DNA metabarcoding and morphological analysis - Assessment of zooplankton biodiversity in transitional waters

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ABSTRACT

Zooplankton biodiversity assessment is a crucial element in monitoring marine ecosystem processes and community responses to environmental alterations. In order to evaluate the suitability of metabarcoding for zooplankton biodiversity assessment and biomonitoring as a fast and more cost-effective method, seasonal zooplankton sampling was carried out in the Venice Lagoon and the nearby coastal area (Northern Adriatic Sea). The molecular analysis showed higher taxa richness compared to the classical morphological method (224 vs. 88 taxa), discriminating better the meroplanktonic component, morphologically identified only up to order level. Both methods revealed a similar spatio-temporal distribution pattern and the sequence abundances and individual counts were significantly correlated for various taxonomic groups. These results indicate that DNA metabarcoding is an efficient tool for biodiversity assessments in ecosystems with high spatial and temporal variability, where high sampling effort is required as well as fast alert systems for non-native species (NIS).

1. Introduction

Transitional waters (estuaries, deltas, lagoons) belong to the most productive ecosystems and provide important habitats for a plethora of species, particularly during their juvenile and reproductive life stages (Milardi et al., 2018). Such waters also act as important nursery areas for many (commercially) important fish species (Tournois et al., 2017), to which zooplankton is a very important food source. These distinct coastal ecosystems are characterised by composite gradients (Tagliapietra et al., 2009) that have a prominent role in the organization of biological communities (Reizopoulou et al., 2014), as they directly influence productivity, colonization and dispersal processes (Ghezzo et al., 2015). In addition, they are strongly characterized by temporal variability of hydrodynamic (fresh water inputs, meteo-marine conditions) and thermo-haline factors that lead to a high natural instability, consequently resulting in wide seasonal variations of pelagic species diversity (Reizopoulou et al., 2014). Zooplankton in transitional environments occupies a variety of niches and significantly contributes to key ecosystem functions due to its high functional diversity (Morabito et al., 2018), not only as prey for juvenile fish species, but also as consumers of primary production. In general, in transitional environments the species are adapted to high environmental variability and show a decrease in species richness, an increase in abundance and a greater importance of small taxa along a confinement gradient (Belmonte et al., 2013; Riccardi, 2010). Due to its pronounced degree of unpredictability, however, the impact of local and large-scale environmental changes on planktonic population dynamics is hard to evaluate (Morabito et al., 2018).

In this framework, high taxonomic resolution assessments of zooplankton biodiversity accompanied with species richness estimations are essential. As accurate morphological assessments are labour-intensive, the characterization of the spatio-temporal variability of zooplankton assemblages is scarcely investigated despite their ecological importance (Djurhuus et al., 2018). Moreover, the complexity of zooplankton assemblages, including cryptic and sibling species, and the lack of diagnostic characters for immature (larval) stages are key

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impediments to understand patterns of biodiversity with classical taxonomic identification methods (Bucklin et al., 2016). Regardless of the rising necessity for taxonomic information across trophic levels to support ecological research and ecosystem-based management, morphological taxonomic expertise is in decline and its importance often underestimated (Hopkins and Freckleton, 2002; Kim and Byrne, 2006). Additionally, in transitional waters, monitoring requires high sampling effort in order to take into account the above-mentioned spatial and temporal variability, even more so regarding zooplankton, which is especially sensitive to altering environmental conditions (Hays et al., 2005; Richardson, 2008).

The estimation of biodiversity with DNA metabarcoding (Taberlet et al., 2012) using high-throughputsequencing (HTS) is becoming an important tool for surveying biodiversity thanks to the broad taxonomic coverage and the possibility of increased sample processing speed allowing to increase the sampling effort (frequency and spatial coverage) with sustainable costs (Brannock et al., 2014; Coissac et al., 2012). An additional significant advantage is the prospective to detect the 'hidden diversity' of zooplankton assemblages, including holo-, mero- and ichthyoplankton (Lindeque et al., 2013). As most marine species are planktonic at some point in their life cycle, this will give us new insights into the overall marine biodiversity (Bucklin et al., 2016). Several studies have shown that DNA metabarcoding can be used as an efficient tool for zooplankton biodiversity assessments in various marine environments (e.g. Bucklin et al., 2019; Deagle et al., 2018; Harvey et al., 2017; Stefanni et al., 2018). With constant progress in this technology, metabarcoding will be extremely helpful in the study of community changes e.g. driven by climate change or other habitat alterations and studies of the ecology of cryptic taxa within zooplankton assemblages (Sommer et al., 2017).

The present study aims at evaluating the suitability of DNA metabarcoding for assessments of zooplankton diversity patterns in transitional waters using a fragment (313 bp) of the cytochrome *c* oxidase subunit 1 (COI) corresponding to the second half of the universal animal DNA barcode (Leray et al., 2013), a DNA metabarcoding marker for which several studies have demonstrated its high value when studying marine metazoans (Carroll et al., 2019; Clarke et al., 2017; Stefanni et al., 2018; Zhang et al., 2018). Two important Mediterranean heterogeneous ecosystems were chosen as study area: the Gulf of Venice and the Venice Lagoon, located in the north-western Adriatic Sea, both of which are subject of investigation within the Long Term Ecological Research (LTER) network (LTER_EU_IT_016 and LTER_EU_IT_057, respectively), the Biodiversity and Ecosystem Research LifeWatch-ERIC, and the European WFD and MSFD directives. In this context, there is a need to increase the knowledge on the zooplankton biodiversity and non-indigenous species (NIS), in order to support the definition of Good Environmental Status (GES) and the identification of management strategies.

This study compares the molecular and morphological approach along environmental gradients and over the year, evaluating it as a tool for zooplankton biodiversity investigations in ecosystems with high spatial and temporal variability and where high frequency monitoring is preferable.

2. Material and methods

2.1. Study area

The Venice Lagoon is located in the Northern Adriatic Sea, a shallow coastal area (mean depth of 35 m) strongly influenced by the inputs of large rivers bringing water from the Alps and characterized by meso-eutrophic conditions and by a remarkable spatial and temporal variability of trophic and physico-chemical gradients (Bernardi Aubry et al., 2006) (Fig. 1). The Gulf of Venice in the Northern Adriatic is a highly productive ecosystem and important nursery area, especially for fish species. Nonetheless, it is also highly impacted by human activities

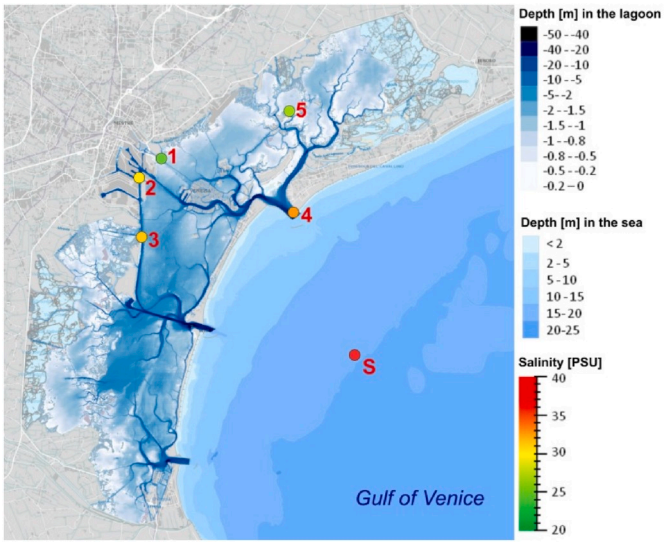

Fig. 1. Study area (Venice Lagoon and Gulf of Venice, Italy). Overview and bathymetry and mean salinity (recorded during seasonal sampling activity) and location of the six sampling stations: 1, 2, 3, 4, 5 and S (www.atlantedellalaguna.it).

(Lotze et al., 2006; Solidoro et al., 2010) and a hot-spot of maritime traffic. Ballast water was recognized as a global vector in human-mediated invasions, inadvertently providing a fast and reliable dispersal mechanism for many marine taxa and therefore massively increasing the risk of NIS introduction (Marchini et al., 2015; Vidjak et al., 2018). The Venice Lagoon has a surface area of about 550 km^2 with a north-south length of ~50 km, a mean horizontal width of 10 km and three inlets on the western side connecting the lagoon with the Adriatic Sea (Fig. 1) (Ghezzo et al., 2011). The lagoon is a heterogeneous and complex system, characterized by a number of environmental gradients involving salinity, marine water renewal (e.g. residence time), nutrients, depth and sediment structure (Tagliapietra et al., 2009) and a mosaic of habitats and landforms (e.g. intertidal marshes, intertidal mudflats, and natural and navigation channels) that are the result of complex natural and man-induced drivers (Sigovini, 2011). The Venice Lagoon is characterized by a semi-diurnal microtidal regime with a mean range of 0.40 m during neap tides and about 0.80 m during spring tides. The amount of seawater that is exchanged during each tidal cycle is about one third of the total volume of the lagoon (Gačić et al., 2004). The residence times range from few days, in the proximity of the three inlets, to over 60 days in the inner lagoon areas (Cucco and Umgiesser, 2006). General hydrodynamics in the lagoon are regulated mainly by tidal currents and affect basic parameters such as water exchange, dissolved oxygen, salinity, nutrients and sediment distribution. The mean depth of the tidal flats is −1.2 a.m.s.l., while it reaches −10/−15 m a.m.s.l. in the natural tidal channels (Ghezzo et al., 2010; Molinaroli et al., 2007). The year averaged salinity ranged from 20 PSU at sites influenced by freshwater to 33 PSU close to the inlets, with high temporal fluctuations due to tides and river discharge (Zirino et al., 2015). The total freshwater discharge from the Lagoon river basin is about 35 m^3s^{-1} (Zuliani et al., 2005). In the Venice Lagoon, about 80% of the total community is composed by copepods (Camatti et al., 2006) with *Acartia* as the most representative genus, while cladocerans and other taxa (e.g. appendicularians), with more marine affinity, can be found more frequently in the areas nearby the inlets (Solidoro et al., 2010).

2.2. Sample and metadata collection

Mesozooplankton community composition was seasonally investigated, from April 2016 to February 2017, at five stations in the Venice

Lagoon (4 inner stations and 1 inlet station) and in the near shore coastal area in the Gulf of Venice (station S) (Fig. 1, Table 1), all belonging to the LTER network. Surface horizontal hauls using an Apstein plankton net (0.4 m opening diameter, 200 μm mesh) and vertical hauls, from the bottom to the surface, using a WP2 net (0.57 m diameter, 200 μm mesh) were performed, at lagoon stations and at the marine station S, respectively. The samples were divided in two equal parts: one was preserved in 4% borax-buffered formalin for taxonomic and quantitative determinations performed by stereomicroscope, and the other part in 96% ethanol for genetic zooplankton community analysis. In the same stations, environmental data were measured by a multiparameter probe.

2.3. Morphological and molecular analysis

For the morphological analysis, taxonomic and quantitative zooplankton determinations at the lowest possible taxonomic level (mostly species level for copepods and cladocerans) were performed using a Zeiss stereomicroscope. According to the International Council for the Exploration of the Sea (ICES) protocols (Harris et al., 2000), representative aliquots of the samples were analysed, ranging from 1/3 to 1/40 of the total sample, while the entire samples were analysed in regard to species not present in the subsample. Other zooplankton were identified to phylum and, where possible, to class, order, or family level.

Genomic DNA was extracted using the E.Z.N.A.® Mollusc DNA kit (Omega Bio-Tech) following the manufacturer's instructions by taking about one third of the total sample and increasing the initial reagents (lysis and binding buffer) provided by the kit proportionally to the sample volume. As we previously noticed an increased concentration of PCR inhibitors, the samples were not grinded, but the cell lysis was done overnight instead. The quality and quantity of the extracted DNA was assessed with a NanoDrop 2000 Spectrophotometer (ThermoScientific) and the amplification of the COI fragment was performed for each sample individually using a combination of degenerated primers: mlCOIintF, dgHCOI2198 and jgHCOI2198 following a two-step protocol after Stefanni et al. (2018). The library was prepared for HTS by pooling an equimolar amount of all products after the secondary PCR. Next, the library was size-selected (range 150–400 bp) and purified using E.Z.N.A. ® Size Select-IT kit (Omega Bio-Tech). Emulsion PCR was conducted using the Ion One Touch System (Life Technologies) following the manufacturer's recommendations and DNA was bound to Ion Sphere particles (Life Technologies) for clonal amplification automatically enriched with the Ion OneTouch ES system (Life Technologies). For sequencing, the library was loaded on a 316™ chip with 650 flows in a PGM (Life Technologies).

For the creation of new local reference sequences, the DNA was extracted from taxonomically identified individuals collected in station 5 and S and COI Folmer region (Folmer et al., 1994) was amplified using the LCO1490 and HCO2198 primers following the manufacturer's instructions for the Polymerase ready mix (2x PCR Bio HS Taq Mix) by PCR Biosystems. DNA Sanger sequencing was performed at Macrogen (Macrogen Europe, Amsterdam, Netherlands).

Table 1
Sampling dates and coordinates of sampling sites.

Station	Spring	Summer	Autumn	Winter	Coordinates
1					45° 27′ 55.44″ N; 12° 16′ 58.26″ E
2					45° 27′ 25.56″ N; 12° 15′ 40.20″ E
3	29-Apr-2016	14-Jul-2016	13-Nov-2016	20-Feb-2017	45° 25′ 04.74″ N; 12° 15′ 34.50″ E
4					45° 25′ 32.94″ N; 12° 25′ 34.44″ E
5					45° 29′ 57.12″ N; 12° 25′ 02.58″ E
S	4-May-2016	12-Jul-2016	22-Nov-2016	12-Jan-2017	45° 18′ 83.00″ N; 12° 30′ 53.00″ E

2.4. Bioinformatic analyses

Raw COI reads were demultiplexed, truncated (tags and primers) and processed using the split_libraries.py script from QIIME 1 v. 1.9.0 pipeline (Caporaso et al., 2010) allowing 2 nucleotide mismatches in primers, a maximum length of homopolymers run of 8, while all other parameters were left as by default. The processing stage also included the removal of low quality reads (minimum average Phred quality score >25) and sequences <200 bp or >1000 bp. Afterwards, the sequences were demultiplexed and dereplicated in QIIME 2 v. 2018 (Rideout et al., 2018), chimeric feature sequences were identified and filtered with q2-vsearch (Rognes et al., 2016) excluding chimeras and "borderline" chimeras (Rideout et al., 2018). Taxonomic assignment of the COI dataset was done by aligning the quality filtered reads against an in-house reference database for "marine metazoa" constructed from metazoan COI sequences that belong to major metazoan groups that appear in marine environments deposited in GenBank (Table S1) with a naive LCA-assignment algorithm implemented in the MEGAN6 alignment tool (MALT) (Huson et al., 2016).

First, an optimal similarity threshold was determined in order to provide a reliable basis for downstream analysis (Mohrbeck et al., 2015; Stefanni et al., 2018). Therefore, taxonomy assignment was conducted in a stepwise manner over a series of similarity thresholds (from 100% to 90%) decreasing by 1% at a time against the "marine metazoa" database (Table S1). According to the relative abundance of assigned reads for various taxonomic groups at different similarity thresholds, the similarity thresholds of 97% and 94% were chosen (Fig. S1 and S3). Above 97% similarity threshold hits were considered as species level operational taxonomic unit (OTUs) ("recovery 97%" dataset), however, if a taxon was first recovered between 97% and 94% similarity threshold, it was considered as less certain and so a "cf." was added to their taxonomy ("recovery 94%" dataset). Afterwards, both datasets were pooled together ("recovery 97% + 94%" dataset).

For the downstream analyses of the community diversity we followed the suggestion of Stefanni et al. (2018) to include putative metazoan OTUs that could not be recovered above the 94% similarity threshold. For this we compared the remaining unassigned reads at the 85% similarity threshold. Reads not matching any metazoan reference sequence at this threshold were considered as non-metazoan and were discarded, while reads with a hit were considered as putative metazoan reads (Fig. S2). In the successive step, all the putative metazoan sequences were clustered *de-novo* with VSEARCH (Rognes et al., 2016) using UCHIME *de-novo* approach at 97% similarity ("de-novo-recovery" dataset). VSEARCH was also used to select representative sequences for both the "de-novo-recovery" and for the "recovery 97% + 94%" datasets. To further deflate the number of OTUs and to fix erroneous OTUs, the two datasets were pooled together and curated with the LULU algorithm (Frøslev et al., 2017). After manually checking the LULU-curated OTUs, those *de-novo* OTUs that still remained unclustered (hence taxonomically unassigned) were blasted against the GenBank database with BlastN. The blasted taxonomy was checked manually and only considered when both the query coverage was above 90% and Max score above 100. If any of the blasted taxonomies matched taxonomies recovered in prior steps, they were pooled together, while blasted taxonomic assignments appearing here for the first time were only considered as a best match ("best match <94%"). All datasets were manually checked regarding the known distribution of the corresponding taxa in the Adriatic Sea or in the Mediterranean Sea and regarding the reliability of the reference (e.g. UNPUBLISHED sequences from GenBank). A flowchart of the bioinformatic pipeline is present in the Supplementary (Fig. S2).

Finally, additional reference COI sequences of the local community of three copepod species, *Labidocera brunescens*, *Centropages ponticus* and *Acartia margalefi* and for the cladoceran *Penilia avirostris* were created. Indeed, these species are expected to appear in and around the Venice Lagoon (and were also recovered with the morphological identification),

but are missing or are not geographically well represented in GenBank. The GenBank accession numbers are: MN604219 and MN604220 for *Labidocera brunescens*, MN604215 and MN604216 for *Acartia margalefi*, MN604217 and MN604218 for *Centropages ponticus* and MN604221 and MN604222 for *Penilia avirostris*. The in-house reference database was expanded with these new local reference sequences. After evaluating the variation in the OTU table including and excluding the local reference sequences from the in-house reference database, the amount of the taxonomic assigned reads were manually added to the final OTU table ("local-barcodes recovery").

2.5. Statistical analyses

Diversity analyses were done with R software (R Core Team, 2018). All calculations were done on square-root transformed data unless stated otherwise. Species richness per sample was quantified according to the measure of the first Hill number – MOTU/taxa richness ($q = 0$) using the R package iNEXT (Chao et al., 2014; Hsieh et al., 2019). Alpha diversity of individual communities were quantified according to the Shannon-Wiener Index using the R package vegan (Oksanen et al., 2019). Alpha diversities of DNA metabarcoding data (hereon MBC) and morphological identification (hereon MOI) were compared with Pearson correlation. The same comparison was done collapsing the MBC dataset to the same taxonomic level as the morphological dataset (e.g. all decapod OTUs collapsed to 1 OTU summing the reads) as proposed by Cahill et al. (2018). Finally, in order to evaluate the contribution of copepods to the total diversity, the estimation based on copepods only was compared to the overall diversity estimation.

Differences between relative abundances (in percents) of the most abundant phyla (Arthropoda, Cnidaria, Echinodermata, Chordata, Mollusca and Annelida) and the most abundant classes of arthropods (Hexanauplia, Malacostraca and Branchiopoda) over seasons and locations (lagoon (including inlet) and sea) were assessed using the non-parametric Kruskal-Wallis-Test, while correlations between the two methods were again tested with Pearson correlation. Here, correlations over seasons and locations were done for the most abundant phyla and most abundant arthropod classes, while correlations for all species that were recovered in both datasets (21 species), were done by both pooling all the seasons and locations together and by keeping them separated. Pearson correlations were evaluated from percentages of square-root transformed data after summation.

Beta diversity was evaluated from dissimilarity matrices built according to Bray-Curtis distances using the metaMDS script with the autotransform function (R package vegan) (Oksanen et al., 2019) and plotted using the function ordiplot superimposing the temperature and salinity values at the sampling sites using the function ordisurf, which fits a smooth surface for a given variable plotting it on the ordination diagram. Spatial and temporal patterns in the community composition based on Bray-Curtis similarity values were assessed using repeated-measure permutational analysis of variance (PERMANOVA) with season and location as fixed factors, and station nested within the location level (PRIMER 6+ and PERMANOVA software package; PRIMER-E, Ltd., UK) for both MBC and MOI. The correlation of the similarity matrices was calculated with the software package PRIMER6, utilising the function RELATE (Spearman's correlations based on resemblance matrices) (Clarke and Gorley, 2006). The average dissimilarities between the MOI and the MBC were calculated applying SIMPER (on-way analysis based on Bray-Curtis similarities) using the software package PRIMER6 (Clarke and Gorley, 2006). The calculation of the combination of environmental parameters that explains the community composition was done using BEST (BIOENV) calculating the Spearman's correlation between both similarity matrices.

3. Results

3.1. Taxonomical composition and richness of molecular and morphological data

The raw sequencing data produced more than 4×10^6 raw sequences in the 24 samples analysed. After quality check and chimera removal, the 1.97×10^6 left sequences had a mean length of 311.9 bp and a median length of 313 bp (Fig. 2A).

At the similarity threshold of 97% mostly Arthropoda, followed by Cnidaria, Chordata and Echinodermata were identified, while the alignment at 94% similarity threshold resulted mostly in Sagittidae (Chaetognatha), Branchostomatidae, Percomorphaceae (Chordata) and Echinoidea (Echinodermata) assignments (Fig. S3). The final dataset included 1.5×10^6 assigned reads belonging to 258 OTUs. Of these, 205 OTUs (84% of assigned reads) were identified at 97%, 15 new OTUs were only identified at 94% similarity threshold (6% of assigned reads), 35 new OTUs and 4% of all assigned reads were identified with blasting the de-novo OTUs, and additional 3 OTUs and 6% of all assigned reads were assigned using the local barcodes. From the final dataset four singleton OTUs were removed as they do not have a confirmed presence in the Mediterranean Sea, while 24 singletons were kept. Without considering the putative metazoan OTUs ("best match <94% recovery") we recovered 224 OTUs belonging to a total of 1.4×10^6 sequences.

The number of reads per sample varied from 3×10^3 (st. 5, winter) with 12 OTUs to 132×10^3 reads with 73 OTUs (st. 2, spring) with a mean of $62.8 \times 10^3 \pm 34.3 \times 10^3$ reads and 44.3 ± 15.1 OTUs per sample (Fig. 2B and S4). Out of the 224 assigned OTUS identified with MBC, 188 were assigned at species level (83.6%), 29 at genus level (12.8%), while three OTUs were left unclassified (Bilateria, Protostomia, and Lophotrochozoa). The morphological identification (MOI) resulted in the identification of 88 taxa (level of taxonomic assignment: species: 40 (45.5%); genus: 14 (15.9%); family: 4 (4.5%); order: 12 (13.6%); infraclass: 1 (1.1%); subclass: 1 (1.1%); class level: 9 (10.2%); phylum level: 7 (7.9%) (Fig. 2C).

The taxa richness with MBC was higher compared to the MOI approach (Fig. 3). Using the MBC approach, 188 species, 140 families, 30 classes and 15 phyla were identified (not including "best match <94%"); on the other hand, with MOI, 40 species, 23 families, 14 classes and 11 phyla were recovered. Compared to MBC, some phyla were not documented at all (Nemertea, Bryozoa, Rotifera, Gastrotricha and Platyhelminthes), some phyla were only recovered at phylum level (e.g. Ctenophora, Nematoda and Phoronida) and for some phyla, like Annelida and Mollusca, organisms could be assigned only to class level (Polychaeta and Gastropoda/Bivalvia). Also within Arthropoda, classes like Malacostraca showed much lower diversity according the MOI approach, as for example Amphipoda and Decapoda were assigned only to order level, while the MBC approach assigned seven amphipod OTUs and 24 decapod OTUs at species level. In addition, the MOI approach also performed poor in recovering Chordata and Cnidaria, namely, with MBC approach 28 Chordata OTUs were identified (30 Actinopterygii, 7 Ascidiacea and one Leptocardii, *Branchiostoma lanceolatum*) and only five with MOI approach – *Branchiostoma* larvae, Actinopterygii larvae, *Engraulis encrasicolus* eggs, Ascidiacea larvae, Appendicularia, Thaliacea. Nonetheless, the relatively abundant class Appendicularia could not be assigned molecularly and when not considering the "best matches <94%", this approach also missed the phylum Phoronida (Fig. 3).

In terms of relative abundance (read abundance) assessed by the MBC approach, arthropods were the most abundant group ($67.5 \pm 26.6\%$), followed by cnidarians ($11 \pm 21.8\%$), echinoderms ($7.6 \pm 16.7\%$), chordates ($7.1 \pm 14.8\%$), molluscs ($4 \pm 7.3\%$), annelids ($1.9 \pm 4.6\%$) and other phyla (with less than 1%) ($0.1 \pm 0.6\%$) (Fig. 4A). Also, the morphological analysis resulted in a dominance of arthropods in terms of number of individuals ($83.5 \pm 16.6\%$), followed by chordates ($10.9 \pm 4.1\%$), while all other phyla were much less represented (Fig. 4B). Although holoplankton resulted to be the most abundant

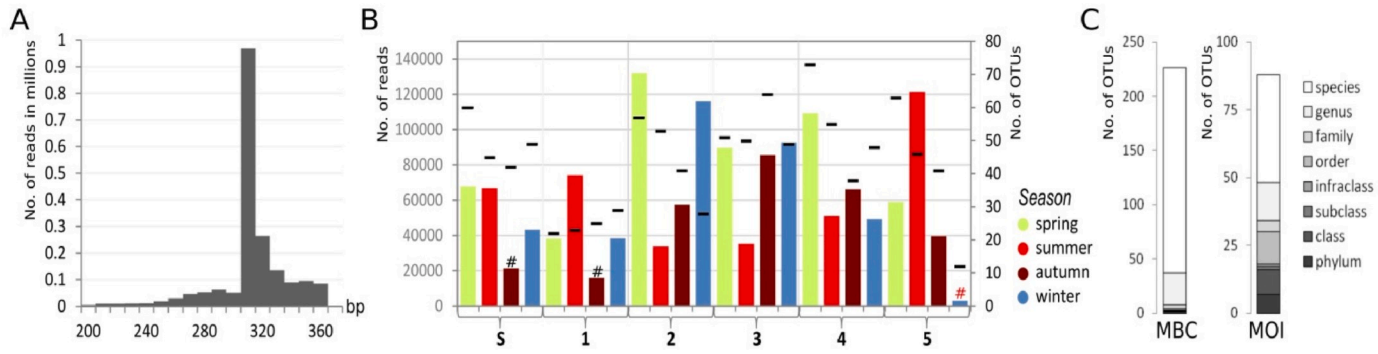

Fig. 2. A) Histogram of read length after quality check and chimera filtering. B) Number of reads per sample of the 24 samples. Two samples (st. S autumn; st. 1, autumn) have less than 20% (black #) and one sample (st. 5, winter) less than 5% (red #) of reads in relation to the sample with the maximum number of reads. C) Taxonomic level of assignment using the molecular approach (MBC) and the morphological approach (MOI). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

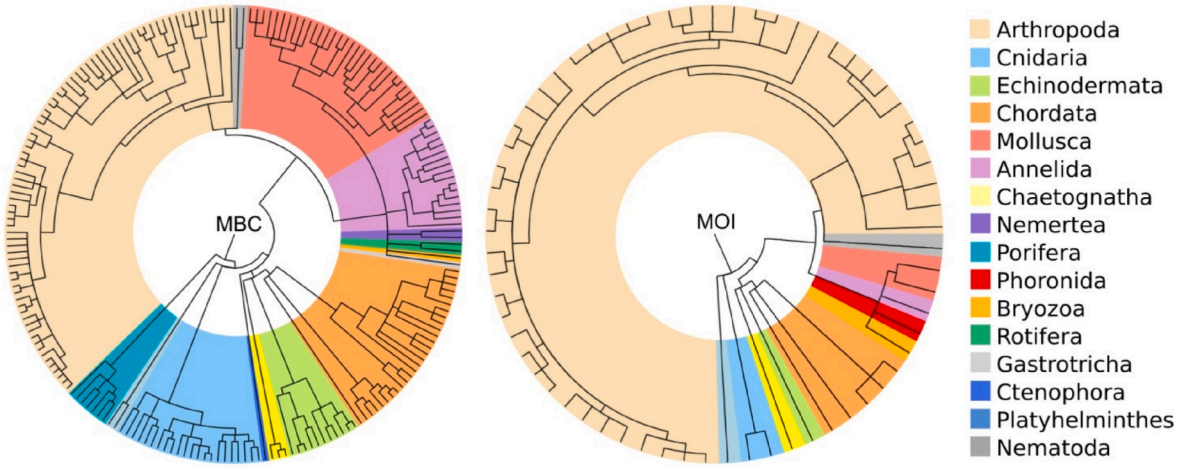

Fig. 3. Taxonomic tree representing the taxonomic richness revealed with molecular approach (MBC) and the morphological approach (MOI), respectively (R package ggtree).

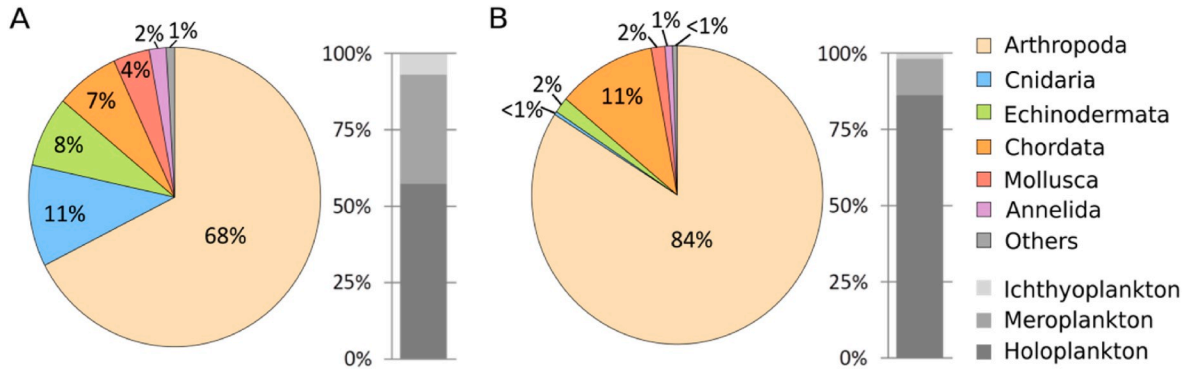

Fig. 4. Relative abundance of main phyla and of zooplanktonic groups by A) the molecular approach (MBC) and B) the morphological approach (MOI).

group with both approaches, MBC (58%) and MOI (86%), in MBC data more than one third (35%) of the retrieved sequences belonged to meroplankton and 7% to ichthyoplankton (Fig. 4A), whereas within MOI data, meroplankton contributed only with 12% to the total abundance and ichthyoplankton only with 2% (Fig. 4B).

In terms of species richness, in MBC data 69% of the OTUs belonged to meroplankton (excluding cnidarians) and 9% to ichthyoplankton, while the morphological analysis was clearly dominated by holoplankton (80%) (mainly copepods and cladocerans) and only 10% were meroplanktonic taxa.

The species richness of copepods was similar with both methods; the molecular analysis allowed the identification of 41 taxa at species level and additional two at genus, one at family and one at order level, while the morphological analysis revealed 35 taxa at species level and additional ten at genus, four at family and four at order level. The contribution of copepods to the whole taxa richness resulted in 17.4% and 60.2% in MBC and MOI, respectively. At species level, only 18 taxa (31%) were shared by both methods; the percentage increased at genus

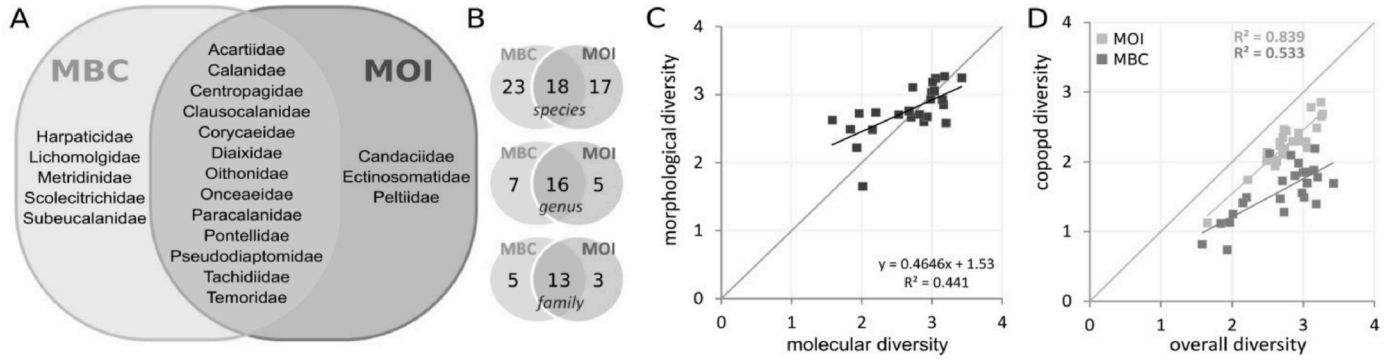

Fig. 5. Venn diagram of A) list of families of copepods; and B) number of taxa (species, genus, family) found with the molecular approach (MBC) and the morphological approach (MOI); and Shannon-Wiener Index of C) MOI vs. MBC; and D) copepods vs. whole dataset.

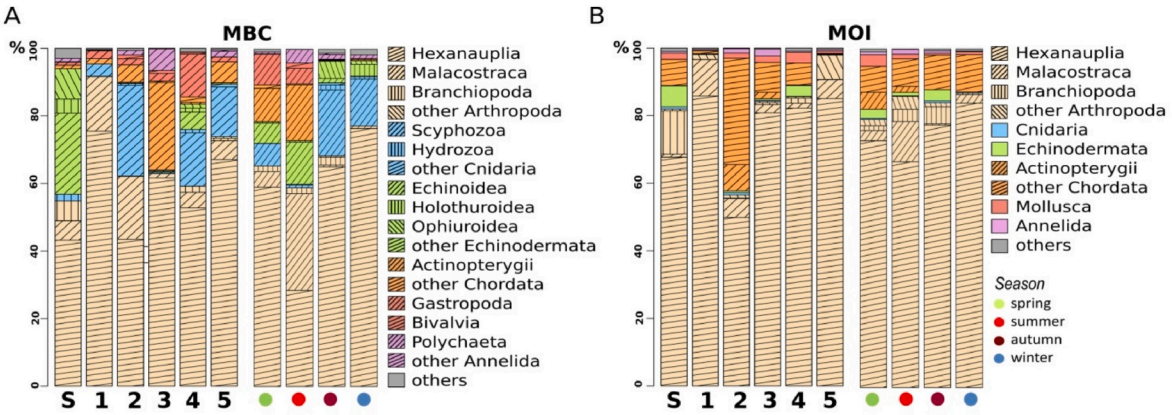

Fig. 6. Barcharts of relative abundances grouped by phyla (colours) and classes (patterns) and averaged by stations (st. S, st. 1–5) and by seasons with A) the molecular approach (MBC) and B) the morphological approach (MOI). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

level (57%; 16 genera) and at family level (62%, 13 families) (Fig. 5). Seventeen species of copepods were identified only with the morphological identification, e.g. *Clytemnestra scutellata, Labidocera wollastoni, Microsetella rosea, Candacia giesbrechti, Centropages kröyeri, Diaixis pygmaea, Oithona setigera, Oithona nana* and *Oithona tenuis*. Moreover, some problematic species discriminations have emerged: *Calanus helgolandicus* has been identified only by MOI and not with MBC where the sequences were assigned to reference sequences annotated as *Calanus euxinus*, known to be a population of *C. helgolandicus*. However, in the MOI dataset the abundance of adults was very low, with only 2 specimens of *C. helgolandicus* found in 1 sample, but probably several not identified juveniles. A similar problem was observed in the *Paracalanus parvus* species complex. Here, MOI could identify only *Paracalanus parvus, Paracalanus nanus* and *Paracalanus sp.*, while MBC also identified *P. quasimodo* and *P. indicus*. However, according to both approaches, *P. parvus* was one of the most dominant species recovered; mean abundance within copepods was 15.36% (up to 56%) in MOI and 31.91% (up to 94%) in MBC. In the case of the genus *Clausocalanus*, two species were identified with both methods (*Clausocalanus furcatus* and *Clausocalanus jobei*), while MBC could identify four more species (*Clausocalanus mastigophorus, Clausocalanus parapergens, Clausocalanus lividus, Clausocalanus paululus*), all reported to be present in the Adriatic Sea. For two copepod families, Oncaeidae and Corycaeidae, the morphological identification stops at family level. For Oncaeidae, with MBC four species could be identified. Three of them reported for the Adriatic Sea (*Oncaea mediterranea, Oncaea scottodicarloi, Oncaea venusta)* and one species, *Oncaea waldemari,* reported for the Mediterranean, but not confirmed to be present in Adriatic Sea. For Corycaeidae, only *Ditrichocorycaeus anglicus* was identified by MBC. However, the morphological identification indicates that at least 2–3 species of Corycaeidae could be present in the sample. Finally, *Pseudocalanus elongatus* was identified only with MBC, but it is known to be present in the Venice Lagoon from other studies (unpublished data). The NIS species *Pseudodiaptomus marinus* was detected by both methodologies (15 samples with both methods, 3 samples only with MBC, 1 sample only with MOI).

The mean zooplankton diversity as measured by the Shannon-Wiener Index was very similar for MBC and MOI data (2.67 ± 0.52 and 2.77 ± 0.36, respectively). The correlation between H'_{MBC} and H'_{MOI} was $R^2 = 0.441$ ($p < 0.001$) with a slope of the line is 0.465 and a mean squared distance to 1:1 correlation line of $R^2 = 0.92$ (Fig. 5C). After collapsing the molecular OTU table in order to match the morphological taxonomic resolution, the correlation between the mean of the two indices was much lower ($R^2 = 0.283$, $p < 0.01$; not shown graphically). When calculating the alpha diversity of only copepods, it resulted significantly higher with MOI data (2.25 ± 0.36) compared to MBC data (1.58 ± 0.39). Furthermore, the overall diversity and the copepod diversity were more correlated in MOI ($R^2 = 0.839$, $p < 0.001$) than in the MBC data ($R^2 = 0.533$, $p < 0.001$) (Fig. 5D).

3.2. Spatial and temporal community patterns

The relative contribution of each phylum and class to the total abundance was highly variable between locations and seasons with both methods (Fig. 6 and S5).

Both, MBC and MOI, indicated that arthropods are the dominant phylum in all six stations and over all seasons, and the abundances, sequence abundance for MBC and individual counts for MOI, were significantly correlated between the two methods (Table 3, Fig. 7A). The

Table 2
Relative abundances in percent (mean value and standard deviation) of main phyla and main classes of arthropods per station, location (sea, inlet lagoon) and per season for the molecular (MBC) and the morphological (MOI) data.

	Taxon	Tax. level	S		1		2		3		4		5		sea		inlet		lagoon		spring		summer		autumn		winter	
			mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
MBC	Arthropoda	phylum	54.8	24.1	91.7	7.9	62.3	32.5	63.0	39.2	59.2	26.1	73.9	17.0	54.8	24.1	59.2	26.1	72.7	27.2	65.4	20.7	58.9	31.1	68.4	24.0	77.2	32.7
	Hexanauplia	class	43.2	24.8	75.5	33.3	43.5	27.4	61.7	40.9	52.7	26.5	67.1	18.8	43.2	24.8	52.7	26.5	61.9	33.0	59.1	20.7	28.5	24.3	65.1	26.1	76.5	32.9
	Malacostraca	class	5.8	7.7	16.1	31.7	18.6	33.1	1.3	1.9	4.7	7.8	5.6	7.9	5.7	7.7	4.7	7.8	10.4	22.0	4.6	6.3	28.7	29.2	0.6	1.2	0.8	0.9
	Branchiopoda	class	5.9	6.4	0.1	0.2	0.2	0.0	0.1	0.1	1.8	2.4	0.7	1.4	5.9	6.4	1.8	2.4	0.2	22.0	1.7	2.1	1.8	3.7	2.4	5.3	0.01	0.0
	Cnidaria	phylum	2.0	1.8	3.7	4.5	27.3	39.1	0.6	0.9	16.8	32.2	15.5	17.2	2.0	1.8	16.8	32.2	11.8	22.1	6.7	11.9	1.0	1.3	21.6	25.0	14.6	33.5
	Echinodermata	phylum	37.1	24.9	0.0	0.0	0.3	0.4	0.5	0.7	7.5	8.1	0.4	0.7	37.1	24.9	7.5	8.1	0.3	0.5	6.4	8.8	12.9	29.1	6.5	15.2	4.8	9.0
	Chordata	phylum	1.0	1.6	1.6	3.1	5.2	22.8	26.3	30.0	2.1	3.6	6.2	7.4	1.0	1.6	2.1	3.6	9.8	17.6	10.9	18.8	16.8	20.3	0.2	0.3	0.3	0.3
	Mollusca	phylum	1.0	1.1	2.5	4.1	2.2	4.0	2.2	3.6	2.3	14.9	1.4	2.7	1.0	1.1	2.3	14.9	2.5	3.1	9.2	8.4	6.2	10.2	0.4	0.4	0.3	0.4
	Annelida	phylum	1.0	1.0	0.5	0.4	0.5	1.3	0.3	10.7	0.3	0.1	0.5	2.8	1.0	1.0	0.3	0.1	2.5	5.5	0.6	0.8	4.0	9.0	1.8	1.2	1.1	2.4
	Other phyla	phylum	3.0	4.7	2.8	0.7	2.8	0.2	2.8	0.2	0.4	1.1	0.9	0.9	3.0	4.7	0.4	1.1	0.4	0.7	0.8	0.9	0.1	0.1	1.2	0.8	1.7	4.1
MOI	Arthropoda	phylum	81.9	9.2	98.1	2.0	56.5	7.5	84.3	9.9	85.6	23.3	97.9	1.1	81.9	9.2	85.6	23.3	84.2	18.4	79.1	21.1	86.0	15.1	84.1	16.9	87.0	19.8
	Hexanauplia	class	67.7	20.9	85.8	17.4	49.9	7.5	80.8	8.6	82.2	24.0	85.0	17.9	67.7	20.9	82.2	26.9	75.4	19.7	72.9	1.4	66.7	13.3	77.5	23.0	84.0	21.1
	Malacostraca	class	0.9	1.1	10.8	19.0	5.6	3.4	2.4	3.2	1.4	1.4	5.6	9.7	0.9	1.1	1.4	19.5	6.1	10.3	3.0	1.4	11.8	15.4	0.4	0.4	2.6	1.9
	Branchiopoda	class	12.8	14.4	0.01	0.0	0.1	0.3	0.6	1.1	1.5	1.7	0.1	0.1	12.8	14.4	1.5	14.8	0.2	0.5	1.4	1.4	3.6	8.0	5.0	12.0	0.04	0.1
	Cnidaria	phylum	0.7	0.4	0.2	0.5	0.7	1.0	0.3	0.3	0.1	0.2	0.1	0.1	0.7	0.4	0.1	0.2	0.3	0.6	0.4	0.3	0.2	0.5	0.6	0.9	0.3	0.3
	Echinodermata	phylum	6.2	8.4	0.0	0.0	0.5	0.6	0.4	0.4	3.2	5.9	0.0	0.0	6.2	8.4	3.2	5.9	0.2	0.4	2.6	4.7	1.0	2.0	3.3	7.4	0.0	0.0
	Chordata	phylum	7.9	6.4	0.9	1.0	39.3	8.8	10.8	6.8	6.7	10.8	0.4	0.3	7.9	6.4	6.7	10.8	12.8	17.1	12.7	14.1	10.0	12.9	10.2	15.1	11.1	20.1
	Mollusca	phylum	1.8	1.1	0.1	0.1	1.5	1.5	1.9	2.0	3.2	5.3	0.7	0.5	1.8	1.1	3.2	5.3	1.1	1.4	3.5	4.0	1.4	1.3	0.5	0.7	0.8	1.2
	Annelida	phylum	0.5	0.3	0.3	0.5	1.3	1.2	2.0	3.5	0.3	0.4	0.4	0.4	0.5	0.3	0.3	0.4	1.0	1.8	0.8	1.1	1.5	2.9	0.8	0.3	0.2	0.4
	Other phyla	phylum	1.0	0.6	0.4	0.4	0.2	0.2	0.3	0.3	0.9	1.2	0.5	0.7	1.0	0.6	0.9	1.2	0.3	0.4	0.9	1.0	0.1	0.1	0.5	0.4	0.6	0.7

annual mean of relative abundances of arthropods per station (averaged over the 4 seasons per station) calculated for MBC was 67.4± 26.6% and 84.1± 17.4% for MOI (Fig. 6, Table 2). The relative abundance of arthropods was slightly higher, yet insignificantly, during winter with both methods compared to other seasons (Fig. 6A, Table 2). High seasonal fluctuations in relative arthropod abundance were observed in MBC, while in MOI it was less variable (Table 2, Fig. S5).

Comparing the mean seasonal abundance of the different classes of arthropods, MOI and MBC showed similar spatial and temporal patterns. Overall, the abundance of dominant classes of arthropods (Hexanauplia, Malacostraca and Branchiopoda) was highly correlated between the two methods and statistically significant (Table 3, Fig. 7B). Hexanauplia were the most abundant class within arthropods, both with MBC (83.7± 24.5% of arthropods) and MOI (89.3 ± 13.1% of arthropods). During summer, MBC showed a decrease of Hexanauplia and a high increase of Malacostraca (Table 2). MOI showed somewhat smaller seasonal fluctuations: relative abundance of Hexanauplia was 66.7 ± 13.3% and 78.1 ± 21.4% and of Malacostraca 11.8 ± 15.4% and 2.0 ± 2.4% in the summer and other seasons respectively (Table 3, Fig. 6 and S5). In contrast to MOI, MBC showed significantly higher abundances in Malacostraca during spring-summer (Table 3).

The molecular analysis resulted in a dominance of cnidarians in some samples (lagoon and inlet station), in contrast to the MOI data, where the abundance of cnidarians was overall very low (Fig. 6, Table 2). However, in MOI, the relative abundance was significantly higher during summer. Indeed, the abundance of cnidarians did not result to be correlated between the two methods (Table 3, Fig. 7A). Echinoderms were more abundant in the station located in the sea (st. S) than and the lagoon (st. 1, 2, 3, 4, 5), but the significance for MOI was weak (Fig. 6A and B, Tables 2 and 3). While in the MBC dataset echinoderms were present in all seasons, in the MOI dataset they were not present during winter (Table 2). Nonetheless, their abundance was significantly correlated between both methods (Table 3, Fig. 7A). Chordates were mostly present in the lagoon stations in spring and summer in the MBC data (Fig. 6A, Table 2), and mainly composed by Actinopterygii (76.6 ± 36.4% of chordates), while with MOI, they were mostly composed by appendicularians (55.6 ± 44.5% of chordates; Table 2). In fact, chordate abundances were not correlated between both methods (Table 3, Fig. 7A). Although appendicularians were a well-represented class in the MOI data, MBC was not able to detect this class. The relative abundance of molluscs in the two methods was significantly correlated and higher in spring-summer according to both methods (Tables 2 and 3, Fig. 7A). Also for annelids, the two methods resulted to be correlated and showed the highest relative abundance in the same sample, st. 3 during summer, with 7.4% in MOI and 22.3% in MBC (Table 3, Fig. 7A).

The number of sequences and the abundance counts based on morphological taxonomic identifications of selected species that are present in both datasets (19 copepods, 1 cladoceran, 1 fish species), show a significant correlation (Fig. 7C and S6), which is especially high for the most dominant of these 21 species: *A. tonsa* ($R = 0.84$, $p < 0.01$), *A. clausi* ($R = 0.8$, $p < 0.01$), *A. margalefi* ($R = 0.67$, $p < 0.01$), *C. ponticus* ($R = 0.8$, $p < 0.01$), *P. marinus* ($R = 0.89$, $p < 0.01$), *P. parvus* ($R = 0.51$, $p < 0.05$), *Temora stylifera* ($R = 0.75$, $p < 0.01$), *P. avirostris* ($R = 0.97$, $p < 0.01$), *Engraulis sp.* ($R = 0.55$, $p < 0.01$).

The beta diversity visualised with non-metric multidimensional scaling plots (Fig. 8) showed that the sample communities of MBC were clearly separated by season (PERMANOVA: Pseudo-F = 1.753, P(perm) 0.001), but not by stations (nested within location) or by locations (Table 4). However, a separation of the categories, sea, inlet, and lagoon was evident. The dissimilarities between lagoon (excluding the inlet st. 4) and sea stations were the largest (90.83%), followed by lagoon-inlet with 86.19% and sea-inlet (82.18%) (SIMPER on-way analysis based on Bray-Curtis similarities). Furthermore, this pattern was consistent with the ordination plot based on abundance counts (MOI), showing a separation between seasons (PERMANOVA: Pseudo-F = 2.205, P(perm) 0.001), but also location (PERMANOVA: Pseudo-F = 1.851, P(perm)

Table 3
Differences, assessed with the non-parametric Kruskal-Wallis-Test on molecular (MBC) and morphological (MOI) data, between relative abundances (square rooted data in percents) of the most abundant phyla and the most abundant classes of arthropods over seasons and locations (lagoon (st. 1, 2, 3, 4) and sea (st. S)) and in specific cases, additional test were perfomed. Correlations between the two methods were assessed with Pearson correlation coefficient. * p-values lower than 0.05 are highlighted in red.

taxa	method	location (df=1)		season (df=3)		additional test (df=1)			Pearson correlation between methods (df=22)		
		χ^2	*p*	χ^2	*p*	χ^2	*p*	*test*	*r*	*t*	*p*
Arthropoda	MBC	2.646	0.104	1.58	0.664	-	-	-	0.538	2.994	0.007
	MOI	1.015	0.314	0.89	0.848	-	-	-			
Cnidaria	MBC	0.096	0.757	6.77	0.08	3.24	0.072	summer vs other seasons	0.223	1.074	0.294
	MOI	1.8	0.18	4.84	0.184	4.578	0.032	summer vs other seasons			
Echinodermata	MBC	9.617	0.002	0.65	0.885	-	-	-	0.680	4.350	<0.001
	MOI	3.787	0.052	5.57	0.135	-	-	-			
Chordata	MBC	0.6	0.439	10	0.019	7.524	0.006	spring/summer vs autumn/winter	0.117	0.553	0.586
	MOI	0.096	0.757	0.1	0.992	0.0033	0.954	spring/summer vs autumn/winter			
Mollusca	MBC	0.054	0.816	12.8	0.005	12	0.001	spring/summer vs autumn/winter	0.628	3.783	0.001
	MOI	1.944	0.163	5.53	0.137	5.333	0.021	spring/summer vs autumn/winter			
Annelida	MBC	0.024	0.877	4.05	0.256	-	-	-	0.755	5.399	<0.001
	MOI	0.096	0.757	5.67	0.129	-	-	-			
Hexanauplia	MBC	3.174	0.075	8.06	0.045	6.084	0.014	summer vs other seasons	0.560	3.169	0.005
	MOI	0.486	0.486	5.42	0.144	3.24	0.072	summer vs other seasons			
Branchiopoda	MBC	3.462	0.063	6.23	0.101	3.419	0.064	spring/summer vs autumn/winter	0.752	5.350	<0.001
	MOI	2.988	0.084	6.29	0.098	3.341	0.068	spring/summer vs autumn/winter			
Malacostraca	MBC	0.096	0.757	15.1	0.002	9.818	0.002	summer vs other seasons	0.943	13.253	<0.001
	MOI	5.4	0.02	7.45	0.059	2.155	0.143	summer vs other seasons			

** p-values lower than 0.05 are highlighted in red*

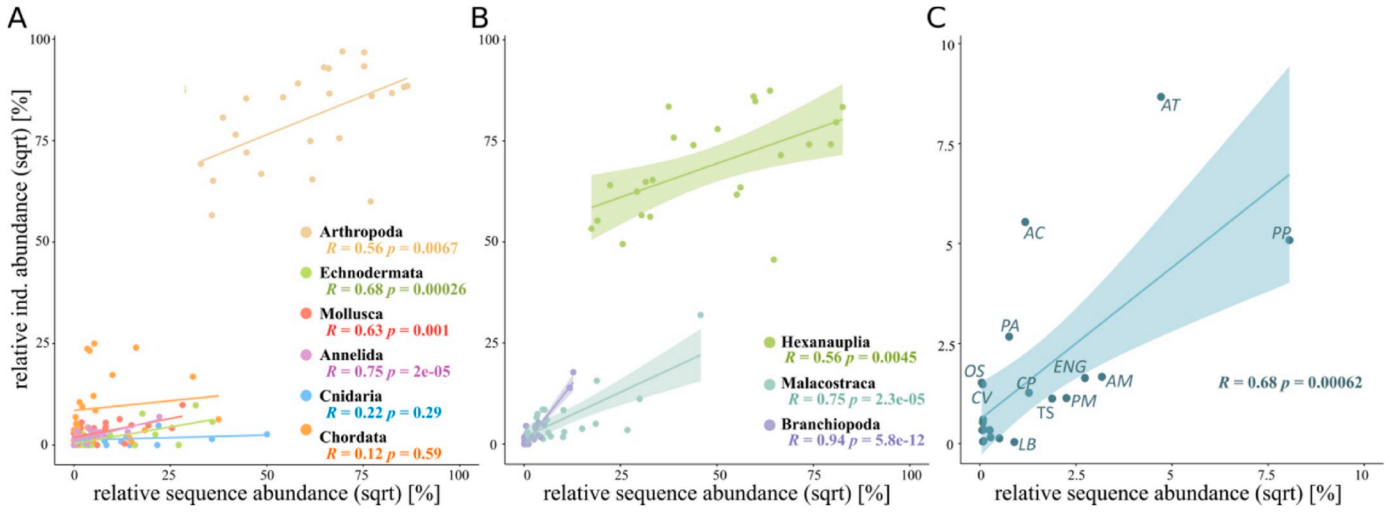

Fig. 7. Relative abundance of reads for molecular data (MBC) and of individual counts for morphological data (MOI) (% based on square rooted data) of (A) most abundant phyla; (B) most abundant classes of Arthropoda (Hexanauplia, Malacostraca, Branchiopoda) and (C) 21 selected species present in both datasets (AT: *Acartia tonsa*, AC: *Acartia clausi*, PP: *Paracalanus parvus*, PA: *Penilia avirostris*, Eng: *Engraulis sp.*, OS: *Oithona similis*, CV: *Ctenocalanus vanus*, CP: *Centropages ponticus*, TS: *Temora stylifera*, PM: *Pseudodiaptomus marinus*, LB: *Labidocera brunescens*; species with lowest abundances are not labelled (*Centropages typicus*, *Clausocalanus jobei*, *Oithona plumifera*, *Nannocalanus minor*, *Paracartia latisetosa*, *Temora longicornis*, *Clausocalanus furcatus*, *Diaixis sp.*). Pearson correlations between the two methods are given in the corresponding colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.013) (Table 4). In fact, both similarity matrices, for MBC and MOI, were correlated with the environmental data (PRIMER RELATE - MBC: Spearman's rho = 0.494, p = 0.001; MOI: Spearman's rho = 0.308, p = 0.003). The dissimilarities of the MOI dataset were around 17% lower compared to the MBC data, with dissimilarities of 76.8% for lagoon-sea, followed by lagoon-inlet with 69.25% and sea-inlet (62.48%) (SIMPER on-way analysis based on Bray-Curtis similarities). The similarity between the two distance matrices (Bray-Curtis) could be confirmed as they were significantly positively correlated (Mantel statistic based on Spearman's rank correlation rho = 0.611, p = 0.001).

Even though the stations were not significantly different, a differentiation following a salinity gradient was evident. Especially in the MBC ordination plot, the stations 2 and 3 were more similar to the sea station and inlet station, as they were under higher marine influence being located in one of the main traffic channels for industrial transport with elevated depths compared to the two inner stations (st. 1 and 5). The MOI ordination plot did not show such a clear discrimination (Fig. 8). The combination of environmental parameters that was best explaining the community composition was salinity, temperature and Chlorophyll-*a* for the MBC data (Spearman's rho = 0.625) and salinity and temperature for MOI data (Spearman's rho = 0.495) (PRIMER BEST).

The temporal changes of relative zooplankton composition differed between MBC and MOl data. In the morphological data, most groups

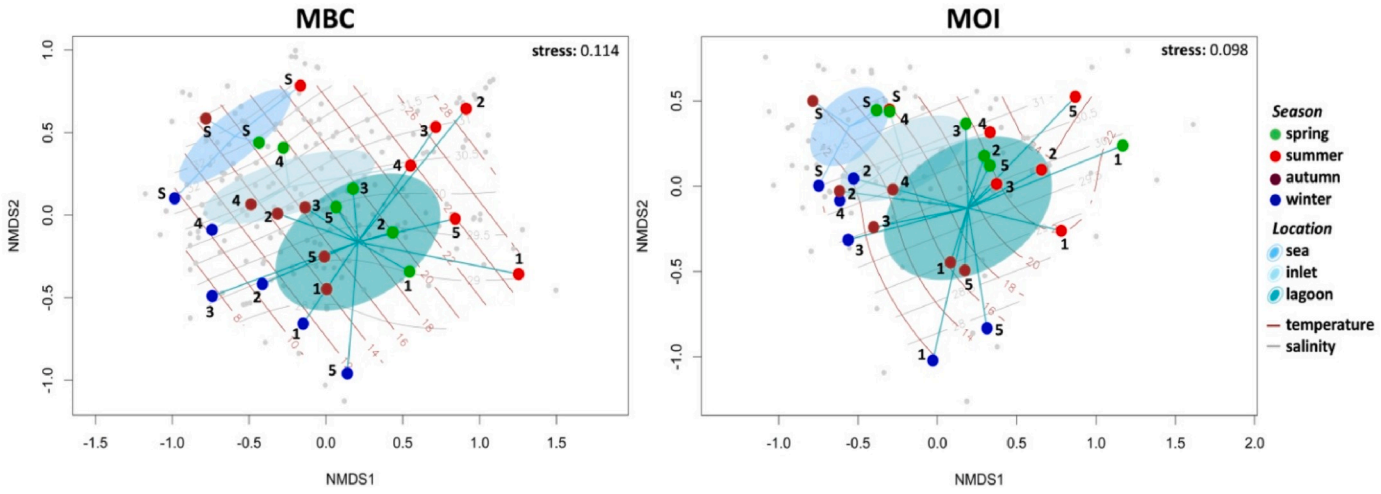

Fig. 8. Beta diversity estimates based on Bray-Curtis similarities plotted on NMDS plots based on molecular (MBC) and morphological (MOI) data, respectively. Colours of points refer to the sampling season of each sample. The three locations (sea, inlet, lagoon) are highlighted plotting the distance to their centroid and the standard deviations of the points per location with the respective colours. Salinity and temperature are superimposed (brown and grey contour lines) on the NMDS plots according to the CTD measurements during sampling. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
PERMANOVA comparing community composition based on the molecular approach (MBC) and the morphological approach (MOI) with season and location as fixed factors, and station nested within location. Prior to analysis data were square-root transformed and normalized using Wisconsin double standardization. * p-values lower than 0.05 are highlighted in red

	MBC (sqrt wis)				MOI (sqrt wis)			
Source	df	MS	Pseudo-F	P(perm)	df	MS	Pseudo-F	P(perm)
season	3	5348.2	1.753	**0.001**	3	3631.4	2.205	**0.001**
location	2	5159.7	1.458	0.145	2	4520.9	1.483	0.209
station(location)	3	3539.8	1.160	0.144	3	3048.4	1.851	**0.013**
Res	9	305.1			9	1647		

(typically meroplanktonic assemblages) presented an evident peak of abundance in the summer samples, over 85% for decapods, Actinopterygii and polychaetes and for molluscs almost 70% of their total abundance (Fig. 9B). Differently, the other groups showed smoother fluctuations in relative abundance; high relative abundances were found also in spring (Mollusca 70%, Actinopterygii 50%, Copepoda 35%) and in autumn (Cnidaria 37%, Copepoda 25%) and winter (Cnidaria 50%, Copepoda 28%) (Fig. 9A). MBC data confirmed the summer peak found in MOI of decapod and polychaete abundance, while copepods and molluscs showed during summer their highest abundances in the MOI data and its lowest abundance in the MBC data (Fig. 9A and B, Table S2).

The analysis of the three zooplanktonic groups, holo-, mero- and ichthyoplankton, showed a high peak in relative abundance during summer with MOI (close to 90%) (Fig. 9D, Table S2). Also MBC revealed mostly higher relative abundances of these groups in summer, but much less prominent (57% and 46%, respectively). In contrast, the holoplanktonic component showed an antagonistic seasonal oscillation comparing both methods, following the abundance of copepods (Fig. 9C and D, Table S2).

4. Discussion

This study demonstrates that COI metabarcoding can be successfully applied to follow zooplankton biodiversity in such complex and seasonally changing environments as transitional waters. In this study, the effectiveness of DNA metabarcoding was confirmed on three levels. First, this approach revealed a substantial level of often overlooked diversity of zooplankton, mostly due its ability in detecting the diversity of mero- and ichthyoplankton. Second, the ecological analysis revealed that DNA metabarcoding approach gives similar spatio-temporal patterns as the morphological approach. Third, our study revealed highly significant positive correlations between total abundance counts from morphological taxonomic identification and metabarcoding sequence number for all species recorded by both approaches.

4.1. Molecular diversity and methodological concerns

In this study, MBC was able to detect more taxa than MOI. The lower species richness in the MOI dataset was largely due to the difficulty of morphological identification of several taxa during larval stages (e.g. the larvae of decapods and molluscs, fish eggs) and to the lack of specific taxonomic expertise for some zooplankton groups; by contrast, the molecular method was able to detect sequences from cryptic early life stages (Djurhuus et al., 2018; Lindeque et al., 2013; Zaiko et al., 2015). Therefore, in MBC, a large proportion of the resulted species richness was composed by meroplankton (69%, excluding cnidarians) and ichthyoplankton (9%), while in MOI holoplankton (80%) was the dominant group (mainly copepods and cladocerans). In fact, the ability of metabarcoding to identify mero- and ichthyoplankton enables to study e.g. their spatial and temporal pattern and larval dispersion e.g. of bivalves or fishes of economic interest (e.g. the three bivalves *Mytilus galloprovincialis*, *Ruditapes philippinarum, Chamelea gallina* or the fishes *Engraulis encrasiocolus*, *Atherina boyeri* and *Zosterisessor ophiocephalus* that were identified with MBC).

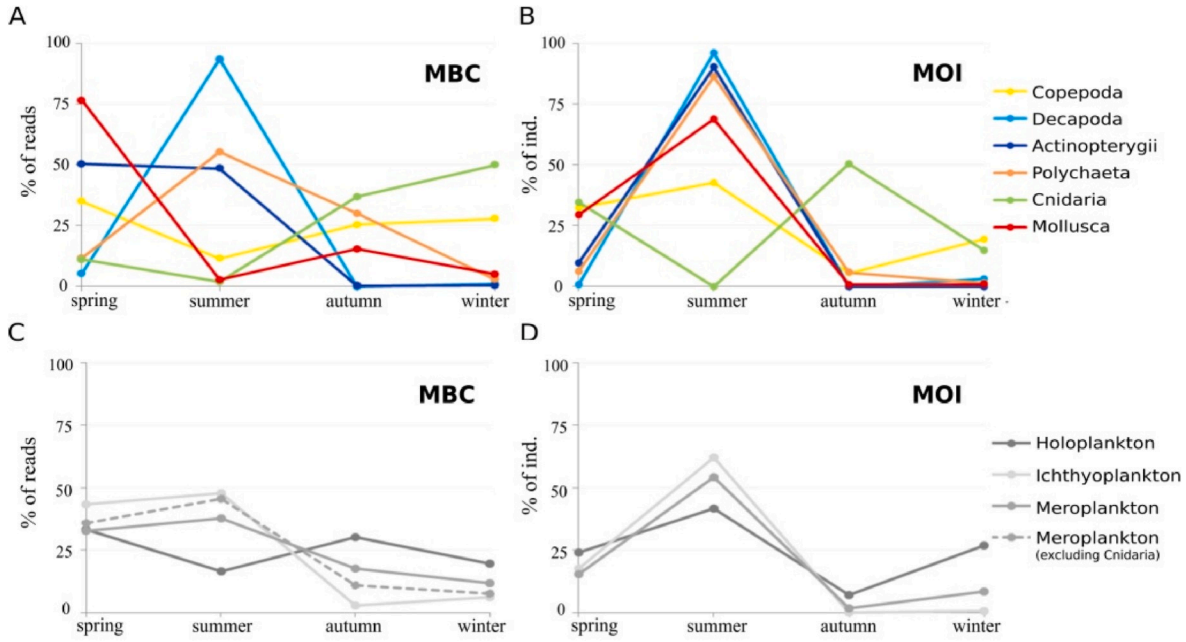

Fig. 9. Relative abundance within specific taxa with A) DNA metabarcoding (MBC) and B) morphological identification (MOI); and of zooplankton groups with C) MBC and D) MOI divided by seasons calculating the fluctuation in abundances for each taxon along the year.

The "taxonomic bias" was especially evident when comparing the contribution of copepod diversity to the overall diversity estimated with the MBC and MOI approach. With MBC, more copepod species overall (41) were detected, but they accounted for only 22% of all recovered taxa at species level; while the 35 copepod species recovered with MOI represent as much as 88% of all recovered taxa at species level. Furthermore, this bias was also observed when comparing the Shannon-Wiener Index based on only copepod diversity and overall diversity, as they were highly correlated and differed only slightly in the MOI dataset and were less correlated and considerably different according to the MBC dataset. Nonetheless the above-mentioned differences, both methods show that copepods dominate the zooplankton community and *Paracalanus* and *Acartia* are the most abundant genera in this study. This is in compliance with the finding by Bucklin et al. (2019) that metabarcoding analysis aligns with the morphological one. Even though only 31% of the detected copepod species were shared by both methods, those taxa comprise 98.5% of all copepod sequences obtained with MBC. For some species, within the holoplanktonic copepods, the morphological identification of juveniles (nauplii and copepodites (C1–C4)) is not always possible at species level, e.g. for the highly abundant genera *Acartia* and *Clausocalanus*. Differently, MBC offers the detection and relative abundance including also the juveniles. However, it cannot distinguish between life stages. In our particular study, this may explain for example the presence of *Oithona davisae* in the MBC data, without the presence in MOI data, as the individuals could have been larval stages and therefore identified as copepod nauplii indet. Moreover, for some species, like *C. helgolandicus*, *C. euxinus* and the *P. parvus* complex, where the species status in not ultimately clarified (Kasapidis et al., 2018; Unal et al., 2006), MBC could give us new insights into the complexity of species discrimination. *Calanus helgolandicus* and *C. euxinus* are morphologically and genetically very similar, and therefore Unal et al. (2006) raised doubt about the species status of *C. euxinus* proposing that it may be a Black Sea population of *C. helgolandicus*. For the *P. parvus* species complex, Kasapidis et al. (2018) indicated that the morphological taxonomic characters are not adequate to discriminate between these species. This may have led to an inaccurate morphological identification, but at the same time the deposited sequences on NCBI may be misidentifications. This would explain why *P. parvus* is the dominant species within this complex, even though the dominant species in the Northern Adriatic may be *P. quasimodo* unlike previously thought (Kasapidis et al., 2018).

Also deficient preservation of specific groups, like some cnidarians, can limit their identification with morphological analysis, in addition to the missing expertise regarding specific groups (Zaiko et al., 2015). Comparing the taxonomic resolution of Cnidaria for example, MBC was able to detect 29 taxa belonging to this phylum while MOI indentified only four groups (Cnidaria indet., Hydrozoa indet., Scyphozoa indet. and Siphonophorae indet.). Nevertheless, it has to be taken into account that the detection of taxa only by MBC could also result from sequences derived of sloughed cells or faecal material (including organic material from adult benthic organisms) (Berry et al., 2019). Moreover, in contrast to morphological data, MBC analyses can sometimes fail or the sequencing depth be too low and therefore, the obtained species richness and relative abundances are less reliable. In this study, three samples resulted in minor sequencing depth, but they have been kept even though probably under-sampled to not interrupt the time series.

Thanks to the bioinformatic multilevel approach used in this study, the taxonomic assignment could be improved. For example, in this way, the abundant copepod *A. margalefi* would have been recovered as "best match <94%" from "de-novo recovery" even without the new local reference barcode. This also improves the alpha and beta diversity estimations, as in this way the diversity estimations are based also on putative metazoans, OTUs that were not assignments, but only "best matches". While adding as much information as possible, this approach is still cautious enough, when it comes to taxonomic considerations as it considers the 94%-only species as cf., and the blast hits only as best-match and not as a proper taxonomic identification.

MBC has the capability of identifying the taxa at lower taxonomic levels (188 vs. 40 OTUS at species level). However, identifications by MBC to species level should be interpreted carefully as the quality of the reference database is one of the most impacting aspects regarding the reliability of this method. In this study, missing reference sequences made it impossible to identify some taxa observed by microscopy, as for example eight copepod species identified only by MOI were not assigned by MBC as no species reference sequence was present on NCBI. For six of them only reference sequences of other species of the same genus were present on NBCI (*C. giesbrechti, C. kröyeri, D. pygmaea, O. setigera* and *O. tenuis, and L. wollastoni*), while for two of them not even the genus

(*Clytemnestra, Microsetella)*. In this study, three species, *C. ponticus*, *L. brunescens* and *A. margalefi* could only be identified with MBC after local barcodes were added to the reference database ("local-barcodes recovery") (except from *A. margalefi* that would have been also identified at 90% in the "best match <94% recovery"), highlighting again the rising need to improve and adjust to the own needs the reference database.

Missing reference sequences of closely related species could also mislead us to consider an erroneous hit at species level, which might generate a genus level assignment if closely related species would hit at the same similarity threshold. This was probably the case for the copepod *D. pygmaea*. As Stefanni et al. (2018) already mentioned, this species is missing in the NCBI reference database, but confirmed for the Adriatic and present in the MOI dataset. However, as a reference sequence of *Diaixis hibernica* was available, in the MBC approach, the sequences have been erroneously assigned to later. And it might also be the case for the assignments of the echinoderm *Psammechinus miliaris* (found by MBC), which is the only species of that genus present on the reference database, while it could also have been *Psammechinus microtuberculatus*. This highlights that metabarcoding requires taxonomically complete and geographically comprehensive reference databases (Bucklin et al., 2016). In fact, reference databases are often not representative of all taxonomic groups (Ardura et al., 2013; Ratnasingham and Hebert, 2013; Zaiko et al., 2015) resulting in possibly biased or hindered taxonomic assignments. In order to maximize phylogenetic representativeness and to provide an interim proximate taxonomic assignment, Weigand et al. (2019) proposed to fill the gaps producing reference barcodes of representative species first from missing orders, then missing families, and so forth down to genera in order to guarantee a broad taxonomic representation.

Apart from the missing assignments due to absent reference sequences, nine copepod species were not detected by MBC even though reference sequences were available (*Mesocalanus tenuicornis*, *Isias clavipes*, *Calocalanus styliremis*, *Calocalanus pavo*, *Calanipeda aquaedulcis*, *O. nana*, *C. helgolandicus*, *M. rosea* and *Goniopsyllus rostratus*), maybe due to high intraspecific variability and the fact that in most cases the reference sequences belong to specimen form the Atlantic or the Pacific Ocean or due to low abundances in the sample. However, two of them were not identified by MBC even though highly abundant (1124.3 ind/m^3 for *O. nana* and 499.1 ind/m^3 for *C. styliremis*) and other two, *C. pavo* and *G. rostratus,* have been correctly assigned in another station nearby st. S in the sea (not part of this study), but not in the samples presented in this study. However, these two species showed low abundance also in the MOI data (0.52 ind/m^3 and 4.95 ind/m^3, respectively).

It is also essential that reference specimens are correctly identified as inaccurate identifications (including identification errors and sequence contaminations) remain a persistent impediment to the reliable use of metabarcoding for analysis of species-level zooplankton biodiversity (Bucklin et al., 2016). In some databases, including NCBI, the submission of sequences does not require to prove species identification. Therefore, special care must be taken with interpretation of the results when detecting rare or unexpected species (Djurhuus et al., 2018).

In fact, when detecting a potential NIS with DNA metabarcoding, the reference sequence should be verified, as it could be a result of errors in the reference database. In this study, this was the case for example for the bony fish belonging to the family of Gobiidae, *Proterorhinus semilunaris*, a well-known highly invasive species, but not yet recorded in Venice Lagoon and adjacent coastal waters. More than 10,000 sequences were assigned to a reference sequence associated to this species. Investigating on that reference sequence (ID: EU444673), this sequence resulted to be more similar to sequences of the family of Blenniidae than to other Gobiidae. In fact, it has as second best-match the Blenniidae *Salaria pavo,* a non-NIS fish species often recorded in Venice Lagoon. This is another example of the importance of a reliable reference database, especially when investigating on non-indigenous species. However, in this case, the risk of misidentification by MBC might have been due to the choice of the marker, as COI is probably not the best marker for the assessment of fish (ichthyoplankton) diversity as it does not offer sufficient resolution, while e.g. CytB or the ribosomal markers 12S and 16S are probably more reliable (Evans et al., 2016; Hänfling et al., 2016; Vences et al., 2016).

In fact, the choice of a specific barcode will alter the results in biodiversity (Clarke et al., 2017; Piñol et al., 2019). A barcoding primer pair, which amplifies a marker sequence of short length for HTS for as many target taxa in the samples as possible, is the most critical component for successful assessments of bulk samples with DNA metabarcoding. However, finding appropriate primers for marine zooplankton assessment is difficult as most of them are prone to severe primer biases that prevent the detection of all taxa from the sample and limit the precise quantification of taxon biomass and/or abundances. Such primer biases might be even more common in the case of marine zooplankton as this group is composed by animals from almost all phyla. To describe the diversity of mixed zooplankton assemblages using metabarcoding different marker gene regions were used in the past. Frequently used gene regions to characterize zooplankton biodiversity patterns across different systematic levels are: 18S rRNA (Chain et al., 2016; De Vargas et al., 2015; Hirai et al., 2015; Lindeque et al., 2013; Pearman et al., 2014), 28S rRNA (Hirai et al., 2014, 2013), the mitochondrial genes 16S rRNA (Goetze, 2010; Lindeque et al., 2006, 1999) and COI (Bourlat et al., 2013; Bucklin et al., 2010b, 2010a; Carroll et al., 2019; Machida et al., 2009; Stefanni et al., 2018; Zaiko et al., 2015). Indeed, several studies used a multi-marker approach for accurate species identification and discrimination, including the usage of group-specific primers (e.g. Bucklin et al., 2010b) in order to reduce the bias resulting from differing amplification success between different taxonomic groups. The 18S V9 region is usually the marker of choice in DNA metabarcoding studies (Bucklin et al., 2019; Stefanni et al., 2018) of marine zooplankton as this hypervariable region is flanked by highly conserved sections, meaning it has a very broad amplification range (Amaral-Zettler et al., 2009; Medlin et al., 1988) and can be considered as a "truly" universal marker for eukaryotes. Nevertheless, a very big draw of using this region for DNA metabarcoding is its low taxonomic resolution allowing family level identification at best. Therefore, more and more DNA metabarcoding studies of marine zooplankton are also relying on the COI marker, which shows great taxonomic resolution, but with a drawback of reduced amplification success. This may explain the number of copepod species that could not be identified despite the presence of reference sequences. Its limitations in quantification power, however, have so far not been evaluated thoroughly.

4.2. Ecological evaluation of morphological taxonomic identification and DNA metabarcoding

The analysis of alpha diversity measured by the Shannon-Wiener Index gave similar results for MBC and MOI that were significantly correlated, similarly as reported by Bucklin et al. (2019) for 18S (V9). Collapsing the molecular OTU table in order to match the morphological taxonomic resolution, as proposed by Cahill et al. (2018), did not increase the correlation between the two methods regarding the alpha diversity. This is probably due to a compensation of different taxonomic groups in their contribution to biodiversity. In MBC, the contribution to the diversity resulted to be more equally shared by different groups than in MOI, coherently to the above discussed results concerning the effectiveness of MBC in detecting meroplankton and ichthyoplankton. In particular, the major contribution of the copepod diversity to the overall diversity in MOI, was a result of the minor proportion of mero- and ichthyoplankton compared to the holoplanktonic copepods.

According to Bucklin et al. (2019) for 18S (V9), stating a distinction among geographic regions, both the metabarcoding data and the morphological abundance counts revealed an evidence of variation among the three locations and between seasons based on NMDS analysis. As stated also by Harvey et al. (2017), in this study both methods

show a similar spatio-temporal pattern, showing a separation by seasons in the NMDS analysis, following a gradient in temperature. The seasonality is slightly clearer with MBC, due to its capability to better detect the seasonal presence of e.g. the decapods larvae peak during summer and the continuous decrease of molluscs larvae from spring to autumn/winter. The spatial pattern shows a noticeable differentiation along the sea-lagoon gradient (sea, inlet, lagoon) with both methods following a salinity gradient typical for transitional waters even if not statistically significant due to the high variability within the lagoon (Bianchi et al., 2004; Camatti et al., 2008; Solidoro et al., 2010). Also within the lagoon the variability of zooplankton community composition follows the salinity gradient, from sites with the higher marine influence sites (st. 2 and 3) to the inner sites (st. 1 and 5). However, for both biodiversity measures it has to be taken into account that the MBC data is based on number of reads as a proxy of biomass (Harvey et al., 2017; Lindeque et al., 2013), while MOI is based on individual counts. Therefore, as for example some taxa may be larger in size (e.g. crustacean larvae) compared to others (e.g. small copepods), its proportion in the MBC data might result greater compared to the individual based morphological data. This could result in different dominance of taxa and, mostly, in different species evenness.

Correlation between sequence data and species abundance has been the focus of a number of studies (Hirai et al., 2014; Lindeque et al., 2013; Mohrbeck et al., 2015). In general, low associations between abundance or biomass and read number have been obtained (Evans et al., 2016; Harvey et al., 2017). But, similarly to the findings of Bucklin et al. (2019) for 18S (V9), where abundance counts were significantly correlated for Gastropoda, Calanoida and Chaetognatha, in this study, the COI marker was also shown to be very promising when it comes to the quantification of important taxonomic groups and a variety of taxa. The numbers of sequences and abundance counts based on morphological taxonomic identifications were significantly correlated for selected species (present in both datasets), for most abundant classes of arthropods and for most phyla, except from two, cnidarians (which seem to be overestimated by MBC) and chordates, which are composed mostly by fish sequences in MBC and by appendicularians in MOI. As mentioned above, it has to be taken into account that the number reads are supposed to better correlate to the biomass than to the number of counts, as for example copepod nauplii are significantly smaller than adults and also as the sizes between copepod species do differ. In fact, for example the copepod *L. brunescens*, relatively large in size, results to be overestimated with MBC in comparison to smaller species. A reliable estimation of biomass or abundance data is still a critical issue which is a fundamental aspect in the suitability of MBC in the framework of biodiversity assessment related to water management.

4.3. NIS detection

The Venice Lagoon is a hotspot of introduction of NIS (e.g. Tagliapietra et al., 2012; Vidjak et al., 2018; Wolf et al., 2018), as it is a transitional water body with high anthropogenic activities (Occhipinti Ambrogi, 2000). Combined with special local environmental conditions, the Venice Lagoon becomes a highly "invadable" site (Camatti et al., 2019; Marchini et al., 2015). Several studies based on MBC successfully detected NIS, both from bulk samples (e.g. Darling et al., 2018; Flynn et al., 2015; Stefanni et al., 2018) and from eDNA (Comtet et al., 2015; Zaiko et al., 2015). In this survey, metabarcoding was able to reveal the possible presence of several NIS, among others *Paranais frici*, *Polydora cornuta*, *A. tonsa*, *O. davisae*, *P. marinus*, *Paracaprella pusilla*, *Palaemon macrodactylus, Dyspanopeus sayi*, *Tiaropsis multicirrata*, *Mnemiopsis leidyi*, *Ostrea stentina, Arcuatula senhousia*). With MOI, only two non-indigenous copepod species, *P. marinus* and *A. tonsa,* have been detected in this study, and vice versa no NIS has been detected only with MOI. However, even though missing in the MOI data in this study, the non-indigenous copepod *O. davisae* has been regularly reported also with MOI in other studies in the Venice Lagoon (Vidjak et al., 2018; unpublished data). These findings confirm that metabarcoding is a promising alternative to traditional methods for early detection of NIS (Abad et al., 2016) and might be a useful tool when assessing and predicting the secondary spread and the effect on recipient communities and for assessing the environmental status within the Marine Strategy Framework Directive (MSFD) (Lehtiniemi et al., 2015).

5. Conclusion

Safeguarding the biodiversity of coastal and transitional waters is an environmental priority and a main objective of European legislation frameworks (Water Framework Directive, WFD, 2000/60/EC; Marine Strategy Framework Directive, MSFD, 2008/56/EC). Metabarcoding became a promising tool for biodiversity assessment, but protocol biases and issues regarding the reference database reduce its reliability. In this study, an innovative bioinformatic pipeline has been applied. In addition to the improvement of the quality of the reference database, solving technical issues, especially regarding the improvement of estimation of biomass or species abundances, should be one of the main future objectives in this field e.g. creating calibration curves for different seasons and different stations.

Both techniques are highly informative, but the comparison highlights that both methods give information of different nature and rather than alternative they should be considered as complementary, as proposed by Bucklin et al. (2016). However, the method of choice may depend on the objectives of the study: While MOI, identifying developmental state and sex, enables the analyses of population structure, MBC offers the possibility of high spatial and temporal coverage, higher taxonomic resolution and broader taxonomic coverage (Bik et al., 2012; Brannock et al., 2014; Hajibabaei et al., 2007; Harvey et al., 2017; Stefanni et al., 2018), which is particularly useful when studying e.g. invasive species, the ecology of larval dispersion or where the high spatio-temporal coverage is preferred over the information on population structure.

Declaration of competing interest

None.

CRediT authorship contribution statement

Anna Schroeder: Data curation, Visualization, Investigation, Formal analysis, Writing - original draft. **David Stanković:** Formal analysis, Data curation, Methodology, Writing - review & editing. **Alberto Pallavicini:** Conceptualization, Supervision, Data curation, Funding acquisition, Writing - review & editing. **Fabrizia Gionechetti:** Formal analysis. **Marco Pansera:** Data curation, Formal analysis, Investigation. **Elisa Camatti:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing - review & editing.

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