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# 1 **Unlocking foraminiferal genetic diversity on estuarine mudflats with eDNA metabarcoding**

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## 8 **Highlights**

- 9 - eDNA detect massive Foraminifera propagules diversity
- 10 - 43% of community variance is explained by physical, chemical, and climate factors
- 11 - New metabarcoding workflow adapted to estuarine foraminiferal diversity

## 12 **Abstract**

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

21 We conducted an environmental DNA survey of estuarine mudflats along the French Atlantic coast,  
22 using a metabarcoding approach targeting foraminifera. Our results demonstrate that estuarine  
23 environments have only a few active OTUs dominating the community composition and a large stock

24 of dormant or propagule stages. This last genetic diversity components constitute an important  
25 reservoir, with different species which can potentially develop in response to the temporal variability  
26 of the multiple stressors. In fact, different OTUs were dominant in the studied estuaries. Our statistical  
27 model shows that the physical and chemical characteristics of the sediment and the climatic conditions  
28 explain only 43% of the community composition variance. This suggests that other, less easily  
29 quantifiable factors, such the history and use of the estuaries or the ecological drift could play an  
30 important role as well. Environmental DNA biomonitoring opens new perspectives to better  
31 characterize the genetic diversity in estuaries.

32 **Keywords:** Foraminifera, environmental DNA, propagules, mudflat, high-throughput sequencing

### 33 **1. Introduction**

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

46 In view of the ecological and economical value of coastal areas, the European Commission adopted  
47 the Water Framework Directive (WFD, 2000/60/EC) in October 2000. This directive commits the  
48 Member States of the European Union to achieve a good quality status of all water bodies, including

49 transitional and coastal waters. To achieve the objectives of the WFD, it is crucial to develop new  
50 approaches and methods to monitor environmental quality. Over the last 50 years many indicators of  
51 environmental quality have been developed (Diaz et al., 2004). Some of these indicators are based on  
52 chemical or physical parameters (Tueros et al., 2008), but these indices concern often only a single  
53 source of pollution or stress. To evaluate the impact of all stressors together, indicators based on  
54 organisms have been elaborated. Among them we can cite indices based on fishes (Lepage et al., 2016),  
55 macrofauna (van Loon et al., 2015), phytoplankton (Revilla et al., 2009) or vegetation composition  
56 (Juanes et al., 2008). In this context, the phylum of Foraminifera (Rhizaria, Eukaryotes) represents  
57 excellent model organisms to evaluate environmental quality. First, they have a short life cycle of some  
58 months up to a year (Murray, 1983). Next, different species are sensitive to organic enrichment  
59 (Jorissen, 1987; Parent et al., 2021), heavy metal pollution (Alve, 1991), or ocean acidification (Haynert  
60 et al., 2014; Keul et al., 2013; Kuroyanagi et al., 2021). Finally, the production of propagules and resting  
61 stages is a well-known adaptive mechanism to temporary adverse conditions (Geisen et al., 2018).  
62 Foraminifera have the ability to form such inactive stages (i.e., dormant adults or propagules), allowing  
63 them to survive prolonged periods of adverse conditions and rapidly respond when the environment  
64 becomes more favorable (Alve & Goldstein, 2002). For example, propagules of sensitive species (e.g.,  
65 porcelaneous taxa) appear to be able to withstand short periods of low pH conditions and to “bloom”  
66 once the conditions are close to their optimum (Weinmann et al., 2021).

67 Over the last decades, many studies have put forward foraminiferal biotic indices for open marine  
68 environments, especially for the shelf area. On the one hand, several indices based on morphological  
69 observations were developed, using different metrics such as alpha diversity (Bouchet et al., 2012) or  
70 indicator species concepts (Barras et al., 2014; Jorissen et al., 2018). On the other hand, more recently,  
71 studies based on environmental DNA (eDNA) have provided indices based on assignments to a  
72 reference database (Cavaliere et al., 2021) or on taxonomy free and machine learning methods  
73 (Cordier et al., 2017). Nevertheless, these indices have their limits in estuarine environments. There,  
74 high amplitude spatial and temporal variability of natural environmental parameters (salinity,

75 temperature, moisture, organic matter, oxygen contents, etc.) as well as superimposed anthropogenic  
76 stressors impact the community composition. Consequently, the alpha diversity found in estuaries is  
77 low in comparison with fully marine environments (Elliott & McLusky, 2002; McLusky & Elliott, 2004).  
78 Even in predominantly natural settings, species living in estuaries are highly adapted to extreme and  
79 highly variable environmental conditions requiring opportunistic life strategies. This strongly biases the  
80 results of environmental quality indices created in fully marine habitats, systematically leading to  
81 negative scores in estuaries (M. P. A. Fouet et al., 2022). This apparent contradiction (low  
82 environmental quality values in apparently natural ecosystems) is known as the “estuarine quality  
83 paradox” (Dauvin, 2007; Elliott & Quintino, 2007). Consequently, assessing the environmental quality  
84 of estuarine ecosystems remains a major challenge (Elliott & Quintino, 2007; Tweedley et al., 2015).

85 Here, we present a survey of foraminiferal genetic diversity (diversity obtained through eDNA  
86 approaches) in estuarine mudflats based on high throughput sequencing (Illumina, MiSeq). To assess  
87 the patterns and drivers of genetic diversity, we used a metabarcoding approach using specific  
88 foraminiferal primers, targeting the V9 region of the 18S rRNA gene. Our dataset consists of 25 sites  
89 on various mudflats in six estuaries of the French Atlantic coast. At each site we measured the major  
90 environmental parameters (sediment grain size, trace metals and organic matter) and retrieved  
91 climatic data (temperature and precipitation). With our HTS eDNA strategy, we address several  
92 fundamental questions, which are essential in a context of environmental biomonitoring: (1) What are  
93 the advantage and issues of eDNA approaches to capture foraminifera genetic diversity in comparison  
94 with the species diversity obtained through morphological approaches? (2) What are the patterns of  
95 genetic diversity along the French Atlantic coast estuaries? And, finally, does eDNA genetic diversity  
96 show the same response to the combination of natural and anthropogenic environmental gradients as  
97 traditional inventories?

98

99

## 100 **2. Materials and Methods**

### 101 *2.1 Studied estuaries, sampling sites and environmental parameters*

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

### 112 *2.2 Morphological dataset*

113 Morphological dataset was based on the methods and results described in detail in Fouet, 2022.  
114 Briefly, sediment samples were collected using a 96mm corer, and the top centimeter of sediment was  
115 preserved in 96% ethanol with Rose Bengal staining (2g/L). The samples were then washed on 125µm  
116 sieves. While the >125 µm fraction does not capture the entire foraminiferal community, it is a time-  
117 efficient compromise for biomonitoring studies (Schönfeld et al., 2012). Foraminifera specimens were  
118 observed under a Leica MZ16 stereomicroscope, and living individuals were identified by the presence  
119 of bright pink Rose Bengal staining in most chambers (Table B.1).

120 The physical properties of each site were further characterized using the Marine Influence Index  
121 (Jorissen et al., 2022). The index includes the normalized measurements of the distance to the sea, the  
122 emergence time at low tide and the river discharge (Table A.1). The detailed protocol and the rationale  
123 behind this index is explained in Jorissen et al. (2022). Climatic data were obtained by using the  
124 coordinates of each site to extract the value of the 19 variables extrapolated from monthly

125 measurements of temperature and precipitation (Table A.1). For this purpose, we used the finest 30  
126 arc second resolution grids of the WorldClim database (Fick & Hijmans, 2017).

127 Sediment grain size, trace metal concentrations and organic matter content were assessed to obtain  
128 sediment properties. Protocols for these methods are detailed in Fouet *et al.*, (2022). In brief, the  
129 sediment grain size was analyzed (non-decarbonated) with a *Mastersizer 3000 laser diffraction particle*  
130 *size analyser* (Malvern Pananalytical Ltd, Malvern, UK). In this study three different fractions are  
131 considered: clay (<4 $\mu$ m), silt (4-63 $\mu$ m) and sand (>63 $\mu$ m) (Table A.1). Three samples (LAI\_1A, ELO\_1A  
132 and CRA\_1A) are considered as outliers because of their high sand percentage (> 80%). These sites  
133 cannot be defined as mudflat environments and will not be included in the ecological models. Trace  
134 metal concentrations were analyzed with an Inductively Coupled Plasma Mass Spectrometer (Thermo  
135 Scientific® X-Series 2 ICP-MS) on freeze-dried sediment, after total acid digestion (HCl, HNO<sub>3</sub> and HF)  
136 (Coynel *et al.*, 2016). Seventeen chemical elements (V, Cr, Co, Ni, Cu, Zn, As, Sr, Mo, Ag, Cd, Sn, Sb, Ba,  
137 Pb, Th and U) were measured. Enrichment Factors (EF) were calculated by comparison with an  
138 unpolluted reference sample (M. P. A. Fouet *et al.*, 2022; Larrose *et al.*, 2010) (Table A.1). Elemental  
139 carbon (%Corg) and nitrogen (%N) contents were measured on decarbonated freeze-dried sediment  
140 with a CHONS Elemental Analyser (EA Vario PYRO cube; Elementar®, Langenselbold, Germany) (Table  
141 A.1).

### 142 *2.3 DNA extraction, amplification, sequencing*

143 Total DNA was extracted from the sediment with the FastDNA Spin Kit for Soil (MP Biomedicals)  
144 according to the manufacturer's instructions. The choice of this specific DNA extraction kit was  
145 motivated by the high quantity of raw material to be analyzed (5g). As the density of Foraminifera on  
146 mudflats can be highly variable (Fouet *et al.* 2022), this should guarantee a good coverage of the  
147 genetic diversity. In our case, as the morphology-based species diversity was low (M. P. A. Fouet *et al.*,  
148 2022), we expected to have deleterious effects of highly homogeneous nucleotide composition. This  
149 limits the number of high-quality reads generated per Illumina run. In order to reduce these effects

150 and increase the overall quality of our sequencing, we included 0 to 4 nucleotides between the tags  
151 and the primers to increase the heterogeneity (Jensen et al., 2019). To reduce the tag jumping effect,  
152 we used a dual-indexing approach (Taberlet et al., 2018). Primers, tag sequences and library  
153 information can be found in Additional file A.1, and Table A.1.

154 PCR was carried out with AccuPrime™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific).  
155 The 37f hypervariable region (Pawlowski et al., 2014) (amplicon size: 135-190 bp) was amplified using  
156 the specific foraminiferal primers s14F1 (Pawlowski, 2000) and s15r (Lejzerowicz et al., 2014). In  
157 metabarcoding studies, PCR replication is important to obtain a correct value of genetic diversity  
158 (Shirazi et al., 2021). Therefore, for each DNA extraction, three PCR replicates were done and pooled  
159 (for cost efficiency) before the quantification and sequencing. The PCR conditions consist of an initial  
160 denaturation of 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing  
161 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  
162 pooled replicates were quantified using the QuBit HS dsDNA (Invitrogen). Each sample was then pooled  
163 with the same amount of DNA and purified using the NucleoSpin™ Gel and PCR Clean-up XS kit  
164 (Macherey-Nagel). Library preparation and Illumina MiSeq (paired-end, 2x250bp) sequencing were  
165 performed at ID-Gene Ecodiagnostics (Geneva, Switzerland). Sequences are available on European  
166 Nucleotide Archive via project number PRJEB55114.

#### 167 *2.4 Bioinformatics and taxonomic assignment*

168 Tags and primers were removed from the reads using cutadapt v. 3.4 (Martin, 2011). Clustering of the  
169 reads was done using the R package DADA2 (v. 1.16; (Callahan et al., 2016)). Raw reads were quality  
170 controlled by truncating (forward and reverse length of 120 bp) and filtering them to a maximum  
171 number of 'expected errors' of two. Amplicon sequence variants (ASV) were dereplicated if identical,  
172 clustered and pair-end reads merged using a minimum overlap of 12 bp and maximum mismatch of 0  
173 bp. Chimeras were removed using the 'pooled' method (Callahan et al., 2016, p. 2). The ASVs were first  
174 automatically assigned taxonomically using VSEARCH v. 2.18.0 (Rognes et al., 2016) and our



175 foraminifera reference database based on the sequences present in the NCBI database. This first  
176 assignation allowed removing ASVs that do not belong to foraminifera (percentage of identity below  
177 70%). Then, the remaining ASVs were compared with the GenBank database using BLAST and assigned  
178 to species when the percentage of identity was superior to 99%. If the percentage of identity was  
179 below this threshold the ASVs were assigned as “environmental clades”. Based on this information a  
180 second clustering where ASVs assigned to the same species, or the same environmental clade were  
181 done. Therefore, the ASVs produced by the first clustering (DADA2, algorithm based on model error  
182 rate) will be renamed into operational taxonomic units (OTUs, algorithm based on the database  
183 similarity) after the second clustering. Finally, a table with these new OTUs including only the species  
184 and environmental clades related to foraminifera was built for further analyses.

## 185 2.5 Data analysis

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

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

207 Then we performed a partial redundancy analysis (RDA) on Hellinger transformed data (Legendre &  
208 Gallagher, 2001). This transformation will standardize the dataset and help to minimize effects of vastly  
209 different total abundances per sample (Legendre & Gallagher, 2001). The significance of the variables  
210 and first and second ordination axes was assessed using a one-way analysis of variance (ANOVA; 1000  
211 permutations, p value threshold = 0.05) (Chambers & Hastie, 1991). Finally, in order to evaluate the  
212 percentage of variance in foraminiferal communities explained by sediment characteristics and  
213 climatic variable, we performed variation partitioning (*varpart* (Peres-Neto et al., 2006)).

### 214 **3. Results**

215 A total of 6,224,249 raw reads were obtained after the bioinformatic clustering steps. After removing  
216 non foraminiferal, low quality and chimeric reads, a final dataset of 4,133,414 reads and a total of 162  
217 ASVs remained (Table C.1). Half of the reads (53.1%) and about a quarter of the ASVs (22.6%) were  
218 assigned with 100% confidence to the reference database (Fig. A.1). The overlap of ASVs between two  
219 replicates was moderate (63%), while the overlap of reads between two replicates was high (90%) (Fig  
220 A.3). This indicate that a substantial part of ASVs were only present in one replicate (Fig. A.2) but that  
221 the dominant ASVs (those that have a high number of reads) are present in both replicates (Fig A.2, Fig  
222 A.3). To reduce this sampling bias, further statistical analyses were therefore carried out on combined  
223 replicates. The rarefaction curves (Fig. A.4) demonstrated that the sequencing depth per site was  
224 sufficient, and the ASVs accumulation curve (Fig. A.5) showed that the whole study adequately

225 presented the total foraminiferal genetic diversity. The final table count based on the 162 ASVs consists  
226 of 99 OTUs (18 OTUs related to monothalamids, 14 OTUs related to Globothalamea, 2 OTUs related to  
227 Tubothalamea and 65 unassigned OTUs considered as environmental clades) (Fig. 2, Table D.1).

228 The proportion of the 65 environmental clades was uneven between the samples and estuaries with a  
229 particularly high number in Auray estuary. The proportion of Rotaliida (Globothalamea) was low,  
230 except for some samples where high numbers of reads of *Ammonia* and *Haynesina* were observed  
231 (Auray ST02\_A, Crac'h STO4\_A, STO5\_A and Odet STO4\_A). Most of the monothalamids were affiliated  
232 to the Saccaminidae, which were dominant in most of the assemblages. A notable exception was the  
233 Laïta estuary where unclassified monothalamids and *Vellaria pellucida* were dominant.

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

245 The NMDS analysis based on the dominant OTUs (Fig. 4) showed that each estuary occupies a specific  
246 part of the ordination space. Both the Auray and Crac'h and the Elorn and Aulne estuaries plot in the  
247 same part of the ordination space. Conversely, the Odet and Laïta estuaries did not overlap with other  
248 estuaries. Although most of the OTUs were in the center of the ordination space, some had a stronger  
249 influence on the ordination of the samples and estuaries. This was for instance the case for env\_18,

250 *Elphidium margaritaceum*, *Haynesina depressula* (Auray and Crac'h estuaries) and two monothalamids  
251 (GenBank accession numbers KP984731 and EU213249).

252 About the chemical composition of the sediment, most samples showed low enrichment factors,  
253 suggesting predominantly natural conditions, except in the Aulne estuary where a strong enrichment  
254 of Pb (>5) was observed (Table A.1). Percentage of organic carbon and nitrogen content are in the  
255 range of values usually found in mudflat environments (Dubois et al., 2012). The RDA analysis (Fig. 5a)  
256 showed that foraminiferal communities are significantly influenced by nine environmental variables  
257 (clay, Corg, distance to the sea, river discharge, Pb, Sn, U, PDM, and isothermality,  $p < 0.05$ ). The first  
258 two axes explained 43.3% of the variance. The variance partitioning showed that the sediment and  
259 physical properties explained 15.9% of the variance, whereas the climatic data explained 8.2% (Fig.  
260 5b). When both were combined, the percentage reached 17.1%. The residuals of this analysis  
261 correspond to 58.7%.

## 262 **4. Discussion**

### 263 *4.1 Sampling, methods, and dataset consistency*

264 This is the first published attempt to study foraminiferal eDNA sampled on estuarine mudflats with  
265 specific markers, and consequently, our sampling strategy was not yet optimized for this purpose. In  
266 order to assess every component of the genetic diversity (active as well as inactive stages) we chose  
267 to focus on the DNA-based HTS and not on RNA-based HTS (Qiao et al., 2022). The downside of this  
268 approach is that some dead cells with preserved DNA inside the shell may also be sequenced.  
269 Seasonality and temporality are also important factors to consider in ecological studies, also for  
270 Foraminifera in estuarine environments (Debenay et al., 2006; Murray, 1983). Therefore, to reduce  
271 potential impacts induced by these factors, we decided to focus on a single season (fall 2020).  
272 Although on average one replicate represents only 63% of the total genetic diversity at the sampling  
273 site (Fig. A.2), the rarefaction and amplification curves clearly reach the "plateau" (Fig. A.4 Fig. A.5).  
274 This indicates that the sequencing depth is sufficient to cover the total genetic diversity of a single

275 replicate. Moreover, in terms of abundance, the shared reads between the replicates account for 90%  
276 (Fig.A.3). This confirms that the dominant ASVs are detected consistently in both replicates. However,  
277 some of the rarer taxa may also be missed in two replicates so that genetic diversity obtained through  
278 an eDNA approach should be described with caution. Several explanations can be provided to explain  
279 the fairly large differences in the ASV's observed in the two replicates: 1) The DNA extraction kit does  
280 not allow perfect DNA extraction of the cells (i.e., bead beating steps could be unable to break properly  
281 organisms provided with calcareous tests), 2) The quantity of sediment used as input may not cover  
282 the total genetic diversity of the site and finally 3) the sampled area (for one replicate) is potentially  
283 too small to have a complete picture of the genetic diversity of the site. A recent study on metazoans  
284 showed a similar pattern with a potential bias toward the largest species (Klunder et al., 2022). To draw  
285 sound ecological conclusions, it is crucial to cover the total genetic or/and species diversity.  
286 Consequently, more methodological work is needed to define the most efficient strategy (Lara et al.,  
287 2022; Zinger et al., 2019). To reduce sampling artifacts in our study we decided to pool the replicates  
288 and consider them as single samples. The total genetic diversity of the environment was approached  
289 with this strategy.

290 To obtain a robust dataset that allows answering ecological hypotheses, different known biases  
291 (contamination, tag jumping, reads quality, assignation...) must be addressed (Pawlowski et al., 2014).  
292 In this study, we chose to adopt the most stringent possible approach. To reduce the "tag jumping"  
293 effect we used a unique double tag combination and limited the number of samples per library to 35  
294 (Esling et al., 2015). We also improved the overall quality of our sequencing by including 1-4  
295 nucleotides as spacers to increase the heterogeneity of the libraries (Fadrosh et al., 2014). Next, we  
296 checked the assignation of each ASV with the reference database to avoid potential bioinformatic  
297 misidentification. To remove genetic diversity biases due to multiple variants that exist for some  
298 foraminiferal species (Weber & Pawlowski, 2014), we decided to pool the ASVs belonging to the same  
299 species or environmental clades to a single OTU. And finally, for our ecological model (Figs. 4 and 5),  
300 we decided to consider only OTUs accounting for >1% of the total number of reads. By considering

301 only OTUs present in fair numbers in many samples, we expected to reduce the noise in our ecological  
302 model caused by the rare biosphere (Schiaffino et al., 2016). All these steps tend to increase the  
303 robustness of our dataset and strengthen our interpretation and conclusions.

#### 304 *4.2 Estuarine foraminiferal eDNA diversity*

305 We were able to assign most of our ASVs to species level (Table C.1), demonstrating the advantage to  
306 use specific markers for specific clade investigation. As expected, due to the extreme environmental  
307 conditions in estuaries, the genetic diversity of foraminifera is low compared to open ocean habitats  
308 (Lecroq et al., 2011). The number of foraminiferal species observed at the investigated sites based on  
309 the morphological observation of the 125µm fraction of the sediment (only species with a mineralized  
310 or agglutinated shell were observed) ranges from two to 19 (M. P. A. Fouet et al., 2022). In our study  
311 we observed 99 OTUs, about five times more that observed in morphological studies. The high  
312 proportion of monothalamids found in the eDNA dataset can be explained by a combination of factors.  
313 For example, the shorter barcode size in some monothalamids (i.e., saccaminids), compared to  
314 Globothalamea or Tubothalamea would promote their amplification and sequencing. Another  
315 explanation could be that monothalamids are easier to extract with the DNA kit as they have an organic  
316 test (Santos et al., 2017). Primer or sequencing biases in favor of this group could also be possible.  
317 Finally, a naturally higher abundance of monothalamids compared to mineral shelled foraminifera  
318 (Globothalamea and Tubothalamea) could also be hypothesized (Laroche et al., 2018; Lecroq et al.,  
319 2011; Lejzerowicz et al., 2021). The much lower abundance or total absence of monothalamids in  
320 morphological studies is explained by the fact that organic shelled foraminifera (dominant in  
321 monothalamids) are usually ignored in this methodology. To overcome some of these limitations, e.g.  
322 the primer/sequencing biases, the use of other markers such as the COI (Macher et al., 2022) can be  
323 envisaged. However, in that case, other biases can be expected and the reference database for these  
324 other barcodes is still far too poor to be efficient for an ecological study like this one.

325 Even if the number of environmental clades found in our study is high (representing 65,6% of the total  
326 number of OTUs), they represent a marginal fraction of the total number of unassigned reads (30,9%,  
327 Fig. A.1). Moreover, two dominants environmental OTUs (ENV\_01 and ENV\_02) represent respectively  
328 61,2% and 14,2% of the unassigned reads. Further investigations are needed to discover which species  
329 are hidden behind these environmental clades. Nevertheless, our study demonstrates overall that the  
330 effort of single cell barcoding is sufficient to reliably assign the majority of ASVs on estuarine mudflats.  
331 Consequently, the reference database for these ecosystems can be considered as accurate.  
332 Monothalamids remain poorly documented in comparison to their multichambered relatives.  
333 Nevertheless, this group profits from a renewed interest, with new descriptions of species belonging  
334 to this polyphyletic clade published recently (Gooday et al., 2022; Holzmann et al., 2022). An additional  
335 effort should be made on intertidal mudflats to describe monothalamids morphologically and  
336 genetically. Thereafter, the intra-specific genetic variance will be better known, and it is highly  
337 probable that several species will be represented by more than one ASV, like is the case for many of  
338 the well-studied species. This will lead to a decrease of alpha diversity.

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

346 Generally, only a few OTUs dominate each sample, which is in line with the conclusion of morphology-  
347 based studies (Fouet et al., 2022). Environmental DNA based studies have the particularity to also  
348 detect propagules and dormant stages. Additionally, they present a semi-quantitative evaluation of  
349 the community composition. In our dataset, the distribution of dominant species that represent more

350 than one percent of the total of reads is very different between the samples, much more than in the  
351 morphological inventories (Fouet et al., 2022). Together with the low alpha diversity of the morphology  
352 based analyses of the same sites (i.e., 2-19 (M. P. A. Fouet et al., 2022)), this suggests that only a limited  
353 number of OTUs is able to occupy the estuarine mudflat environments with adult specimens, and that  
354 a large part of the eDNA comes from dormant stages (and eventually some dead forams). In fact, due  
355 to the strong hydrodynamics caused by tidal and fluvial currents and waves, dormant stages will be  
356 massively introduced in estuaries. Many of these introduced taxa will not be able to colonize mudflats  
357 due to the environmental conditions which are unfavorable to them.

#### 358 *4.3 Environmental parameters*

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

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

370 Two physical parameters, namely the distance to the sea and river discharge, significantly impact the  
371 first axis of the RDA (Fig. 5). Both variables are linked to the salinity of the estuary (Jorissen et al., 2022),  
372 which is considered a critical factor in driving species diversity for fishes (Whitfield, 2015),  
373 phytoplankton (Nche-Fambo et al., 2015), and foraminifera (M. P. A. Fouet et al., 2022). Additionally,  
374 two climate-related variables, Isothermality and Precipitation of the Driest Month (PDM), are also



375 significant in the model. Isothermality quantifies the extent of temperature differences between day  
376 and night compared to seasonal oscillations (O'Donnell & Ignizio, 2012). Estuarine mudflats experience  
377 substantial temperature gradients on both a daily (tidal effect) and seasonal (spring and neap tides)  
378 scale, resulting in low isothermality. PDM represents extreme conditions with minimal freshwater  
379 input into the system. Both PDM and river discharge reflect the influence of freshwater input,  
380 underlining its importance in the model. Finally, the Pb enrichment factor potentially affects estuarine  
381 foraminiferal communities (Fig. 5, Table C.1). Previous studies have indicated that high Pb  
382 concentrations can significantly impact the abundance of dominant foraminiferal species (Brouillette  
383 Price et al., 2019).

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

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#### 400 *4.4 Putative impact of anthropogenic stressors*

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

#### 407 *4.5 Advantage and limitations of eDNA approaches*

408 One major difference between HTS and classical morphological studies is that HTS does not only take  
409 into account adult stages, but also monothalamids, propagules and dormant stages (and potentially  
410 also some dead individuals), thereby potentially strongly increasing genetic diversity. As HTS data are  
411 semi-quantitative, we can assume that due to the intrinsic mudflat proprieties, the dominant OTUs  
412 (corresponding to >1% of the total of reads) in our study correspond to the adult and the most active  
413 stages of foraminifera. Nevertheless, the distribution of the reads of these dominant OTUs is very  
414 different between the studied estuaries. It appears that the dominant species are present in all  
415 estuaries, but they do not fully develop everywhere; they have a high density of reads only at some of  
416 the sites. Environmental DNA approaches are therefore a powerful tool, which does not only assess  
417 the living and dominant species but also species that are potentially present and await more favorable  
418 conditions to develop. However, a limitation of the eDNA approach is the impossibility to distinguish  
419 the different categories (adults, juveniles, propagules, dormant stages). A dual approach, combining  
420 morphological as well as eDNA studies, to assess the various stages, seems therefore the ideal strategy  
421 to obtain a more complete overview of the foraminiferal communities. In the future, new molecular  
422 methods such as single cells transcriptomics (Sierra et al., 2022) or long-read metabarcoding (Jamy et  
423 al., 2020) can be envisaged. Nevertheless, more research and development are requested to assess  
424 ecological questions with such new approaches.

## 425 **5. Conclusion**

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

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### 730 **Data Availability Statement**

731 Raw sequences (fastq files) are available on European Nucleotide Archive (ENA) via the project number  
732 PRJEB55114. The data generated or analyzed during this study are included in this published article:  
733 the ASV table counts, ASV taxonomy and sample metadata are provided as additional files.

### 734 **Benefits Generated**

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

### 737 **Authors Contributions**

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

742 **Tables**743 **Table 1)** General information about the estuaries, localities and coordinates of the stations.

<b>Samples</b>	<b>Estuary</b>	<b>Locality</b>	<b>Nb of stations</b>	<b>Nb of samples</b>	<b>Sampling date</b>	<b>Latitude</b>	<b>Longitude</b>
AUR_1	Auray	Locmariaquer	3	6	16.09.2020	47.5701	-2.9422
AUR_2	Auray	Kerouarch	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 Trinite 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	Keradenec	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 a 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 SA 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

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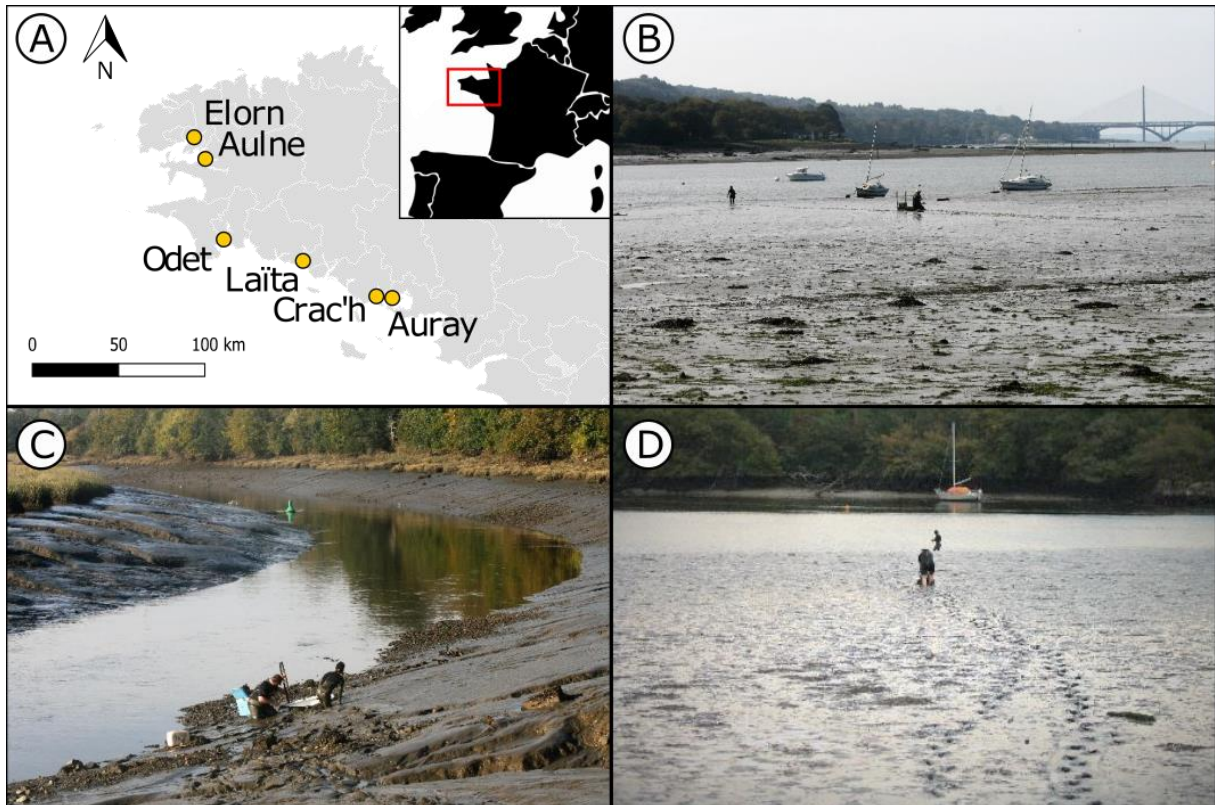
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752 **Fig. 1)** A) Map of the six estuaries (Elorn, Aulne, Odet, Laïta, Crac'h and Auray), B) Picture of Elorn  
753 ST03\_A and ST03\_B C) Picture of Elorn ST04\_A D) Picture of Laïta ST03\_A.

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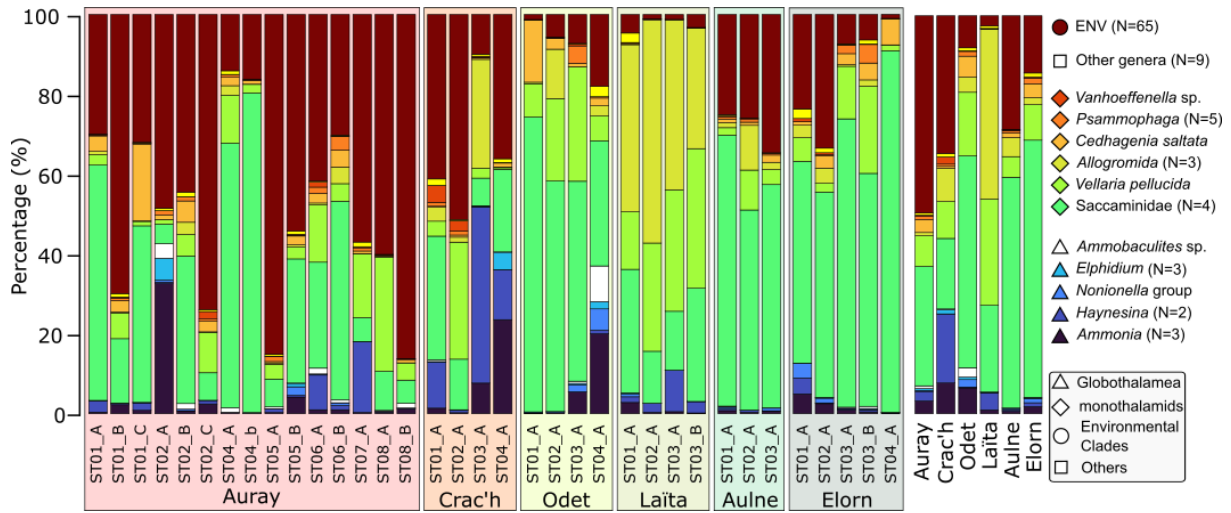
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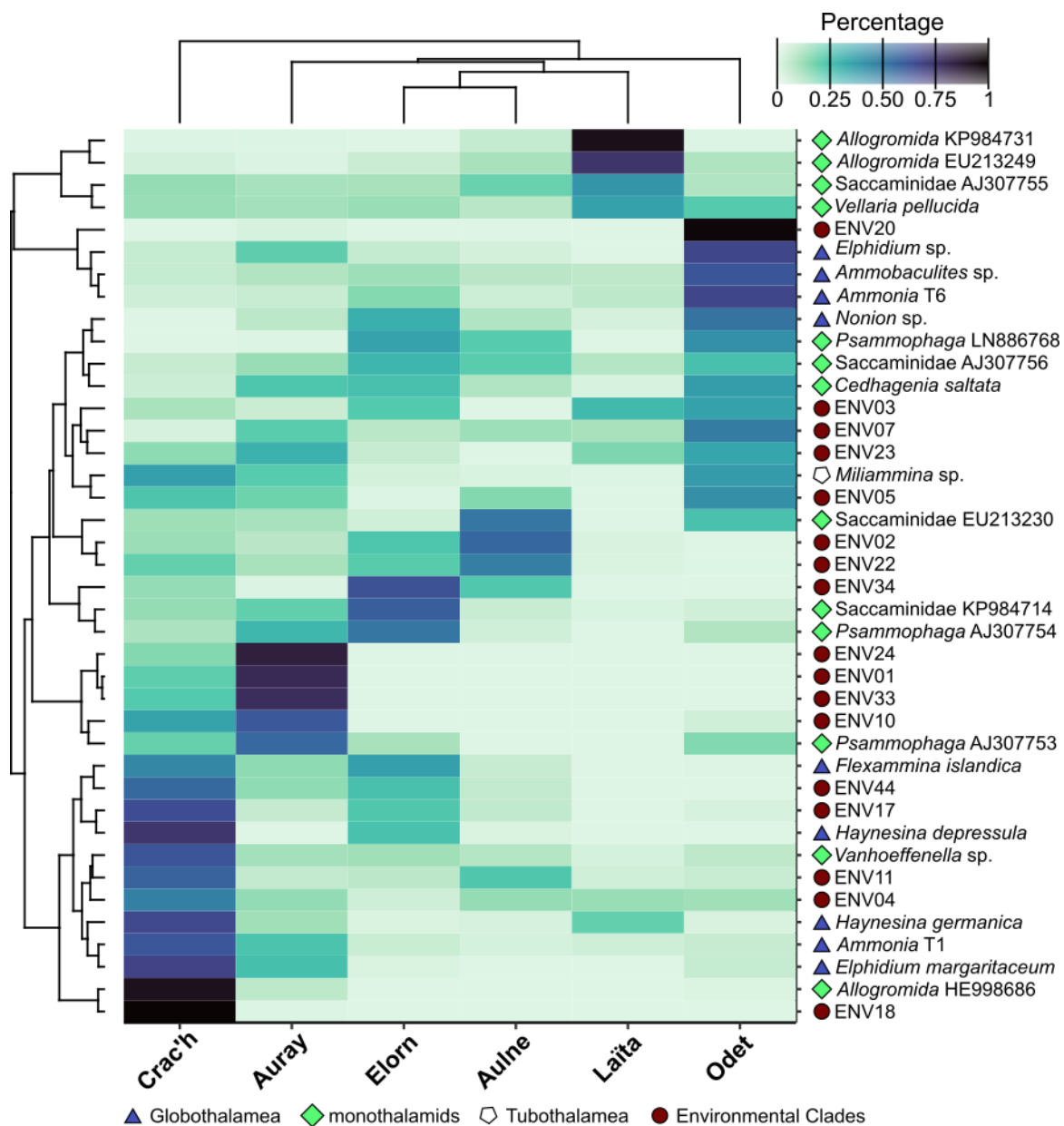
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763 **Fig. 2)** Distribution of the foraminifera taxa living in 6 French coast Estuaries. Taxa were obtained using  
 764 an eDNA metabarcoding approach targeting specifically foraminifera. Sequencing was performed  
 765 using a MiSeq platform. The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

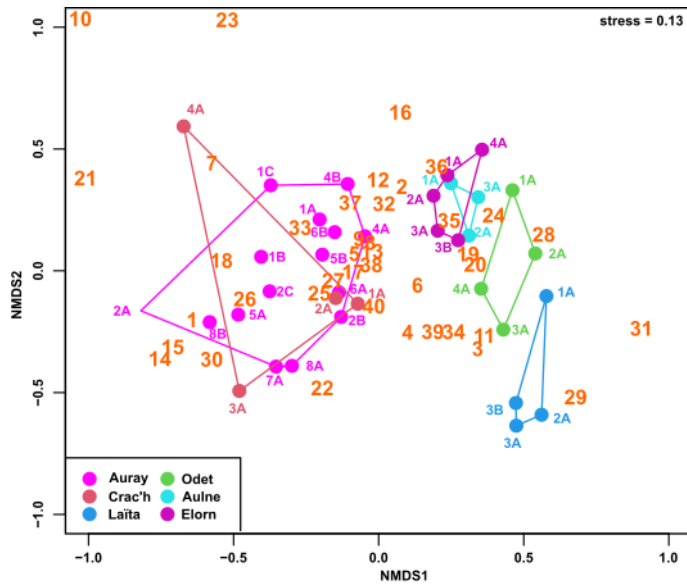


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769 **Fig. 3)** Heatmap depicting the dominant foraminiferal genetic diversity and relative abundance in  
 770 French coast estuaries. The taxa were obtained with an eDNA metabarcoding approach using specific  
 771 primers for Foraminifera. Sequencing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

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- 1 ENV 1
  - 2 ENV 2
  - 3 ENV 3
  - 4 ENV 4
  - 5 ENV 5
  - 6 ENV 7
  - 7 ENV 10
  - 8 ENV 11
  - 9 ENV 17
  - 10 ENV 18
  - 11 ENV 20
  - 12 ENV 22
  - 13 ENV 23
  - 14 ENV 24
  - 15 ENV 33
  - 16 ENV 34
  - 17 ENV 44
  - ▲ 18 *Ammonia* T1
  - ▲ 19 *Ammonia* T6
  - ▲ 20 *Elphidium* sp.
  - ▲ 21 *Elphidium margaritaceum*
  - ▲ 22 *Haynesina germanica*
  - ▲ 23 *Haynesina depressula*
  - ▲ 24 *Nonion* sp.
  - ▲ 25 *Ammobaculites* sp.
  - ◆ 26 *Psammophaga* AJ307753
  - ◆ 27 *Psammophaga* AJ307754
  - ◆ 28 *Psammophaga* LN886768
  - ◆ 29 Allogromida EU213249
  - ◆ 30 Allogromida HE998686
  - ◆ 31 Allogromiid KP984731
  - ◆ 32 *Cedhagenia saltata*
  - ◆ 33 *Flexammina islandica*
  - ◆ 34 Saccaminidae AJ307755
  - ◆ 35 Saccaminidae AJ307756
  - ◆ 36 Saccaminidae EU213230
  - ◆ 37 Saccaminidae KP984714
  - ◆ 38 *Vanhoeffenella* sp.
  - ◆ 39 *Vellaria pellucida*
  - ◇ 40 *Miliammina* sp.
- ▲ Globothalamea   ◆ monothalamids   ◇ Tubothalamea   ● Environmental Clades

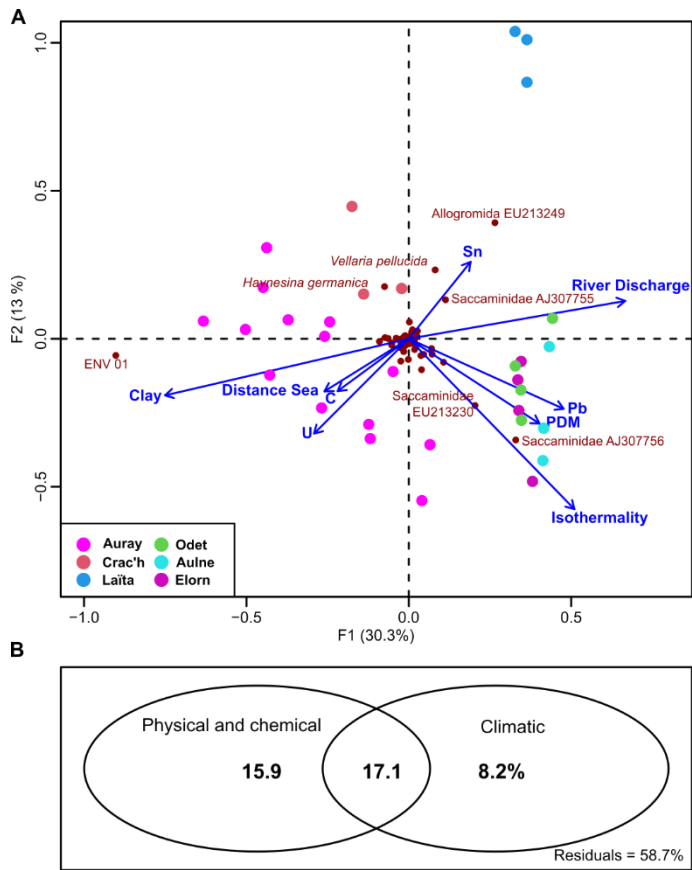
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777 **Fig. 4)** Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of 35 samples  
 778 from Auray, Odet, Crac'h, Aulne, Laita and Elorn estuary. Stress value = 0,13. The NMDS is constructed  
 779 with the dominant taxa that represent more than 1% of the total number of reads.

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784 **Fig. 5)** A) Redundancy analysis (RDA) of dominant foraminifera taxa extracted from sediment sample  
 785 collected from six estuaries from the French Atlantic coast. Significant environmental variables ( $P <$   
 786  $0.05$ ) are represented by arrows. B) The variance partitioning results for community composition  
 787 among the components of the physical and chemical parameters and the climatic data. Residual values  
 788 are also displayed.

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797 **Caption for supporting information**

798 **Fig A.1)** Barplots representing the similarity of the reads (A) and the ASVs (B) to the taxonomic  
799 reference database. Table (C) represents the percentage of the reads and ASVs that are assigned  
800 according to different thresholds (99.99, 99. 95. 90 and 80 percent).

801 **Fig A.2)** Barplot representing the numbers of common ASVs (grey) and unique ASVs in replicates A  
802 (grey green) and B (turquoise blue) of each sample. The core ASVs present in both replicates A and B  
803 are narrow and most of the genetic diversity is present in only one replicate. On average one replicate  
804 represents 63 percent of the genetic diversity.

805 **Fig A.3)** Barplot representing the numbers of common ASVs (grey) and unique ASVs in replicates A  
806 (grey green) and B (turquoise blue) of each sample. The core ASVs present in both replicates A and B  
807 are narrow and most of the genetic diversity is present in only one replicate. On average one replicate  
808 represents 63 percent of the genetic diversity.

809 **Fig A.4)** Rarefaction curves for each replicate according to estuary. Reads were obtained using Illumina  
810 MiSeq platform. ASVs were based on the specific primers (s14F1 and s15r) targeting foraminifera.

811 **Fig A.5)** Species accumulation curve of samples issue from environmental DNA metabarcoding study  
812 targeting Foraminifera. The study pertains to six French coast estuaries.

813 **Fig A.6)** UpSetR plot of the dominant taxa (representing more than >1% of the dataset) found in an  
814 environmental DNA metabarcoding study targeting Foraminifera. The study pertains to six French  
815 coast estuaries. The intersections represent the numbers of taxa that are common in the estuaries.  
816 Most taxa (16) were found in all estuaries and no dominant taxa were present in only one estuary.

817 **Additional file A.1)** Primers Forward and Primers reverse (Fasta file)

818 **Table A.1)** Environmental variables

819 **Table B.1)** Morphological dataset

820 **Table C.1)** ASVs assignation

821 **Table D.1)** Count table



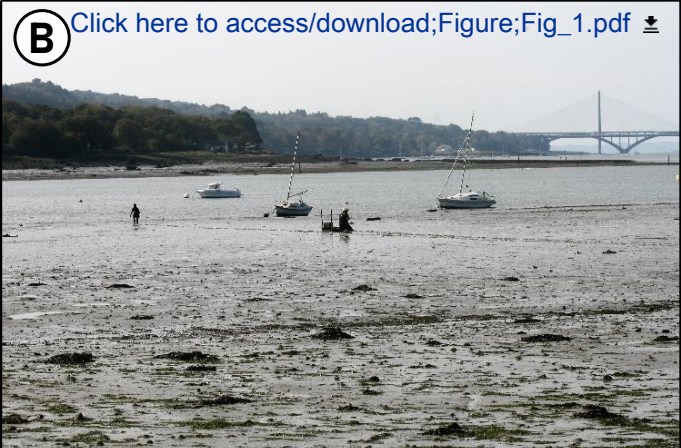
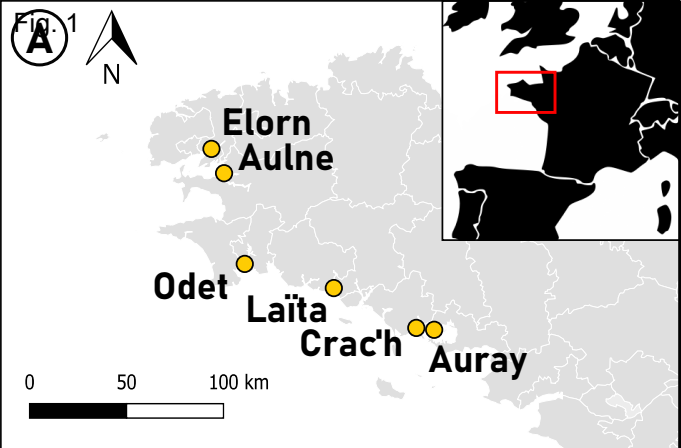
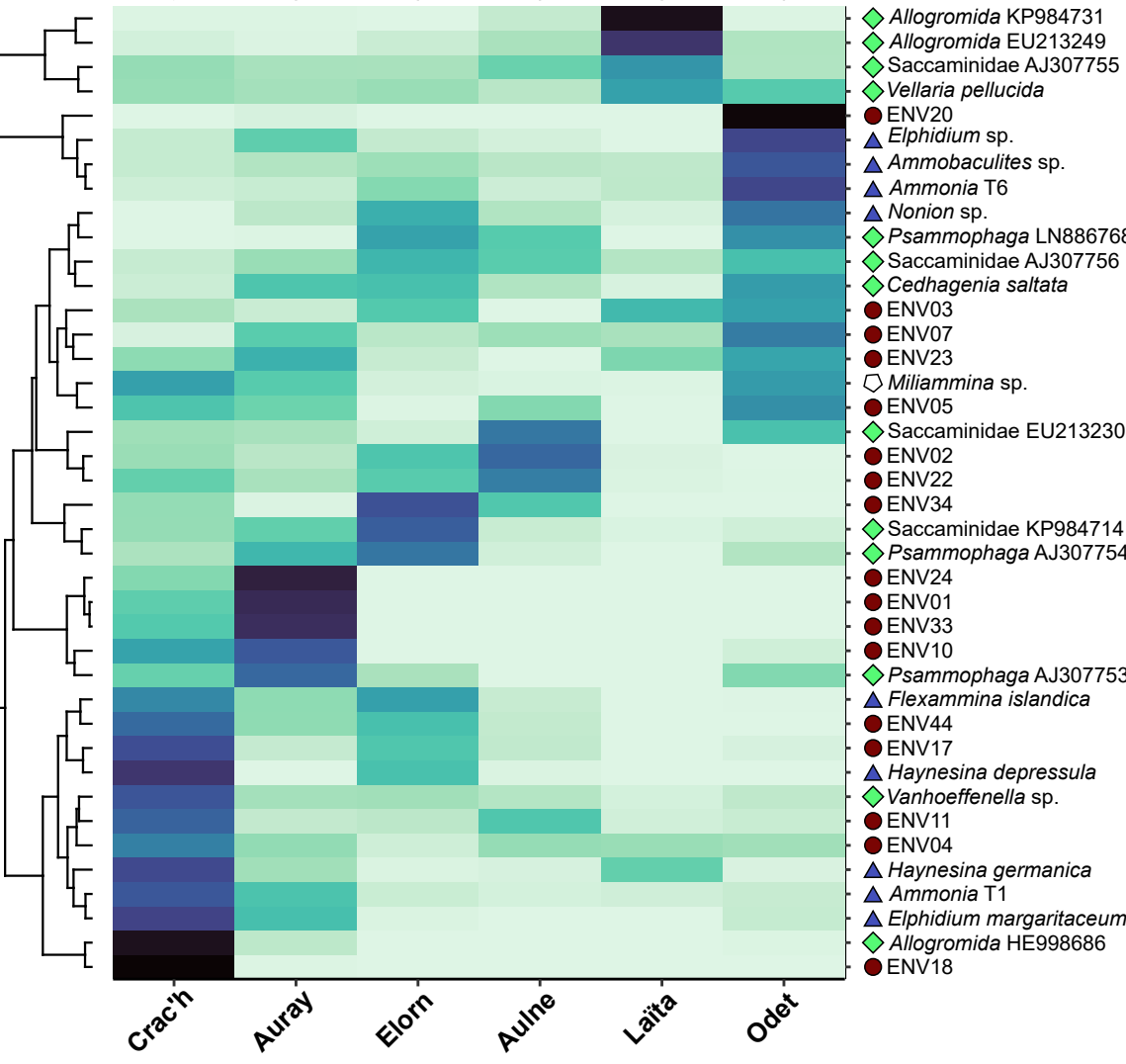
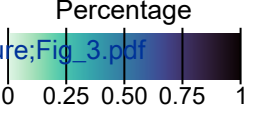




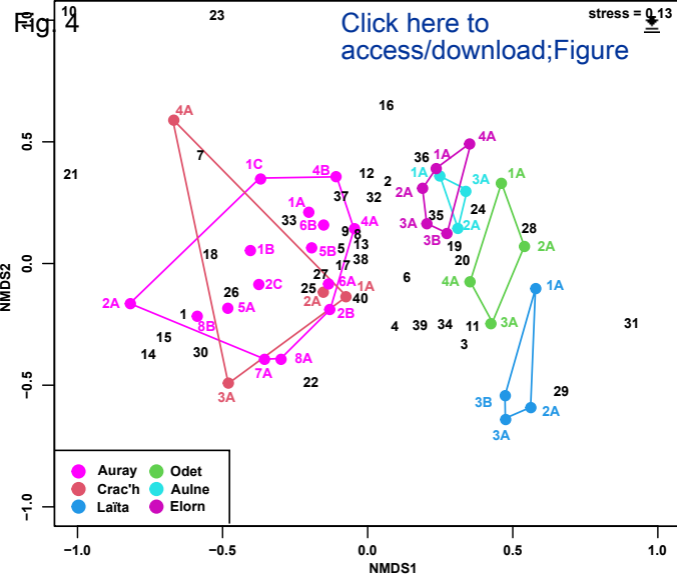
Fig. 3

Click here to access/download:Figure, Fig\_3.pdf



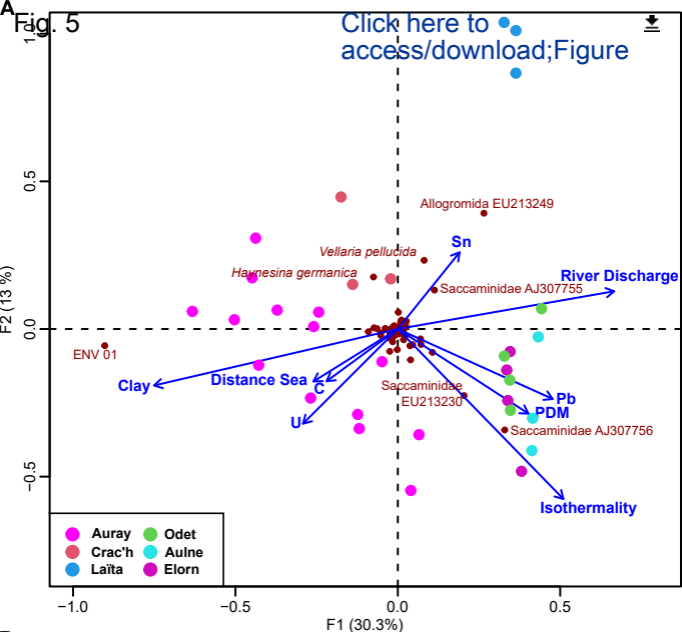
▲ Globothalamea    ◆ monothalamids    ○ Tubothalamea    ● Environmental Clades

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- |             |                                     |                                  |
|-------------|-------------------------------------|----------------------------------|
| ● 1 ENV 1   | ● 15 ENV 33                         | ◆ 29 Allogromida EU213249        |
| ● 2 ENV 2   | ● 16 ENV 34                         | ◆ 30 Allogromida HE998686        |
| ● 3 ENV 3   | ● 17 ENV 44                         | ◆ 31 Allogromiid KP984731        |
| ● 4 ENV 4   | ▲ 18 <i>Ammonia</i> T1              | ◆ 32 <i>Cedhagenia saltata</i>   |
| ● 5 ENV 5   | ▲ 19 <i>Ammonia</i> T6              | ◆ 33 <i>Flexammina islandica</i> |
| ● 6 ENV 7   | ▲ 20 <i>Elphidium</i> sp.           | ◆ 34 Saccaminidae AJ307755       |
| ● 7 ENV 10  | ▲ 21 <i>Elphidium margaritaceum</i> | ◆ 35 Saccaminidae AJ307756       |
| ● 8 ENV 11  | ▲ 22 <i>Haynesina germanica</i>     | ◆ 36 Saccaminidae EU213230       |
| ● 9 ENV 17  | ▲ 23 <i>Haynesina depressula</i>    | ◆ 37 Saccaminidae KP984714       |
| ● 10 ENV 18 | ▲ 24 <i>Nonion</i> sp.              | ◆ 38 <i>Vanhoeffenella</i> sp.   |
| ● 11 ENV 20 | ▲ 25 <i>Ammobaculites</i> sp.       | ◆ 39 <i>Vellaria pellucida</i>   |
| ● 12 ENV 22 | ◆ 26 Psammophaga AJ307753           | ◻ 40 <i>Miliammina</i> sp.       |
| ● 13 ENV 23 | ◆ 27 Psammophaga AJ307754           |                                  |
| ● 14 ENV 24 | ◆ 28 Psammophaga LN886768           |                                  |

▲ Globothalamea    ◆ monothalamids    ◻ Tubothalamea    ● Environmental Clades



**B**

