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Unlocking foraminiferal genetic diversity on estuarine mudflats with eDNA metabarcoding

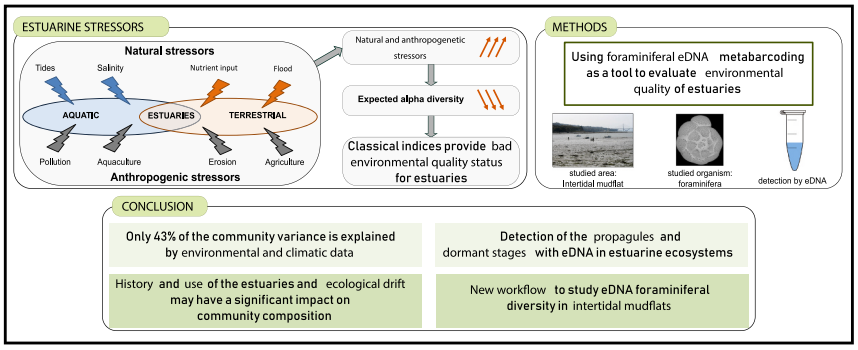
David Singer [a,b,*], Marie P.A. Fouet [a], Magali Schweizer [a], Aurélia Mouret [a], Sophie Quinchard [a], Frans J. Jorissen [a]

[a] Université d'Angers, Nantes Université, Le Mans Université, CNRS, Laboratoire de Planétologie et Géosciences, LPG UMR 6112, 49000 Angers, France
[b] Changins College for Viticulture and Enology, University of Sciences and Art Western Switzerland, Route de Duillier 60, 1260 Nyon, Switzerland

HIGHLIGHTS

- eDNA detect massive Foraminifera propagules diversity.
- 43 % of community variance is explained by physical, chemical, and climate factors.
- New metabarcoding workflow adapted to estuarine foraminiferal diversity.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental biomonitoring is a prerequisite for efficient evaluation and remediation of ecosystem degradation due to anthropogenic pressure or climate change. Estuaries are key habitats subject to multiple anthropogenic and natural stressors. Due to these multiple stressors, the detection of anthropogenic pressure is challenging. The fact that abundant natural stressors often lead to negative quality assessments has been coined the "estuarine quality paradox". To solve this issue, the application of molecular approaches with successful bioindicators like foraminifera is promising. However, sampling protocols, molecular procedures and data analyses need to be validated before such tools can be routinely applied.

We conducted an environmental DNA survey of estuarine mudflats along the French Atlantic coast, using a metabarcoding approach targeting foraminifera. Our results demonstrate that estuarine environments have only a few active OTUs dominating the community composition and a large stock of dormant or propagule stages. This last genetic diversity components constitute an important reservoir, with different species which can potentially develop in response to the temporal variability of the multiple stressors. In fact, different OTUs were dominant in the studied estuaries. Our statistical model shows that the physical and chemical characteristics of the sediment and the climatic conditions explain only 43 % of the community composition variance. This suggests that other, less easily quantifiable factors, such as the history and use of the estuaries or the ecological drift could play an

* Corresponding author at: UMR CNRS 6112 LPG-BIAF, Angers University, 2 Boulevard Lavoisier, CEDEX 01, 49045 Angers, France.
E-mail address: david.singer@changin.ch (D. Singer).

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important role as well. Environmental DNA biomonitoring opens new perspectives to better characterize the genetic diversity in estuaries.

1. Introduction

Estuarine intertidal areas correspond to the ecotone where aquatic (oceans and rivers) and terrestrial ecosystems merge. They represent highly productive ecosystems (Day Jr et al., 2012) that provide numerous ecological (Cave et al., 2003) and economical (Sudhakaran et al., 2021; Wolanski et al., 2019) services. Due to their unique properties, estuarine ecosystems are subject to both natural and anthropogenic environmental pressures resulting from continental (nutrient input, pollution, floods…) as well as oceanic (tides, salinity…) drivers. For example, the tidal cycle will cause high variability of salinity and moisture contents of different coastal habitats (Jorissen et al., 2022). River flow (low water levels, floods, etc.) will influence salinity as well, but may also cause erosion of banks and mudflats (Jorissen et al., 2022). To integrate these different parameters in a single index, we have recently developed and tested the "Marine Influence Index" (MII) which aims to provide an integrated assessment of the natural environmental conditions of estuarine sites (Fouet et al., 2022; Jorissen et al., 2022).

In view of the ecological and economical value of coastal areas, the European Commission adopted the Water Framework Directive (WFD, 2000/60/EC) in October 2000. This directive commits the Member States of the European Union to achieve a good quality status of all water bodies, including transitional and coastal waters. To achieve the objectives of the WFD, it is crucial to develop new approaches and methods to monitor environmental quality. Over the last 50 years many indicators of environmental quality have been developed (Diaz et al., 2004). Some of these indicators are based on chemical or physical parameters (Tueros et al., 2008), but these indices concern often only a single source of pollution or stress. To evaluate the impact of all stressors together, indicators based on organisms have been elaborated. Among them we can cite indices based on fishes (Lepage et al., 2016), macrofauna (van Loon et al., 2015), phytoplankton (Revilla et al., 2009) or vegetation composition (Juanes et al., 2008). In this context, the phylum of Foraminifera (Rhizaria, Eukaryotes) represents excellent model organisms to evaluate environmental quality. First, they have a short life cycle of some months up to a year (Murray, 1983). Next, different species are sensitive to organic enrichment (Jorissen, 1987; Parent et al., 2021), heavy metal pollution (Alve, 1991), or ocean acidification (Haynert et al., 2014; Keul et al., 2013; Kuroyanagi et al., 2021). Finally, the production of propagules and resting stages is a well-known adaptive mechanism to temporary adverse conditions (Geisen et al., 2018). Foraminifera have the ability to form such inactive stages (i.e., dormant adults or propagules), allowing them to survive prolonged periods of adverse conditions and rapidly respond when the environment becomes more favorable (Alve and Goldstein, 2002). For example, propagules of sensitive species (e.g., porcelaneous taxa) appear to be able to withstand short periods of low pH conditions and to "bloom" once the conditions are close to their optimum (Weinmann et al., 2021).

Over the last decades, many studies have put forward foraminiferal biotic indices for open marine environments, especially for the shelf area. On the one hand, several indices based on morphological observations were developed, using different metrics such as alpha diversity (Bouchet et al., 2012) or indicator species concepts (Barras et al., 2014; Jorissen et al., 2018). On the other hand, more recently, studies based on environmental DNA (eDNA) have provided indices based on assignments to a reference database (Cavaliere et al., 2021) or on taxonomy free and machine learning methods (Cordier et al., 2017). Nevertheless, these indices have their limits in estuarine environments. There, high amplitude spatial and temporal variability of natural environmental parameters (salinity, temperature, moisture, organic matter, oxygen contents, etc.) as well as superimposed anthropogenic stressors impact the community composition. Consequently, the alpha diversity found in estuaries is low in comparison with fully marine environments (Elliott and McLusky, 2002; McLusky and Elliott, 2004). Even in predominantly natural settings, species living in estuaries are highly adapted to extreme and highly variable environmental conditions requiring opportunistic life strategies. This strongly biases the results of environmental quality indices created in fully marine habitats, systematically leading to negative scores in estuaries (Fouet et al., 2022). This apparent contradiction (low environmental quality values in apparently natural ecosystems) is known as the "estuarine quality paradox" (Dauvin, 2007; Elliott and Quintino, 2007). Consequently, assessing the environmental quality of estuarine ecosystems remains a major challenge (Elliott and Quintino, 2007; Tweedley et al., 2015).

Here, we present a survey of foraminiferal genetic diversity (diversity obtained through eDNA approaches) in estuarine mudflats based on high throughput sequencing (Illumina, MiSeq). To assess the patterns and drivers of genetic diversity, we used a metabarcoding approach using specific foraminiferal primers, targeting the V9 region of the SSU rRNA gene. Our dataset consists of 25 sites on various mudflats in six estuaries of the French Atlantic coast. At each site we measured the major environmental parameters (sediment grain size, trace metals and organic matter) and retrieved climatic data (temperature and precipitation). With our HTS eDNA strategy, we address several fundamental questions, which are essential in a context of environmental biomonitoring: (1) What are the advantage and issues of eDNA approaches to capture foraminifera genetic diversity in comparison with the species diversity obtained through morphological approaches? (2) What are the patterns of genetic diversity along the French Atlantic coast estuaries? And, finally, does eDNA genetic diversity show the same response to the combination of natural and anthropogenic environmental gradients as traditional inventories?

2. Materials and methods

2.1. Studied estuaries, sampling sites and environmental parameters

Samples were collected in six estuaries located along the north French Atlantic coast: Elorn, Aulnes, Odet, Laïta, Crac'h and Auray (Fig. 1, Table 1, Table A.1). These estuaries are subjected to a meso- to low macrotidal regime with a tidal range of about 4 to 5.5 m at the inlet. The sampling was done during the low tide. To obtain a maximum of genetic diversity, 3 to 7 sites were sampled per estuary, on intertidal mudflats along the riverbanks. A total of 25 sites were sampled between September and October 2020. On the largest mudflats, at some sites, up to three stations ($n = 35$) were sampled at different elevations (A to C from upper to lower mudflat). To cover a maximum of genetic diversity at each station, two replicate samples of 5 g of the first centimeter of the sediment were collected and stored rapidly at −20 °C prior to DNA extraction. Furthermore, samples were collected at each site for sediment grain size analysis, organic matter and heavy metal measurements.

2.2. Morphological dataset

Morphological dataset was based on the methods and results described in detail in Fouet, 2022. Briefly, sediment samples were collected using a 96 mm corer, and the top centimeter of sediment was preserved in 96 % ethanol with Rose Bengal staining (2 g/L). The samples were then washed on 125 μm sieves. While the >125 μm fraction does not capture the entire foraminiferal community, it is a time-efficient compromise for biomonitoring studies (Schönfeld et al., 2012). Foraminifera specimens were observed under a Leica MZ16

stereomicroscope, and living individuals were identified by the presence of bright pink Rose Bengal staining in most chambers (Table B.1).

The physical properties of each site were further characterized using the Marine Influence Index (Jorissen et al., 2022). The index includes the normalized measurements of the distance to the sea, the emergence time at low tide and the river discharge (Table A.1). The detailed protocol and the rationale behind this index is explained in Jorissen et al. (2022). Climatic data were obtained by using the coordinates of each site to extract the value of the 19 variables extrapolated from monthly measurements of temperature and precipitation (Table A.1). For this purpose, we used the finest 30 arc second resolution grids of the WorldClim database (Fick and Hijmans, 2017).

Sediment grain size, trace metal concentrations and organic matter content were assessed to obtain sediment properties. Protocols for these methods are detailed in Fouet et al. (2022). In brief, the sediment grain size was analyzed (non-decarbonated) with a *Mastersizer 3000 laser diffraction particle size analyser* (Malvern Panalytical Ltd., Malvern, UK). In this study three different fractions are considered: clay (<4 μm), silt (4-63 μm) and sand (>63 μm) (Table A.1). Three samples (LAI_1A, ELO_1A and CRA_1A) are considered as outliers because of their high sand percentage (>80 %). These sites cannot be defined as mudflat environments and will not be included in the ecological models. Trace metal concentrations were analyzed with an Inductively Coupled Plasma Mass Spectrometer (Thermo Scientific® X-Series 2 ICP-MS) on freeze-dried sediment, after total acid digestion (HCl, HNO_3 and HF) (Coynel et al., 2016). Seventeen chemical elements (V, Cr, Co, Ni, Cu, Zn, As, Sr, Mo, Ag, Cd, Sn, Sb, Ba, Pb, Th and U) were measured. Enrichment Factors (EF) were calculated by comparison with an unpolluted reference sample (Fouet et al., 2022; Larrose et al., 2010) (Table A.1). Elemental carbon (%Corg) and nitrogen (%N) contents were measured on decarbonated freeze-dried sediment with a CHONS Elemental Analyser (EA Vario PYRO cube; Elementar®, Langenselbold, Germany) (Table A.1).

2.3. DNA extraction, amplification, sequencing

Total DNA was extracted from the sediment with the FastDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The choice of this specific DNA extraction kit was motivated by the high quantity of raw material to be analyzed (5 g). As the density of Foraminifera on mudflats can be highly variable (Fouet et al., 2022), this should guarantee a good coverage of the genetic diversity. In our case, as the morphology-based species diversity was low (Fouet et al., 2022), we expected to have deleterious effects of highly homogeneous nucleotide composition. This limits the number of high-quality reads generated per Illumina run. In order to reduce these effects and increase the overall quality of our sequencing, we included 0 to 4 nucleotides between the tags and the primers to increase the heterogeneity (Jensen et al., 2019). To reduce the tag jumping effect, we used a dual-indexing approach (Taberlet et al., 2018). Primers, tag sequences and library information can be found in Additional file A.1, and Table A.1.

PCR was carried out with AccuPrime™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific). The 37f hypervariable region (Pawlowski et al., 2014) (amplicon size: 135–190 bp) was amplified using the specific foraminiferal primers s14F1 (Pawlowski, 2000) and s15r (Lejzerowicz et al., 2014). In metabarcoding studies, PCR replication is important to obtain a correct value of genetic diversity (Shirazi et al., 2021). Therefore, for each DNA extraction, three PCR replicates

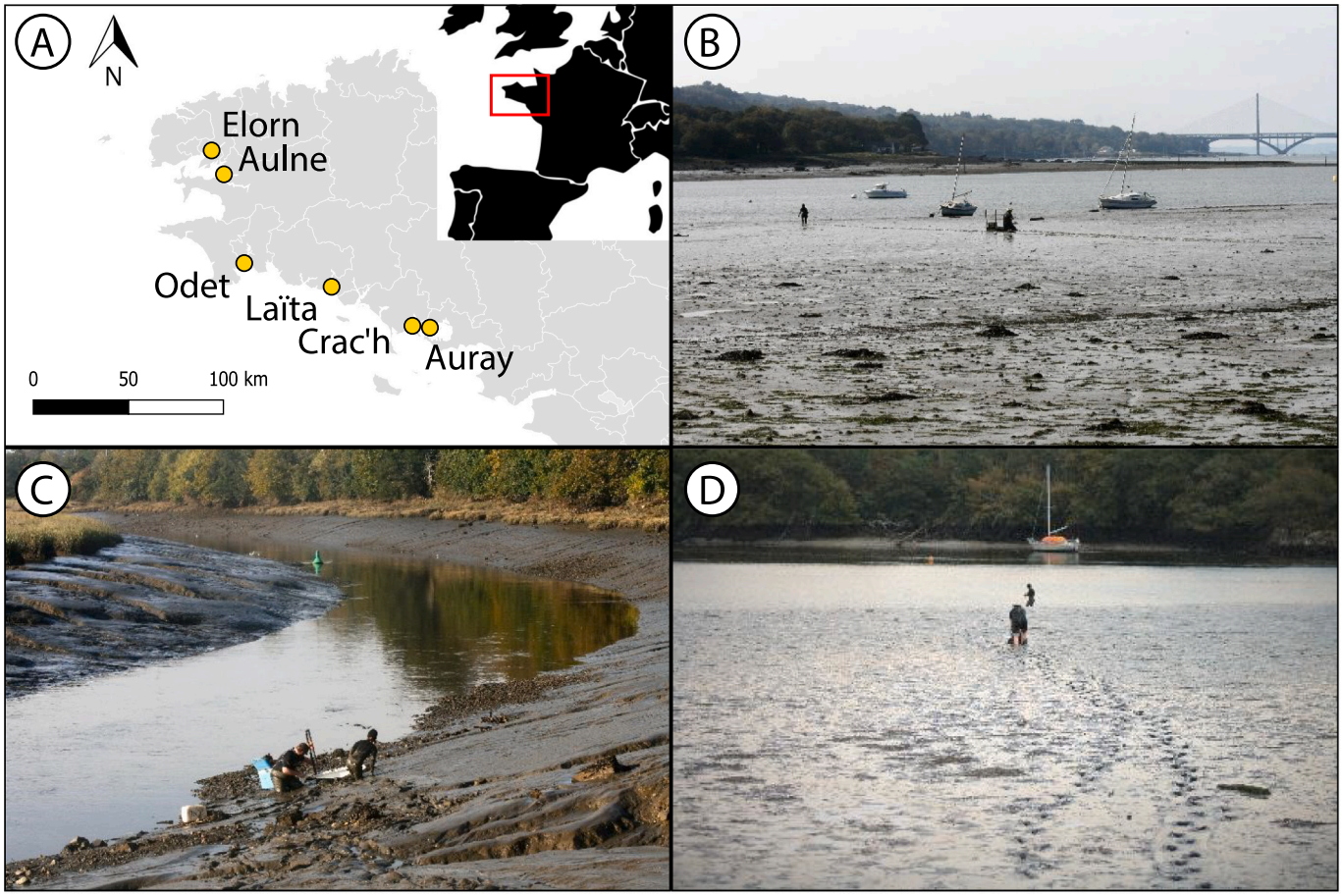

Fig. 1. A) Map of the six estuaries (Elorn, Aulne, Odet, Laïta, Crac'h and Auray), B) picture of Elorn ST03_A and ST03_B, C) picture of Elorn ST04_A, D) picture of Laïta ST03_A.

Table 1
General information about the estuaries, localities and coordinates of the stations.

Samples	Estuary	Locality	Nb of stations	Nb of samples	Sampling date	Latitude	Longitude
AUR_1	Auray	Locmariaquer	3	6	16.09.2020	47.5701	−2.9422
AUR_2	Auray	Kerouarc'h	3	6	17.09.2020	47.5845	−2.9618
AUR_3	Auray	Fort Espagnol	2	4	17.09.2020	47.6162	−2.9534
AUR_4	Auray	Berly	2	4	17.09.2020	47.6348	−2.9645
AUR_6	Auray	Plessis	2	4	17.09.2020	47.6348	−2.9645
AUR_7	Auray	Reclus	1	2	18.09.2020	47.6561	−2.9791
AUR_8	Auray	Pont d'Auray	2	4	16.09.2020	47.6678	−2.9711
CRA_1	Crac'h	La Trinité sur mer	1	2	20.10.2020	47.5835	−3.0248
CRA_2	Crac'h	Kerguirone	1	2	20.10.2020	47.6251	−3.0323
CRA_3	Crac'h	Kervilor	1	2	20.10.2020	47.6101	−3.0246
CRA_4	Crac'h	Kerguet	1	2	20.10.2020	47.6006	−3.0293
ODET_1	Odet	Benodet	1	2	18.10.2020	47.8827	−4.1155
ODET_2	Odet	Pois Keraigr	1	2	18.10.2020	47.9074	−4.1439
ODET_3	Odet	Pois Meillon	1	2	18.10.2020	47.9319	−4.1137
ODET_4	Odet	Keradennec	1	2	18.10.2020	47.9724	−4.1003
LAI_1	Laïta	Kerbrest	1	2	19.10.2020	47.7716	−3.5290
LAI_2	Laïta	Abbaye	1	2	19.10.2020	47.8046	−3.5268
LAI_3	Laïta	St Germain	2	4	19.10.2020	47.7904	−3.5312
AUL_1	Aulne	Landenevec	1	2	16.10.2020	48.2922	−4.2617
AUL_2	Aulne	Moulin à la mer	1	2	16.10.2020	48.2755	−4.2828
AUL_3	Aulne	Kerbastard	1	2	16.10.2020	48.2461	−4.2006
ELO_1	Elorn	Camfront	1	2	17.10.2020	48.3958	−4.3825
ELO_2	Elorn	Kermeur St Yves	1	2	17.10.2020	48.4066	−4.3452
ELO_3	Elorn	Beg ar Groaz	2	4	17.10.2020	48.4246	−4.3050
ELO_4	Elorn	Landernau	1	2	17.10.2020	48.4448	−4.2723

were done and pooled (for cost efficiency) before the quantification and sequencing. The PCR conditions consist of an initial denaturation of 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 45 s and extension at 68 °C for 90 s plus a final extension at 68 °C for 10 min. PCR product pooled replicates were quantified using the QuBit HS dsDNA (Invitrogen). Each sample was then pooled with the same amount of DNA and purified using the NucleoSpin™ Gel and PCR Clean-up XS kit (Macherey-Nagel). Library preparation and Illumina MiSeq (paired-end, 2 × 250 bp) sequencing were performed at ID-Gene Ecodiagnostics (Geneva, Switzerland). Sequences are available on European Nucleotide Archive via project number PRJEB55114.

2.4. Bioinformatics and taxonomic assignment

Tags and primers were removed from the reads using cutadapt v. 3.4 (Martin, 2011). Clustering of the reads was done using the R package DADA2 (v. 1.16; Callahan et al., 2016). Raw reads were quality controlled by truncating (forward and reverse length of 120 bp) and filtering them to a maximum number of 'expected errors' of two. Amplicon sequence variants (ASV) were dereplicated if identical, clustered and pair-end reads merged using a minimum overlap of 12 bp and maximum mismatch of 0 bp. Chimeras were removed using the 'pooled' method (Callahan et al., 2016, p. 2). The ASVs were first automatically assigned taxonomically using VSEARCH v. 2.18.0 (Rognes et al., 2016) and our Foraminifera reference database based on the sequences present in the NCBI database. This first assignation allowed removing ASVs that do not belong to foraminifera (percentage of identity below 70 %). Then, the remaining ASVs were compared with the GenBank database using BLAST and assigned to species when the percentage of identity was superior to 99 %. If the percentage of identity was below this threshold the ASVs were assigned as "environmental clades". Based on this information a second clustering where ASVs assigned to the same species, or the same environmental clade were done. Therefore, the ASVs produced by the first clustering (DADA2, algorithm based on model error rate) will be renamed into operational taxonomic units (OTUs, algorithm based on the database similarity) after the second clustering. Finally, a table with these new OTUs including only the species and environmental clades related to foraminifera was built for further analyses.

2.5. Data analysis

Statistical analyses were performed using R (v4.0.3) (R. Core Team, 2014) and the package vegan (v2.5-7) (Oksanen et al., 2021), if not specified otherwise. We constructed distribution plots showing the number of reads and ASVs versus the similarity value with the reference sequences (single cell barcoded species) (Mahé et al., 2017). Then, to determine whether the sequencing depth is sufficient to obtain an accurate estimate of ASV richness, we constructed rarefaction and accumulation curves with the *rarecurve* and *specaccum* functions, respectively. Representation of the genetic diversity of the species representing >1 % of the dataset (Schiaffino et al., 2016) was computed using the *ggheatmap* function of the heatmaply (v1.3.0) package (Galili et al., 2018) and the *upset* function of the UpSetR (v4.0.5) package (Conway et al., 2017). We assessed the beta diversity (similarity patterns) among foraminiferal communities by non-metric multidimensional scaling (NMDS). NMDS was based on Bray-Curtis dissimilarities retrieved from the sequence relative abundance. Even if the relative abundances derived from the number of reads should be interpreted with caution (Lara et al., 2022), this information can provide additional information compared to presence/absence data.

To assess relationships between the OTUs and the environmental variables, we first assessed the collinearity between the explanatory variables. We performed a stepwise selection based on the variance inflation factors (VIF) with the recommended threshold of ten (Belsley, 1980). The following variables were selected for further analyses: organic carbon, clay (%), distance to the sea, altitude, river discharge, trace elements (V, Co, Cu, As, Mo, Sn, Ba, Pb, U) and bioclimatic variables (Isothermality, Mean Temperature of Wettest Quarter (MTWQ) and Precipitation of Driest Month (PDM)).

Then we performed a partial redundancy analysis (RDA) on Hellinger transformed data (Legendre and Gallagher, 2001). This transformation will standardize the dataset and help to minimize effects of vastly different total abundances per sample (Legendre and Gallagher, 2001). The significance of the variables and first and second ordination axes was assessed using a one-way analysis of variance (ANOVA; 1000 permutations, p value threshold = 0.05) (Chambers and Hastie, 1991). Finally, in order to evaluate the percentage of variance in foraminiferal communities explained by sediment characteristics and climatic variables, we performed variation partitioning (*varpart*; Peres-Neto et al.,

2006).

3. Results

A total of 6,224,249 raw reads were obtained after the bioinformatic clustering steps. After removing non foraminiferal, low quality and chimeric reads, a final dataset of 4,133,414 reads and a total of 162 ASVs remained (Table C.1). Half of the reads (53.1 %) and about a quarter of the ASVs (22.6 %) were assigned with 100 % confidence to the reference database (Fig. A.1). The overlap of ASVs between two replicates was moderate (63 %), while the overlap of reads between two replicates was high (90 %) (Fig. A.3). This indicate that a substantial part of ASVs were only present in one replicate (Fig. A.2) but that the dominant ASVs (those that have a high number of reads) are present in both replicates (Fig. A.2, Fig. A.3). To reduce this sampling bias, further statistical analyses were therefore carried out on combined replicates. The rarefaction curves (Fig. A.4) demonstrated that the sequencing depth per site was sufficient, and the ASVs accumulation curve (Fig. A.5) showed that the whole study adequately presented the total foraminiferal genetic diversity. The final table count based on the 162 ASVs consists of 99 OTUs (18 OTUs related to monothalamids, 14 OTUs related to Globothalamea, 2 OTUs related to Tubothalamea and 65 unassigned OTUs considered as environmental clades) (Fig. 2, Table D.1).

The proportion of the 65 environmental clades was uneven between the samples and estuaries with a particularly high number in Auray estuary. The proportion of Rotaliida (Globothalamea) was low, except for some samples where high numbers of reads of *Ammonia* and *Haynesina* were observed (Auray ST02_A, Crac'h STO4_A, STO5_A and Odet STO4_A). Most of the monothalamids were affiliated to the Saccaminidae, which were dominant in most of the assemblages. A notable exception was the Laïta estuary where unclassified monothalamids and *Vellaria pellucida* were dominant.

In terms of the presence and absence of OTUs in estuaries, we generated an UpSetR plot (an enhanced Venn diagram) focusing on the dominant OTUs, which constitute >1 % of the total number of reads. Our results show that 67.5 % of the OTUs were present in five or six estuaries (Fig. A.6). >80 % of the OTUs were present in at least four estuaries (Fig. A.6). Finally, no dominant OTUs were present in only one estuary. However, the relative abundance of the number of reads of the dominant OTUs was highly variable among the studied estuaries (Fig. 3). A dominance of three to 12 specific OTUs was typical for each of the six estuaries (Fig. 3). The communities often showed a dominance of one or two monothalamid species that were different in the various estuaries. For the Crac'h and Odet estuaries, the relative abundance of Globothalamea species was higher (Fig. 2) in comparison with the other estuaries. Even if every dominant species had a favorite estuary, they were also present in most of the other estuaries (Fig. 3).

The NMDS analysis based on the dominant OTUs (Fig. 4) showed that each estuary occupies a specific part of the ordination space. Both the Auray and Crac'h and the Elorn and Aulne estuaries plot in the same part of the ordination space. Conversely, the Odet and Laïta estuaries did not overlap with other estuaries. Although most of the OTUs were in the center of the ordination space, some had a stronger influence on the ordination of the samples and estuaries. This was for instance the case for env_18, *Elphidium margaritaceum*, *Haynesina depressula* (Auray and Crac'h estuaries) and two monothalamids (GenBank accession numbers KP984731 and EU213249).

About the chemical composition of the sediment, most samples showed low enrichment factors, suggesting predominantly natural conditions, except in the Aulne estuary where a strong enrichment of Pb (>5) was observed (Table A.1). Percentage of organic carbon and nitrogen content are in the range of values usually found in mudflat environments (Dubois et al., 2012).The RDA analysis (Fig. 5a) showed that foraminiferal communities are significantly influenced by nine environmental variables (clay, Corg, distance to the sea, river discharge, Pb, Sn, U, PDM, and isothermality, $p < 0.05$). The first two axes explained 43.3 % of the variance. The variance partitioning showed that the sediment and physical properties explained 15.9 % of the variance, whereas the climatic data explained 8.2 % (Fig. 5b). When both were combined, the percentage reached 17.1 %. The residuals of this analysis correspond to 58.7 %.

4. Discussion

4.1. Sampling, methods, and dataset consistency

This is the first published attempt to study foraminiferal eDNA sampled on estuarine mudflats with specific markers, and consequently, our sampling strategy was not yet optimized for this purpose. In order to assess every component of the genetic diversity (active as well as

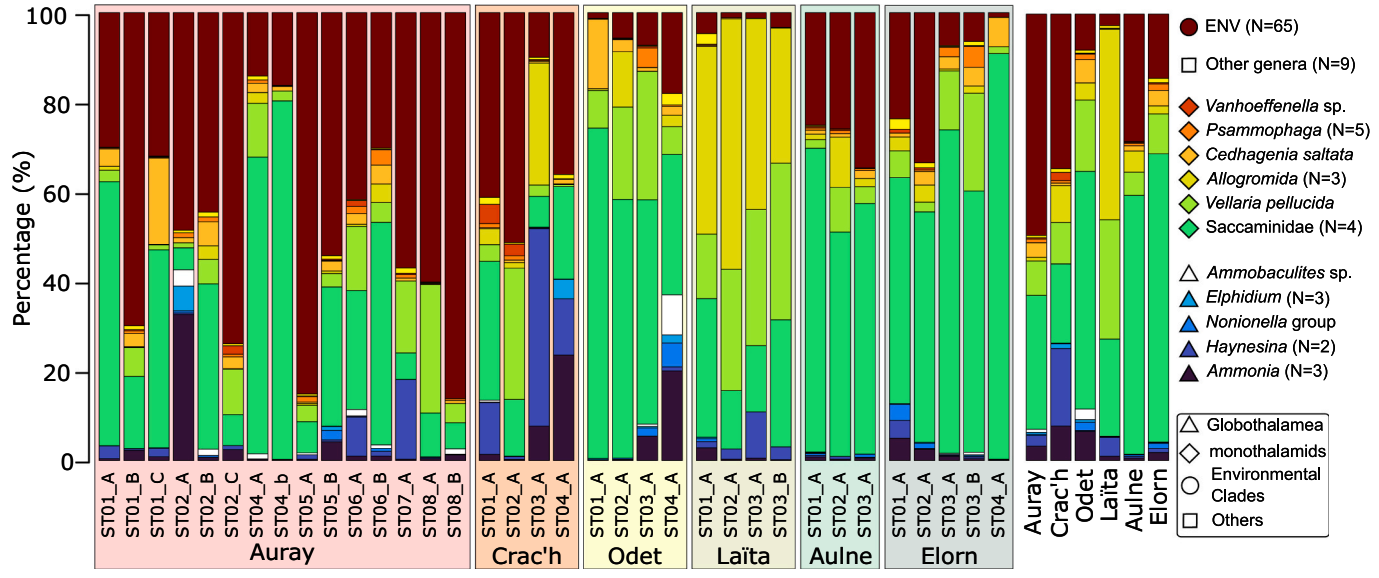

Fig. 2. Distribution of the foraminifera taxa living in 6 French coast Estuaries. Taxa were obtained using an eDNA metabarcoding approach targeting specifically foraminifera. Sequencing was performed using a MiSeq platform. The taxa in blue are related to Rotaliida, in dark red the environmental clades that were not assigned to a known reference. Other colors represent the taxa affiliated to the Monothalimids.

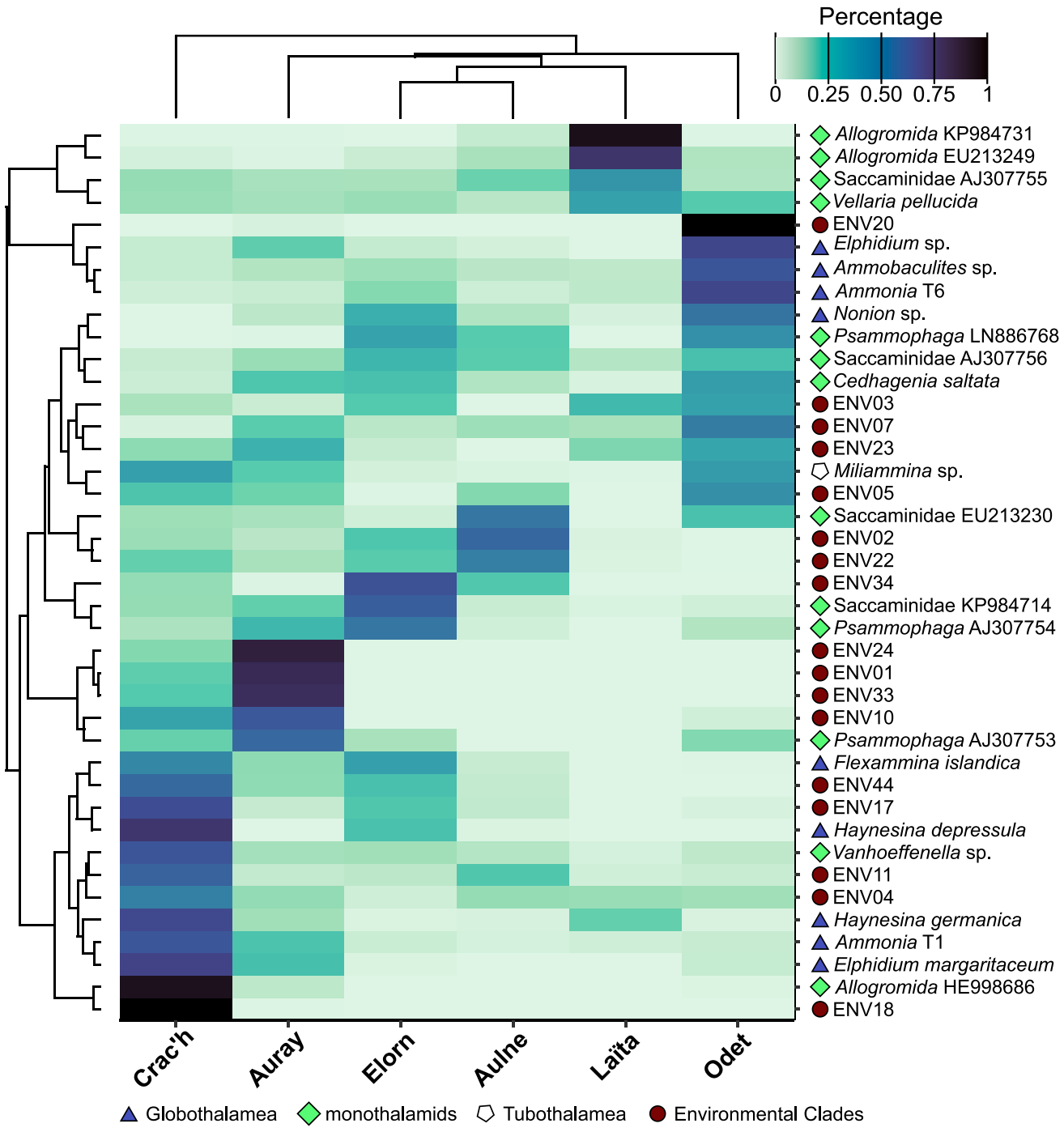

Fig. 3. Heatmap depicting the dominant foraminiferal genetic diversity and relative abundance in French coast estuaries. The taxa were obtained with an eDNA metabarcoding approach using specific primers for Foraminifera. Sequencing was done with a MiSeq platform. The distribution of the relative abundance of the reads across the estuaries is uneven and specific taxa are strongly related to a certain estuary.

inactive stages) we chose to focus on the DNA-based HTS and not on RNA-based HTS (Qiao et al., 2022). The downside of this approach is that some dead cells with preserved DNA inside the shell may also be sequenced. Seasonality and temporality are also important factors to consider in ecological studies, also for Foraminifera in estuarine environments (Debenay et al., 2006; Murray, 1983). Therefore, to reduce potential impacts induced by these factors, we decided to focus on a single season (Fall 2020). Although on average one replicate represents only 63 % of the total genetic diversity at the sampling site (Fig. A.2), the rarefaction and amplification curves clearly reach the "plateau" (Fig. A.4, Fig. A.5). This indicates that the sequencing depth is sufficient to cover the total genetic diversity of a single replicate. Moreover, in terms of abundance, the shared reads between the replicates account for 90 % (Fig. A.3). This confirms that the dominant ASVs are detected consistently in both replicates. However, some of the rarer taxa may also be missed in two replicates so that genetic diversity obtained through an eDNA approach should be described with caution. Several explanations can be provided to explain the fairly large differences in the ASV's observed in the two replicates: 1) The DNA extraction kit does not allow perfect DNA extraction of the cells (i.e., bead beating steps could be unable to break properly organisms provided with calcareous tests), 2) The quantity of sediment used as input may not cover the total genetic

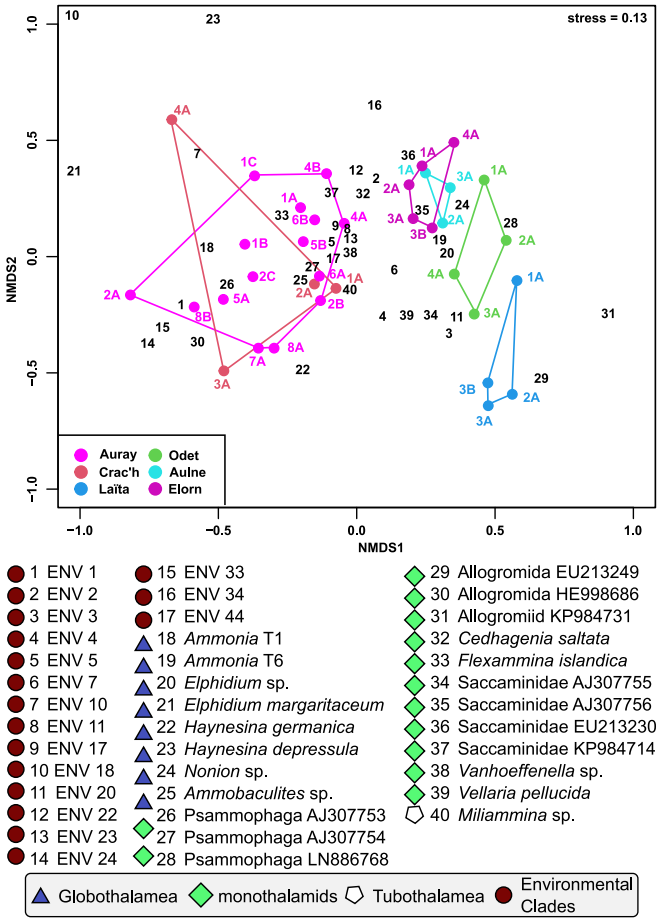

Fig. 4. Non-metric multidimentional scaling (NMDS) based on Bray-Curtis dissimilarities of 35 samples from Auray, Odet, Crac'h, Aulne, Laïta and Elorn estuary. Stress value = 0.13. The NMDS is constructed with the dominant taxa that represent >1 % of the total number of reads.

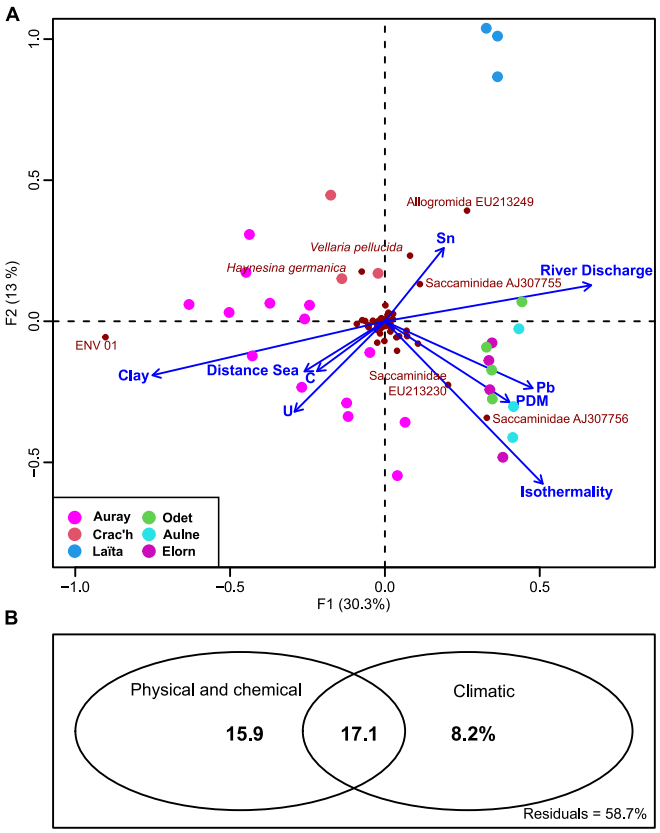

Fig. 5. A) Redundancy analysis (RDA) of dominant foraminifera taxa extracted from sediment sample collected from six estuaries from the French Atlantic coast. Significant environmental variables ($P < 0.05$) are represented by arrows. B) The variance partitioning results for community composition among the components of the physical and chemical parameters and the climatic data. Residual values are also displayed.

diversity of the site and finally 3) the sampled area (for one replicate) is potentially too small to have a complete picture of the genetic diversity of the site. A recent study on metazoans showed a similar pattern with a potential bias toward the largest species (Klunder et al., 2022). To draw sound ecological conclusions, it is crucial to cover the total genetic or/and species diversity. Consequently, more methodological work is needed to define the most efficient strategy (Lara et al., 2022; Zinger et al., 2019). To reduce sampling artifacts in our study we decided to pool the replicates and consider them as single samples. The total genetic diversity of the environment was approached with this strategy.

To obtain a robust dataset that allows answering ecological hypotheses, different known biases (contamination, tag jumping, reads quality, assignation…) must be addressed (Pawlowski et al., 2014). In this study, we chose to adopt the most stringent possible approach. To reduce the "tag jumping" effect we used a unique double tag combination and limited the number of samples per library to 35 (Esling et al., 2015). We also improved the overall quality of our sequencing by including 1–4 nucleotides as spacers to increase the heterogeneity of the libraries (Fadrosh et al., 2014). Next, we checked the assignation of each ASV with the reference database to avoid potential bioinformatic misidentification. To remove genetic diversity biases due to multiple variants that exist for some foraminiferal species (Weber and Pawlowski, 2014), we decided to pool the ASVs belonging to the same species or environmental clades to a single OTU. And finally, for our ecological model (Figs. 4 and 5), we decided to consider only OTUs accounting for >1 % of the total number of reads. By considering only OTUs present in fair numbers in many samples, we expected to reduce the noise in our ecological model caused by the rare biosphere (Schiaffino et al., 2016). All these steps tend to increase the robustness of our dataset and strengthen our interpretation and conclusions.

4.2. Estuarine foraminiferal eDNA diversity

We were able to assign most of our ASVs to species level (Table C.1), demonstrating the advantage to use specific markers for specific clade investigation. As expected, due to the extreme environmental conditions in estuaries, the genetic diversity of foraminifera is low compared to open ocean habitats (Lecroq et al., 2011). The number of foraminiferal species observed at the investigated sites based on the morphological observation of the 125 μm fraction of the sediment (only species with a mineralized or agglutinated shell were observed) ranges from two to 19 (Fouet et al., 2022). In our study we observed 99 OTUs, about five times more that observed in morphological studies. The high proportion of monothalamids found in the eDNA dataset can be explained by a combination of factors. For example, the shorter barcode size in some monothalamids (i.e., saccaminids), compared to Globothalamea or Tubothalamea would promote their amplification and sequencing. Another explanation could be that monothalamids are easier to extract with the DNA kit as they have an organic test (Santos et al., 2017). Primer or sequencing biases in favor of this group could also be possible. Finally, a naturally higher abundance of monothalamids compared to mineral shelled foraminifera (Globothalamea and Tubothalamea) could also be hypothesized (Laroche et al., 2018; Lecroq et al., 2011; Lejzerowicz et al., 2021). The much lower abundance or total absence of monothalamids in morphological studies is explained by the fact that

organic shelled foraminifera (dominant in monothalamids) are usually ignored in this methodology. To overcome some of these limitations, e.g. the primer/sequencing biases, the use of other markers such as the COI (Macher et al., 2022) can be envisaged. However, in that case, other biases can be expected and the reference database for these other barcodes is still far too poor to be efficient for an ecological study like this one.

Even if the number of environmental clades found in our study is high (representing 65.6 % of the total number of OTUs), they represent a marginal fraction of the total number of unassigned reads (30.9 %, Fig. A.1). Moreover, two dominants environmental OTUs (ENV_01 and ENV_02) represent respectively 61.2 % and 14.2 % of the unassigned reads. Further investigations are needed to discover which species are hidden behind these environmental clades. Nevertheless, our study demonstrates overall that the effort of single cell barcoding is sufficient to reliably assign the majority of ASVs on estuarine mudflats. Consequently, the reference database for these ecosystems can be considered as accurate. Monothalamids remain poorly documented in comparison to their multichambered relatives. Nevertheless, this group profits from a renewed interest, with new descriptions of species belonging to this polyphyletic clade published recently (Gooday et al., 2022; Holzmann et al., 2022). An additional effort should be made on intertidal mudflats to describe monothalamids morphologically and genetically. Thereafter, the intra-specific genetic variance will be better known, and it is highly probable that several species will be represented by more than one ASV, like is the case for many of the well-studied species. This will lead to a decrease of alpha diversity.

Our results show that natural environmental gradients have a strong impact on species composition. In function of the habitat properties, a specific selection of often opportunistic species will be present in each estuary. Our analyses suggest that the community composition is similar in the estuaries of Auray and Crac'h as well as in the estuaries of the Aulne and Elorn (Figs. 4 and 5), even if the dominant OTUs are not the same in both estuaries (Fig. A.6, Fig. 3). It appears that the small geographic distance between these two estuaries, and/or the comparable environmental conditions, could have led to more similar communities compared to other estuaries (Fig. 1).

Generally, only a few OTUs dominate each sample, which is in line with the conclusion of morphology-based studies (Fouet et al., 2022). Environmental DNA based studies have the particularity to also detect propagules and dormant stages. Additionally, they present a semi-quantitative evaluation of the community composition. In our dataset, the distribution of dominant species that represent >1 % of the total of reads is very different between the samples, much more than in the morphological inventories (Fouet et al., 2022). Together with the low alpha diversity of the morphology based analyses of the same sites (i.e., 2–19 (Fouet et al., 2022), this suggests that only a limited number of OTUs is able to occupy the estuarine mudflat environments with adult specimens, and that a large part of the eDNA comes from dormant stages (and eventually some dead forams). In fact, due to the strong hydrodynamics caused by tidal and fluvial currents and waves, dormant stages will be massively introduced in estuaries. Many of these introduced taxa will not be able to colonize mudflats due to the environmental conditions which are unfavorable to them.

4.3. Environmental parameters

The community composition of foraminifera in estuarine mudflats is influenced by various environmental properties, sediment characteristics, and climatic factors. Our RDA model (Fig. 5a) demonstrates that these factors collectively account for 43 % of the variance. Notably, nine variables within the model significantly impact the community composition.

One crucial factor is sediment grain size, specifically the clay percentage, which has a known influence on foraminiferal communities (Armynot du Châtelet et al., 2009). Our analysis (Fig. 5) reveals that also the distribution of ASVs on intertidal mudflats is affected by this parameter. Similarly, also the percentage of organic carbon, often used as an indicative parameter in morphology-based quality indices in fully marine environments (Alve et al., 2016; Jorissen et al., 2018), appears to influence the community distribution (Fig. 5). However, it is worth noting that this influence may be partly attributed to the strong negative correlation with grain size.

Two physical parameters, namely the distance to the sea and river discharge, significantly impact the first axis of the RDA (Fig. 5). Both variables are linked to the salinity of the estuary (Jorissen et al., 2022), which is considered a critical factor in driving species diversity for fishes (Whitfield, 2015), phytoplankton (Nche-Fambo et al., 2015), and foraminifera (Fouet et al., 2022). Additionally, two climate-related variables, Isothermality and Precipitation of the Driest Month (PDM), are also significant in the model. Isothermality quantifies the extent of temperature differences between day and night compared to seasonal oscillations (O'Donnell and Ignizio, 2012). Estuarine mudflats experience substantial temperature gradients on both a daily (tidal effect) and seasonal (spring and neap tides) scale, resulting in low isothermality. PDM represents extreme conditions with minimal freshwater input into the system. Both PDM and river discharge reflect the influence of freshwater input, underlining its importance in the model. Finally, the Pb enrichment factor potentially affects estuarine foraminiferal communities (Fig. 5, Table C.1). Previous studies have indicated that high Pb concentrations can significantly impact the abundance of dominant foraminiferal species (Brouillette Price et al., 2019).

Even if these variables undoubtedly influence the community composition, their contribution appears to be limited. In fact, together these factors explain only 43 % of the total variance of the dataset, and therefore 57 % remains unexplained in the RDA model (Fig. 5). This means that other factors are important drivers of genetic diversity as well. This is underlined by the important differences observed between estuaries (Figs. 3 and 4), which are not corresponding to equally large differences in the adult community (as shown by morphological studies) and appear unrelated to the environmental characteristics discussed before. Several additional factors can be involved. For instance, more qualitative environmental factors such as the morphology, history and anthropogenic use of the estuaries could affect the community characteristics. However, such factors should also affect the composition of the adult assemblages, which is not evident. Therefore, we hypothesized that ecological drift could explain a significant part of the variance (Fodelianakis et al., 2021), as previously reported for rare bacteria (Shi et al., 2023). In fact, stochastic processes are expected to become more significant with low population size and genetic diversity (Vellend, 2010), therefore this process can be especially pertinent in estuarine ecosystems.

4.4. Putative impact of anthropogenic stressors

Due to the cumulative effects of natural and anthropogenic stressors as well as potential qualitative environmental factors and ecological drift, the development of bioindicators in estuarine environments is still a challenge. Even if some metallic trace enrichment factors (i.e., Pb) and organic carbon can have an anthropogenic source, it was impossible to evaluate the impact of anthropogenic pollutants individually in our model (Fig. 5). Therefore, we can assume that the foraminifera are not substantially influenced by anthropogenic stressor.

4.5. Advantage and limitations of eDNA approaches

One major difference between HTS and classical morphological studies is that HTS does not only take into account adult stages, but also monothalamids, propagules and dormant stages (and potentially also some dead individuals), thereby potentially strongly increasing genetic diversity. As HTS data are semi-quantitative, we can assume that due to the intrinsic mudflat proprieties, the dominant OTUs (corresponding to

>1 % of the total of reads) in our study correspond to the adult and the most active stages of foraminifera. Nevertheless, the distribution of the reads of these dominant OTUs is very different between the studied estuaries. It appears that the dominant species are present in all estuaries, but they do not fully develop everywhere; they have a high density of reads only at some of the sites. Environmental DNA approaches are therefore a powerful tool, which does not only assess the living and dominant species but also species that are potentially present and await more favorable conditions to develop. However, a limitation of the eDNA approach is the impossibility to distinguish the different categories (adults, juveniles, propagules, dormant stages). A dual approach, combining morphological as well as eDNA studies, to assess the various stages, seems therefore the ideal strategy to obtain a more complete overview of the foraminiferal communities. In the future, new molecular methods such as single cells transcriptomics (Sierra et al., 2022) or long-read metabarcoding (Jamy et al., 2020) can be envisaged. Nevertheless, more research and development are requested to assess ecological questions with such new approaches.

5. Conclusion

Our study demonstrates that eDNA is particularly well adapted to assess the foraminiferal genetic diversity in estuarine mudflats. The sampling strategy and the choice of eDNA extraction kits still need optimization. We suggest increasing the number or replicates and sites or/and the quantity of raw material and optimizing the bead biting step to extract more DNA from calcareous foraminifera. Several crucial factors (tag jumping, assignation, reference database…) must be carefully assessed to obtain a reliable dataset. The environmental parameters, sediment characteristics as well as the climatic data partly explain the community composition. A larger dataset, including more sites and samples would be needed to assess the importance of other factors like ecological drift or the anthropogenic occupation history of the estuary. Finally, eDNA investigation allows to assess other foraminiferal genetic diversity components such as juveniles, propagules and dormant stages, that are usually not considered or underestimated in morphology-based studies. Therefore, the combination of morphological information, mainly concerning the adult living community, and HTS of eDNA allows obtaining a more complete picture of the different components of the genetic diversity. By monitoring the evolution of the genetic diversity, we can detect shifts in the community due to major environmental changes and anthropogenic impact.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165983.

Benefits generated

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

CRediT authorship contribution statement

Conceptualization of the study, D.S. and F.J.J.; Sampling, D.S., S.Q. and M.P.A.F. DNA extraction, PCR and samples preparation, D.S. and S. Q. Bioinformatic and statistical analyses D.S.; interpretation of the ASVs M.S. and D.S., visualization, D.S. writing, D.S. and F.J.J. supervision and project administration, F.J.J.; All authors have read, commented and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Fastq files are available on ENA via the project number PRJEB55114. The data generated/analyzed are included: the ASV table counts, ASV taxonomy and sample metadata are provided as additional files.

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