ECOLOGICAL RESPONSE OF BENTHIC FORAMINIFERAL COMMUNITY TO ENVIRONMENTAL FACTORS IN OUJIANG ESTUARY, CHINA

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Abstract. Estuaries are among the most productive and valuable ecosystems on the planet, greatly affected by human activities. The decrease of benthic foraminifera community diversity, the increase in the abundance of tolerant species and the decrease in the abundance of sensitive species were often used as indicators of marine environmental pollution. In this study, we focused on the Oujiang Estuary to investigate the degree of heavy metal pollution and potential ecological risks. The results showed the degree contamination of Cd was higher, while that of Cu, Zn, Pb, Cr, Hg, As and Ni were moderate. Cd concentrations meant considerable and even high ecological risks, and Hg concentration was at moderate or considerable ecological risks. Concentrations of Cu, Zn, Pb, Cr, As and Ni meant low potential ecological risks. There was considerable contamination and moderate ecological risk of heavy metals in Oujiang Estuary. Based on high-throughput sequencing technology, a total of 51 genera of benthic foraminifera were identified. *Operculina* was the most dominant, followed by *Allogromiina*, *Nummulites* and *Parasorites*. Correlations between heavy metal concentrations and foraminiferal diversity, species tolerance were analysed. It was found that Chla, Pb, Cu, grain size and Hg might be key factors affecting the distribution and composition of benthic foraminiferal communities. *Rotaliella* was tolerant to Zn, and *Nummulites* was tolerant to Pb. *Ovammina* and *Crithionina* might prefer sediment environments with smaller particle sizes.
Keywords: *high-throughput sequencing, environmental DNA, diversity, heavy metals, grain size*

Introduction

Estuary is the main hub of the interaction between ocean and river basin, which is a fragile and environmentally sensitive zone under the comprehensive influence of human activities, land and ocean (Wang et al., 2023; Chai, 2020). Located in the south of Zhejiang Province, Oujiang Estuary is rich in ports, beaches, tourism and other resources, and it is a region with active social economy and significant influence of human activities (Wang et al., 2023; Zhang et al., 2016). With the acceleration of industrialization and urbanization, the discharge of industrial and agricultural wastewater and domestic sewage posed a serious threat to the ecological health of the Oujiang Estuary water environment, resulting in increasingly serious heavy metal pollution problems in the area (Zhang et al., 2023; Che et al., 2017). Sediments are both sinks and sources of water pollutants. On the one hand, pollutants accumulated in sediments through various physical, chemical and biological processes, and the sediment became a reservoir of marine pollutants (Che et al., 2017; Liu et al., 2024). On the other hand, the release of pollutants in sediments increased the pollutant loads of marine waters, causing important impacts on marine organisms and human health (Che et al., 2017; Liu et al., 2024).

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Benthic foraminifera are single-celled protozoa, which inhabited all possible marine and brackish water environments from shallow water intertidal regions to deep trenches (Saraswati, 2021). Benthic foraminifera are very sensitive to the changes in the ambient environment, including temperature, salinity, dissolved oxygen, pH, pollutants, and anthropogenic activities, which are often used as indicators of changes in the marine environment (Polovodova and Schönfeld, 2008; Frontalini et al., 2009). Zalesny (Zalesny, 1959) initially proposed the utilization of benthic foraminifera as an indicator of pollution in the marine environment. Since then, researchers around the world have conducted studies on the relationship between heavy metal pollution and species distribution, test morphology, community structure of benthic foraminifera in different sea areas (Coccioni et al., 2009; Ferraro et al., 2006; Frontalini and Coccioni, 2008).

In this study, the concentrations and distribution characteristics of heavy metals in the Oujiang Estuary sediments were investigated, and benthic foraminifera were identified based on high-throughput sequencing (HTS). The main objectives are to (1) assess the pollution levels and ecological risks of heavy metal; (2) evaluate the molecular diversity and community composition of benthic foraminifera; (3) explored the relationships between benthic foraminiferal community and environmental factors in Oujiang Estuary.

Materials and methods

Sampling

Sediment samples were collected using a Petersen grab sampler from Oujiang Estuary on August 13, 2021 (*Fig. 1*). The water depth information of the sampling station is shown in *Table 1*. The top ~2 cm of the surface sediment was carefully collected and three parallel samples were collected at each station. The three samples were mixed evenly with glass rods in the sample bottle and then divided into three 50 ml centrifuge tubes, respectively. One tube was stored at room temperature for sediment particle size analysis, and the others were frozen in liquid nitrogen and then stored in a refrigerator at -80°C for DNA extraction and heavy metal determination, respectively. Seawater was collected and fixed with magnesium carbonate suspension for chlorophyll a (Chla) determination.

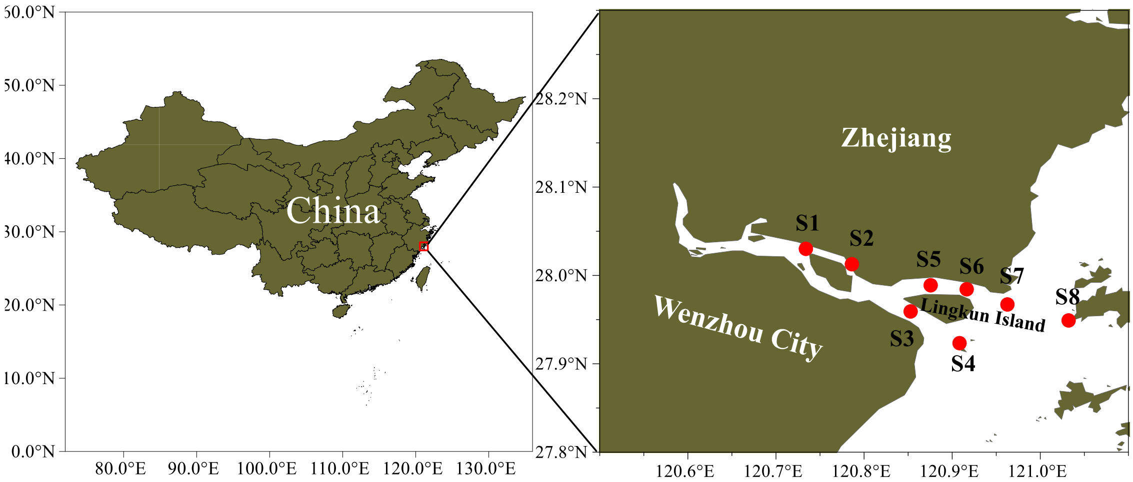

Figure 1. *Location map of the study area and sampling stations*

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Table 1. *Coordinates and water depth of sampling stations*

Station	Longitude	Latitude	Depth (m)
S1	120°44′2″	28°1′48″	15
S2	120°47′10″	28°0′46″	17
S3	120°51′10″	27°57′33″	7
S4	120°54′30″	27°55′24″	6
S5	120°52′32″	27°59′20″	8
S6	120°54′59″	27°59′4″	14
S7	120°57′46″	27°58′2″	7
S8	121°1′56″	27°56′57″	7

Sample measurements

The concentrations of Chla in seawater was determined using a ultraviolet spectrophotometer (Cary50, Agilent Technologies Co. Ltd). Grain size fractions of surface sediments were detected using a Microtrac S3500 Particle Size Analyzer (Microtrac S3500, Microtrac Ltd). The sediment samples used for heavy metal analysis were freeze-dried, ground and sieved, and digested before determination. The concentrations of Pb and Cd using an atomic absorption spectrometer (280Z AA, Agilent Technologies Co. Ltd). The concentrations of Cu, Zn, Cr and Ni using an atomic absorption spectrometer (AA240, Agilent Technologies Co. Ltd). Hg concentration was analyzed using a Milestone DMA-80 direct mercury analyzer (DMA-80, Milestone), and the concentration of As was measured and analyzed using atomic fluorescence spectrometer (Kylin S12, Beijing Jitian Instrument Co., Ltd).

Pollution assessments of heavy metals in sediments

In this study, Hakanson index method was used to assess the degree of contamination and ecological risks of heavy metal in the Oujiang Estuary (Hakanson, 1980). The formulas are as follows:

$$C_f^i = \frac{C_i}{C_n^i} \quad \text{(Eq.1)}$$

$$C_d = \sum_{i=1}^{8} C_f^i \quad \text{(Eq.2)}$$

$$E_{\mathrm{r}}^i = T_r^i \times C_f^i \quad \text{(Eq.3)}$$

$$PERI = \sum_{i=1}^{8} E_r^i \quad \text{(Eq.4)}$$

where C_{i} is the measured concentrations of heavy metals, and C_{n}^i is the background concentrations of heavy metals. The background values of Cu, Zn, Pb, Cd, Cr, Hg, As

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and Ni in the East China Sea were 14, 66, 21, 0.032, 61, 0.025, 8 and 25 mg/kg, respectively (Chi and Yan, 2007). C_f^i is the monomial contamination factor of heavy metal, C_d is the polynomial contamination factor of eight heavy metals. T_r^i is the biological toxicity response factor of heavy metals. The toxicity factors of Cu, Zn, Pb, Cd, Cr, Hg, As and Ni were 5, 1, 5, 30, 2, 40, 10 and 5, respectively (Hakanson, 1980; Xu et al., 2008; Lu et al., 2012). E_r^i is the monomial potential ecological risk index of heavy metal. $PERI$ is the sum of all risk factors of heavy metals in sediments. The degree of contamination and potential ecological risk classification standard referred to Hakanson (1980).

Extraction and sequencing of DNA

Genomic DNA was extracted from sediment samples using Fast DNA® SPIN Kit for Soil (MP Biomedicals, USA) according to manufacturer's instructions. The foraminiferal small subunit (SSU) rDNA sequences were amplified using foraminiferal-specific primers s14F3 (5′-ACGCAMGTGTGAAACTTG-3′) and s17 (5′-CGGTCACGTTCGTTGC-3′) (Pawlowski, 2000; Lejzerowicz et al., 2013). PCR reaction system and amplification procedure referred to Qiao et al. (2024). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Data quality control and processing

Raw reads were quality filtered using QIIME (version 1.9.1; http://qiime.org/install/index.html), and merged by FLASH (version 1.2.11, https://ccb.jhu.edu/software/FLASH/index.shtml). Then the optimized sequences were clustered into operational taxonomic units (OTUs) using UPARSE (version 7.0.1090, http://www.drive5.com/uparse/) based on 97% sequence similarity. The taxonomy of each OTU representative sequence was assigned to the National Center for Biotechnology Information (NCBI) database. The OTUs that could not be assigned to foraminifera were eliminated from the dataset. To enable statistical analyses and comparisons of sequencing data across all samples, the numbers of foraminifera rDNA sequences were rarefied to 66,873 to normalize the data of each sample. The subsequent benthic foraminiferal diversity and community composition analysis were carried out based on the data after this standardization.

Statistical analysis

OTU-level alpha diversity indices including Chao1 richness and Shannon index were calculated with Mothur (version 1.30.2; https://www.mothur.org/wiki/Download_mothur) using the Majorbio Cloud platform (https://cloud.majorbio.com). The relative abundance of benthic foraminifera was defined as the percentage of species sequences in the total sequences. The dominant genus was benthic foraminifera with a relative abundance greater than 10%. Redundancy analysis (RDA) was conducted using the CANOCO (version 5.0) to identify the main factors that influenced benthic foraminiferal community structure. Spearman correlation analysis was performed to determine the correlation between dominant genus of benthic foraminifera and environmental factors using SPSS (version 20). $p < 0.05$ was considered as statistically significant.

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Results

Environmental parameters

The concentrations of Chla in seawater samples ranged from 0.082 to 2.512 μg/L (*Fig. 2*). The highest Chla concentration appeared at station S1, and the lowest concentration was found at station S8. In summary, the average Chla concentration in the upper reaches of Oujiang was higher than that in the lower reaches.

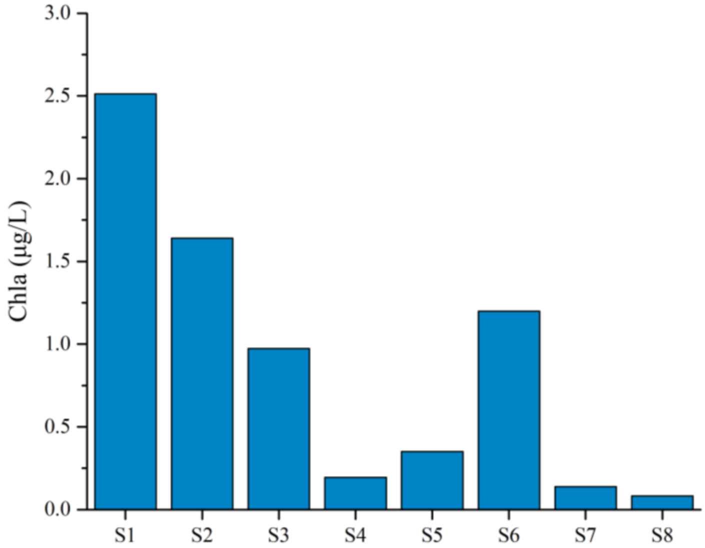

Figure 2. *The concentrations of Chla in the seawater of the Oujiang Estuary*

The range of grain sizes of surface sediments in the study area was 7.71 to 72.56 μm (*Fig. 3*). The grain size at station S5 was the largest, and at the other stations were all less than 21 μm. Silt was the main component of surface sediments in Oujiang Estuary, followed by clay and sand. In the study area, the percentage of clay were higher at stations S3 and S4, with contents of 40.53% and 36.94%, respectively. Spatially, the grain sizes on the northern of Lingkun Island were larger than those on the southern of Lingkun Island.

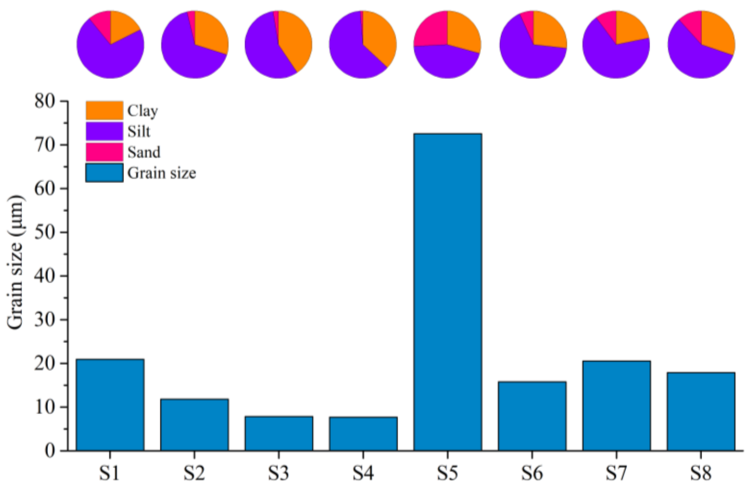

Figure 3. *The grain sizes and percentages of clay, silt, and sand in the Oujiang Estuary*

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Eight heavy metals including Cu, Zn, Pb, Cd, Cr, Hg, As and Ni were measured, and their concentration and spatial distributions were showed in *Figure 4*. Their concentration ranges were 13.62-56.63 mg/kg, 87.75-146.81 mg/kg, 31.41-50.04 mg/kg, 0.10-0.19 mg/kg, 42.15-104.35 mg/kg, 0.03-0.06 mg/kg, 4.00-12.42 mg/kg and 28.90-48.83 mg/kg, respectively. Except for Ni, the concentrations of the other 7 heavy metals were the lowest at station S1. The highest concentrations of Cu and Cd were found at station S2. The concentrations of Zn, Pb, Cr and Ni were higher in the southern of Lingkun Island than those in the northern. In addition, the concentration of Hg was higher in the lower reaches of the Oujiang River than in the upper reaches.

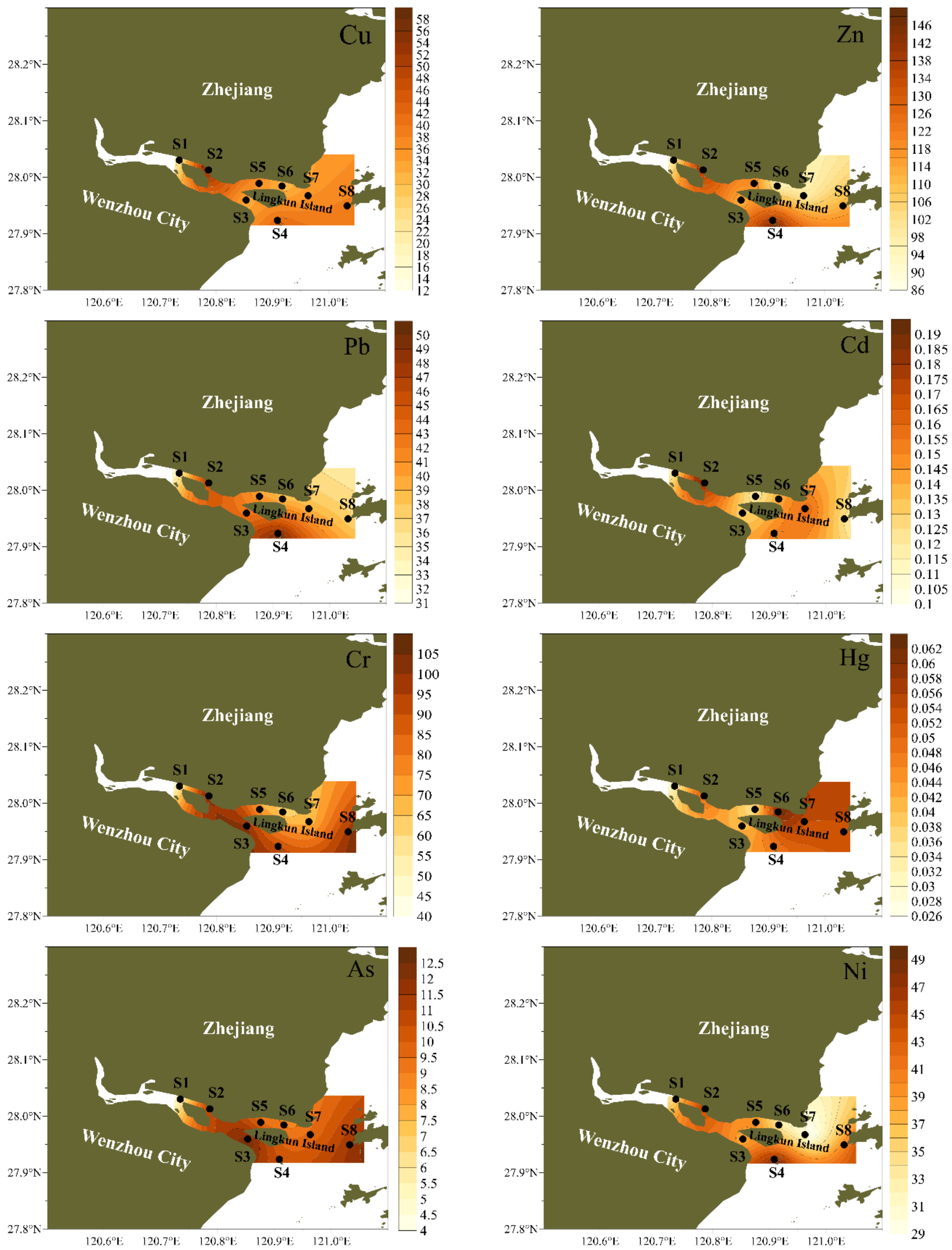

Figure 4. *Spatial distributions of heavy metals in surface sediments of the Oujiang Estuary (concentration unit: mg/kg)*

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The result of Hankanson index method showed that the degree of contamination of Cd was higher, while other heavy metals were moderate contamination (*Fig. 5a*). The range of C_d values was from 10.68 to 21.13, and the highest value appeared at station S2, and the lowest value appeared at station S1 (*Fig. 5a*). The average value of C_d was 16.54. Thus, there was considerable contamination with heavy metals in Oujiang Estuary. The values of E_r^i indicated that Cd was at considerable and even high ecological risks, and Hg was at moderate or considerable ecological risks. The other six heavy metals were at low potential ecological risks (*Fig. 5b*). The *PERI* values were 168.74-318.32, and the highest value appeared at station S2, and the lowest value appeared at station S1 (*Fig. 5b*). The average value of *PERI* was 251.84, suggesting that there was moderate ecological risk of heavy metals in Oujiang Estuary.

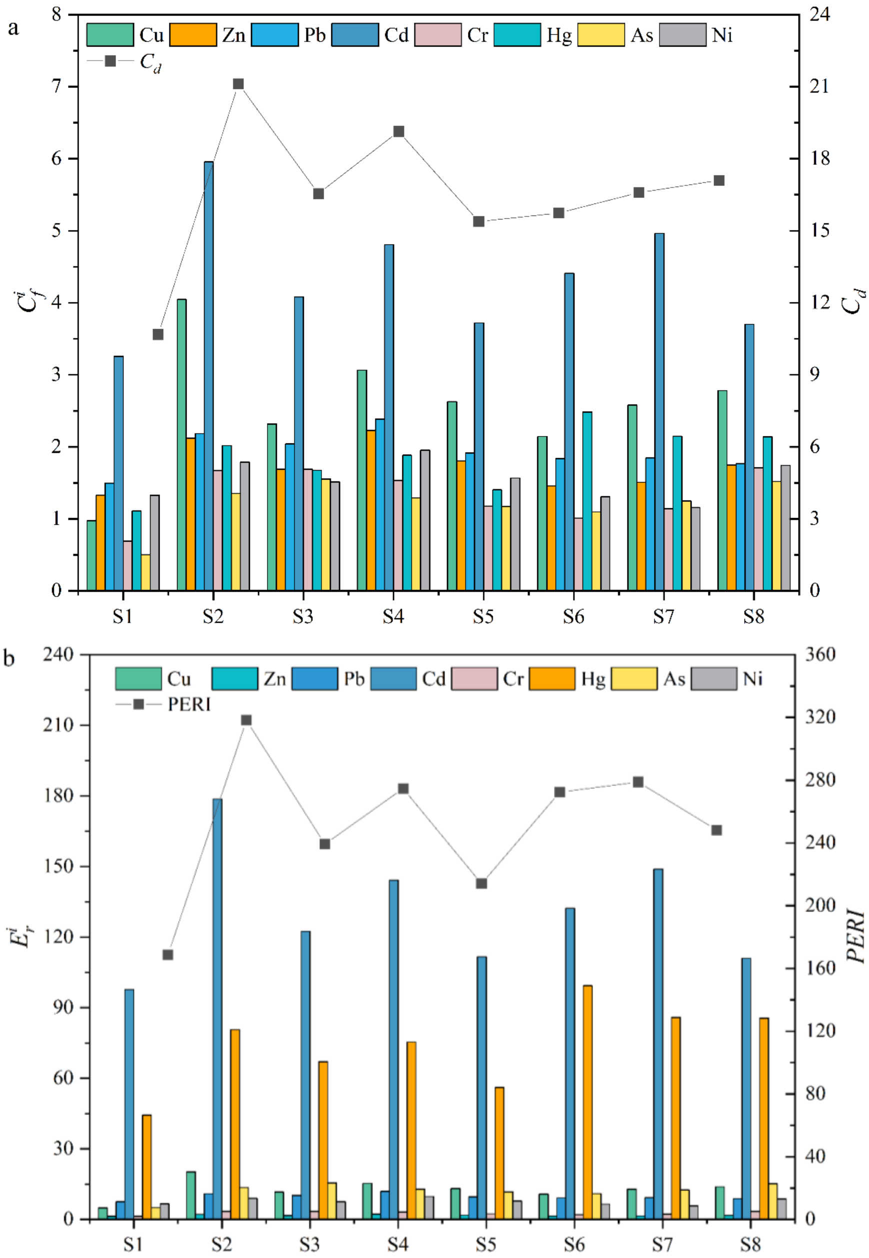

Figure 5. *The degree of contamination (a) and potential ecological risk index (b) of heavy metals in surface sediments of Oujiang Estuary*

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Benthic foraminiferal diversity

Benthic foraminiferal Chao 1 richness index and Shannon diversity are shown in *Figure 6*. The results indicated that benthic foraminiferal richness and diversity on the northern of Lingkun Island were lower than those on the southern of Lingkun Island. On the northern of Lingkun Island, the richness and diversity of benthic foraminiferal community decreased gradually along the Oujiang River.

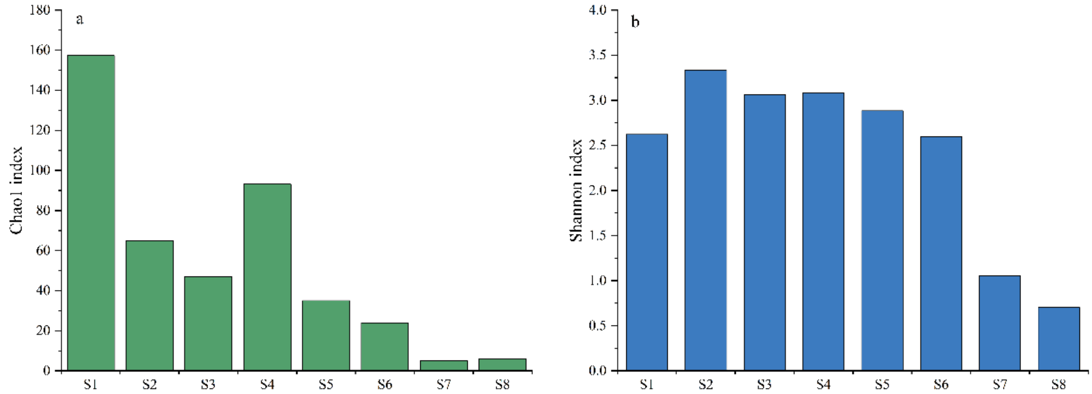

Figure 6. *OTU-level Chao1 index (a) and Shannon index (b) of benthic foraminiferal community*

Benthic foraminiferal community composition

A total of 4 classes including Globothalamea, Monothalamea, Nodosariata and Tubothalamea were identified (*Fig. 7a*). At station S1, Nodosariata was the most abundant class. However, this class was not found in other stations. Globothalamea was the most dominant, followed by Monothalamea and Tubothalamea at stations S2, S3, S5 and S7. Monothalamea was the most dominant at stations S4 and S6. At station S8, only two classes including Globothalamea and Monothalamea were identified.

At the order level, a total of 7 orders of benthic foraminifera were found (*Fig. 7b*). At station S1, Polymorphinida was the most abundant order, followed by Rotaliida and Miliolida. At station S2, the dominant taxa changed to Rotaliida, Miliolida, Astrorhizida and Allogromiida. Along the Oujiang River, on the southern of Lingkun Island, the dominant taxa were Rotaliida, Miliolida and Astrorhizida; and on the northern of Lingkun Island, the dominant taxa changed to Rotaliida, Miliolida and Allogromiida.

At the family level, 33 families of benthic foraminifera were identified (*Fig. 7c*). At station S1, Glandulinidae was the most abundant family, followed by Nummulitidae. At station S2, the dominant taxa changed to Nummulitidae, Allogromiidae, Soritidae and Saccamminidae. On the southern of Lingkun Island, the dominant taxa were Nummulitidae, Saccamminidae, Soritidae, Rotaliidae and Crithioninidae; and on the northern of Lingkun Island, the dominant taxa were Nummulitidae, Allogromiidae, Alveolinidae, Soritidae and Rotaliidae.

At the genus level, 51 genera of benthic foraminifera were found (*Fig. 7d*). *Operculina* was the most dominant, with average relative abundance of 25.04%, followed by *Allogromiina* (12.41%), *Nummulites* (9.35%) and *Parasorites* (6.29%). At station S1, *Glandulina* was the most abundant genus, followed by *Operculina*. At station S2, the dominant genera were changed to *Nummulites*, *Operculina*, *Allogromiina*

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and *Parasorites*. On the southern of Lingkun Island, the dominant genera were *Operculina*, *Ovammina*, *Parasorites*, *Nummulites*, *Ammonia* and *Crithionina*. On the northern of Lingkun Island, the dominant genera included *Alveolinella*, *Allogromiid*, *Operculina*, *Nummulites*, *Vellaria* and *Rotaliella* at stations S5 and S6, and changed to *Operculina*, *Allogromiina* and *Amphisorus* at stations S7 and S8.

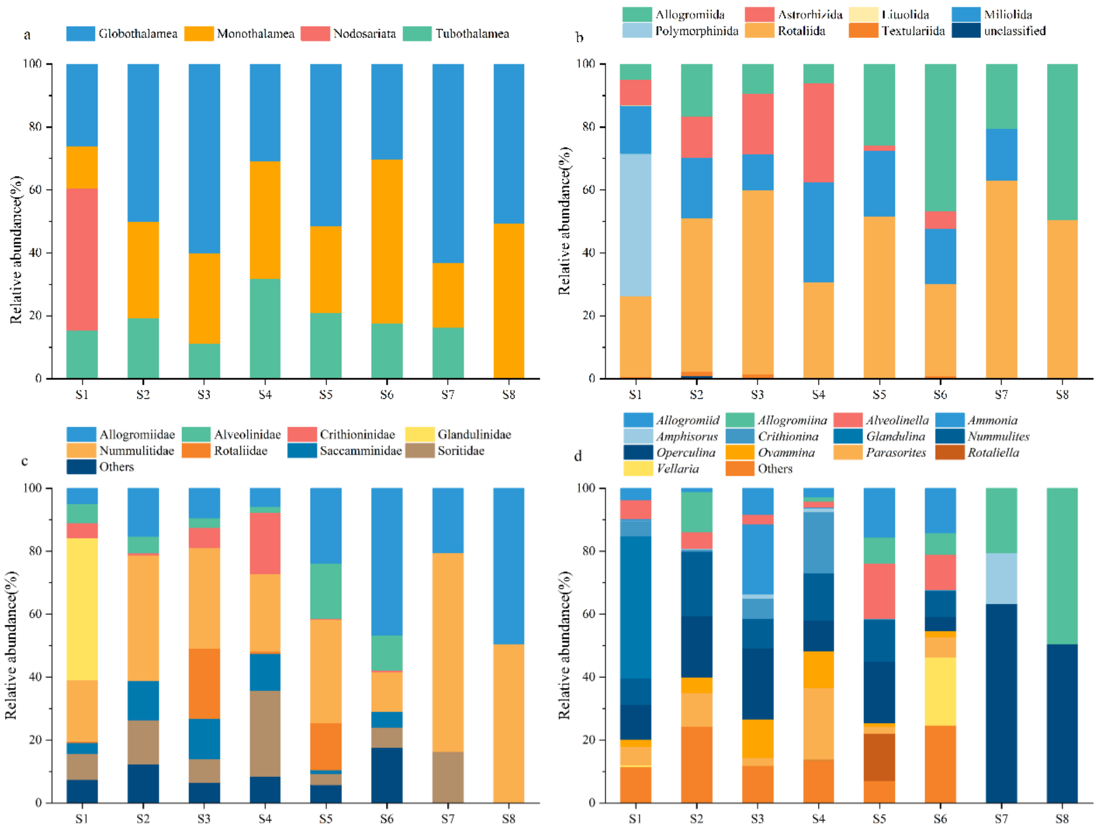

Figure 7. *Benthic foraminiferal community composition in the sediment samples at the class (a), order (b), family (c) and genus (d) levels*

Relationships between benthic foraminiferal community and environmental factors

Thirteen dominant genera were selected for RDA and Spearman correlation analysis. RDA results showed that the first and the second axes explained 53.25% and 18.07% of the total variation, respectively (*Fig. 8*). Chla was the most important variable, explaining 25.6% of benthic foraminiferal community variation, followed by Pb (20.5%), Cu (16.2%), grain size (11.2%) and Hg (10.7%). Therefore, Chla, Pb, Cu, grain size and Hg might be key factors affecting the distribution and composition of benthic foraminiferal communities. The heatmap plots based on Spearman's rho correlations (*Fig. 9*) showed that *Ovammina* exhibited negative correlations with the percentages of sand and grain size ($p \leq 0.05$). *Crithionina* was also negatively correlated with the percentages of sand ($p \leq 0.05$). *Rotaliella* showed a positive correlation with Zn ($p \leq 0.05$), and *Nummulites* was positively correlated with Pb ($p \leq 0.05$). Chao 1 richness index exhibited negative correlations with Hg ($p \leq 0.05$). Shannon index was positively correlated with Pb.

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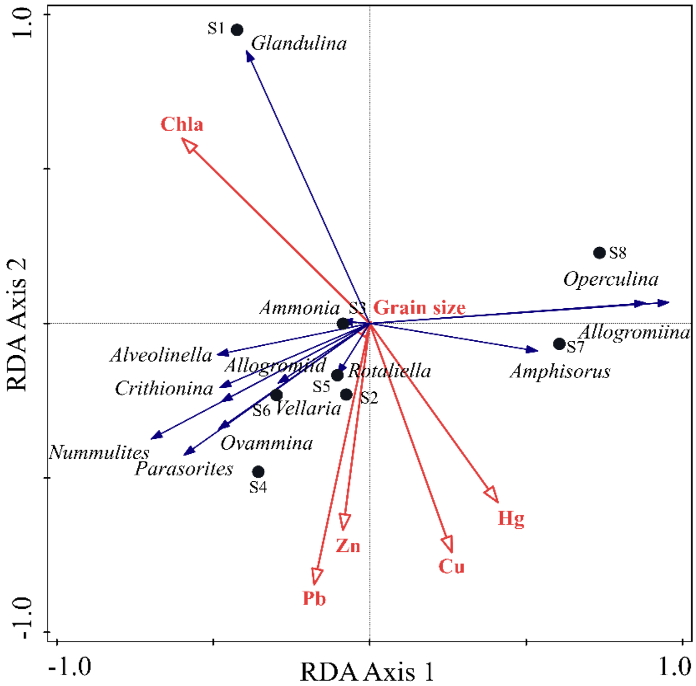

Figure 8. *Redundancy analysis (RDA) of benthic foraminiferal community considering genus level and environmental factors*

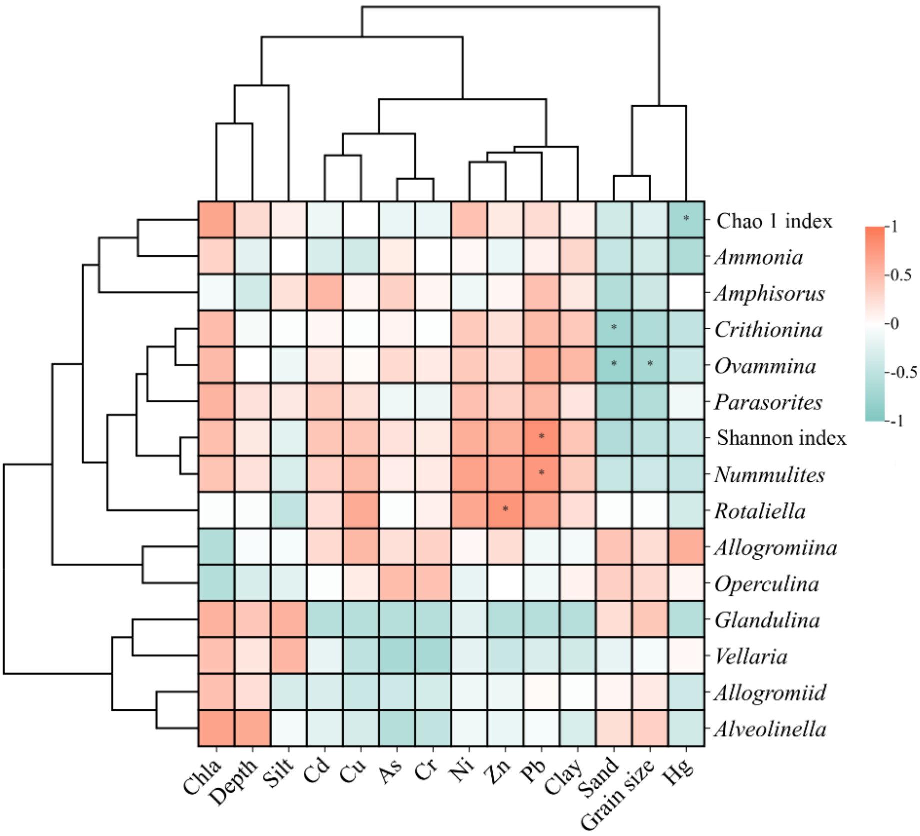

Figure 9. *Heatmaps indicating the correlations between benthic foraminiferal community and environmental parameters (*0.01 < p ≤ 0.05)*

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Discussion

With the port construction and urban development, a large amount of industrial, agricultural and domestic wastewater discharge has caused heavy metal pollution to the Oujiang Estuary and its adjacent waters (Chai, 2020). In this study, we found the degree contamination of Cd was higher, while that of Cu, Zn, Pb, Cr, Hg, As and Ni were moderate. There was considerable contamination with heavy metals in Oujiang Estuary. Cd was at considerable and even high ecological risks, and Hg was at moderate or considerable ecological risks. Cu, Zn, Pb, Cr, As and Ni were at low potential ecological risks. There was moderate ecological risk of heavy metals in Oujiang Estuary. Heavy metals are important pollutants in the marine environment due to their characteristics of bioaccumulation, environmental persistence, high toxicity and long-distance migration (Yang et al., 2021). The accumulation of heavy metals in marine sediments can adversely affect benthic organisms (Almeida et al., 2002; Gale et al., 2006).

Benthic foraminifera are unicellular eukaryotic organisms, characterized by high diversity and abundance. In this study, a total of 51 genera belonging to 33 families, 7 orders, 4 classes of benthic foraminifera were identified. *Operculina* was the most dominant, followed by *Allogromiina*, *Nummulites* and *Parasorites*. The results of RDA revealed that Chla, Pb, Cu, grain size and Hg might be key factors affecting the distribution and composition of benthic foraminiferal communities. Food supply is one of the important factors controlling the dynamics of benthic foraminiferal communities (Schmiedl et al., 2000). Investigation found that the Chla concentrations were significantly positively correlated with the foraminifera abundance, which indicated that the phytodetritus content and freshness were key factors affecting the vertical distribution of foraminifera in sediments of the Yellow Sea and the East China Sea (Xu et al., 2017). The effects of heavy metals on benthic foraminiferal community mainly included the decrease of richness and diversity, the decrease or even disappearance of sensitive species, and the increase of tolerant species (Tarasova, 2006; Frontalini and Coccioni, 2011; Bouchet et al., 2020). In this study, Spearman correlation analysis showed that Chao 1 richness index exhibited negative correlations with Hg ($p \leq 0.05$). *Rotaliella* showed a positive correlation with Zn ($p \leq 0.05$), and *Nummulites* was positively correlated with Pb ($p \leq 0.05$). Therefore, *Rotaliella* was tolerant to Zn, and *Nummulites* was tolerant to Pb. Previous studies of the ecological response of benthic foraminifera community structure to heavy metals in the Oujiang River estuary showed that *Alveolinella*, *Nummulites*, *Parasorites*, *Globorotalia* and *Calcarina* exhibited a significantly negative correlation with Cd, indicating that these species might be sensitive to Cd pollution (Zhao et al., 2024). In this study, *Alveolinella* was negatively correlated with Cd ($p > 0.05$), while *Nummulites* and *Parasorites* showed positive correlations with Cd ($p > 0.05$). The differences in the results might be caused by different sampling times and sampling stations. Therefore, in future studies, sampling time and stations should be fixed for long-term monitoring, so as to fully understand the response of benthic foraminifera community to marine environmental changes in the Oujiang Estuary.

Previous studies have shown that grain size was one of the important factors affecting the distribution and community structure of benthic foraminifera (Jonkers, 1984). On the one hand, the grain size of sediments was related to oxygen content. The sandy sediment had large pore space, which was conducive to maintaining a high oxygen content in the sediment (Li et al., 2021). On the other hand, the grain size of

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sediments also affected the contents of organic matters in sediments. Clay and sandy sediments had strong adsorption capacity, resulting in high organic matter contents and rich nutrition (Li et al., 2021). Organic matters could be used as food for benthic foraminifera, thus providing nutrients for benthic foraminifera. Investigations revealed that *Adercotryma*, *Alveolophragmium*, *Ammonia*, *Borelis*, *Cribrostomoides*, *Discorbis*, *Eggerelloides*, *Elphidiella*, *Elphidium*, *Lepidodeuterammina*, *Pararotalia*, and *Saccammina* were mainly distributed in sandy sediment environments. *Haynesina*, *Hyalinea*, *Islandiella*, *Jadammina*, *Karreriella*, *Melonis*, *Miliammina*, and *Nonion* are mainly distributed in silty sediment environments. Some species, including *Ammobaculites*, *Ammotium*, *Arenoparrella*, *Astrononion*, *Bolivina*, *Bolivinella*, *Bolivinellina*, *Brizalina*, *Buccella*, *Buliminella*, *Cassidella*, *Chilostomella*, *Cibicidoides*, *Cycloclypeus*, *Discorinopsis*, *Ehrenbergina*, *Eilohedra*, *Epistominella*, *Fursenkoina*, *Glabratella*, *Globobulimina*, *Globocassidulina*, *Gyroidina*, *Haplophragmoides*, *Heterostegina*, *Hoeglundina*, *Hormosina*, *Lenticulina*, *Martinottiella*, *Nonionella*, *Nonionoides*, *Oridorsalis*, *Ovammina*, *Pullenia*, *Uvigerina*, and *Vellaria* were mainly distributed in caly sediment environments (Rohling, 2008; Qiao et al., 2022). In this study, Spearman correlation analysis showed that *Ovammina* and *Crithionina* exhibited negative correlations with the percentages of sand ($p \leq 0.05$) and positive correlation with the percentages of clay ($p > 0.05$). This suggested that *Ovammina* and *Crithionina* might prefer sediment environments with smaller particle sizes.

Conclusions

Monitoring and assessment of heavy metals in marine ecosystems have attracted worldwide attention due to serious ecological and biodiversity issues and associated human health concerns. In this study, the pollution levels and ecological risks of heavy metals in the sediments of Oujiang Estuary were assessed. The results showed that the degree of contamination of Cd was higher, while that of Cu, Zn, Pb, Cr, Hg, As and Ni were moderate. There was considerable contamination with heavy metals in Oujiang Estuary. Cd was at considerable and even high ecological risks, and Hg was at moderate or considerable ecological risks. Cu, Zn, Pb, Cr, As and Ni were at low potential ecological risks. There was moderate ecological risk of heavy metals in Oujiang Estuary. The decrease of benthic foraminifera community diversity, the increase in the abundance of tolerant species and the decrease in the abundance of sensitive species were often used as indicators of marine environmental pollution. Benthic foraminifera were identified based on HTS in this study. A total of 51 genera belonging to 33 families, 7 orders, 4 classes of benthic foraminifera were identified. *Operculina* was the most dominant, followed by *Allogromiina*, *Nummulites* and *Parasorites*. The relationships between benthic foraminiferal community and environmental factors in Oujiang Estuary were explored. We found that Chla, Pb, Cu, grain size and Hg might be key factors affecting the distribution and composition of benthic foraminiferal communities. *Ovammina* and *Crithionina* might prefer sediment environments with smaller particle sizes. *Rotaliella* was tolerant to Zn, and *Nummulites* was tolerant to Pb. In the future research, laboratory culture experiments should be conducted to confirm field-observed foraminiferal tolerance to heavy metals. In addition, future studies can also use omics technology to explore the genetic mechanism of benthic foraminifera response to pollutants, so as to improve the utilization level of benthic foraminifera as a biological indicator of marine environmental quality.

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