 2019; Blanco-Bercial, 2020).

In most cases, metabarcodes have been analysed using DNA extracted from zooplankton communities collected with traditional net sampling (bulk samples, Blanco-Bercial, 2020; Schroeder *et al.*, 2020) and at times in species mixtures assembled in mock samples (Brown *et al.*, 2015; Hirai *et al.*, 2017). However, the DNA of a range of pelagic metazoans, from micro- to macro-organisms, can also be found in filtered seawater samples, along with that of unicellular organisms. The analysis of environmental DNA from filters has recently contributed to disclose the diversity and distribution of aquatic animals from micrometazoans (Djurhuus *et al.*, 2018; Berry *et al.*, 2019) to vertebrates (Valentini *et al.*, 2016) or through the complete marine tree of life (Stat *et al.*, 2017).

The aim of this article was to investigate the potential of eDNA metabarcoding to study the metazoan diversity in water samples collected at a Long-Term Ecological Research (LTER) site in the Gulf of Naples (Mid Tyrrhenian Sea, Mediterranean Sea). The study was conducted during a three-year period (2011–2013), as an integration of the zooplankton community analysis and as a proof of concept of the advantages and limitations of the approach. Through this study we tried to answer the following questions: (i) what is the diversity of metazoan community in the water column; (ii) how it varies through the seasons; and (iii) to what extent the picture emerging from the analysis of eDNA metabarcoding matches the knowledge accumulated over the years for zooplankton communities in the area.

Material and methods

Study site

The long-term ecological research site *MareChiara* (LTER-MC) is sampled for plankton and environmental variables since 1984 (Figure 1). The site, located 2 nautical miles off the city of Naples (40°48.5′N, 14°15′E, depth ca. 75 m), is influenced by the eutrophic coastal zone and the oligotrophic waters of the mid Tyrrhenian Sea (Cianelli *et al.*, 2017). A strong seasonal signature characterizes the environmental and biological features of the site (Ribera d'Alcalà et al., 2004). From December to March, temperatures of the fully mixed water column can be as low as 13°C. Starting from April, a thermocline establishes and sea surface temperature increases up to 28°C in August, with frequent influx of nutrients from municipal runoff reaching the site during the stratification period. Currents are relatively weak especially during the summer, while eddies and plumes can mix the water masses in intricate patterns (Uttieri *et al.*, 2011; Iermano *et al.*, 2012). Phytoplankton show minima in winter, increasing from late winter over the water column and reaching maximum value (2.2×10^5 cells ml^{-1}) at surface in late spring–summer. Zooplankton (≥200 µm) present two periods of highest abundance: the first one generally in mid-spring (April–May) and the main one in summer (July–August) with a maximum abundance of 2.3×10^3 ind. m^{-3}. The latter is followed by a gradual decline in autumn until the minimum (1.1×10^2 ind. m^{-3}) that typically occurs in late December–January (Ribera d'Alcalà et al., 2004; Zingone *et al.*, 2019). Zooplankton communities are very diversified throughout the year (Mazzocchi *et al.*, 2011). The holoplanktonic component is dominated by copepods, followed by cladocerans, tunicates (mainly appendicularians), cnidarians, chaetognaths, and ostracods. Meroplankton are mainly represented by decapod larvae, which generally peak in spring, and by larvae of cirripedes and echinoderms, which peak in winter.

Sampling and DNA extraction and sequencing

For metabarcoding analysis, water samples were collected at the long-term time station LTER-MC since 2010 at nearly weekly frequency with a 10-l Niskin bottle deployed at 0.5 m depth. To avoid contamination, the bottle was thoroughly washed with fresh water after each sampling event and was left open at the sampling depth for at least 10 min during the sampling. Samples were placed in acid-cleaned tanks and transported to the laboratory, where they were processed within a couple of hours from their collection. Generally, 3 l of sample, or less in case of plankton-rich samples, were filtered on each of two cellulose ester filters (47-mm diameter, 1.2-µm pore-size, EMD Millipore, USA), which were cut in two parts, immediately frozen and stored in the laboratory at −80°C. For the present study, filters from 48 dates over a three-year period (11 January 2011 to 30 December 2013) were selected based on plankton abundance, to ensure a good seasonal coverage and inclusion of peaks and lows.

The workflow applied in the analyses is illustrated in Supplementary Figure S1. For DNA extraction, a half of each of the two filters was processed with the DNeasy Plant Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The hypervariable V4 region of eukaryote SSU rRNA gene was amplified using the primers TAReuk454FWD1 and TAReukREV3 (Stoeck *et al.*, 2010) modified as in Piredda *et al.* (2017) (5′-CCAGCASCYGCGGTAATTCC-3′ 5′-ACTTTCGTTCTTGATYRATGA-3′).

Sequencing was performed using the Illumina MiSeq platform (2 × 250 bp) following the Illumina Nextera's protocol (Illumina, San Diego, CA, USA) and subsequent modifications (Kozich *et al.*, 2013; Manzari *et al.*, 2015). Briefly, in the first amplification

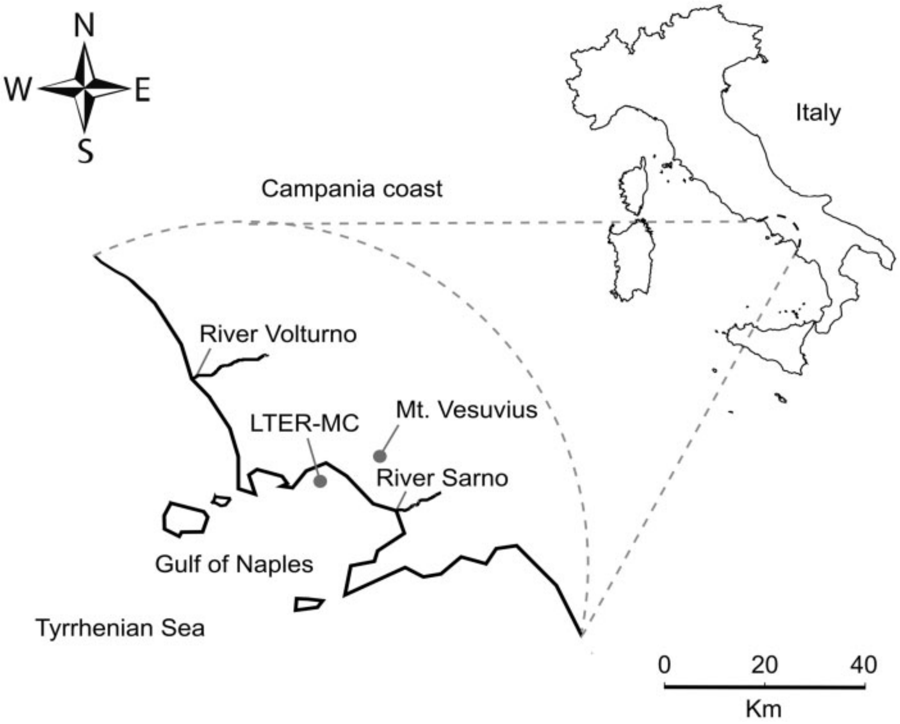

Figure 1. The sampling site, station LTER-MC, in the Gulf of Naples (Mediterranean Sea). Reproduced from Zingone *et al.* (2019).

step the V4 region of the 18S rRNA gene was amplified using the selected V4 universal primers having a 5′ end overhang sequence, corresponding to Nextera transposase primer. Amplifications were performed in a reaction mixture containing 5 ng of extracted DNA, 1× Buffer HF, 0.2 mM dNTPs, 0.5 µM of each primer, and 1 U of Phusion High-Fidelity DNA polymerase (New England Biolabs Inc, Ipswich, MA, USA) in a final volume of 25 µl. The cycling parameters for PCR were standardised as follows: initial denaturation at 98°C for 30 s, followed by 10 cycles of denaturation at 98°C for 10 s, annealing at 44°C for 30 s, extension at 72°C for 15 s, and subsequently 15 cycles of denaturation at 98°C for 10 s, annealing at 62°C for 30 s, extension at 72°C for 15 s, with a final extension step of 7 min at 72°C. All PCRs were performed in the presence of a negative control (RNase/DNase-free water). The PCR products (∼470 bp for V4) were visualized on 1.2% agarose gel and purified using the AMPure XP Beads (Agencourt Bioscience Corp., Beverly, MA, USA), at a concentration of 1.2× vol/vol, according to manufacturer's instructions. Further details are reported in Piredda *et al.* (2017).

The purified V4 amplicons were used as templates in the second PCR step, which was performed with the Nextera index primers and Illumina P5 and P7 primers as required by the Nextera dual index approach. The dual index strategy consists of incorporating unique indices into both ends of the library molecules to allow sample identification for the subsequent bioinformatics analysis (Kozich *et al.*, 2013). The 50 µl reaction mixture was made up of the following reagents: template DNA (40 ng), 1× Buffer HF, dNTPs (0.1 mM), Nextera index primers (index 1 and 2), and 1 U Phusion DNA Polymerase. The cycling parameters were those suggested by the Illumina Nextera protocol. The dual indexed amplicons obtained (∼530 bp for V4) were purified using AMPure XP Beads, at a concentration of 0.6× vol/vol, then checked for quality control on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and quantified by fluorimetry using the Quant-iTTM PicoGreen-dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a NanoDrop 3300 (Thermo Fisher Scientific). Equimolar quantities of V4 amplicons were pooled and subjected to 2 × 250 bp sequencing on a MiSeq platform.

Data processing and taxonomic assignment

Paired-end reads were processed using mothur v.1.33.0 (Schloss *et al.*, 2009). Contigs between read pairs were assembled and differences in base calls in the overlapping region were solved using ΔQ parameter (Kozich *et al.*, 2013). Primer sequences were removed (pdiffs = 3), and no ambiguous bases were allowed; the maximum homopolymer size was 8 bp. The remaining sequences were de-replicated and screened for chimaeras using UCHIME in *de novo* mode (Edgar *et al.*, 2011). Taxonomic assignment of the de-replicated reads was performed using standalone BLAST in BLAST+ suite (Altschul *et al.*, 1990; Camacho *et al.*, 2009) against the PR2 database (v.4.10.0; https://doi.org/10.6084/m9.figshare.5913181; Guillou et al., 2013), discarding the assignments with similarity ≤90% and query coverage ≤70% of the sequence length. Haplotypes assigned to metazoans were extracted and a new local blast was performed using a custom version of PR2 including metazoan sequences present in GenBank at April 2018. From the total metazoan table, non-marine taxa (Arachnida, Insecta, Amphibia, Craniata, and terrestrial Mammalia) were removed manually screening the OTU table after the assignments.

For the analyses of the whole metazoan community, including description at high taxonomic level and exploration of alpha- and beta-diversity, OTUs at 97% were generated using vsearch (Rognes *et al.*, 2016) (method=dgc) through mothur (Supplementary Figure S1). Singletons were removed and the

OTU table was subsampled to the lowest number of reads ($n = 23\,477$) to normalize the dataset using the function rrarefy from vegan R package (Oksanen *et al.*, 2018).

For the analysis at lower taxonomic level, ribotypes assigned to Copepoda were ranked into three levels based on similarity with references: (i) species level for assignments in the 99–100% similarity range; (ii) genus level for assignments in the 98–95% similarity range; and (iii) higher levels for assignments in the 94-90% similarity range. These arbitrary similarity thresholds have previously been used in other eukaryote diversity studies in order to assign names to anonymous OTUs (e.g. Holovachov, 2016). OTUs including a number of reads ≥ 10 were aligned and a Maximum Likelihood tree was build using Fastree (Price *et al.*, 2010) and visualized in iTOL (www.itol.embl.de) (Letunic and Bork, 2019). Morphological taxonomic classification of Copepoda followed those of Razouls *et al.* (2005–2020) and WoRMS Editorial Board (2020).

Statistical analyses were performed and results plotted using several R packages (R version 3.5.2; R Core Team, 2014). Alpha-diversity was explored using several descriptors, including observed OTUs, Shannon entropy index, Berger-Parker dominance index and Pielou evenness index, generated using the diverse R package (Guevara *et al.*, 2016). Hellinger transformation was applied to the OTU table and non-metric multidimensional scaling (NMDS) was performed using phyloseq R package (McMurdie and Holmes, 2013) based on a Bray-Curtis dissimilarity matrix. Boxplots, barplots and bubble plots were obtained with ggplot2 (Wickham, 2016).

In addition, three MAME1 (MArine MEtazoan group 1) OTUs belonging to a potential novel group of tunicates discovered in the Gulf of Naples during the BioMarKs project (López-Escardó et al., 2018) were blasted against our dataset. All reads assigned to MAME1 (similarity 100–97%) were extracted and their temporal distribution visualized using a bubble plot.

Metazoan sequences from this study are available in GenBank (MW140087–MW142014).

Results

The V4-18S rRNA metazoan dataset from eDNA collected at station LTER-MC in 2011–2013 contained 5 011 047 total reads with 667 265 unique non-redundant ribotypes (Supplementary Material S1). Clustering at 97% similarity, removal of singletons and subsampling generated a normalized dataset of 1928 OTUs including a total of 1 099 701 reads (Supplementary Material S2).

Metazoan OTUs were affiliated to 17 high-level holoplanktonic and meroplanktonic taxonomic groups (Figure 2). Holoplanktonic Copepoda covered the large majority of the reads (over 60%), while meroplanktonic Annelida were the second most abundant taxon (35%), with the dominance of a single species, the benthic polychaete *Hydroides elegans*, covering ca. 25% of the OTUs and 34% of total reads. Planktonic and benthic Cnidaria and Tunicata represented 2.6 and 1.1% of the reads, respectively. Among the other taxa, Mollusca, Rotifera, and Chaetognata were represented by <1% reads each, while each of the remaining seven taxa by less than 0.05% of the reads. Altogether the latter ten groups covered only 5% of the reads, but constituted a relatively high proportion of the OTUs (19%); among them Vertebrata were assigned only to teleost fishes.

Community composition and seasonal patterns

The temporal distribution of the metazoan community highlighted the overall prevalence of Copepoda and of the polychaete *H. elegans* (Figure 3). Copepoda were the most abundant holoplanktonic group on almost all dates (Figure 3), and were largely dominated by calanoids (24–97% of total copepods).

Non-copepod holoplankton (NCHolo) varied at the interannual and seasonal scales (Figures 3 and 4a and b). Oikopleuridae (Appendicularia), Siphonophorae, and Rotifera comprised the largest part of NCHolo across the 3 years. Oikopleuridae were represented by a single species, *Oikopleura dioica*, and attained higher abundance in summer-autumn (Figure 4a). Siphonophorae included six genera, with *Muggiaea* and *Nectopyramis* as the most abundant ones, with no clear seasonality. Rotifera were mainly represented by the marine genus *Cephalodella*, which was more abundant in spring and summer. Thaliacea occurred with four genera, i.e. *Doliolum*, *Pegea*, *Salpa*, and *Thalia*, the latter showing the highest contribution (11% of NCHolo in 2011), with *T. democratica* observed manly in July in the three years. Among Thaliacea, it is worth noting the distribution of a novel taxon of tunicates (named MAME1) that was discovered at station LTER-MC in a metabarcoding dataset collected in European coastal areas (López-Escardó et al., 2018). In the present dataset, this taxon consisted of three co-occurring OTUs showing a bimodal distribution over the three years, with a longer period of occurrence in late autumn and winter and a shorter one in full summer (Supplementary Figure S2). Cnidaria mainly included the jellyfish *Pelagia noctiluca*, which showed a high contribution (99% of the NCHolo reads) on two consecutive winter dates (December 2012 and January 2013). The other Cnidaria were represented by tiny Hydroidomedusae, with highest contributions (up to 31% of NCHolo reads) in summer (mainly Leptothecata) and winter (mainly Narcomedusae). Cladocera, found in summer, accounted for less of 1% of NCHolo. The remaining NCHolo reads belonged to Brachypoda, Eumalacostraca, Gastrotricha, Xenacoelomorpha, and Ctenophora, of which only the latter were occasionally abundant (January 2011 and October 2013, as "Others" in Figure 4a).

Whitin meroplankton, *H. elegans* was present in almost all samples with an increase from September 2012 onwards (Figure 3). In the rest of meroplankton, four taxa, i.e. Anthozoa, Bivalvia, other Annelida, and Gastropoda, emerged as the most abundant in the 3 years and were recurrent over the seasons (Figure 5a and b). Anthozoa dominated in autumn–winter and Bivalvia in spring–summer. Other Annelida and Gastropoda showed a less regular occurrence across the seasons (Figure 5a). The other meroplanktonic groups comprised 10 taxa (Bryozoa, Chordata, Eleutherozoa, Enteropneusta, Nematoda, Nemertea, Porifera, Platyhelminthes, and Vertebrata). The subphylum Eleutherozoa with the class Echinoidea and the phylum Arthropoda with Cirripedia became occasionally abundant, respectively, in October 2012 and 2013 and in August 2011 (as "Others" in Figure 5a).

Focus on Copepoda

Assignment of individual ribotypes of the non-normalized dataset to Copepoda references (90–100% similarity) resulted in 2 399 478 total reads (343 756 ribotypes) representing six orders: Calanoida, Cyclopoida, Poecilostomatoida, Canuelloida, Harpacticoida, and Siphonostomatoida (Table 1 and

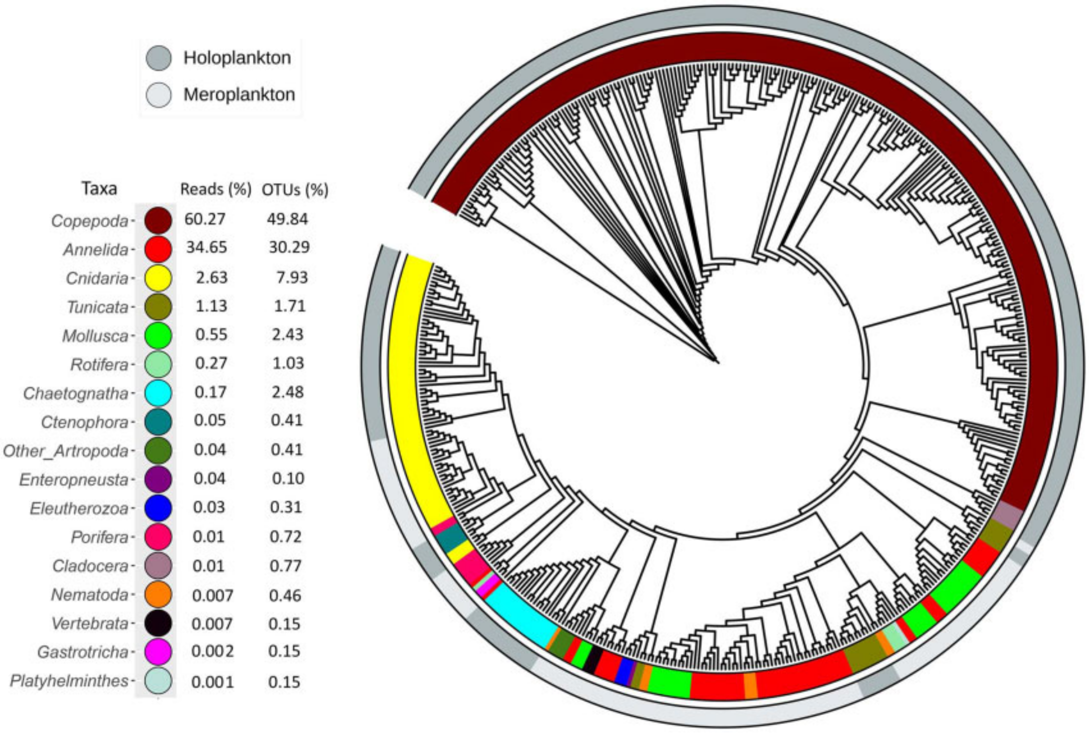

Figure 2. Maximum likelihood tree of the 481 most abundant Metazoa ($\geq$10 reads) recorded at station LTER-MC in the years 2011–2013, and their contribution to the normalized dataset.

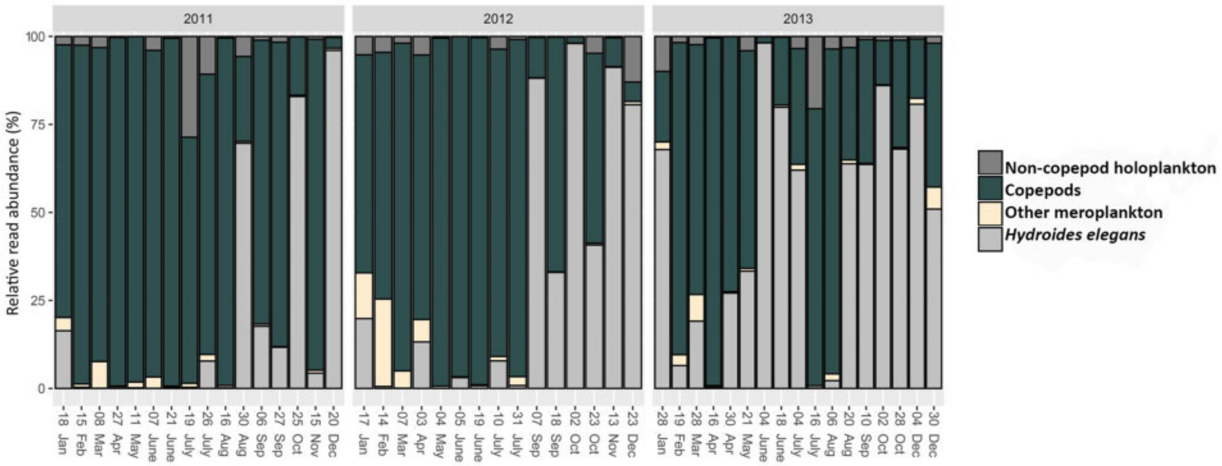

Figure 3. Temporal variability of the four main groups of the metazoan community at station LTER-MC.

Supplementary Material S3). Almost half of the reads (45%, 104 809 ribotypes) showed 99–100% similarity to 21 species: 17 Calanoida, 1 Cyclopoida, 1 Poecilostomatoida, 1 Canuelloida, and 1 Harpacticoida. Other copepod reads with 98–95% similarity to reference sequences were assigned to 32 genera. The genus *Acartia* covered about half of these reads, none of which 100% matching the *A. clausi* reference, and only 44 being 99–100% similar to it. With the exception of *Acartia*, taxa assigned with 99–100% similarity mostly corresponded to dominant copepod genera and species identified based on morphology in samples collected by vertical (50–0 m) net hauls at station LTER-MC on the same sampling dates (data not shown).

In addition to *Acartia*, Calanoida included the genera *Paracalanus* (29% of Calanoida reads), *Clausocalanus* (13%), *Centropages* (7%), *Neocalanus* (6%), and *Temora* (5%). Among the other genera, the most represented was *Oithona*, which was the only Cyclopoida, while other genera accounted for <5% of the copepod reads each. The remaining genera contributed with very low percentages (4.1 ± 9.9% of the total copepod reads). Among the Calanoida, *Acartia* and *Centropages* showed recurrent

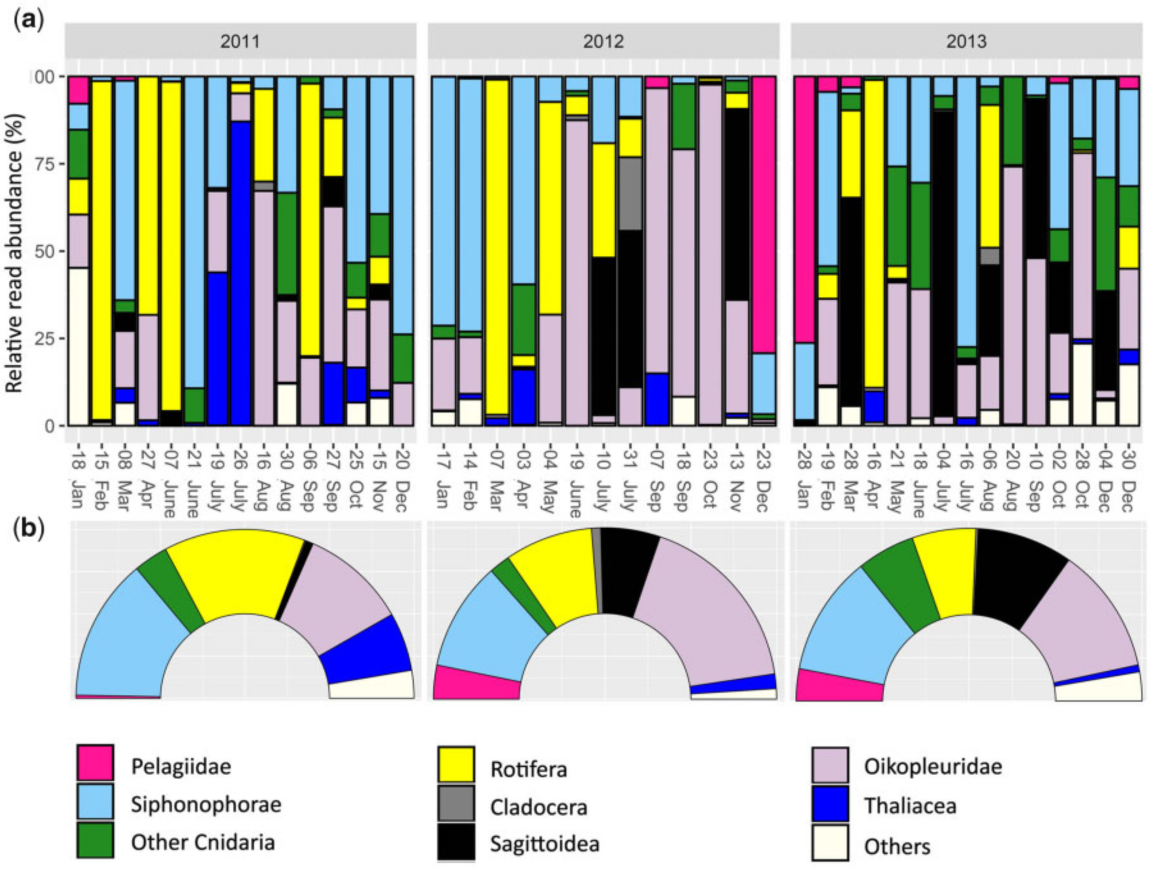

Figure 4. Temporal variability of non-copepod holoplankton taxa at station LTER-MC. (a) Variations across the sampling dates and (b) interannual variations. Five samples with <21 reads each were excluded.

peaks in the spring–early summer period, *Paracalanus* in summer–early autumn, *Temora* in late summer–autumn, and *Clausocalanus* in autumn–winter (Figure 6). The *Oithona* cyclopoids were frequent but without any clear seasonal pattern, while the rest of the copepod genera showed a sporadic occurrence.

Alpha- and beta-diversity

Metazoan diversity was analysed on the normalized dataset, but *H. elegans* reads were excluded because their pervasive and often dominant presence in the dataset would have masked the general pattern for the rest of the community. The alpha-diversity indexes showed all a clear seasonal signal (Figure 7). Richness (as number of observed OTUs) had the highest values in the first months of the year (Figure 7a) which, along with November–December samples, were also characterized by maximum Shannon diversity and evenness values coupled with lower signals of dominance (Figure 7b–d). The minimum Shannon diversity value was found in June samples due to the presence of dominant taxa as indicated by the highest values of Berger–Parker index (Figure 7c).

Beta-diversity showed an even clearer seasonal pattern, with metazoan communities generally grouped by months along the seasonal progression (Figure 8). One group included late autumn–winter samples (December–March), with a clearly defined seasonal gradient and quite marked transitions towards the other group. The next group clustered samples from April through June, the latter month with a high interannual variability. July stand alone to represent full summer and showed quite diverse metazoan communities compared to other months. The last group included samples collected in August to November, with quite weak boundaries and partial overlaps in the transition from summer to autumn.

Discussion

In metazoan-focused studies, sampling is generally performed using nets rather than filters or, in case of the use of filters, large seawater volumes are collected across the water column. Our samples of a few liters of surface waters, originally aimed at exploring the protist community, were not expected to account for metazoan distribution across the whole water column. Surprisingly, we were not only able to trace reasonably the main metazoan plankton players, but we also detected taxa that are hardly found or recognized with traditional methods, including some rare species, as detailed below. These results match the findings from several recent efforts that have addressed invertebrate and vertebrate communities using eDNA, in some cases along with protist communities, which have been able to detect spatial and temporal differences in pelagic communities (Bakker *et al.*, 2017; Berry *et al.*, 2019). In our study, we aimed at assessing the suitability of the eDNA approach to capture seasonal variability of the planktonic metazoan community based on the knowledge acquired through morphology-based methods at the LTER-MC site over >30 years investigation (Zingone *et al.*, 2019).

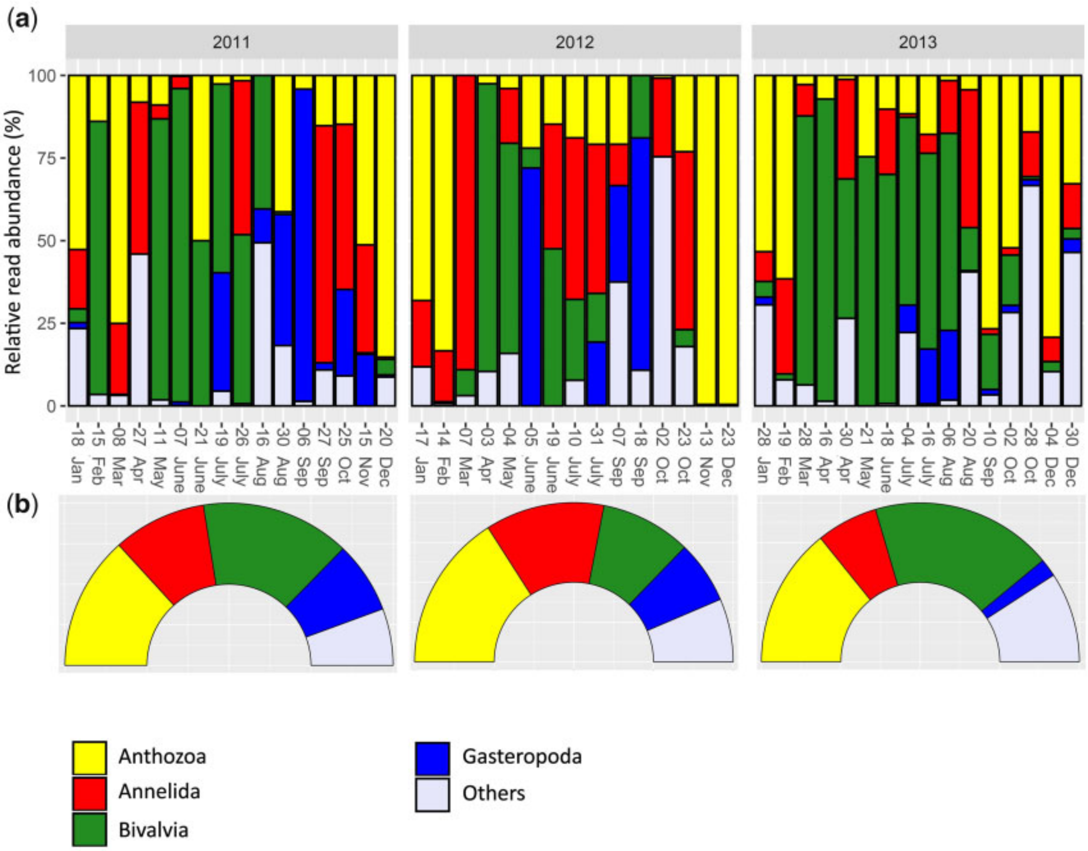

Figure 5. Temporal variability of meroplankton taxa (*Hydroides elegans* excluded) at station LTER-MC. (a) Variations across the sampling dates and (b) interannual variations. Two samples with <22 reads each were excluded.

We did not apply any pre-filtering to our samples, but it is unlikely that entire animals or small larval stages could regularly be trapped in the few liters of waters of the Niskin bottles and deposited onto the filters. In fact, pre-filtering has been shown not to be effective in decreasing metazoan abundance and diversity detected through sea-water filtration (Liu *et al.*, 2017). It is also unlikely that the DNA analysed was dissolved in seawater as we used a 1.2-µm-pore-size filter, while large organisms have been detected using similar or even larger pore-size filters in other cases (Takahara *et al.*, 2012). Therefore, the largest part of metazoan eDNA in our samples probably derived from (i) body fragments and digested or excreted material and (ii) gametes and early reproductive stages (and their fragments). While both origins are possible for holoplankton, the latter is likely the case for most benthic organisms that spend the initial part of their life cycles in the water column. Species detection through extra-organismal eDNA may not ensure the co-presence in time and space of the corresponding whole live organisms or their gametes. However, the scarce persistence of DNA in the natural environment (Thomsen *et al.*, 2012; Collins *et al.*, 2018) supports the potential to collect ecological community and diversity data of high local fidelity using extra-organismal DNA.

Along with the sampling strategy, the choice of the molecular marker, target fragment, and primers are all crucial steps in metabarcoding studies. The mitochondrial gene COI would be the natural choice in studies targeting planktonic metazoans, in which it has been actually largely applied (e.g. Bucklin *et al.*, 2010, 2016; Stefanni *et al.*, 2018). On the other hand, universal eukaryotic primers commonly used in protist studies also permit the co-amplification of metazoan taxa, allowing to exploit environmental 18S datasets to address a wide range of organisms. As a matter of fact, the high amount of metazoan reads analysed in this study were retrieved in a dataset originally planned to address the protists of the Gulf of Naples (Piredda *et al.*, 2017; Gaonkar *et al.*, 2020).

With respect to COI, the V4-18S region underestimates the number of metazoan species (Tang *et al.*, 2012; Stefanni *et al.*, 2018), which could be due to low resolution, but also to the lower number of reference sequences available for 18S rRNA. In our study, the V4 region missed or resolved insufficiently a couple of conspicuous groups, i.e. decapods, whose larvae are abundant at station LTER-MC typically in spring (Mazzocchi *et al.*, 2011), and cladocerans, which dominate the summer zooplankton (Mazzocchi and Ribera d'Alcalà, 1995). Unknown still remain the reasons why V4-18S fails to detect these groups. In the case of cladocerans, lack of reference sequences (Stefanni *et al.*, 2018), as well as length polymorphism of the V4 fragment (Clarke *et al.*, 2017), have been suggested as possible reasons.V4

We explored the performance of the V4-18S region in more details for copepods, exploiting the information obtained on simultaneous samples along with the long-term knowledge of the Gulf of Naples zooplankton (Table 1). With some exceptions, most genera identified by metabarcoding were also found in light microscopy-identified samples obtained through net sampling

Table 1. Copepoda genera and/or species identified in this study based on V4-18S rRNA (years 2011–2013) and their presence in the morphology-based dataset (light microscopy, 1984 to date).

	Molecular identification			**Morphological identification**	
Taxa	***Best hit***	**Similarity (%)**	**Accession no.**	**Genus**	**Species**
Calanoida					
Acartiidae	*Acartia* spp.			×	6
	Acartia clausi	99.2	JX995282	×	×
Calanidae	*Calanus* sp.			×	1
	Calanus helgolandicus	100	JX995315.1	×	×
	Limnocalanus spp.	99	HQ407006	–	–
	Neocalanus sp.	99	AF514339	×	1
Eucalanidae	*Subeucalanus* spp.			×	2
	Subeucalanus crassus	99.5	AY335862	×	×
Calocalanidae	*Calocalanus* spp.			×	9
	Calocalanus plumulosus	99.9	JQ911946	×	×
Candaciidae	*Candacia* spp.	98	HM997063	×	8
Centropagidae	*Centropages* spp.			×	4
	Centropages typicus	98.2	GU594639	×	×
	Centropages violaceus	100	HM997060	×	×
	Isias sp.			×	1
	Isias clavipes	99.2	JX995302	×	×
Clausocalanidae	*Clausocalanus* spp.			×	8
	Clausocalanus arcuicornis	100	HM997079	×	×
	Pseudocalanus spp.	96	GU594644	–	–
Lucicutiidae	*Lucicutia* spp.			×	2
	Lucicutia flavicornis	100	HM997055	×	×
Mecynoceridae	*Mecynocera* sp.			×	1
	Mecynocera clausi	99.5	JQ911953	×	×
Megacalanidae	*Elenacalanus* spp.	97.6	HM997069	–	–
Paracalanidae	*Paracalanus* spp.			×	3
	Paracalanus aculeatus	99.5	JQ911955	×	–
	Paracalanus aff. indicus	99.2	JQ911959	×	–
	Paracalanus parvus	100	JX995311	×	×
	Paracalanus quasimodo	100	JQ911957	×	–
	Parvocalanus sp.			×	1
	Parvocalanus crassirostris	100	KU861810	–	–
Pontellidae	*Anomalocera* spp.	97.1	JX995305	×	1
Pseudodiaptomidae	*Pseudodiaptomus* sp.			×	1
	Pseudodiaptomus marinus	99	KR048712	×	×
Temoridae	*Temora* spp.			×	1
	Temora stylifera	99.2	GU969210.1	×	×
Cyclopoida					
Oithonidae	*Oithona* spp.			×	9
	Oithona davisae	99.5	KJ814022	×	–
Cyclopoida incertae sedis	*Pachos* spp.	97.9	AY627014	×	1
Oithonidae	*Dioithona* sp.	98	KR048726	–	–
Poecilostomatoida					
Oncaeidae	*Oncaea* spp.	98	MF077765	×	5
Corycaeidae	*Ditrichocorycaeus* spp.			×	3
	Ditrichocorycaeus anglicus	99.7	MF077730	×	×
Lichomolgidae	*Lichomolgus* spp.	98	JF781543	–	–
Pseudanthessiidae	*Pseudanthessius* sp.	95	AY627007	–	–
Canuelloida					
Canuellidae	*Canuella* sp.			×	1
	Canuella perplexa	99.7	EU370432	×	–
Longipediidae	*Longipedia* sp.	95.9	MF077724	–	–

Continued

Table 1 continued

	Molecular identification			Morphological identification	
Taxa	***Best hit***	**Similarity (%)**	**Accession no.**	**Genus**	**Species**
Harpacticoida					
Euterpinidae	*Euterpina* sp.			×	1
	Euterpina acutifrons	95	KJ193738	×	×
Tachidiidae	*Tachidius* spp.	95.1	JQ315760	–	–
Tisbidae	*Tisbe* spp.	97.4	JQ315751	–	–
Siphonostomatoida					
Dirivultidae	*Aphotopontius* spp.	95.1	DQ538508	–	–

×: presence; –: absence. Numbers in the last column indicate the total number of species of each genus at LTER-MC.

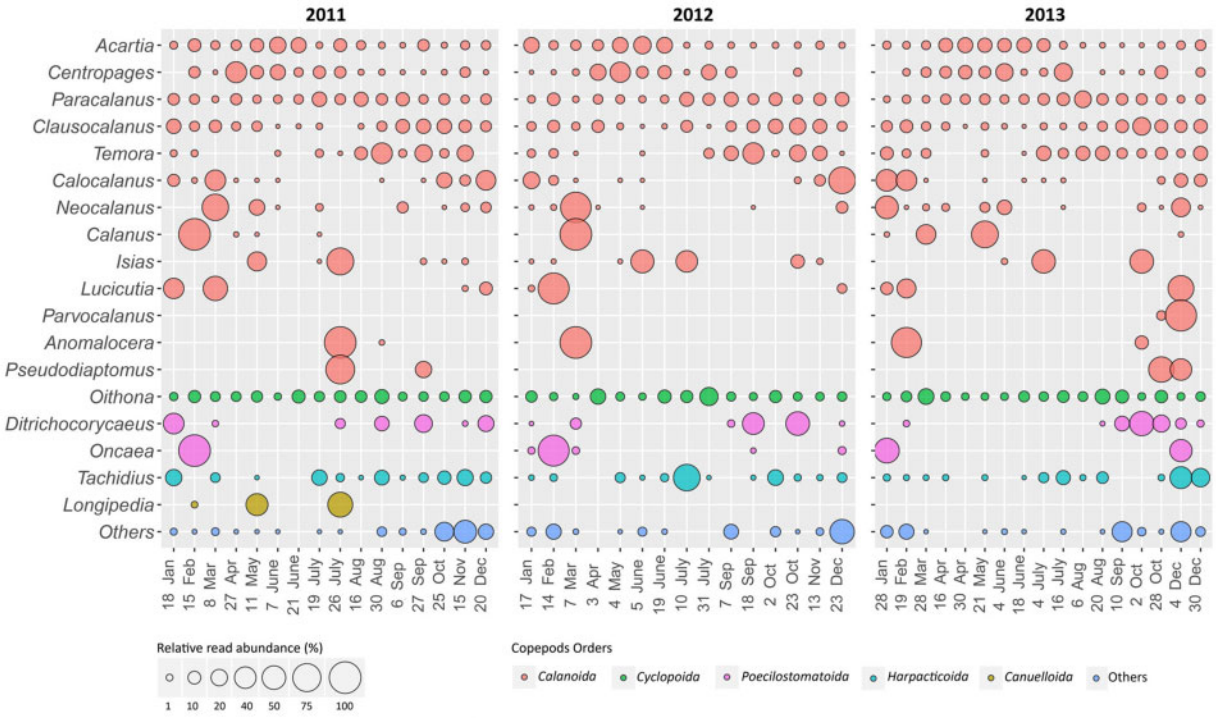

Figure 6. Variability of the annual cycles of the main copepod genera at station LTER-MC. Each circle represents the percentage contribution in that sample over the total reads of the genus in the given year.

over the water column, including most calanoid copepods that were assigned to reference sequences with 99–100% similarity. These results match the view that the V4 fragment allows for a good family- and genus-level identification for copepods, e.g. compared to the shorter and widely used V9 fragment (Wu *et al.*, 2015). On the other hand, some of the 65 genera common in the area were under-represented or not found at all in the metabarcoding dataset. The lack of reference sequences for 28% of the genera known for the Gulf of Naples (Mazzocchi and Di Capua, unpublished), along with a possible lack of recovery of those taxa through a surface seawater sampling, do not allow for a sound comparison between the morphological and molecular datasets, also hindering an assessment of the primer/marker performances. In the case of Oncaeidae, which are common components of the copepod assemblages at station LTER-MC (Mazzocchi *et al.*, 2011), their low abundance confirms the difficulty to detect this family through molecular approaches with any of the several markers and primers used (Clarke *et al.*, 2017; Di Capua *et al.*, 2017; Stefanni *et al.*, 2018; Schroeder *et al.*, 2020). Other mismatches between microscopy and molecular information were the finding of the calanoid copepods *Limnocalanus* and *Elenacalanus*, which do not occur in the Mediterranean Sea (Razouls *et al.*, 2005–2020). Taxonomic assignment at species level was at times ambiguous because the same V4 region may be shared by multiple species, as in the case of *Calanus helgolandicus*/*Calanus pacificus* and *Temora stylifera*/*Temora discaudata*. These cases clearly reflect the low resolution of V4 region for those genera.

Nevertheless, the degree of mismatches in our study is within the expected levels of errors in plankton metabarcoding (Santoferrara, 2019). Despite the limitations highlighted above, this approach disclosed the presence of a wide diversity of

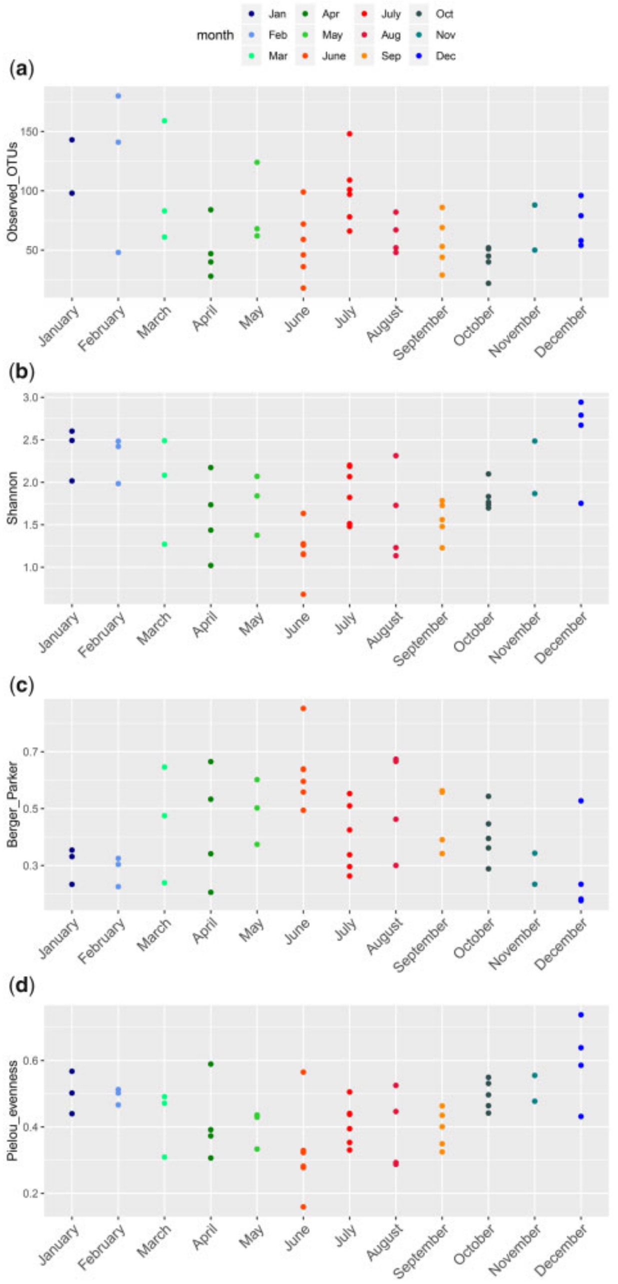

Figure 7. Monthly variations of alpha-diversity based on metazoan OTUs (*Hydroides elegans* excluded) at station LTER-MC. (a) Number of observed OTUs, (b) Shannon entropy index, (c) Berger–Parker dominance index, and (d) Pielou eveness index.

metazoan taxa that are typical components of mesozooplankton assemblages in the upper 50 m water column of the Gulf of Naples (Mazzocchi *et al.*, 2011). Many taxa recurred in the 3 years of the study with the same timing as that generally observed in net zooplankton samples. This is the case, for example of the meroplanktonic groups of bivalves and anthozoans, which in the Gulf of Naples are more important in spring-summer and autumn-winter, respectively. In other cases, seasonal patterns were instead less clear and possibly blurred by the lack of an in-depth study of the individual taxa and their identification at species level. This was especially the case of meroplankton, which were highly diversified in our dataset, covering all 11 animal phyla expected in the plankton, from Porifera to Craniata. The knowledge of diversity and occurrence of these organisms in the plankton of the study area is scarce, because of their relatively low abundance compared to holoplankton, but also and mainly due to the difficult morphology-based identification of larval stages of benthic species.

One limitation of our dataset was the low number of reads available for a sound exploration of the diversity of the less abundant planktonic groups, which was caused by the large dominance of copepods and of the benthic polychaete *H. elegans*. While the dominance of copepods is a typical feature in the plankton of the area, the great contribution of *H. elegans* in different periods of the year and with an increasing trend over the sampling period was totally unexpected. This hard bottom, non-indigenous species typically inhabits stressed environments such as harbours (Langeneck *et al.*, 2020), where it may be invasive and dominate the fouling community, and is widespread in the Mediterranean Sea, including the Italian coasts and the Gulf of Naples (Zenetos *et al.*, 2010). A reason that can explain how *H. elegans* got to represent little less than half of the planktonic metazoan reads is the peculiar reproductive biology of the species, which can release large amounts of gametes in the water column (Qiu and Qian, 1998) and has a reproductive season ranging from late summer to early winter, or even continuous through the year under favourable conditions (Udhayakumar and Karande, 1996). In the study period, *H. elegans* peaks occurred annually between August/September and December/January, with additional peaks and reads detected all year-round, suggesting that the environmental conditions of the Gulf of Naples are particularly suitable for this species.

Among copepods, *Acartia*, *Centropages*, *Temora*, and *Paracalanus* prevailed in terms of quantity and frequency during the entire period of the study, matching their relevant contribution to the neritic zooplankton communities in the Gulf of Naples (Ribera d'Alcalà et al., 2004), as well as their regular seasonal cycle (Di Capua and Mazzocchi, 2004; Mazzocchi *et al.*, 2012). Other conspicuous genera of the area as well as of the entire Mediterranean Sea were *Clausocalanus* and *Oithona* (Mazzocchi *et al.*, 2014), which occurred in several periods of the year reflecting the peaks of their numerous congeneric species that succeed one another over the seasons (Mazzocchi and Ribera d'Alcalà, 1995). While sampling only surface waters may have limited the coverage of the whole copepod diversity, remarkably metabarcoding revealed since July 2011 the presence of *Pseudodiaptomus marinus*, a non-indigenous species recently introduced in the Tyrrhenian Sea (Sabia *et al.*, 2015) and not observed in net samples from LTER-MC until July 2014. Other interesting new findings were the small copepods *Parvocalanus crassirostris*, *Oithona davisae*, and *Dioithona*, which are reported in coastal Mediterranean regions (https://copepodes.obs-banyuls.fr/en/) but, because of their tiny size, may have been missed regularly by the 200-µm mesh net used in vertical hauls at LTER-MC. Different is the case of meiobenthic copepods such as the harpacticoids *Canuella perplexa*, *Tachidius*, and *Tisbe*, and the canuelloid *Longipedia*, or the symbiotic cyclopoid *Lichomolgus* associated with ascidians, also never recorded before in the LTER-MC plankton. They could occasionally be brought to the upper layer from the bottom by the hydrodynamics of the area but are probably pooled as unidentified harpacticoids in the microscopy analyses.

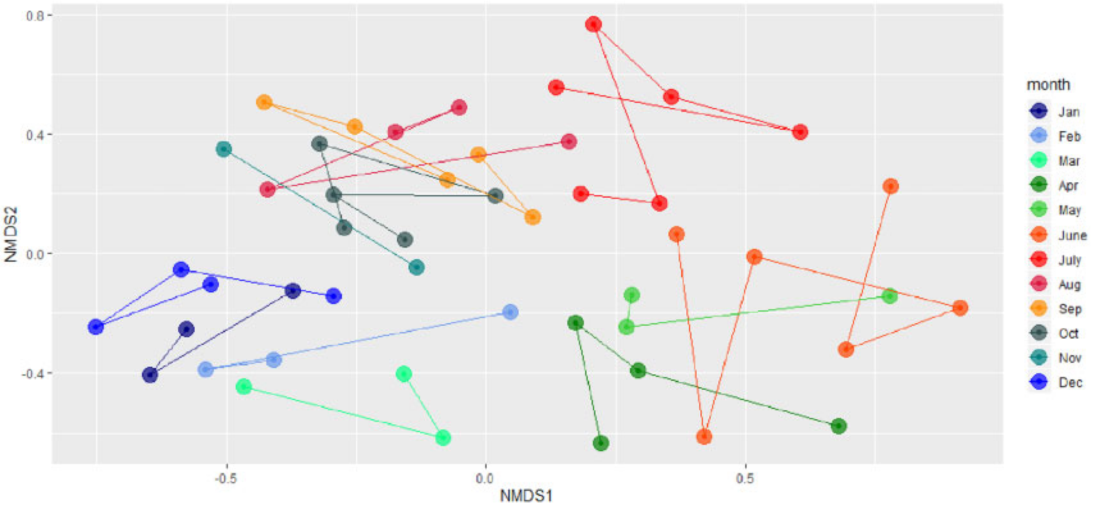

Figure 8. NMDS of the total LTER-MC metazoan community on the 48 dates sampled between 2011 and 2013. Samples from the same month are represented with the same colour and are linked by a line. Stress 0.22.

Jellyfish and small Hydromedusae found in our dataset are also largely missed by the traditional zooplankton sampling because of their patchy and ephemeral occurrence or because of their fragile bodies, highlighting the possibility of eDNA metabarcoding to fill an existing knowledge gap by assessing the abundance and temporal variations of this important component of the pelagic food web (Boero *et al.*, 2008).

Among other interesting results, our investigation highlights the regular occurrence of MAME1, a taxon possibly related to tunicates that was discovered during the EU-BioMarKs project and found in many sites around the world seas (López-Escardó et al., 2018). While mysteries remain about this group of organisms, their regular seasonal signal in the molecular dataset could guide to targeted sampling aimed at clarifying their taxonomic identity.

Seasonality was indeed one the most evident signal in our 3-year dataset, which emerged clearly for both copepods and the whole community. Despite the remarkable heterogeneity of the latter, the seasonal variations in diversity with maxima in winter and minima in summer match those highlighted for both prokaryotes (Gilbert *et al.*, 2012) and eukaryotic communities in other areas (Genitsaris *et al.*, 2015) and in the Gulf in the Naples (Piredda *et al.*, 2017). This typical pattern can be interpreted as the resultant of both hydrographic and ecological forcings. In autumn–winter, hydrologic instability and fast water mass displacement make single-point, surface samples representative of larger water masses, allowing a more exhaustive detection of the whole community of an area. On the other hand, favourable spring/summer conditions allow for the massive development of many planktonic organisms and the outbreaks of single taxa, enhancing dominance and lowering diversity. The even clearer signal of recurrent patterns across the years highlighted by the analysis of our dataset reveals a high resilience of the whole community composition over time and an overall high stability in the system despite its coastal nature, which could be driven by strong evolutionary constraints over the temporal niches acting on all components of the plankton communities.

Conclusions

Despite some limitations, the V4-18S fragment metabarcoding analysis captured a high number of metazoan taxa in the plankton of the Gulf of Naples, overall ensuring a good coverage of the extant diversity. The results of our study definitely confirm DNA metabarcoding as a powerful approach to be integrated at LTER sites, which points at the need to identify standardized procedures that ensure reproducibility and comparability both within a time series and among different time series (Stern *et al.*, 2018; Jeunen *et al.*, 2019; Santoferrara, 2019). In addition, considering the huge taxonomic variety of marine zooplankton, multi-markers strategies (Stat *et al.*, 2017) along with the expansion of the available reference datasets are warranted in order to increase the taxonomic coverage and resolution of the eDNA-metabarcoding approach.

The overview of metazoan diversity obtained from filtered eDNA from a relatively small seawater sample corroborates the finding that detection rate does not always increase with the water volume sampled (Mächler *et al.*, 2016), supporting a wider application of sample filtering, which is logistically simpler compared to net sampling of bulk populations.

Our background knowledge of the holoplankton of the area confirms that most taxa identified through their molecular signatures actually belong to the local zooplankton community, while the marked seasonal signal in metazoan occurrence mostly coincides with that detected through morphological-based studies. The seasonal alternation of taxa and the retrieval of species known or likely for the area indicate a high temporal and spatial fidelity of extra-organismal DNA, confirming that the eDNA analysis can actually be used to trace phenological and biogeographical patterns of a wide range of organisms.

As for the new findings, the presence of fragile and tiny Hydromedusae, invasive species, and other taxa likely for the area but not detected beforehand, along with the need to clarify the identification of some recurrent yet mysterious, non-labelled taxa, are all results that will stimulate further studies to complement and increase the knowledge of the Mediterranean plankton biodiversity.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Data availability statement

The data underlying this article are available in in GenBank Nucleotide Database at https://www.ncbi.nlm.nih.gov/genbank/ and can be accessed with accession numbers MW140087 and MW142014.

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