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1 **High throughput shotgun sequencing of eRNA reveals taxonomic and derived functional**
2 **shifts across a benthic productivity gradient**

3

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17

18 Running title: Using eRNA to assess biodiversity

19

20 **Abstract**

21 Benthic macrofauna is regularly used in monitoring programmes, however the vast majority of
22 benthic eukaryotic biodiversity lies mostly in microscopic organisms, such as meiofauna
23 (invertebrates < 1 mm) and protists, that rapidly responds to environmental change. These
24 communities have traditionally been hard to sample and handle in the laboratory, but DNA
25 sequencing has made such work less time consuming. Compared to DNA sequencing that
26 captures both alive and dead organisms, environmental RNA (eRNA) can be used to better
27 target living organisms or organisms of recent origin in the environment. Here, we assessed the
28 biodiversity of three known bioindicator microeukaryote groups (nematodes, foraminifera, and
29 ciliates) in sediment samples collected at seven coastal sites along an organic carbon (OC)
30 gradient. We aimed to investigate if eRNA shotgun sequencing can be used to simultaneously
31 detect differences in 1) biodiversity of multiple microeukaryotic communities, and 2)
32 functional feeding traits of nematodes. Results showed that biodiversity was lower for
33 nematodes and foraminifera in high OC (6.2–6.9 %), when compared to low OC sediments
34 (1.2–2.8 %). Dissimilarity in community composition increased for all three groups between
35 Low OC and High OC, as well as the classified feeding type of nematode genera (with more
36 non-selective deposit feeders in high OC sediment). High relative abundant genera included
37 nematode *Sabatieria* and foraminifera *Elphidium* in high OC, and *Cryptocaryon*-like ciliates
38 in low OC sediments. Considering that future sequencing technologies are likely to decrease
39 in cost, the use of eRNA shotgun sequencing to assess biodiversity of benthic microeukaryotes
40 could be a powerful tool in recurring monitoring programmes.

41

42 **Introduction**

43 Biodiversity is decreasing globally due to human alteration and pollution of terrestrial and
44 aquatic environments (Brondizio, Settele, Díaz, & Ngo, 2019). Essential ecosystem services
45 affiliated with human health, such as availability of food, clean water, and recreational areas
46 are dependent on biodiversity (Cardinale et al., 2012; Pan, Marcoval, Bazzini, Vallina, &
47 Marco, 2013). In addition to the provision of ecosystem services, biodiversity losses have also
48 been linked to a decrease in ecosystem stability (McCann, 2000). Anthropogenic pressure on
49 coastal aquatic ecosystems by e.g. climate change, eutrophication and contaminant pollution
50 threatens the diversity of many organisms in these systems (Pan et al., 2013). Such threats on
51 coastal ecosystems should be taken seriously because coastal zones are transitional areas
52 directly adjacent to human settlements between land and sea, and impacted areas are predicted
53 to increase in both number and area with a continued climate change scenario (Levin et al.,
54 2001; Rabalais, Turner, Díaz, & Justić, 2009). It is therefore essential to understand how the
55 diversity of organisms living in coastal zones respond to changes in environmental gradients
56 and anthropogenic pressure (Snelgrove, Thrush, Wall, & Norkko, 2014).

57 Biodiversity assessments of benthic macrofauna are commonly used in national
58 monitoring programs, including coastal zones, to determine various ecological indices (Pinto
59 et al., 2009). However, microeukaryotes present in sediment such as meiofaunal nematodes (<
60 1 mm body size) are also known to react to e.g. eutrophication status (Ristau, Spann, &
61 Traunspurger, 2015), and the composition and quantity of organic carbon (OC) (Ingels,
62 Kiriakoulakis, Wolff, & Vanreusel, 2009; Pusceddu, Gambi, Zeppilli, Bianchelli, & Danovaro,
63 2009). Furthermore, because nematodes are known to have different feeding behaviors such as
64 bacterivory, detritivory or algal feeding (Moens, Traunspurger, & Bergtold, 2006; Wieser,
65 1953) changes in nematode assemblages are therefore likely to affect food web dynamics and
66 ecosystem function (e.g. Nascimento et al., 2019; Nascimento, Karlson, & Elmgren, 2008;

67 Nascimento, Näslund, & Elmgren, 2012). In sediment with high quantity of labile OM non-
68 selective deposit feeding nematodes have been observed to be prevalent (e.g. Ingels et al.,
69 2009). Other arguments for including meiofauna such as nematodes in national monitoring
70 systems include their high diversity, short generation time, and ubiquitous distribution
71 (Kennedy & Jacoby, 1999). However, these organisms are often neglected in monitoring
72 studies (Kennedy & Jacoby, 1999), likely due to financial reasons derived from time
73 consuming activities such as sieving, sorting, and microscopic morphological analyses.

74 In addition, the protist phyla Foraminifera (henceforth forams) and Ciliophora (i.e. ciliates)
75 are well-studied as bioindicators of environmental state of aquatic ecosystems. The diversity
76 and community composition of forams are known to change with anthropogenic pollution, fish
77 farming, and decreasing water quality (Damak, Frontalini, Elleuch, & Kallel, 2019; Frontalini
78 & Coccioni, 2011; Pawlowski, Esling, Lejzerowicz, Cedhagen, & Wilding, 2014; Raposo et
79 al., 2018; Uthicke & Nobes, 2008), and similar to nematodes, OC enrichment of the sediment
80 also influences the diversity of forams (Alve et al., 2016; Martins et al., 2015; Murray, 2006).
81 Ciliates are used as bioindicators in e.g. aquaculture (Stoeck, Kochems, Forster, Lejzerowicz,
82 & Pawlowski, 2018), wastewater treatment plants, and monitoring of eutrophication and
83 chemical pollution (Chen, Xu, Tam, Cheung, & Shin, 2008; Foissner, 2016; Pawlowski,
84 Lejzerowicz, Apotheloz-Perret-Gentil, Visco, & Esling, 2016). In natural aquatic
85 environments, the diversity and community composition of ciliates are influenced by e.g.
86 salinity, pH, and anthropogenic pollution (e.g. Gong et al., 2015; Jiang, Xu, Hu, Warren, &
87 Song, 2013). One of the main merits of assessing the diversity of protists as bioindicators is
88 their documented rapid change to environmental conditions (Payne, 2013). The assessment of
89 microeukaryotic biodiversity is therefore a good proxy in monitoring programmes to study
90 changes in ecosystems. However, these communities are rarely studied together and challenges
91 still include being able to investigate multiple communities from bulk sediment samples

92 without time consuming activities involved in studying the benthic microeukaryotic fraction
93 such as sieving, sorting, and microscopy.

94 In the last ten years, environmental DNA (eDNA) and RNA (eRNA) metabarcoding
95 studies targeting the 18S rRNA marker gene (or 18S rRNA for eRNA) have been extensively
96 conducted to study microeukaryotes (Creer et al., 2016; Forster et al., 2019; Pochon et al.,
97 2015). Such tools have drastically reduced the time needed to taxonomically classify organisms
98 compared to morphological taxonomic techniques that also involves sieving and sorting of
99 organisms (Carugati, Corinaldesi, Dell'Anno, & Danovaro, 2015). However, limitations exist
100 with metabarcoding such as non-optimized PCR protocols and primer bias when targeting
101 multiple taxa (Kelly, Shelton, & Gallego, 2019), and limitations of available species in
102 reference databases when taxonomically classifying sequences. Compared to metabarcoding
103 that typically yields ~60 000 sequences per sample (Singer, Fahner, Barnes, McCarthy, &
104 Hajibabaei, 2019) shotgun sequencing can generate millions of sequences per sample. New
105 bioinformatic tools that can today taxonomically classify these large datasets within minutes
106 to hours (e.g. Wood, Lu, & Langmead, 2019) and estimate relative abundances at species or
107 genus level (e.g. Lu, Breitwieser, Thielen, & Salzberg, 2017). However these methods rely on
108 the reference databases to classify taxonomy and are therefore likely to become more precise
109 over time when databases grow. While eDNA makes it possible to assess the biodiversity of
110 both living organisms plus non-degraded DNA from dead organisms, eRNA is targeting mainly
111 living organisms or RNA derived from organisms of recent origin in the environment
112 (Cristescu, 2019; Wood et al., 2020). It is therefore valuable to investigate if eRNA combined
113 with shotgun sequencing, thereby bypassing PCR limitations of metabarcoding, is a useful
114 approach to assess differences in the biodiversity of active multiple communities from highly
115 diverse and densely inhabited environments such as sediments.

116 Here we assessed the biodiversity and community composition of three microeukaryotic
117 groups in sediment samples: nematodes, forams, and ciliates, along an OC gradient in a coastal
118 archipelago in the Gulf of Finland, Baltic Sea. The aim was to investigate if eRNA shotgun
119 sequencing, without any sieving or sorting of samples (i.e. bulk sediment), could be used to
120 detect differences in biodiversity of multiple microeukaryotic communities for biomonitoring
121 purposes. This is possible because this method is not based on amplification of known markers
122 and avoids common limitations of metabarcoding such as: i) PCR primers only targeting certain
123 species; ii) amplifying certain species more than others, and iii) the amount of cycles and type
124 of polymerase used has been shown to influence diversity and community composition (Kelly
125 et al., 2019; Nichols et al., 2018). Additionally, we assessed if changes in nematode functional
126 ecology (feeding type) as a response to the OC gradient could be detected. We expected that
127 nematode deposit feeders would have higher relative abundance in stations with higher OC.
128 This approach was coupled to the latest sequencing platform (Illumina NovaSeq S4, yielding
129 ~87 million read-pairs per sample in our study) which has been demonstrated to detect
130 significantly more taxa compared to Illumina MiSeq sequencing that is the most used
131 technology for metabarcoding studies (yields ~60 000 read-pairs per sample) (Singer et al.,
132 2019). To analyze this large dataset, we used new bioinformatic tools to estimate taxonomic
133 classifications and relative abundances (Kraken 2 + Bracken 2.5 combination). The Gulf of
134 Finland is characterized by strong environmental gradients associated with eutrophication
135 (Andersen et al., 2015; Villnäs et al., 2019). This contributes to spatially heterogenous benthic
136 macro-communities in terms of diversity and composition in this ecosystem (Bonsdorff, Laine,
137 Hanninen, Vuorinen, & Norkko, 2003). The Gulf of Finland is therefore a well-suited system
138 to investigate if a similar heterogeneity exists in active microeukaryotic communities.

139

140 **Materials and methods**

141 *Field sampling*

142 Sediment was collected on board R/V Electra during 2018 September 20–23 in the Gulf of
143 Finland (Baltic Sea) close to the Tvärminne Zoological Station, Finland. A total of seven
144 stations were visited along coastal gradients in depth and OC (0–4 km, 10–45 m water depth;
145 Figure 1). The bottom water in the study areas at the time of sampling was oxic with 7.6–8.7
146 ml/l O₂ measured by oxygen probes equipped on a CTD instrument (full details in Broman,
147 Sun, et al., 2020). The stations were divided into four low % OC shallow sites (stations 11, 12,
148 15, 16; 1.2–2.8 % OC) and three sites with higher % OC and depth (stations 7, 10, 13; 6.2–
149 6.9 % OC), following a station labelling system used during reoccurring monitoring in the
150 Tvärminne region (Table 1). Triplicate sediment cores (labelled A, B, C), retrieved in rinsed
151 acrylic core liners, were collected from each station with a GEMAX twin gravity corer (height:
152 80 cm, inner diameter: 90 mm). The top 0–2 cm sediment surface layer was sliced into
153 autoclaved 215 ml polypropylene containers (Noax Lab). After slicing, the sediment was
154 directly aseptically homogenized inside the containers and 2 cm³ sediment transferred into 2
155 ml cryogenic tubes (VWR) that were immediately flash frozen at -196°C. The samples were
156 transported on dry ice and stored at -80°C until RNA extraction. The remaining sediment in
157 the 215 ml containers were stored at -20°C for sediment C and N content and pore water
158 chemistry analyses.

159

160 *Sediment and pore water chemistry analyses*

161 The remaining sediment in the frozen 215 ml containers were thawed, homogenized, and 15
162 cm³ sediment from each sample was dried at 60°C for seven days for C/N analysis. In addition,
163 20 cm³ of sediment from each sample was centrifuged at 2200 × g to extract the pore water for
164 ammonium (NH₄⁺) and phosphate (PO₄³⁻) analyses. The dried sediment was ground,
165 homogenized, and 1 cm³ dry weight sediment per sample stored in a desiccator prior to freeze

166 drying, re-grinding, re-homogenization and treated with HCl to remove inorganic carbon.
167 Samples were subsequently weighed into tin capsules. Concentrations of total OC and total
168 nitrogen were determined on an elemental analyzer (Flash 2000, Thermo Scientific). The pore
169 water was collected after centrifugation by filtering 10 ml of the supernatant through a 0.45
170 μm polyethersulfone membrane filter (Filtropur S 0.45, Sarstedt). NH_4^+ and PO_4^{3-} were
171 determined colorimetrically (Multiskan GO spectrophotometer, Thermo Scientific) and NH_4^+
172 analysis followed the modified salicylate-hypochlorite method by Bower and Holm-Hansen
173 (1980), and PO_4^{3-} analysis followed the standard methods for seawater analyses (Grasshoff,
174 Kremling, & Ehrhardt, 2009). NH_4^+ values were first reported in Broman, Sun, et al. (2020).

175

176 *RNA extraction and sequencing*

177 Sediment was thawed within minutes inside the cryotubes and ~2 g of material was added into
178 the RNeasy PowerSoil bead tubes and was extracted using the same kit (RNeasy PowerSoil,
179 QIAGEN). After RNA extraction, any remaining DNA was removed with DNase treatment
180 using the TURBO DNA-free kit (Invitrogen), followed by bacterial rRNA depletion using the
181 RiboMinus Transcriptome Isolation Kit (bacteria version, ThermoFisher Scientific). A 2100
182 Bioanalyzer (Agilent) was used to confirm that no DNA contamination was present in the
183 samples. Library preparation followed the TruSeq RNA Library Prep v2 kit (Illumina) without
184 including the poly-A selection step. This procedure does not include an amplification of a
185 marker gene and therefore avoids PCR limitations common for metabarcoding studies as
186 mentioned in the introduction. The samples were sequenced at the Science for Life Laboratory,
187 Stockholm on a single Illumina NovaSeq6000 S4 lane using paired-end 2×150 bp read
188 technology. A full list of sample names, sequences yielded, quality scores, read lengths etc. are
189 available in Supplementary Data 1.

190

191 *Bioinformatics*

192 The sequencing yielded on average 87.3 million paired-end sequences per sample (range 77.7–
193 97.8 million sequences). Illumina adapters were removed with SeqPrep 1.2 (St John, 2011)
194 following default settings with parameters -A and -B targeting the adapter sequences with
195 identical selection. Any remaining PhiX sequences in the raw data were removed by mapping
196 the reads using bowtie2 2.3.4.3 (Langmead & Salzberg, 2012) against the PhiX genome (NCBI
197 Reference Sequence: NC_001422.1). Final quality trimming of the data was conducted with
198 Trimmomatic 0.36 (Bolger, Lohse, & Usadel, 2014) with the following parameters:
199 LEADING:20 TRAILING:20 MINLEN:50. The final quality of the trimmed reads were
200 checked with FastQC 0.11.5 (Andrews, 2010) and MultiQC 1.7 (Ewels, Magnusson, Källér, &
201 Lundin, 2016). On average 86.8 million sequences remained (range 77.3–97.2 million
202 sequences) with a Phred quality score of 36–37 per base, and an average read length of 144 bp
203 (range 139–147 bp).

204 Small subunit (SSU) rRNA sequences were extracted from the quality trimmed data using
205 SortMeRNA 2.1b (Kopylova, Noé, & Touzet, 2012) with the databases supplied with the
206 software. Taxonomic classification was conducted with Kraken2 2.0.7 (Wood et al., 2019)
207 using paired-end reads against the SILVA SSU r132 NR99 (Quast et al., 2013) (database
208 downloaded 1 March 2019) and NCBI NT database (database downloaded 12 March 2019).
209 Kraken2 uses a k-mer based approach to classify sequences, and a lowest common ancestor
210 (LCA) algorithm to determine where unclassified sequences belong on a taxonomic tree (Wood
211 et al., 2019). To estimate the relative abundance of each taxon Bracken 2.5 was used on the
212 Kraken2 outputs with default settings set to genus level (i.e. a count threshold of 10) (Lu et al.,
213 2017). Bracken 2.5 uses a Bayesian algorithm method to estimate the genus level read
214 abundance (or species, we chose genus for higher accuracy) of Kraken2 sequences classified
215 higher up on the taxonomic tree (Lu et al., 2017; Wood et al., 2019). This is important, because

216 without estimating read abundance to genus the unclassified reads on higher taxonomic levels
217 will underestimate relative abundances of genera (Lu et al., 2017). The Bracken output reports
218 were combined into a biom-format file with the python package kraken-biom 1.0.1 (using
219 parameters: ---fmt hdf5 -max D --min G), and the biom-format file was converted to a tax table
220 using the python package biom-format 2.1.7 (McDonald et al., 2012). The 18S rRNA
221 eukaryotic data was extracted, normalized as relative abundances (%), and analyzed in the
222 software Explicet 2.10.5 (Robertson et al., 2013). Results for i) Nematoda (NCBI NT
223 classifications, on average 478,331 sequences per sample); ii) Foraminifera (NCBI NT,
224 average 13,913 sequences), and iii) Ciliophora (SILVA, average 774,027 sequences) were
225 extracted and analyzed separately. The NCBI NT database was used for the Nematoda and
226 Foraminifera data because, 1) the SILVA database is known to contain errors in the nematode
227 classifications (Broman et al., 2019; Holovachov, Haenel, Bourlat, & Jondelius, 2017), and the
228 NCBI NT has previously been used to discern differences in nematode communities on a
229 spatial scale in the Baltic Sea (Broman et al., 2019); and 2) the SILVA database gave inaccurate
230 classifications for Foraminifera, resulting in the identification of taxa never discovered in the
231 Baltic Sea (more details in the discussion).

232

233 *Nematoda functional ecology analyses*

234 Nematode genera were classified into feeding types based on their known buccal cavity
235 morphology in available literature according to Wieser (1953). Each genus was categorized
236 into the four feeding types described by Weiser: 1A) selective deposit feeder, 1B) non-selective
237 deposit feeder, 2) epistrate feeder, and 2B) predator/omnivore. In addition, the maturity index
238 (MI) of each nematode community was calculated to infer changes in the life history
239 characteristics of nematode genera. MI was calculated according to Bongers, Alkemade, and
240 Yeates (1991) by assigning colonizer–persister (cp) values to nematode genera on a scale from

241 1 to 5 based in available literature. Low cp-values indicate nematode genera that can be
242 classified as colonizers (short life cycle, high reproduction rates, high colonization ability and
243 tolerance to disturbance) while high cp-values represent persisters (nematode genera that
244 display long life cycles, few offspring, low colonization ability and high sensitivity to
245 disturbance). MI could then be calculated from:

$$246 \quad MI = \sum_{i=1}^n v(i) \times f(i)$$

247 where $v(i)$ is the cp-value of genus i and $f(i)$ is the frequency of genus i .

248

249 *Statistics*

250 Rarefaction curves of sequence counts versus the taxonomic classifications were conducted in
251 the R package *vegan* 2.5.6 (Oksanen et al., 2018) using the *rrarefy* function with default
252 settings. Species richness (Chao1) and alpha diversity (Shannon's H) were calculated in the
253 software *Explicit* 2.10.5 for each taxonomic group (Nematoda, Foraminifera, and Ciliophora).
254 Before calculating Shannon's H index the data was sub-sampled to the lowest sample size and
255 bootstrapped $\times 100$ (Nematoda 79,815 counts, Foraminifera 2473 counts, Ciliophora 299,504
256 counts). Non-metric multidimensional scaling (NMDS) plots showing beta diversity were
257 based on the presence/absence (Sørensen dissimilarity index) and Bray-Curtis dissimilarity
258 index (based on relative abundance) using the software *past* 3.26 (Hammer, Harper, & Ryan,
259 2001). The difference in read abundance between the high and low OC stations for Nematoda
260 feeding type data was normalized and statistically tested using the R package *DESeq2* 1.26
261 with default settings (Love, Huber, & Anders, 2014). The *DESeq2* output was plotted using
262 the *ggplot2* package in R (Wickham, 2016). Differences between groups on alpha diversity
263 metrics (Chao1, Shannon's H), relative abundance of taxonomic groups, and maturity index
264 for nematodes were tested with univariate statistics conducted in the software IBM SPSS
265 Statistics 26. First, Shapiro-Wilk tests were used to check if the data was normally distributed.

266 Differences between groups in normally distributed data were tested with One-Way ANOVA
267 tests, while non-parametric data were tested with Mann-Whitney U tests. PERMANOVA tests
268 (9999 permutations) were conducted in the software past 3.26 and used to identify differences
269 in beta diversity between stations, based on presence/absence, relative abundance, and
270 Hellinger transformed data (i.e. square rooted relative abundances) (Legendre & Gallagher,
271 2001). To investigate if the abiotic variables (% OC, % N, PO_4^{3-} , NH_4^+ , and water depth) were
272 associated with the community composition canonical correspondence analysis (CCA) was
273 conducted in the R package *vegan* 2.5.6 with the *cca* function and plotted using the *ggplot2*
274 3.2.1 package. The input data for the CCAs were the measured abiotic variables and relative
275 abundances of the different taxa. Significant associations between abiotic variables and
276 community compositions were tested for CCA axis 1 and axis 2 with PERMANOVA tests
277 (9999 permutations) using the function *envfit* included in the *vegan* package. To detect if
278 measured abiotic variables (rather than solely water depth) significantly explained community
279 compositions of the three studied groups the *adonis2* function in the R package *vegan* 2.5.6
280 was used. The Bray-Curtis dissimilarity matrix for each study group was loaded with the abiotic
281 data and the abiotic variables were added in sequential order after water depth. Mantel tests
282 (Mantel, 1967) of the of Bray-Curtis dissimilarity distances were used to test the correlation
283 with OC by using the function *mantel.rtest* in the R package *ade4* 1.7.13 (Dray & Dufour,
284 2007), after turning the OC data to a distance matrix with the *dist* function with default settings.

285

286 **Results**

287 *Field data and sediment characteristics*

288 Both OC and N content in the sediment were higher at the deeper stations, i.e. > 30 m water
289 depth (6.5 ± 0.2 % OC and 0.9 ± 0.02 % N) when compared to the shallow stations, i.e. < 30
290 m water depth (1.8 ± 0.1 % OC and 0.2 ± 0.02 % N; Mann-Whitney U tests, $U = 0$, $P = 0.000007$

291 for both; Table 1). Hereafter the shallow stations will therefore be referred to as “Low OC” and
292 the deep stations as “High OC”. Pore water NH_4^+ and PO_4^{3-} extracted from the top 0–2 cm
293 sediment layer at the seven sampled stations (Table 1 & Figure 1, $n = 3$ for each station)
294 correlated positively with water depth (both $P < 0.01$, Pearson correlations, $r = 0.83$ and $r =$
295 0.64 , respectively). NH_4^+ was significantly higher at High OC stations ($308 \pm 19.8 \mu\text{g/l}$, $n =$
296 12) compared to the Low OC stations ($196 \pm 14.3 \mu\text{g/l}$ (mean \pm SE, $n = 9$; Mann-Whitney U
297 test, $U = 10$, $P = 0.002$). Similarly, pore water PO_4^{3-} was significantly higher at High OC
298 stations ($32.2 \pm 7.0 \mu\text{g/l}$) compared to the Low OC ($4.4 \pm 0.5 \mu\text{g/l}$; Mann-Whitney U test, $U =$
299 1 , $P = 0.000140$). A full list of abiotic data for all stations is available in Supplementary Data
300 2.

301

302 *Sediment 18S rRNA community*

303 The most abundant microeukaryotic taxonomic groups in our sediments included nematodes,
304 arthropoda (mainly copepods), rotifers, and single-celled eukaryotes such as Bacillariophyta
305 (mainly diatoms), ciliates, and Kraken2 unclassified eukaryotic sequences that Bracken2
306 distributed to protists Malawimonadidae and Hemimastigophora (Figure 2a-b and
307 Supplementary Data 3). There was a significant difference in community composition when
308 testing the stations grouped as Low OC against High OC (Sørensen dissimilarity index test
309 (presence/absence data), PERMANOVA, pseudo $F = 6.71$, $P = 0.0001$; Figure 2c). This was
310 also significant when tested with Bray-Curtis dissimilarity index based on relative abundance
311 data (PERMANOVA, pseudo $F = 4.73$, $P = 0.0007$), as well as Hellinger transformed data
312 (pseudo $F = 5.36$, $P = 0.0001$). For this study we focused on three microeukaryotic groups used
313 in biomonitoring: Nematoda (average 4% of all eukaryotes), Foraminifera (average 0.15% of
314 all eukaryotes), and Ciliophora (average 7% of all eukaryotes).

315

316 *Alpha and beta diversity for nematodes, foraminifera, and ciliates*

317 Rarefaction analyses showed that the majority of the genera had been detected in the samples
318 (Supplementary Figure 1). The species richness Chao1 index and Shannon's H alpha diversity
319 index were significantly lower for Nematoda and Foraminifera at the High OC stations
320 compared to Low OC (Chao1: One-Way ANOVA test for each group; Nematoda, $F_{(1,19)} = 32.7$,
321 $P = 0.000016$; Foraminifera, $F_{(1,19)} = 57.0$, $P = 0.0000004$; Figure 3a-b; Shannon's H:
322 Nematoda, $F_{(1,19)} = 24.8$, $P = 0.000083$; Foraminifera, $F_{(1,19)} = 48.2$, $P = 0.000001$; Figure 3d-
323 e). No significant difference in species richness or Shannon's H alpha diversity was observed
324 for Ciliophora when comparing High OC stations with Low OC (Figure 3c). A full list of
325 Shannon's H values is available in Supplementary Data 4.

326 Beta diversity was also significantly different between stations for all three groups, with
327 the presence/absence Sørensen dissimilarity index test (PERMANOVA) when testing the
328 stations grouped as Low OC against High OC (PERMANOVA) test for each group, Nematoda,
329 pseudo $F = 11.4$, $P = 0.0001$; Foraminifera, pseudo $F = 25.5$, $P = 0.0001$; Ciliophora, pseudo
330 $F = 5.1$, $P = 0.0001$; Figure 4). Similar results were also observed when using the Bray-Curtis
331 dissimilarity index based on relative abundances as well as Hellinger transformed data
332 (Supplementary Figure 2)

333 CCAs based on the relative abundance of genera showed that the measured abiotic
334 variables (water depth, sediment % C and % N, plus pore water NH_4^+ and PO_4^{3-}) were
335 associated with the High OC stations for all of the three studied taxonomic groups, i.e.
336 Nematoda, Foraminifera, and Ciliophora (Figure 5). The CCA analysis showed that 67 %, 77
337 % and 66 % of the total constrained inertia for nematodes, foraminifera, and ciliates, was
338 explained with the five environmental variables here studied, respectively. There was also a
339 significant association between all five abiotic variables and the community composition for
340 each studied group (PERMANOVA test, Nematoda, $R^2 = 0.76\text{--}0.83$, $P < 0.001$; Foraminifera,

341 $R^2 = 0.52\text{--}0.94$, $P < 0.05$; Ciliophora, $R^2 = 0.53\text{--}0.85$, $P < 0.01$; Supplementary Data 5).
342 Moreover, adonis PERMANOVA tests showed that OC was a significant variable determining
343 community composition for all three taxonomic groups, even when accounting for the variance
344 explained by depth (Nematoda pseudo $F = 6.06$, Foraminifera pseudo $F = 12.85$, Ciliophora
345 pseudo $F = 6.01$; all $P < 0.001$; see Supplementary Data 5 for results of all variables). OC was
346 also tested separately with mantel tests with the Bray-Curtis dissimilarity distances for each of
347 the three taxonomic groups, and was positively correlated with the community composition
348 (Nematoda $r = 0.58$; Foraminifera, $r = 0.76$; Ciliophora, $r = 0.54$; all $P = 0.0001$).

349

350 *Differences in Nematoda community structure*

351 The Nematoda 18S rRNA dataset showed differences in community composition along the OC
352 gradient. This included e.g. the nematode genus *Sabatieria* that was detected at all stations and
353 had a significantly higher relative abundance at the High OC stations when compared to Low
354 OC ($52.2 \pm 7.9\%$ compared to $22.2 \pm 3.5\%$ (% denote portion of Nematoda community, Mann-
355 Whitney U test, $U = 16$, $P = 0.006$; Figure 6a)). Similarly, the genus *Axonolaimus* had a higher
356 relative abundance at High OC stations ($8.9 \pm 2.3\%$ compared to $0.7 \pm 0.2\%$, Mann-Whitney
357 U test, $U = 0$, $P = 0.000007$; Figure 6a). In contrast, in the Low OC stations the genera
358 *Daptonema* ($19.7 \pm 3.0\%$) and *Desmolaimus* ($4.8 \pm 0.7\%$) had a significantly higher relative
359 abundance when compared to High OC with *Daptonema* ($4.0 \pm 0.7\%$, $U = 2$, $P = 0.000027$)
360 and *Desmolaimus* ($0.7 \pm 0.2\%$, $U = 1$, $P = 0.000014$) (Mann-Whitney U tests; Figure 6a).

361

362 *Differences in Foraminifera community structure*

363 Looking closer at Foraminifera, the genera with a high relative abundance such as *Elphidium*
364 had a significantly higher relative abundance among the Foraminifera at the High OC stations
365 ($66.3 \pm 2.7\%$) compared to Low OC ($16.2 \pm 2.1\%$ (% denote portion of Foraminifera

366 community); Mann-Whitney U test, $U = 0$, $P = 0.000007$; Figure 6b). On the other hand, the
367 genus *Rhizammina* had a significantly higher relative abundance at the Low OC stations (17.9
368 ± 3.1 %) when compared to High OC (1.0 ± 0.8 %; Mann-Whitney U test, $U = 2$, $P = 0.000027$;
369 Figure 6b). In addition, we also detected genera with a low relative abundance that showed a
370 significant difference, although with high variation, between Low OC and High OC stations.
371 For example, *Globobulimina* had a higher relative abundance at Low OC (0.8 ± 0.7 %
372 compared to 0.0 ± 0.0 % at High OC), while both *Nonionella* and *Virgulinella* had a higher
373 relative abundance at High OC sites (1.5 ± 0.6 % and 2.6 ± 1.7 % compared to 0.4 ± 0.3 % and
374 1.7 ± 2.7 % at Low OC, respectively; Mann-Whitney U tests, $P < 0.05$; Fig. 6).

375

376 *Differences in Ciliophora community structure*

377 Examples of Ciliophora genera that were significantly different between the Low OC and High
378 OC stations included *Cryptocaryon* that had a significantly higher relative abundance at the
379 Low OC stations (17.4 ± 1.4 % compared to High OC 12.4 ± 1.4 % (% denote portion of
380 Ciliophora community), Mann-Whitney U tests, $U = 21$, $P = 0.018$; Figure 6c). Instead, the
381 genus *Spirotrachelostyla* had a significantly higher relative abundance at High OC (2.9 ± 0.5
382 %) when compared to Low OC (0.8 ± 0.1 %, Mann-Whitney U test, $U = 9$, $P = 0.00066$; Figure
383 6c). Ciliophora with low relative abundance that had significant difference, although with high
384 variation, between Low OC and High OC stations included e.g. *Bresslaueria* and *Epiphyllum*
385 having a higher relative abundance at Low OC (2.0 ± 2.5 % and 1.8 ± 0.8 % compared to 0.1
386 ± 0.1 and 0.9 ± 0.5 % at High OC, respectively), and *Zosterodasys* having a higher relative
387 abundance at High OC sites (2.8 ± 1.0 % compared to 1.3 ± 1.0 % at Low OC; Mann-Whitney
388 U tests, $P < 0.05$; Fig. 6). A full list of taxonomic classifications and sequence counts for all
389 three studied groups are available in Supplementary Data 6.

390

391 *Nematoda functional ecology*

392 The maturity index calculated from classified Nematoda genera showed no difference between
393 High OC and Low OC stations (1.9 ± 0.1 maturity index for all samples, One-Way ANOVA
394 test, Supplementary Data 7). Considering that values closer to one indicate habitat colonizers
395 (and values closer to five indicate habitat persisters) the nematode communities in this study
396 are considered colonizers. Looking closer at the classified feeding type of the nematodes the
397 Genera classified as non-selective deposit feeders (1B, following the classification systems by
398 Wieser (1953)) had a significantly higher number of reads in the High OC stations when
399 compared to Low OC (log₂ fold change 1.79, DESeq2 analysis, false discovery rate (*FDR*) <
400 0.01; Figure 7). In contrast, the Low OC stations had significantly more genera classified as
401 selective deposit feeders (1A, log₂ fold change 1.62) and predator/omnivores (2B, log₂ fold
402 change 1.40) (*FDR* < 0.01 and *FDR* < 0.05, respectively; Figure 7). A full list of all maturity
403 index and feeding type classifications and their relative abundance per Nematoda genera is
404 available in Supplementary Data 7.

405

406 **Discussion**

407 In this study we investigated if current sequencing technology and eRNA shotgun sequencing
408 has the power to differentiate changes in biodiversity of multiple microeukaryotes in bulk
409 sediment samples. We focused on nematodes, forams, and ciliates which are useful
410 bioindicators and known to change in diversity and community composition in relation to
411 environmental change (Gong et al., 2015; Ingels et al., 2009; Martins et al., 2015; Pawlowski
412 et al., 2014; Ristau et al., 2015). The results showed a difference in community structure for
413 each of the communities along the OC gradient in the study area. For example, the non-
414 selective deposit feeding nematode genera *Sabatieria* and *Axonolaimus* (Schratzberger, Warr,
415 & Rogers, 2007) had a higher relative abundance at the High OC stations. Potentially this could

416 be a beneficial feeding strategy at the deeper stations where the sediment consists mainly of
417 decayed organic particles and bacteria as food (and is reflected in the nematode feeding type
418 analysis; Figure. 7). *Sabatieria* are typical nematodes found in organic rich sediments, and have
419 been identified in sediments also containing other non-selective deposit feeders such as
420 *Daptonema* (Armenteros et al., 2009; Broman et al., 2019; Montagna & Harper, 1996;
421 Schratzberger, Warr, & Rogers, 2006). Interestingly, the genera *Daptonema* and *Desmolaimus*
422 (also a non-selective deposit feeder (Schratzberger et al., 2007)) had a higher relative
423 abundance at the Low OC stations. The Low OC stations had more nematodes classified as
424 selective deposit feeders and predator/omnivores, suggesting different kinds of food and
425 increased competition for the available food in these sediments. Nematodes of the genus
426 *Sabatieria* are known to also inhabit deeper layers of the sediment in the Baltic Sea
427 (Nascimento et al., 2008) and it is possible that such a response to increased competition in the
428 top sediment layer influenced the relative abundance of this genus in Low OC sediments. In
429 addition, the chemistry data indicate that the High OC sediments had higher concentrations of
430 dissolved phosphate compared to the Low OC stations, which indicate more reduced conditions
431 and generally a thinner oxic zone (Bonaglia, Deutsch, Bartoli, Marchant, & Brüchert, 2014).
432 This could be beneficial for *Sabatieria* which is known to be resistant to low oxygen conditions
433 (Broman, Bonaglia, et al., 2020). These nematode genera (*Axonolaimus*, *Daptonema*, and
434 *Sabatieria*) have previously been detected in other basins of the Baltic Sea using 18S rRNA
435 gene metabarcoding (Broman et al., 2019), and here their presence was confirmed by shotgun
436 sequencing.

437 The foram genera *Elphidium* and *Rhizammina* showed contrasting patterns in the dataset,
438 with *Elphidium* having higher relative abundance at High OC stations, and *Rhizammina* at the
439 Low OC stations. Both *Elphidium* and *Rhizammina* are known to exist in the south-western
440 Baltic Sea (Frenzel, Tech, & Bartholdy, 2005; Schweizer, Polovodova, Nikulina, & Schönfeld,

2011), and to our knowledge, this is the first study using molecular data to investigate diversity of forams in the Gulf of Finland. Many benthic forams depend on high saline conditions because they build shells (so called tests) with calcium carbonate (Charrieau, Filipsson, Nagai, et al., 2018), while some species instead agglutinate sediment particles (Charrieau, Filipsson, Ljung, et al., 2018). Considering the low saline conditions in our study area it is likely difficult for calcitic forams to fully develop calcified tests. In a study by Charrieau, Filipsson, Ljung, et al. (2018) species belonging to the calcitic foram genera *Elphidium* and *Ammonia* were found in the Southern Baltic Sea at slightly higher salinities, but with dissolved tests. It is therefore possible that many of the calcite forming forams found in our study had none, partly developed, or dissolved tests. Previous morphological studies have shown that the community composition of forams change in response to OC enrichment as observed in the north Atlantic (Alve et al., 2016) and Mediterranean Sea (Jorissen et al., 2018). Even though such studies are missing for the Baltic Sea, our data indicate that *Elphidium* increased in relative abundance to OC enrichment. The morphospecies *Elphidium excavatum* has been found in OC-rich, brackish sediments in Japan (Takata, Takayasu, & Hasegawa, 2006), however it is not certain that the same species is present in our study. Similarly to nematodes in our study, forams also showed a lower alpha diversity at the High OC stations. This finding is in accordance with previous metabarcoding work by Pawlowski et al. (2014) that also found benthic forams to have a lower alpha diversity and different community composition as a response to high organic matter areas (fish farms, North Atlantic, Scotland). For taxonomic classification of protists, SILVA is one of the recommended options when classifying 18S rRNA sequences (Creer et al., 2016). However, we were still surprised to see many differences in classified Foraminifera genera between the SILVA and NCBI NT databases. For example, SILVA reported a high relative abundance of genera (e.g. *Calcarina*, up to 35% in the offshore stations) never previously detected in the Baltic Sea (Supplementary Data 6). Almost 100 foram species have been

466 reported from the south-western Baltic Sea, but very few studies investigating forams in the
467 central and north Baltic Sea are available (Frenzel et al., 2005). Hard-shelled (calcitic and
468 agglutinated) forams have low densities in our study area and soft-shelled (organic) forams are
469 not often studied morphologically. The NCBI NT data also reported potential alien species
470 such as *Planoglabratella* previously detected in shallow New Zealand sediment (Hayward,
471 1999) and this could also be due to database limitations (Fig. 6). It is therefore possible that the
472 differences we observed between databases are due to a limited number of 18S rRNA foram
473 sequences in the databases. There are specific foram databases such as the PFR² (Morard et al.,
474 2015). However, this database is focusing on oceanic planktonic forams which are absent from
475 the Baltic Sea. Nevertheless, we report good results with the NCBI NT database.

476 Regarding the ciliate community, Stoeck et al. (2018) used metabarcoding and showed
477 that benthic ciliate communities in the vicinity of fish farms (i.e. areas with high organic matter)
478 had a lower alpha diversity and different community composition compared to non-affected
479 reference sites. In our study we also observed a significant difference in ciliate community
480 composition between the Low OC and High OC sites, and although there was a decrease in
481 alpha diversity it was not significant. Neither was there a difference between ciliate genera with
482 a high relative abundance (except for *Cryptocaryon*). Therefore, the difference observed in beta
483 diversity is likely due to differences in low abundant genera. The higher variance in ciliate
484 alpha diversity (compared to nematodes and forams) indicates that higher replication and
485 associated statistical power is required to detect differences in ciliate diversity between the
486 stations. The ciliate genus *Cryptocaryon* was more prominent at the Low OC stations. This is
487 a marine genus known to include parasitic species targeting fish (Wright & Colorni, 2002).
488 However, low-saline (5–7 ppt) variants of *Cryptocaryon* have previously been described
489 (Yamamoto, Song, & Sung, 2003). *Cryptocaryon*-like ciliates have previously been detected
490 phylogenetically in the more saline (~14 ppt) deeper waters of the Baltic Sea (Stock, Jürgens,

491 Bunge, & Stoeck, 2009), and both SILVA and NCBI NT (and manually checking classified
492 sequences via BLAST) confirmed *Cryptocaryon*-like ciliates in our samples. Potentially
493 because the Low OC stations were located in shallow areas closer to the shore, the
494 *Cryptocaryon*-like ciliates detected in this study might be adapted to lower salinities (~7 ppt)
495 and related to host organisms residing in these more diverse and euphotic habitats. In addition,
496 considering that the summer heatwave of 2018 was one of the most intense ever recorded in
497 the study area (Humborg et al., 2019), the warmer waters might attract *Cryptocaryon*-like
498 ciliates which are typically more common at temperate and tropical temperatures (Colorni &
499 Burgess, 1997). As far as we are aware, this is the first time such species have been reported
500 from the Gulf of Finland based on molecular data. Finally, measurement of more geochemistry
501 variables such as iron and sulfate, and sediment microprofiles (e.g. oxygen profiles) could help
502 to further explain differences in nematode, foram, and ciliate diversity and taxonomy between
503 low and high OC stations.

504 Limitations of this study include the relatively small sample size for RNA extraction (2 g).
505 A previous study investigating the effect of sample size on diversity estimations of
506 microeukaryotes and metazoans using metabarcoding, found that larger volumes of sediments
507 are necessary to accurately estimate small-scale spatial heterogeneity in biodiversity
508 (Nascimento, Lallias, Bik, & Creer, 2018). Here we used the available commercial extraction
509 kit that could process the largest input of sediment volume. It will be useful for future studies
510 to develop larger kits for eRNA extraction and investigate similar effects of sample size on
511 biodiversity assessment based on eRNA. Nevertheless, even with this sample size we report
512 clear differences in biodiversity of multiplied communities including metazoans along the
513 environmental gradients. We also used a kit to deplete bacterial rRNA in the laboratory, and
514 this might have influenced the eukaryotic rRNA results. However, this influence should be
515 similar among all samples and have a negligible impact in the results here shown. Previous

516 studies focusing on eukaryotic RNA data backed up with microscopy has shown that bacterial
517 rRNA depletion did not change the main findings as determined by microscopy (Broman,
518 Bonaglia, et al., 2020; Broman, Varvara, Dopson, & Hylander, 2017). Organisms contain
519 multiple ribosomes and while this is not an issue when analyzing species richness or beta
520 diversity with a presence/absence index, for community composition organisms with a large
521 number of ribosomes could skew the proportions. This issue also exists in DNA metabarcoding
522 studies, with many eukaryotic organisms carrying multiple genome copies per cell (i.e.
523 polyploidy) (Edgar, Zielke, & Gutierrez, 2014) and for prokaryotes that can also be polyploid
524 (Soppa, 2017). With the shotgun sequencing approach there is not a specific region of the 18S
525 rRNA targeted by PCR and instead all regions are sequenced randomly. This could potentially
526 influence the classification for certain taxa such as Foraminifera where some 18S rRNA regions
527 have been shown to be more precise than others (Pawlowski & Lecroq, 2010). However, in
528 this study the goal was to investigate if changes in biodiversity of multiple communities along
529 environmental gradients could be detected using eRNA shotgun sequencing. For this purpose
530 getting a large number of reads covering as much as possible of the 18S rRNA region for
531 different taxa is likely a benefit.

532 Methods used in this study (and metabarcoding studies as well) relies heavily on the
533 information in the reference databases when classifying taxonomy. In this study we set the
534 lower limit to “genus” during taxonomic classification to compare relative abundance, as
535 species would likely decrease the accuracy. However, we discovered that a large portion of
536 unclassified eukaryotic sequences by Kraken2 had been distributed to protists groups
537 Malawimonadidae and Hemimastigophora. These two groups have been found in soil, as well
538 as freshwater for Malawimonadidae (Adl et al., 2019; Lax et al., 2018). Considering Kraken2
539 only detected a few hundred sequences for these taxa, while Bracken2 distributed millions of
540 unclassified eukaryotic sequences to these taxa it is possible that this is an effect of the

541 information available in the databases. For example, Hemimastigophora was recently
542 phylogenetically placed outside all eukaryote supergroups (Lax et al., 2018), and this could
543 explain why Bracken2 would distribute unclassified sequences to such taxa. When databases
544 grow with more reference species for microeukaryotes tools such as Kraken2 are likely to
545 become more accurate for both genus and species level. The cost of shotgun sequencing on the
546 latest platform is still quite high (several thousands of USD per Illumina NovaSeq S4 lane,
547 yielding ~2000 million read-pairs) compared to metabarcoding of marker genes (a few
548 thousand USD per Illumina MiSeq flowcell, yielding ~18 million read-pairs; yields are based
549 on information from SciLifeLab, Stockholm). However, there has been a large decrease in
550 sequencing cost over the past 20 years (Wetterstrand, 2020) and if this trend continues,
551 alongside streamlined bioinformatic protocols, large scale eRNA shotgun studies could be a
552 future possibility in biomonitoring programmes.

553 Shotgun sequencing “catches” all organisms in the sample including both prokaryotes and
554 eukaryotes (Zepeda Mendoza, Sicheritz-Pontén, & Gilbert, 2015). rRNA has a relatively short
555 lifespan in the environment (Blazewicz, Barnard, Daly, & Firestone, 2013) and sediment
556 surfaces in shallow water systems are highly active environments The approach used in this
557 study therefore likely targeted active organisms present in the study area, as well as eRNA
558 derived from other motile organisms that were originally not located in the collected sediment
559 (up to ~13h or longer in biofilm, see Wood et al., 2020). However, there have been studies that
560 indicate that rRNA might be stable for long periods, potentially up to several years in deep sea
561 sediments, therefore potentially failing to reflect live communities better than eDNA in such
562 stable environments (Brandt et al., 2019; Orsi, Biddle, & Edgcomb, 2013). It is possible that
563 rRNA is also prevalent for some time in other sediments including shallow systems. However,
564 because coastal ecosystems are highly active environments rRNA degradation is expected to
565 be faster. eRNA sequencing is therefore a potential useful method to study benthic

566 communities, especially considering that a substantial portion of sediment consists of long-
567 lasting dead organic matter (Burdige, 2006). For example, forams are a known microfossil
568 group and with the use of DNA extraction it has been possible to study the ancient DNA of
569 these organism (Lejzerowicz et al., 2013). However, limitations such as rRNA stability,
570 especially below the oxic sediment surface, cannot be ruled out. Nevertheless, for
571 biomonitoring studies changes in biodiversity and taxonomy are studied over time from the
572 same stations and this might be less problematic. Shotgun sequencing of eRNA has been used
573 in a wide variety of marine studies, including investigations of prokaryotic communities
574 (Broman, Sachpazidou, Pinhassi, & Dopson, 2017; Broman, Sjöstedt, Pinhassi, & Dopson,
575 2017; Klindworth et al., 2014; Urich et al., 2014), marine viruses, (Culley, Lang, & Suttle,
576 2006), sediment eukaryotic metatranscriptomes (Broman, Varvara, et al., 2017), nematodes in
577 oxygen deficient sediment (Broman, Bonaglia, et al., 2020), old marine groundwaters in the
578 deep terrestrial biosphere (Lopez-Fernandez et al., 2018), and has also been used in similar
579 environments such as soil ecosystems (Urich et al., 2008). Many studies have used eRNA to
580 study prokaryotes (as mentioned above and e.g. Cottier et al., 2018), however there is paucity
581 of studies using eRNA to assess the biodiversity of microeukaryotes in sediment. In addition,
582 active bacterial communities could also be an interesting method to monitor changes in OC
583 content. Although 18S rRNA metabarcoding has gained popularity to investigate such
584 communities (see e.g. Birrer et al., 2018; Comeau, Lagunas, Scarcella, Varela, & Lovejoy,
585 2019; Rodríguez-Martínez et al., 2020), we have here shown that eRNA shotgun sequencing is
586 also a viable approach to detect differences in diversity and community compositions for
587 multiple communities as a response to different environmental conditions. Even though not
588 directly compared in this study, shotgun sequencing avoids PCR limitations of metabarcoding
589 such as i) PCR primers only targeting certain species; ii) amplifying certain species more than
590 others, and iii) the amount of cycles and type of polymerase used has been shown to influence

591 diversity and community composition (Kelly et al., 2019; Nichols et al., 2018). In addition,
592 eRNA shotgun studies also provide information on all organisms over a large range of trophic
593 stages in the sediment, and it is also possible to study the RNA transcripts of expressed genes
594 to estimate oxidation and reduction processes from prokaryotic metabolism (see e.g. Broman,
595 Sjöstedt, et al., 2017). Finally, common shotgun sequencing bioinformatic pipelines are
596 intricate and include many different software which increases the complexity of the data
597 analysis. Here, conversely, we followed a protocol with few and straightforward steps,
598 including: 1) Quality trimming (removal of Illumina adapters and phiX control sequences,
599 quality trimming, and verifying final quality); 2) extraction of SSU rRNA sequences from the
600 dataset; 3) taxonomic classification of the SSU rRNA sequences using Kraken2 against the
601 NCBI NT and SILVA databases; and 4) estimation of relative abundance at genus level using
602 Bracken2 (for more details on the kraken2+bracken2 combination see Wood et al., 2019).
603 These new bioinformatic tools make it less daunting and possible to classify large datasets
604 containing hundreds of millions of sequences within minutes to hours which would previously
605 have taken several weeks using traditional aligners. As such this approach is closer to current
606 metabarcoding bioinformatic pipelines with relatively straightforward steps (see e.g. the
607 DADA2 pipeline Callahan et al., 2016). Moreover, ongoing developments in machine learning
608 could make eRNA shotgun sequencing a powerful tool for future biomonitoring programmes.
609 Such innovative approaches combined with eRNA shotgun sequencing would allow to bypass
610 some of the database limitations when assigning taxonomy as the data can be incorporated into
611 taxonomy-free models (Cordier et al., 2018; Cordier, Lanzén, Apothéloz-Perret-Gentil, Stoeck,
612 & Pawlowski, 2019).

613

614 **Conclusions**

615 Here we have shown that eRNA shotgun sequencing is a useful tool to study the biodiversity
616 of benthic microeukaryotes. The latest sequencing technology yields tens of million sequences
617 per sample and this makes it possible to investigate the biodiversity of multiple communities.
618 In our study we focused on three microeukaryotic groups (nematodes, forams, and ciliates).
619 We were able to detect a decrease in biodiversity for nematodes and forams in sediments with
620 higher OC, when compared to low OC sediments. Moreover, we detected differences in beta
621 diversity for all three groups between the stations along the OC gradient, as well as in the
622 functional ecology of nematodes (i.e. feeding type). Considering that future sequencing
623 technologies are likely to develop and decrease in cost, shotgun sequencing of eRNA to assess
624 biodiversity of benthic microeukaryotes could be a useful method in recurring monitoring
625 programmes. Taken together, eRNA shotgun sequencing and new bioinformatic tools give the
626 opportunity to simultaneously study a large diversity of microeukaryotes within a reasonable
627 time frame. These methods also make it possible to avoid any biases introduced by PCR
628 amplification, and thus captures the whole environmental diversity in the samples.

629

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639

640 **Competing interests**

641 We have no competing interests.

642

643 **Author contributions**

644 EB designed the study, sampled in the field, conducted molecular laboratory work,
645 bioinformatics, analyzed molecular data, and drafted the manuscript. SB conducted chemistry
646 laboratory work, analyzed chemistry data, and gave feedback on the manuscript. AN sampled
647 in the field and gave feedback on the manuscript. SC provided input on the study and
648 feedback on the manuscript. FJAN designed the study, helped with data analyses, and gave
649 feedback on the manuscript. All authors gave final approval for publication.

650

651 **Data Accessibility**

652 The raw sequence data have been uploaded and are available on the NCBI database with the
653 following BioProject number PRJNA541422 ([dataset] Broman, Bonaglia, Norkko, Creer, &
654 Nascimento, 2020).

655

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1016 **Figures**

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1019 **Figure 1.** Map showing the location of the stations sampled during September 20–23, 2018.

1020 At each station triplicate sediment cores were collected and the top 0–2 cm sediment surface

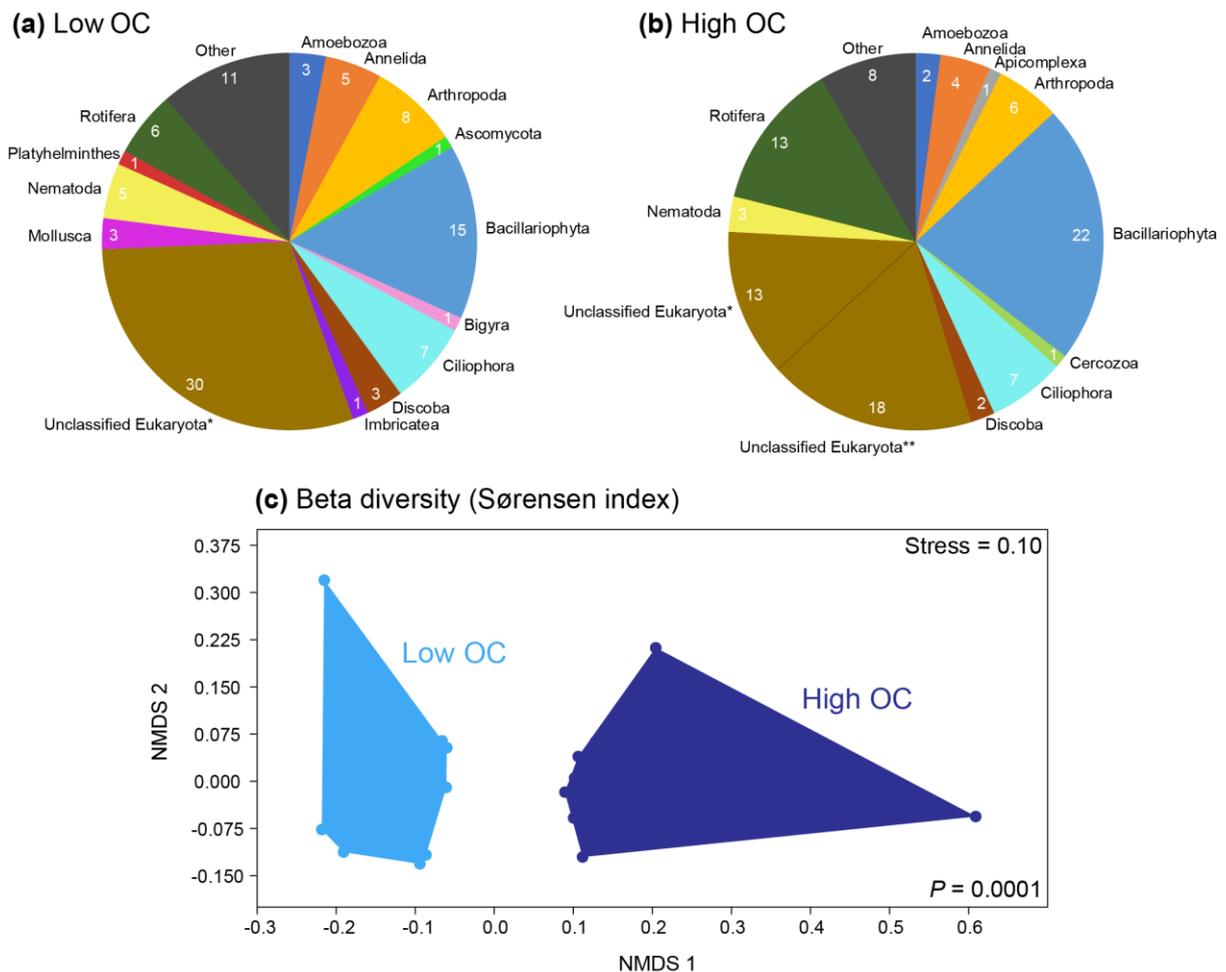
1021 sliced. The study area is located in the Gulf of Finland (Baltic Sea) nearby the Tvärminne

1022 Zoological Station (TZS). The numbers denote each station name. Stations 11, 12, 15, 16 were

1023 grouped as “Low OC”, and stations 7, 10, 13 as “High OC” based on the % OC content. The

1024 map layer is © OpenStreetMap contributors.

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Figure 2. Pie charts showing eukaryotic taxonomic groups on the highest level (based on all 18S rRNA sequences classified against NCBI NT) with an average of > 1% for the (a) Low OC or (b) High OC stations. The pie chart sums to 100%, and the group “Other” shows the total of all groups < 1%. The white numbers inside the charts shows the relative abundance (%) for each slice. Labels with stars denote sequences classified by Kraken2 as Unclassified Eukaryota that were distributed by Bracken2 to protists groups (*) Malawimonadidae and (**) Hemimastigophora. (c) NMDS plots showing the beta diversity of whole eukaryotic community in the sediment surface. The beta diversity was based on the 18S rRNA data and the Sørensen index (presence/absence, labels show station numbers). The light blue shaded area denotes Low OC stations, while dark blue shaded area denotes High OC stations. The P values show the results from PERMANOVA tests between the Low OC and High OC stations.

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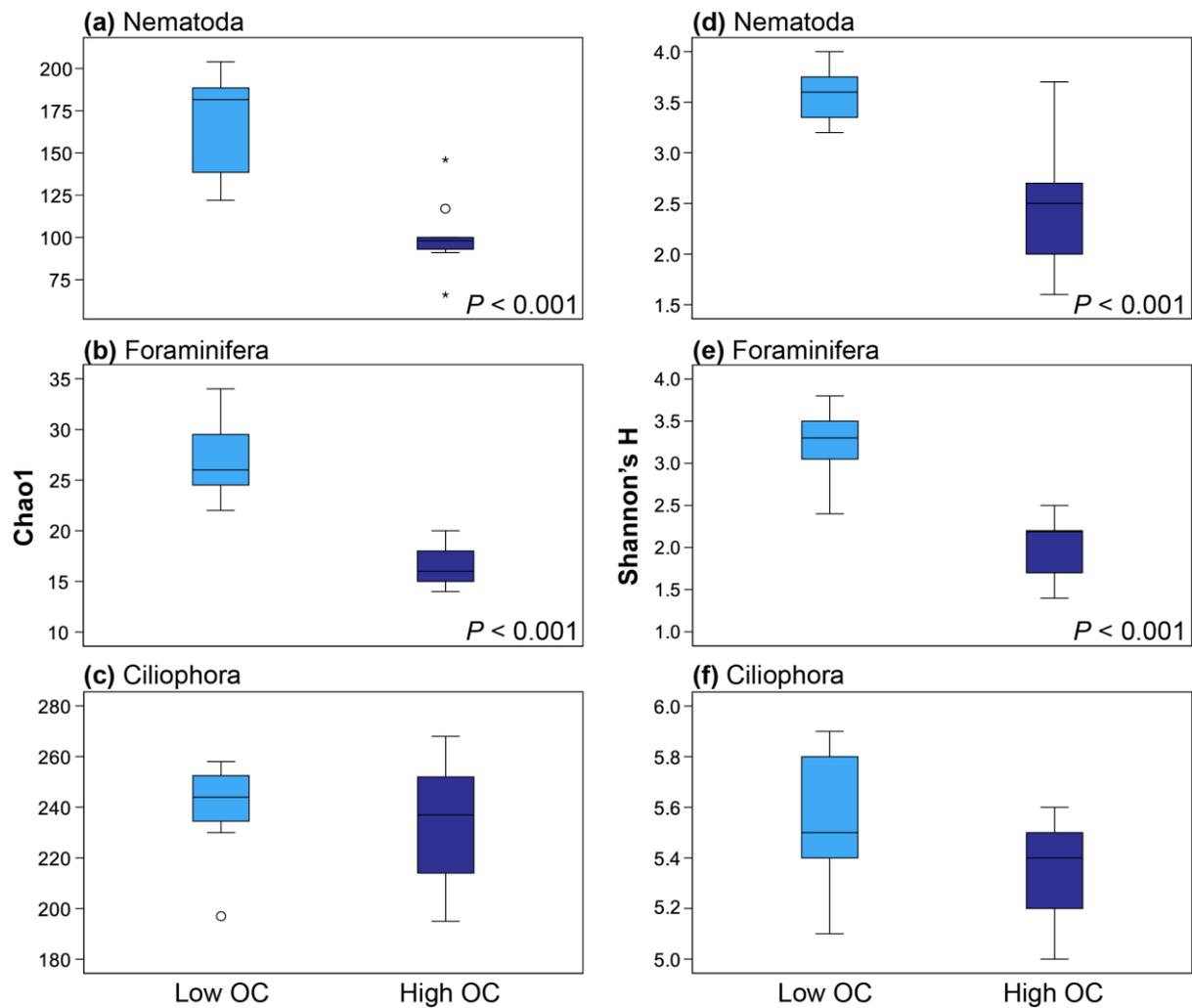
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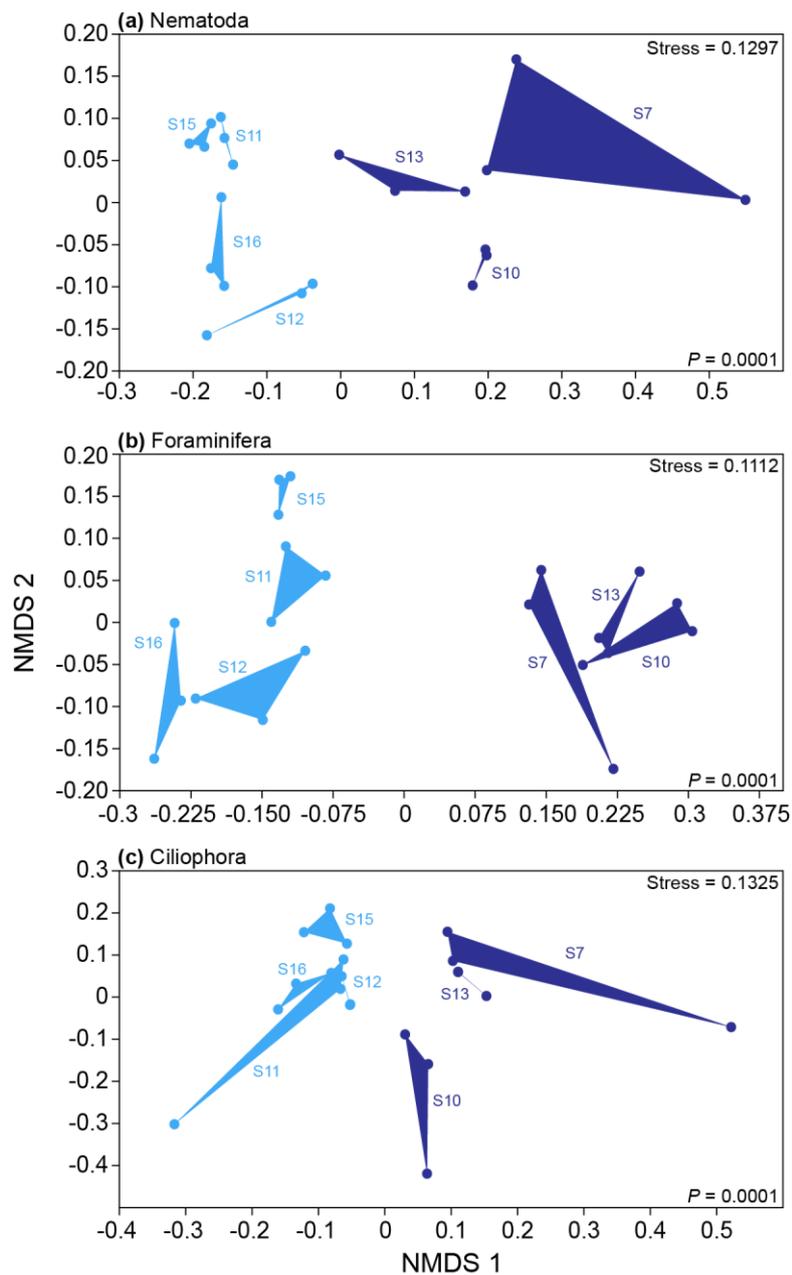
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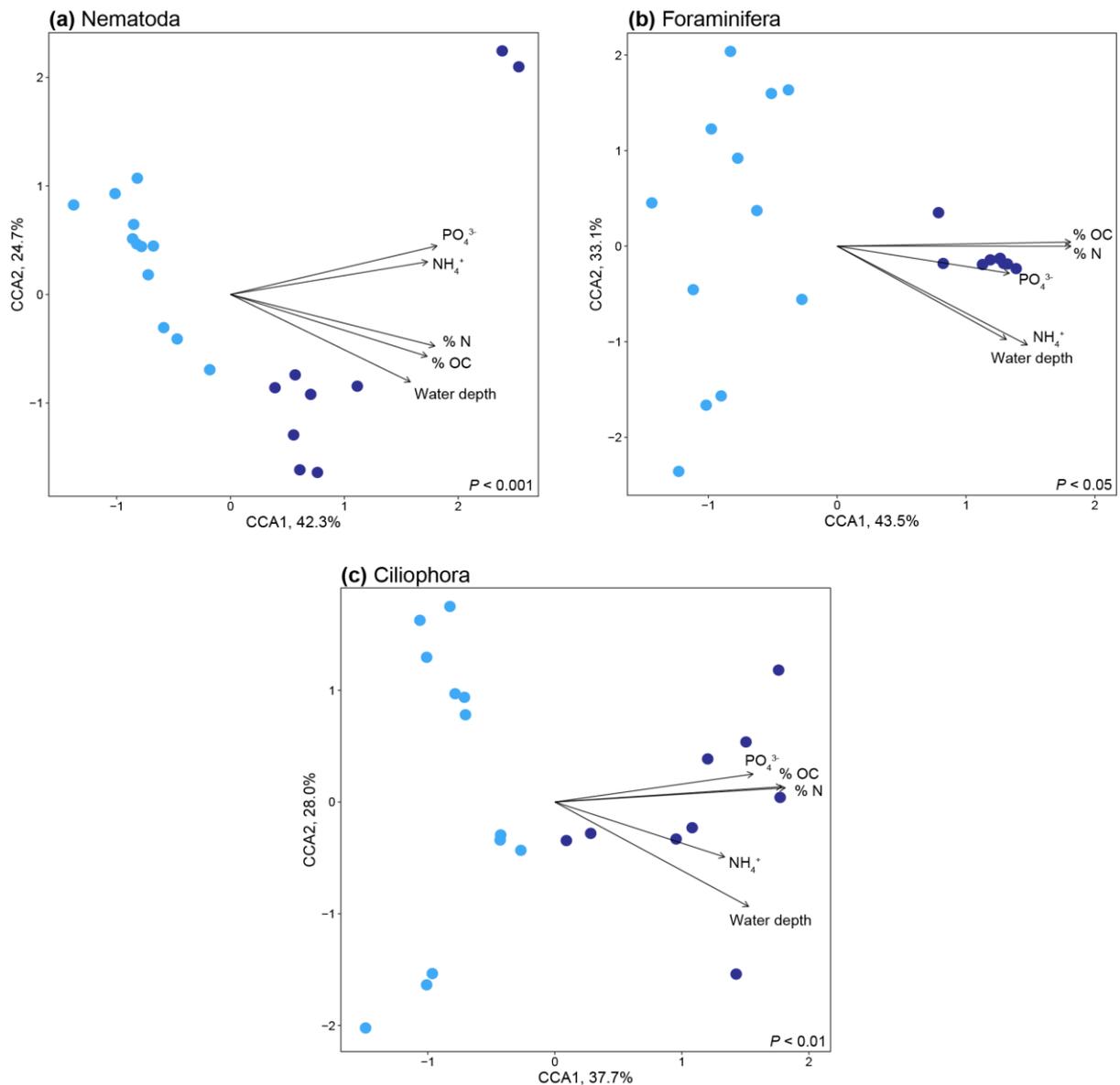
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1039 **Figure 3.** The boxplots show the species richness Chao1 and Shannon's H alpha diversity index
 1040 for the three taxonomic groups studied in the sediment surface in the Low OC and High OC
 1041 stations. The data are based on 18S rRNA sequences extracted from the RNA-seq data, with
 1042 (a–c) showing Chao1 and (d–f) showing Shannon's H. Note the different scale on the y-axes
 1043 between the three taxonomic groups. The *P* values show the results from One-Way ANOVA
 1044 tests between the Low OC and High OC (only shown if statistically significant). The outliers
 1045 denote, circles: 1.5–3 box lengths from the median, and stars: 3 or more box lengths from the
 1046 median.



1047

1048 **Figure 4.** NMDS plots showing the beta diversity of the three studied taxonomic groups in the
 1049 sediment surface, featuring (a) Nematoda, (b), Foraminifera, and (c) Ciliophora. The beta
 1050 diversity was based on the 18S rRNA data and the Sørensen index (presence/absence, labels
 1051 show station numbers). The light blue shaded areas denote Low OC stations, while dark blue
 1052 shaded areas denote High OC stations. The *P* values show the results from PERMANOVA
 1053 tests between the Low OC and High OC stations.



1054

1055 **Figure 5.** CCAs showing the distribution of (a) Nematoda, (b) Foraminifera, and (c) Ciliophora

1056 among the Low OC stations (light blue circles) and High OC stations (dark blue circles). The

1057 data was based on the relative abundances of genera for each taxonomic group. The grey

1058 triplots shows the direction of the measured abiotic variables (water depth, sediment % OC and

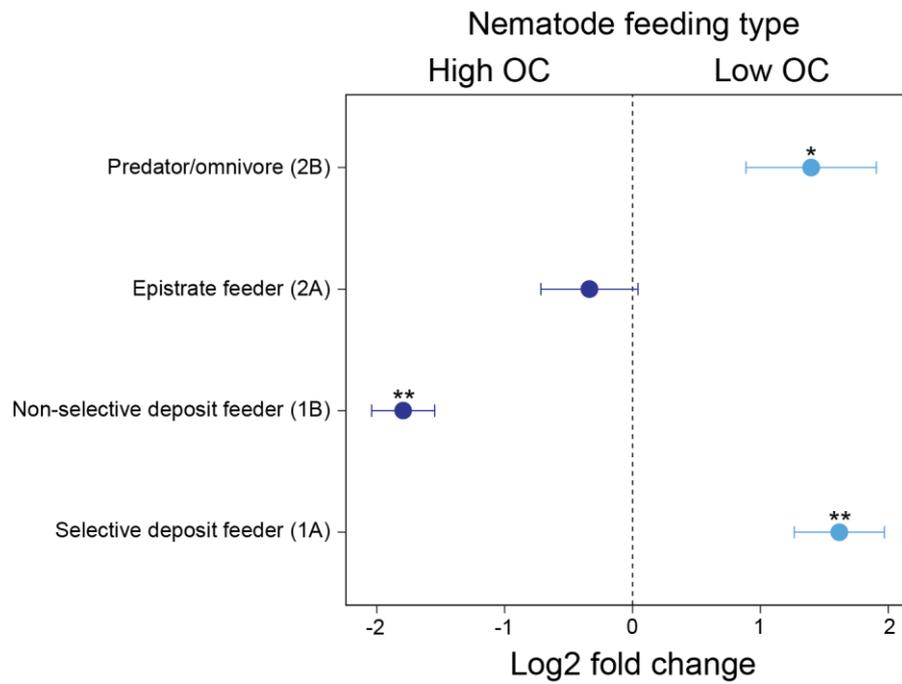
1059 % N, plus pore water NH_4^+ and PO_4^{3-}) in relation to the community composition. NH_4^+ values

1060 were first reported in (Broman, Sun, et al., 2020). Each circle represents one sediment core.

1061 The *P* values shows the statistical significance (PERMANOVA) between the abiotic data and

1062 community composition when tested between Low OC and High OC stations.

1063



1073

1074 **Figure 7.** Nematoda genera were classified into a feeding type category according to Wieser
 1075 (1953) and the plot is based on the sum of all classifications between the Low OC and High
 1076 OC stations. DESeq2 statistical analyzing showed significant differences for all feeding types
 1077 ($FDR < 0.05 = *$, $FDR < 0.01 = **$). Negative log2 fold change values indicate a higher
 1078 prevalence at the High OC stations (dark blue circles), while positive values indicate a higher
 1079 prevalence at Low OC (light blue circles). The errors bars show the standard error.