Environmental RNA outperforms eDNA metabarcoding in assessing impact of marine pollution: a chromium-spiked mesocosm test

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Abstract

Environmental (e)DNA metabarcoding holds great promise for biomonitoring and ecotoxicological applications. However, few studies have compared the performance of eDNA versus eRNA metabarcoding in assessing organismal response to marine pollution, in experimental conditions.

Here, we performed a chromium (Cr)-spiked mesocosm experimental test on benthic foraminiferal community to investigate the effects on species diversity by analysing both eDNA and eRNA metabarcoding data across different Cr concentrations in the sediment.

Foraminiferal diversity in the eRNA data showed a significant negative correlation with the Cr concentration in the sediment, while a positive response was observed in the eDNA data. The foraminiferal OTUs exhibited a higher turnover rate in eRNA than in the eDNA-derived community. Furthermore, in the eRNA samples, OTUs abundance was significantly affected by the Cr gradient in the sediment (Pseudo-R^2= 0.28, p = 0.05), while no significant trend was observed in the eDNA samples. The correlation between Cr concentration and foraminiferal diversity in eRNA datasets was stronger when the less abundant OTUs (<100 reads) were removed and the analyses were conducted exclusively on OTUs shared between eRNA and eDNA datasets. This indicates the importance of metabarcoding data filtering to capture ecological impacts, in addition to using the putatively active organisms in the eRNA dataset. The comparative analyses on foraminiferal diversity revealed that eRNA-based metabarcoding can better assess the response to heavy metal exposure in presence of subtle concentrations of the pollutant. Furthermore, our results suggest that to unlock the full potential for ecosystem assessment, eDNA and eRNA should be studied in parallel to control for potential sequence artifacts in routine ecosystem surveys.

Key-words: ecotoxicology, eDNA, eRNA, foraminifera, mesocosm, metabarcoding,

1. Introduction

Benthic foraminifera, single-celled eukaryotes, have been applied as proxies for paleoceanographic and paleoenvironmental reconstructions (Jorissen, Fontanier, and Thomas 2007). Their application has also been extended to biomonitoring in marine and transitional environments (Bouchet et al. 2018). Despite the recent advances in foraminiferal biomonitoring, the application of standard morphological technique is time-consuming, neglects important communities (i.e., monothalamids), and requires skilled specialists that make it impractical for large-scale biomonitoring surveys (Keeley et al. 2018; Pawlowski et al. 2014a, b, 2016). The development of high-throughput sequencing (HTS) technologies has deeply revolutionized the way of assessing biodiversity and biomonitoring (Guardiola et al. 2016; Cordier et al. 2020; Valentini et al. 2016; Cavaliere et al., 2021). In this context, metabarcoding of environmental DNA or RNA using specific gene region(s) amplified from sediment represents a complementary, possibly alternative, reliable, time- and cost-effective methodology to apply in routine biomonitoring surveys (Lejzerowicz et al. 2015; Pawlowski et al. 2014b). The eDNA comprises intra- and extra-organismal DNA from living and dead organisms, while eRNA is generally considered to originate mainly from metabolically active organisms (Cristescu 2019; Wood et al. 2020). The preservation of eDNA is controlled by a set of environmental factors (i.e., organic matter content, oxygen availability, temperature, sedimentation rate) (Corinaldesi et al. 2018) and important fractions of extracellular DNA might escape degradation processes and be preserved in sediments (Dell'Anno and Danovaro 2005). The lower stability of eRNA has been related to its single-stranded and the presence of hydroxyl groups that enhanced the abiotic chemical breakdown (Li and Breaker 1999; Marshall et al. 2021). The higher-preservation capability of eDNA might therefore reduce the β-diversity (i.e., diversity across sites) and overestimate the α-diversity (i.e., diversity at a local site) by detecting unviable organisms. Indeed, Cristescu (2019) underlines the problematic high rates of false positives and negatives in the eDNA analyses (Veilleux et al. 2021). Diversity surveys based on eDNA would therefore be more biased, while analyses using eRNA better mirror metabolic active or dormant but viable organisms (Pochon et al. 2017) and reflect the real

community response to environmental conditions (Pawlowski et al. 2014; Adamo et al. 2020). Nevertheless, working with RNA rather than DNA is more complicated as well as economically (higher costs) and technically challenging (Laroche et al. 2017; Zaiko et al. 2018).

Foraminiferal metabarcoding has been used for monitoring the impact of fish farms, and oil and gas drilling activities in the field (Pawlowski et al. 2014b; 2016; Pochon et al. 2015; Frontalini et al. 2020; Cordier et al. 2019) and in laboratory experiments (Frontalini et al. 2018a). Some of these studies have simultaneously considered eDNA and eRNA (Pawlowski et al. 2014b; Pochon et al. 2015; Laroche et al. 2018; Keeley et al. 2019) and documented a higher sensitivity of eRNA over eDNA for detecting environmental changes (Pawlowski et al. 2014b; Pochon et al. 2015). In contrast, evidence from other environmental impact studies have shown a better response of eDNA, but only in terms of β-diversity, when data were trimmed by shared OTUs with eRNA (Laroche et al. 2017, 2018). This supports eDNA as a better molecular proxy of community turnover (β-diversity), and eRNA for species diversity (α-diversity) (Laroche et al. 2017). As suggested by Wood et al. (2020), the identification of a molecule (i.e., eRNA) characterized by a fast turnover rate could represent an essential proxy in monitoring populations (i.e., endangered or invasive species) but also for biomonitoring. Indeed, the application of eRNA in environmental biomonitoring might constrain the observed changes to a relatively higher spatial and temporal resolution (Veilleux et al. 2021; Yates et al. 2021).

Laboratory experiments (e.g., microcosm or mesocosm) have been proven as an effective and direct method to assess the effect of a single parameter (i.e., pollutant at different concentrations and exposure time-length) on biota (Frontalini et al. 2018a,b; Chariton et al. 2014), but they might also enable to test the accuracy of eDNA and eRNA outcomes. In an innovative experimental study, Wood et al. (2020) revealed that DNA persisted in water longer (up to 94 h) than RNA (up to 13 h), after the organism removal, but both eDNA and eRNA were detected in biofilms after 21 days. To our knowledge, no laboratory experiment has been used to assess the effectiveness of eDNA and eRNA metabarcoding as indicators of heavy metal pollution in the sediments.

Here, we evaluate the sensitivity of benthic foraminifera in a spiked-sediment toxicity test with subtle variations of chromium (Cr), a metal known to become toxic at high concentrations (Stankovic, Kalaba, and Stankovic 2014). To this end, we use a metabarcoding approach to parallelly assess the foraminiferal community response extracting both eDNA and eRNA from the sediment. We use the resulting molecular datasets to (i) compare the α-diversities response to Cr, (ii) investigate variation in β-diversities, and (iii) identify foraminiferal molecular operational taxonomic units (OTUs) with high indicator potential.

2. Materials and Methods

2.1 Sediment sampling

Sediment samples were collected from a coastal site off Mt. Conero (central Adriatic Sea) in early autumn 2014. This site was chosen as it falls in a natural area and is considered in two previous studies (Frontalini et al. 2018a,b). At the collection site, temperature, pH, Salinity, Eh and dissolved oxygen of seawater were measured using a multiparametric probe. Sediment was sampled by Van Veen grab and only the uppermost part of the sediment (2 cm) was retained. Once on board, the collected sediment was immediately homogenized and sieved over a 500 μm mesh with natural seawater (NSW). The resulting <500 μm sediment-fraction was placed in an insulated box covered by NSW.

2.2 Experimental set-up and subsampling

Artificial Sea Water (ASW) was prepared following the methods of Ciacci et al. (2012), stored in the dark, aerated and mixed under *in-situ* temperature. Three Cr-ASW solutions with different metal concentrations (1 ppb, 100 ppb and 10 ppm that correspond to 1 μg L^{-1}, 100 μg L^{-1} and 10 mg L^{-1}, respectively) and control (no Cr added) were considered. Chromium (III) nitrate ($Cr(NO_3)_3$, CAS Number 13548-38-4, Sigma-Aldrich) 98% pure was used for preparing stock solutions. The details of the methodology have been reported in (Maccotta et al. 2016).

Mesocosms (15 cm x 8 cm x 3 cm) containing 1 cm-thick sediment (i.e., <500 μm) were placed

inside 20-L-tank (60 cm x 40 cm x 20 cm) that reflects different Cr concentrations plus control. Sediment (2 mg) from each mesocosm was sampled after 1, 2, 4, 8 and 12 weeks for metabarcoding analyses, using disposable spoons (SteriPlast, Burkle). During each sampling event, two aliquots of sediment were collected, one for eRNA and the other for eDNA analyses. Samples were preserved in 5 mL of LifeGuard Soil Preservation Solution (Qiagen), frozen at −20 °C and sent to the Department of Genetics & Evolution, University of Geneva (Switzerland). Additionally, one sediment aliquot (ca. 40 cm^3) was used for geochemical analysis at the Department of Earth and Marine Sciences (DISTEM), Palermo University (Italy). Details of geochemical characterizations and analyses are reported in Maccotta et al. (2016). The Cr concentration in the sediment at which foraminifera were exposed ranged from 33 to 43 ppb (i.e., 33 to 43 mg kg^{-1}).

2.3 eDNA and eRNA extraction, amplification and sequencing

The total RNA and DNA content of sediment samples were extracted using the PowerSoil™ Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBio, USA) in RNase- free conditions and following the manufacturer instructions. Extracted RNA was reverse transcribed as described in Langlet et al. (2015) using the SuperScript® III reverse transcriptase (Life Technologies). Amplification protocols and sequencing steps were described in (Frontalini et al. 2018a). Briefly, DNA and cDNA samples were amplified in triplicates using foraminiferal-specific forward primer s14F1 (5' - AAGGGCACCACAAGAACGC - 3') and reverse primer s17 (5' – CGGTCACGTTCGTTGC - 3') (Pawlowski et al. 2002). An additional, nested PCR step was performed using tagged versions of the same primers to label the PCR products of each of our samples to a combination of tag sequences. Every tag consists of a unique sequence of 8 nucleotides appended to the 5'-end of the specific amplification primer sequence. Tagged-primers combinations were selected following an optimized multiplexing design (Esling et al. 2015). Reactions were performed in a total volume of 25 µl including 1 Unit of Taq DNA polymerase (Roche), 2.5 µl of 10× PCR Reaction Buffer (Roche), 0.2 mM of each dNTP, 0.2 µM of each primer, and 1 µl of DNA / 5 µl of

153 cDNA extracts. The conditions for the first amplification consisted of a pre-denaturation step at 95°C
154 for melting the complex genomic DNA mixture, followed by 20 cycles of denaturation at 94°C for
155 30 s, annealing at 50°C for 30 s and extension at 72°C for 1:30 min, followed by a final extension
156 step at 72°C for 5 min. From the first PCR products, 10 ng were used for the nested PCR. The nested
157 PCR conditions consisted of pre-denaturation step at 95°C for 1 min, followed by 14 cycles of
158 denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 2 min, followed
159 by a final extension step at 72°C for 5 min. The PCR products were then purified using High Pure
160 PCR Cleanup Micro Kit (Roche) and then quantified using the fluorometric quantitation method
161 based on the Qubit HS dsDNA Kit (Invitrogen). Successively, purified samples were pooled in equal
162 amounts and the total volume was concentrated using a Speed Vac (30 min at medium temperature).
163 The sequencing library was prepared using the reagents of the PCR-free TruSeq kit (Illumina)
164 following manufacturer instructions.
165
166 *2.4. Sequence data pre-processing and taxonomic assignment*
167 The obtained raw reads for the DNA and RNA samples were analysed in parallel using a publicly
168 available pipeline written in C language (https://github.com/esling/illumina-pipeline). Briefly, raw
169 reads containing at least one ambiguous base ("N"), quality below 30, and more than 5 mismatches
170 in an overlap region of at least 50 positions between the paired reads were filtered out. The
171 demultiplexing step was performed based on the combination of tags associated with the forward and
172 reverse primers. The clustering and assignment of the sequencing were preceded by a pre-clustering
173 step based on the 30 positions of the 5′-end of the 37f hypervariable region based on which
174 foraminiferal species can be determined (Pawlowski and Lecroq 2010). This step is followed by a
175 complete linkage clustering based on the pairwise Needleman-Wunsch distances computed for the
176 complete 37f sequences of each pre-cluster, using a floating taxon-specific threshold for each pre-
177 cluster, as extensively described in Lejzerowicz et al. (2014) and Pawlowski et al. (2014b). The
178 resulting sequences were then clustered into OTUs and for each OTU, the sequence represented by

179 the highest number of reads was assigned a taxonomy based on a manually-curated database
180 comprising 1069 non-redundant foraminiferal species sequences, as described in Pawlowski et al.
181 (2014b). Chimeric OTUs originating from the artificial recombination of different sequences during
182 the PCR steps were detected using Uchime v.4.232 based both on comparisons of OTUs sequences
183 amongst them or against the reference database, and every detected candidate OTU was removed from
184 the dataset.

185 The resulting OTU tables were explored using six different read abundance thresholds in both
186 DNA and RNA datasets: Total, Filter 1, Filter 10, Filter 100, Filter 500 and Filter 1000. The Total
187 dataset considers all sequences in DNA and RNA samples also including those represented by only
188 one read (singletons) in the entire dataset. The Filter 1 dataset was obtained removing the singletons.
189 Analogously, in the datasets Filter 10, Filter 100, Filter 500, and Filter 1000, all sequences represented
190 in the entire dataset by less than 10, 100, 500, or 1000 reads were removed, respectively. As a result,
191 twelve datasets (six for the DNA samples and six for the RNA samples) were generated.

192

193 *2.5 Biostatistics*

194 Alpha diversity indexes were calculated for each sample of the twelve datasets using the *abdiv*
195 package (https://github.com/kylebittinger/abdiv) in R version 4.0.2 (R core Team, 2017). To test for
196 differences in diversity between the two molecular proxies (eDNA and eRNA), Wilcoxon tests were
197 performed on each dataset pair and results were visualised using the R package ggpubr
198 (https://github.com/kassambara/ggpubr).

199 The α diversity response of the benthic foraminiferal community to Cr concentrations in the
200 sediment was explored with Spearman correlation using the *rcorr* function from the Hmisc R package
201 (https://github.com/harrelfe/Hmisc/).

202 Next, we investigated the response of the benthic community captured by the eDNA and eRNA
203 in terms of β-diversity. To this end, we analysed the dataset pair at Filter 100 and in parallel, tested
204 the effect of analysing only taxa present in the eDNA and eRNA from the same environmental sample

205 (trimming by shared OTUs). The OTU turnover rate for each of the datasets obtained was assessed
206 by calculating Jaccard distance matrices based on OTU presence/absence with the *vegdist* function
207 from the R package vegan (Oksanen et al. 2018). Changes in the community structure of foraminiferal
208 OTUs in the eDNA and eRNA dataset pairs were then analysed performing a non-metric
209 multidimensional scaling (NMDS) using the *metaMDS* function from the R package vegan. The
210 obtained ordination was used to assess the response of foraminiferal OTUs to Cr gradient by
211 performing BIOENV analysis that allows to link multivariate community structure and environmental
212 variables by calculating a correlation coefficient that is then subjected to a permutation test to
213 determine its significance (Clarke and Ainsworth 1993). Based on the results of this analysis, we
214 selected the dataset that performed best (BIOENV significance level) and extracted the individual
215 sample scores on the NMDS axis parallel to the observed pollutant gradient. To identify potential
216 ecologically important OTUs, the sample scores were used to perform a differential abundant analyses
217 with the R package DESeq2 using unrarefied data and parametric estimation of the count-variance
218 relationship (Love, Huber, and Anders 2014). We used a threshold of $p < 0.05$ to select significantly
219 different taxa. To visualize differences in response to the pollutant gradient of different OTUs, raw
220 counts were normalized ($\log_2$ (x+1)) and heatmaps were created with the pheatmap R package (Kolde
221 2019).
222
223 **3. Results**
224 *3.1. Sequence data and taxonomic composition*
225 The final amount of reads and taxonomic composition of each of the obtained datasets are
226 summarized in Table 1. In total 567,209 high-quality reads have been obtained. They were clustered
227 into 2562 OTUs for eDNA and 2057 OTUs for eRNA. By applying different filters, the number of
228 OTUs was progressively decreasing to 40 and 43, for Filter 1000 of eDNA and eRNA, respectively
229 (Table 1). For further analyses, we selected Filter 100, for which the OTUs number was reduced to
230 141 and 156 for eDNA and eRNA datasets, respectively. At the same time, the number of reads was

231 reduced by about 5-7% suggesting that the majority of OTUs were represented by less than 100 reads.

232 A great percentage of the OTUs retrieved could not be assigned to any known reference sequence,
233 particularly in the eDNA datasets, where the observed proportion of unassigned reads was higher than
234 55% (Table 1). Among assigned OTUs, Monothalamea class dominated the community in both eDNA
235 and eRNA datasets accounting for more than 30% and 50% of the total assemblage, respectively
236 (Table 1). The most represented monothalamid OTUs in the eDNA datasets belonged to the clades
237 ENFOR6 (25-27%) and Clade Y (24-25%), while in the eRNA datasets OTUs from BM
238 (*Bathysiphon*/*Micrometula*) Clade were predominant (56-58%). Less than 5% of the reads in both
239 eDNA and eRNA datasets were assigned to the classes Globothalamea and Tubothalamea (Table 1).
240 Indeed, within these two classes, the composition varied greatly among datasets. OTUs belonging to
241 the family Buliminidae were dominant in the eDNA datasets (61-100%), whereas OTUs assigned to
242 order Textulariida made up to 93% of the Globothalamea reads in the eRNA datasets. The quasi
243 totality of the Tubothalamea reads (82-100%) were assigned to Hauerinidae, but none of
244 Tubothalamea OTU passed the abundance threshold in the eDNA Filter 100, 500 and 1000 datasets
245 (Table 1).

246

247 *3.2. Foraminiferal diversity and Cr concentration*

248 The observed response of foraminiferal diversity to Cr concentration was not univocal in eDNA
249 and eRNA datasets. Spearman correlation between Cr in the sediment and diversity measured in
250 eDNA datasets yielded positive but not significant relationships (Fig. 1). The opposite response was
251 observed in the eRNA datasets where benthic foraminiferal diversity decreased with increasing Cr
252 concentrations. However, the correlation in the eRNA datasets was significant for Filters 10 and 100,
253 only (Fig. 1).

254 To properly assess benthic foraminiferal response to Cr in terms of β diversity while controlling
255 for sequencing artefacts, we proceeded by comparing eDNA Filter 100 and eRNA Filter 100 datasets
256 (abundance trim). We furtherly controlled for PCR errors by deriving from the aforementioned

datasets two new datasets including solely the shared OTUs observed in both eDNA and eRNA samples (trim by shared OTUs). Of the 78 shared OTUs, more than half could not be taxonomically assigned (55%), 42% belonged to Monothalamids and 3% were assigned to Globothalamea (Rotaliida and Textularida) (Fig. 2). No Tubothalamea was observed in the shared eRNA/eDNA dataset (Table 1, Fig. 2).

We compared the foraminiferal communities across eDNA and eRNA datasets based on OTUs presence/absence. This comparison was based on Jaccard distances independently determined for eDNA and eRNA samples. The turnover in OTU composition across different Cr concentrations was more evident in eRNA than in eDNA samples as indicated by the distance range in the violin plots (Fig. 3). In particular, the eRNA Filter 100 dataset showed the highest turnover rate across samples.

We then proceeded by investigating the single OTUs response to the Cr concentration based on read abundance and visualized the results in a multivariate space (i.e., NMDS) for each of the datasets produced (Fig. 4). The BIOENV analyses performed on the obtained ordinations revealed that, in the eRNA shared dataset, Cr gradient correlated significantly with the obtained ordination (R^2=0.28, p = 0.05) indicating that assemblage shifts could, at least in part, be attributed to changes in the pollutant concentration. No significant correlation between Cr concentrations and OTUs abundance was instead observed in the ordination obtained on the other datasets.

3.3. Potential new bioindicators

To identify potential foraminiferal bioindicator species, the eRNA shared OTUs sample scores on the second dimension of the ordination in Figure 4 were extracted and used for a differential abundance analysis (Fig. 5).

Less than half of the retrieved OTUs (38%) could be identified as Monothalamids. The DESeq2 analyses pointed at potential indicators foraminiferal OTUs (Fig. 5). In particular, Monothalamids belonging to Clade Y (Allogromiid, ICEMON 7, OTU 5829), Clade O (Allogromiid, OTU 3166, OTU 6719, OTU 6740, OTU 6269), Clade BM (OTU 146) and unassigned monothalamid (OTU 79)

appeared to be negatively affected by increasing concentration of Cr in the sediment. The only OTU found as tolerant to the highest values of Cr in the sediment could not be taxonomically identified (OTU 5150). To verify that the identified OTUs were solely to respond to the concentration of Cr in the sediment and to rule out the presence of an 'ageing of the sediment' signal, we plotted the abundance change in the Control samples at each time of sampling (Fig. S3). The plot shows that the reads trend of the 21 OTUs in absence of a Cr gradient differs from the one presented in the heatmap in Figure 5b.

4. Discussion

4.1 eRNA better captures ecological information in metabarcoding surveys

The observed eDNA diversity was significantly higher for all the datasets generated (Fig. S1). Correlation analysis showed unilaterally that the increase of Cr concentration, though subtle, negatively impacted α-diversity measured on eRNA derived data (Fig. 1). In contrast, even if the eDNA datasets showed higher α-diversity (Fig. S1), no significant relationship with the Cr gradient was present (Fig. 1). The diversity inflation recorded from the DNA template indicates that DNA was more spread among samples and that the data reflected the presence of extra-organismal and extracellular molecular components in the sediment (Dell'Anno and Danovaro 2005; Corinaldesi et al. 2011). These findings agree with previous studies that showed how RNA-based diversity surveys tend to be less biased (Not et al. 2009) and to better reflect environmental changes (Pawlowski et al., 2014b; Pochon et al., 2015; Visco et al., 2015) as well as macrofauna-based biotic indices (Pawlowski et al. 2016) since they more accurately return only the active fraction of the biomass (Guardiola et al. 2016).

Across all the datasets investigated, our analyses show that eRNA samples were characterized by a higher OTUs turnover resulting in an increased difference in composition across samples, especially in the eRNA filter 100 dataset (Fig. 3). This indicates that the longer persistence of the DNA molecule in the sediment can possibly increase the rates of false positives due to the detection of non-viable

OTUs (Veilleux et al. 2021), resulting in a reduced inter-sample variability and artificially decreasing β-diversity estimates (Lejzerowicz et al. 2015). On the other hand, the OTU turnover in the eRNA samples suggests that the response to the pollutant is measured on individuals that are viable at the time of sampling, entailing that the RNA molecule is better suited for studying its short-term community response (Novitsky 1986; Stoeck et al. 2007). Indeed, eRNA has been suggested to potentially increase the level of spatial and temporal resolution provided by environmental nucleic acid assays (Yates, Derry, and Cristescu 2021). This is because, unlike DNA, the RNA molecule is considered to be less stable, breaking down rapidly after cell death, and is therefore expected to persist in the sediment for shorter periods of time (Eigner et al. 1961; Mengoni et al. 2005). This has been recently confirmed in an experiment where a comparison of the decay rates of the two molecules showed a significantly faster degradation of RNA compared to DNA across both mitochondrial and nuclear genes (Marshall, Vanderploeg, and Chaganti 2021). Importantly, other factors can influence the detection and persistence of the two molecules, such as the presence of microbial activity (Strickler et al. 2015), sediment conditions (Corinaldesi et al. 2011; Orsi et al. 2013) and even the seafloor topography (Lejzerowicz et al. 2021).

4.2 Data filtering increases metabarcoding surveys accuracy

To properly assess the differences in foraminiferal β-diversity responses registered in eDNA and eRNA samples and identify potential ecological relevant OTUs, we needed to control/reduce the effect of potential sequence artifacts. To this end, we based our analyses on the Filter 100 datasets (trimming by abundance threshold) and on the datasets including only the shared OTUs (composition filter). These methods have been shown to increase the accuracy of the assessment of anthropogenic impacts in metabarcoding-based studies (Laroche et al. 2017; Pawlowski et al. 2014b).

After trimming we found that 78 OTUs were present only in the eRNA dataset while 63 OTUs were only observed in the eDNA dataset (Fig. 2). The presence of additional DNA OTUs could be explained by the occurrence of rare species occurring sporadically in the mesocosms integrating the

335 history of the several communities across seasons present as extracellular DNA deposited in the
336 marine sediment initially sampled (Dell'Anno and Danovaro 2005; Corinaldesi et al. 2011). The
337 RNA-only OTUs observed in our dataset could instead represent rare taxa possibly stemming from
338 DNA under-sampling (given the higher heterogeneity/diversity of the DNA dataset, Fig. S1). Thus,
339 the complete absence of the Tubothalamea in the eDNA dataset (Filter 100) (Fig. 2) suggests that this
340 particular group is rare but highly active in the sediment and could not be detected in the eDNA dataset
341 because of its overall low abundance (Table 1) (Laroche et al. 2017). Another possibility is that OTUs
342 observed only in the eRNA dataset represent PCR artefacts originated from the reverse transcription
343 of RNA to cDNA (Egge et al. 2013; Ficetola et al. 2015). This process is based on the usage of a
344 reverse transcriptase enzyme that, lacking proofreading activity, can introduce point mutations in
345 some of the cDNA sequences (Houseley and Tollervey 2010). Furthermore, the reverse transcription
346 process can introduce errors like chimeric sequences and isoform sequences that cannot be easily
347 detected and filtered during bioinformatic processes (Laroche et al. 2017).
348 The application of both filtering strategies enabled purifying the foraminiferal response, at the
349 single OTU level, to the Cr concentration in the sediment with statistically significant results
350 observable only in the eRNA shared dataset (Fig. 4).
351
352 *4.3 Identification of new foraminiferal bioindicators*
353 From the differential abundance analysis, we could identify OTUs belonging to Clade Y (OTU
354 5829), Clade O (OTU 3166, OTU 6719, OTU 6740, OTU 6269, OTU 7378), Clade BM (OTU 146)
355 and one unassigned monothalamous species (OTU 79) as more sensitive to Cr increase than other
356 taxa.
357 The prevalence of ecologically informative Monothalamiids emerging from our analyses indicates
358 that taxa belonging to this group, largely ignored in morphological studies, could potentially be
359 adopted as new category of foraminiferal bioindicators in environmental surveys. Especially in
360 experimental settings involving mesocosms, these small and fast reproducing species would represent

a better model for ecotoxicological analyses than large, hard-shelled species.

Finally, the lack of reference sequences hampers the functional assessment of the foraminifera OTUs that could not be taxonomically identified in our experiment. However, the use of protists as bioindicators does not necessarily have to rely on taxonomic identification, instead, a taxonomy-free, sequence-centred approach has been recently invoked to maximize the potential of microbial communities in biomonitoring applications (Pawlowski et al. 2016; Cordier et al. 2020; Cavaliere et al., 2021).

Conclusions

Our analyses indicated that the variations of Cr concentration, even if subtle, negatively affected the α and β diversity measured in the eRNA samples, while no significant effect was observed in the eDNA samples. Foraminiferal taxa showed a higher turnover rate in the eRNA dataset with abundance fluctuations significantly correlated with the Cr concentration gradient when only shared OTUs were considered. Overall, our findings suggest that even if working with eRNA can present some technical and economic challenges, its usage should be implemented in environmental monitoring programs and possibly studied in parallel with eDNA to properly assess microbial diversity response to anthropogenic pollution when in presence of subtle variation of pollutant concentrations.

Data Availability

Raw reads will be deposited in the European Nucleotide Archive (ENA) upon acceptance. The data tables used for the analyses are available at https://figshare.com/s/02b34064c4cd237f6be7 (DOI: 10.6084/m9.figshare.16818421). The geochemical data were previously published in Maccotta et al. (2016).

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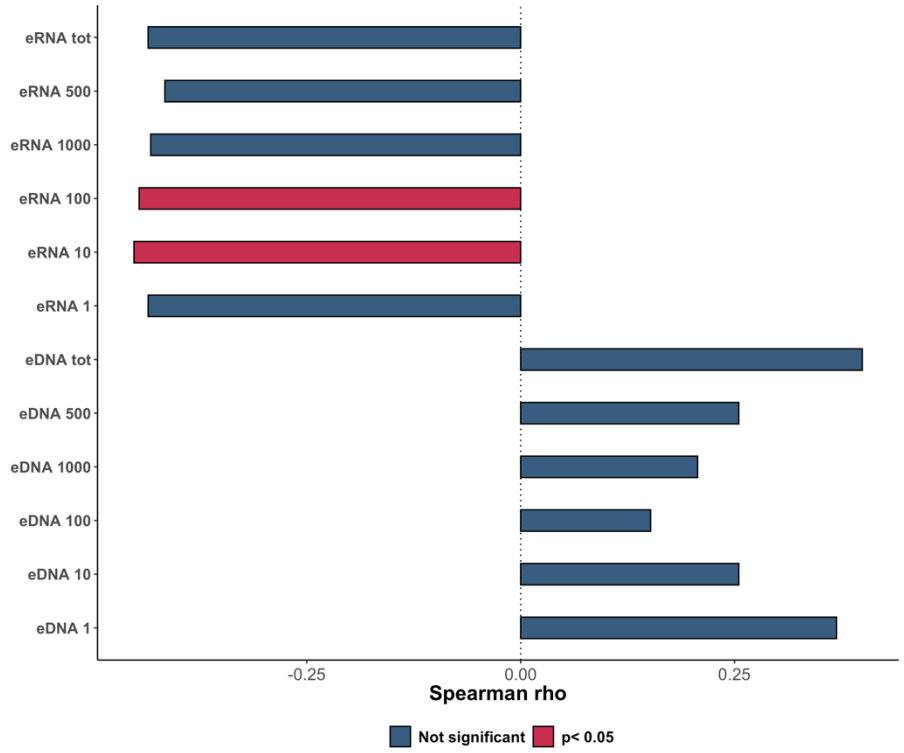
Dataset	*# Reads*	*# OTUs*	*Unassigned (%)*	*Monothalamea (%)*	*Globothalamea (%)*	*Tubothalamea (%)*
eDNA Total	300,751	2,562	60.24	38.49	1.25	0.02
eDNA Filter 1	299,782	1,593	60.12	38.61	1.25	0.02
eDNA Filter 10	296,024	702	59.70	39.05	1.24	0.01
eDNA Filter 100	280,298	141	58.06	41.02	0.92	0.00
eDNA Filter 500	261,977	61	57.53	42.00	0.47	0.00
eDNA Filter 1000	247,096	40	55.82	43.68	0.50	0.00
eRNA Total	266,458	2,057	41.37	53.52	4.49	0.62
eRNA Filter 1	265,852	1,451	41.25	53.64	4.50	0.62
eRNA Filter 10	262,515	718	40.56	54.30	4.51	0.63
eRNA Filter 100	245,533	156	37.45	57.66	4.30	0.59
eRNA Filter 500	226,969	65	34.38	60.92	4.28	0.42
eRNA Filter 1000	211,606	43	32.48	62.93	4.59	0.00

Table 1. Foraminiferal taxonomic composition of the Datasets analysed.



404 **Fig. 1** Correlation between Simpson diversity and Cr concentration in the sediment for each of the
405 datasets investigated.
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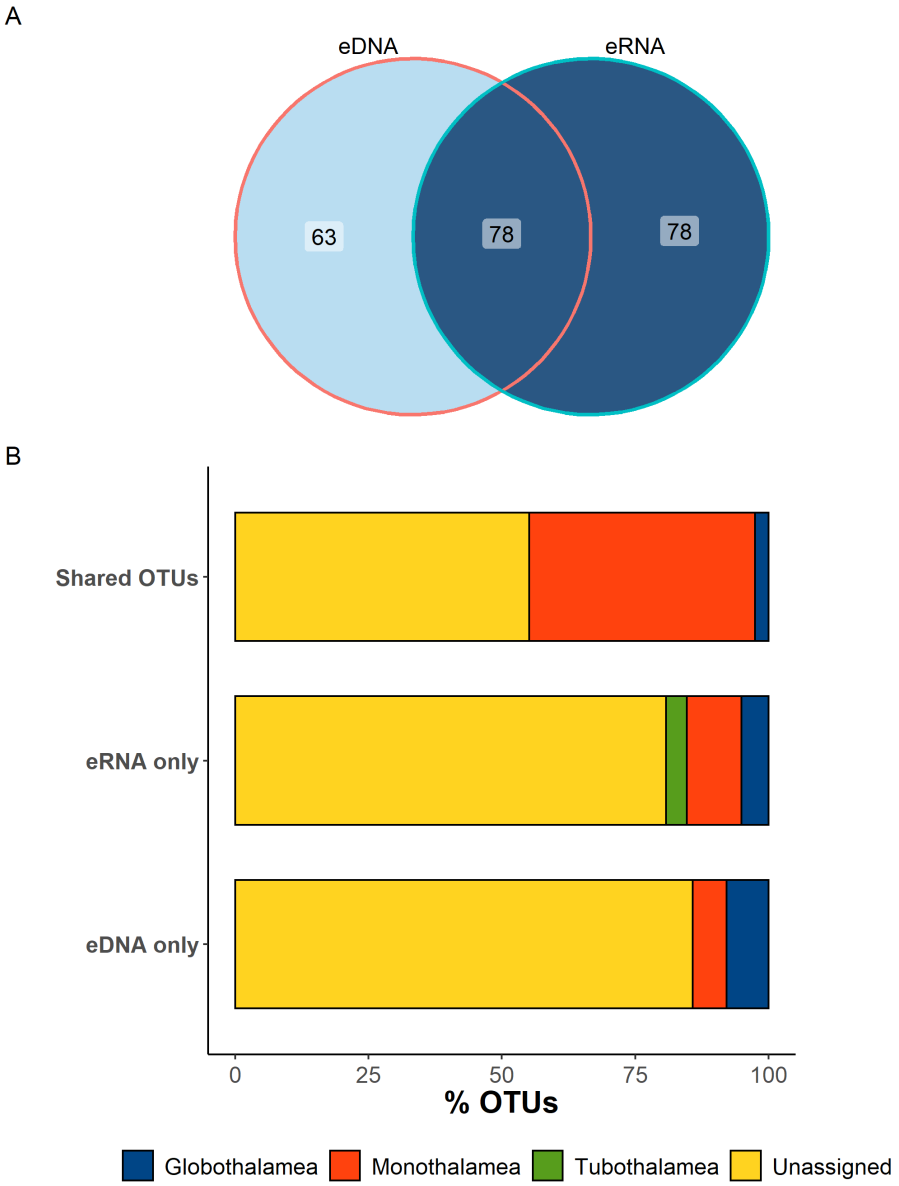
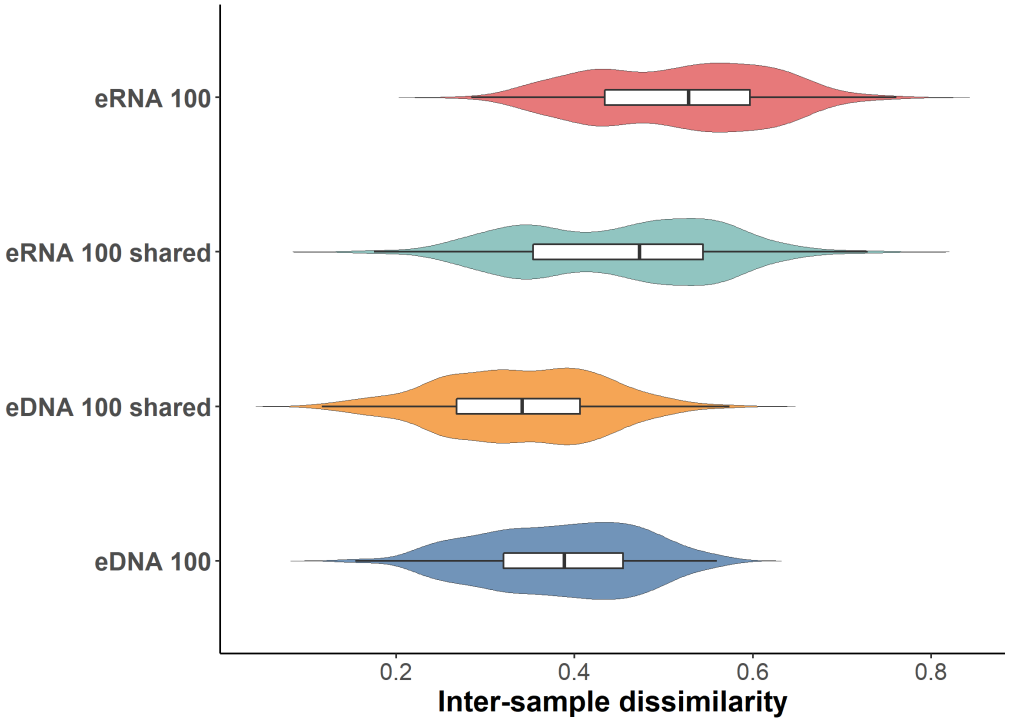


Fig. 2 a) Venn Diagram showing OTUs distribution between the eDNA and eRNA Filter 100 datasets and **b)** their corresponding taxonomic composition. Relative abundances were calculated on the total number of OTUs in each data subset (Shared OTUs, eRNA only and eDNA only).



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424 **Fig. 3** Boxplot showing foraminiferal OTUs turnover based on the Jaccard distance calculated on
425 presence-absence data for each subset. The violin plots show the sample-to-sample pairs distance
426 distribution.

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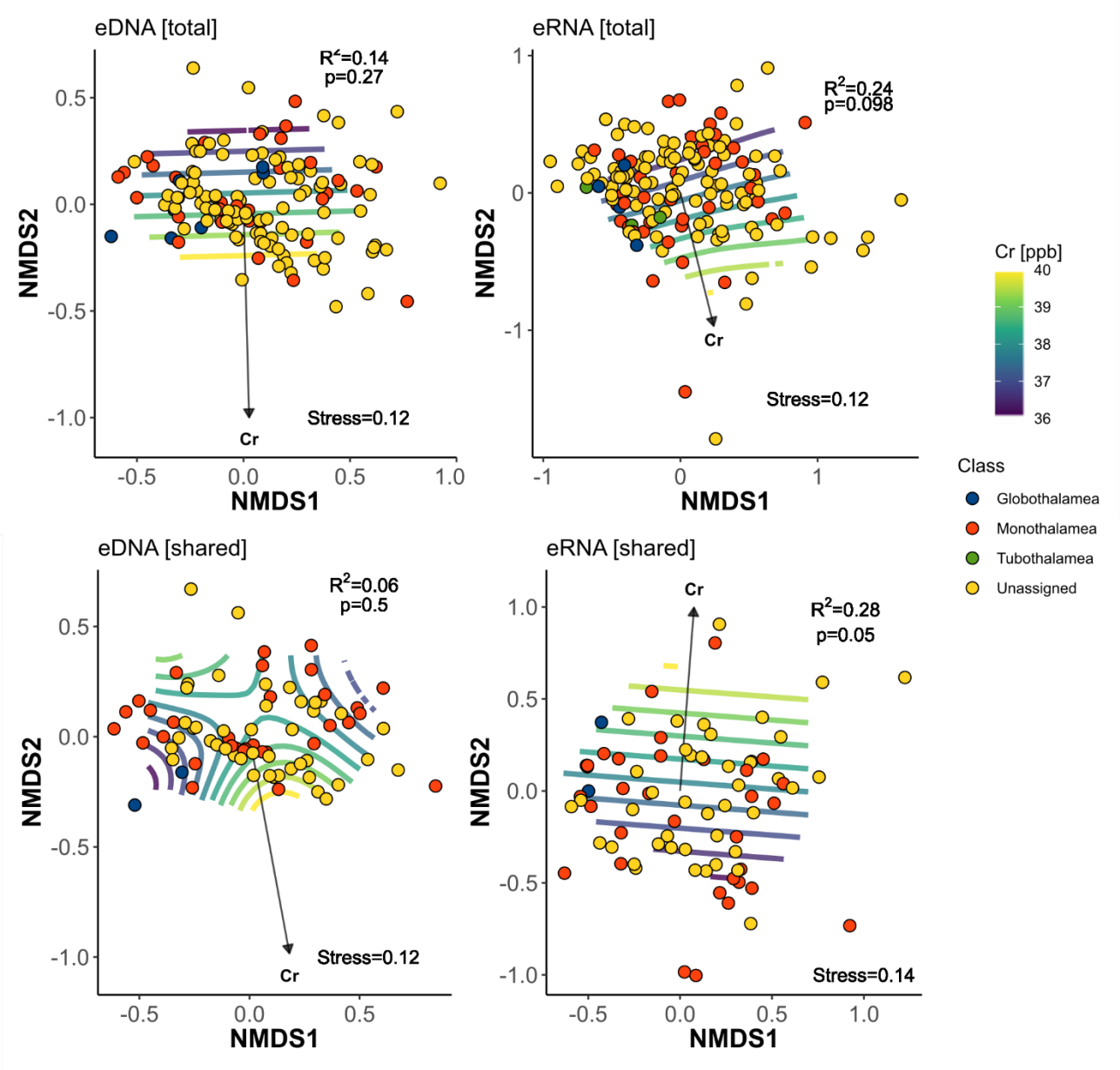

440 **Fig. 4** NMDS ordination performed on the Filter 100 datasets including the results of the BIOENV
441 analysis. Points in the panels represent the different Foraminiferal OTUs color-coded by their
442 taxonomical assignment. Contour lines were derived from surface fitting (GAM) of the Chromium
443 concentration. The results of the BIOENV analyses (R-squared and p value) are given for each dataset
444 along with NMDS stress.
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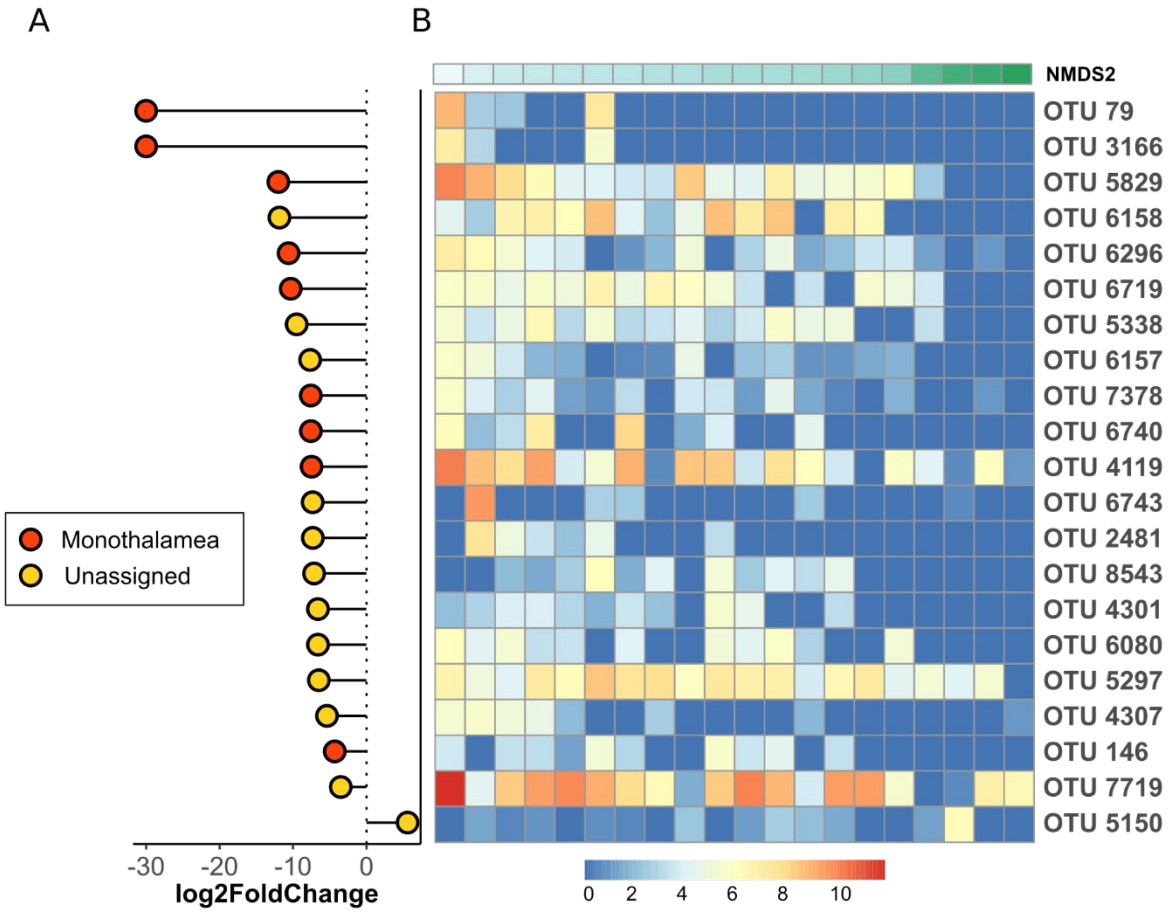

448 **Fig. 5 a**) Log2 Fold Change and **b)** Heatmap showing the response of the ecologically important
449 foraminiferal OTUs exposed to the Cr gradient extrapolated from the NMDS in figure 4. The x-axis
450 in **b)** shows the second dimension of the NMDS in Fig. 4.
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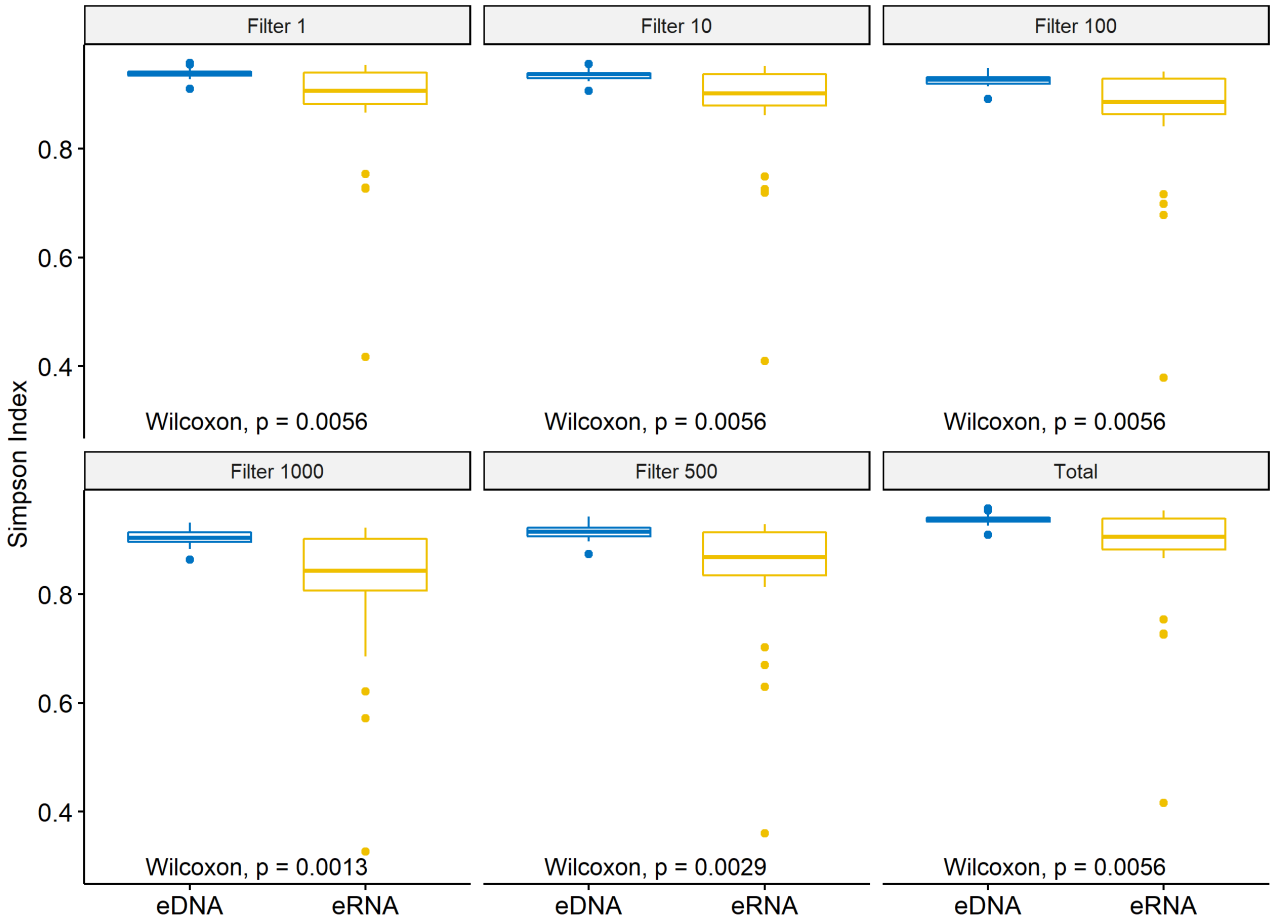

461 **Fig. S1** Simpson diversity index calculated on each datasets pairs. P-values report the results of
462 Wilcoxon test.
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464

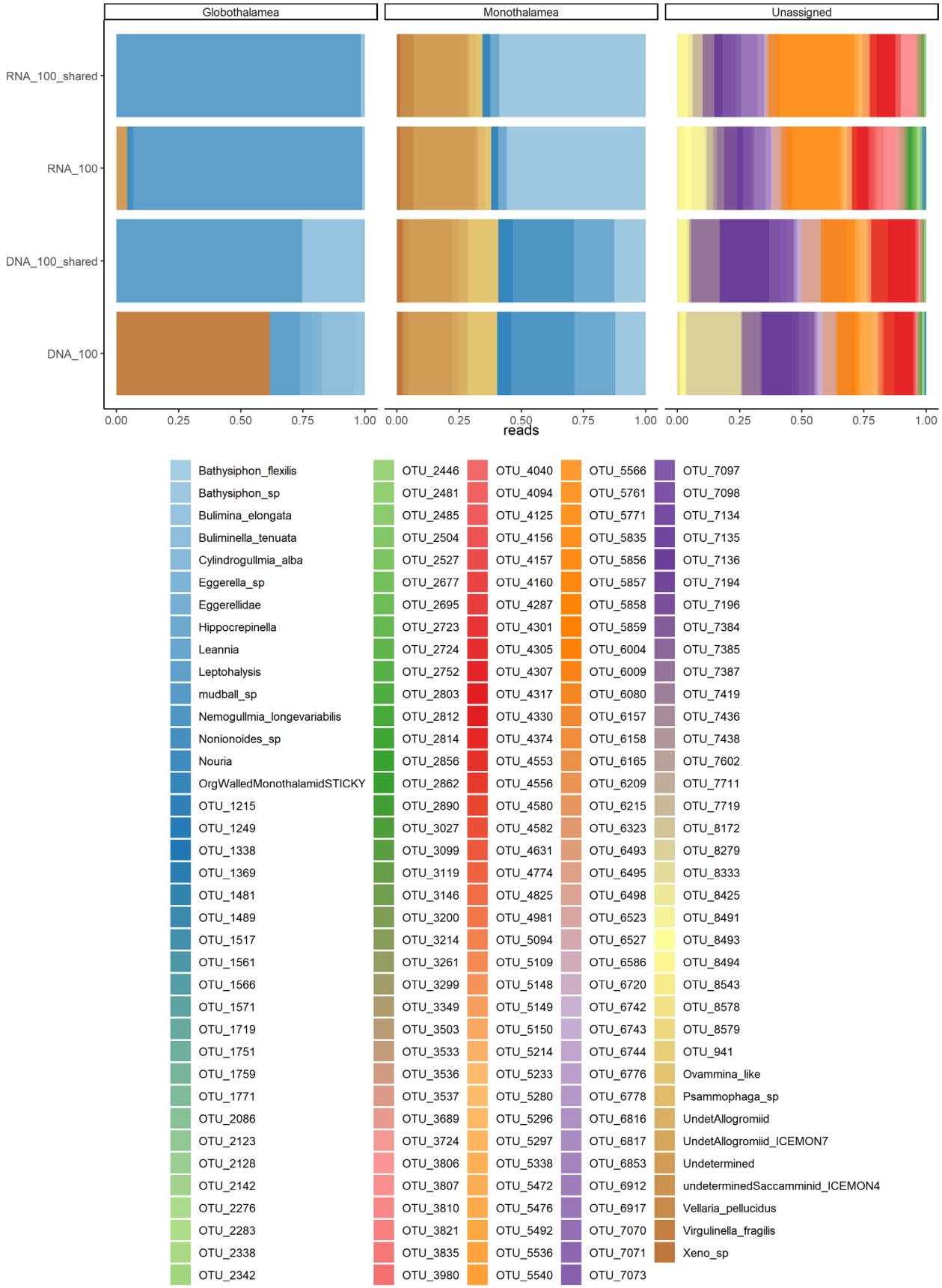

Fig. S2 OTU composition normalised to one of the RNA Filter 100 and DNA Filter 100 datasets and the respective shared subsets.

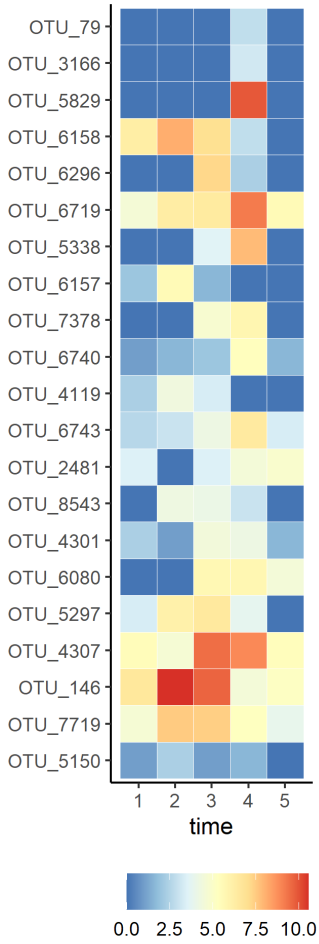

Fig. S3 Heatmap showing changes in abundance of ecologically relevant foraminiferal OTUs showed in Figure 5 in the control samples. Changes of abundance are plotted along a time gradient (weeks 1, 2, 4, 8, and 12).

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