



Original Articles

COI metabarcoding better reveals the seasonal variations in the zooplankton community in the western Pacific Warm Pool

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ABSTRACT

The environment of the western Pacific Warm Pool is considered to be pronouncedly uniform and stable compared with other tropical ocean environments. Knowledge of the seasonal variations in the zooplankton community structure in this region with a high plankton species diversity remains limited. In this study, three methods, including identification using microscopy, ZooScan digital imaging, and DNA metabarcoding, were used to analyze zooplankton samples collected in three seasons, and to detect the seasonal variations in the community. The results show that the metabarcoding method based on the mitochondrial cytochrome *c* oxidase I (COI) marker detected up to 2.3 times more zooplankton taxa than identification using microscopy. In contrast, the small subunit ribosomal RNA (18S) marker revealed fewer taxa than microscopy. Significantly positive correlations were observed between the sequence counts and three types of quantitative data, including the biovolume, dry weight biomass, and carbon biomass in all three seasons. The COI sequence counts exhibited a stronger correlation with the dry weight biomass than the biovolume and carbon biomass. Although both the microscopic and molecular methods detected seasonal variability of the zooplankton community, the COI metabarcoding method was more sensitive in detecting seasonal variations in the community, which was positively correlated with the taxonomic resolution of the different methods. In summary, the results of this study suggest that COI metabarcoding is an ideal tool for monitoring the seasonal variations in the diversity and community structure of zooplankton in the tropical open ocean regions with a stable environment all year round.

1. Introduction

Zooplankton occupy a pivotal position in the marine ecosystem, as they transfer significant amounts of organic matter and energy from primary producers to upper trophic levels via the food web (Steinberg & Landry, 2017; Stock et al., 2014). The processes of nutrient regeneration and dissolved organic matter release by zooplankton regulate the rate of primary production by phytoplankton, particularly in oligotrophic ocean regions (Banse, 1995). Many zooplankton taxa react rapidly to shifts in the environment and are sensitive to climate change (Buttay et al., 2015; Hays et al., 2005; Richardson, 2008). Therefore, information about the biodiversity and community dynamics of zooplankton is fundamental for understanding marine ecological processes.

Conducting biological monitoring in the western tropical Pacific Ocean has long been a challenge, due to its reputation as a biodiversity hotspot with remarkably high zooplankton species richness (Rombouts et al., 2010; Tittensor et al., 2010). The systematic complexity and high diversity of zooplankton make the traditional microscopy-based identification method time-consuming and laborious. Digital imaging analysis offers a semi-automated approach for determining the biomass and size structure, which is valuable in ecological studies. However, this method could only identify zooplankton at a coarse taxonomic level (Gorsky et al., 2010). In recent years, DNA metabarcoding has emerged as an economical and accurate method for detecting higher marine zooplankton diversity and for better characterizing the spatial-temporal distribution patterns than morphological approaches in various

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environments (e.g., Berry et al., 2019; Chain et al., 2016; de Vargas et al., 2015; Deagle et al., 2018; Kiemel et al., 2022; Lindeque et al., 2013; Robinson et al., 2022; Xie et al., 2021). However, linking read counts to actual abundance data obtained using morphological methods has been a recurring challenge in metabarcoding (Bucklin et al., 2016). Meta-analysis of 22 metabarcoding articles targeting various organisms suggests that this method possesses some quantitative value (Lamb et al., 2019). For zooplankton, the sequence numbers are more closely correlated to biomass than abundance due to significant size variations among different groups (Ershova et al., 2021). Matthews et al. (2021) found that there is a significant positive correlation between the reads number and carbon biomass. However, other studies did not find such a correlation due to bias from interindividual variation and the metabarcoding process (Piñol et al., 2015; Sun et al., 2015). The quantification ability of metabarcoding can also be affected by the characteristics of samples, and this method is more quantitative when analyzing samples from tropical ecosystems with high diversity and low evenness compared to those from temperate ecosystems (Piñol et al., 2019). In the highly diverse western Pacific Ocean ecosystem, the adequacy of DNA metabarcoding to identify components of the zooplankton community and estimate their composition from read counts has not been fully evaluated. Given the importance of species richness and quantitative data to understanding zooplankton community dynamics, it is necessary to assess the suitability of DNA metabarcoding prior to ecological analysis in the western tropical Pacific Ocean.

The subtropical and tropical oceans are commonly regarded as low-seasonality areas compared to temperate and subarctic areas. Nonetheless, strong seasonal variations in the zooplankton community have been reported in the South China Sea (Li et al., 2021; Ren et al., 2021; Tseng et al., 2008) and the Arabian Sea (Aparna et al., 2022; Pieper et al., 2001; Smith et al., 1998), which are greatly influenced by the monsoons. Even at ALOHA station in the North Pacific Subtropical Gyre, well-known for its stable hydrography and environment, seasonal fluctuations in zooplankton have been reported (Hannides et al., 2020; Huntley et al., 2006; Landry et al., 2001; Sheridan & Landry, 2004; Valencia et al., 2016). The western tropical Pacific has been considered to be the region with the lowest seasonal variability in the Pacific Ocean (Delcroix, 1993). There is a large stable water body known as the western Pacific Warm Pool (WPWP), which has a permanent surface temperature exceeding 28–29 °C (Cravatte et al., 2009; de Garidel-Thoron et al., 2005). The WPWP is critical to the global climate because its high surface temperature facilitates evaporation and precipitation, resulting in the strongest ocean–atmosphere interaction worldwide (Deckker, 2016). The intense rainfall results in low salinity of the sea surface water, thereby resulting in a robust and consistent barrier layer between the shallow halocline and the deeper thermocline. This barrier persists regardless of the season, leading to remarkably low seasonality of the hydrography and productivity in the WPWP compared to other areas in the western tropical Pacific Ocean (Longhurst, 2007; Lukas & Lindstrom, 1991; Mignot et al., 2007). Understanding the seasonal variations in the zooplankton community in this pronouncedly uniform region is essential to predicting the effects of climate change and the related ecological processes. Since the seasonal shifts in the zooplankton community in this region are assumed to be more gradual, the high-resolution DNA metabarcoding method is required to obtain detailed biodiversity and community composition information.

In this study, we utilized DNA metabarcoding techniques that rely on the mitochondrial cytochrome oxidase I gene (COI) and the small subunit ribosomal RNA gene (18S) to analyze zooplankton samples collected during summer, autumn, and winter via 0–200 m vertical hauls in the WPWP. Alongside this approach, we conducted microscopic identification to acquire richness and abundance data while also utilizing ZooScan digital imaging to estimate three distinct types of quantitative data (i.e., biovolume, dry weight biomass, and carbon biomass). The first aim of this study was to assess the adequacy of metabarcoding to monitor the zooplankton community in the WPWP. We expected that

metabarcoding would reveal a greater zooplankton richness than microscopy and that the composition of the zooplankton community in terms of read counts would be comparable to that in terms of the ZooScan data. The second aim was to examine the variability of the zooplankton community across different seasons within this notably stable environment. Our earlier research in the western tropical Pacific demonstrated that the COI gene, with a superior resolution, was more effective at describing the spatial variability of zooplankton communities compared to the 18S and morphology (Feng et al., 2022). Therefore, we expected that the COI metabarcoding method would better capture the seasonal variability of the zooplankton community compared to other approaches.

2. Materials and methods

2.1. Field sampling

Three cruises were conducted in summer, autumn, and winter in the WPWP. The summer investigation was carried out on the R/V Dayanghao in July–August 2020, while the winter and autumn investigations were carried out on the R/V Shenhaiyihao in January and November 2021, respectively. At a total of 18 stations (6 per season) located on the Kyushu–Palau Ridge (Fig. 1), zooplankton samples were collected by towing from 200 m depth to the surface using a WP2 net with a mesh size of 200 µm. The volume of filtered water was measured by a flowmeter (Hydro-Bios, Germany) installed on the mouth of the net. All samples were split evenly into two subsamples. One was preserved in 5 % (v/v) buffered formalin-seawater solution for morphological analysis and the other was preserved in 95 % ethanol for metabarcoding analysis. Temperature and salinity were obtained in situ using a Sea-Bird conductivity-temperature-depth (CTD) sampler (SBE 911 plus, Seabird, United States). The Chlorophyll α (Chl α) concentrations were determined using a Trilogy Laboratory Fluorometer (Model 7200, Turner Designs, United States) after overnight pigment extraction with 90 % aqueous in darkness (Parsons et al., 1984), based on water samples collected every 25 m from 200 m depth to the surface. The monthly average surface Chl α concentrations of the western trophic Pacific Ocean in August 2020, January 2021, and November 2021 were obtained through E.U. Copernicus Marine Service Information (<https://doi.org/10.48670/moi-00015>) for analysis purposes. For comparison, sea surface temperature (SST) of the ALOHA station in August 2020, January 2021, and November 2021 were downloaded from the HOT website (<https://hahana.soest.hawaii.edu/hot/hot-dogs/i>

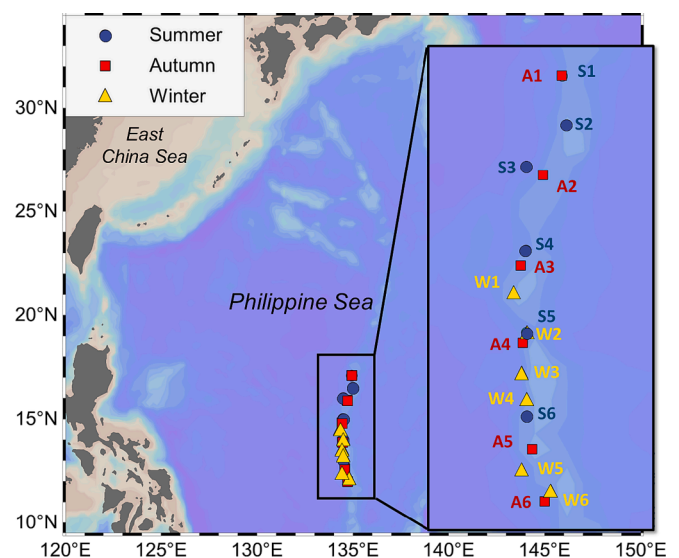


Fig. 1. Location of the sampling stations during summer, autumn, and winter.

ndex.html), a repository of Hawaii Ocean Time-series observations supported by the U.S. National Science Foundation under Award #1756517".

2.2. Morphology analysis

2.2.1. Microscopic identification

After large particles were removed, each formaldehyde-fixed zooplankton sample was resuspended in 100 mL of sterile water. Following this, 1–4 % fractions of each zooplankton sample (200–300 individuals) were extracted for visual analysis. Each individual was identified to the lowest taxonomic level possible (Razouls et al., 2005–2023; Zheng, et al., 1984) and counted under a stereomicroscope (ZEISS SteREO Discovery V20, German). The entire sample was then screened for large zooplankton such as medusae, chaetognaths, and euphausiids. Zooplankton abundance was expressed as individuals m^{-3} (ind. m^{-3}).

2.2.2. ZooScan imaging

After microscopic identification, zooplankton samples were imaged by a ZooScan digital imaging system (Hydroptic, France), following the details described by Chen et al. (2020). The detected organisms were then automatically sorted into nine zooplankton groups (including Calanoida, Cyclopoida, Medusa, Ostracoda, Polychaeta, Chaetognatha, Malacostraca, Gastropoda, and Tunicata) and others. Any misidentified particles were moved to the correct folder under manual inspection. The objects identified as “detritus” and bubbles” were removed before further analysis. The biovolume of each object was calculated based on their equivalent spherical diameter (ESD). The dry weight biomass of zooplankton was calculated using the taxon-specific “body area-individual dry mass” conversion equation (Lehette & Hernández-León, 2009). The carbon biomass was calculated using the taxon-specific “dry weight biomass-carbon” conversion equation (Postel et al., 2000). ESD and body area of each project were provided by ZooScan system. Zooplankton biovolume, dry weight biomass, and carbon biomass were standardized for the filtered water volume of each sample and expressed as $mm^3 m^{-3}$, $mg m^{-3}$, and $mg m^{-3}$, respectively.

2.3. Metabarcoding analysis

2.3.1. DNA extraction, amplification, and sequencing

Ethanol-fixed zooplankton samples were fully ground into powder. DNA extraction was done using the EasyPure Marine Animal Genomic DNA Kit (TransGen Biotech, China). For the details, see Li et al. (2022). The primer set mlCOIintF, (5'-GGWACWGGWTGAACWGTWTAAYC-CYCC-3') and jgHCO2198, (5'-TAIACYTCIGGRTGICRAARAAYCA-3') (Geller et al., 2013; Leray et al., 2013) was used to amplify a 313-bp region of the COI gene. Additionally, the primers SSUF04 (5'-GCTTGCTCAAAGATTAAGCC-3') and SSUR22mod (5'-CCTGCTGCTTCCTTRGA-3') (Fonseca et al., 2010; Sinniger et al., 2016) were chosen to amplify the 365-bp V1-V2 region of 18S. PCR amplification, purification, and quantification were conducted following previously described methods (Li et al., 2022). High-throughput sequencing (HTS) was performed on an Illumina NovaSeq-PE250 platform using paired-end sequencing (2 × 250 bp) by Mingke Biotechnology, China.

2.3.2. Bioinformatics

Raw sequences were demultiplexed using the demux plugin followed by primers and tags cut using the Cutadapt (Anderson, 2001) plugin in QIIME 2 (Caporaso et al., 2010). The DADA2 plugin (Callahan et al., 2016) was used for filtering, dereplicating, merging paired-end reads, and chimeras removal. Details were described by Feng et al. (2022). Singletons and doubletons were removed from the datasets. For COI, the generated amplicon sequence variants (ASVs) were annotated using BLAST against the MetaZooGene Barcode Atlas and Database (MZGdb;

<https://metazoogene.org/mzgdbs>). The assignments were then checked and corrected based on a similarity threshold for each taxonomic level described by Laroche et al. (2020). For 18S, ASVs were assigned to the lowest common ancestor of the top five BLAST hits against the NCBI nt database. To standardize the sequencing effort, the ASV tables were subsampled to the lowest obtained sequencing depth (26,804 for COI and 21,382 for 18S) by the “vegan” package (Oksanen et al., 2020) in R ver.4.1.2 (R Development Core Team, 2019). Prior to data analysis, we discarded the COI hits with <80 % sequence similarity following Djurhuus et al. (2020), and the 18S hits that were unclassified at the phylum level. By consulting the World Register of Marine Species (WoRMS Editorial Board, 2023), we removed all freshwater taxa, insects, arachnids, and chordates other than tunicates to focus on marine zooplankton. Rarefaction curves of ASV richness were generated using the “vegan” package for all samples.

2.4. Data analysis

The mean temperature/salinity/Chl α concentration of the top 200 m (Ave-T/S/Chl α) were calculated by means of a trapezoidal integration of the values at the different depths. All environmental variables were $\log_{10}(X+1)$ transformed and normalized prior to analysis. To explore the seasonal variations of environmental conditions, cluster analysis was conducted based on the Euclidean distance, and principal component analysis (PCA) was conducted. Regarding the zooplankton data, unique lowest-level taxa were counted when comparing the richness observed using metabarcoding and microscopy. To compare the quantitative composition of zooplankton communities across methods, the datasets generated using metabarcoding and microscopy were collapsed to the same taxonomic group as the datasets obtained from ZooScan (e.g., all Calanoida species/ASVs collapsed to a group summing the individual abundance/reads). Only the nine main zooplankton groups (Calanoida, Cyclopoida, Medusa, Ostracoda, Polychaeta, Chaetognatha, Malacostraca, Gastropoda, and Tunicata) were included in further comparison. Spearman correlation analysis was carried out on the sequence numbers and ZooScan data (including the biovolume, dry weight biomass, and carbon biomass) with Bonferroni correction after the datasets were square-root transformed.

To fully utilize the data, alpha and beta diversity were analyzed at the lowest-level taxa level for microscopy and ASV level for metabarcoding, respectively. Boxplots containing Kruskal-Wallis tests were plotted for the microscopic abundance, biovolume, dry weight biomass, and Shannon–Wiener diversity index of microscopy, COI, and 18S among the three seasons in the Genescloud platform (<https://www.genescloud.cn>). To avoid bias from rare organisms, only species/ASVs contributing to at least 1 % of the total abundance/reads were used for community structure analysis. Using Bray-Curtis similarity matrices generated from method-specific datasets after square-root transformation, non-metric multidimensional scaling (NMDS) was conducted to explore seasonal variations in zooplankton communities. Permutational multivariate analysis of variance (PERMANOVA) was performed to test for significant differences of zooplankton community structure. To identify key species/ASVs responsible for dissimilarities, similarity percentage analysis (SIMPER) was applied to microscopy and metabarcoding datasets. Finally, redundancy analysis (RDA) was applied to examine the effect of environmental factors on zooplankton communities with forward selection, since the longest gradient lengths were <3 for all zooplankton datasets in the detrended correspondence analysis.

The PCA, NMDS and RDA were implemented using CANOCO v5 (Microcomputer Power, USA). The SIMPER analysis, cluster analysis and PERMANOVA tests were conducted using the “vegan” package and “factoextra” package (Lê et al., 2008) in R ver.4.1.2.

3. Results

3.1. Environmental conditions

The temperature was the highest at the surface (Summer: 30.22 ± 0.12 °C; Autumn: 29.52 ± 0.14 °C; Winter: 28.79 ± 0.07 °C), and it gradually decreased with depth (Fig. 2). Our study area had a lower seasonal variability in the SST compared to that at ALOHA station (Table S2). The Ave-T were 26.75 ± 0.29 °C, 26.31 ± 0.42 °C, and 26.16 ± 0.48 °C in summer, autumn, and winter, respectively. The salinity was the lowest at the surface and the highest at around 150–170 m (Fig. 2). The surface salinity was the highest in summer (34.52 ± 0.30), followed by that in autumn (34.21 ± 0.02), and it was the lowest in winter (33.90 ± 0.16). The Ave-S were 34.77 ± 0.06 , 34.63 ± 0.03 , and 34.48 ± 0.07 in summer, autumn, and winter, respectively. The western trophic Pacific Ocean is highly oligotrophic, with surface Chl α concentrations of less than $0.05 \mu\text{g L}^{-1}$ all year round (Fig. S1). In our study area, the Ave-Chl α was the highest in summer ($0.12 \pm 0.02 \mu\text{g L}^{-1}$), followed by that in autumn ($0.10 \pm 0.01 \mu\text{g L}^{-1}$), and it was the lowest in winter ($0.09 \pm 0.01 \mu\text{g L}^{-1}$). Generally, the temperature, salinity, and Chl α varied slightly with the season in the survey area. The results of the cluster analysis and PCA indicated the presence of significant seasonal variations in the environmental conditions (Fig. 3).

3.2. Richness

For COI, the HTS process produced a total of 891,812 raw sequences. The sequencing number of each sample ranged from 33,461 (W1) to 61,301 (A1), with an average of 49,545. After all of the filtering steps and subsampling, 455,448 reads clustered into 2,983 zooplankton ASVs were included in the final dataset. The ASV number of each sample ranged from 221 (A4) to 450 (S6), with an average of 316. For 18S, 906,569 raw sequences were obtained. The sequencing depth of the samples ranged from 32,645 (S3) to 61,242 (A4), with an average of 50,365. A total of 950 ASVs comprising 380,752 reads passed all of the filtering and subsampling steps. The ASV number of the samples varied from 111 (W4) to 247 (A2), with an average of 177. The numbers of raw sequences and ASVs per sample are shown in Table S1. The rarefaction curves for both markers demonstrate that the sequence depths were sufficient for all of the samples (Fig. S2).

Out of the 2,983 COI ASVs analyzed, 41 %, 14 %, and 19 % were assigned to the species, genus, and family levels, respectively. In total, 441 unique taxa were identified, belonging to 10 phyla, 22 classes, 60 orders, 122 families, 176 genera, and 216 species. Calanoida was the most dominant group, accounting for 22 % of the total unique taxa and 48 % of the total reads. It was followed by Medusa and Malacostraca

(Table 1). For the 950 18S ASVs analyzed, 9 %, 37 %, and 22 % were assigned to the species, genus, and family levels, respectively. In total, 164 unique taxa were identified, belonging to 13 phyla, 23 classes, 56 orders, 81 families, 79 genera, and 31 species. Calanoida was also the most dominant group, accounting for 19 % of the total unique taxa and 56 % of the total reads. It was followed by Medusa and Gastropoda (Table 1). The microscopic analysis identified 191 unique taxa, belonging to 6 phyla, 11 classes, 21 orders, 55 families, 105 genera, and 142 species. Calanoida was once again the most dominant group, accounting for 33 % of the total unique taxa and 53 % of the total counts. Medusa accounted for 14 % of the total unique taxa but for only 1 % of the total abundance. Cyclopoida accounted for 13 % of the total unique taxa and 33 % of the total abundance (Table 1).

3.3. Morphology versus metabarcoding

3.3.1. Comparison of richness detected via microscopy and metabarcoding

A total of 21 zooplankton taxonomic groups were identified using a combination of metabarcoding and microscopic identification. All of the groups detected by using microscopy were also identified by using COI metabarcoding, but the 18S metabarcoding failed to detect Ostracoda, Mormonilloida, and Cephalopoda. Eight additional groups, predominantly meroplankton, were only identified via metabarcoding. Hemichordata, Phoronida, and Platyhelminthes were exclusively identified via 18S metabarcoding (Table 1). The taxa richness detected via COI (441) metabarcoding was 2.3 times higher than that detected via microscopy (191), while the taxa richness detected via 18S (164) metabarcoding was slightly lower than that detected via microscopy. In all three seasons, the COI metabarcoding identified more unique taxa for most groups, particularly Medusa and Malacostraca, but it detected fewer taxa for Cyclopoida and Tunicata compared to microscopy; while 18S metabarcoding identified a lower taxa richness than microscopy or COI metabarcoding, with the exception of Polychaeta and Tunicata (Fig. S3). For the most dominant group (Calanoids), 14 families (74 %) were detected via the two markers and microscopy. This overlap percentage decreased to 21 % at the genus level, with only 10 out of 48 genera shared by all three methods. The overlap percentage was higher between COI metabarcoding and microscopy than between 18S metabarcoding and microscopy (Fig. S4). Specifically, *Clausocalanus* was the most abundant genus detected via both COI metabarcoding and microscopy, but it had a low sequence occurrence in the 18S dataset. Four genera were only detected via microscopy: *Euaugaptilus*, *Eucalanus*, *Lophothrix*, and *Metridia*. At the species level, none were shared by the three approaches. The most abundant Calanoid species in the microscopy dataset (>1% of the total abundance) were *Acartia negligens* (71.62 ind.m^{-3}), *Cosmocalanus darwinii* (50.61 ind.m^{-3}), and *Lucicutia flavicornis* (33.19 ind.m^{-3}). They were also detected via COI metabarcoding with a high sequence abundance, but not via 18S metabarcoding. In addition, 25 Calanoid species were identified exclusively via microscopy, e.g., *Scolecithricella nicobarica* (7.22 ind.m^{-3}), *Scaphocalanus longifurca* (6.13 ind.m^{-3}), *Pleuromamma quadrangulata* (5.56 ind.m^{-3}), *Haloptilus paralonicirrus* (5.23 ind.m^{-3}), and *Centropages elongatus* (5.13 ind.m^{-3}).

3.3.2. Comparisons of relative composition in terms of microscopic abundance, ZooScan data, and sequence counts

The relative composition of the zooplankton groups in terms of microscopic abundance and other data varied greatly (Fig. 4). Calanoida and Cyclopoida were the dominant groups in the microscopic abundance dataset in all three seasons, and the other groups only accounted for ≤ 15 %. In terms of the ZooScan data (including the biovolume, dry weight biomass, and carbon biomass) and sequence data, Calanoida was consistently the most dominant group, while the proportions of Cyclopoida were considerably low. Chaetognatha also emerged as a significant contributor to the biovolume, accounting for 20–40 % among the different seasons, but it contributed less to the biomass and sequence

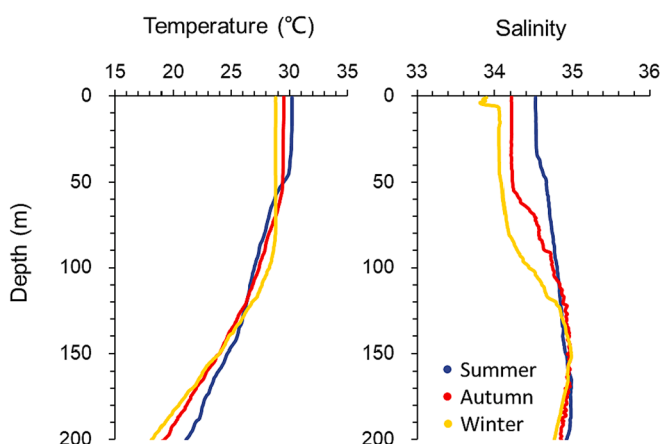


Fig. 2. Vertical profiles of temperature and salinity from surface to 200 m deep. Each line represents the average value for each season.

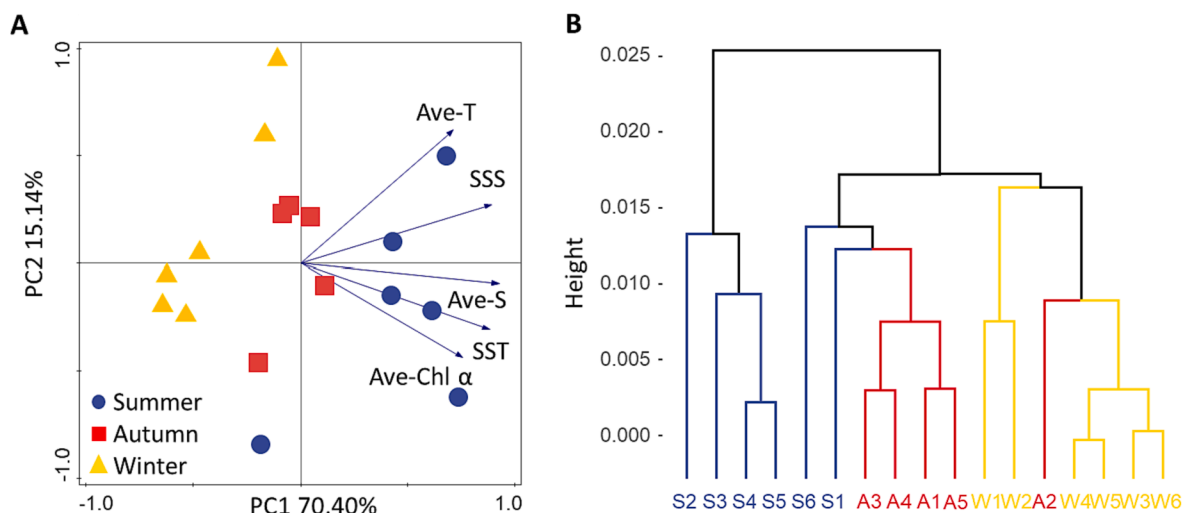


Fig. 3. Results of the (A) principal component analysis (PCA) and (B) cluster analysis of the environmental variables. Ave-T/S/Chl α refer to the average concentration of T/S/Chl α of the upper 200 m, respectively.

Table 1

The numbers of unique taxa and proportions of reads/abundance resolved by metabarcoding (COI and 18S) and microscopy.

Group	COI		18S		Microscopy	
	No. unique taxa	% Reads	No. unique taxa	% Reads	No. unique taxa	% Abundance
Calanoida	95	47.65	31	55.91	63	53.03
Cyclopoida	23	3.66	4	3.76	25	32.72
Medusa	68	11.34	21	7.22	27	0.98
Ostracoda	22	3.64	–	–	12	2.57
Polychaeta	12	1.10	15	4.37	11	0.80
Chaetognatha	16	3.62	5	4.20	7	1.71
Malacostraca	57	11.49	16	10.09	25	1.54
Gastropoda	51	3.28	22	2.44	4	0.53
Tunicata	3	0.01	15	10.92	6	4.87
Harpacticoida	1	<0.01	2	<0.01	3	0.09
Mormonilloida	1	0.04	–	–	2	0.26
Siphonostomatoida	1	<0.01	1	<0.01	–	–
Branchiopoda	2	0.63	1	0.05	1	0.17
Anthozoa	5	0.02	4	0.11	–	–
Cephalopoda	8	0.01	–	–	1	<0.01
Echinodermata	2	0.56	7	0.12	–	–
Nemertea	6	0.12	1	<0.01	–	–
Sipuncula	6	0.04	4	0.01	–	–
Hemichordata	–	–	2	0.07	–	–
Phoronida	–	–	1	<0.01	–	–
Platyhelminthes	–	–	1	<0.01	–	–

data. The COI sequence dataset featured a higher proportion of Ostracoda compared to the other datasets, while the 18S sequence data indicated that Polychaeta and Tunicata were more abundant. Generally, the relative composition of each zooplankton group in terms of the sequence data was more similar to that of the dry weight biomass than to that of the carbon biomass owing to the much lower proportion of Medusa in the latter (Fig. 4). The results of the Spearman correlation analysis indicate that the sequence reads from both markers were positively correlated to the ZooScan data in all three seasons (Fig. 5). In particular, for COI, the sequence reads had the strongest correlation with the dry weight biomass and the weakest correlation with the biovolume, whereas for 18S, the sequence reads were most closely related to the dry weight biomass in summer and to carbon biomass in autumn and winter (Fig. 5).

3.4. Seasonal variations of zooplankton

The zooplankton abundance was the highest in summer ($150.94 \pm 50.84 \text{ ind.m}^{-3}$), followed by that in autumn ($88.00 \pm 49.74 \text{ ind.m}^{-3}$),

and it was the lowest in winter ($61.16 \pm 11.4 \text{ ind.m}^{-3}$) (Fig. S5). The relative abundance of Calanoida decreased from summer to winter. Tunicata had the highest relative abundance in autumn (Fig. 4). The zooplankton biovolume was also the highest in summer ($63.54 \pm 18.24 \text{ mm}^3 \text{ m}^{-3}$), followed by that in autumn ($36.85 \pm 19.99 \text{ mm}^3 \text{ m}^{-3}$), and it was the lowest in winter ($28.51 \pm 28.12 \text{ mm}^3 \text{ m}^{-3}$). The dry weight biomass of zooplankton was also the highest in summer ($3.15 \pm 1.02 \text{ mg m}^{-3}$), followed by that in autumn ($1.84 \pm 1.09 \text{ mg m}^{-3}$), and it was the lowest in winter ($1.52 \pm 0.54 \text{ mg m}^{-3}$) (Fig. S5). The contribution of Malacostraca to the dry weight biomass was the highest in summer and the lowest in autumn, while that of Chaetognatha was the lowest in summer (Fig. 4). The Shannon-Wiener diversity index obtained via COI metabarcoding and that obtained via microscopy were the highest in summer and the lowest in autumn, while that obtained via 18S metabarcoding was the highest in autumn and the lowest in winter. The results of the Kruskal-Wallis tests indicate that there were significant seasonal variations in the abundance, dry weight biomass, and COI diversity index (Fig. S5).

Both the metabarcoding and microscopy methods identified notable

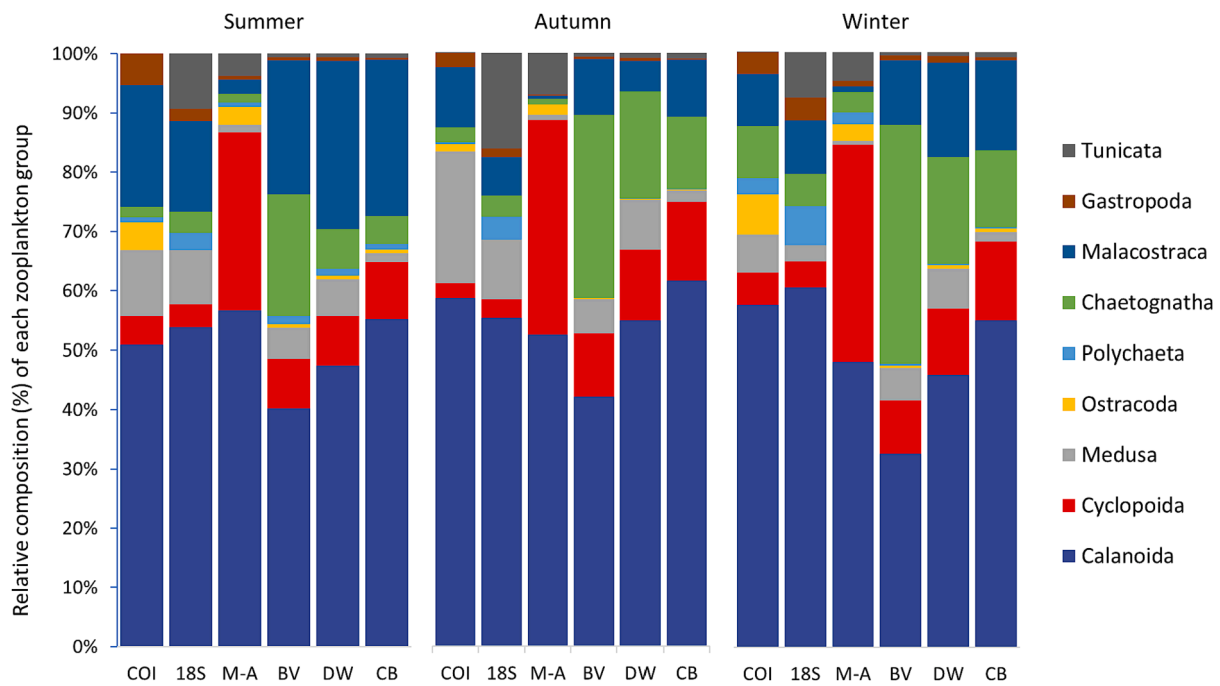


Fig. 4. Relative composition of the zooplankton groups in terms of the metabarcoding sequence data (COI and 18S), microscopic abundance (M–A), and ZooScan data including the biovolume (BV), dry weight biomass (DW), and carbon biomass (CB).

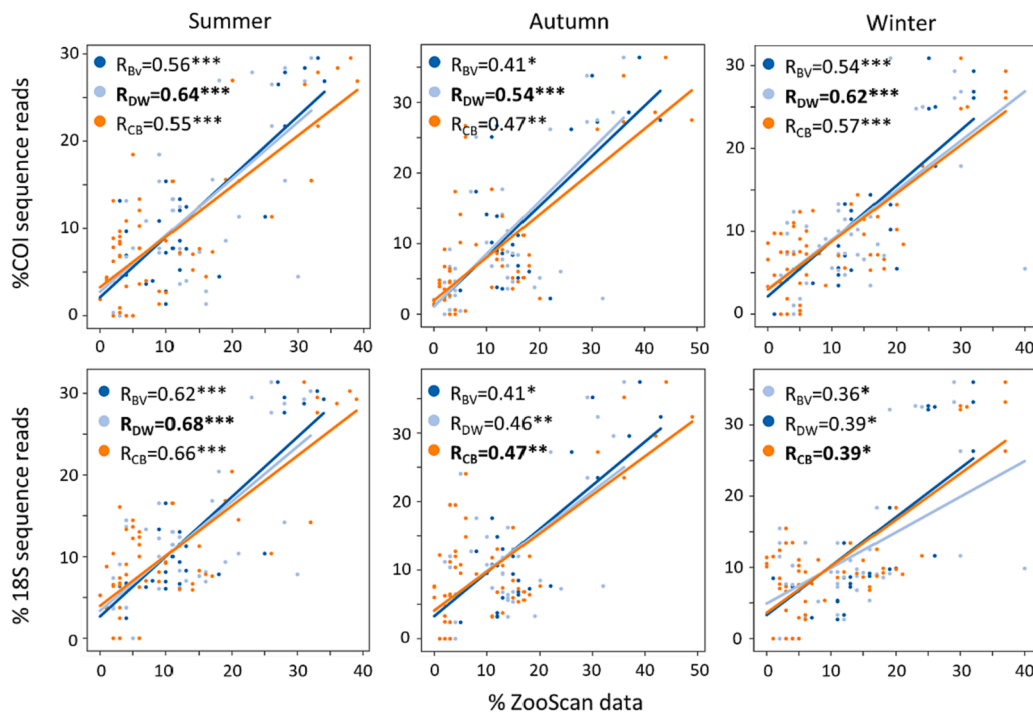


Fig. 5. Linear relationship between the square-root transformed proportion of the sequence reads (COI and 18S) and the proportion of ZooScan data (biovolume, dry weight biomass, and carbon biomass) of nine zooplankton groups at each station. The sequence reads that were used represent a summation of the reads of all of the ASVs identified in each group. The blue, gray, and orange symbols represent the biovolume (BV), dry weight biomass (DW), and carbon biomass (CB), respectively. The Spearman correlation coefficient (R) between every two methods is noted in the corresponding colors. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$. The largest R value in each plot is marked in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seasonal variations within the zooplankton community (Fig. 6). The sequence reads obtained via metabarcoding based on both markers revealed clear separations between each pair of seasons (Fig. 6A and B). It should be noted that the PERMANOVA tests revealed that the seasonal

separation of the COI community was greater than that of the 18S community (Fig. 6D). The results of NMDS ordination analysis and PERMANOVA tests based on microscopic abundance also revealed significant differences in zooplankton community among the seasons, but

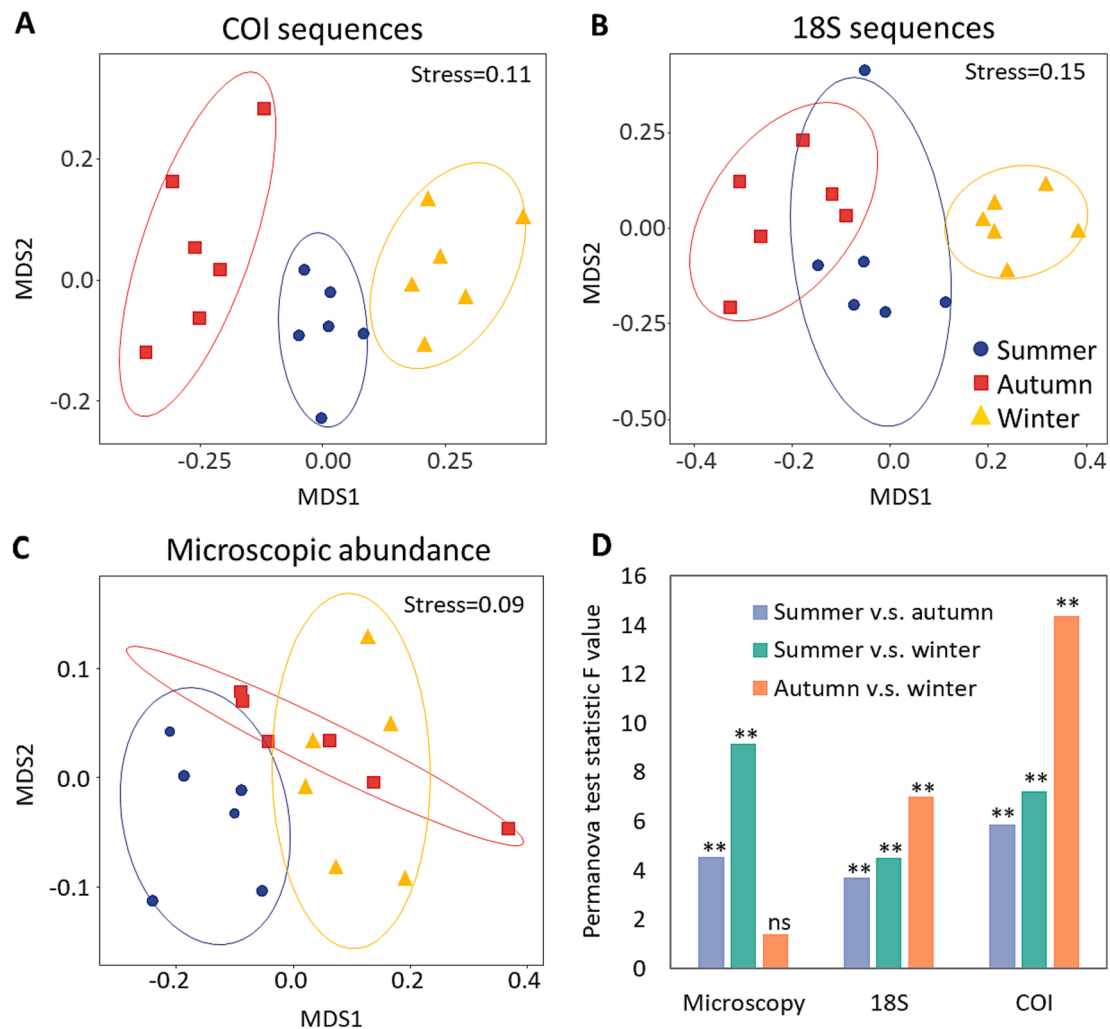


Fig. 6. Nonmetric multidimensional scaling (NMDS) plots of the zooplankton community in terms of the (A) COI metabarcoding sequences, (B) 18S metabarcoding sequences, and (C) microscopic abundance based on Bray-Curtis similarities; (D) Permutational multivariate analysis of variance (PERMANOVA) tests results for the zooplankton community across seasons.

the autumn and winter groups were not distinguishable from one another (Fig. 6C). However, the variation between the summer and winter groups in terms of the microscopic abundance was more significant than that in terms of the metabarcoding sequences (Fig. 6D).

The SIMPER analysis was utilized to identify the top two taxa/ASVs that contributed significantly to the zooplankton community variations between the seasons (Table S3). By combining all the methods, 12 taxa/ASVs were identified and their relative abundances were represented using the heatmap (Fig. 7). In the COI dataset, ASV1 (*Acartia negligens*) and ASV2 (*Clausocalanus furcatus*) were the most prevalent in autumn, while ASV3 (*Paracalanidae* sp.) was predominantly present in winter. In the 18S dataset, ASV1 (*Calanidae* sp.) and ASV22 (*Alciopidae* sp.) were prominently present in winter, while ASV14 (*Euphausia* sp.) was mostly present in summer. In the microscopic abundance dataset, *Acartia negligens* and *Euchaeta* larvae were more abundant in summer than in the other seasons.

According to the results of RDA, the explained variation of each environmental variable to the zooplankton communities varied depending on the type of data. Specifically, Ave-S was selected first in the two RDAs based on sequence data from COI and 18S, which explained 10.6 % and 11.3 % of the total variations, respectively. However, Ave-Chl α was identified as the most important contributor in the RDA models based on microscopic abundance, with an explained variation of 29.1 %. The total amount of explained variations was

observed to be higher in the analyses based on the microscopic abundance than those based on sequence data.

4. Discussion

4.1. Evaluation of the adequacy of the metabarcoding method

To mitigate the potential for overestimation of the richness resulting from intraspecific sequence variations, in this study, we compared the numbers of unique lowest-level taxa obtained using the metabarcoding and microscopy methods. We found that the COI metabarcoding detected all of the major zooplankton groups and produced up to 2.3 times greater taxa richness compared to microscopy (Table 1), even though 26 % of the COI sequence was assigned only to the order and higher taxonomic ranks. This finding is consistent with those of other studies (Cicala et al., 2022; Djurhuus et al., 2018; Schroeder et al., 2020; Suter et al., 2021), which demonstrates the superior performance of COI metabarcoding in detecting zooplankton richness compared to microscopy. This could be due to many reasons. First, the subsampling required for microscopy, given the large sample sizes, resulted in the underestimation of rare taxa (Blanco-Bercial, 2020; Meredith et al., 2021). In the metabarcoding analysis, homogenizing samples prior to DNA extraction increased the detection of low-abundance taxa (Pagenkopp Lohan et al., 2019). Second, the microscopy method greatly

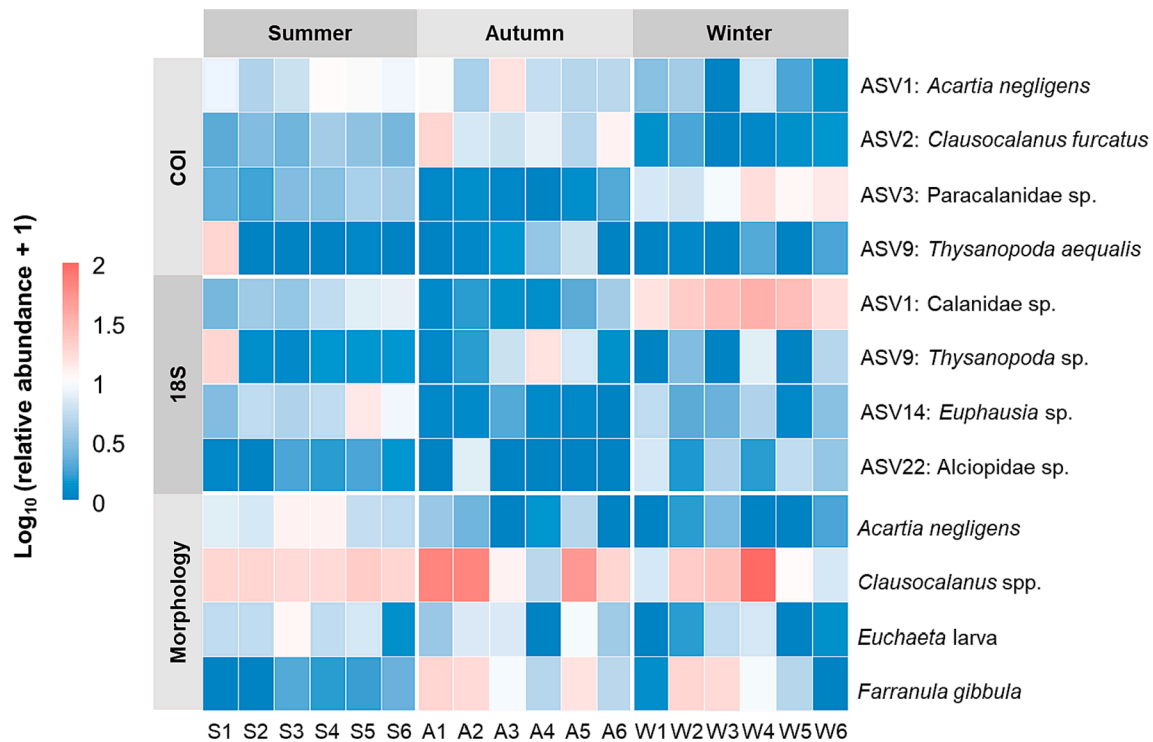


Fig. 7. Heatmap of the key taxa or ASVs contributing to the seasonal variation in the zooplankton community, which were identified via similarity percentage analysis (SIMPER). The relative abundance of each taxon was scaled via $\log_{10}(X+1)$ transformation.

depends on the expertise of the researchers, which could explain the few types of many zooplankton groups identified in this study. Even for the most well-studied and diverse group of copepods, misidentification and underestimation by the morphological method would exist. However, DNA metabarcoding is deemed to be an accurate method as it can detect zooplankton species with few sufficient morphological characteristics, including cryptic species and larvae (Bucklin et al., 2016). For example, organisms from the highly abundant genus *Clausocalanus* presented identification challenges at the species level, particularly in their early life stages, resulting in them being grouped as *Clausocalanus* spp. during microscopic identification in this study. In comparison, the COI metabarcoding method identified 10 *Clausocalanus* species. Third, it is important to note that the efficiency of metabarcoding greatly depends on the quality and completeness of the reference database. The MZGdb database utilized in this study is a reliable and taxonomically-complete database that provides high-quality COI sequences for a wide range of marine zooplankton (Bucklin et al., 2021).

The metabarcoding method also has some inadequacies. First, the reference sequences are primarily obtained from organisms caught and identified using traditional plankton nets, which may introduce a net bias. Some errors of reference sequences (Bidartondo, 2008) will also lead to incorrect assignments and a discrepancy between the results of morphological and molecular methods. Second, even in the well-explored calanoids copepods group, which has rich reference sequences, more than half of the species detected via microscopy were not identified via metabarcoding (Fig. S4). This outcome is likely due to the low abundance and high intraspecific variability of these species (Schroeder et al., 2020). Third, the taxa recovered through metabarcoding may originate from environmental DNA (eDNA), i.e., genetic materials released by organisms. This eDNA can be transported from other areas by currents or predators, leading to an overestimation of the taxa richness identified via metabarcoding. However, the eDNA concentration is typically low relative to that of full specimens, and thus, it has little effect on the overall pattern of the zooplankton community structure. By comparing the two markers, it was found that the 18S

method identified significantly fewer taxa at the species level and failed to recover certain dominant species compared to the COI method, likely due to its highly conservative nature (Lindeque et al., 2013; Tang et al., 2012; Wu et al., 2015). However, in some instances, the COI method underperformed the 18S method due to a lack of sufficient reference sequences and difficulties in the amplification of several groups (Djuruhuus et al., 2018; Sommer et al., 2017; Zhan et al., 2014). As the methodology of metabarcoding continues to mature and the reference database is expanded, the superiority of the COI biomarker will become more evident, particularly when analyzing highly diverse communities.

Our results revealed that there were significant correlations between the sequence reads and the three types of quantitative data (i.e., biovolume, dry weight biomass, and carbon biomass) estimated via ZooScan imaging (Fig. 5). The correlation between the sequence counts and biovolume was the weakest, likely due to the overestimation of chaetognaths in terms of the biovolume. Chaetognaths are semi-gelatinous plankton that have a lower dry mass per unit area than other zooplankton groups (Lehette & Hernández-León, 2009). The large number of Chaetognatha in our samples contributed to the strong discrepancy between their proportional contributions to the biovolume and biomass. When comparing the two types of biomass data, we noted that the sequence reads exhibited a closer relationship to the dry weight biomass than to the carbon biomass (Fig. 5) since medusae with a low carbon content were underestimated in terms of the carbon biomass (Fig. 4). Metabarcoding has the potential to estimate the carbon flux associated with zooplankton, as ZooScan does, as long as proper conversion factors are applied to specific groups such as medusae. Notably, the cyclopoids with a high microscopic abundance were strongly underestimated in the sequence counts (Fig. 4), likely due to their small body size. Nevertheless, the proportions of their reads were also lower than those of the biovolume and dry weight biomass (Fig. 4). These small copepods are essential in the food web in the oligotrophic area as they feed on microzooplankton (Frangoulis et al., 2016; Paffenhöfer, 1993), which are the main primary consumers when the phytoplankton biomass is low (Calbet & Landry, 2004; Sherr & Sherr, 2002). This

underestimation of cyclopoids reflects the inadequacy of metabarcoding to some extent. Many steps in the metabarcoding protocols can introduce uncertainty, such as the primer bias, uneven amplification efficiency, and variabilities in the copy numbers of different zooplankton groups (Lamb et al., 2019; McLaren et al., 2019; Piñol et al., 2019). On the other hand, the ZooScan system can only provide accurate body area measurements (Gorsky et al., 2010). Therefore, the conversions from body area to dry weight biomass and carbon biomass also produce biases. For example, chaetognaths and gastropods have size-dependent conversion factors since the water content of their body composition increases as their size increases (Kjørboe, 2013). Furthermore, due to the resolution limitation of the ZooScan system, we could only obtain the average quantitative data of each taxonomic group. However, individual difference of substances and DNA copies of zooplankton species is quite large within each group. Our results demonstrate that metabarcoding is a semiquantitative method that could reveal broad quantitative patterns of zooplankton between sites. Further research with larger sample sizes and more accurate quantitative estimation is necessary to address these biases.

4.2. Seasonality of zooplankton community

Compared to the extensive research conducted at ALOHA station in the North Pacific Subtropical Gyre, the zooplankton seasonality in the WPWP remains largely understudied. Our findings reveal that the zooplankton abundance and biomass in summer were twice those in winter, echoing similar trends observed at ALOHA station (Hannides et al., 2020; Huntley et al., 2006; Landry et al., 2001; Valencia et al., 2016). However, at time series Station S1 (30°N, 145°E) located in the subtropical western Pacific, the surface zooplankton biomass peaks in deep winter and spring and is the lowest in autumn (Kitamura et al., 2016; Kobari et al., 2016). The bloom of zooplankton observed at time series Station S1 is driven by enhanced winter mixing, which is analogous to the seasonal cycles observed in temperate oceans such as at BAT station in the Sargasso Sea (Blanco-Bercial, 2020; Ivory et al., 2019). Unlike temperate oceans, tropical and subtropical oceans have a shallow mixing layer that restricts the flow of nutrients from the deep water layer, contributing to a low primary productivity and zooplankton biomass throughout the year. The maxima primary productivity observed in summer are related to the enhanced irradiance and nitrogen fixation in the oligotrophic North Pacific Ocean (Dore et al., 2008; Hannides et al., 2020). In this study, we recorded a balanced seasonality of the Chl α and zooplankton biomass, as expected for the zooplankton seasonal cycle in tropical oceans (Heinrich, 1962).

Our research findings reveal the significant seasonal variation in the zooplankton community in the WPWP identified via both microscopic and metabarcoding methods involving two markers. Notably, only the high-resolution metabarcoding method was able to depict the distinct separations between each pair of seasons (Fig. 6). The metabarcoding method enabled the identification of key community components at the ASV level, which can represent haplotypes of the same species with different ecological preferences (Cordier et al., 2017). In spite of certain ASVs not being assigned to the species level owing to inadequate reference sequence data, metabarcoding may offer more precise insights than the microscopic approach (Tapolczai et al., 2021). Metabarcoding also identified large non-copepod zooplankton as critical elements driving the seasonal variation in the community, with particular emphasis on high biomass euphausiids that tend to be underestimated in microscopic assessments (Fig. 7). Regarding the two biomarkers used, the key community components identified via the 18S method were completely different from those identified via the COI and microscopic methods, demonstrating that COI was a more reliable marker than 18S in capturing the seasonal variations in the zooplankton community.

We discovered that the seasonal variation in the zooplankton community in the WPWP, determined using microscopic abundance and metabarcoding data, was associated with different environmental

factors (Table S4). Our results are consistent with those of Yebrá et al. (2022), who observed discrepancies between different methodologies in a region characterized by complex mesoscale hydrodynamics. The microscopy-based dataset relied on individual zooplankton abundances, with small copepods dominating the population. Given their dependence on phytoplankton and microzooplankton, these small copepods are highly sensitive to bottom-up control in the Pacific (Vinogradov & Shushkina, 1989; Yang et al., 2017). As such, the Ave-Chl α was the primary factor shaping the seasonal structure of the microscopy-based zooplankton community. In comparison, the metabarcoding-based datasets were dominated by both small copepods and large zooplankton, such as *Scolecithrix danae*, *Thysanopoda aequalis*, and *Euphausia mutica*, which exhibited greater sensitivity to environmental fluctuations due to their longer development periods and more intricate development pathways (Farjalla et al., 2012). For example, for the copepod *S. danae*, the optimal salinity level is >34 ; whereas the euphausiid *T. aequalis* prefers a salinity of <33 (Xu, 2007; Xu & Gao, 2011). This might explain why Ave-S was the dominant factor shaping the structure of the metabarcoding-based zooplankton community. These findings suggest that the excessive influence of larger organisms must be considered when determining the relationships between the metabarcoding-derived zooplankton community variability and environmental variables.

5. Conclusions

Evaluating the seasonal variations in the zooplankton community in the extremely stable environment of the WPWP is crucial for gaining insights into the long-term effects of environmental perturbations such as climate change. Our results demonstrate that the COI metabarcoding method is a highly effective tool for achieving this objective. Our findings indicate that it achieves a higher detection of zooplankton taxa richness with a finer resolution compared to microscopy and 18S sequencing. Additionally, the metabarcoding approach was found to have a good potential for estimating the biovolume, dry weight biomass, and carbon biomass at a coarse taxonomic level. Significant seasonal variations in the zooplankton community were detected in the WPWP using all three methods. The COI marker revealed more pronounced seasonal distinction compared to the 18S and microscopy methods. Nevertheless, some limitations of the metabarcoding method, such as the underestimation of small cyclopoids and overestimation of some large zooplankton, may produce biases in our understanding of the trophic relationships and environmental interactions. Additional efforts to refine the quantitative capacity of COI metabarcoding and extend time-series sampling will contribute to the attainment of more valuable assessments of the zooplankton community dynamics and will enhance our comprehension of the related ecological processes.

CRediT authorship contribution statement

Yunzhi Feng: Conceptualization, Data curation, Writing – original draft. **Dong Sun:** Conceptualization, Methodology, Writing – review & editing. **Qianwen Shao:** Conceptualization, Writing – review & editing. **Chen Fang:** Investigation, Resources. **Chunsheng Wang:** Investigation, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

All raw sequence data used in this study and the related metadata have been deposited in the Sequence Read Archive (SRA) repository of

NCBI under the bioproject ID: PRJNA954122.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2023.111183>.

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