

Experimental assessment of benthic foraminifera as salinity bioindicators: Integrating morphological and eDNA approaches

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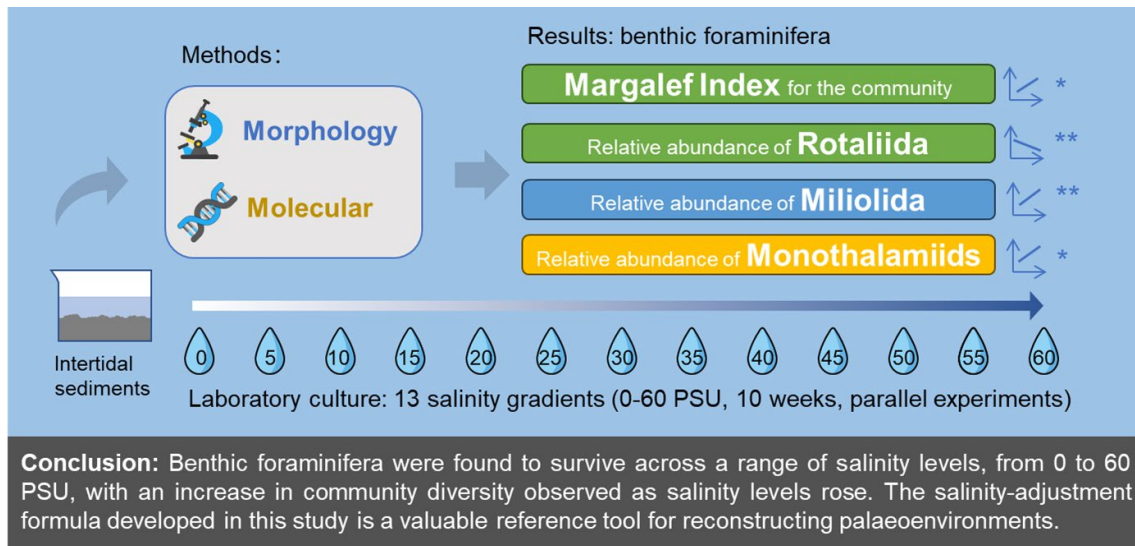
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10 **Abstract.** Benthic foraminifera are extensively used as bioindicators for paleoenvironmental reconstruction, and environmental DNA (eDNA) analysis provides a powerful lens to uncover their community diversity and environmental responses. Foraminifera are commonly used to assess salinity changes in estuary settings, but quantitative experimental studies of their responses to salinity gradients are scarce. Here, sediments from the intertidal zone of Qingdao Bay were subjected to a 10-week controlled culture across 13 salinity levels (0–60 Practical Salinity Units, PSU), and community dynamics were
15 analysed using both morphological and eDNA approaches. Foraminifera exhibited broad salinity tolerance (0–60 PSU). Analyses of both morphological and molecular datasets revealed a significant increase in community diversity with salinity ($p < 0.05$), accompanied by a marked decrease in the relative abundance of calcified Rotaliida ($p < 0.01$). The inverse relationship for Rotaliida was quantitatively robust and well-described by a linear regression model. The eDNA analysis revealed that soft-bodied Monothalamiids—often overlooked morphologically—reached up to 76.2% relative abundance (average 56.1%). In
20 contrast, the salinity-driven increase in high-Mg calcite-shelled Milioliida was observed only through morphological analysis. These results demonstrate the distinct but complementary nature of morphological and molecular methods. This study addresses the scarcity of experimental constraints on salinity responses, offering a calibrated reference for applying foraminifera in both ecological assessment and paleo-reconstruction in marginal marine environments.



25 1 Introduction

Foraminifera are cosmopolitan unicellular protists widely distributed across marine environments. Highly sensitive to environmental changes, they preserve extensive environmental information within their tests and community structures, making them valuable proxies for environmental monitoring and paleoclimatic reconstructions. (Bouchet et al., 2012; Ying et al., 2024). In coastal and shelf environments, seawater conditions are highly variable, and salinity is closely linked to local monsoons, precipitation, and continental runoff. The mixing of freshwater alters the physicochemical properties of seawater, thereby influencing the growth of foraminiferal populations and the preservation of their tests (Murray et al., 1999; Nigam et al., 1992; Sen Gupta, 1999). Field investigations into salinity have revealed the presence of living foraminifera in environments ranging from extremely low salinity levels (down to 0.1 PSU in the Paraná River in South America) to hypersaline conditions (up to 92 PSU in the saline-alkaline regions of southern Europe) (Boltovskoy et al., 1991). However, both abnormally high and low salinities can induce morphological deformities, including changes in test size, increased proportions of abnormal individuals, loss of surface ornamentation, and delayed or even inhibited reproduction (Boltovskoy et al., 1991).

Laboratory culture experiments on salinity have traditionally focused on species-level responses, such as growth, reproduction, and test morphology, whereas the collective responses of entire benthic foraminiferal communities remain poorly understood. Bradshaw (1957) investigated the salinity tolerance of *Ammonia beccarii* (Linné) var. *tepida* (Cushman) and found that the optimal range for reproduction and growth lies between 20 and 40 PSU. When salinity dropped below 13 PSU, the time required for reproduction doubled compared to normal conditions, and growth ceased at salinities above 67 PSU. Similarly, Nigam et al. (2006) reported that both excessively high and low salinities adversely affect the growth rate of foraminifera; however, low salinity poses a greater threat than hypersaline conditions, as test dissolution tends to occur under reduced salinity.

45 Traditional studies have typically relied on morphological identification of foraminiferal taxa and statistical analyses of
community composition to infer past or present environmental conditions. However, this approach is time-consuming and
requires considerable taxonomic expertise. With the advancement of molecular biology, environmental DNA (eDNA)
techniques have become increasingly popular. High-throughput sequencing (HTS) enables more efficient assessments of
foraminiferal diversity, substantially reducing both time and labour costs (Pawlowski et al., 2020; Pawlowski et al., 2022).
50 Recently, eDNA-based approaches for foraminiferal research have been successfully applied to monitor biodiversity and to
further assess environmental conditions (Maeda et al., 2024; O'Brien et al., 2024). In addition, analyses of foraminiferal ancient
DNA (aDNA) extracted from sediment cores provide valuable complementary information on soft-bodied Monothalamiids
taxa that are widespread in modern environments but rarely preserved in the fossil record, thereby enabling the reconstruction
of past biodiversity and offering a more comprehensive understanding of paleoenvironmental changes (Demianiuk et al., 2025;
55 Pawłowska et al., 2014). However, applications of the eDNA approach to investigate the responses of foraminiferal
communities to environmental changes under controlled laboratory conditions remain very limited, which constrains the
potential of foraminifera as reliable indicators for tracking short-term and long-term environmental variability.

In this study, sediment samples were used for culture experiments rather than single-species cultivation, allowing a more
realistic simulation of the natural habitats of benthic foraminifera. Based on this setup, environmental salinity levels were
60 manipulated to assess the ecological effects of environmental variability under controlled conditions. Intertidal sediment
samples were collected from Qingdao Bay and cultured for ten weeks under 13 salinity levels ranging from 0 to 60 PSU. Both
morphological and eDNA approaches were employed to analyse community parameters and taxonomic composition, and their
relationships with salinity were quantitatively examined. The objectives of this study are to evaluate the consistency between
morphological and eDNA-based estimates of benthic foraminiferal diversity, to identify the differential responses of distinct
65 taxonomic groups to salinity variation, and to establish a practical framework for linking community dynamics with
environmental levels, providing a valuable reference for modern environmental assessment and paleoenvironmental
reconstruction.

2 Materials and methods

2.1 Sample collection and laboratory culture

70 Sediment samples were collected from the intertidal zone of Qingdao Bay (36.03°N, 120.19°E, Fig. 1), a region where
annual salinity typically ranges from 31 to 38 PSU (Lei et al., 2017). At the time of sampling, the *in situ* water temperature
was 17.8 °C, and the seawater salinity was 33.1 PSU (measured by handheld refractometer, precision ±1‰, interpolated to 0.1
PSU). Ambient seawater collected for the experiment showed a pH of 8.21.

To ensure community representativeness, surface sediments (top 0–2 cm) were collected from approximately 20 random
75 locations within the sampling area using a sterile spoon and pooled into a single composite sample. Upon transfer to the
laboratory, the sediment was gently homogenized. Prior to aliquoting, the sediment was wet-sieved through a 300-mesh (~48
µm) silk screen using ambient seawater. This pre-treatment was performed to remove fine clay particles and excess organic

detritus, thereby improving pore-water oxygenation and preventing bacterial overgrowth during the subsequent static culture. From this processed material, an initial subsample (~50 g) was preserved as the baseline (T0), and the remaining sediment was divided into 13 equal aliquots (approximately 50 g wet weight each) for the salinity gradient experiment.

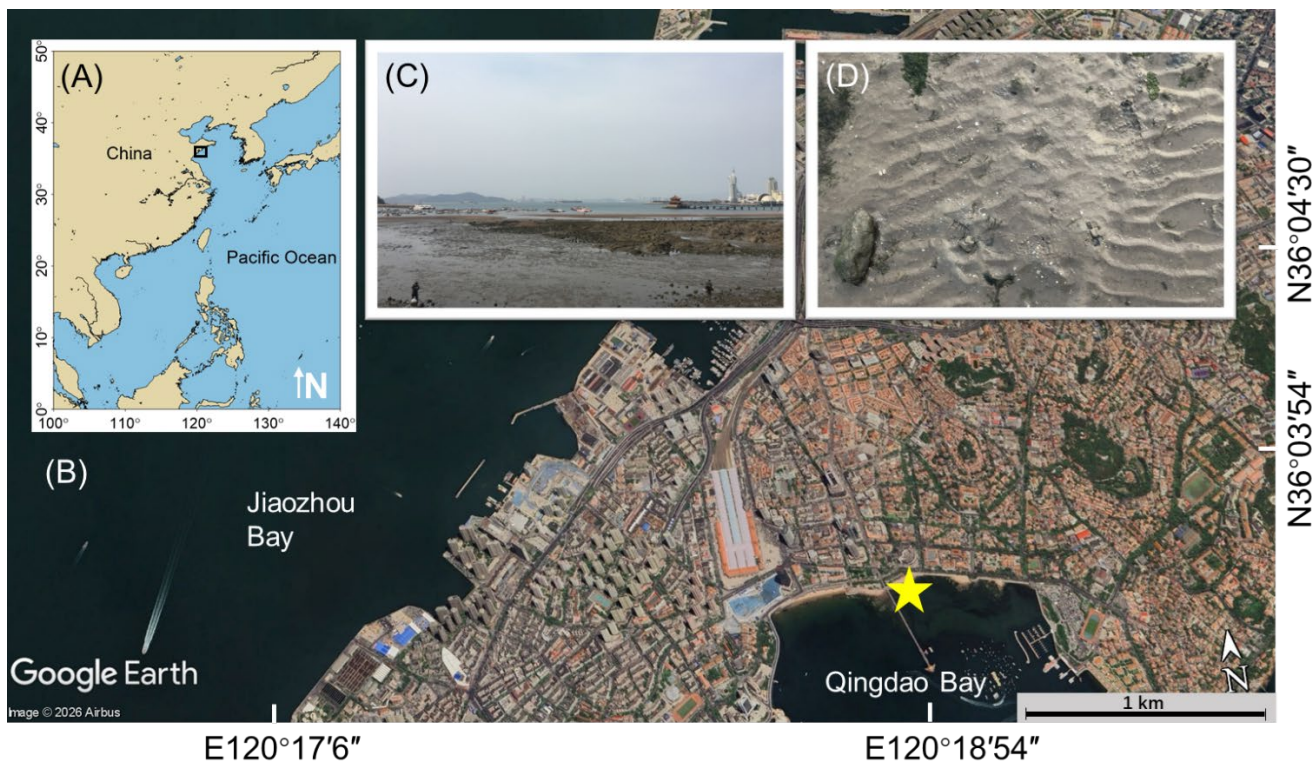


Figure 1. Map of the sampling area. (A) Regional map with a black rectangle indicating the location of the detailed view. (B) Close-up map view of the sampling site (satellite imagery: © 2026 Airbus; image date: May 18, 2025; map data: © Google, and respective data providers); (C) Photograph of the sampling area; (D) Photograph of sediment substrate conditions at the sampling site.

Sediment samples were cultured in clean, sterilized glass crystallizing dishes (90 mm in diameter) under 13 salinity levels of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 PSU. The dishes were incubated in a temperature-controlled chamber maintained at 18 °C, with a light: dark cycle of 12 h:12 h and an illumination intensity of approximately 6000 lx. Seawater with different salinities was prepared using in situ seawater as the base. Prior to salinity adjustment, the seawater was filtered through a 0.45 µm membrane to remove impurities. High-salinity water was obtained by evaporative concentration in an oven, whereas low-salinity water was prepared by dilution with distilled water (Bradshaw, 1955; Dissard et al., 2010). During the culture experiment, an overlying water volume approximately equal to that of the sediment was maintained in each crystallization dish, which was covered with a gas-permeable membrane to prevent salinity fluctuations caused by evaporation. Throughout the 10-week culture, half of the overlying water was replaced every three days, and 20 µL of concentrated algal suspension was added as food at the same interval. The algal suspension of *Phaeodactylum tricornutum* Bohlin, cultivated using Guillard's f/2 medium, was pre-concentrated by centrifugation, homogenized, aliquoted into sterile 1.5 mL centrifuge

tubes, and stored at -20°C until use. During the culture period, the pH of the overlying water was regularly monitored and maintained at 7.7 ± 0.16 . After 10 weeks of culture, half of each sediment sample was used for morphological analysis, while the other half was reserved for eDNA extraction.

2.2 Sample processing by morphology

100 For morphological analysis, the samples were fixed for 48 hours in a solution of 95% ethanol mixed with 1 g/L Rose Bengal to distinguish living from dead foraminiferal individuals (Lei et al., 2017; Schönfeld et al., 2012). Subsequent morphological processing and analysis were conducted following ISO 23040:2021. After staining, samples were dried at 50°C for 12 hours, weighed, and wrapped in 300-mesh sieve netting, then soaked and rinsed in seawater. Foraminifera were concentrated by flotation using carbon tetrachloride (CCl_4), followed by a second drying step. The residues were then dry-sieved through
105 0.150 mm and 0.063 mm mesh screens and split into two size fractions: >0.150 mm and 0.063–0.150 mm (Fontanier et al., 2002; Jian et al., 1999). The 0.063–0.150 mm fraction, consisting mainly of juveniles and very small specimens, was not picked. All stained (i.e., living) individuals from the >0.150 mm fraction were hand-picked under a stereomicroscope. Although all specimens were counted, only Rose Bengal-stained individuals were included in the final community analyses.

To provide a baseline for comparison, the original intertidal sediment samples were processed identically. The
110 pre-experimental community composition is shown as T0. Species identification was conducted based on existing literature plates (Lei et al., 2016), with pre-treatment viability determined by observing staining patterns. The identification of all living foraminiferal individuals in the selected samples was conducted to the level of species, and these were then grouped for quantitative analysis. In addition, community parameters were calculated, including total abundance, Margalef diversity index, Shannon–Wiener index, and Simpson index. For morphological data, relative abundance was calculated as the number of Rose
115 Bengal-stained specimens of a given taxon divided by the total number of stained foraminiferal specimens in the sample.

2.3 Sample processing by eDNA metabarcoding

For sediment samples used in DNA extraction, 0.25 g replicates were collected from three distinct locations within each sample. This was done in order to minimize errors arising from sample heterogeneity. The sample was then processed using the QIAGEN (Germany) DNeasy PowerSoil Kit (12888-100), yielding 100 μL of environmental DNA solution. The process
120 of polymerase chain reaction (PCR) was conducted utilizing the foraminifera-specific primers s14F3 and s17, which were designed to target the 37f hypervariable region of the foraminifera SSU ribosomal DNA. Each PCR reaction volume of 25 μL contains 12.5 μL of $2 \times$ High-Fidelity PCR Master Mix, 0.5 μL of each primer at 10 μM , 2 μL of DNA template, and 9.5 μL of ddH_2O . The thermal cycling profile implemented a two-stage amplification strategy: an initial denaturation at 94°C for 90 s, followed by 35 cycles of denaturation (94°C , 60 s), annealing (55°C , 60 s), and extension (72°C , 45 s). This was immediately
125 followed by an additional 10 cycles with modified timing (30 s denaturation, 30 s annealing, and 120 s extension) to ensure complete amplification.

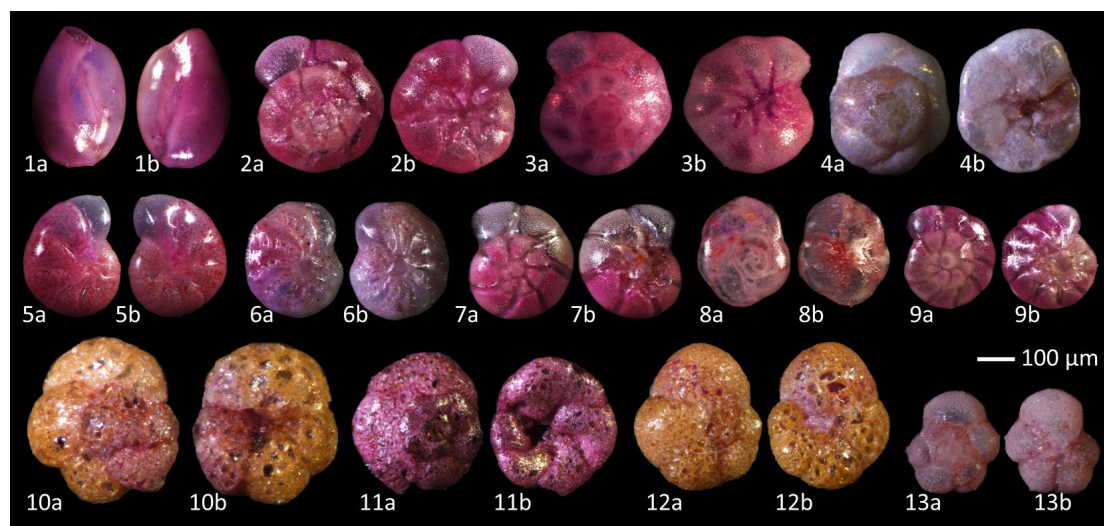
130 Amplicons were purified using a DNA Gel Extraction Kit and verified via 1% agarose gel electrophoresis. Library construction was carried out using the Illumina TruSeq DNA PCR-Free Library Preparation Kit, followed by quantification with Qubit. Sequencing was conducted on the Illumina NovaSeq 6000 platform to generate paired-end reads. The amplified target fragment was 380 bp in length, and paired-end sequencing with 250 bp reads was employed, resulting in a potential overlapping region of approximately 120 bp between forward and reverse reads.

135 Raw paired-end reads were demultiplexed based on unique barcodes and merged using FLASH (v1.2.7) (Magoc and Salzberg, 2011). Data quality was inspected using FastQC (Brown et al., 2017). To ensure high-quality datasets, low-quality sequences (quality score < 30) were filtered out using QIIME (v1.9.1) (Bokulich et al., 2013). Finally, the filtered sequences were processed using the UNOISE3 pipeline (Edgar, 2016) to generate an OTU (Operational Taxonomic Units) table, with the similarity threshold set at 100%. OTUs with fewer than 10 reads or appearing in fewer than 3 samples were removed to eliminate potential contaminants. Representative sequences were extracted from the OTU table and taxonomically annotated against the PR2 database (Protist Ribosomal Reference Database, Guillou et al., 2012) using BLAST (version 2.7.1). OTUs not classified under Foraminifera (rank 3 in the PR2 database) were subsequently discarded. OTUs annotated as Foraminifera yet unassigned at the Order level or lower were collectively categorized as "Others."

140 For eDNA data, relative abundance was defined as the proportion of reads assigned to a given taxon relative to the total number of foraminiferal-derived reads in the sample. The OTU table was used to quantify the species number and relative abundance of each foraminifera taxon under different salinity conditions, as well as the alpha diversity indices of the community. Spearman correlation analysis between each taxon (as well as community diversity) and salinity was performed using SPSS software. For data showing significant correlations, figures were generated in Origin with linear fitting equations, plotted using a 95% confidence interval. The eDNA based phylogenetic tree is available in the Supplementary Materials.

3 Result

3.1 Morphological analysis of benthic foraminifera

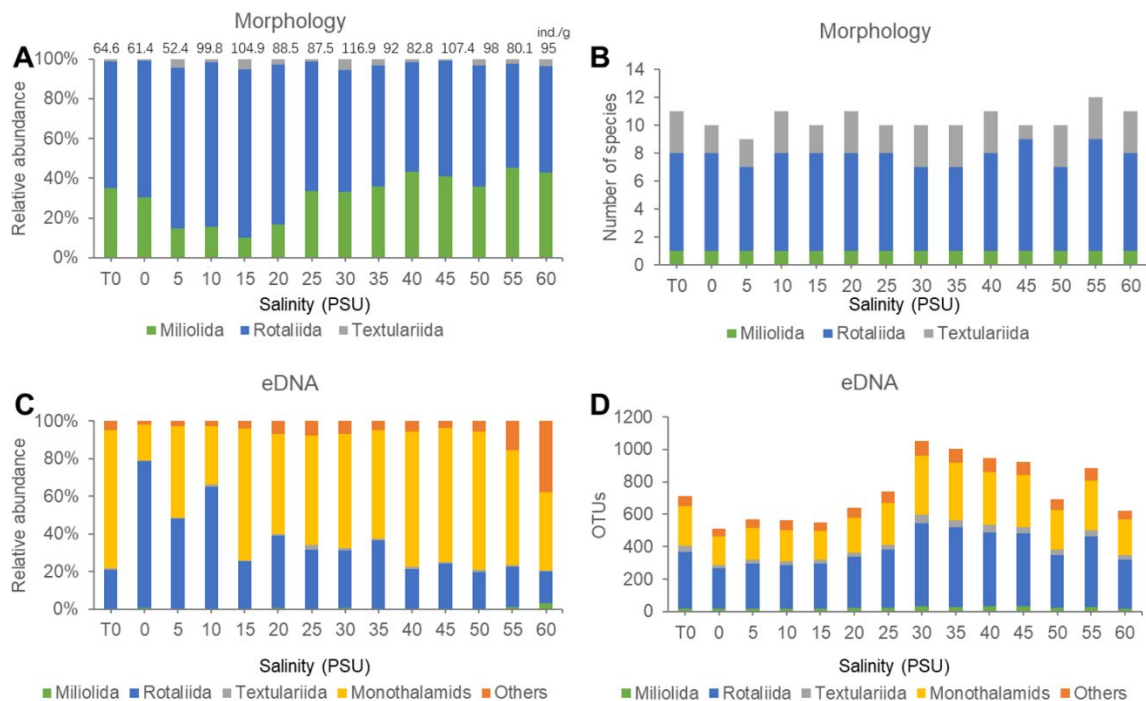


150 **Figure 2. Micrographs of benthic foraminifera.** (The samples were fixed and stained with Rose Bengal dye to differentiate living organisms.) Note: 1. *Quinqueloculina seminula*, 2. *Ammonia tepida*, 3. *Ammonia sobrina*, 4. *Rosalina vilardeboana*, 5. *Cribronion gnythosuturatum*, 6. *Elphidium macellum*, 7. *Ammonia aomoriensis*, 8. *Buccella frigida*, 9. *Ammonia beccarii*, 10. *Ammoglobigerina globigeriniformis*, 11. *Trochammina squamata*, 12. *Trochammina inflata*, 13. *Murrayinella globosa*.

In this study, traditional morphological methods identified a total of 9,077 foraminiferal specimens belonging to 13 species, of which 7,217 were stained live specimens (see Fig. 2, Table S1 in the Supplement). Foraminiferal abundance at each salinity level is shown at the top of the bar chart in Fig. 3A. Overall, foraminiferal abundance exhibited an increasing trend with increasing salinity. Foraminifera species numbers exhibited slight fluctuations across salinity levels, with the highest species count of 13 recorded at the 55 PSU salinity gradient and the lowest count of 10 species documented at 10 PSU. The relative abundance and species number of different taxonomic composition across salinity levels is demonstrated in Fig. 3 (A/B) and Table S3a in the Supplement. The Rotaliida group is comprised of nine species, which account for 66.52% of the total. The relative abundance of these species peaked at 15 PSU, where they constituted 84.70% of the total. The Miliolida group comprised one species, accounting for 30.59% of the total. At its most prevalent relative concentration, it attained 45.20% at a salinity gradient of 55 PSU. The Textulariida group was comprised of three species, accounting for 2.89% of the total. Their maximum relative abundance was recorded as 5.59% at a salinity gradient of 30 PSU.

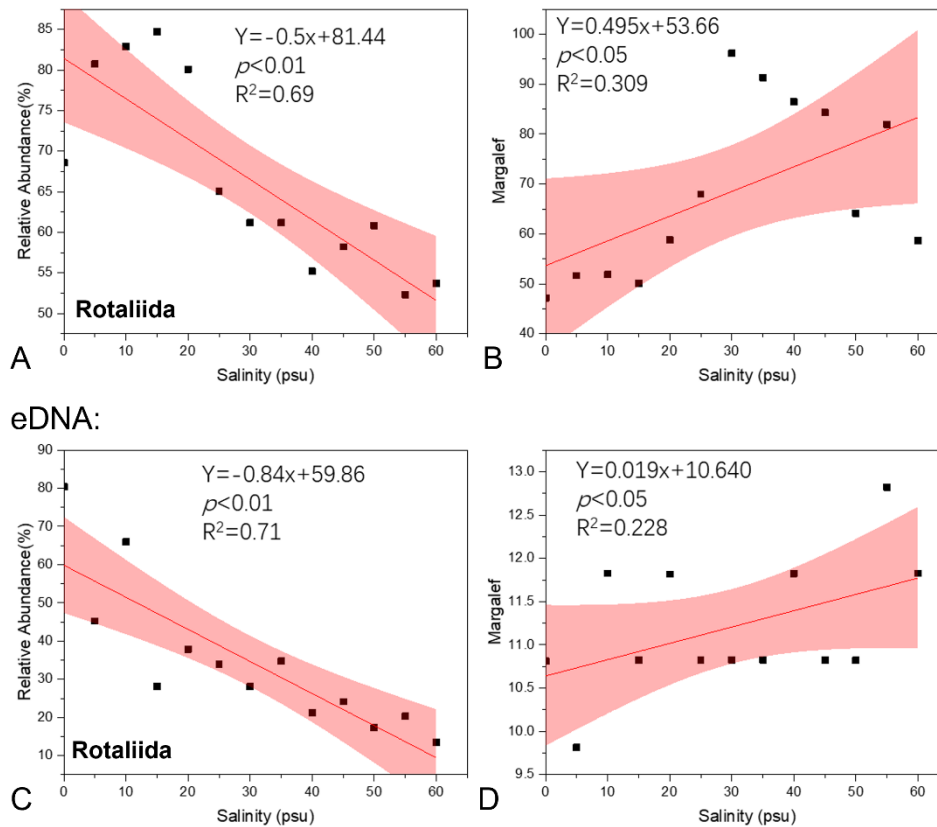
165 The dominant species in the community were *Quinqueloculina seminula*, *Cribronion gnythosuturatum*, *Ammonia aomoriensis*, *Ammonia beccarii*, and *Ammonia tepida*. The combined abundance of these five dominant species accounted for 89.18% of the total foraminifera count. A Spearman correlation analysis was conducted between the relative abundances of the three taxonomic groups and salinity (Table 1). The results demonstrated a statistically significant negative correlation between the relative abundance of Rotaliida and salinity ($p < 0.01$). The relative abundance of the Rotaliida group decreased

170 from approximately 80% at low salinities (5–20 PSU) to around 50% at high salinity (55 PSU). A regression model (Fig. 4A), $Y = -0.5x + 81.44$, was developed to describe the relationship between the variables ($p < 0.01$, $R^2 = 0.69$). The relative abundance of the Milioliida group showed a significant positive correlation with salinity ($p < 0.01$).



175 **Figure 3. Culture experiment results: relative abundance of taxa (A/C) at various salinities using morphological and eDNA methods, the number of species identified by morphological methods (B) and alongside the number of OTUs per taxon in the eDNA method (D). The total abundance of foraminifera is indicated above each bar in panel (A).**

Morphology:



180 **Figure 4. Regression function fitting plots for the relative abundance of Rotaliida (A, C) and the Margalef diversity index of the foraminifera community (B, D) against salinity levels in this study's morphological and eDNA methods, with the pink area indicating the 95% confidence interval.**

Table 1 Spearman correlation coefficients (R values) between foraminiferal taxa detected by morphological and eDNA methods and salinity levels. Bold text indicates statistically significant correlations (*p < 0.05, ** p < 0.01).

	Rotaliida	Milioliida	Monothalamiids	Textulariida
Morphology	- 0.896**	0.874**	\	0.060
eDNA	- 0.912**	- 0.033	0.544	-0.016

185 The alpha diversity of foraminiferal assemblages obtained using morphological methods at various salinities is shown in Table S3b in the Supplement. Based on morphological statistical results, the Spearman correlation analysis between benthic foraminiferal community diversity indices and salinity is presented in Table 2. The results indicate that the Margalef index exhibits a significant positive correlation with salinity, while the Simpson index of the community shows a significant negative correlation with salinity. The Margalef diversity index increased from 9.814 at the lowest salinity of 5 PSU to 12.823 at 55

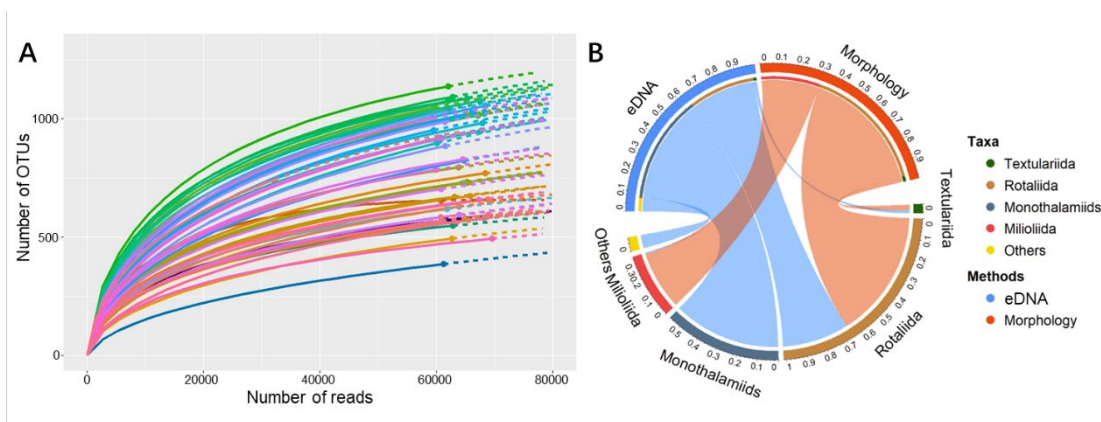
PSU, while the Simpson index decreased from 0.978 to 0.796 over the same salinity range. Consequently, a linear regression function was established between the Margalef index and salinity gradient (Fig. 4B): $Y = 0.495x + 53.66$ ($p < 0.05$, $R^2 = 0.309$).

190 **Table 2 Spearman correlation coefficients between alpha diversity of foraminiferal communities detected by morphological and eDNA methods and salinity levels. Bold text indicates statistically significant correlations (* $p < 0.05$, ** $p < 0.01$).**

	Margalef	Shannon-Wiener	Simpson	Pielou
Morphology	0.619*	0.538	-0.874**	0.407
eDNA	0.517*	0.319	0.269	0.044

3.2 Environmental DNA results of benthic foraminifera

In this eDNA experiment, the amplified fragments utilized for sequencing exhibited an approximate length of 380 base
 195 pairs. Subsequent to the implementation of quality control measures, the results yielded a total of 773,819 DNA sequences,
 which were annotated into 1,977 OTUs and matched to 103 species. Rarefaction analysis was performed on the eDNA data
 (Fig. 5A, Table S2 in the Supplement Materials). The curves for all treatment groups levelled off as the number of reads
 increased, suggesting that the sampling effort was adequate to represent the benthic foraminiferal diversity. The number of
 OTUs in samples at different salinities is shown in Fig. 3D. The results obtained from the study indicate that the total number
 200 of foraminiferal OTUs increased initially and then decreased under experimental conditions ranging from 0 to 60 PSU (Figure
 3A, Table S3b in the Supplement Materials), with a peak observed near 30 PSU. Moreover, the trend was more pronounced
 in the low-salinity range (0–30 PSU) compared to the high-salinity range (35–60 PSU).



205 **Figure 5. (A) Rarefaction curves for eDNA samples, plotting the number of OTUs against sequencing reads. Colored curves represent individual samples from each salinity level, with replicates shown in distinct shades. (B) Chord diagram comparing the community composition of benthic foraminifera obtained by molecular (eDNA) and morphological approaches. Note: Each outer semicircle represents a method's dataset (Molecular/Morphology), independently normalized to 100%. Numbers on the outer ring indicate relative abundance. Inner coloured arcs represent taxa; their widths equal the sum of relative abundances from both methods, showing total detection strength. Ribbons connect taxa to methods; their endpoint widths equal the taxon's relative abundance within that method's dataset.**

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In terms of taxonomic composition (Fig. 5B, Table S3a in the Supplement Materials), the eDNA data detected Monothalamiids, a group typically overlooked by morphological analysis. Additionally, a portion of taxonomically unresolved taxa were grouped as 'Others'. Monothalamiids exhibited the highest read count, accounting for 56.06% of the total. The maximum relative abundance recorded for this group was 76.19%, which was achieved under the 50 PSU salinity gradient (Fig. 3C). The Rotaliida constituted 34.65% of the total, achieving a maximum relative abundance of 80.34% under the 0 PSU salinity gradient. The Textulariida class accounted for 1.67% of the total, with a maximum relative abundance of approximately 3.43% at the 30 PSU salinity gradient. The Milioliida class constituted 0.66% of the total, exhibiting a maximum relative abundance of 4.70% at the 10 PSU salinity gradient.

As the salinity gradient increases, there is a concomitant decrease in the proportion of Rotaliida, accompanied by a slight increase in the proportion of Monothalamiids. A Spearman correlation analysis was conducted between each group and salinity, revealing a highly significant negative correlation ($p < 0.01$) between Rotaliida relative abundance and salinity (Table 1). With increasing salinity, Rotaliida abundance dropped from 80.344% (0 PSU) to 13.467% (60 PSU), while Monothalamiids abundance increased from 18.088% (0 PSU) to 76.195% (50 PSU). As shown in Fig. 4C, the following linear regression equation was established: the regression equation can be expressed as $Y = -0.84x + 59.86$ ($p < 0.01$, $R^2 = 0.71$).

The number of reads, OTUs, and alpha diversity of each salinity treatment group obtained using the eDNA method are presented in Table S3b in the Supplement Materials. Spearman's correlation analysis based on eDNA data revealed a significant positive correlation between the Margalef diversity index and salinity (Table 2). With increasing salinity, the foraminiferal community's Margalef diversity index showed an overall increase from 47.195 at 0 PSU to 81.902 at 55 PSU, peaking at 96.170 at an intermediate salinity of 30 PSU. Consequently, a regression model was established to quantify this relationship (Fig. 4D), yielding the following equation: $Y = 0.019x + 10.640$ ($p < 0.05$, $R^2 = 0.228$).

4 Discussion

4.1 Investigation into the salinity adaptability of benthic foraminifera communities

The formation of foraminiferal community structures is driven by multiple environmental factors, including food supply, temperature, salinity, pH, and dissolved oxygen. These factors influence survival, reproduction, and shell preservation, thereby shaping sedimentary records (Goody, 2003). For planktonic foraminifera, community dynamics are more strongly governed by hydrological conditions and variations in primary productivity. In contrast, for benthic foraminifera, particularly within marginal sea environments, abiotic factors such as temperature and salinity frequently exert a dominant influence (Chaabane et al., 2024; Groeneveld et al., 2018). This study, which was conducted through multi-gradient salinity culture experiments on intertidal sediments, found that both molecular and morphological methods could detect viable foraminifera even under extreme low-salinity (0 PSU) and high-salinity (60 PSU) conditions. Furthermore, the Margalef index demonstrated a significant increase in community diversity with rising salinity, indicating that salinity is a key driver influencing community structure, with high salinity promoting greater complexity in community composition.

Foraminifera exhibit a remarkably broad tolerance of salinity levels. In the Burullus Lagoon, large populations of *Ammonia tepida* flourish across a salinity range of 1.5 to 14.6 PSU, accounting for over 97 per cent of the total foraminiferal community (Orabi et al., 2017). *Ammonia beccarii* has been found in lagoons in southern California with a salinity range of 15–68 PSU and in salt marshes along the southern European coast with a salinity range of 7–92 PSU. It exhibits no discernible morphological variation in response to environmental conditions (Boltovskoy et al., 1991). However, such broad tolerances reported for single morphospecies should be interpreted with caution, as they may partly reflect the cumulative response of multiple cryptic species within the *Ammonia* genus—a group now recognized for its extensive genetic diversity (Goetz et al., 2025). This taxonomic complexity underscores the need for molecular identification to fully understand species-specific ecological responses. We recognize that integrating high-resolution genetic identification with traditional morphological analyses in future research is essential to fully resolve the ecological responses of distinct cryptic species within the *Ammonia* complex. This constitutes a key direction for subsequent studies. Nonetheless, changes in salinity levels often result in the deformation or dissolution of foraminifera shells, as well as alterations to the structure of the community (Amao et al., 2018; Saraswat et al., 2015).

Although foraminifera exhibit a broad tolerance to salinity variations, low-salinity environments cause a demonstrable decline in feeding and metabolic efficiency (Lintner et al., 2020; Nigam et al., 2006; Sujata et al., 2011). Conversely, the collective of foraminifera exhibits a higher degree of tolerance to physiological stress induced by elevated levels of salinity (Debenay, 1990; Gull et al., 2025). The present study also revealed a significant positive correlation between the Margalef index of foraminiferal communities and salinity, with the diversity of benthic foraminifera increasing as salinity rises. This finding aligns with in situ observations in the Yellow Sea, where diversity is primarily limited by freshwater input (Lei et al., 2017). However, it is crucial to contextualize this linear trend within broader global patterns. As noted by Poag (2015) in the Gulf of Mexico, extreme hypersaline environments (e.g., Laguna Madre) often exhibit low-diversity assemblages dominated by Miliolida, mirroring the stress response seen in hyposaline waters. The continuous increase in diversity observed in our experiment suggests that for the Qingdao Bay community, hyposaline stress exerts a more severe physiological filter than the hypersaline levels tested (up to 60 PSU). In order to apply salinity parameters to paleoenvironmental reconstructions, multiple experimental runs are required. Increases in both density and breadth of experimental setups, in conjunction with extended cultivation periods, would yield more precise results.

4.2 The Relationship Between the Biomineralizations of Two Calcareous Foraminiferal Groups and Salinity

The biomineralization mechanisms of different foraminiferal taxa are closely linked to their ecological environments (Weinkauff et al., 2013). In conditions of reduced seawater salinity, there is an increase in the solubility of carbon dioxide, resulting in calcium carbonate being placed in a low-concentration, unsaturated state. Porcelaneous foraminifera (Miliolida), representing an early-diverging lineage, utilize supersaturated seawater as a mother liquor (Bentov et al., 2006). The formation of acicular calcite crystals within cellular vesicles is a direct process, and these crystals are assembled at the site responsible for shell formation through a process of disordered precipitation (Nooijer et al., 2009). At higher salinities, the solubility of

carbon dioxide is known to decrease, leading to calcium carbonate supersaturation. This environment is conducive to the formation of Miliolida, characterized by compact structures and disordered calcium carbonate crystal arrangements. Given the relatively elevated magnesium-to-calcium ratio characteristic of seawater, calcite formed under the shell-building mechanism of Miliolida exhibits elevated magnesium content (Nooijer et al., 2009). The magnesium content of different species of hyaline foraminifera (Rotaliida) varies considerably. In seawater of moderate salinity, where calcium carbonate approaches saturation or is slightly supersaturated, Rotaliida can gradually transform seawater into vesicles (Bentov et al., 2009), chemically modifying the seawater within the cell. This process involves the pumping out of hydrogen ions and magnesium ions, thereby raising the solution's pH. This process has been shown to facilitate the formation of more robust, less soluble, and orderly arranged low-magnesium calcite. This process has its origins in calcification genes that are unique to Rotaliida (Toyofuku et al., 2017; Ujjié et al., 2023).

Our quantitative data directly show that, in conditions of low salinity, there was a sharp decline in the abundance of foraminifera. Among these, the high-magnesium calcite shells of porcelaneous Miliolida were prone to both physiological mortality and test dissolution, whereas the low-magnesium calcite shells of hyaline Rotaliida resisted dissolution more readily, leading to an increase in the relative abundance of Rotaliida. At higher salinities, the formation of shells was found to be more favourable for Miliolida, thereby explaining the higher relative abundance observed at elevated salinities in classical morphological analyses (Fig. 3A). Research undertaken hitherto has indicated that hyaline Rotaliida, for example *Elphidium* and *Ammonia*, exhibit a high level of tolerance to low-salinity environments, whereas porcelaneous Miliolida become more prevalent in highly saline conditions (Amao et al., 2018; Charrieau et al., 2018; Orabi et al., 2017).

Foraminifera, indicator organisms of great utility in the field of geology, are frequently employed to deduce paleo-oceanic temperatures by means of their shell magnesium-calcium ratios. Significant variations in the responses of different foraminiferal taxa are observed across salinity levels. In circumstances where species assemblages are divergent, the resultant community changes may manifest distinct patterns. Therefore, after controlling for temperature effects, foraminifera also serve as amplifiers of salinity variations, enabling salinity determination based on specific species assemblages (Murray, 2006; Strachan et al., 2015). Furthermore, when employing foraminifera as paleo-oceanic thermometers in geological studies, we recommend conducting faunal composition analyses beforehand to enhance the accuracy of results. Given that salinity influences Mg/Ca-based thermometry (e.g., Lea et al., 1999; Katz et al., 2010), our results further underscore the necessity of conducting foraminiferal composition analyses to constrain salinity backgrounds, thereby enhancing the accuracy of paleo-oceanic temperature reconstructions. Regarding the direct reconstruction of paleo-salinity, the quantitative regression models derived in this study provide a specific reference framework. While our experimental results establish robust linear models for Qingdao Bay, we acknowledge that local environmental factors (e.g., temperature, substrate) vary across global estuaries. Thus, while the physiological trends (e.g., low-salinity sensitivity of Miliolida) are likely broadly applicable, the specific quantitative coefficients should be applied with caution when reconstructing paleo-salinity in geographically distant regions.

4.3 Traditional morphology and molecular biology methods

4.3.1 Complementarity of morphological and eDNA approaches

310 This study employed a combined approach of traditional morphology and environmental DNA (eDNA) analysis to examine the relationship between benthic foraminiferal communities and salinity response. The findings from both methodologies concurred on several conclusions: foraminiferal diversity exhibited a positive correlation with salinity, while the relative abundance of Rotaliida taxa demonstrated a significant negative correlation with salinity.

315 Molecular methods are a powerful tool for describing species diversity (Pawlowski et al., 2014). In this experiment, morphological analysis identified only 13 species, whereas eDNA detected 1,977 OTUs annotated to 103 species, thereby better reflecting the diversity within the foraminiferan group. Moreover, soft-shelled Monothalamiids are often damaged during morphological preparation and lack distinct morphological characteristics, leading to their exclusion from traditional morphological studies due to the absence of relevant identification knowledge (Schönfeld et al., 2012). Molecular methods can detect these organic-walled groups, which constitute dominant taxa in eDNA analyses across all salinity levels. The
320 discovery of this “hidden majority” has profound ecological implications. Relying solely on morphology leads to severe underestimation of benthic standing stock and biodiversity, and may result in misinterpretation of ecosystem resilience: while calcified taxa decline under stress, the persistence of soft-bodied forms represents a functional shift rather than a system collapse. Consequently, excluding this group limits our understanding of energy flow in the benthic food web.

325 Conversely, morphological methods provide valuable complementary insights that eDNA cannot fully capture. In this experiment, to avoid controversies arising from primer bias and sampling volume (Pawlowski et al., 2014), statistical analyses were conducted based on OTU counts and proportions rather than read abundances. In the eDNA dataset, the porcelaneous Milioliida group constituted a negligible proportion of total reads, making their community response to salinity gradients undetectable. In contrast, morphological analysis revealed that although the Milioliida assemblage was monospecific (represented only by *Quinqueloculina seminula*), its abundance was substantial, constituting a high proportion of total counts.
330 Morphological quantification captured the dynamic response of this group to salinity stress: Milioliida are sensitive to low salinity (maintaining < 20% relative abundance at 5–20 PSU) but exhibit remarkable tolerance to hypersaline conditions, with relative abundances remaining high (~40%) at 40–60 PSU and peaking at 55 PSU. Notably, the slightly higher relative abundance at 0 PSU compared to 5 PSU is an artifact resulting from the sharp decline in total community density under extreme freshwater stress rather than a preference for freshwater. Additionally, traditional morphology enables observation of shell
335 damage and deformities, providing insights into specific environmental impacts on foraminifera that molecular methods cannot offer.

Thus, integrating morphological and eDNA methods provides a holistic understanding of foraminiferal community responses to salinity, with morphology offering the essential reference for fossilizable taxa and eDNA revealing the full extent of biodiversity.

340 4.3.2 Methodological considerations and future directions

Both approaches are subject to inherent uncertainties when determining viability. Morphological identification is susceptible to factors such as staining efficiency and subjective judgment (Bernhard et al., 2006; Fontanier et al., 2002), while eDNA is unable to distinguish genetic signals originating from living, dead, or environmental residues (Pawlowski et al., 2014).

To minimize the potential impact of relic DNA, a well-recognized limitation of sediment based eDNA approaches, we
345 homogenized the original sediment and split it equally across all culture dishes prior to the start of the experiment. Thus, all salinity treatments began with the same initial sediment pool and an identical baseline eDNA signal, allowing post incubation differences to be primarily attributed to differential community responses to salinity. Nevertheless, relic DNA may still introduce background noise and could dampen the detection of rapid community shifts. This may partly explain the discrepancy between morphological and eDNA data for the Miliolida. In the morphological dataset, Miliolida showed a clear increase in
350 relative abundance with increasing salinity (Fig. 3A), consistent with their known tolerance to hypersaline conditions. In contrast, their eDNA signal was consistently low across most salinity treatments. A closer examination reveals that the relative abundance of Miliolida increased from 0.29% at 0 PSU to 1.23% at 60 PSU, indicating that the same directional trend, though much weaker, remains detectable. The attenuated signal may reflect lower DNA extraction efficiency, faster DNA degradation, or lower ribosomal copy numbers in this group, compounded by the masking effect of relic DNA from other taxa.

To address these limitations, environmental RNA (eRNA) has emerged as a promising tool for identifying metabolically
355 active communities, due to its rapid degradation outside living cells, which better reflects contemporary biological activity (Chen et al., 2025; Qiao et al., 2025). Although eRNA methodology in foraminiferal research is still evolving and not yet standardized, recent studies highlighting anaerobic metabolic adaptations in subseafloor foraminifera and the role of primary production in shaping benthic eukaryotic interactions underscore its potential to uncover physiological and ecological
360 responses inaccessible to traditional approaches. The continued development and integration of eRNA with controlled laboratory culture systems will help directly resolve how salinity fluctuations influence active foraminiferal community structure and function, thereby extending the findings of this study toward more mechanistic, ecophysiological meaningful insights.

5 Conclusions

This study involved collecting intertidal sediments for salinity gradient culture experiments. A combination of traditional
365 morphological and molecular biological methods revealed that benthic foraminifera can survive under salinity conditions ranging from 0 to 60 PSU. The Margalef diversity index for benthic foraminifera increased markedly with increasing salinity, while the relative abundance of Rotaliida taxa decreased significantly. Specifically, robust linear regression models were established linking salinity to both the relative abundance of Rotaliida and community diversity. Moreover, the relative
370 abundance of the Milioliida group, which dominates in morphological analyses, exhibits a positive correlation with salinity. Molecular methods employing high-throughput sequencing have detected a diverse array of Monothalamiids groups, whose

relative abundance exceeds 50%. The salinity-related linear functions derived in this study provide a critical quantitative baseline for calibrating proxies, supporting paleo-environmental reconstruction in the Yellow Sea and similar temperate marginal marine systems.

375 **Author contributions**

YC and YL conceptualized the study and curated the data, and YC carried out the investigation and methodology. YC and FW developed the software and created the visualizations, with FW performing the formal analysis. YC prepared the original draft, and YL reviewed and edited the manuscript. YL also acquired funding, provided resources, and supervised the project.

Competing interests

380 The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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