Title: **Global mesozooplankton communities show lower connectivity in deep oceanic layers**

Running title*:* **Low deep-sea mesozooplankton connectivity**

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Abstract

Mesozooplankton is a key component of the ocean, regulating global processes such as the carbon pump, and ensuring energy transfer from lower to higher trophic levels. Yet, despite the importance of understanding mesozooplankton diversity, distribution and connectivity at global scale to predict the impact of climate change in marine ecosystems, there is still fragmented knowledge. To fill this gap, we applied DNA metabarcoding to mesozooplankton samples collected during the Malaspina-2010 circumnavigation expedition across temperate and tropical oceans from the surface to bathypelagic depths. By conducting a hidden diversity analysis, we highlight the still scarce knowledge on global mesozooplankton diversity and identify the Indian Ocean and the deep sea as the most understudied areas. By analysing mesozooplankton community spatial distribution, we confirm global biogeographical patterns across the temperate to tropical oceans both in the vertical and horizontal gradients. Additionally, we reveal a consistent increase in mesozooplankton beta-diversity with depth, indicating reduced connectivity at deeper layers, and identify a water mass type-mediated structuring of bathypelagic communities, instead of an oceanic basin-mediated as observed at upper layers. This suggests limited dispersal at deep ocean layers, most likely due to weaker currents and lower mixing of water mass types. Overall, our work supports the neutral theory of biodiversity and thus the importance of oceanic currents and barriers in dispersal in shaping global plankton communities, and provides key knowledge for predicting the impact of climate change in the deep-sea.

Keywords: deep ocean, zooplankton, connectivity, dispersal, oceanic currents, environmental selection.

Introduction

Marine mesozooplankton is comprised of a wide range of functionally, phylogenetically, and morphologically diverse organisms whose size range from 0.2 to 20 mm (Bucklin et al., 2021; Steinberg & Landry, 2017) and which include some of the most abundant animals on Earth, such as copepods or euphausiids (Turner, 2004). Mesozooplankton taxa are key components of marine ecosystems, ensuring energy transfer from lower to higher trophic levels (Zeldis & Décima, 2020) as main predators of producers and primary consumers (Calbet, 2001) and as main food source of a number of organisms, including many relevant commercial fish species (Hays, Richardson, & Robinson, 2005; Turner, 2004). Additionally, many mesozooplankton species perform diel vertical migrations, significantly contributing to trophic connectivity, and are deeply involved in global biogeochemical cycles such as the biological carbon pump through recycling sinking organic matter and enhancing carbon sequestration in the deep ocean (Bode, Koppelmann, Teuber, Hagen, & Auel, 2018; Hernández-León et al., 2020; Kelly et al., 2019; Liszka, Manno, Stowasser, Robinson, & Tarling, 2019; Steinberg & Landry, 2017; X. Zhang & Dam, 1997). Specific plankton community composition and trophic networks have been related to the local intensity of the carbon pump (Ducklow, Steinberg, & Buesseler, 2001; Guidi et al., 2016) and thus increasing knowledge on plankton diversity and on how they are spatially distributed and connected along the horizontal and vertical ocean gradients is essential to monitor and predict the impacts derived from climate change or other anthropogenic perturbations (Chiba et al., 2018; Hays et al., 2005; Ratnarajah et al., 2023). Yet, mesozooplankton

63 diversity still has far to go to be fully described (Bucklin et al., 2021), as well as its global
64 structuring and the factors shaping it along the ocean.

65 Global structuring of planktonic groups in the ocean is assumed to be determined by the
66 interaction between dispersal, speciation, drift, and selection (Vellend, 2010). Dispersal
67 refers to organismal transport, and tends to homogenise community composition among
68 sites (i.e., to decrease beta-diversity; see Whittaker (1960)) (Soininen, Lennon, & Hillebrand,
69 2007). Speciation refers to the appearance of new variants and ultimately species and,
70 contrarily to dispersal, contributes to increasing beta-diversity between sites (Casteleyn et
71 al., 2010). On the other hand, drift and selection act at the alpha-diversity level, shaping the
72 relative abundance of the different species in a community and defining the local diversity
73 (Gilbert & Levine, 2017; Hellweger, van Sebille, & Fredrick, 2014). The interaction between
74 these processes typically results in a distance-decay pattern between biological
75 communities, which is represented by an increase in beta-diversity with increasing
76 geographical distance (Nekola & White, 1999). Distance-decay patterns have been reported
77 from microbes to larger plankton (Cermeño, de Vargas, Abrantes, & Falkowski, 2010; Chust,
78 Irigoien, Chave, & Harris, 2013; Villarino et al., 2022; Villarino et al., 2018). Beta-diversity
79 measurements are also a proxy of connectivity between communities (i.e., the rate of
80 migration of individuals and species between two communities) (Giner et al., 2020; Villarino
81 et al., 2018), so that the higher the beta-diversity the less connected the communities are.

82 The primary factors shaping global plankton distribution and community composition
83 patterns are still unclear. Global plankton structuring in the ocean has been largely
84 considered to follow the classical niche differentiation hypothesis ("everything is

85 everywhere but the environment selects"; Hutchinson (1957)), which considers selection as
86 the main factor determining plankton distribution. Recently, the importance of
87 geographical barriers and oceanic currents in shaping plankton community distribution has
88 been further acknowledged (Chust et al., 2017), and the role of drift and barriers to dispersal
89 has been recognised, in line with the neutral theory of biodiversity (Dornelas, Connolly, &
90 Hughes, 2006; Hubbell, 2001; Pueyo, 2006). In the upper oceanic layers, global plankton
91 dispersal and community assembly rules have been related to oceanic currents and
92 environmental factors (Richter et al., 2020; Villarino et al., 2018; Watson et al., 2011), and
93 to other causes such as body size (Villarino et al., 2018). Knowledge on planktonic spatial
94 distribution patterns at deeper layers is even much scarcer than at upper depths (Chust et
95 al., 2017; St. John et al., 2016). The deep ocean is environmentally more homogeneous
96 (Bode et al., 2018; Danovaro, Dell'Anno, & Pusceddu, 2004) and with generally weaker
97 oceanic currents (Reid, 1969, 1994). Hence it is expected that plankton dispersal and spatial
98 patterns of community composition are differently influenced by dispersal and selection
99 than in upper layers, as recently reported for prokaryotes and picoeukaryotes (Giner et al.,
100 2020; Villarino et al., 2022).

101 In the horizontal oceanic gradient, epipelagic mesozooplankton diversity and community
102 composition have been reported to vary latitudinally and to be linked to variations in
103 productivity, temperature, salinity and to phytoplankton community composition (Brandão
104 et al., 2021; Domínguez, Garrido, Santos, & dos Santos, 2017; Ibarbalz et al., 2019; Saporiti
105 et al., 2015; Soviadan et al., 2022), in addition to oxygen concentration at mesopelagic
106 depths (Soviadan et al., 2022). Yet, global horizontal mesozooplankton patterns below the

mesopelagic zone remain unexplored. Regarding the vertical oceanic gradient, mesozooplankton communities are known to be strongly structured along the water column, with many species showing a clear preference for specific depths (Fernández de Puelles et al., 2019; Hirai, Tachibana, & Tsuda, 2020; Pearman & Irigoien, 2015; Sommer, Van Woudenberg, Lenz, Cepeda, & Goetze, 2017). Because many mesozooplankton organisms perform vertical migrations (Ohman, 1990) and transport direction is related to depth (Fiksen, Jørgensen, Kristiansen, Vikebø, & Huse, 2007), the resulting distribution pattern of mesozooplankton is a complex combination of such processes together with adaptation to water mass environment, demographic traits and stochasticity.

Studies analysing the global distribution and connectivity of mesozooplankton communities and the ecological mechanisms shaping them are limited partly due to the scarcity of globally scaled surveys. Additionally, exploring the deep ocean has added challenges related to the sampling at high depths. To date, most studies on mesozooplankton have been carried out at local (Domínguez et al., 2017; Ershova & Kosobokova, 2019; Kim, Lee, Lee, Oh, & Kim, 2020; Pearman & Irigoien, 2015) or regional (Carlotti et al., 2018; Cheng et al., 2022; Feliú, Pagano, Hidalgo, & Carlotti, 2020; Landry, Hood, & Davies, 2020; Siokou et al., 2019) scales, and only some studies covered large oceanic transects (Bode et al., 2018; Hirai et al., 2020; Vereshchaka, Abyzova, Lunina, & Musaeva, 2017) or global oceanic areas (Fernández de Puelles et al., 2019; Soviadan et al., 2022). Another limitation lies on the taxonomic identification of mesozooplankton being a time-consuming task that greatly depends on often lacking taxonomic expertise and information of the targeted organisms (Hirai & Tsuda, 2015). Thus, many studies only consider abundant crustaceans (mainly

129 copepods) or identify mesozooplankton groups at higher taxonomic levels (Domínguez et
130 al., 2017; Ershova & Kosobokova, 2019; Siokou et al., 2019; Soviadan et al., 2022). Also,
131 some mesozooplankton groups such as gelatinous organisms are usually under sampled or
132 damaged while sampling with traditional methods (i.e., plankton nets), so that they cannot
133 be identified. The combination of these issues has made it difficult to gather knowledge on
134 the structuring and distribution patterns of mesozooplankton on a global scale.

135 Combining global oceanographic surveys and DNA metabarcoding, i.e., large-scale
136 taxonomic identification of complex samples via analysis of one or few orthologous DNA
137 regions (Bucklin, Lindeque, Rodriguez-Ezpeleta, Albaina, & Lehtiniemi, 2016), is a promising
138 approach for plankton research. Applying DNA metabarcoding to plankton virtually
139 overcomes the need of taxonomic expertise, ensures accurate taxonomic classification of
140 organisms difficult to identify (Bucklin et al., 2016; Govindarajan et al., 2021; Hirai & Tsuda,
141 2015) and allows the detection of hidden diversity, i.e., diversity that remains to be
142 discovered, described, and/or sequenced (Lindeque, Parry, Harmer, Somerfield, & Atkinson,
143 2013).

144 Here, we aim to increase the knowledge on mesozooplankton biodiversity, community
145 structuring, and connectivity in the global ocean along both horizontal and vertical oceanic
146 gradients by i) identifying the oceanic regions—both in the vertical and horizontal scales—
147 with a higher amount of hidden diversity and thus needing more taxonomic efforts, ii)
148 testing whether patterns in mesozooplankton alpha- and beta-diversity and community
149 structure differ along the vertical and horizontal gradients at a global oceanic scale, and iii)
150 unveiling the factors determining mesozooplankton spatial distribution and connectivity at

different oceanic depths. To achieve these goals, we applied DNA metabarcoding to mesozooplankton samples collected during the Malaspina-2010 circumnavigation expedition (Duarte, 2015) covering a large temperate to tropical oceanic area comprising the Atlantic, Indian and Pacific Oceans, and four depth ranges, including the epipelagic, upper mesopelagic, lower mesopelagic and bathypelagic layers (down to 3000 m depth). We hypothesise: i) that unexplored oceanic regions, such as the deep sea, harbour a higher proportion of hidden mesozooplankton diversity than those from upper layers, ii) that mesozooplankton communities are subjected to vertical and horizontal oceanic gradients at a global scale, which generate global biogeographic patterns, and iii) that mesozooplankton spatial distribution and connectivity differ at the different ocean layers, with higher dissimilarity between deep-sea communities than between communities at upper layers due to the average weaker deep-sea currents compared to surface ones (Manral et al., 2023; Reid, 1994).

Material and methods

Sampling and environmental data collection

Mesozooplankton samples were collected during the Malaspina 2010 circumnavigation expedition (from December 2010 to July 2011; Duarte (2015)) from 43 different stations (Figure 1) using a 0.5 m^2 Hydrobios MultiNet (300 µm mesh size) programmed to open at regular depths (0–200, 200–500, 500–1000, 1000–2000 and 2000–3000 m depth) from the surface to 3,000 m depth for a total of 133 samples. All samples were collected during

daytime (10:00 to 14:00 am local time). Additional details on the sampling and stations can be found in Fernández de Puelles et al. (2019). On the cruise, each net was softly rinsed with filtered seawater to capture all organisms, which were stored in 50 ml flasks filled with absolute ethanol. At each sampling station a Rosette sampling system fitted with a Seabird 0911Plus CTD probe was deployed (Duarte, 2015), measuring seawater temperature (°C), conductivity (S/m), salinity (PSU), fluorescence (Seapoint), photosynthetically active radiation (PAR), and oxygen (ml/l) along the water column. Samples were grouped according to their depth range into 0-200 m (epipelagic layer), 200-500 m (upper mesopelagic), 500-1000 m (lower mesopelagic) and 1000-3000 m depth (bathypelagic); when two samples covered a unique depth range, they were pooled after sequencing into one unique integrated sample by summing up their absolute number of reads (i.e., samples collected at 1000-2000 and 2000-3000 m depth were merged into a unique 1000-3000 m sample). Similarly, a unique value of each environmental variable was used for each depth range, which corresponded to the average of all measurements for that depth range (Table S1). It should be noted that we used the term mesozooplankton although the mesh size used for the sampling (300 µm) did not exactly correspond to the size range expected for mesozooplankton (from 200 µm to 2 mm length). This decision responded to the fact that most reads and OTUs corresponded to metazoans that are known to belong to this planktonic fraction.

DNA extraction, quantity, and quality check

Samples were centrifuged (3,500 g; 10 min) to remove ethanol and resulting zooplankton pellets were grinded with a mortar in 1-2 ml lysis buffer (10 mM Tris-HCl, 100 mM EDTA,

200 mM NaCl, 1% SDS) until no integer organism could be appreciated. After an overnight incubation with proteinase K (0.2 mg/ml, final concentration) at 56 °C, samples were centrifuged (3,500 g; 15 min) and supernatant was incubated with RNAse (37 °C; 30 min). Extracted total DNA was purified using a phenol-chloroform-isoamyl alcohol (25:24:1, vol:vol:vol) mixture followed by ethanol 95% ammonium acetate 0.5 M precipitation. DNA was suspended in 100 µl Milli-Q water and stored at -20 °C until further use. DNA concentration was measured with the Quant-iT dsDNA HS assay kit using a Qubit® 2.0 Fluorometer (Life Technologies, California, USA), while DNA purity was inferred from 260/280 and 260/230 absorbance ratios with the ND-1000 Nanodrop (Thermo Scientific, Massachusetts, USA). Integrity of extracted genomic DNA was assessed by electrophoresis in 0.7% agarose. Eighteen of the samples did not yield gel-visible DNA.

Library preparation and sequencing

110 samples were amplified using the #1/#2RC primer pair (Machida & Knowlton, 2012) targeting the hypervariable V4 region of the 18S rRNA gene (henceforth *mac18S*) and 85 were amplified using the mlCOIintF/dgHCO2198 primer pair (Leray et al., 2013) targeting a 313 bp length region of the cytochrome oxidase I (COI) gene (henceforth *mlCOI*). For the first PCR reaction, 2 µl of genomic DNA (5 ng/µl) were added to a mix consisting of 10 µl of 1X Phusion Master Mix (ThermoScientific, Massachusetss, USA), 0.4 µl of each primer (0.2 µM) and 7.2 µl of MilliQ water. For the *mlCOI* primer pair, annealing was performed for 1 min at 46 °C, and for the *mac18S* primer pair annealing was performed for 30 s at 55 °C and only 22 cycles were used. PCR products were purified using AMPure XP beads (Beckman Coulter, California, USA) following manufacturer's instructions and used as templates for

the generation of the dual-indexed amplicons in the second PCR reaction following the "16S Metagenomic Sequence Library Preparation" protocol (Illumina, California, USA) using the Nextera XT Index Kit (Illumina, California, USA). Multiplexed PCR products were purified using the AMPure XP beads, quantified using Quant-iT dsDNA HS assay kit using a Qubit® 2.0 Fluorometer (Life Technologies, California, USA) and adjusted to 4 nM. Then, 5 µl of each sample were pooled, checked for size and concentration using the Agilent 2100 bioanalyzer (Agilent Technologies, California, USA), sequenced using the 2 x 300 paired end protocol on the Illumina MiSeq platform (Illumina, California, USA) and demultiplexed based on their barcode sequences. Four and one samples in *mlCOI* and *mac18S*, respectively, produced less than 5,000 reads and were not considered for further analyses. In addition, four and five pairs of samples in *mlCOI* and *mac18S*, respectively, belonged to the same depth range and were pooled into unique depth range samples. At the end, the *mlCOI* and *mac18S* datasets consisted of a total of 77 and 104 samples (for sample details, see Table S2).

Pre-processing, clustering, and taxonomic assignment of amplicon sequences

The *mlCOI* barcode is 313 bp length, while the *mac18S* barcode has a variable length that ranges between 537 to 595 (5 to 95th percentile) in eukaryotes (Figure S1). In order to accommodate these differences, alternative read pre-processing pipelines had to be applied for each marker (Figure S2). In both cases, raw demultiplexed reads were quality checked with FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). *mlCOI* forward and reverse reads were merged using FLASH (Magoč & Salzberg, 2011) with an allowed overlap range of 217 to 257 bp (20 nucleotides more and less of the expected

238 overlap). *mac18S* forward and reverse reads were merged with a minimum overlap of 162,
239 and non-merged pairs were trimmed at 220 bp (based on a median Phred score lower than
240 30 after these positions) and the forward and the reverse complement of the reverse reads
241 were pasted introducing an ambiguous base (N) in between; this was done so that no k-
242 mers including fragments of the forward and reverse reads are used for taxonomic
243 assignment (Jeraldo et al., 2014). Using Trimmomatic (Bolger, Lohse, & Usadel, 2014), for
244 both barcodes only those resulting contigs with a minimum average Phred score of 20 and
245 containing the appropriate primer sequence were retained for subsequent analyses.
246 Sequences with at least one (for *mlCOI*) or two (for *mac18S*) ambiguous bases were
247 discarded using mothur (Schloss et al., 2009). Chimeras were detected and removed using
248 UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011). Clustering of sequences into
249 operational taxonomic units (OTUs) was performed using SWARM (Mahé, Rognes, Quince,
250 de Vargas, & Dunthorn, 2014) with *d*=1, and singletons (i.e., OTUs with one unique read in
251 the dataset) were removed. Taxonomic assignment was performed according to the naïve
252 Bayesian classifier method from (Wang, Garrity, Tiedje, & Cole, 2007) implemented in
253 mothur against the BOLD (http://www.boldsystems.org) and SILVA (release 132) (Quast et
254 al., 2013) databases as references for *mlCOI* and *mac18S* barcodes, respectively. For *mlCOI*
255 dataset, the sequences assigned to metazoans in the previous step were taxonomically
256 reassigned using the more recent, curated MetaZooGene database (MZGdb) (Bucklin et al.,
257 2021) for a more accurate classification. To compare the results between barcodes, the
258 taxonomic ranks of both databases were adjusted. It should be noted that, in most clades,

SILVA database lacked detailed taxonomy for levels below Class, preventing some analyses for the *mac18S* dataset (specified along the manuscript).

Hidden diversity, alpha-, and beta-diversity analyses

To determine the amount of hidden diversity in each oceanic basin and depth layer we relied on the sequence similarity values obtained by comparing the representative sequence of each OTU against the reference sequences in MZGdb using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990). To assess horizontal and vertical alpha-diversity patterns we used the OTU richness (number of OTUs) and H index (*diversity* function, *vegan* package version 2.5.6; Oksanen et al. (2019)) measurements.

To infer patterns in mesozooplankton community composition similarity and connectivity between sites (beta-diversity), we used Bray-Curtis distances (*vegdist* function, *vegan* package) and phylogenetic community dissimilarities (PCD) (*pcd* function, *picante* package version 1.8.2; Kembel et al. (2010)). For PCD, we only considered the 100 most abundant OTUs due to computational requirements for the analysis. Vertical and horizontal structuring of mesozooplankton communities was examined by applying nonmetric multidimensional scaling (NMDS) (*metaMDS* function, vegan package) analysis based on Bray–Curtis and PCD distance matrices, followed by ANOSIM test (Clarke, 1993) (*anosim* function, vegan package) to test for statistical significance of communities ordination according to predefined sample groups. Finally, to unveil the factors driving mesozooplankton spatial distribution we relied on the correlations between mesozooplankton community composition distances and environmental and least cost

oceanic distances, by using the Mantel test *(mantel* function, *vegan* package). Environmental distances were based on the Euclidean distance (*vegdist* function, *vegan* package) between pairs of sites and included all environmental variables measured after previous standardization to the same scale. The least cost oceanic distances were obtained using the *marmap* package (*lc.dist* function, version 1.0.6; Pante and Simon-Bouhet (2013)). All statistical analyses and plots were conducted in R statistical environment (R version 4.0.4; R Core Development Team (2013)).

Results

Global mesozooplankton composition

Most of the *mlCOI* and *mac18S* dataset reads and OTUs were assigned to Metazoa, from which over 75% and 100% of both reads and OTUs in *mlCOI* and *mac18S*, respectively, were successfully assigned to phylum and used for further analysis (Figure 2a). In both datasets, Arthropoda appeared as the most abundant and diverse group (Figure 2b). Chaetognatha, Cnidaria, Mollusca, Annelida, Vertebrata (Chordata) and Tunicata (Chordata) also exhibited relevant richness and/or abundance in at least one of the datasets. The Class Hexanauplia (Arthropoda), which includes copepods, was the dominant component of the mesozooplankton community in both datasets; other abundant Classes were Malacostraca (Arthropoda; 16.4% and 6.6% of reads in *mlCOI* and *mac18S*, respectively), Hydrozoa (Cnidaria; 7.9% and 18.5%) and Actinopterygii (Vertebrata; 6.0% and 1.0%). It should be noted that some taxa were not (or hardly) amplified by one of the markers. For instance,

Ostracoda, Chaetognatha and Cephalopoda (Mollusca) were rarely or not detected by *mac18S* despite being relatively abundant in *mlCOI* (11.4%, 6.3% and 1.9% of reads, respectively), while Annelida, Gastropoda and Tunicata (Chordata), which represented 3.3%, 3.5%, and 8.5% of *mac18S* metazoan reads, respectively, were clearly underrepresented in *mlCOI* dataset (0.5%, 0.8%, and 0.02% of reads).

At the Class level, mesozooplankton taxonomic composition—and differences in community composition between markers—was overall consistent along the water column (Figure 2b), especially in *mac18S* dataset. Yet, i) an overall trend to increase both the proportion of reads and OTUs assigned to Arthropoda with depth, ii) a peak of Vertebrata reads at lower mesopelagic depths, and iii) a peak of Ostracoda reads and OTUs at the upper mesopelagic layer in the *mlCOI* dataset, was observed. Note that these results represent the average taxonomic composition by depth range, and that mesozooplankton community composition differed between sites (Figure S3).

Remarkably, just about half of OTUs (representing two thirds of the reads) were successfully assigned to the species level in *mlCOI* dataset—analysis not performed in *mac18S* due to SILVA reference database not specifying taxonomic levels below Class. Yet, this percentage greatly varied between and within taxonomic groups (Table S3).

Hidden diversity

We inferred the amount of hidden diversity globally and in the different oceanic basins and depths under study. This inference was based on the sequence similarity values obtained by comparing the representative sequence of each OTU against the reference sequences in

MZGdb, i.e., the lower the sequence similarity the farther the retrieved sequence is to an already known (sequenced) organism. The Indian Ocean resulted as the oceanic basin presenting a higher proportion of unknown mesozooplankton diversity (with approximately half of the OTUs displaying less than 90% of sequence similarity to described species), followed by the Pacific and Atlantic basins (Figure 3). In the vertical gradient, we observed a general trend to increase the proportion of hidden diversity with depth (from the epipelagic to mesopelagic and bathypelagic layers) while decreasing the proportion of well-known OTUs (with >98% similarity to MZGdb sequences) below the upper mesopelagic layer. This vertical pattern was observed both globally and at each oceanic basin separately (except for the bathypelagic layer of the Pacific Ocean, whose sequence similarity values were comparable to those from epipelagic depths).

Horizontal and vertical structuring of mesozooplankton community composition

Ordination of communities using NMDS analysis based on Bray-Curtis and PCD dissimilarities followed by ANOSIM test evidenced a strong vertical mesozooplankton structuring (according to depth) in both datasets (Figure 4E-H), which was consistently observed in each oceanic region separately (Table 1). Horizontal structuring (according to ocean basin) was clearly supported in *mlCOI* dataset but was not (or weakly) supported in *mac18S* (Figure 4A-D). Interestingly, mesozooplankton communities exhibited horizontal structuring at epipelagic and mesopelagic layers (excepting in *mac18S*-PCD, where mesozooplankton structuring was only observed at the epipelagic layer) but not at bathypelagic depths by any combination of marker and beta-diversity parameter (Table 1).

343 Further, we found that bathypelagic communities were structured according to deep-water
344 mass type (as defined in Catalá et al. (2015)) rather than to oceanic basin.

345 *Horizontal and vertical patterns in mesozooplankton alpha- and beta-diversity*

346 We analysed how mesozooplankton alpha- and beta-diversity are globally structured along
347 the temperate to tropical global ocean. We did not observe consistent vertical nor
348 horizontal patterns in mesozooplankton alpha-diversity measurements among markers,
349 neither analysing the data globally (Figure S4A) nor by oceanic region (Figure S4B).

350 On the other hand, we observed a recurrent pattern of increasing beta-diversity between
351 mesozooplankton communities with depth (from the surface to the bathypelagic zone) in
352 both *mlCOI* (Figure 5A) and *mac18S* datasets (Figure 6A), i.e., mesozooplankton
353 communities from the upper layers are more similar to each other than communities from
354 the lower mesopelagic and bathypelagic depths, indicating a greater connectivity between
355 mesozooplankton assemblages at the surface than at deeper ocean. This trend was evident
356 in *mlCOI* dataset using Bray-Curtis distances and in *mac18S* using both Bray-Curtis and PCD
357 but less clear in *mlCOI* using PCD. The pattern of increasing beta-diversity with depth turned
358 out more robust for each combination of marker and beta-diversity measurement when the
359 different oceanic regions were analysed separately (Figure 5B, 6B), and still quite apparent
360 when considering only the stations with all four depth ranges sampled (Figure S5).

361 *Relative contribution of environment and oceanic distance to mesozooplankton spatial*
362 *structuring*

To determine the factors driving mesozooplankton spatial distribution at each depth range, we performed Mantel correlations between variations in mesozooplankton community composition and variations in oceanic and environmental distances (Table 2, Figure S6). Mesozooplankton community dissimilarities correlated significantly with oceanic distances in both *mlCOI* and *mac18S* datasets regardless of the beta-diversity measurement used (except for 500-1000 m depth in *mac18S*), indicating the existence of distance-decay patterns in mesozooplankton communities at all depth ranges. It was especially noticeable at the upper oceanic layers, where oceanic distance was the main contributor to mesozooplankton community composition. Less consistency was found regarding the contribution of environmental distances to mesozooplankton spatial distribution among markers—the environment noticeably determined mesozooplankton community composition in *mlCOI* but had limited influence in *mac18S* dataset—and among beta-diversity measurements—with particularly different results in *mac18S* using Bray-Curtis and PCD distances. In the bathypelagic layer, the relative contribution of the environment to mesozooplankton community composition was overall higher than the oceanic distance, yet not always statistically significant (Table 2). Among the parameters measured, oxygen, temperature, conductivity, and salinity emerged as the ones most influencing mesozooplankton communities. Oceanic and environmental distances were consistently correlated at all depths, with remarkably high correlation values at bathypelagic depths (Table 2).

Discussion

Overview of mesozooplankton community composition across the tropical to temperate global ocean

Our findings identified Arthropoda—specifically Hexanauplia (copepods), and to a lesser extent Ostracoda and Malacostraca (group including euphausiids and decapods)—as the most abundant and diverse groups in the tropical to temperate global ocean, in agreement with previous studies (Fernández de Puelles et al., 2019; La et al., 2015; Sommer et al., 2017; Stefanoudis et al., 2019). Due to their high abundance and worldwide distribution, these organisms are recognised as a central component of epipelagic marine ecosystems, playing a key role as main link between lower (producers and primary consumers) and higher trophic levels (Steinberg & Landry, 2017) and being the main food source of many commercial fishes, thus sustaining a number of fisheries worldwide (Hays et al., 2005; Turner, 2004). Our data suggest that marine arthropods are also dominant among mesozooplankton at mesopelagic and bathypelagic depths, thus supporting a central role of these organisms in the deep-ocean trophic web as well (Kelly et al., 2019), in addition to their relevance modulating global biogeochemical processes such as the biological carbon pump (Bode et al., 2018; Steinberg & Landry, 2017).

Although the taxonomic composition retrieved by both markers (*mlCOI* and *mac18S*) was very similar, some taxonomic groups were retrieved differently by one of the markers. This fact highlights the importance of choosing an adequate barcode when designing metabarcoding-based studies (Bucklin et al., 2016) and reinforces the need for multi-marker approaches to get comprehensive insights on the zooplankton taxonomic diversity (Stefanni et al., 2018; van der Loos & Nijland, 2021; G. K. Zhang, Chain, Abbott, & Cristescu, 2018). A

407 remarkable proportion of reads in both datasets at all depths under study were attributed
408 to gelatinous organisms (e.g., cnidarians or tunicates), which are normally underestimated
409 in morphologically based surveys using nets due to their fragility, and for which DNA-based
410 methods may be more effective (Bucklin et al., 2019; Govindarajan et al., 2021). Otherwise,
411 we acknowledge that we are probably missing some of the most abundant
412 mesozooplanktonic organisms in the ocean, such as *Oithona* spp. and other small-sized
413 Cyclopoids (Turner, 2004), most of which probably escaped our detection due to having a
414 body size smaller than the 300 µm mesh size used during the Malaspina sampling.
415 Additional studies including other size fractions could complement our findings by
416 confirming whether the global patterns observed here also apply for the smallest
417 mesozooplankton fraction.

418 *Focusing on the unknown – identifying hotspot areas of hidden diversity*

419 The presence of a high number of OTUs that could not be assigned to species level and that
420 were so distant to sequences from MZGdb (Bucklin et al., 2021), suggests that our
421 knowledge of the organisms inhabiting the pelagic open ocean it is still scarce, especially
422 beyond the epipelagic layer as also reported by other authors (Sommer et al., 2017), and
423 evidence that zooplankton molecular reference databases are far from completion (Bucklin
424 et al., 2010; Bucklin et al., 2021). According to our data, the Indian Ocean and the lower
425 mesopelagic and bathypelagic layers are the regions requiring further taxonomic and/or
426 sequencing efforts along the tropical to temperate latitudes. Our findings agree with Bucklin
427 et al. (2021), who placed the Indian Ocean among the oceanic basins with lower species
428 coverage by DNA barcoding initiatives (with only 29% of copepod species barcoded) and

429 considered the deep-sea ecosystems as an immediate priority for DNA barcoding and
430 metabarcoding studies. Additional initiatives to the ones from (Bucklin et al., 2010) and
431 other barcoding projects detailed therein are thus required in order to obtain these
432 references—while increasing our knowledge on zooplankton biodiversity—to ensure a
433 reliable application of DNA-based methods for the study of mesozooplankton.

434 *Mesozooplankton community composition exhibits vertical and horizontal biogeographic*
435 *patterns at a global scale*

436 Our results indicate that mesozooplankton community composition is structured across
437 both vertical and horizontal oceanic gradients. Vertical structuring was particularly strong
438 in both *mlCOI* and *mac18S* datasets either analysing the data globally or at each oceanic
439 basin separately, thus adding evidence for a global, solid vertical structuring of
440 mesozooplankton in the ocean, corroborating many previous observations (Cheng et al.,
441 2022; Fernández de Puelles et al., 2019; Hirai et al., 2020; Pearman & Irigoien, 2015;
442 Sommer et al., 2017; Stefanoudis et al., 2019). Horizontal mesozooplankton structuring (i.e.,
443 according to oceanic basins) was also overall supported in both datasets, although it was
444 strongly supported in *mlCOI* than in *mac18S*, most likely due to a higher capability of the
445 former to detect intraspecific genetic variants (Turon, Antich, Palacín, Præbel, &
446 Wangensteen, 2020), and thus better detect regional diversity and dissimilarities between
447 distant communities and populations (Chust et al., 2016).

448 Horizontal structuring of mesozooplankton communities has been widely reported at the
449 epipelagic layer and highlights the existence of biogeographic regions responding to

productivity, hydrology, environmental characteristics of water, and connectivity barriers (Becker, Eiras Garcia, & Freire, 2018; de Vargas et al., 2015; Domínguez et al., 2017; Ershova, Wangensteen, Descoteaux, Barth-Jensen, & Præbel, 2021; Feliú et al., 2020; Gaard et al., 2008; Hirai & Tsuda, 2015), but few studies to date have assessed horizontal structuring of mesozooplankton at meso- or bathypelagic depths (Hirai et al., 2020; Siokou et al., 2019). Here, we observed that horizontal structuring of mesozooplankton community composition along the temperate to tropical global ocean is unevenly supported across depth; it was strongly supported at the epipelagic layer, moderately at mesopelagic depths, and low supported in the bathypelagic zone. Although the latter finding was unexpected considering the low connectivity of deep-sea mesozooplankton communities reported here—which should lead to a more evident horizontal structuring, we observed that structuring of mesozooplankton communities at bathypelagic depths was not determined by the oceanic basin but by the deep-water mass type (as defined in Catalá et al. (2015)) from which they were collected. Similar findings have been previously reported for prokaryotes (Agogué, Lamy, Neal, Sogin, & Herndl, 2011; Salazar et al., 2016) and picoeukaryotes (Pernice et al., 2016). Our results also indicate the existence of a distance-decay pattern (i.e., the farther the communities the more different the community composition) for mesozooplankton assemblages at all depths under study. Since this result may somehow indicate a relationship between the oceanic distance and the deep-water mass type from which bathypelagic samples were collected, further studies covering more samples and additional deep-water mass types should be carried out in order to verify our findings.

Vertical mesozooplankton alpha-diversity patterns are not ruled globally

Previous studies on microzooplankton point to a general pattern of decreasing alpha-diversity (richness and diversity indices) along the vertical oceanic gradient (Canals, Obiol, Muhovic, Vaqué, & Massana, 2020; Countway et al., 2007; Giner et al., 2020); however, to date there is no clear consensus on whether mesozooplankton alpha diversity increases or decreases with depth. For instance, while a decreasing trend in mesozooplankton richness and/or H index has been observed in Fernández de Puelles et al. (2019), Vereshchaka et al. (2017), and Pearman and Irigoien (2015), among others, peaks in alpha-diversity at mesopelagic or/and bathypelagic depths have also been reported for copepods (Hirai et al., 2020; Kosobokova & Hirche, 2000; Stefanoudis et al., 2019) and for the whole mesozooplankton community (Cheng et al., 2022; Sommer et al., 2017). Here, we did not observe any consistent pattern in mesozooplankton alpha diversity with depth, but our results seem to support the deep sea (down to the bathypelagic layer) as an ecosystem harbouring a level of diversity comparable to the ones at upper depths. Based on the discrepancies between the different studies, it is most likely that there is not a unique, global pattern of mesozooplankton alpha diversity along the vertical profile in the ocean, but that it is region specific. Further studies are thus needed to determine the factors regulating mesozooplankton alpha diversity patterns along the vertical oceanic scale, such as primary productivity and water column mixing.

In DNA-based studies, alpha diversity values in the deeper layers could be accounting for the capture of mesozooplankton DNA sinking from upper layers (e.g., carcasses, attached to sinking particles; Preston, Durkin, and Yamahara (2020)) and the stomach contents of diel vertical migratory species, which move upward the water column to feed during the

night, returning to the depths at sun (Steinberg & Landry, 2017). Yet, this downward-transported or prey material is expected to be less abundant and more degraded than the one from the alive individuals comprising the samples, thus representing a neglecting proportion of OTUs and reads. Also, it is interesting to note that DNA-based approaches are known to yield higher diversity values (especially in richness) than morphologically based surveys (Ershova et al., 2021; Schroeder et al., 2020; Sommer et al., 2017). In the deep ocean, this bias between methods could be even magnified due to the notably lesser knowledge on deep-sea mesozooplankton diversity—hampering its taxonomic classification—and its overall lower abundance—making it less likely to be sampled.

The deeper the lower the connectivity between mesozooplankton communities

Results derived from the beta-diversity analyses indicated a higher dissimilarity between mesozooplankton communities from the ocean deep layers (especially at the bathypelagic zone) than between communities from the upper layers. These findings are in line with those obtained by Siokou et al. (2019) in the Mediterranean Sea, who observed differentiation between Eastern and Western Mediterranean mesozooplankton communities at lower mesopelagic and bathypelagic depths, but no differentiation at epipelagic and upper mesopelagic layers. These results point to lower connectivity between deep-sea mesozooplankton communities than between communities from upper oceanic layers, which may be driven by limitations in the dispersal of mesozooplankton assemblages at the ocean depths due to prevailing weaker oceanic currents and water mixing in the deep sea compared to the surface (Manral et al., 2023; Reid, 1981, 1994)—in agreement with previous findings for picoeukaryotes (Villarino et al., 2022). Our findings add further

516 evidence on the major role of oceanic currents in shaping zooplankton dispersal and
517 connectivity at a global scale, not only at epipelagic layers as previously reported (Richter
518 et al., 2020; Villarino et al., 2018; Watson et al., 2011), but, for the first time for
519 mesozooplankton, also at the ocean depths. Yet, it should be noted that the relative
520 contribution of oceanic distance in shaping mesozooplankton communities at the ocean
521 depths was overall lower relative to the contribution of the environment. While oceanic
522 distance can be assumed as a proxy of oceanic currents at the epipelagic layer, this
523 assumption could lose strength deeper in the water column, since deep oceanic currents
524 may follow not only horizontal but also vertical and/or oblique routes due to the
525 thermohaline circulation.

526 *Contribution of dispersal and selection to mesozooplankton community composition in the*
527 *deep ocean*

528 The contribution of dispersal and environmental selection on plankton spatial distribution
529 has been reported to differ among groups and oceanic depths, as recently reported by
530 (Villarino et al., 2022). Here, our results suggested dispersal as the main contributor to
531 mesozooplankton distribution at the upper oceanic layers, attributing a secondary role to
532 environmental selection. At bathypelagic depths, selection was the main driver of
533 mesozooplankton community composition together with dispersal, even though the
534 bathypelagic zone is much more homogeneous in terms of environmental conditions than
535 the layers above it (Bode et al., 2018; Danovaro et al., 2004). Considering that dispersal of
536 plankton in the ocean is globally constrained by environmental selection (Ward, Cael,
537 Collins, & Young, 2021), our results indicate that little environmental variations in the

bathypelagic layer may generate more marked differences in mesozooplankton beta-diversity than at upper depths. Yet evaluating the specific contribution of environmental and oceanic distances (i.e., selection and dispersal, respectively) in the present study is challenging due to the significant, consistent correlation between both factors, specially at the bathypelagic layer. Between 1000 and 3000 m depth, environmental and oceanic distances appeared to be markedly correlated, thus somehow blurring the boundary between dispersal and environmental selection when aiming to interpret the results. As observed in the present study and in previous works (Agogué et al., 2011; Pernice et al., 2016; Salazar et al., 2016), plankton community composition in the deep ocean appears to be highly related to the deep-water mass type at which they are found, which are in turn defined according to its environmental characteristics (Catalá et al., 2015) and present limited mixing with the surrounding water masses (Reid, 1981). Thus, although our findings clearly support that mesozooplankton communities show biogeographic patterns in the deep ocean, to elucidate whether these patterns are primarily driven by dispersal or by environmental selection will require further research including the collection of more samples from additional deep-water mass types.

Despite the main role of dispersal in shaping mesozooplankton community composition, correlation between environmental variables and mesozooplankton community composition was also found at all depth ranges under study, which supports the view that global structuring of planktonic communities is vulnerable to climate change-derived effects (Benedetti et al., 2021; Villarino et al., 2015), particularly at the ocean depths, where environmental conditions are more stable (Bode et al., 2018; Danovaro et al., 2004). The

consequences derived from alterations in global mesozooplankton structuring on the whole marine ecosystem services are still uncertain, but they are expected to be significant considering the central role of mesozooplankton in the oceanic tropic web and in biogeochemical processes (Danovaro, Corinaldesi, Dell'Anno, & Snelgrove, 2017; Kelly et al., 2019; Steinberg & Landry, 2017).

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Data Accessibility

Raw sequence data and associated metadata are available on the NCBI SRA ([to be completed upon acceptance]).

Author Contributions

XI and NRE designed research. OC, JC, EV, GC, EA, IM, and NRE performed research. JC, EA, IM, CTM, JIG and NRE contributed new reagents or analytical tools. OC, JC, EV, EA and NRE analysed the data. OC wrote the paper, with insightful contributions from EV, GC, XI and NRE. All authors revised the manuscript and agreed with its publication.

Tables and Figures (with captions)

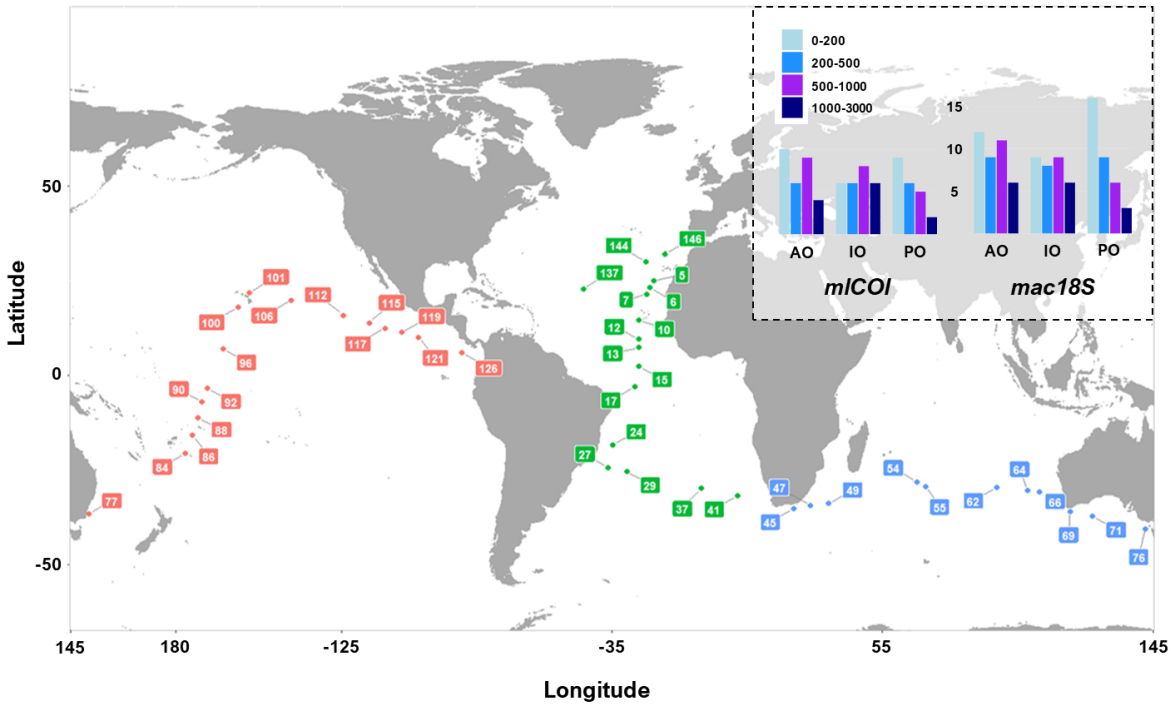
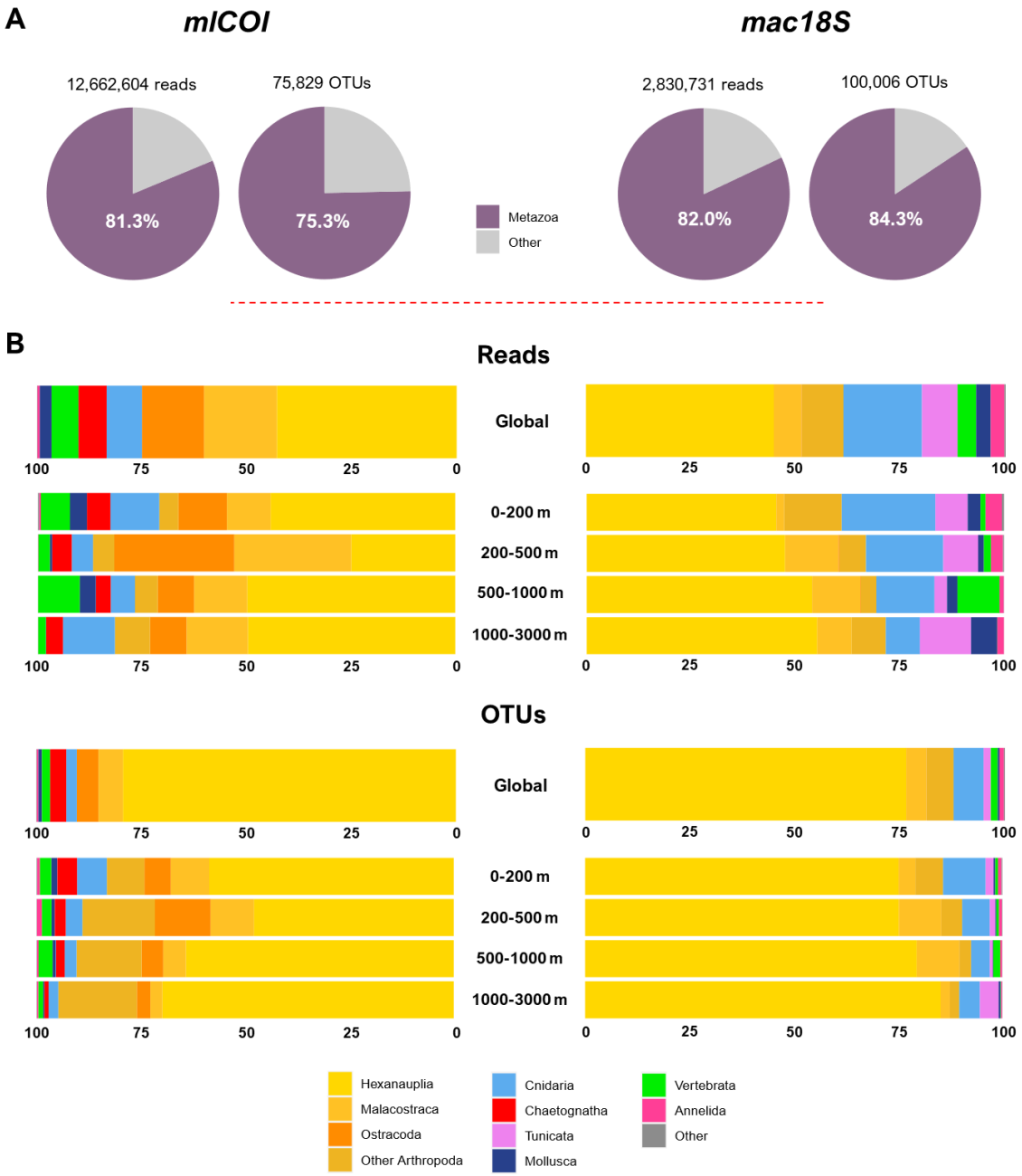


Figure 1. Location of the sampling stations of the Malaspina-2010 expedition from where mesozooplankton samples were analysed in this study (map) and number of samples analysed per depth range in the different oceanic basins for each marker (top right square). AO: Atlantic Ocean, IO: Indian Ocean, PO: Pacific Ocean.



923

924 **Figure 2.** Overview of mesozooplankton taxonomic diversity in *mlCOI* and *mac18S* datasets.
925 **A:** Proportion of reads and OTUs assigned to Metazoa (Phylum level) and other (including
926 unclassified, non-metazoan, and metazoan OTUs not assigned at Phylum). **B:** Proportion of
927 reads and OTUs assigned to each metazoan Phyla and most abundant Classes within
928 Arthropoda, globally and per depth range.

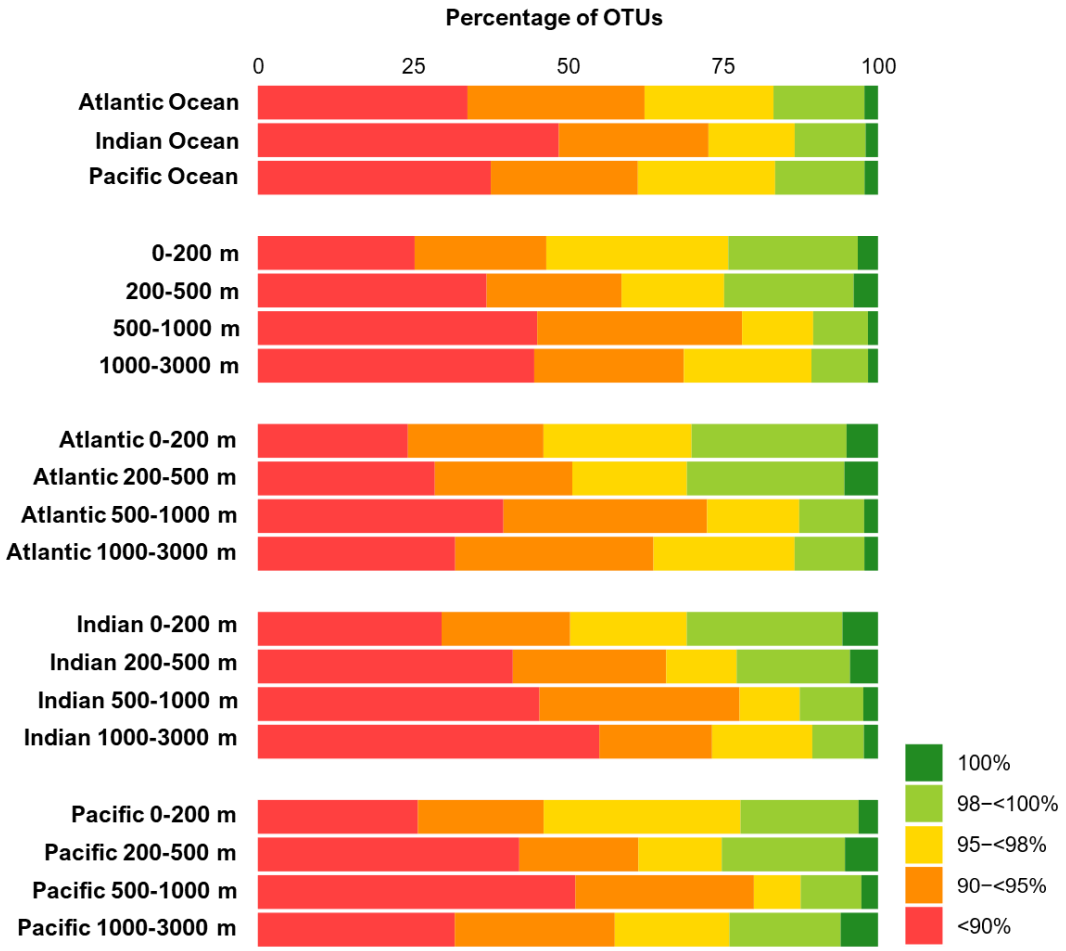


Figure 3. Percentage of *mlCOI* OTUs with 100% (dark green), 98-100% (light green), 95-98% (gold), 90-95% (orange), and less than 90% (red) sequence similarity to any sequence of the MetaZooGene database for the Atlantic, Indian, and Pacific Oceans, for the four depth ranges under study, and for each combination of oceanic basin and depth range.

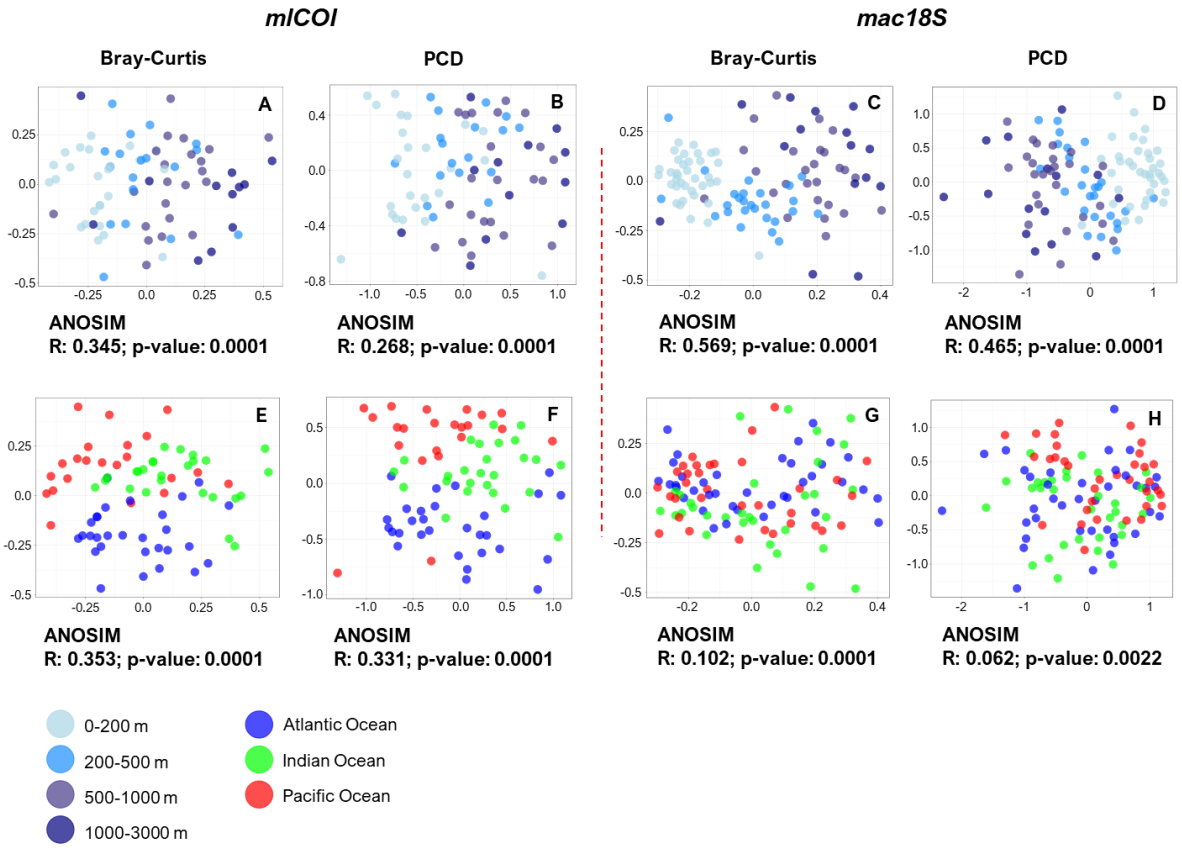
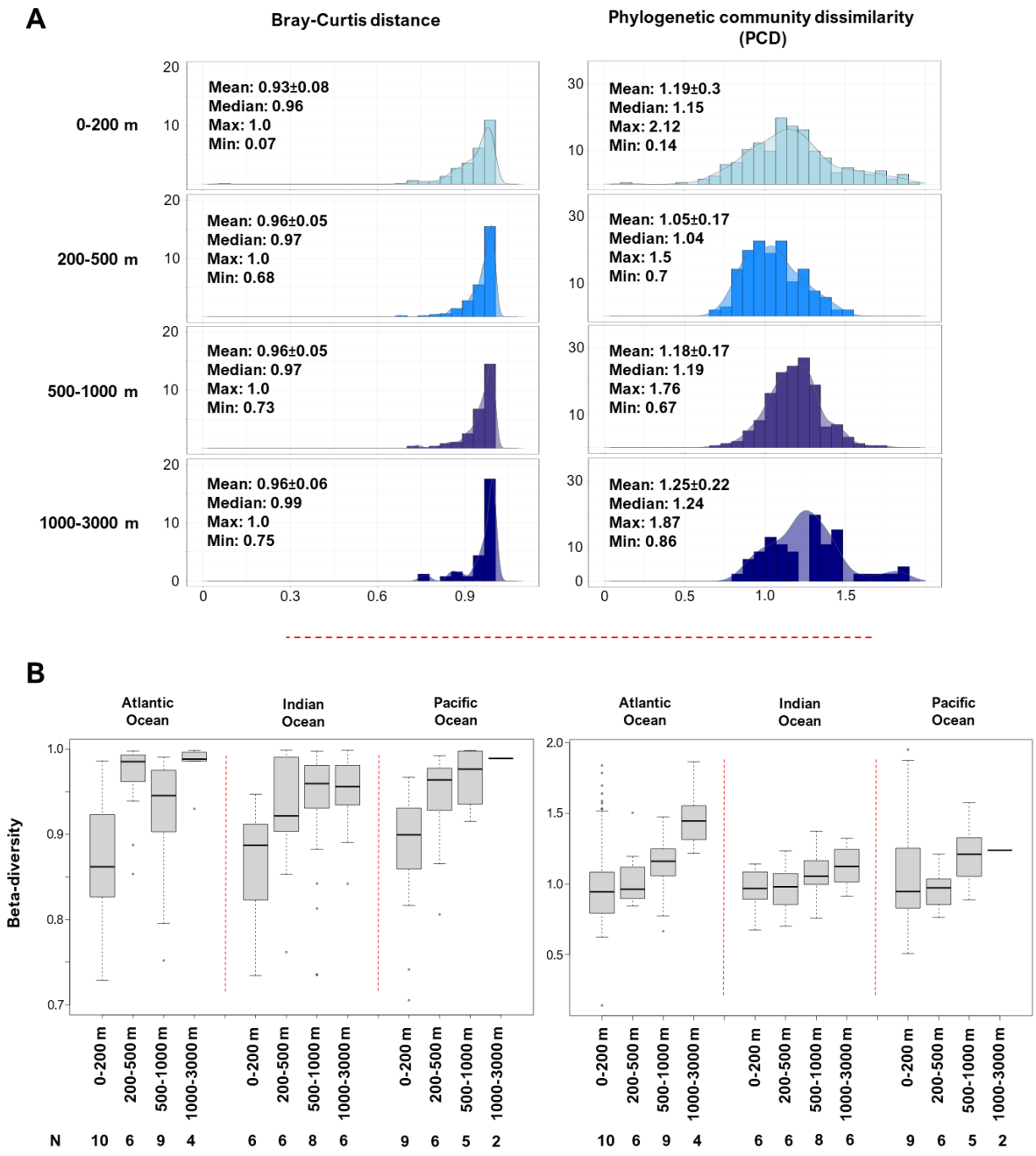


Figure 4. Ordination of mesozooplankton communities by NMDS (non-metric multidimensional scaling) analysis based on Bray-Curtis and PCD beta-diversity measurements for *mlCOI* and *mac18S* datasets; plots A-D coloured by depth, plots E-H coloured by oceanic basin. Stress values of plots A and E: 0.247, B and F: 0.226, C and G: 0.258, D and H: 0.214.



941

942 **Figure 5. A:** Histograms of beta diversity measurements based on Bray Curtis distances and
943 PCD (phylogenetic community dissimilarities) between mesozooplankton communities
944 from each depth range in the *mlCOI* dataset. **B:** Boxplots showing the distribution of beta-
945 diversity measurements (Bray Curtis and PCD) between mesozooplankton communities at
946 each depth range from each oceanic basin separately. N indicates the number of samples
947 considered in each boxplot.

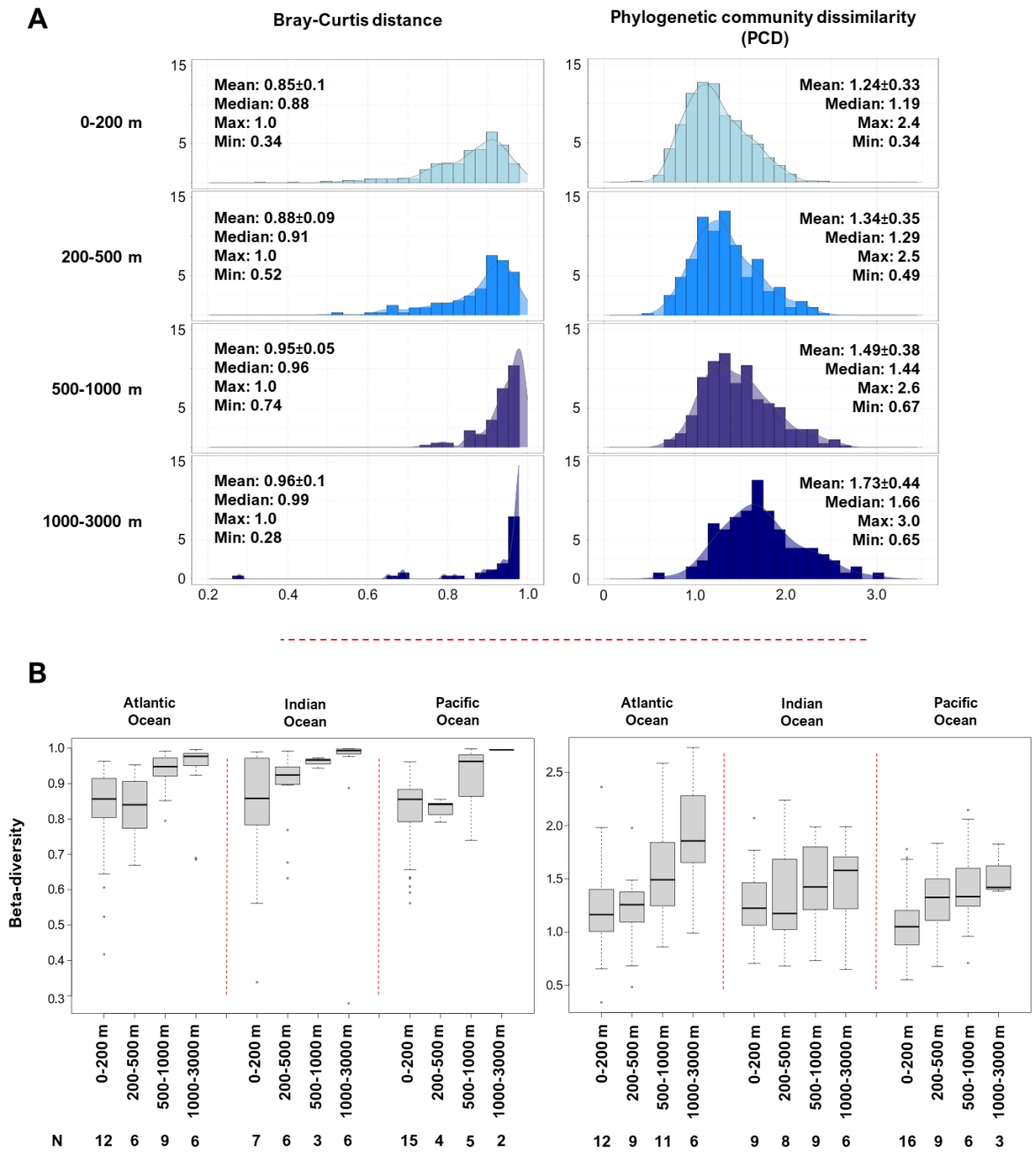


Figure 6. A: Histograms of beta diversity measurements based on Bray Curtis distances and PCD (phylogenetic community dissimilarities) between mesozooplankton communities from each depth range in the *mac18S* dataset. **B:** Boxplots showing the distribution of beta-diversity measurements (Bray Curtis and PCD) between mesozooplankton communities at each depth range from each oceanic basin separately. N indicates the number of samples considered in each boxplot.

955 **Table 1.** ANOSIM test results regarding the grouping of mesozooplankton communities at
956 the different oceanic basins by depth, at the different depths by oceanic basin, and the
957 grouping of bathypelagic mesozooplankton communities by deep-water mass type (DWT;
958 defined according to Catalá et al. (2015)). * p-value<0.1, **<0.05, ***<0.01, n.s. non-
959 significant.

960

	mlCOI				*mac18S*			
	Bray-Curtis		**PCD**		**Bray-Curtis**		**PCD**	
	R statistic	**p-value**	**R statistic**	**p-value**	**R statistic**	**p-value**	**R statistic**	**p-value**
Atlantic by depth	0.42	**	0.30	**	0.52	**	0.46	**
Indian by depth	0.30	**	0.19	**	0.44	**	0.29	**
Pacific by depth	0.60	**	0.29	**	0.82	**	0.70	**
0-200 by ocean	0.76	**	0.53	**	0.40	**	0.25	**
200-500 by ocean	0.22	**	0.39	**	0.27	**	0.11	n.s.
500-1000 by ocean	0.33	**	0.28	**	0.12	*	-0.04	n.s.
1000-3000 by ocean	0.27	†	0.15	n.s.	0.04	n.s.	0.05	n.s.
1000-3000 by DWT	0.51	*	0.79	**	-0.01	n.s.	0.41	**

961

Table 2. Results of Mantel test for each combination of marker, beta-diversity measurement, and depth, between mesozooplankton communities' distances and oceanic distances (log-transformed), environmental distances, and each environmental variable separately (temperature, salinity, oxygen, fluorescence, conductivity, and PAR—photosynthetically active radiation), and between oceanic and environmental distances. † p-value <0.1, *<0.05.

	mlCOI								*mac18S*							
	Bray-Curtis				PCD				Bray-Curtis				PCD			
	0-200 m	200-500 m	500-1000 m	1000-3000 m	0-200 m	200-500 m	500-1000 m	1000-3000 m	0-200 m	200-500 m	500-1000 m	1000-3000 m	0-200 m	200-500 m	500-1000 m	1000-3000 m
Oceanic distance	0.58*	0.37*	0.48*	0.26*	0.45*	0.44*	0.46*	0.28*	0.21*	0.27*	0.13	0.37*	0.21*	0.19*	0.03	0.20*
Environmental distance	0.22*	0.32*	0.03	0.38*	0.35*	0.25*	0.23*	0.40†	0.15*	-0.01	0.18†	0.13†	0.05	0.21*	-0.2	0.35*
Temperature	0.19*	0.28*	-0.06	0.28†	0.13	0.18*	0.02	0.44*	0.11†	-0.12	0.24*	0.11	0.01	0.14*	-0.08	0.30*
Salinity	0.15*	0.18†	-0.05	0.36*	0.16†	0.19†	0.1	0.51*	0.04	0.11	0.17†	0.12	0.02	0.13†	-0.22	0.32*
Oxygen	0.20*	0.41*	0.35*	0.32*	0.43*	0.35*	0.41*	0.06	0.27*	0.17†	0.07	0.11	0.18*	0.25*	-0.04	0.29*
Fluorescence	-0.01	0.15	-0.08	0.2	0.22	0.26*	0.09	0.1	0.1	0.07	-0.03	0.09	0.01	0.23*	-0.2	0.22†
Conductivity	0.16*	0.26*	-0.07	0.30†	0.11	0.19*	0.02	0.46*	0.09	-0.11	0.24*	0.11	-0.001	0.12†	-0.12	0.32*
PAR	-0.04	-0.2	-	-	0.02	-0.24	-	-	-0.05	-0.06	-	-	-0.07	0.002	-	-
Oceanic *vs* environmental	0.26*	0.24*	0.17*	0.70*	0.26*	0.24*	0.17*	0.70*	0.16*	0.26*	0.12	0.66*	0.19*	0.21*	0.21*	0.68*