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Molecular diversity and biogeography of benthic microeukaryotes in temperate seagrass (*Zostera japonica*) systems of northern China

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Abstract

The productivity and health of seagrass depend on the combined inputs of nutrients from the water and sediments in which they grow and the microbiota with which they live intimately. However, little is known about the composition and diversity pattern of single-celled benthic eukaryotes in seagrass meadows. Here, we investigated how the structure and diversity of the benthic microeukaryotic community vary with respect to season, location, and seagrass colonization, by applying 18S rRNA gene amplicon sequencing for 96 surface sediment samples that were collected from three different seagrass habitats through four seasons. We found that benthic microeukaryotic communities associated with seagrass *Zostera japonica* exhibited remarkable spatial and seasonal variations, as well as differences between vegetated and unvegetated sediments. Diatoms and dinoflagellates predominated in the benthic microeukaryotic communities, but they were inversely correlated and displaced each other as the dominant microbial group in different seasons or habitats. Mucoromycota was more prevalent in vegetated sediments, whereas Lobulomycetales and Chytridiales had higher proportions in unvegetated sites. Total organic carbon and total organic nitrogen were the most important environmental factors in driving the microeukaryotic assemblages and diversity. Our study expands the available knowledge on the biogeographic distribution patterns and niche preferences for benthic microeukaryotes in seagrass systems.

Key words: biogeochemistry, spatiotemporal variation, seasonality, microeukaryote, seagrass meadow, seagrass-colonized effect, microbial ecology

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1 Introduction

Seagrasses, flowering aquatic plants, play a crucial role in the equilibrium of coastal ecosystems where they provide high primary productivity and marine biodiversity (Short et al., 2011). Seagrasses could enhance the organic matter contents in the rhizosphere by releasing dissolved organic carbon (DOC) produced by themselves through the roots and by trapping organic particles from seawater (Duarte et al., 2005). Furthermore, photosynthesis-produced oxygen could also be released into sediments from seagrass roots, which creates relatively high oxygen concentration, less-reducing conditions, and less toxic metabolite accumulation (e.g., sulfides) compared with unvegetated sediments (Jensen et al., 2005; Frederiksen and Glud, 2006). As one of microbial hotspots (Hansen et al., 2000; Smith et al., 2004), sediments harbor a diversity of microbial organisms which have been increasingly recognized as a critical role in regulating the interactions between seagrass and its surroundings (Hansen et al., 2000; Lehnen et al., 2016; Crump et al., 2018). However, the diversity and composition of single-celled eukaryotes, including fungi and protists, remain poorly understanding in this ecologically important ecosystem, despite their functional importance (e.g., decomposing macrophyte debris, grazing on bacteria, promoting biogeochemical cycles and primary production) in the microbial loop (Azam and Malfatti, 2007). A better understanding of the spatiotemporal distribution of microeukaryotes and the underlying mechanisms that shape these communities, could provide a sound clue about the global loss of seagrass habitats and guide the bioprospecting of desired microbes for seagrass restoration (Devereux, 2005; Duarte et al., 2005; Behera et al., 2019).

Advances in molecular (especially rRNA gene-based) tech-

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niques have shed new light on the diversity and biogeographical patterns of microeukaryotic distribution in coastal waters and sediments (Martiny et al., 2006; Gong et al., 2015; Massana et al., 2015). Previous studies have revealed that shifts in microeukaryotic community structure (MCS) and diversity can be influenced by various environmental variables, including temperature (or seasonal variations) (Marquardt et al., 2016), dissolved oxygen (DO) concentration (Orsi et al., 2012), nutrients (e.g., nitrogen, phosphorous) contents (Liu et al., 2020), and concentrations of pollutants (Zettler et al., 2002). While spatial factor (e.g., geographic distance) is another crucial determinant in shaping microeukaryotic α- and β-diversities in coastal ecosystems (Gong et al., 2015). There are at least two potential factors involved in the spatial distribution of microeukaryotes in the surface sediments of seagrass meadows. One lies in the vegetation of seagrass, which may alter the physicochemical properties of rhizosphere sediments (Sun et al., 2015; Lin et al., 2021). And the other factor concerns spatial distance, which may cause the seagrass-habitat changes. Some recent studies have investigated the eukaryotic assemblages associated with seagrasses, including rhizosphere, endosphere, and phyllosphere (Ugarelli et al., 2017; Hurtado-McCormick et al., 2019). These niches have been characterized to be dominated by members of macroalgae, metazoa, and fungi, which shift with seasons and geographic locations (Medina-Pons et al., 2009; Bengtsson et al., 2017; Wainwright et al., 2018; Petersen et al., 2019). Nonetheless, most of these studies focused on discrete microenvironments within seagrass plants, and the diversity and the composition of microeukaryotic populations in sediments of seagrass meadows remain unknown. Besides, studies suggested that both spatiotemporal and environmental factors should be taken into considerations when investigating microbial biogeography (Wang et al., 2015; Zhang et al., 2018). Therefore, it is still a great challenge to assess the relative importance of environment variables, seasonal variation, and habitat heterogeneity on the distribution of microeukaryotes associated with seagrass meadows.

The bacterial biogeography in rhizospheres and/or in seagrass-vegetated sediments has been found strongly influenced by spatially structured environmental gradients (Ikenaga et al., 2010; Sun et al., 2015; Arasamuthu et al., 2017) and varies with seasons (James et al., 2006). By contrast, the biogeographical patterns of benthic microeukaryotes associated with seagrasses have lagged far behind. In the present study, surficial sediments (0–5 cm) of seagrass (*Zostera japonica*) meadows were collected from three distinct coastal habitats (muddy coast, sandy coast, and lagoon) of northern China through four seasons in 2018. A range of environmental parameters of sediment was determined, and microeukaryotic diversity and relative abundance were characterized using high-throughput sequencing. We are particularly interested in the following questions: (1) which microeukaryotic phyla or groups are residing in the temperate seagrass systems; (2) how does the microeukaryotic community shift with sediment type (seagrass colonization or not), spatial and seasonal changes; and (3) what are the major environment influential factors regulating these microeukaryotes?

2 Materials and methods

2.1 *Study area and sampling*

Three *Z. japonica* meadows at the coast of the Bohai Sea and the Yellow Sea (Fig. 1) were selected for sampling: Stations at Dalian (DL, 39.23°N, 122.70°E), Dongying (DY, 37.85°N, 119.10°E), and Weihai (WH, 37.35°N, 122.58°E), where seasonal variations of climate are pronounced. These meadows represent three typical temperate ecological habitats (sandy coast, muddy coast, and lagoon). The sandy coastal habitat (DL) is located at one of the numerous islands of Liaoning Province, which is characterized by a sandy beach, while the muddy coast (DY) in Shandong Province features mainly muddy sediments and rich in organic matters. The surveyed lagoon (WH) is located at the easternmost tip of the Shandong Peninsula, connecting to the Yellow Sea through a narrow inlet, and differed from the other habitats (i.e., DY and WH) in grain size (GS) and sediment nutrient contents. A total of 96 samples (8 samples×3 locations×4 seasons) were obtained in 2018 (Table S1). Five surface sediments (top 5 cm, V1–V5) were randomly collected approximately 10 m away from each other within seagrass-vegetated regions. Another three sites (U1–U3) were collected from the adjacent bare regions approximately 10–20 m from the seagrass-covered regions. Samplings were performed with a custom-made corer (1-cm diameter) during low-tide conditions. All samples were well-mixed by stirring and stored at −80°C until DNA extraction and measurement of physicochemical parameters.

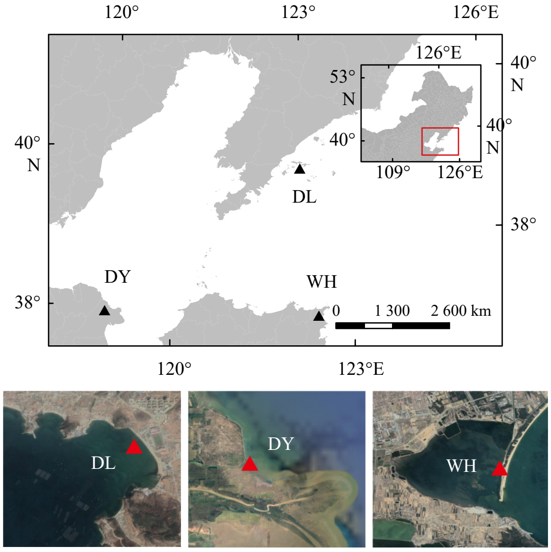

Fig. 1. Geographical locations of the three *Zostera japonica* seagrass meadows (DL: Dalian; DY: Dongying; WH: Weihai) distributing along the coast of the Yellow Sea and the Bohai Sea.

2.2 *Determination of environmental parameters*

Environmental parameters (i.e., temperature, pH, salinity, and concentration of DO) of the overlying water at each site were measured using YSI 556 (YSI, USA). The homogenized sediments were freeze-dried, ground, and sieved for subsequent determinations of sedimentary parameters. The distributions of GS in sediments were measured using a Mastersizer 2000F laser diffraction particle size analyzer (Malvern, England). The contents of nitrate (NO_3^--N), nitrite (NO_2^--N), and ammonium (NH_4^+-N) were analyzed in 2 mol/L KCl soil extracts (1:10 sediment/extractant) using a nutrient autoanalyzer (Seal, Germany). To determine the contents of sulfate (SO_4^{2-}), the sediments were pretreated with ultrapure water (1:5 sediment/water) and then analyzed by an ion chromatography Dionex ICS 3000 (USA). Before total organic nitrogen (TON) content and total organic carbon (TOC) content analysis, the carbonates were removed from sediments with 1 mol/L HCl. Subsequently, these samples were washed with ultrapure water three times before drying at 60°C, and the contents of TON and TOC were then measured by a Vario Micro Cube elemental analyzer (Elementar, Germany). To determine the contents of metals, the sediments were dissolved in 1 mol/L HCl, after which the contents were analyzed using an ELAN DRC II plasma mass spectrometer (PerkinElmer, China).

2.3 *DNA extraction, PCR amplification, and high-throughput sequencing*

Total DNA was extracted from 0.5 g to 1.0 g of sediments using a FastDNA SPIN Kit for soil (MP Biomedical, USA) according to the manufacturer’s instructions. The DNA integrity was checked in 1% agarose gel, and contents were determined using a Nanodrop 2000c spectrophotometer (ThermoFisher, USA). The V4 hypervariable region of 18S rDNA was amplified using the universal eukaryotic primers 528F (5′-GCGGTAATTCCAGCTCCAA-3′) and 706R (5′-AATCCRAGAATTTCACCTCT-3′) (Elwood et al., 1985) in ABI GeneAmp 9700 PCR system (PerkinElmer, USA). Each sample was distinguished by a paired specific 6-bp barcode. The thermal cycling program consisted of an initial denaturation of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C, and a final extension of 5 min at 72°C. Amplicon sequencing was performed on the Illumina MiSeq platform at a commercial sequencing company (Majorbio Bio-Pharm Technology Co. Ltd., China).

2.4 *Sequence processing and diversity analysis*

Raw data of 5 703 092 reads were quality-filtered and processed using QIIME (v.1.8.0) (Caporaso et al., 2010) and MOTHUR software package v.1.34.4 (Schloss et al., 2009). Clean reads satisfied the following criteria: (1) quality score>20, (2) no ambiguous bases, (3) no primer sequence mismatches, (4) length of overlap sequence⩾10 bp, (5) minimum sequence⩾300 bp, and (5) homopolymers⩽6 bp. Both primers and barcode bases were trimmed using the pcr.seqs command within MOTHUR. USEARCH v.61 (Edgar, 2010) was used to remove the putative chimeric sequences based on the reference of the SILVA SSU Ref database (release 132). Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UCLUST v.1.2.2 (Edgar, 2010). Singletons (OTUs containing a single read) across all samples were discarded. The taxonomy of representative sequences was assigned with UCLUST according to the SILVA database.

To investigate the microeukaryotes, the reads related to unassigned, metazoa, and plants (Ulvophyceae, Rhodophyta, and Streptophyta) were discarded before subsequent analysis, as suggested by Zhu et al. (2018). The α-diversity estimators included OTU richness, Shannon, Simpson, and Chao1, which were calculated based on a random resampling of 400 reads (one autumn vegetated-sediment sample of WH was discarded due to the extremely low reads number). To determine the dissimilarity of microeukaryotic communities between samples, the OTU table was subjected to normalization by using the edgeR package (Robinson et al., 2010), rather than rarefying the sequencing data as suggested by McMurdie and Holmes (2014). The non-metric multidimensional scaling (NMDS) was conducted based on Bray-Curtis similarity using the Vegan package (Paradis et al., 2004).

2.5 *Statistical analysis*

Student’s *t*-tests (two-tailed) and one-way ANOVA testing with least significance difference post hoc were performed using the software SPSS v.20 (SPSS, USA) to test the spatial, seasonal, and type-wise differences in physicochemical parameters, α-diversity estimators, and relative abundances of taxonomic groups. To examine the dissimilarity in community structure among sample groupings, the analysis of similarity (ANOSIM) (Clarke, 1993) based on the Bray-Curtis distance was calculated using the Anosim function of the Vegan package in R.

To determine the degree to which physicochemical data explained the variations in microbial eukaryotic communities, the following two methods were used. Firstly, redundancy analysis (RDA) was conducted to assess the relative importance of environmental variables on the MCS. The OTU table was Hellinger-transformed using the decostand function (Vegan package) before RDA, and geochemical parameters were selected to reduce multicollinearity using the vif.cca function (Vegan package). Secondly, the Spearman’s correlations were calculated to examine the associations of microeukaryotic diversity and major taxa with physicochemical factors by using the Hmisc package in R. The *P* values of Spearman analysis were adjusted using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) in the base R stats package.

2.6 *Accession number and data availability*

FASTQ files containing all raw sequences have been deposited in the NCBI Sequence Read Archive database under the accession number SRR10752480–SRR10752575.

3 Results

3.1 *Environmental setting characteristics*

Environmental factors (temperature, salinity, DO concentration and pH) of the overlying water significantly differed among the four seasons (P<0.043, one-way ANOVA, Tables S1 and S2). Compared with DL and DY, higher temperature and salinity, but lower DO concentration and pH were observed in WH, despite no significant differences in temperature and DO concentration (P>0.279). In addition, no statistical differences were detected in the overlying water between vegetated and unvegetated areas (*t*-test, P>0.425; Tables S1 and S2).

The analysis of the physicochemical properties of sediments showed sand was richer both in DL and WH, whereas DY contained more silt and trace metals (P<0.036, Table S2). Among seasonal comparisons, the majority of environmental factors exhibited significant differences (P<0.043), except GS and metal Mn content (P>0.192). In particular, the contents of NO_3^--N and NO_2^--N in the spring and summer were approximately 2–9 times higher than those in the autumn and winter, whereas the highest mean of NH_4^+-N contents was found in the winter (P<0.001). The contents of TOC, SO_4^{2-}, and metal elements appeared to be higher in summertime (Table S2). Compared with the properties in unvegetated sediments, the seagrass vegetated sediments were significantly much finer (P<0.047), and exhibited higher contents of TOC, SO_4^{2-} and metal Cu (P<0.027, Table S2).

3.2 *Community composition of microeukaryotes*

After quality control and chimeric and multicellular-eukaryotic sequence removal, 1 356 929 clean reads were remained (Table S3). A total of 4 568 OTUs were generated across all samples, with 19% and 23% of OTUs being shared among three locations and four seasons, respectively (Figs 2a, b). However, a much higher proportion (61%) of shared OTUs existed between seagrass-vegetated and unvegetated areas (Fig. 2c). Ochrophyta, Dinoflagellata, and Rhizaria dominated the microeukaryotic assemblages across all sediment samples (Fig. 2d). Specifically, Ochrophyta (principally diatoms) was the greatest taxon in DY and WH (54% and 60%), but was less abundant in DL (32%). In contrast to Ochrophyta, Dinoflagellata was the most abundant

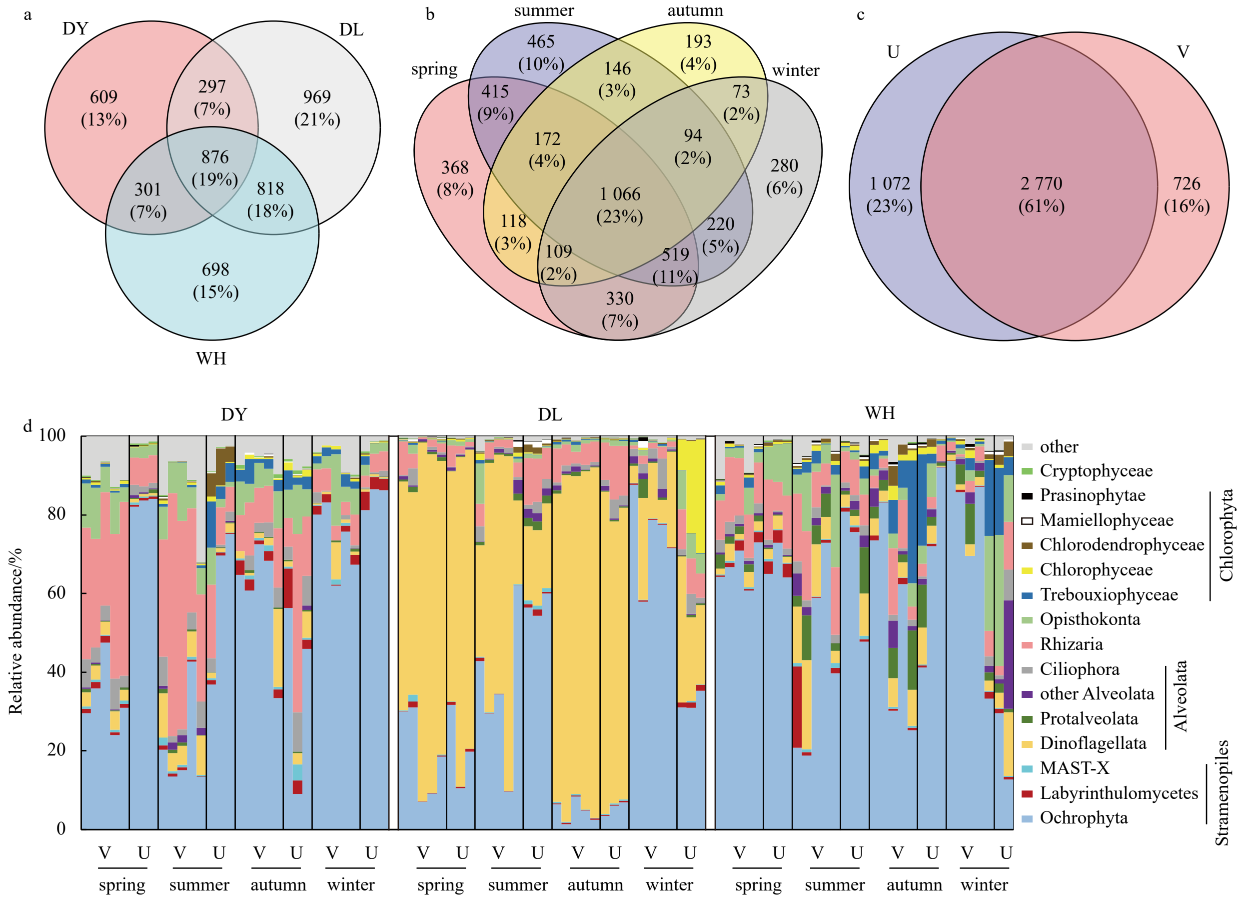

Fig. 2. Venn diagrams showing the shared and unique OTU number in sediments among three sampling locations (a), four seasons (b), and seagrass vegetated (V) and unvegetated (U) samples (c). Taxonomic composition of seagrass *Zostera japonica*-associated microeukaryotic communities revealed by Miseq sequencing of 18S rRNA genes (d). The percentages in parentheses indicate the proportion of a given OTU number to the total. MAST: marine stramenopile.

taxon in DL (53%) and mainly consisted of Peridiniphycidae, Gymnodiniphycidae, and Syndiniales. Rhizaria was the third dominant group, accounting for 10.8% of total sequences, whereas other groups (i.e., Prasinophytae, Cryptophyceae, Mamiellophyceae) appeared minor (Fig. 2d).

3.3 *Alpha diversity of microeukaryotic community*

The α-diversity estimators of microeukaryotes associated with seagrass meadows were high and fluctuated greatly on spatial and seasonal scales, with OTU richness ranging from 27 to 135, Chao1 indices from 83 to 344, Simpson indices from 0.17 to 0.98, and Shannon indices from 0.85 to 6.28 (Fig. 3, Table S3). Among four seasons, the α-diversity estimators in summer were generally the highest (ANOVA, $P<0.047$), whereas there were no significant differences among the other three seasons ($P>0.05$; Fig. 3). Among three locations, all α-diversity estimators in DL were significantly lower than the other two sites (ANOVA, $P<0.001$; Fig. 3). Although differences between unvegetated and vegetated sediments were not statistically significant (except the Chao1 index), the unvegetated sediments generally had higher mean values of α-diversity estimators (*t*-tests; Fig. 3).

3.4 *Beta-diversity of microeukaryotic community*

As illustrated in the NMDS plot (Fig. 4a), samples were largely clustered according to the locations of seagrass meadows. This result was supported by ANOSIM, which showed the differences of MCS among DY, DL, and WH were significant based on Bray-Curtis distance ($R=0.51$, $P=0.001$, Table S4). To furtherly visualize the dissimilarity of community structure between sediment types and seasons, the samples from each location were then analyzed separately (Figs 4b–d, Table 1). The NMDS plots (Figs 4b–d) showed a result in line with ANOSIM (Table 1), i.e., in each habitat there was a significant difference in the MCS between vegetated and unvegetated sediments ($R>0.195$, $P<0.005$; Table 1). Seasonal separation was also distinct (Figs 4b–d), with the only exception for autumn-winter comparison in WH ($R=0.081$, $P=0.125$; Table 1).

Distinct spatial or seasonal separations of the community structure were observed through the variations in relative abundances of microeukaryotic taxa (Table S5). Specifically, the relative abundances of Rhizaria, Ciliophora, and Cryptophyceae were significantly higher in DY ($P<0.004$), while Syndiniales and Chlorophyta were abundant in WH ($P<0.028$). DL was characterized by the sharp increase of dinoflagellate reads as well as the decrease of diatoms and fungi reads ($P<0.001$). The reads of dinoflagellates and Rhizaria exhibited lower proportions of occurrence in wintertime ($P<0.032$), but these declines coincided with an increment in the relative abundance of diatoms ($P=0.008$). Chytridiomycota was distinctly higher in spring than those in other seasons ($P=0.045$). Additionally, several microeukaryotic taxa were found with significant differences in vegetated and unvegetated sediments. For example, the Mucoromycota

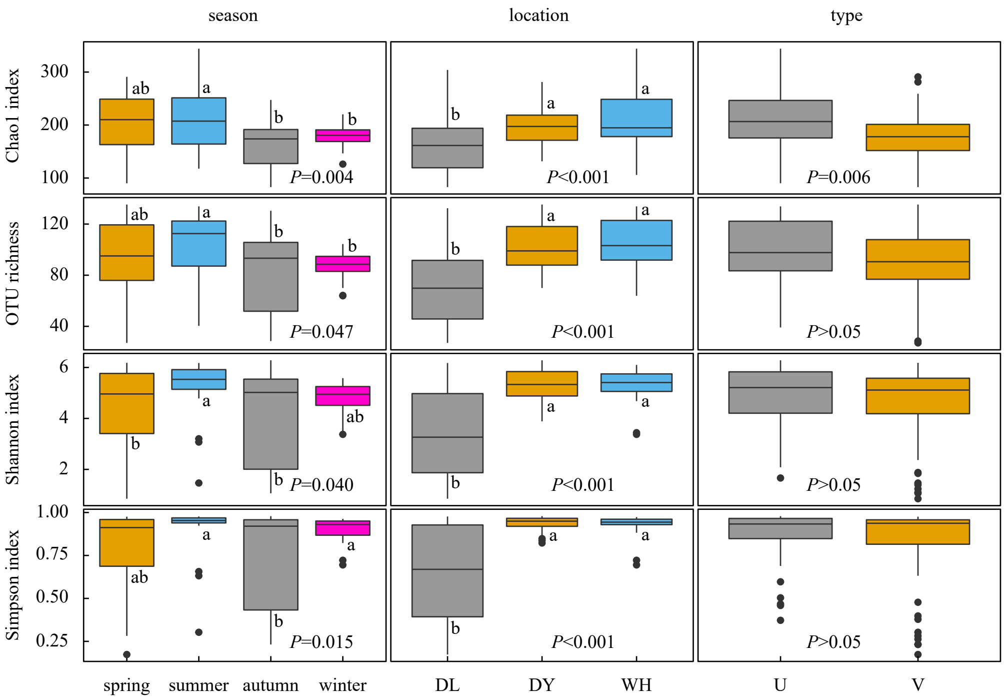

Fig. 3. Comparisons of microeukaryotic α-diversity estimators of seagrass-vegetated (V) and unvegetated (U) sediments collected from Dongying (DY), Dalian (DL), and Weihai (WH) across four seasons. The *P* values are given for the comparison among seasons and locations using one-way ANOVA with least significance difference post hoc, while difference between vegetated and unvegetated was examined using *t*-test. Different letters above the box indicate significant differences among groups.

presented with much higher proportions in seagrass-colonized sediments (P=0.046), while Chlorodendrophyceae, Chytridiales, and Lobulomycetales occurred more frequently in the unvegetated sediments (P<0.045; Table S5).

3.5 *Correlations of microeukaryotic diversity and relative proportions with environment variables*

Spearman's correlation analysis (Table 2) was conducted to decipher the relationships between environmental factors and microeukaryotic diversity. All α-diversity estimators were positively correlated with temperature, TOC content, and TON content (ρ>0.22, P<0.031), but negatively correlated with NH_4^+-N content (ρ<−0.23, P<0.026), pH (except for Chao1; ρ<−0.21, P<0.044) or DO concentration (except for Simpson; ρ<−0.21, P<0.046). Both Shannon and Simpson indices showed significantly positive correlations with the contents of SO_4^{2-} and Cr (ρ>0.21, P<0.045). Additionally, Chao1 and Simpson indices were positively correlated with GS (ρ=0.22, P=0.034) and metal V content (ρ=0.21, P=0.047), respectively.

The RDA plot revealed that the community structure of microeukaryotes co-varied significantly with the sedimentary GS, TOC content, Mn content, and the salinity, temperature, and pH of overlying water (P<0.01; Fig. 5a). Spearman's correlation analysis was also calculated to explore the possible influence of geochemical parameters on the relative proportions of major taxonomic groups (Fig. 5b). The relative abundances of Marine stramenopile and Rhizaria were positively correlated with both temperature and metal As content, while the proportion of Ciliophora exhibited a positive relationship with pH. Chlorodendrophyceae and Protalveolata had positive relationships with salinity and TOC content, but negative relationships with DO concentration and pH. The relative abundances of Nephroselmidophyceae and Trebouxiophyceae showed opposite responses to several factors (i.e., pH, NO_3^--N content, and TOC content); besides, Nephroselmidophyceae was more abundant in higher metal (Fe, Co, and Ni) contents and richer NO_3^--N conditions, while Trebouxiophyceae was relatively more abundant in the sediments with more clay and silt. However, for the most abundant taxa, Ochrophyta and dinoflagellates, no strong correlations were observed with all environmental variables measured in this study.

4 Discussion

Previously, information on the genetic diversity of microeukaryotic community associated with seagrass came mainly from traditional gene cloning sequencing and culture-dependent approaches under laboratory conditions or field surveys at local or regional scales (Newell, 1981; Medina-Pons et al., 2009; Gnavi et al., 2014; Ling et al., 2015). Nevertheless, these methods were limited by the cloning bias, low throughput, and neglect of non-culturable. In the present study, we expanded the sampling areas and selected three different seagrass habitats, better representing the temperate seagrass ecosystems. We explored the seasonal dynamics of microeukaryotic composition in seagrass-colonized sediments for the first time. Using high-throughput sequencing technology, 4 568 OTUs from different microeukaryotic taxa were detected, revealing much higher diversity than traditional culture-dependent approaches.

4.1 *The driving effects of physicochemical factors on microeukaryotic diversity*

In terms of α-diversity, conspicuous differences existed based on sampling season, location, and sediment type (Fig. 3). Seasonally, the highest microeukaryotic diversity was detected in summer, while the lowest generally occurred in winter, which is in line with previous observations (Oberbeckmann et al., 2014).

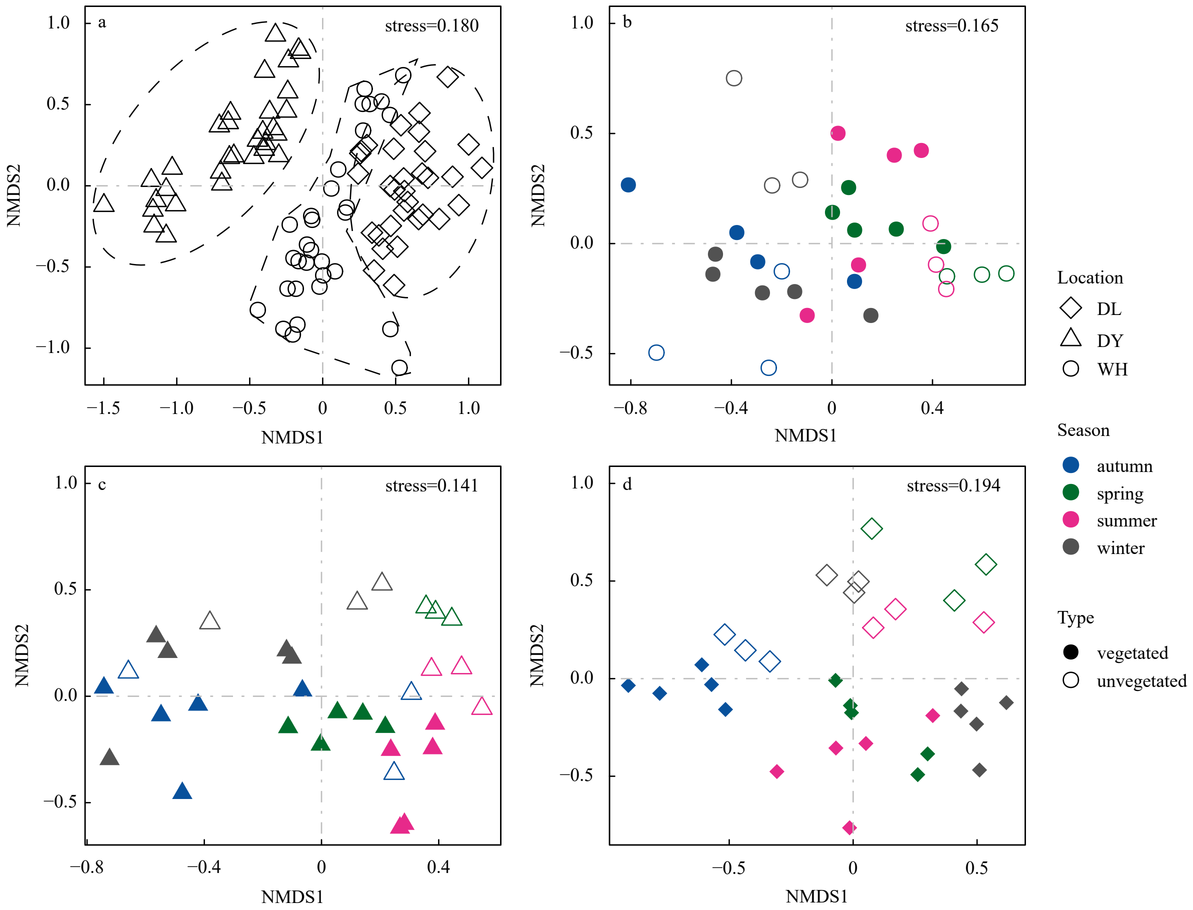

Fig. 4. Plots of non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distance, showing the variations of β-diversity of the microeukaryotic community from seagrass-vegetated (solid, V) and unvegetated (hollow, U) sediments in spatial scale (a), as well as seasonal patterns within each habitat: b in Weihai (WH); c in Dongying (DY), d in Dalian (DL). The shapes of points represent different location samples: DL, diamond; DY, triangle; and WH, round. And samples are colored by their corresponding seasons: spring, green; summer, pink; autumn, blue; and winter, black.

Table 1. ANOSIM testing the differences of benthic microeukaryotic communities between the two sediment types and four seasons within three seagrass beds, based on Bray-Curtis distance

Groups	DL		DY		WH	
	R	*P*	*R*	*P*	*R*	*P*
Global test	0.553	**0.001**	0.445	**0.001**	0.381	**0.001**
Spring vs. Summer	0.185	**0.040**	0.301	**0.008**	0.199	**0.039**
Spring vs. Autumn	0.568	**0.001**	0.451	**0.001**	0.658	**0.001**
Spring vs. Winter	0.486	**0.002**	0.491	**0.002**	0.455	**0.001**
Summer vs. Autumn	0.751	**0.001**	0.547	**0.001**	0.440	**0.002**
Summer vs. Winter	0.316	**0.011**	0.693	**0.001**	0.458	**0.002**
Autumn vs. Winter	0.901	**0.001**	0.248	**0.015**	0.081	0.125
Vegetated vs. Unvegetated	0.195	**0.001**	0.235	**0.005**	0.221	**0.002**

Note: Significant *P*-values ($\leqslant$0.05) are highlighted in bold.

Table 2. Spearman rank correlations between microeukaryotic α-diversity estimators and environment variables

Variable	OTU richness		Chao1		Simpson		Shannon	
	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*
Temperature	0.35	0.001	0.23	0.029	0.33	0.001	0.37	<0.001
DO concentration	−0.22	0.034	−0.21	0.046	–	–	−0.21	0.043
pH	−0.23	0.028	–	–	−0.21	0.044	−0.22	0.032
Grain size	–	–	0.22	0.034	–	–	–	–
NH_4^+-N content	−0.33	0.001	−0.34	0.001	−0.23	0.026	−0.28	0.007
SO_4^{2-} content	–	–	–	–	0.21	0.044	0.26	0.012
TOC content	0.29	0.005	0.22	0.031	0.28	0.005	0.31	0.003
TON content	0.33	0.001	0.26	0.010	0.33	0.001	0.36	<0.001
V content	–	–	–	–	0.21	0.047	–	–
Cr content	–	–	–	–	0.21	0.045	0.21	0.038

Note: Only the significant correlations ($P\leqslant$0.05) are shown. OTU is the abbreviation of operational taxonomic unit. ρ represents Spearman rank correlation coefficient; –, no data.

Spearman analysis between α-diversity and environmental factors revealed that organic matter (TOC, TON) contents and temperature might be the most important environmental factors in driving microeukaryotic diversity. This observation could be attributed to a large fraction of organic carbon converted into DOC during the thermal summertime, which was taken up almost exclusively by heterotrophic bacteria (Azam and Malfatti, 2007), and the diversity of microeukaryotic heterotrophic communities grazing bacteria through the microbial food loop was thereby improved (Glücksman et al., 2010).

Spatially, except for the eutrophic lagoon (WH), the muddy coast (DY) also had a high α-diversity (Fig. 3). In DY seagrass meadows, large inputs of sediment load, freshwater, agrochemicals, and fertilizers through the Yellow River contributed to the in-

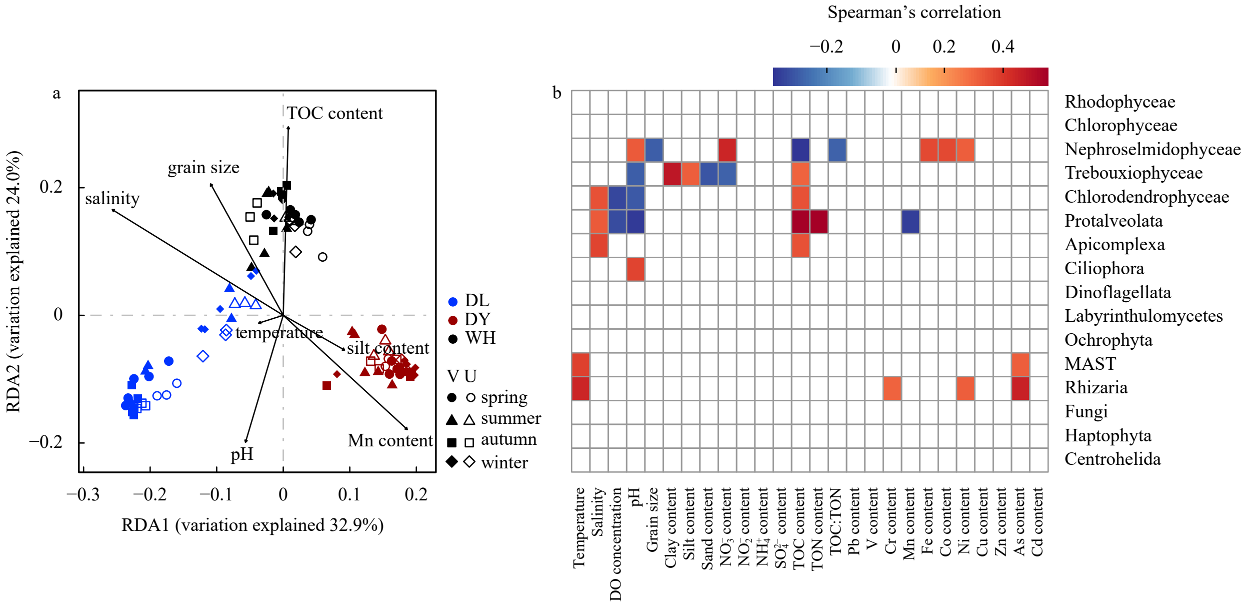

Fig. 5. Redundancy analysis (RDA) ordination diagram (a) and heatmap of Spearman’s correlation (b) showing the relationships of geochemical variables with microeukaryotic community structure and relative abundances of major taxa, respectively. Letters in sample IDs mean the vegetated (V, solid) and unvegetated (U, hollow) samples, and shapes of points represent different season samples: spring, round; summer, triangle; autumn, square; and winter, diamond. Samples are colored by habitat types: DL, Dalian (sandy coast, blue); DY, Dongying (muddy coast, red); and WH, Weihai (lagoon, black). Only vectors for environmental variables with *P*-values of 0.05 or smaller are shown in a and b. The values of Spearman’s correlation coefficients are indicated according to the scale bar. MAST is the abbreviation of marine stramenopile; TOC:TON, the contents ratio between total organic carbon and organic nitrogen.

creased contents of finer particles and trace metals in sediments, as well as relatively lower salinity levels (Table S2). Studies have reported that species richness increased significantly with smaller sediment particles, especially in coastal systems (Lallias et al., 2015). Moreover, high contents of heavy metals (especially Pb and As) detected in DY seagrass sediments may be directly related to the nearby crude-oil production processes. Meanwhile, finer GS contributes to the enrichment of heavy metals due to its larger surface-area-to-volume ratio (Maslennikova et al., 2012). Spearman analysis showed that heavy metals (V and Cr) had significant positive correlations with α-diversity (Table 2). Usually, stressors and disturbances can break the dominance of abundant taxa and promote the emergence of opportunistic species (Virta et al., 2020). As Lin and colleagues (Lin et al., 2016) highlighted, the health of seagrass habitats distributed in the Yellow River Estuary has already been at serious risk. Thus, the high community evenness in DY seagrass sediments is likely attributed to a relatively harsh condition.

Lower α-diversity of microeukaryotes was detected in seagrass-colonized sediments than in surrounding bulk sediments, which was unexpected in our study. Because the less-reducing condition in vegetated sediments is not only favorable to the growth of bacteria but also microeukaryotes. A possible reason is that microeukaryotes are suppressed by metabolic products of bacteria and seagrasses through regulatory mechanisms (Onishi et al., 2014; Hurtado-McCormick et al., 2019).

4.2 *Dominance of diatoms and dinoflagellates in seagrass sediments*

Our results showed that the microeukaryotic communities in *Z. japonica* sediments were mostly represented by diatoms and dinoflagellates. This finding is highly consistent with the recent work (Trevizan Segovia et al., 2021), which observed that diatoms and dinoflagellates were the core microeukaryotes on *Z. marina* leaves in ten meadows in the Northeast Pacific. These two groups are considered to be of global significance in primary production (Malviya et al., 2016). Meanwhile, their heterotrophic utilization of organic substrates, such as picoplankton (Sherr and Sherr, 2002) and DOC decomposed from seagrass fibers (Trevizan Segovia et al., 2021), is likely a vital survival strategy when levels of solar radiation are too low to support autotrophic metabolism (photosynthesis) in sediments (Tuchman et al., 2006). In addition, these heterotrophic metabolisms perform a significant impact on bacterial biomass and composition (Wu et al., 2020). Thus, the dominance of diatoms and dinoflagellates has potential implications for microbial food web and biogeochemical processes in coastal ecosystems.

Diatoms serve as the dominant benthic microeukaryotic group in seagrass meadows, playing a major role in marine carbon and silicon cycles (Nelson et al., 1995). Cox et al. (2020) reported that diatoms contributed up to 85.7% to benthic production in a seagrass habitat, which pushed the benthic system into net autotrophy. Therefore, these productive diatom assemblages were allowed for colonization in abundance within seagrasses habitats (Cox et al., 2020). Dinoflagellates as the second most abundant microeukaryotic group in *Z. japonica* systems included both exclusively parasitic groups, such as Syndiniales (dominated by Syndiniales Group I), and free-living groups, such as the Dinophycea (mainly Peridiniphycidae and Gymnodiniphycidae). Similarly, these two taxa (Syndiniales and Gymnodiniales) also represented the major portions of dinoflagellate sequences on *Z. marina* leaves (Trevizan Segovia et al., 2021). Combining with previous data, we speculated that seagrass-associated microeukaryotes communities, like bacteria, may also harbor core taxa, which are prevalent and/or abundant in seagrass meadows (Trevizan Segovia et al., 2021) and are important functionally for hosts. However, it should be noted that potential high rDNA copy number and dormant cysts may lead to the overestimation of abundance (or relative abundance) of major taxa (Gong et al., 2013; Yamada et al., 2014).

4.3 *Selectively enrichment of microeukaryotic populations in seagrass vegetated and unvegetated sediments*

There was obvious differentiated MCS between vegetated and unvegetated sediments within each location (Table 1), possibly due to the contrasting physicochemical properties between the two sediment types, such as increased organic compounds and oxygen around the seagrass rhizosphere (Duarte et al., 2005). This finding is generally consistent with previous studies on seagrass-associated microbiomes (Sun et al., 2015; Ettinger et al., 2017; Zheng et al., 2019). However, the MCS dissimilarity between the two sediment types turned to be less conspicuous after pooling all samples (Table S4), indicating the large-scale (habitat and season) environment fluctuations are likely to mask the effects of seagrasses colonization on the benthic microeukaryotic assemblages.

Although the MCS of the two sediment types appeared to be similar across all samples compared with the overall spatial or seasonal differences, each still contained a large number of unique OTUs (23% and 16%; Fig. 2c, Table S5). In this study, the members of phylum Mucoromycota were frequently detected in seagrass-colonized sediments, which are known for capable of decomposing oleaginous substrates (Magdouli et al., 2014; Kosa et al., 2018). Thus, the degradation of oil-rich seagrass seeds could be favorable to the growth of Mucoromycota in the vegetated sediments. Nevertheless, linages Lobulomycetales and Chytridiales affiliated with phylum Chytridiomycota were more frequently found in the unvegetated sediments. This may be related to the high abundance of diatom in the unvegetated samples, since most members of Lobulomycetales were parasites of diatoms and algae (Frenken et al., 2017; Van den Wyngaert et al., 2018).

4.4 *Seasonal and spatial distribution patterns of microeukaryotic communities*

Our results showed that benthic microeukaryotic communities associated with *Z. japonica* meadows were spatiotemporally assembled (Fig. 4; Table 1). Diatoms and dinoflagellates were highly dynamics, contributing significantly to the spatiotemporal variations in benthic MCS (Fig. 2; Table S5). Seasonally, diatoms and dinoflagellates showed opposite seasonal trends across entire samples. In the cold seasons, winter and spring were dominated by diatoms, and their relative abundances receded in summer and autumn (Table S5). A long-term study proved that diatoms prefer low temperature and may have a particular growth strategy to adapt to low-temperature environments (Xiao et al., 2018). In comparison, autumn-dominant members belonged to dinoflagellates which are known to have wide ecological tolerance, which is consistent with a previous study showing dinoflagellates were the dominant microplankton during autumn in a Turkey Bay (Küçük and Ergül, 2011). Spatially, the two microeukaryotic groups showed a preference for different habitats. Dinoflagellates constituted an incredibly high proportion in the sandy coast DL, whereas diatoms were prominent in the semi-enclosed lagoon WH and the muddy coast DY (Fig. 2d). Previous studies demonstrated that the habitat preference of the two taxa could be described in terms of nutrient availability and nutrient ratios (Smayda and Trainer, 2010; Malviya et al., 2016; Xiao et al., 2018). Dinoflagellates prefer higher N:P ratio and lower P concentration conditions, but diatoms prefer to live in coastal areas with high nutrient levels and low N:Si ratio (Xiao et al., 2018). This explains well that the dominance of dinoflagellates in DL is due to its higher inorganic nitrogen contents (NO_3^--N, NO_2^--N, and NH_4^+-N). Furthermore, reports have described the succession of dominant species of microeukaryotic communities from diatoms to dinoflagellates when the allochthonous nutrient supply is limited (Smayda and Trainer, 2010). Therefore, in the organic-rich DY and WH seagrass meadows, more diatoms will be promoted when silicon is not limited.

Except for the diatoms and dinoflagellates, other minor groups also showed apparent seasonal or spatial variations. The Spearman correlation analysis indicated that Rhizaria was highly correlated with temperature and metal contents (Fig. 5). Thus, high proportions of Rhizaria (mainly Cercozoa) were presented during the warm spring and summer, as well as in DY seagrass meadows with rich heavy metals (Fig. 2; Table S5). However, most of the Cercozoa members have been reported to perform an adverse effect on seagrass reproductions, even causing uprooting (Den Hartog, 1989; Walker and Campbell, 2009), and aggravating declination of seagrass habitats. In addition, the lowest proportion of fungal sequences was detected in DL, primarily assigned to Pezizomycotina (e.g., Dothideomycetes, Eurotiomycetes, and Sordariomycetes) which are known as the endophytic fungi of seagrass (Sakayaroj et al., 2010).

Overall, the differences in community composition among the lagoon, the sandy beach, and the muddy coast reflect OTU-specific environmental adaptations to local conditions. However, it is not easy to determine how much of these spatial heterogeneities are caused by sampling site features (landform and/or geochemical variables) (Hanson et al., 2012), and how much are attributed to dispersal limitations (Lear et al., 2014; Zhang et al., 2018), and interplay between species (Krabberød et al., 2017). More work will be needed to resolve these biotic and abiotic contributions in the future. Nevertheless, our findings indicate that major microeukaryotic taxa are well adapted to the seagrass sediments with high productivity, and that the MCS variations in seagrass meadow seem to be governed by the spatial and seasonal shifts of physicochemical properties of sediments and overlying water.

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Supplementary information:

Table S1. Summary of environmental variables in vegetated (V1-5) and unvegetated (U1-3) sediments collected from three habitats across four seasons.

Table S2. Comparisons of physicochemical properties of vegetated and unvegetated sediment samples collected from three seagrass meadows across four seasons.

Table S3. Summary of sequence analyses and α-diversity estimators (rarefied at 400) of microeukaryotic OTUs in vegetated (V1-5) and unvegetated (U1-3) sediments from DY, DL and WH across four seasons.

Table S4. ANOSIM testing the spatiotemporal differences of microeukaryotic communities among three locations and four seasons, and between seagrass vegetated and unvegetated sediments based on the Bray-Curtis distance.

Table S5. Comparisons of relative abundances (mean±SE) of major microeukaryote taxa among three locations and four seasons and between vegetated (V) and unvegetated (U) sediments.

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