Article

Composition and Distribution of Microeukaryotic Communities in the Surface Sediments of Five Geographic Regions of Bohai Sea Based on 18S rDNA Amplicon Sequencing

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Abstract: The Bohai Sea is a semi-enclosed shallow water that is influenced by both natural and anthropogenic stressors. However, the microeukaryotic communities and environmental factors that affect them in different regions remain largely unclear. We investigated microeukaryotic communities in surface sediments from five geographic regions using high-throughput sequencing of the 18S rDNA gene. The Miaodao Archipelago, Yellow River Estuary, and Central Bohai Sea had the highest Shannon and Simpson indices of the eukaryotic communities, while the Yellow River Estuary exhibited the highest Chao1 index. The microeukaryotic communities in surface sediments were mainly composed of Dinoflagellata, Bacillariophyta, Ciliophora, Cercozoa, and Protalveolata. *Thalassiosira* has a relatively high abundance at the Liaodong Bay and Central Bohai Sea, possessing the proportion of 41.70% and 38.10%, respectively, while *Gonyaulax* was the most abundant taxa in the Bohai Bay, occupying a proportion of 57.77%. Moreover, a negative correlation between diatoms and dinoflagellates was observed. Phosphorus, nitrogen, salinity, temperature, and silicate were major environmental determinants of microeukaryotic composition. Microeukaryotic communities in the surface sediments, especially for the composition and ratio of diatoms to dinoflagellates, reflected the environmental quality of marine ecosystems. Overall, these microeukaryotic community compositions provide a reliable indicator for monitoring the level of marine eutrophication in the Bohai Sea.

Keywords: surface sediment; microeukaryote; biological diversity; community structure; marine environment; 18S rDNA amplicon sequencing

1. Introduction

The Bohai Sea is surrounded by the Shandong Peninsula and Liaodong Peninsula. It is the only semi-enclosed inland sea in China and has an area of approximately 77,000 km^2, with an east–west width of approximately 346 km and a northeast–southwest length of about 555 km [1,2]. The Bohai Sea's coastline spans 2796 km and is composed of five parts: Liaodong Bay in the north, Bohai Bay in the west, the central shallow basin, Laizhou Bay in the south, and the Bohai Strait in the east [3]. The Bohai Sea is enclosed by land on three sides and is only adjacent to the Yellow Sea via the 109 km wide Bohai Strait. The sea's highest depth is 83 m, with an average depth of about 18 m [1]. The Bohai Sea receives abundant freshwater, sediment, and nutrients from numerous rivers, including the Yellow River, Haihe River, and Liaohe River. These inputs make the Bohai Sea an important spawning ground, nursery area, and feeding ground for many marine organisms. The Miaodao Archipelago, located at the junction of the Yellow Sea and the Bohai Sea, consists of 32 islands and serves as an important habitat and migratory corridor for fishery resources as well as a hotspot of biodiversity [4]. The areas surrounding the Miaodao Archipelago,

Liaodong Bay, and the southern side of the Dalian Peninsula are habitats for species such as spotted seals. The Bohai Sea has a rich variety of marine organisms and high biodiversity, making it ecologically valuable [5].

The Bohai Sea is located at the central region of Beijing–Tianjin–Hebei. The Bohai Sea ecosystem exhibits distinct regional and closed characteristics. Its high dependence on coastal habitat conditions makes the ecosystem vulnerable and highly susceptible to human activities. With the rapid growth of population and economy, nitrogen and phosphorus fertilizers from agriculture and aquaculture, industrial wastewater discharges, and urban sewage emissions produced by frequent human activities introduce a large amount of nutrients and pollutants, alter the natural balance of water bodies, and exacerbate eutrophication [3,6,7]. Although the Chinese government has implemented extensive efforts in recent years to limit land-based pollutant discharge, eutrophication continues to frequently occur in the Bohai Sea, particularly in the Bohai Bay and the Laizhou Bay [6,7]. Currently, the marine resources and the ecological environment of the Bohai Sea are facing severe challenges, including resource constraints, serious environmental pollution [8,9], and ecosystem degradation [7]. Frequent occurrences of harmful algal blooms have significantly reduced species diversity in the Bohai Sea, disrupted the ecosystem balance, and affected aquaculture and tourism industries. Harmful algal blooms have become one of the major marine disasters in the Bohai Sea [10]. Additionally, climate change and human activities have caused increased water temperature, sea-level rise, ocean acidification, and disruption of ecological balance, directly affecting the survival and reproductive capacity of marine organisms and having profound impacts on species distribution and migration patterns in the Bohai Sea [11,12].

Benthic organisms represent one of the most diverse ecological groups in marine ecosystems, encompassing a wide range of animal phyla, large and small algae, as well as marine seed plants [1]. Many benthic organisms are utilized for food and serve as targets for fishing, aquaculture, and mariculture, such as crustaceans, bivalves, flatfish, and various economically important seaweeds [13]. Moreover, the diverse array of benthic organisms plays a crucial role in the trophic and material flow within marine ecosystems, serving as natural prey for economically valuable fish and shrimp species [1]. Most species of benthic organisms have limited activity capacity, strong regional characteristics, and poor ability to avoid adverse environmental conditions. Therefore, the species composition, quantity changes, and community structure of benthic organisms can accurately reflect the long-term changes in their habitat environment and are often used as biological monitoring indicators for marine bottom environment [1,4,14,15]. Marine sediments support a more varied variety of eukaryotic organisms than does the water column; however, research on the geographic distribution and richness of benthic microeukaryotes in the Bohai Sea is limited due to methodological restrictions, and their diversity distribution patterns in marine sediments remain little understood. 18S rDNA amplicon sequencing offers advantages in providing comprehensive coverage, sensitivity to rare species, and quantitative analysis; facilitating comparative studies; and enhancing our understanding of marine ecology [16,17]. This study aims to investigate the diversity, distribution, and correlations with the environmental conditions of benthic microeukaryotes in the five geographic regions of the Bohai Sea to better understand the impacts of global climate change and human activities on the Bohai Sea ecosystem.

2. Materials and Methods

2.1. Description of Sampling Sites and Sample Collection

The study area is in the five geographic regions of the Bohai Sea in China. In May 2023, sediment samples from nine sampling sites were collected during the survey voyage of a research vessel (RV), called Xiangyanghong 18 (37°07′–41°0′ N, 116°35′–122°15′ E). These sampling sites were distributed among the five geographic regions [1,3]: Bohai Bay (BB) including NS-24; Liaodong Bay (LB) including NS-13; Central Bohai Sea (CS) including NS-27; Yellow River Estuary (YE) including NS-29, NS-30, and NS-31; and

Miaodao Archipelago (MA) including NS-33, NS-34, and NS-35 (Figure 1). At least three parallel sediments (0–5 cm) were collected from each sampling site by a box-type sediment sampler (Figure 2A). Samples were sealed and placed in sterile plastic bags, stored at −80 °C until the extraction of DNA.
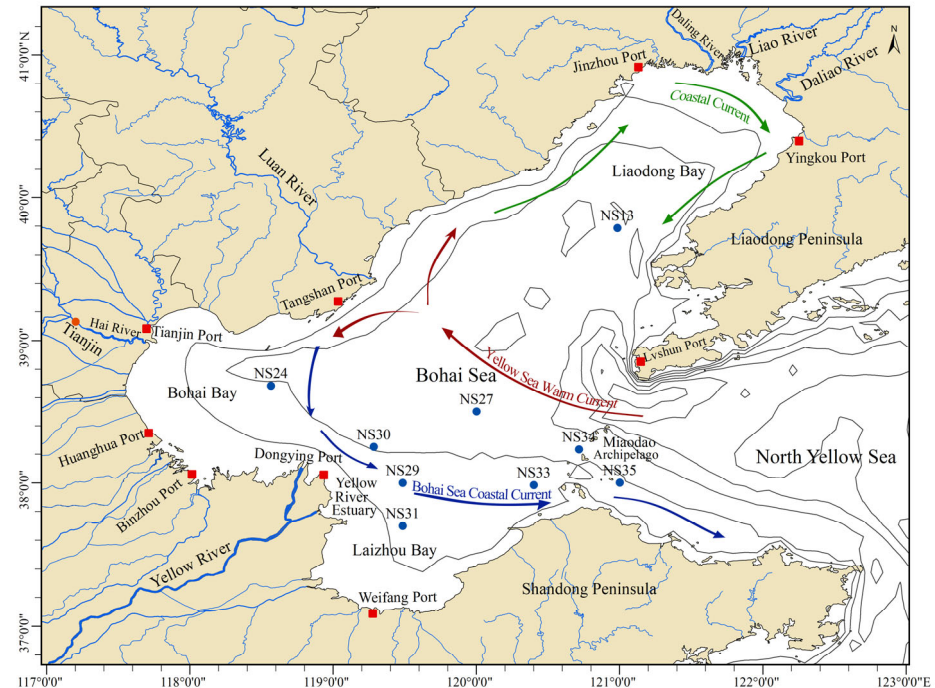
Figure 1. The geographic location of sampling sites. Samples were collected from the surface sediments (0–5 cm) of five geographic regions in Bohai Sea, China. NS-24 samples were obtained from Bohai Bay; NS-13 samples from Liaodong Bay; NS-27 samples from Central Bohai Sea; NS-29, NS-30, and NS-31 samples from Yellow River Estuary; and NS-33, NS-34, and NS-35 samples from Miaodao Archipelago.
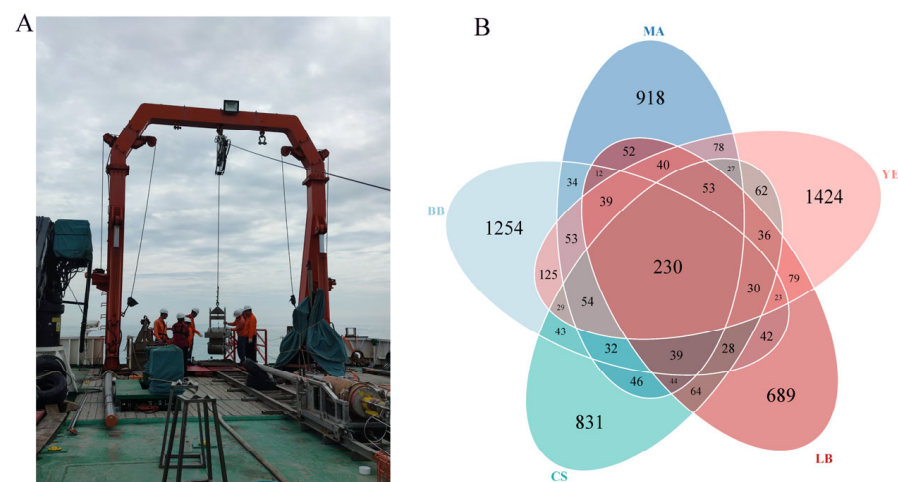
Figure 2. Sediment collection and ASV grouping. (**A**) Photos of sediment collection operations. (**B**) Flower diagrams showing the common and unique ASVs between different groups. Miaodao Archipelago (MA), Bohai Bay (BB), the Central Bohai Sea (CS), Liaodong Bay (LB), and Yellow River Estuary (YE).

2.2. DNA Extraction and Amplicon Sequencing

According to the manufacturer's instructions, the total DNA from sediment samples was extracted using a Qiagen DNeasy® PowerSoil Pro Kit (Qiagen, Hilden, Germany, Catalog: 47014). The quality of the DNA was assessed through 1% agarose gel electrophoresis. DNA concentrations range from 21 to 57 ng/uL, which was measured using a QubitTM dsDNA Assay Kit on a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Subsequently, the V4 hypervariable region of the 18S rDNA was amplified using PCR primers 18SV4f (5′-GCGGTAATTCCAGCTCCAA-3′) and 18SV4r (5′-TAATCCRAGAATTTCACCTCT-3′), which included a barcode [18]. Each PCR consisted of 0.2 µM paired primers, 10 ng template DNA, and 15 µL Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The PCR protocol included an initial denaturation step at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. A final extension step at 72 °C for 10 min concluded the process. PCR products were observed on a 2% agarose gel, purified using a Universal DNA Purification Recovery Kit (TianGen, Beijing, China, Catalog: DP214), and subsequently subjected to library preparation using a NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs, Ipswich, MA, USA, Catalog: E7430L) following the manufacturer's recommendations. Library quality and quantification were verified using Qubit and real-time PCR, while size distribution was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The constructed libraries were sequenced on an Illumina NovaSeq6000 PE250 platform at Novogene Co., Ltd. (Beijing, China). All sequencing data utilized in this study have been deposited in the Genome Sequence Archive of the National Genomics Data Center of China under accession number PRJCA022295.

2.3. Paired-End Reads Assembly and Species Annotation

FLASH software was used to integrate paired-end reads (Version 1.2.11). fastp software was used to do quality-filtering on the raw tags in order to produce high-quality Clean Tags (Version 0.23.1). To obtain high-quality sequences and subsequent generation of amplicon sequence variants (ASVs), the raw data were subjected to denoising, dereplication, and removal of chimeras using DADA2 v1.1.6 plugin in Qiime2 software (Version 2022.2) [19]. The sequences were classified and annotated using the Silva databases, filtering out ASVs with confidence values below 0.8 and those with a sequence count fewer than 8. Compared with typical OTU methods, the DADA2 method is more specific and sensitive, producing fewer spurious sequences while detecting true biological differences that OTU methods ignored [20]. The accuracy, thoroughness, and repeatability of marker gene data analysis were all enhanced when ASVs took the place of OTUs [21].

Using QIIME2′s classify-sklearn algorithm, pre-trained naïve Bayes classifiers were used for species annotation for ASVs [19]. The taxonomic database of Silva v138.1 was used to annotate sequences, while the NCBI dmp file was used to supplement the taxonomic information. Based on the annotation results of ASVs and the characteristic tables (sequence abundance in each sample) of each sample, species abundance tables at the levels of kingdom, phylum, class, order, family, genus, and species were obtained. To reduce the impact of macrobenthos, we removed the sequencing data annotated as the Animalia kingdom. A standard of sequence number from the sample with the fewest sequences was used to standardize the absolute abundance of ASVs. Based on the output normalized data, alpha and beta diversity analyses were subsequently carried out. The statistics of sequencing output (raw reads) and annotation are listed in Table S1.

2.4. Cluster and Community Composition Analysis

For the benthic microeukaryotic diversity, alpha diversity indices, including Shannon–Weaver, Simpson, and Chao indices, were generated with Qiime2 software (version 2022.2) [19]. The β diversity and its components were computed using the adespatial package in R, utilizing the Jaccard dissimilarity index. Box plots were used to statis-

tically analyze and visualize the alpha diversity indices of surface sediment biological communities. In addition, Tukey and Kruskal–Wallis rank sum analyses were performed to determine the presence of significant variations in species diversity between the groups. Non-metric multidimensional scaling (NMDS) was also implemented for data dimension reduction. NMDS is a non-linear model that utilizes distance metrics such as weighted unifrac and unweighted unifrac to assess differences in the community structure [22]. The weighted unifrac and unweighted unifrac distances were calculated based on the abundance information of amplicon sequence variants (ASVs) in the samples. Using QIIME2 software (version 2022.2), multiple sequence alignment was carried out to examine the evolutionary relationship of each ASV and find variations in dominant species among different groups. The top ten taxa in each sample at the taxonomic levels of phylum and genus were chosen in order to plot the relative abundance distribution histogram. Flower diagrams visually displayed the common and unique information between different groups. To construct species relationship networks, microeukaryotic genera were selected for calculating Spearman correlation coefficients. For the purpose of creating the network graph, links that had correlation coefficients larger than 0.6 or less than −0.6 and were statistically significant (*p*-value < 0.05) were deemed legitimate.

2.5. Environmental Correlation Analysis

Total phosphorus (TP), total nitrogen (TN), and total organic carbon (TOC) of surface sediments were measured using established methods in the Specification for Marine Monitoring of China—Part 5: Sediment Analysis [23]. Silicate was measured using the standard method of Chemical reagent—General Method for the Determination of Silicate [24]. Sediment grain size (SGZ) was measured using the standard method in the Specifications for Oceanographic Survey—Part 8: Marine Geology and Geophysics Survey [25]. Temperature, salinity, and pH are monitored on-site using bottom seawater values. To explore the symbiotic relationship between species and to reveal the environmental factor influence on community structures, network diagrams were drawn for visualization. Further analyses such as the Spearman correlation test and canonical correspondence analysis (CCA) were used to reflect the correlation between species abundance and environment variables. All these diagrams and analyses were completed in R studio.

3. Results

3.1. Microeukaryotic Communities of Five Typical Regions in Bohai Sea

After clustering the paired-end reads and quality control, a total of 2,890,316 high-quality sequences (ASVs) were obtained from nine sediment sites (Table S1). The number of sequences averaged 85,009 and ranged from 63,946 to 95,891 for each sample. Using QIIME2 software (version 2022.2), 94.35% of total ASVs were annotated and were further classified and divided to the levels of kingdom, phylum, class, order, family, genus, and species. We calculated the total number of annotated ASVs in the five geographic regions of the Bohai Sea, including 2067 in the Bohai Bay, 1500 in the Liaodong Bay, 1648 in the Central Bohai Sea, 2382 in the Yellow River Estuary, and 1751 in the Miaodao Archipelago (Figure 2). In addition, 1254 of ASVs were unique to the Bohai Bay among the five geographic regions, 689 in the Liaodong Bay, 831 in the Central Bohai Sea, 1424 in the Yellow River Estuary, and 918 in the Miaodao Archipelago. There were 78 ASVs shared by the Miaodao Archipelago and the Yellow River Estuary. The sequencing data annotated as the Animalia kingdom were removed, and the remaining sequences were used for the analyses of composition of microeukaryotic communities.

To compare the inter-group differences of microeukaryotic communities, the alpha diversity indices (Shannon index, Simpson index, and Chao1 index) of the five geographic regions in the Bohai Sea were calculated (Figure 3 and Table S2). As shown in Figure 3A–C, we found that the microeukaryotic communities in the Miaodao Archipelago, Yellow River Estuary, and Central Bohai Sea had higher Shannon and Simpson indices, while the Bohai Bay had the lowest values for the microeukaryotic communities. Among the five groups,

the Yellow River Estuary exhibited the highest Chao1 index value for eukaryotes, indicating higher species richness compared with other regions (Figure 3C). The Central Bohai Sea also has relatively high species richness. In contrast, the Miaodao Archipelago showed the lowest Chao1 index value among these five groups, implying lower species richness (Figure 3C).
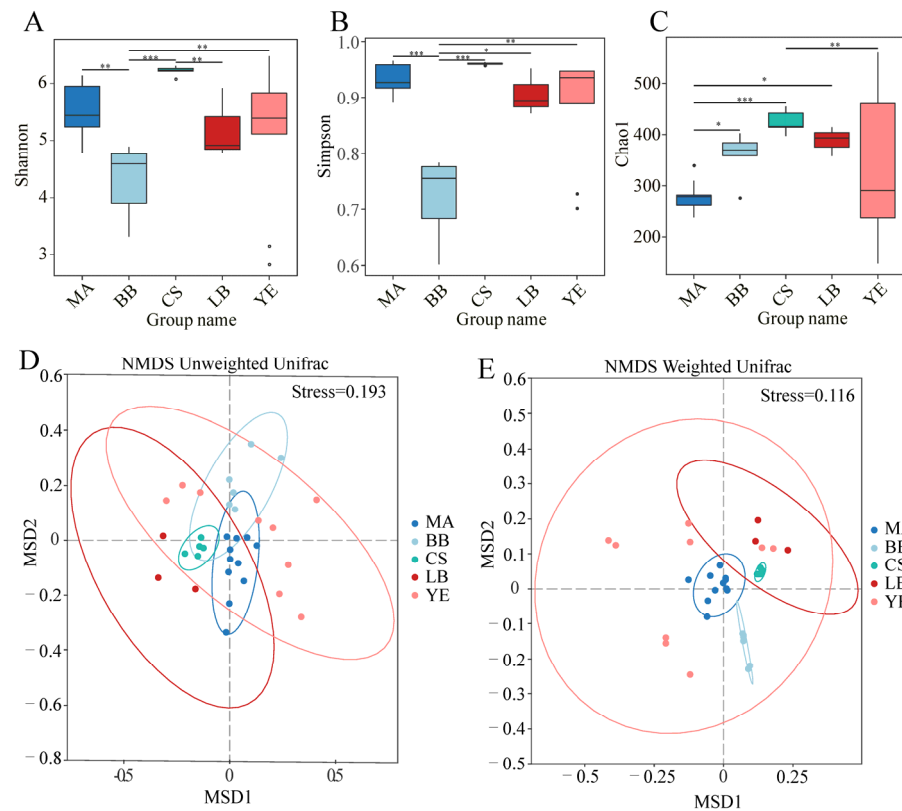

Figure 3. Biodiversity analysis of microeukaryotic communities. (**A–C**) Alpha diversity (Shannon index, Simpson index, and Chao1 index) indices of microeukaryotic communities among I, II, III, IV, and V groups. Asterisk above each box in the same subfigure represents significant differences between different groups (Tukey's HSD test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). (**D,E**) Non-metric multidimensional scaling (NMDS) plots based on weighted unifrac distance and unweighted unifrac distance derived from standardized ASV abundance for microeukaryotic communities among 34 samples from Bohai Sea. Ellipses represent the 95% confidence interval. Each color circle represents the clustering degree of samples within its group.

To identify the similarity and dissimilarity of the spatial distribution for microeukaryotic communities, beta diversity analysis was carried out. We conducted the non-metric multidimensional scaling (NMDS) analysis to visualize the similarity of eukaryote communities among these five geographic regions. The stress values of the NMDS ordination plots were found to be 0.119 and 0.165, both below 0.2, indicating their effectiveness in describing the similarity relationships between samples and reflecting differences in the eukaryotic community structure among them (Figure 3D,E). This clustering of the 34 samples into five distinct groups suggests significant variations in the microeukaryotic community structure among these five geographic regions in the Bohai Sea. These results collectively indicate disparities in spatial differences in community composition in the Bohai Sea.

3.2. Composition and Distribution of Dominant Microeukaryotic Taxa

According to the species annotation results, the microeukaryotic communities in surface sediments were mainly composed of Dinoflagellata, Bacillariophyta, Ciliophora, Cercozoa, Protalveolata, Labyrinthulomycetes, Apicomplexa, Chytridiomycota, Oomycota,

and Marine Stramenopiles-1 among these five geographic regions (Figure 4A). Specifically, Dinoflagellata had the highest relative abundance, with an average proportion of 37.80% across the whole Bohai sea. Dinoflagellata was the greatest taxa in the Bohai Bay, accounting for 78.67% of the total species, but was less abundant in the Liaodong Bay. Except for Dinofllagellata, Bacillariophyta, Ciliophora, Cercozoa, and Protalveolata had a high abundance in the Miaodao Archipelago, accounting for 16.20, 9.69, 12.47, and 13.46 of the total species, respectively. Meanwhile, Bacillariophyta was the major microeukaryotic group in the Central Bohai Sea, Liaodong Bay, and NS29 site (Yellow River Estuary). In contrast, the abundance of diatoms is lower in the Bohai Bay (NS24) and the Laizhou Bay (NS31). Ciliophora has a high abundance in the Yellow River Estuary, especially for the sampling site of NS30; its proportion ranges from 43.74% to 73.92% of the total species among the three parallel samples.
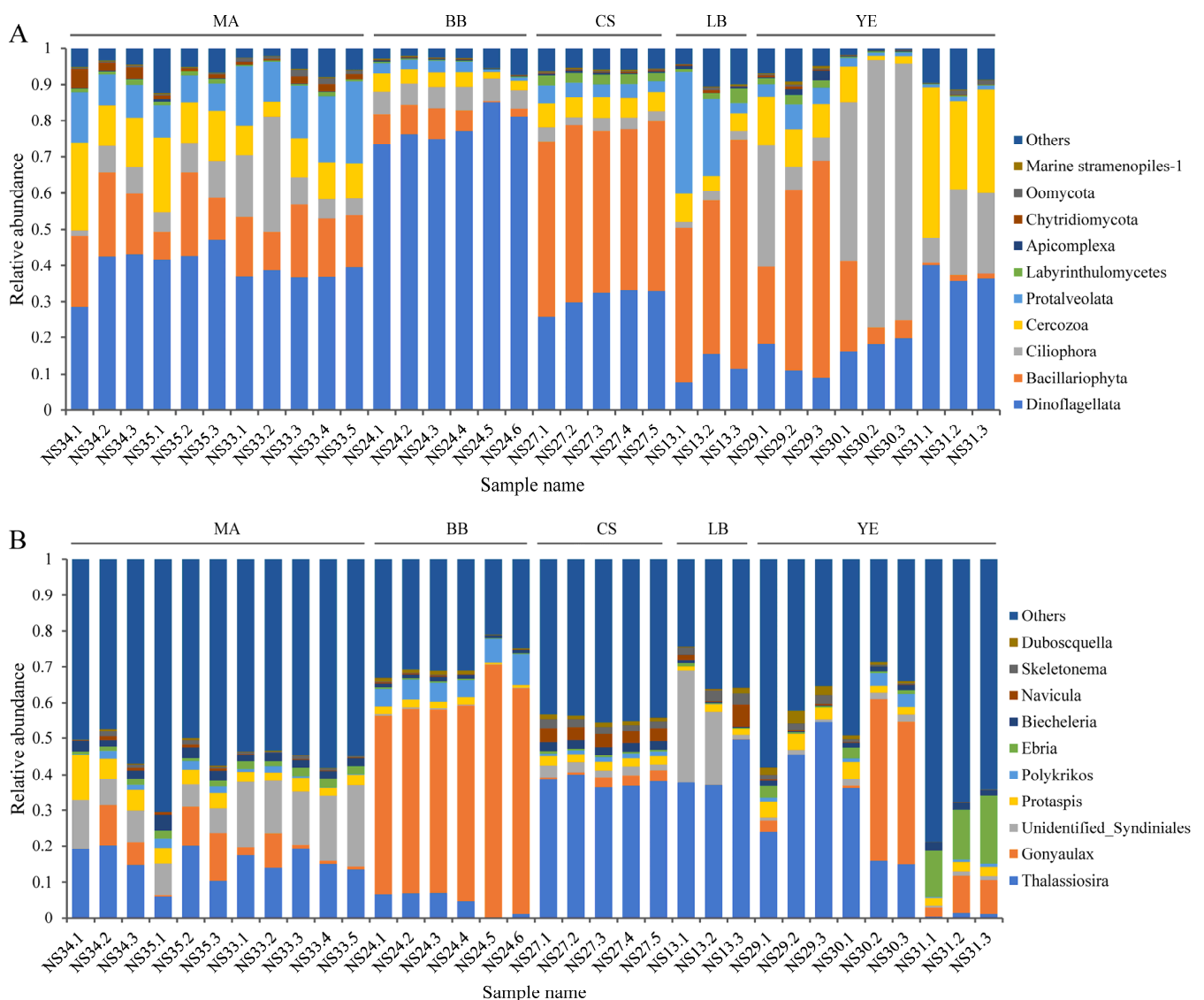

Figure 4. Relative abundances of dominant microeukaryotic organisms in the sediments of Bohai Sea. (**A**) The phylum level. (**B**) The genus level. Miaodao Archipelago (MA), NS33, NS34, and NS13; Bohai Bay (BB), NS24; the Central Bohai Sea (CS), NS27; Liaodong Bay (LB), NS13; Yellow River Estuary (YE), NS29, NS30, and NS31.

According to the genus level, *Thalassiosira*, *Gonyaulax*, *Unidentified_Syndiniales*, *Protaspis*, *Polykrikos*, *Ebria*, *Biecheleria*, *Navicula*, *Skeletonema*, and *Duboscquella* dominated the

microeukaryotic communities across the whole Bohai Sea (Figure 4B). Among them, *Thalassiosira*, belonging to the phylum Bacillariophyta, has a relatively high abundance at the Central Bohai Sea and Liaodong Bay, possessing the proportion of 38.10% and 41.70%, respectively. *Gonyaulax*, belonging to the phylum Dinofllagellata, occupied a proportion of 57.77% in the Bohai Bay, which was the most abundant taxa (Figure 4B).

In these five geographic regions, we identified *Gonyaulax*, *Polykrikos*, *Biecheleria*, *Islandinium*, *Pyrophacus*, *Pelagodinium*, *Grammatodinium*, *Protoperidinium*, *Alexandrium*, and *Noctiluca* as the primary genera of dinoflagellates (Figure 5A). Except for the Bohai Bay, the diversity of the dinoflagellate composition was observed in the other four regions. However, the composition of diatoms remained relatively consistent across all five regions. Among the diatom species, *Thalassiosira* exhibited a significant dominance, accounting for an average proportion exceeding 86.70% of all diatoms (Figure 5B). Among them, the sampling sites with the highest abundance of diatoms (average ASV number) are NS13 (21,722.33), NS29 (15,785.33), and NS27 (11,829.20). The sampling site with the highest abundance of dinoflagellate (average ASV number) is NS24 (29,246.6), followed by NS30 (3669.33) and NS27 (3526.33). In addition, we calculated the ratios of diatoms to dinoflagellates in the Bohai Bay, Liaodong Bay, Central Bohai Sea, Yellow River Estuary, and Miaodao Archipelago, which were 0.07, 24.95, 5.59, 2.19, and 1.29, respectively.
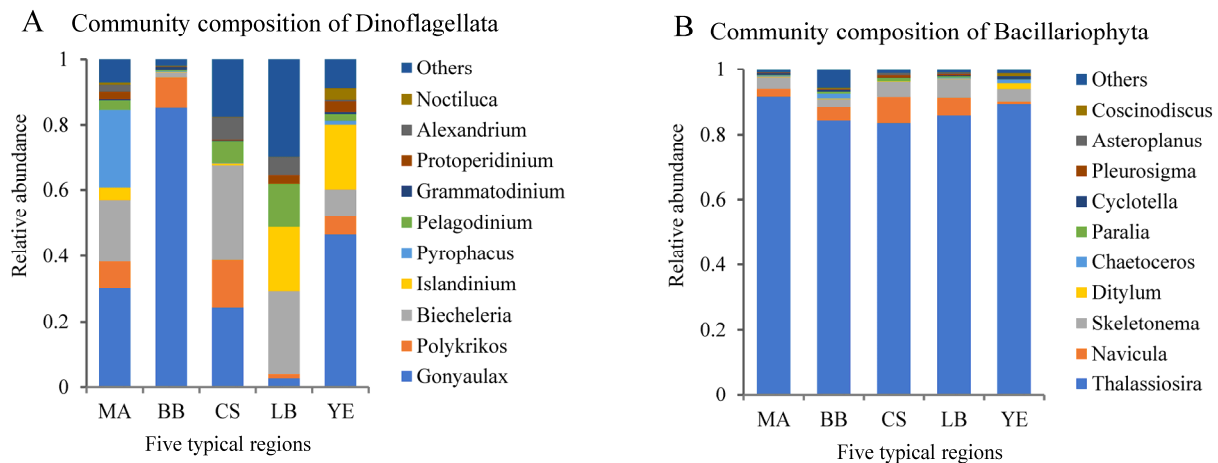

Figure 5. Relative abundances of community composition of Dinoflagellata and Bacillariophyta in the sediments of Bohai Sea. (**A**) Community composition of Dinoflagellata. (**B**) Community composition of Bacillariophyta. Miaodao Archipelago (MA), Bohai Bay (BB), the Central Bohai Sea (CS), Liaodong Bay (LB), and Yellow River Estuary (YE).

3.3. Species Relationship Networks of Microeukaryotic Communities

For the species relationship network, Spearman correlation coefficients were calculated for all samples. Following the acquisition of the species correlation coefficient matrix, connections with coefficients below 0.6 were filtered out, along with self-connections and connections with low node abundance. Nodes denote distinct genera, whereas node size indicates the average relative abundance of the genus. The correlation between species interactions is favorably connected with the thickness of the connecting lines between nodes, while the connection between species interactions and the color of the connecting lines is either positively or negatively correlated (red positive correlation; blue negative correlation). In the correlation analysis, we retained the phylum Ciliophora data. As shown in Figure 6, *Thalassiosira*, *Gonyaulax*, *Unidentified_Syndiniales*, *Cryptocaryon*, *Parastrom bidinopsis*, *Protaspis*, and *Polykrikos* were the dominant species in the microeukaryotic communities. An interesting finding was that there was a clear competitive relationship between Bacillariophyta and Dinoflagellata. Meanwhile, Ciliophora had a positive relationship with Dinoflagellata, but it had a negative relationship with Bacillariophyta. There is a teamwork in the oceanic microscale that nutrient input to a diatom cell surface can be increased four to

ten times by fluid flows produced by ciliary beating compared with a stationary cell lacking ciliate epibionts [26]. The correlation analysis established a total of 177 connecting lines, with 130 red lines for positive correlation and 47 blue lines for negative correlation. These results indicated that mutualistic relationships dominate in microeukaryotic communities.
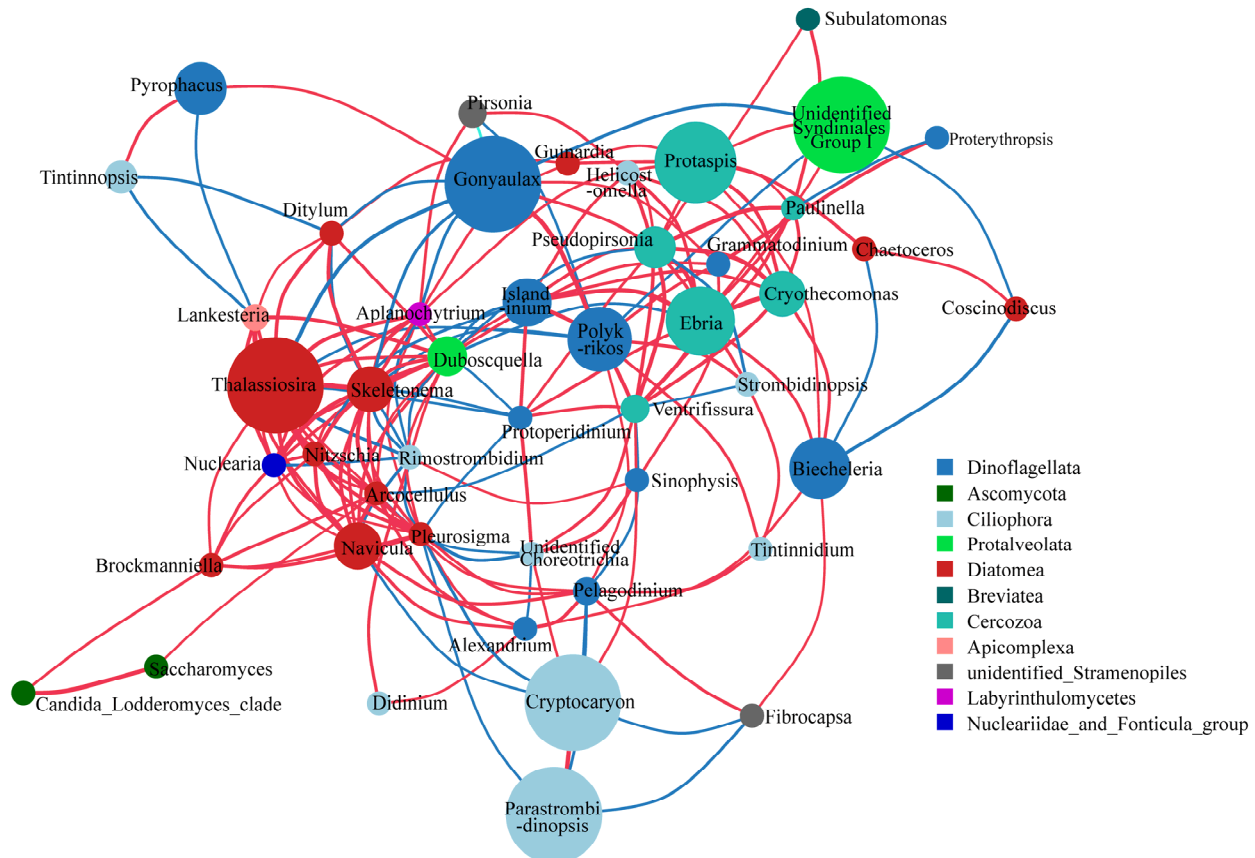
Figure 6. The species correlation network on genus level. Different nodes represent different genera, and the size of each node represents the average relative abundance of that genus. Nodes belonging to the same phylum share the same color (as indicated in the legend). The thickness of the connecting lines between nodes is positively correlated with the absolute value of the correlation coefficient indicating species interactions. The color of the connecting lines corresponds to the positive or negative nature of the correlation (red for positive correlation; blue for negative correlation).

3.4. Correlation Analysis between Microeukaryotic Communities and Environmental Factors

The correlation analysis between microeukaryotic abundance and environment variables in the Bohai Sea is presented in Figure 7. The primary environmental variables that determined the number of microeukaryotic organisms were phosphorus, nitrogen, salinity, temperature, and silicate. Environmental variables, particularly phosphorus and silicate contents, were highly correlated with the Bacillariophyta abundance. In contrast, Dinoflagellata showed significant positive correlations with salinity, temperature, and TOC, while it showed significant negative correlations with silicate, phosphorus, and nitrogen contents. In addition, Ciliophora showed significant positive correlations with nitrogen, sediment grain size, pH, phosphorus, and silicate, while it showed significant negative correlations with salinity and temperature. Diatoms and dinoflagellates are dominant microeukaryotes at NS27 and NS24 sampling sites, respectively. This revealed that the distribution of species in the CCA plot is consistent with the distribution of sampling sites. Overall, environmental factors accounted for 48.78% of community variation, indicating that the microeukaryotic biogeography of the Bohai Sea was primarily influenced by spatial fluctuations in phosphorus, nitrogen, salinity, temperature, and silicate levels.
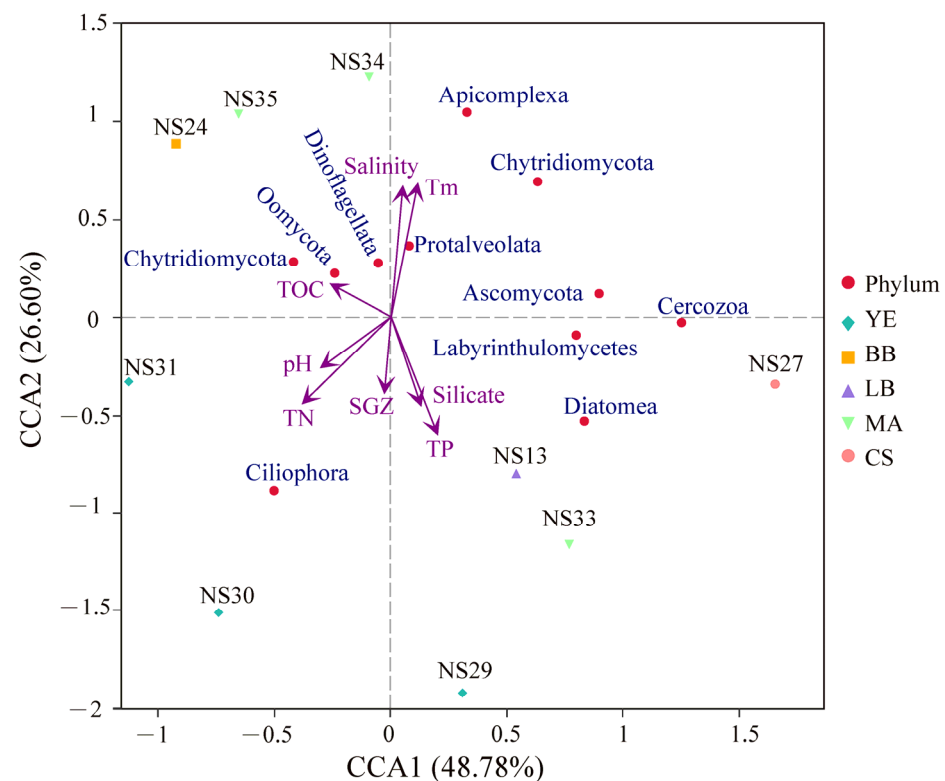
Figure 7. Correlation analysis between community structures and environmental factors. CCA analysis integrating environmental parameters and the relative abundance of eukaryotic taxa from different sediment samples at the phylum level. The x-axis denotes the first principal component, the y-axis represents the second principal component, and the percentage values in parentheses indicate the amount of sample variance explained by each principal component. The phylum is represented by red dots. Dots with color in the diagram correspond to different site samples. Arrows represent environment factors. TP = total phosphorus, TN = total nitrogen, pH = pH value, Tm = temperature, TOC = total organic carbon, and SGZ = sediment grain size.

4. Discussion

4.1. Microeukaryotic Alpha Diversity Affected by Environmental Variables

Global climate change and human activities are currently affecting the distribution and the number of marine creatures through pollution, invasive species, and coastal development, which also provide strong threats to marine biodiversity [27]. The term "alpha diversity" describes the diversity found within a certain area or ecosystem. The Shannon–Wiener diversity index, the Simpson diversity index, and the Chao1 richness index are examples of measuring indicators that are frequently employed [28,29]. The Chao1 index is widely used to estimate species richness, with higher values indicating greater richness [29]. The Chao1 index analysis provides insights into the variation in species richness among different regions in the Bohai Sea. The alpha diversity indices (Shannon, Simpson, and Chao1) were calculated to compare inter-group differences in microeukaryotic communities in the Bohai Sea. Microeukaryotic communities in the Miaodao Archipelago, Yellow River Estuary, and Central Bohai Sea had higher Shannon and Simpson indices, while the Bohai Bay had the lowest values (Figure 3A,B). We demonstrated that the Shannon index varies from 2.82 to 6.48 among these five geographic regions in the Bohai Sea (Table S2). Morphological identification using a light microscope showed that the Shannon index varies (diatoms and silicoflagellates) from 1.71 to 4.64, with higher values in the sea areas adjacent to the mouth of the Yellow River [2]. The Yellow River Estuary exhibited the highest Chao1 index, indicating greater species richness, while the Miaodao Archipelago showed the lowest Chao1 index, implying lower species richness.

Archipelagos' littoral zones are recognized as highly productive coastal habitats and biodiversity hotspots [4,14,30]. However, intensive aquaculture practices, including the cultivation of algae, finfish, and shellfish, often lead to habitat alteration and the simplification of ecosystems [31,32]. The Miaodao Archipelago is renowned for its well-developed marine aquaculture industry, earning it the titles of "township of abalone", "township of scallop", and "township of seaweed" [33]. Additionally, frequent fishing activities, transportation, and ocean currents have moderately disturbed the benthic ecological quality of the Miaodao Archipelago. The Chao1 index was employed to assess the diversity of eukaryotic communities in different regions of the Bohai Sea. Our findings indicate that the microeukaryotic communities in the Miaodao Archipelago exhibit higher Shannon and Simpson indices but the lowest Chao1 index value among the five groups, suggesting lower species richness (Figure 3C). This observed trend may be attributed to the intense aquaculture practices in the Miaodao Archipelago, which likely influence the richness of benthic eukaryotes in the region.

Microeukaryotic alpha diversity in marine ecosystems can be significantly influenced by various environmental variables. One important factor is nutrient availability. High concentrations of nitrogen and phosphorus have been associated with increased microeukaryotic diversity. These nutrients serve as essential resources for the growth and metabolism of microeukaryotes. The Yellow River Estuary has a significant role in the organization of fisheries resources and biological reproduction in the northern China Sea. It is a crucial spawning ground for numerous species that are found in the Bohai Sea and Yellow Sea [34]. The Yellow River Estuary demonstrated higher species richness compared with the Miaodao Archipelago, highlighting the importance of regional differences in shaping eukaryotic community diversity and richness. The use of NMDS analysis and stress values to assess community similarities and differences is a well-established approach in ecological studies [22,35]. We conducted NMDS analysis to identify similarities and differences in the spatial distribution of microeukaryotic communities. The clustering of samples into five distinct groups suggests significant variations in the microeukaryotic community structure among the regions, reflecting spatial disparities in community composition in the Bohai Sea.

4.2. Dominant Microeukaryotic Assemblages Alter Spatially with Environmental Variation

Local environmental factors in typical subtropical coastal waters were linked to the habitat-specific profiles of microeukaryotes and influenced the microeukaryotic biogeography [36]. Functional groups of benthic organisms provide a comprehensive reflection of marine habitats and environmental quality [37,38]. The microeukaryotic communities in the surface sediments of the Bohai Sea were mainly dominated by Dinoflagellata, Bacillariophyta, Ciliophora, Cercozoa, Protalveolata, and Labyrinthulomycetes (Figure 4). In addition, our results indicated that there were significant differences in the relative abundance of dominant species among the five geographic regions (Figure 3). A previous report demonstrated that diatoms and dinoflagellates, which play a crucial role in coastal ecosystems, undergo restructuring due to eutrophication and warming, making them a significant concern in coastal waters [39]. In the present study, we identified a total of 380 ASVs of diatoms, annotated with 41 genera and 51 species from nine sites of the five geographic regions in the Bohai Sea. Morphological identification using a light microscope identified a total of 156 diatoms from 63 sites of surface sediments in the Bohai Sea, including 74 centric diatoms and 82 pennate diatoms [2]. Through data comparison, we have also clearly identified the advantages (high throughput and coverage) and disadvantages (lacking reference sequence and insufficient resolution according to amplification sequence) of species identification based on molecular biology methods.

Over the past ten years, the dominance of dinoflagellates has been more apparent [38,40]. Dinoflagellates had the highest relative abundance, accounting for 37.80% on average. It dominated the Bohai Bay (78.67%) but was less abundant in the Liaodong Bay. The presence of dinoflagellates and associated cysts in coastal areas acts as an indicator to track the process of anthropogenic eutrophication [41,42]. Dinoflagellate dynamics were sensitive to water

temperature, and the optimal growth temperature for most dinoflagellates was above 20 °C. *Gonyaulax*, belonging to the phylum Dinofllagellata, occupied a proportion of 57.77% in the Bohai Bay, which was the most abundant taxa (Figure 4B). Many species in *Gonyaulax* belong to harmful algal blooms and produce toxins. For example, *Gonyaulax polygramma*, a bloom-forming dinoflagellate, accounted for 99.4% of the phytoplankton abundance at the bloom location of the southeastern Arabian Sea [43]. A temperature higher than 25 °C is suitable for *G. polygramma* blooming [44]. The abundance of *Gonyaulax* and *Polykrikos* was markedly higher in the Bohai Bay compared with other marine areas (Figure 4B). We deduced that the high abundance of dinoflagellates in the Bohai Bay can potentially be associated with both eutrophication and warm water temperatures. Moreover, the Bohai Sea Coastal Current may further contribute to the high abundance of *Gonyaulax* in the Yellow River Estuary and Miaodao Archipelago (Figures 1 and 5A). *Thalassiosira*, belonging to the phylum Bacillariophyta, has a relatively high abundance at the Central Bohai Sea, Liaodong Bay, and NS29 site (Yellow River Estuary). The Yellow Sea Warm Current, Yellow River, and Liaohe River runoff bring abundant nutrients such as silicon, nitrogen, and phosphorus to these sea areas [3]. Diatoms, especially in spring and autumn, can rapidly reproduce and become dominant species in water bodies.

At the niche level, biotic interactions like competition, predation, and niche creation are essential for biodiversity [45]. Networks are a potent tool for comprehending and visualizing the ecological and evolutionary relationships between different species [46]. This approach allows us to understand the complex structure and functionality of biological communities, highlighting the importance of biotic interactions for biodiversity [47]. We constructed a species relationship network graph to analyze the benthic eukaryotes in the Bohai Sea. Spearman correlation coefficients were calculated to determine species relationships within the network. A total of 177 connecting lines were found by correlation analysis, 130 of which showed positive association and 47 of which showed negative correlation (Figure 6). It was a highly intriguing observation that dinoflagellates and diatoms clearly possessed a competitive relationship. These observations further support the idea that biotic interactions play a crucial role in influencing the biodiversity patterns observed in this region. These findings align with the statement suggesting the essential role of biotic interactions such as competition and predation in maintaining ecosystem stability and functioning [48].

Although many studies have emphasized the significance of both spatial and environmental factors in microeukaryotic communities, it is still difficult to determine which mechanism predominates [3,36]. The Bohai ecosystem has undergone a transition from an N-limited oligotrophic state prior to the 1990s to a potentially P-limited eutrophic state. Between the late 1950s and mid-2010s, dissolved inorganic nitrogen (DIN) concentrations increased approximately sevenfold, while dissolved inorganic phosphorus (DIP) and dissolved silicon (DSi) concentrations declined [34]. A two-year survey conducted from 2020 to 2021 revealed significant spatial variations in the parameter distribution [3]. The Liaodong Bay, Bohai Bay, and Laizhou Bay exhibited elevated levels of chemical oxygen demand (COD), DIN, and soluble reactive phosphate (SRP), primarily due to terrestrial inputs as the primary source of pollution. Phosphorus is gradually becoming the main factor limiting the growth of phytoplankton in the Bohai Sea [38,39], and higher concentrations of petroleum pollutants were detected in port areas, offshore oilfields, and their surrounding regions [3].

Canonical correlation analysis (CCA) is a sorting method that combines correspondence analysis with multivariate regression analysis [49]. This analysis method is mainly used to reflect the relationship between benthic organisms and environmental factors. Our CCA results showed that phosphorus, nitrogen, salinity, temperature, and silicate were the main factors influencing the distribution of benthic communities (Figure 7). Similarly, CCA results demonstrated that the most significant environmental variables affecting the variance of the phytoplankton community structure in a scallop cultivation sea area of the Bohai Bay were phosphate, salinity, temperature, silicate, and the DIN/SiO_2 ratio [50]. We found that phosphorus, nitrogen, silicate, sediment grain size, and pH are environmental

factors positively correlated with the microeukaryotic communities in sediment samples in the Yellow River Estuary, while salinity, temperature, and TOC positively correlated with the microeukaryotic communities in the Bohai Bay and Miaodao Archipelago (NS34 and NS35 sites) (Figure 7). Silicate and phosphate concentrations were closely associated with the abundance of diatoms, while salinity, temperature, and TOC showed significant positive correlations with Dinoflagellata (Figure 7). RDA and Spearman analysis showed that temperature and phosphorus (P) differentiated the spatial distributions of diatoms and dinoflagellates in the Bohai Bay [39]. Many dinoflagellates are heterotrophic, meaning they may graze on other tiny creatures in an oligotrophic environment to supplement their diet [51]. Previously, total phytoplankton and dinoflagellates also both had a negative correlation with salinity in the Bohai Sea according to an examination of the structural traits of phytoplankton communities and their interaction with environmental conditions [52]. We suggested that environmental variables may be essential in shaping the spatial variation of microeukaryotic communities of surface sediments in the Bohai Sea.

4.3. Regime Shifts in Diatom–Dinoflagellate Composition Reflected Eutrophication

Over the past 50 years, the composition of the microeukaryote community in many coastal marine ecosystems has changed due to anthropogenic pressures such as global warming and nutrient variations [39,53]. During the recent warming reacceleration stage, the spatial mode of the circulation anomalies in East Asia and the western Pacific has moved westward and has an increased inshore effect compared with previous periods due to higher fluctuations in the warming rate [54]. The surface warming rate of offshore China, particularly in the Bohai Sea, has rapidly increased and considerably exceeds that of the global ocean. In addition to warming, the Bohai Sea's ecological environment is mostly impacted by inland pollution inputs, including heavy metals, petroleum, phosphorus, and nitrogen [9,40]. The Bohai Rim region's population has been quickly growing since the 1970s, which has caused significant amounts of phosphorus and nitrogen being released into the sea. Throughout the Bohai Sea, the N/P ratio has risen from 3.3 to 14.0 over the past 50 years, whereas the Si/N ratio has dropped from 10.0 to 1.0 [39]. Lately, P limitation, or P-Si limitation, has replaced the N constraint as the primary nutrient limitation [38,39].

Eutrophication has had significant impacts on the composition and biomass of microeukaryotes in coastal regions, particularly leading to an increase in harmful algal bloom (HAB) incidents accompanied by severe silicon (Si) limitation [39,40]. Diatoms and dinoflagellates play crucial roles in coastal waters as key components of aquatic ecosystems [39,53]. Diatoms have decreased in proportion, while non-diatoms like dinoflagellates and cyanobacteria have become more prevalent [40]. A recent study revealed distinct seasonal shifts in the phytoplankton distribution in the Bohai Sea, with dinoflagellates dominating during spring/summer and diatoms dominating during autumn/winter [50,55]. In the Bohai Sea, the abundance of dinoflagellates increased from 16% to 30%, while the abundance of diatoms decreased from 82% to 68% between summer and autumn. Eutrophication-driven regime shifts in the diatom–dinoflagellate composition have been observed in the Baltic Sea and Bohai Sea, causing detrimental effects on the regulatory, provisioning, cultural, and supporting functions of marine ecosystems, as well as the overall coastal environment [40]. We found that the proportion of diatoms and dinoflagellates is relatively high in the Bohai Bay and Laizhou Bay, further indicating that these two bays are strongly influenced by human activities.

Numerous terrestrial and marine environments contribute to pollution in the Bohai Bay. These include ship pollution in the waters of Tianjin Port, river discharges (such as the Yellow and Haihe Rivers), seawater aquaculture discharge, domestic sewage, pollution from offshore oil and gas extraction, pollution from dredging, and pollution from ship ballasts in the bay [39,50]. The Bohai Bay, in particular, has received a lot of attention because of its significance to the coastal economy and its susceptibility to both human activities and unpredictable climate conditions [39]. Due to climate warming and eutrophication, the microeukaryotic community structure in the Bohai Bay has significantly changed in

recent decades [50]. We found that the ratio of diatoms to dinoflagellates in the Bohai Bay was 0.07, the lowest among the five geographic regions of the entire Bohai Sea. We speculate that the abnormal ratio of diatoms to dinoflagellates can be connected to changes in the Bohai Bay's nutrition cycle and surface warming. Currently, long-term datasets are needed to examine how the dynamics of nutrient ratios and warming affect the aberrant succession of dinoflagellates and diatoms [39]. We also should further distinguish between active cells and cysts in the microeukaryote assemblies identified from the surface sediment. In summary, our results reveal that spatial microeukaryote variation and environmental determinants are reliable indicators of the level of eutrophication in marine environments caused by global climate change and human activities.

5. Conclusions

We found that dominant microeukaryotic assemblages spatially alter with environmental variation in the surface sediments of the five geographic regions of the Bohai Sea. The Miaodao Archipelago, Yellow River Estuary, and Central Bohai Sea had high Shannon and Simpson indices, while the Yellow River Estuary had the highest Chao1 index. Dinoflagellata, Bacillariophyta, Ciliophora, Cercozoa, Protalveolata, and Labyrinthulomycetes dominated these regions. *Thalassiosira* prevailed in the Central Bohai Sea and Liaodong Bay, while *Gonyaulax* dominated in the Bohai Bay. Environmental factors like phosphate, nitrate, salinity, temperature, and silicate influenced the community composition. The ratio of diatoms to dinoflagellates reflected the anthropogenic eutrophication of coastal ecosystems of the Bohai Sea. Our research offers crucial information for forecasting microeukaryote responses, adaptations, and feedback to spatially environmental variation, as well as for evaluating the sustainability of the ecosystem in the Bohai Sea. However, future studies should consider categorizing microeukaryotes based on cell size and addressing differential assembly mechanisms to more comprehensively understand microeukaryotic biogeography.

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