ECOLOGICAL RESPONSE OF BENTHIC FORAMINIFERA TO HEAVY METALS: A CASE STUDY FROM OUJIANG ESTUARY, CHINA

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Abstract. As the connection between river and ocean, the estuaries are greatly affected by human activities and has sensitive and fragile ecological environment. In this study, the degree of contamination and potential ecological risk level of 8 heavy metals in the surface sediments of Oujiang Estuary were investigated. The results showed that Oujiang Estuary was moderately contaminated with heavy metals, and Cd was moderate even considerable contamination and potential ecological risks. The molecular diversity and community composition of total and active benthic foraminifera were revealed using high-throughput sequencing based on small subunit rDNA and rRNA amplification. A total of 19 genera for total benthic foraminifera were identified, while only 11 genera for active benthic foraminifera were found. The total benthic foraminifera community had higher species diversity and richness than those of active communities. *Alveolinella*, *Nummulites*, *Parasorites*, *Globorotalia* and *Calcarina* exhibited a significantly negative correlation with Cd, indicating that these species might be sensitive to Cd pollution.
Keywords: *high-throughput sequencing, eDNA and eRNA, active foraminifera, molecular diversity, heavy metals*

Introduction

Estuary is the hub of rivers and oceans, which is greatly affected by human activities and has sensitive and fragile ecological environment. The Oujiang Estuary, located in Zhejiang Province, southeastern of China, is the fifth largest estuary after the Yangtze River Estuary, the Yellow River Estuary, the Pearl River Estuary, and the Hangzhou Bay in China (Editorial Board of China Bay Survey, 1992). The main functions of Oujiang Estuary and its adjacent waters are used by port transportation, coastal tourism, and marine fisheries (Zhang et al., 2023). In recent years, with the development of urban construction, port construction and reclamation projects, a large number of industrial, agricultural and domestic pollutants have been discharged into Oujiang and its adjacent waters, which has brought great pressure to the marine ecological environment in the region (Feng et al., 2015).

Heavy metal pollution has the characteristics of toxicity, persistence, biological accumulation, and biological amplification, which is one of the most worldwide concerning environmental problems in the past decades (Qu et al., 2020; Yang et al., 2021; Wang et al., 2022). In marine ecosystems, sediments play an important role in the transportation and storage of heavy metals, which act as both the sink and potential source of heavy metals (Wang et al., 2020, 2022). On one hand, heavy metals in seawater can be deposited to surface sediments via adsorption and sedimentation; on the other hand, heavy metals in sediments also can be released to seawater via particle resuspension and

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desorption processes (De Souza Machado et al., 2016). The enrichment of heavy metals in sediments had a serious effect on the growth and reproduction of benthic organisms (Solami and Satheesh, 2022).

Benthic foraminifera are unicellular protozoa that are widely distributed in the marine ecosystems (Saraswati, 2021). Owing to their high biodiversity, short life cycle, and high sensitivity to changing environmental conditions, benthic foraminifera have been used as environmental bioindicators, especially in polluted coastal marine environments (Tarasova, 2006; Frontalini and Coccioni, 2011; Youssef et al., 2021). Both field and experimental studies have found that heavy metals could affect the growth, abundance, biodiversity, community composition, spatial distribution, shell morphology and chemical composition of benthic foraminifera (Ayadi et al., 2016; El-Kahawy et al., 2018; Boehnert et al., 2020; Qiao et al., 2022).

In this study, high-throughput sequencing (HTS) based on small subunit (SSU) rDNA and rRNA amplifications was used to investigate the total and active benthic foraminifera. The main objectives are to (1) assess the degree of contamination and ecological risks of heavy metal; (2) evaluate the molecular diversity and community composition of total and active benthic foraminifera; and (3) explore the response of benthic foraminiferal community to heavy metals.

Materials and methods

Sampling

This survey was conducted in Oujiang Estuary and its adjacent waters in May 2021 (*Fig. 1*, *Table 1*). The top ~2 cm of surface sediment was sampled. Three duplicate samples were collected at each station for RNA extraction, DNA extraction and heavy metal determination, respectively. All sediments were frozen in liquid nitrogen and brought back to the laboratory for subsequent analysis.

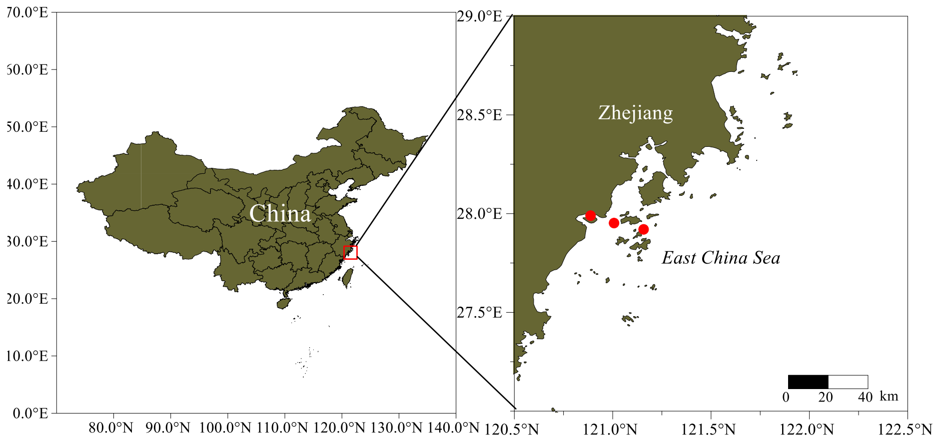

Figure 1. *Location map of the study area and sampling stations*

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

Station	Longitude	Latitude	Depth (m)
OJ1	120°53′15″	27°59′23″	11.3
OJ2	121°00′25″	27°57′08″	5.7
OJ3	121°09′26″	27°55′13″	4.3

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Sample measurements

The sediment samples for chemical analysis were freeze-dried to a constant weight. Subsequently, samples were ground and homogenized with a glass mortar and pestle. Finally, the samples were then passed through a 100-mesh nylon sieve and stored in polyethylene bags. Organic carbon (OC) contents were measured using the potassium dichromate volumetric method. Total nitrogen (TN) and total phosphorus (TP) contents were determined by the potassium persulfate oxidation method using ultraviolet spectrophotometer. The concentrations of Cu, Zn, Pb, Cr, Cd and Ni were analyzed using were determined by atomic absorption spectrometer (Agilent Technologies Co. Ltd., USA). The concentration of As was measured using atomic fluorescence spectrometer (Beijing Jitian Instrument Co., Ltd., China). The concentration of Hg was analyzed using a Milestone DMA-80 direct mercury analyzer (Milestone, Italy).

Pollution assessments of heavy metals in sediments

In this study, pollution assessments of heavy metals in sediments were conducted using the geoaccumulation index (I_{geo}) (Müller, 1969) and Hankanson potential ecological risk index (*PERI*) (Hakanson, 1980). The background values of Cu, Zn, Pb, Cd, Cr, Hg As 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).

The I_{geo} is calculated using the following formula:

$$I_{geo} = \log_2 \frac{C_n}{kB_n} \quad \text{(Eq.1)}$$

where C_n is the measured concentration of heavy metals, B_n is the geochemical background values of heavy metals, k is the background matrix correction factor due to lithospheric effects, which is usually defined as 1.5 (Jiang et al., 2014; Wang et al., 2022). The *PERI* is calculated using the following formulas:

$$C_f^i = \frac{C_i}{C_n^i} \quad \text{(Eq.2)}$$

$$C_d = \sum_{i=1}^{8} C_f^i \quad \text{(Eq.3)}$$

$$E_r^i = T_r^i \times C_f^i \quad \text{(Eq.4)}$$

$$PERI = \sum_{i=1}^{8} E_r^i \quad \text{(Eq.5)}$$

where C_i and C_n^i are the measured and background concentrations of heavy metals, respectively. C_f^i and C_d are the monomial and the polynomial contamination factors of heavy metals, respectively. E_r^i is the monomial potential ecological risk index of 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). *PERI* is the sum of all risk factors for heavy metals in sediments. The evaluation criteria of the I_{geo}, C_f^i, C_d, E_r^i and *PERI* were shown in *Table 2*.

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Table 2. *Corresponding relationships between evaluation indices and pollution degree and potential ecological risks*

I_{geo}		**C_f^i**		**C_d**		**E_r^i**		**PERI**	
Range	Degree of contamination	Range	Degree of contamination	Range	Degree of contamination	Range	Potential ecological risk	Range	Potential ecological risk
$I_{geo}≤0$	practically uncontaminated	$C_f^i<1$	low	$C_d<8$	low	$E_r^i<40$	low	PERI<150	low
$0<I_{geo}≤1$	uncontaminated to moderately contaminated	$1≤C_f^i<3$	moderate	$8≤C_d<16$	moderate	$40≤E_r^i<80$	moderate	150≤PERI<300	moderate
$1<I_{geo}≤2$	moderately contaminated	$3≤C_f^i<6$	considerable	$16≤C_d<32$	considerable	$80≤E_r^i<160$	considerable	300≤PERI<600	considerable
$2<I_{geo}≤3$	moderately to heavily contaminated	$C_f^i≥6$	very high	$C_d≥32$	very high	$160≤E_r^i<320$	high	PERI≥600	very high
$3<I_{geo}≤4$	heavily contaminated					$E_r^i≥320$	very high		
$4<I_{geo}≤5$	heavily to extremely contaminated								
$I_{geo}>5$	extremely contaminated								

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Extraction and sequencing of DNA and RNA

Genomic DNA and RNA were extracted from the surficial sediment samples using a Fast DNA® SPIN Kit for Soil (MP Biomedicals, USA) and a Fast RNA® Pro Soil Direct Kit (MP Biomedicals, USA) according to the manufacturer’s protocol, respectively. The concentrations and purities of DNA and RNA were determined using a NanoDrop 2000 (Thermo Scientific, USA), and the qualities of them were examined using gel electrophoresis with 1% agarose gels. The purified RNA was then reverse-transcribed to cDNA using the SuperScript First-Strand Synthesis System with random hexamers for RT-PCR (Invitrogen, USA). The DNA, RNA and cDNA samples were stored at -80 °C for subsequent experiments.

The foraminiferal SSU rDNA and cDNA sequences were amplified using foraminiferal-specific primers s14F3 (5′-ACGCAMGTGTGAAACTTG-3′) and s17 (5′-CGGTCACGTTCGTTGC-3′) (Pawlowski, 2000; Lejzerowicz et al., 2013). PCR reaction was performed in a total volume of 20 μL that contained 10 μL 2× Pro Taq, 0.8 μL of each primer (5 μM), 1 μL of DNA template (10 ng/μL) and 7.4 μL of ddH_2O. The PCR reaction consisted of a pre-denaturation at 94 °C for 90 s, followed by 25 cycles of denaturation at 94 °C for 60 s, annealing for 60 s at 55 °C and extension at 72 °C for 45 s, immediately followed by an additional 10 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C and extension at 72 °C for 2 min (Lejzerowicz et al., 2013; Li et al., 2020). The amplicons were purified and then sequenced on an Illumina MiSeq PE250 platform at Majorbio Bio-Pharm Technology Co., Ltd, Shanghai, China.

Bioinformatic analysis

The paired-end reads from the original DNA/cDNA fragments of each sample were merged using FLASH (version 1.2.11, https://ccb.jhu.edu/software/FLASH/index.shtml). Raw reads were subjected to strict quality control using QIIME (version 1.9.1; http://qiime.org/install/index.html). The operational taxonomic units (OTUs) were generated with 99% similarity as the threshold using UPARSE (version 7.0.1090, http://www.drive5.com/uparse/). OTU containing less than 200 reads was removed. The representative sequences for each OTU were assigned to the National Center for Biotechnology Information (NCBI) database. After taxonomic assignment, OTUs belonging to foraminifera were retained for subsequent analysis.

Statistical analysis

To enable statistical analyses and comparisons of data across all samples, standardization among samples was performed by randomly subsampling the OTU table to the number of sequences present in the sample with the lowest amount. The subsequent benthic foraminiferal diversity and community composition analysis were carried out based on the data after this standardization. OTU-level alpha diversity indices such as Chao1 and Shannon were calculated using Mothur (version 1.30.2; https://www.mothur.org/wiki/ Download_mothur). The relative abundance of foraminifera at various taxonomic levels was defined as the percentage of species sequences in the total sequences. Spearman bivariate correlation analysis was then undertaken to determine the correlation between benthic foraminifera and environmental factors using SPSS (version 20).

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Results

Environmental parameters

The OC, TN, TP and 8 heavy metals including Cu, Zn, Pb, Cd, Cr, Hg, As and Ni were measured, and their concentrations are presented in *Table 3*. The concentrations of OC, TN, Cu, Zn, Cr, Hg and Ni at station OJ2 were highest. The concentrations of Pb and Cd were higher at station OJ1, whereas the concentrations of TP and As were higher at station OJ3.

Table 3. *Concentrations of organic carbon, total nitrogen, total phosphorus and 8 heavy metals in surface sediments of Oujiang Estuary*

Station	OC (%)	TN (mg/kg)	TP (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Pb (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Hg (mg/kg)	As (mg/kg)	Ni (mg/kg)
OJ1	0.691	271	284	21.81	103.37	39.13	0.12	61.76	0.040	5.4	35.12
OJ2	0.858	423	327	35.02	104.14	34.88	0.08	70.35	0.057	11.2	44.71
OJ3	0.414	304	336	29.55	93.11	31.71	0.07	53.94	0.050	11.4	39.28

The results of the geoaccumulation index and Hankanson potential ecological risk index were similar. The degree of contamination of Cd at station OJ1 was higher, while other heavy metals were low or moderate contamination (*Fig. 2a, b*). The C_d values were 13.13-14.73 (*Fig. 2b*). Thus, Oujiang Estuary was moderately contaminated with heavy metals. The values of E_r^i indicated that Cd and Hg were at moderate or considerable ecological risks, while the other six heavy metals were at low potential ecological risks (*Fig. 2c*). The *PERI* values were 189.69-209.63 (*Fig. 2c*), suggesting that Oujiang Estuary was in a moderate ecological risk level.

Benthic foraminiferal diversity

Benthic foraminiferal diversity and richness were evaluated using the Shannon index (*Fig. 3a*) and Chao1 index (*Fig. 3b*), respectively. The results showed that the diversity and richness of total benthic foraminifera community were significantly higher than that than those of active communities ($p < 0.05$). However, there was no significant difference in benthic foraminiferal diversity between stations ($p > 0.05$).

Benthic foraminiferal community composition

The total and active foraminiferal community composition in surface sediments of Oujiang Estuary is shown in *Fig. 4*. A total of 3 classes including Globothalamea, Monothalame and Tubothalamea were identified (*Fig. 4a*). At station OJ1, Monothalamea was the most abundant class, which accounted for 61.34% and 98.13% of the total and active benthic foraminifera, respectively. At station OJ2, the dominant class became Globothalamea, contributing 73.68% and 96.49% of the total and active foraminifera abundance, respectively. At station OJ3, Globothalamea was the most abundant class, followed by Monothalame and Tubothalamea in both total and active assemblages. At the order level, 5 orders of benthic foraminifera were identified (*Fig. 4b*). At station OJ1, Astrorhizida was the most dominant group, accounting for 41.93% and 94.01% of the total and active benthic foraminifera, respectively. At station OJ2, the dominant order became Rotaliida, which contributed 72.98% and 96.49% of the total and active foraminifera abundance, respectively.

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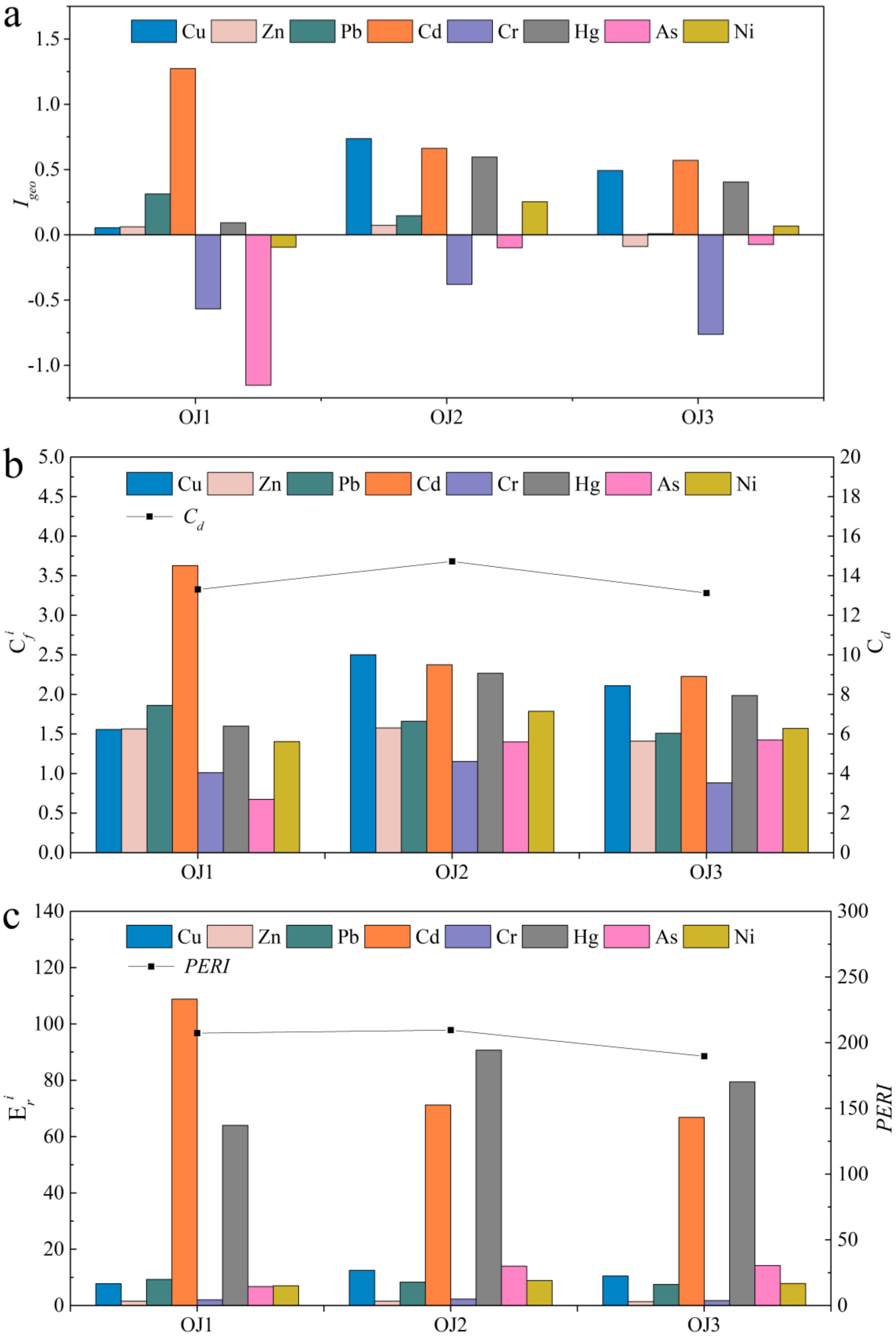

Figure 2. *Geoaccumulation index (a), degree of contamination (b) and potential ecological risk index (c) of 8 heavy metals in surface sediments of Oujiang Estuary*

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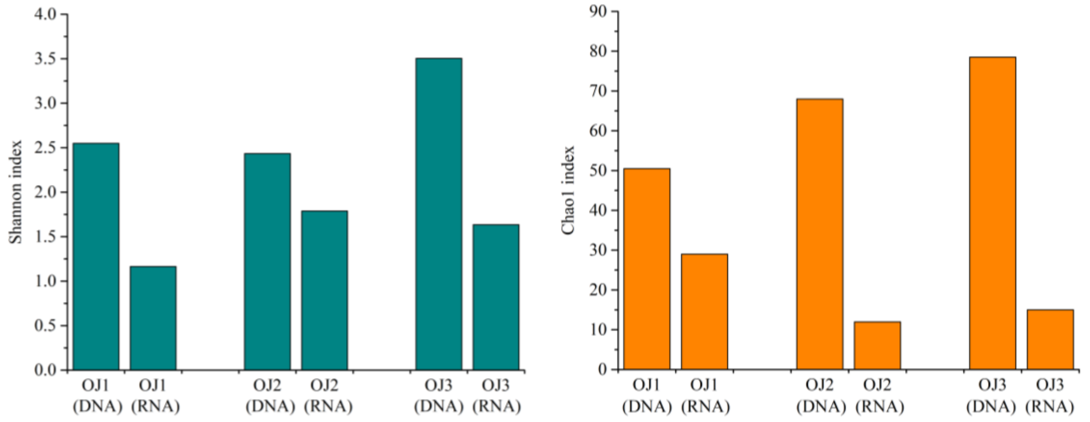

Figure 3. *OTU-level Shannon index (a) and Chao1 index (b) of total and active foraminiferal community*

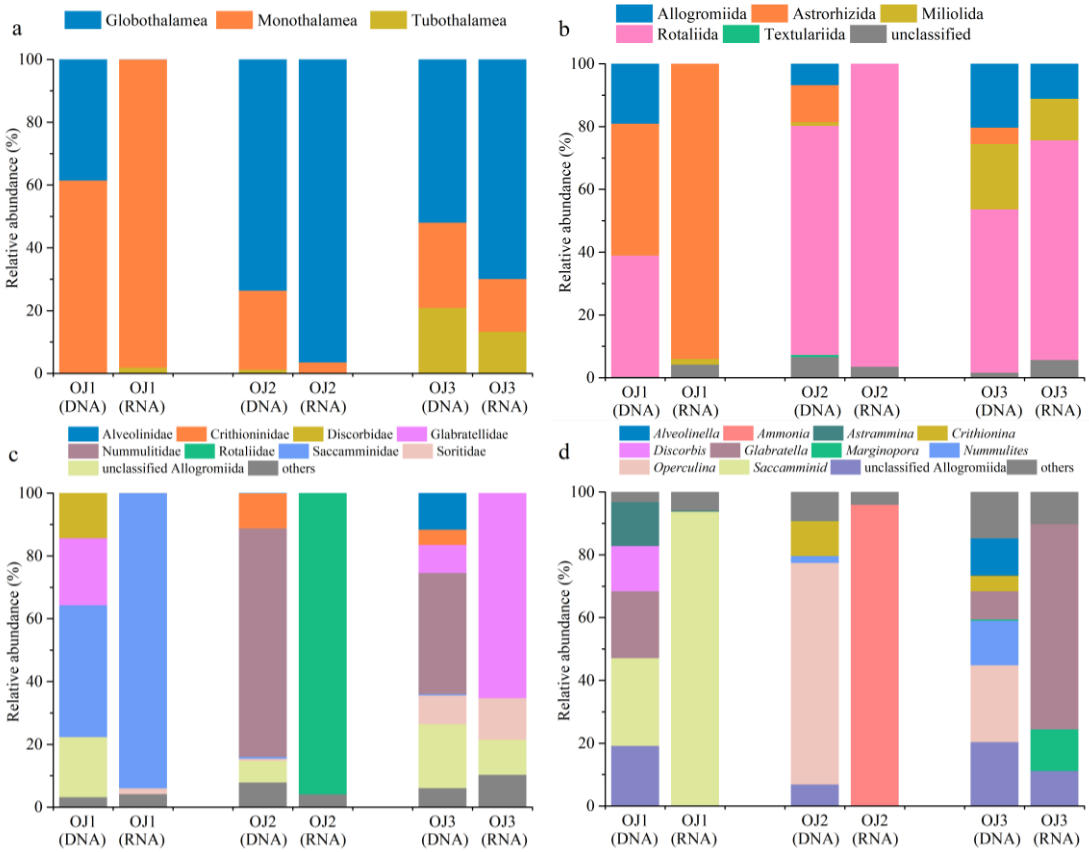

Figure 4. *Total and active foraminiferal community composition in the sediment samples at the class (a), order (b), family (c) and genus (d) levels*

At station OJ3, Rotaliida was the most dominant order, followed by Miliolida and Allogromiida in both total and active foraminiferal communities. At the family level, a total of 16 families of benthic foraminifera were identified (*Fig. 4c*). At station OJ1, Saccamminidae was the most dominant group (41.93%), followed by Glabratellidae (21.33%) and Discorbidae (14.42%) in total assemblages, and only one dominant family,

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Saccamminidae was detected in active assemblages (94.01%). At station OJ2, the dominant group in total assemblages became Nummulitidae and Crithioninidae, which contributed 72.72% and 11.20% of the total benthic foraminifera abundance, respectively. Only one family, Rotaliidae, dominated in active assemblages (95.92%). At station OJ3, the relative abundance of Nummulitidae (38.63%) and Alveolinidae (11.65%) were higher in total foraminiferal communities, while the relative abundance of Glabratellidae (65.32%) and Soritidae (13.26%) were higher in active foraminiferal communities. At the genus level, 19 genera for total benthic foraminifera were identified, while only 11 genera for active benthic foraminifera were found (*Fig. 4d*). At station OJ1, the dominant genera included *Saccamminid* (27.93%), *Glabratella* (21.33%), *Discorbis* (14.42%) and *Astrammina* (14.00%) in total assemblages, and only one dominant genus, *Saccamminid* was detected in active assemblages (93.70%). At station OJ2, the dominant genera in total assemblages became *Operculina* and *Crithionina*, which contributed 70.57% and 11.20% of the total benthic foraminifera abundance, respectively. Only one genus, *Ammonia*, was dominant in active assemblages (93.70%). At station OJ3, the relative abundance of *Operculina* (24.43%), *Nummulites* (14.20%) and *Alveolinella* (11.65%) were higher in total assemblages, while the relative abundance of *Glabratella* (65.32%) and *Marginopora* (13.26%) were higher in active assemblages.

Relationships between benthic foraminifera and environmental parameters

The heatmap plots based on Spearman's rho correlations is shown in *Fig. 5*. In total benthic foraminiferal community, *Alveolinella*, *Nummulites*, *Parasorites*, *Globorotalia* and *Calcarina* were negatively correlated with Cd, Pb and Depth ($p \leq 0.01$), and positively correlated with As and TP ($p \leq 0.01$). *Crithionina* and *Operculina* exhibited a positive correlation with Hg, Cu, Ni and TN ($p \leq 0.01$), while *Pulleniatina*, *Glabratella* and *Astrammina* were significantly negatively correlated with them ($p \leq 0.01$). In active benthic foraminiferal community, only one genus, *Ammonia* showed positive correlations with Cr, Zn and OC ($p \leq 0.01$).

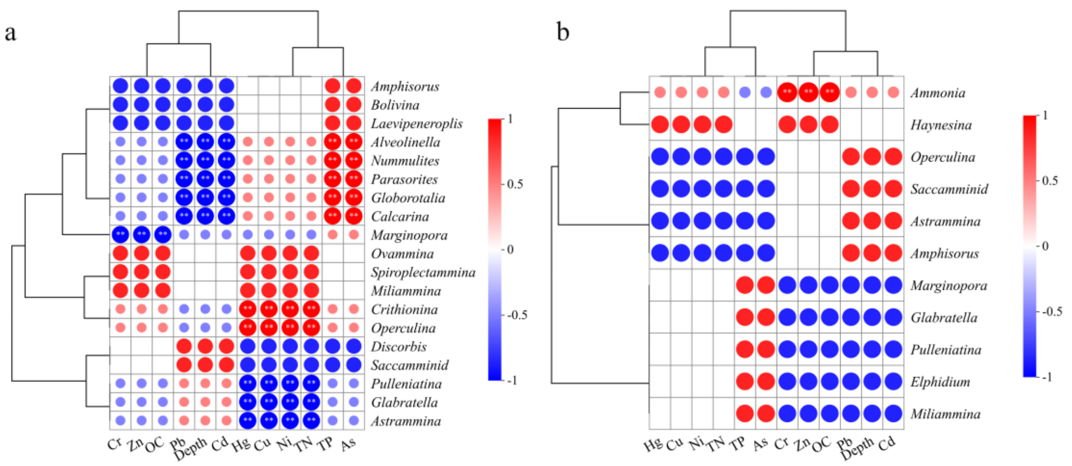

***Figure 5.** Heatmaps indicating the correlations between total (a) and active (b) benthic foraminifera at the genus level and environmental parameters. ** indicates p ≤ 0.01*

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Discussion

In the last decades, numerous studies have demonstrated the value of benthic foraminifera in monitoring marine ecosystem contamination (Tarasova, 2006; Frontalini and Coccioni, 2011). The traditional method for identification and investigation of foraminifera was microscopic examination, which is time-consuming and laborious, requires professional personnel, and is not suitable for large-scale investigations (Vilela et al., 2011; Pawlowski et al., 2014, 2018; Parsaian et al., 2018; Bergamin et al., 2019). In recent years, HTS technology has shown strong advantages and been widely used in evaluating the molecular diversity and community composition of foraminifera (Li et al., 2020, 2023; Shi et al., 2020; Cao et al., 2022). In this study, HTS based on SSU rDNA and rRNA amplification was used to investigate the total and active benthic foraminifera in Oujiang Estuary. A total of 19 genera for total benthic foraminifera were identified using DNA-based HTS, of which *Operculina* was the most abundant genus, followed by *Glabratella* (*Fig. 4d*). However, only 11 genera for active benthic foraminifera were found by RNA-based HTS, in which the average relative abundance of *Ammonia* was the highest, followed by *Saccamminid* and *Glabratella* (*Fig. 4d*). The total benthic foraminifera community had higher species diversity and richness than those of active communities (*Fig. 3*). This finding was in agreement with previous result that DNA could reveal greater taxonomic richness than RNA (Pawlowski et al., 2014). Difference between the total and active foraminiferal communities might be related with the following aspects. Firstly, environmental DNA (eDNA) contains the intracellular DNA from living cells and extracellular DNA from dead cells (Pietramellara et al., 2009; Veilleux et al., 2021), which could not distinguish between living and dead cells. While the eRNA has a faster degradation rate compared to eDNA, which allows eRNA to better reflect the “metabolically active” parts of the community (Pawlowski et al., 2014; Pochon et al., 2017). Secondly, eDNA could detect species at longer spatial scales (Deiner and Altermatt, 2014; Shogren et al., 2017), making it difficult to distinguish between indigenous species and imported species. Thirdly, eRNA metabarcoding has an additional step of reverse transcription of RNA, making it more more error-pron. In summary, combining the eDNA with eRNA will provide a better information of the foraminiferal community.

Heavy metals are the main pollutants in sediments in marine ecosystems (Yang et al., 2021; Wang et al., 2022), which are harmful to benthic organisms, especially benthic foraminifera (Boehnert et al., 2020). In the present study, Oujiang Estuary was moderately contaminated with heavy metals. The degree of contamination and potential ecological risk level of heavy metals at station OJ1 and OJ2 were higher compared to those at station OJ3 (*Fig. 2*), and the relatively lower species diversity and richness of total foraminiferal communities were found at station OJ1 and OJ2 than those obtained at station OJ3 (*Fig. 3*). This was consistent with previous research that high heavy metal concentrations co-occurred with a low diversity and richness of foraminiferal community (Tarasova, 2006; Bouchet et al., 2020). In this study, Cd was moderate even considerable contamination and potential ecological risks (*Fig. 2*). Culture and mesocosms experiment showed that Cd could reduce growth rate and abundance, caused shell abnormalities, alter the pseudopodial activity and the polarity of the lipid content, suppress the respiration rate of benthic foraminifera (Linshy et al., 2013; Smith and Goldstein, 2019; Losada Ros et al., 2020; Andreas and Bowser, 2021). In addition, different species of benthic foraminifera have different tolerance to heavy metal pollution, which might cause changes in benthic foraminiferal community structure (Tarasova, 2006; Klootwijk et al.,

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2021; Qiao et al., 2022). Investigations found that *Ammonia tepida*, *Amphistegina lobifera*, *A. lessonii*, *Elphidium crispum*, *Sorites orbiculus* exhibited tolerance towards heavy metals pollution, while *Adelosina* spp., *A. parkinsoniana*, *Quinqueloculina* spp. were sensitive to heavy metals pollution (Tarasova, 2006; Frontalini and Coccioni, 2008; Coccioni et al., 2009). In this study, Spearman's rho correlation between total foraminifera and 8 heavy metals was conducted, and the results suggested that *Alveolinella*, *Nummulites*, *Parasorites*, *Globorotalia* and *Calcarina* exhibited a significantly negative correlation with Cd ($p < 0.01$). Therefore, we speculated these species might be sensitive to Cd pollution. This guess was based on statistical analysis of field surveys. In the field, all environmental factors are interrelated. It is difficult to accurately determine the causal relationship between individual pollutant and benthic foraminiferal communities through field surveys. Bioassay is a direct and effective method to quantify the effect of a single pollutant or compound pollutants at different concentrations and times to benthic foraminifera (Frontalini and Coccioni, 2012; Frontalini et al., 2018; Losada Ros et al., 2020). Therefore, culture (exposure of a single species to a single metal) or mesocosm (exposure of sediments and the entire community living therein to a single metal) experiments are necessary to be carried out to further investigate the dose-response relationship between benthic foraminifera and heavy metals.

Conclusions

In this study, pollution assessments of heavy metals and molecular diversity and community composition of total and active benthic foraminifera in the surface sediments of Oujiang Estuary were studied. The conclusions were as follows:

(1) Oujiang Estuary was moderately contaminated with heavy metals, and Cd was moderate even considerable contamination and potential ecological risks.

(2) Total of 19 genera for total benthic foraminifera were identified, while only 11 genera for active benthic foraminifera were found. The total benthic foraminifera community had higher species diversity and richness than those of active communities.

(3) *Alveolinella*, *Nummulites*, *Parasorites*, *Globorotalia* and *Calcarina* might be sensitive to Cd pollution.

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