Received: 31 January 2020 | Revised: 29 June 2020 | Accepted: 18 July 2020
DOI: 10.1111/mec.15561

SPECIAL ISSUE

MOLECULAR ECOLOGY WILEY

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

Elias Broman[1,2] | Stefano Bonaglia[1,3,4] | Alf Norkko[2,5] | Simon Creer[6] | Francisco J.A. Nascimento[1,2]

[1]Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden
[2]Baltic Sea Centre, Stockholm University, Stockholm, Sweden
[3]Nordcee, Department of Biology, University of Southern Denmark, Odense, Denmark
[4]Department of Marine Sciences, University of Gothenburg, Gothenburg, Sweden
[5]Tvärminne Zoological Station, University of Helsinki, Hanko, Finland
[6]Molecular Ecology and Fisheries Genetics Laboratory, School of Natural Sciences, Bangor University, Bangor, UK

Correspondence
Elias Broman, Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden
Email: elias.broman@su.se

Funding information
Svenska Forskningsrådet Formas, Grant/Award Number: 2017-01513; Science for Life Laboratory; Knut and Alice Wallenberg Foundation; Stockholm University

Abstract

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

KEYWORDS
biodiversity, community ecology, environmental RNA, organic carbon, sediment, shotgun sequencing

---

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2020 The Authors. Molecular Ecology published by John Wiley & Sons Ltd

1 | INTRODUCTION

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

Biodiversity assessments of benthic macrofauna are commonly used in national monitoring programs, including coastal zones, to determine various ecological indices (Pinto et al., 2009). However, microeukaryotes present in sediment such as meiofaunal nematodes (<1 mm body size) are also known to react to e.g., eutrophication status (Ristau, Spann, & Traunspurger, 2015), and the composition and quantity of organic carbon (OC) (Ingels, Kiriakoulakis, Wolff, & Vanreusel, 2009; Pusceddu, Gambi, Zeppilli, Bianchelli, & Danovaro, 2009). Furthermore, because nematodes are known to have different feeding behaviors such as bacterivory, detritivory, or algal feeding (Moens, Traunspurger, & Bergtold, 2006; Wieser, 1953), changes in nematode assemblages are therefore likely to affect food web dynamics and ecosystem function (e.g., Nascimento et al., 2019; Nascimento, Karlson, & Elmgren, 2008; Nascimento, Näslund, & Elmgren, 2012). In sediment with high quantity of labile OM nonselective deposit feeding nematodes have been observed to be prevalent (e.g., Ingels et al., 2009). Other arguments for including meiofauna such as nematodes in national monitoring systems include their high diversity, short generation time, and ubiquitous distribution (Kennedy & Jacoby, 1999). However, these organisms are often neglected in monitoring studies (Kennedy & Jacoby, 1999), probably due to financial reasons derived from time consuming activities such as sieving, sorting, and microscopic morphological analyses.

In addition, the protist phyla Foraminifera (henceforth forams) and Ciliophora (i.e., ciliates) are well-studied as bioindicators of environmental state of aquatic ecosystems. The diversity and community composition of forams are known to change with anthropogenic pollution, fish farming, and decreasing water quality (Damak, Frontalini, Elleuch, & Kallel, 2019; Frontalini & Coccioni, 2011; Pawlowski, Esling, Lejzerowicz, Cedhagen, & Wilding, 2014; Raposo et al., 2018; Uthicke & Nobes, 2008), and similar to nematodes, OC enrichment of the sediment also influences the diversity of forams (Alve et al., 2016; Martins et al., 2015; Murray, 2006). Ciliates are used as bioindicators in e.g., aquaculture (Stoeck, Kochems, Forster, Lejzerowicz, & Pawlowski, 2018), wastewater treatment plants, and monitoring of eutrophication and chemical pollution (Chen, Xu, Tam, Cheung, & Shin, 2008; Foissner, 2016; Pawlowski, Lejzerowicz, Apotheloz-Perret-Gentil, Visco, & Esling, 2016). In natural aquatic environments, the diversity and community composition of ciliates are influenced by e.g., salinity, pH, and anthropogenic pollution (e.g., Gong et al., 2015; Jiang, Xu, Hu, Warren, & Song, 2013). One of the main merits of assessing the diversity of protists as bioindicators is their documented rapid change to environmental conditions (Payne, 2013). The assessment of microeukaryotic biodiversity is therefore a good proxy in monitoring programmes to study changes in ecosystems. However, these communities are rarely studied together and challenges still include being able to investigate multiple communities from bulk sediment samples without time consuming activities involved in studying the benthic microeukaryotic fraction such as sieving, sorting, and microscopy.

In the last 10 years, environmental DNA (eDNA) and RNA (eRNA) metabarcoding studies targeting the 18S rRNA marker gene (or 18S rRNA for eRNA) have been extensively conducted to study microeukaryotes (Creer et al., 2016; Forster et al., 2019; Pochon et al., 2015). Such tools have drastically reduced the time needed to taxonomically classify organisms compared to morphological taxonomic techniques that also involves sieving and sorting of organisms (Carugati, Corinaldesi, Dell'Anno, & Danovaro, 2015). However, limitations exist with metabarcoding such as nonoptimized PCR protocols and primer bias when targeting multiple taxa (Kelly, Shelton, & Gallego, 2019), and limitations of available species in reference databases when taxonomically classifying sequences. Compared to metabarcoding that typically yields ~60,000 sequences per sample (Singer, Fahner, Barnes, McCarthy, & Hajibabaei, 2019) shotgun sequencing can generate millions of sequences per sample. New bioinformatic tools that can today taxonomically classify these large data sets within minutes to hours (e.g., Wood, Lu, & Langmead, 2019) and estimate relative abundances at species or genus level (e.g., Lu, Breitwieser, Thielen, & Salzberg, 2017). However these methods rely on the reference databases to classify taxonomy and are therefore likely to become more precise over time when databases grow. While eDNA makes it possible to assess the biodiversity of both living organisms plus nondegraded DNA from dead organisms, eRNA is targeting mainly living organisms or RNA derived from organisms of recent origin in the environment (Cristescu, 2019; Wood et al., 2020). It is therefore valuable to investigate if eRNA combined with shotgun sequencing, thereby bypassing PCR limitations of metabarcoding, is a useful approach to assess differences in the biodiversity of active multiple communities from highly diverse and densely inhabited environments such as sediments.

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

2 | MATERIALS AND METHODS

2.1 | Field sampling

Sediment was collected on board R/V Electra during 20–23 September 2018 in the Gulf of Finland (Baltic Sea) close to the Tvärminne Zoological Station, Finland. A total of seven stations were visited along coastal gradients in depth and OC (0–4 km, 10–45 m water depth; Figure 1). The bottom water in the study areas at the time of sampling was oxic with 7.6–8.7 ml/L O_2 measured by oxygen probes equipped on a CTD instrument (full details in Broman, Sun, et al., 2020). The stations were divided into four low % OC shallow sites (stations 11, 12, 15, 16; 1.2%–2.8% OC) and three sites with higher % OC and depth (stations 7, 10, 13; 6.2%–6.9% OC), following a station labelling system used during reoccurring monitoring in the Tvärminne region (Table 1). Triplicate sediment cores (labelled A, B, C), retrieved in rinsed acrylic core liners, were collected from each station with a GEMAX twin gravity corer (height: 80 cm, inner diameter: 90 mm). The top 0–2 cm sediment surface layer was sliced into autoclaved 215 ml polypropylene containers (Noax Laboratory). After slicing, the sediment was directly aseptically homogenized inside the containers and 2 cm^3 sediment transferred into 2 ml cryogenic tubes (VWR) that were immediately flash frozen at −196°C. The samples were transported on dry ice and stored at −80°C until RNA extraction. The remaining sediment in the 215 ml containers were stored at −20°C for sediment C and N content and pore water chemistry analyses.

2.2 | Sediment and pore water chemistry analyses

The remaining sediment in the frozen 215 ml containers were thawed, homogenized, and 15 cm^3 sediment from each

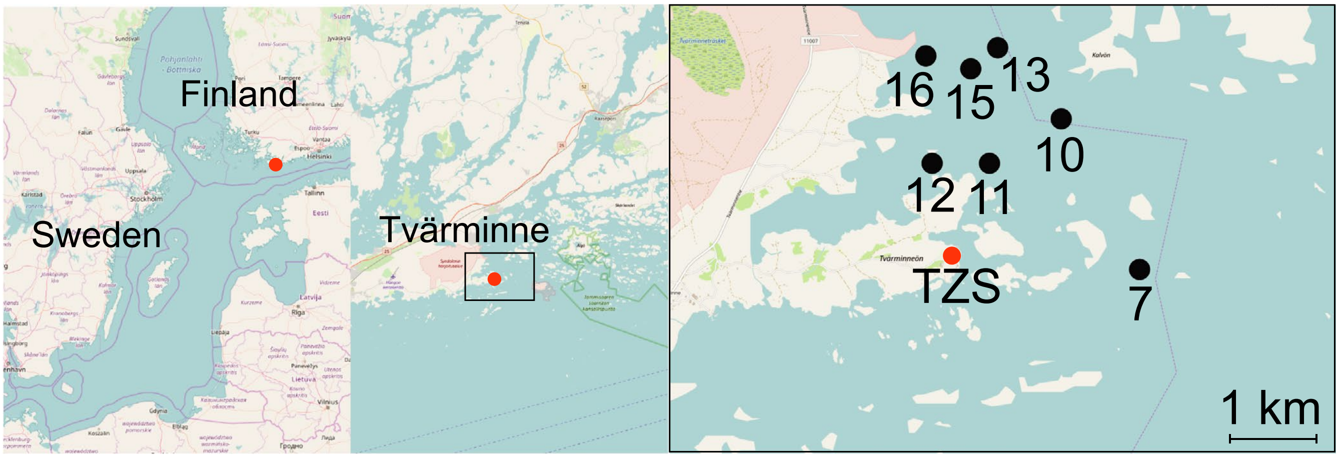

FIGURE 1 Map showing the location of the stations sampled during 20–23 September 2018. At each station triplicate sediment cores were collected and the top 0–2 cm sediment surface sliced. The study area is located in the Gulf of Finland (Baltic Sea) nearby the Tvärminne Zoological Station (TZS). The numbers denote each station name. Stations 11, 12, 15, 16 were grouped as "Low OC", and stations 7, 10, 13 as "High OC" based on the % OC content. The map layer is © OpenStreetMap contributors

Station	Category	*n*	Date	Lat. (dd)	Long. (dd)	Depth (m)	% OC
12	Low OC	3	22 Sep	59.8521	23.24495	10	2.4 ± 0.1
16	Low OC	3	22 Sep	59.8613	23.24387	10	2.1 ± 0.3
11	Low OC	3	20 Sep	59.8521	23.25475	18	1.4 ± 0.0
15	Low OC	3	20 Sep	59.8602	23.25155	28	1.3 ± 0.0
10	High OC	3	20 Sep	59.8559	23.26695	36	6.3 ± 0.1
7	High OC	3	23 Sep	59.8430	23.28035	37	6.6 ± 0.1
13	High OC	3	22 Sep	59.8620	23.25615	40	6.7 ± 0.1

TABLE 1 List of the station numbers, their classified category based on the sediment % OC (Low OC or High OC), the number of sediment cores collected (top 0–2 cm) for RNA extraction and pore water analyses, sampling dates, latitude, longitude, water column depth, and the measured sediment % OC at each station (mean ± SE)

sample was dried at 60°C for seven days for C/N analysis. In addition, 20 cm^3 of sediment from each sample was centrifuged at 2,200 × *g* to extract the pore water for ammonium (NH_4^+) and phosphate (PO_4^{3-}) analyses. The dried sediment was ground, homogenized, and 1 cm^3 dry weight sediment per sample stored in a desiccator prior to freeze drying, regrinding, rehomogenization and treated with HCl to remove inorganic carbon. Samples were subsequently weighed into tin capsules. Concentrations of total OC and total nitrogen were determined on an elemental analyser (Flash 2000, Thermo Scientific). The pore water was collected after centrifugation by filtering 10 ml of the supernatant through a 0.45 μm polyethersulfone membrane filter (Filtropur S 0.45, Sarstedt). NH_4^+ and PO_4^{3-} were determined colorimetrically (Multiskan GO spectrophotometer, Thermo Scientific) and NH_4^+ analysis followed the modified salicylate-hypochlorite method by Bower and Holm-Hansen (1980), and PO_4^{3-} analysis followed the standard methods for seawater analyses (Grasshoff, Kremling, & Ehrhardt, 2009). NH_4^+ values were first reported in Broman, Sun, et al. (2020).

2.3 | RNA extraction and sequencing

Sediment was thawed within minutes inside the cryotubes and ~2 g of material was added into the RNeasy PowerSoil bead tubes and was extracted using the same kit (RNeasy PowerSoil, QIAGEN). After RNA extraction, any remaining DNA was removed with DNase treatment using the TURBO DNA-free kit (Invitrogen), followed by bacterial rRNA depletion using the RiboMinus Transcriptome Isolation Kit (bacteria version, ThermoFisher Scientific). A 2,100 Bioanalyser (Agilent) was used to confirm that no DNA contamination was present in the samples. Library preparation followed the TruSeq RNA Library Prep v2 kit (Illumina) without including the poly-A selection step. This procedure does not include an amplification of a marker gene and therefore avoids PCR limitations common for metabarcoding studies as mentioned in the introduction. The samples were sequenced at the Science for Life Laboratory, Stockholm on a single Illumina NovaSeq6000 S4 lane using paired-end 2 × 150 bp read technology. A full list of sample names, sequences yielded, quality scores, read lengths etc. are available in Data S1.

2.4 | Bioinformatics

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

Small subunit (SSU) rRNA sequences were extracted from the quality trimmed data using SortMeRNA 2.1b (Kopylova, Noé, & Touzet, 2012) with the databases supplied with the software. Taxonomic classification was conducted with Kraken2 2.0.7 (Wood et al., 2019) using paired-end reads against the SILVA SSU r132 NR99 (Quast et al., 2013) (database downloaded 1 March 2019) and NCBI NT database (database downloaded 12 March 2019). Kraken2 uses a k-mer based approach to classify sequences, and a lowest common ancestor (LCA) algorithm to determine where unclassified sequences belong on a taxonomic tree (Wood et al., 2019). To estimate the relative abundance of each taxon Bracken 2.5 was used on the Kraken2 outputs with default settings set to genus level (i.e., a count threshold of 10) (Lu et al., 2017). Bracken 2.5 uses a Bayesian algorithm method to estimate the genus level read abundance (or species, we chose genus for higher accuracy) of Kraken2 sequences classified higher up on the taxonomic tree (Lu et al., 2017; Wood et al., 2019). This is important, because without estimating read abundance to genus the unclassified reads on higher taxonomic levels will underestimate relative abundances of genera (Lu et al., 2017). The Bracken output reports were combined into a biom-format file with the python package kraken-biom 1.0.1 (using parameters: ---fmt hdf5 -max D --min G), and the biom-format file was converted to a tax table using the python package biom-format 2.1.7 (McDonald et al., 2012).

The 18S rRNA eukaryotic data was extracted, normalized as relative abundances (%), and analysed in the software Explicet 2.10.5 (Robertson et al., 2013). Results for (a) Nematoda (NCBI NT classifications, on average 478,331 sequences per sample); (b) Foraminifera (NCBI NT, average 13,913 sequences); and (c) Ciliophora (SILVA, average 774,027 sequences) were extracted and analysed separately. The NCBI NT database was used for the Nematoda and Foraminifera data because (a) the SILVA database is known to contain errors in the nematode classifications (Broman et al., 2019; Holovachov, Haenel, Bourlat, & Jondelius, 2017), and the NCBI NT has previously been used to discern differences in nematode communities on a spatial scale in the Baltic Sea (Broman et al., 2019); and (b) the SILVA database gave inaccurate classifications for Foraminifera, resulting in the identification of taxa never discovered in the Baltic Sea (more details in the discussion).

2.5 | Nematoda functional ecology analyses

Nematode genera were classified into feeding types based on their known buccal cavity morphology in available literature according to Wieser (1953). Each genus was categorized into the four feeding types described by Weiser: (1a) selective deposit feeder; (1b) nonselective deposit feeder; (2) epistrate feeder; and (2b) predator/omnivore. In addition, the maturity index (MI) of each nematode community was calculated to infer changes in the life history characteristics of nematode genera. MI was calculated according to Bongers, Alkemade, and Yeates (1991) by assigning colonizer–persister (cp) values to nematode genera on a scale from 1 to 5 based on available literature. Low cp-values indicate nematode genera that can be classified as colonizers (short life cycle, high reproduction rates, high colonization ability and tolerance to disturbance) while high cp-values represent persisters (nematode genera that display long life cycles, few offspring, low colonization ability and high sensitivity to disturbance). MI could then be calculated from:

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

where $\nu(i)$ is the cp-value of genus *i* and *f(i)* is the frequency of genus *i*.

2.6 | Statistical analysis

Rarefaction curves of sequence counts versus the taxonomic classifications were conducted in the R package vegan 2.5.6 (Oksanen et al., 2018) using the rrarefy function with default settings. Species richness (Chao1) and alpha diversity (Shannon's H) were calculated in the software Explicet 2.10.5 for each taxonomic group (Nematoda, Foraminifera, and Ciliophora). Before calculating Shannon's H index the data was subsampled to the lowest sample size and bootstrapped × 100 (Nematoda 79,815 counts, Foraminifera 2,473 counts, Ciliophora 299,504 counts). Nonmetric multidimensional scaling (NMDS) plots showing beta diversity were based on the presence/absence (Sørensen dissimilarity index) and Bray-Curtis dissimilarity index (based on relative abundance) using the software past 3.26 (Hammer, Harper, & Ryan, 2001). The difference in read abundance between the high and low OC stations for Nematoda feeding type data was normalized and statistically tested using the R package DESeq2 1.26 with default settings (Love, Huber, & Anders, 2014). The DESeq2 output was plotted using the ggplot2 package in R (Wickham, 2016). Differences between groups on alpha diversity metrics (Chao1, Shannon's H), relative abundance of taxonomic groups, and maturity index for nematodes were tested with univariate statistics conducted in the software IBM SPSS Statistics 26. First, Shapiro-Wilk tests were used to check if the data was normally distributed. Differences between groups in normally distributed data were tested with One-Way ANOVA tests, while nonparametric data were tested with Mann-Whitney U tests. PERMANOVA tests (9,999 permutations) were conducted in the software past 3.26 and used to identify differences in beta diversity between stations, based on presence/absence, relative abundance, and Hellinger transformed data (i.e., square rooted relative abundances) (Legendre & Gallagher, 2001). To investigate if the abiotic variables (% OC, % N, PO_4^{3-}, NH_4^+, and water depth) were associated with the community composition canonical correspondence analysis (CCA) was conducted in the R package vegan 2.5.6 with the *cca* function and plotted using the ggplot2 3.2.1 package. The input data for the CCAs included the measured abiotic variables and relative abundances of the different taxa. Significant associations between abiotic variables and community compositions were tested for CCA axis 1 and axis 2 with PERMANOVA tests (9,999 permutations) using the function envfit included in the vegan package. To detect if measured abiotic variables (rather than solely water depth) significantly explained community compositions of the three studied groups the adonis2 function in the R package vegan 2.5.6 was used. The Bray-Curtis dissimilarity matrix for each study group was loaded with the abiotic data and the abiotic variables were added in sequential order after water depth. Mantel tests (Mantel, 1967) of the of Bray-Curtis dissimilarity distances were used to test the correlation with OC by using the function mantel.rtest in the R package ade4 1.7.13 (Dray & Dufour, 2007), after turning the OC data to a distance matrix with the dist function with default settings.

3 | RESULTS

3.1 | Field data and sediment characteristics

Both OC and N content in the sediment were higher at the deeper stations, i.e., >30 m water depth (6.5% ± 0.0% OC and 0.9 ± 0.0% N) when compared to the shallow stations, i.e., <30 m water depth (mean ± SE, 1.8% ± 0.0% OC and 0.2% ± 0.0% N; Mann-Whitney

U tests, $U = 0$, $p = .000007$ for both; Table 1). Hereafter the shallow stations will therefore be referred to as "Low OC" and the deep stations as "High OC". Pore water NH_4^+ and PO_4^{3-} extracted from the top 0–2 cm sediment layer at the seven sampled stations (Table 1; Figure 1, $n = 3$ for each station) correlated positively with water depth (both $p < .01$, Pearson correlations, $r = 0.83$ and $r = 0.64$, respectively). NH_4^+ was significantly higher at High OC stations (308 ± 19.8 µg/L, $n = 12$) compared to the Low OC stations (196 ± 14.3 µg/L ($n = 9$; Mann-Whitney U test, $U = 10$, $p = .002$). Similarly, pore water PO_4^{3-} was significantly higher at High OC stations (32.2 ± 7.0 µg/L) compared to the Low OC (4.4 ± 0.5 µg/L; Mann-Whitney U test, $U = 1$, $p = .000140$). A full list of abiotic data for all stations is available in Data S2.

3.2 | Sediment 18S rRNA community

The most abundant microeukaryotic taxonomic groups in our sediments included nematodes, arthropoda (mainly copepods), rotifers, and single-celled eukaryotes such as Bacillariophyta (mainly diatoms), ciliates, and Kraken2 unclassified eukaryotic sequences that Bracken2 distributed to protists Malawimonadidae and Hemimastigophora (Figure 2a,b; Data S3). There was a significant difference in community composition when testing the stations grouped as Low OC against High OC (Sørensen dissimilarity index test (presence/absence data), PERMANOVA, pseudo $F = 6.71$, $p = .0001$; Figure 2c). This was also significant when tested with Bray-Curtis dissimilarity index based on relative abundance data (PERMANOVA,

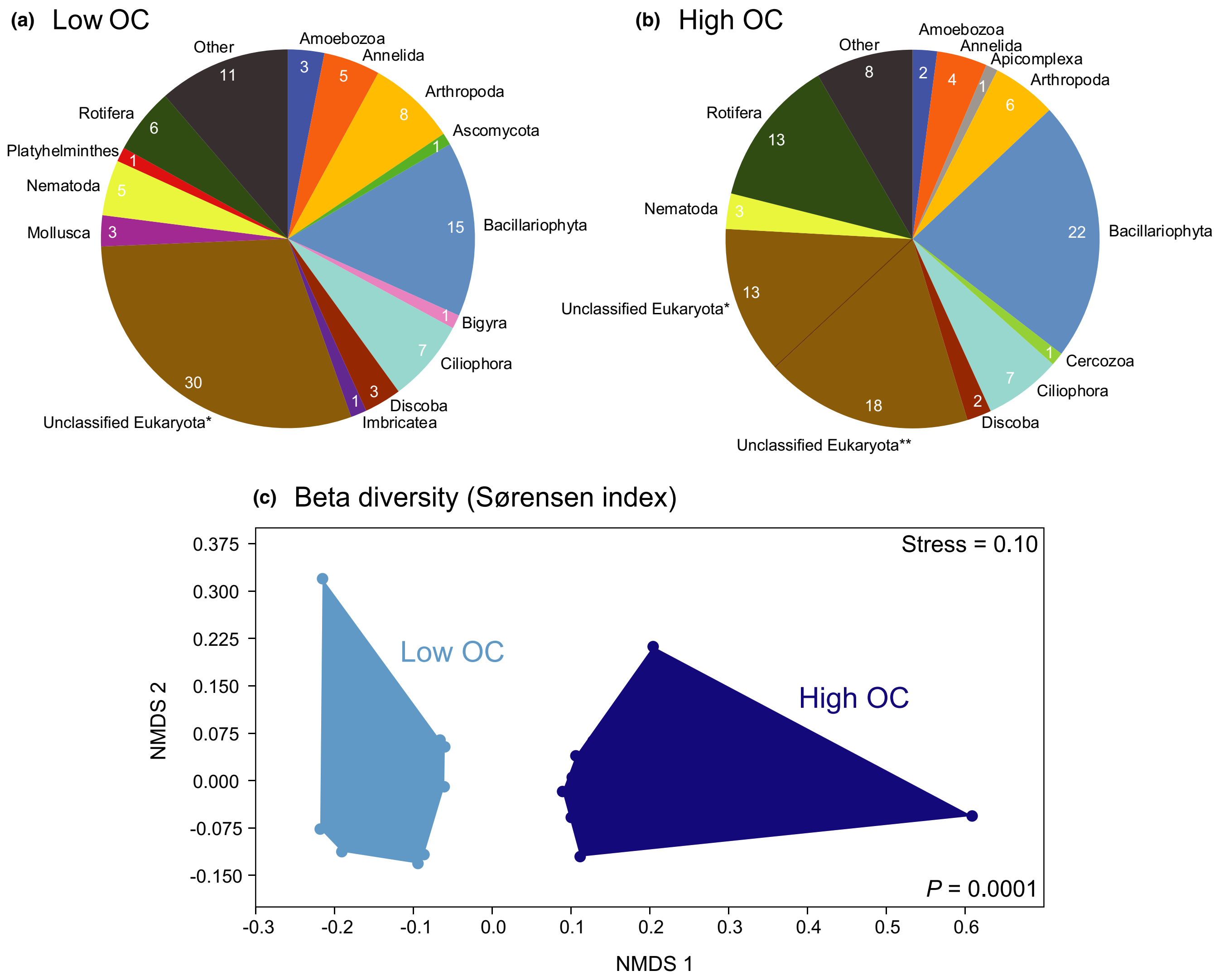

FIGURE 2 Pie charts showing eukaryotic taxonomic groups on the highest level (based on all 18S rRNA sequences classified against NCBI NT) with an average of > 1% for the (a) Low OC or (b) High OC stations. The pie chart sums to 100%, and the group "Other" shows the total of all groups < 1%. The white 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

pseudo $F = 4.73$, $p = .0007$), as well as Hellinger transformed data (pseudo $F = 5.36$, $p = .0001$). For this study we focused on three microeukaryotic groups used in biomonitoring: Nematoda (average 4% of all eukaryotes), Foraminifera (average 0.15% of all eukaryotes), and Ciliophora (average 7% of all eukaryotes).

3.3 | Alpha and beta diversity for nematodes, foraminifera, and ciliates

Rarefaction analyses showed that the majority of the genera had been detected in the samples (Figure S1). The species richness Chao1 index and Shannon's H alpha diversity index were significantly lower for Nematoda and Foraminifera at the High OC stations compared to Low OC (Chao1: One-Way ANOVA test for each group; Nematoda, $F_{(1,19)} = 32.7$, $p = .000016$; Foraminifera, $F_{(1,19)} = 57.0$, $p = .0000004$; Figure 3a,b; Shannon's H: Nematoda, $F_{(1,19)} = 24.8$, $p = .000083$; Foraminifera, $F_{(1,19)} = 48.2$, $p = .000001$; Figure 3d,e). No significant difference in species richness or Shannon's H alpha diversity was observed for Ciliophora when comparing High OC stations with Low OC (Figure 3c). A full list of Shannon's H values is available in Data S4.

Beta diversity was also significantly different between stations for all three groups, with the presence/absence Sørensen dissimilarity index test (PERMANOVA) when testing the stations grouped as Low OC against High OC (PERMANOVA) test for each group,

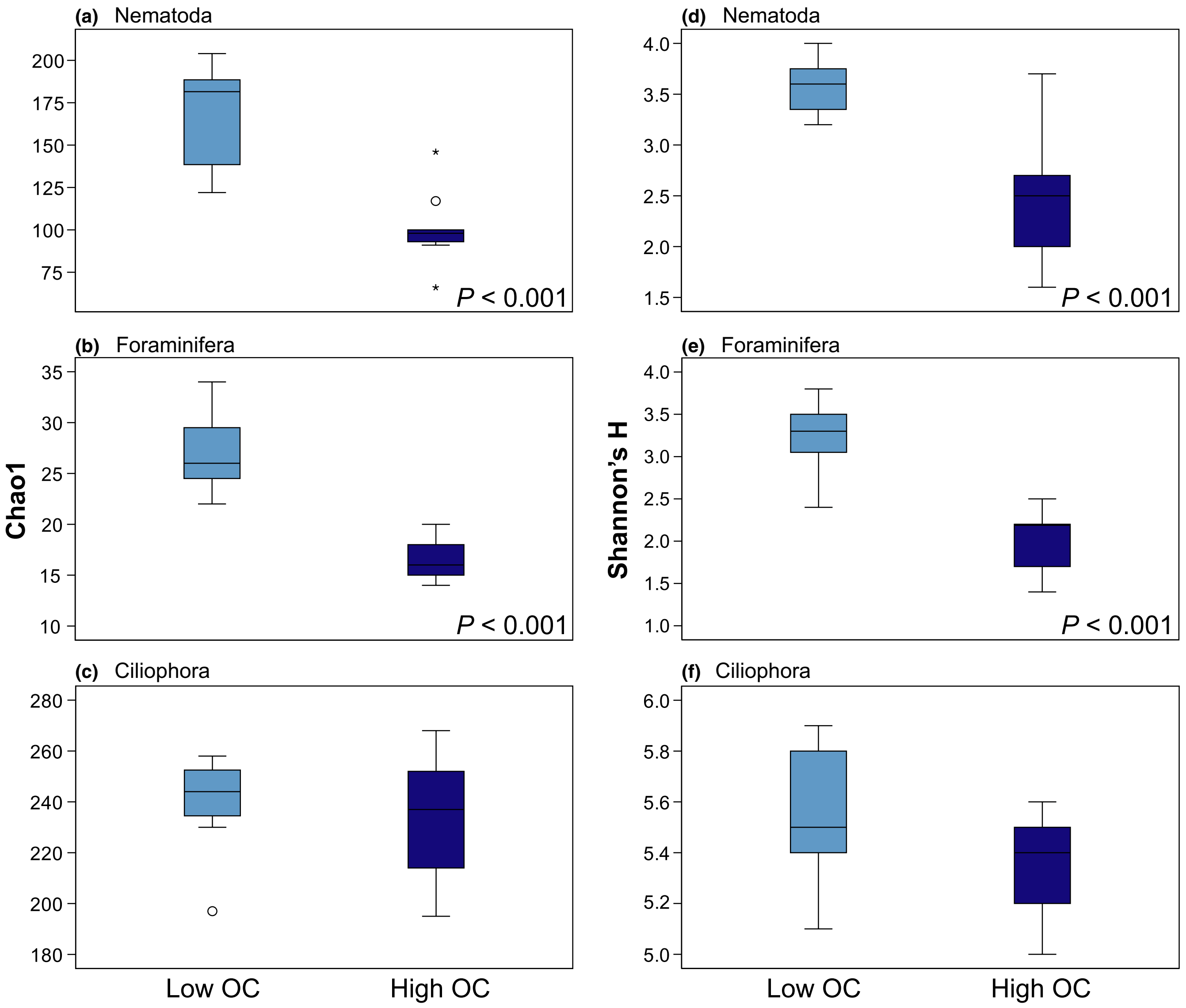

FIGURE 3 The boxplots show the species richness Chao1 and Shannon's H alpha diversity index for the three taxonomic groups studied in the sediment surface in the Low OC and High OC stations. The data are based on 18S rRNA sequences extracted from the RNA-seq data, with (a–c) showing Chao1 and (d–f) showing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

Nematoda, pseudo $F = 11.4$, $p = .0001$; Foraminifera, pseudo $F = 25.5$, $p = .0001$; Ciliophora, pseudo $F = 5.1$, $p = .0001$; Figure 4). Similar results were also observed when using the Bray-Curtis dissimilarity index based on relative abundances as well as Hellinger transformed data (Figure S2).

CCAs based on the relative abundance of genera showed that the measured abiotic variables (water depth, sediment % OC and % N, plus pore water NH_4^+ and PO_4^{3-}) were associated with the High OC stations for all of the three studied taxonomic groups, i.e. Nematoda, Foraminifera, and Ciliophora (Figure 5). The CCA analysis showed that 67%, 77% and 66% of the total constrained inertia for nematodes, foraminifera, and ciliates, was explained with the five environmental variables here studied, respectively. There was also a significant association between all five abiotic variables and the community composition for each studied group (PERMANOVA test, Nematoda, $R^2 = 0.76–0.83$, $p < .001$; Foraminifera, $R^2 = 0.52–0.94$, $p < .05$; Ciliophora, $R^2 = 0.53–0.85$, $p < .01$; Data S5). Moreover, adonis PERMANOVA tests showed that OC was a significant variable determining community composition for all three taxonomic groups, even when accounting for the variance explained by depth (Nematoda pseudo $F = 6.06$, Foraminifera pseudo $F = 12.85$, Ciliophora pseudo $F = 6.01$; all $p < .001$; see Data S5 for results of all variables). OC was also tested separately with mantel tests with the Bray-Curtis dissimilarity distances for each of the three taxonomic groups, and was positively correlated with the community composition (Nematoda $r = 0.58$; Foraminifera, $r = 0.76$; Ciliophora, $r = 0.54$; all $p = .0001$).

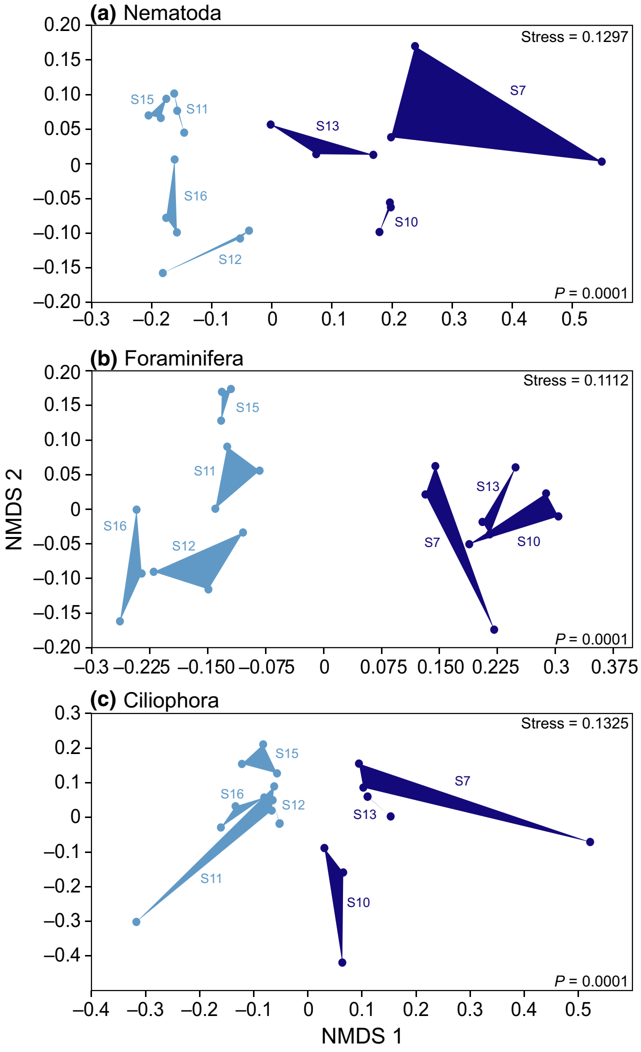

FIGURE 4 NMDS plots showing the beta diversity of the three studied taxonomic groups in the sediment surface, featuring (a) Nematoda; (b) Foraminifera; and (c) Ciliophora. The beta diversity was based on the 18S rRNA data and the Sørensen index (presence/absence, labels show station numbers). The light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

3.4 | Differences in Nematoda community structure

The Nematoda 18S rRNA data set showed differences in community composition along the OC gradient. This included e.g., the nematode genus *Sabatieria* that was detected at all stations and had a significantly higher relative abundance at the High OC stations when compared to Low OC (52.2% ± 7.9% compared to 22.2% ± 3.5% (% denote portion of Nematoda community, Mann-Whitney U test, $U = 16$, $p = .006$; Figure 6a). Similarly, the genus *Axonolaimus* had a higher relative abundance at High OC stations (8.9% ± 2.3% compared to 0.7% ± 0.2%, Mann-Whitney U test, $U = 0$, $p = .000007$; Figure 6a). In contrast, in the Low OC stations the genera *Daptonema* (19.7% ± 3.0%) and *Desmolaimus* (4.8% ± 0.7%) had a significantly higher relative abundance when compared to High OC with *Daptonema* (4.0% ± 0.7%, $U = 2$, $p = .000027$) and *Desmolaimus* (0.7% ± 0.2%, $U = 1$, $p = .000014$) (Mann-Whitney U tests; Figure 6a).

3.5 | Differences in Foraminifera community structure

Looking closer at Foraminifera, the genera with a high relative abundance such as *Elphidium* had a significantly higher relative abundance among the Foraminifera at the High OC stations (66.3% ± 2.7%) compared to Low OC (16.2% ± 2.1% (% denote portion of Foraminifera community); Mann-Whitney U test, $U = 0$, $p = .000007$; Figure 6b). On the other hand, the genus *Rhizammina* had a significantly higher relative abundance at the Low OC stations (17.9% ± 3.1%) when compared to High OC (1.0% ± 0.8%; Mann-Whitney U test, $U = 2$, $p = .000027$; Figure 6b). In addition, we also detected genera with a low relative abundance that showed a significant difference, although with high variation, between Low OC and High OC stations. For example, *Globobulimina* had a higher relative abundance at Low OC (0.8% ± 0.7% compared to 0.0% ± 0.0% at High OC), while both *Nonionella* and *Virgulinella* had a higher relative abundance at High OC sites (1.5% ± 0.6% and 2.6% ± 1.7% compared to 0.4% ± 0.3% and 1.7% ± 2.7% at Low OC, respectively; Mann-Whitney U tests, $p < .05$; Figure 6).

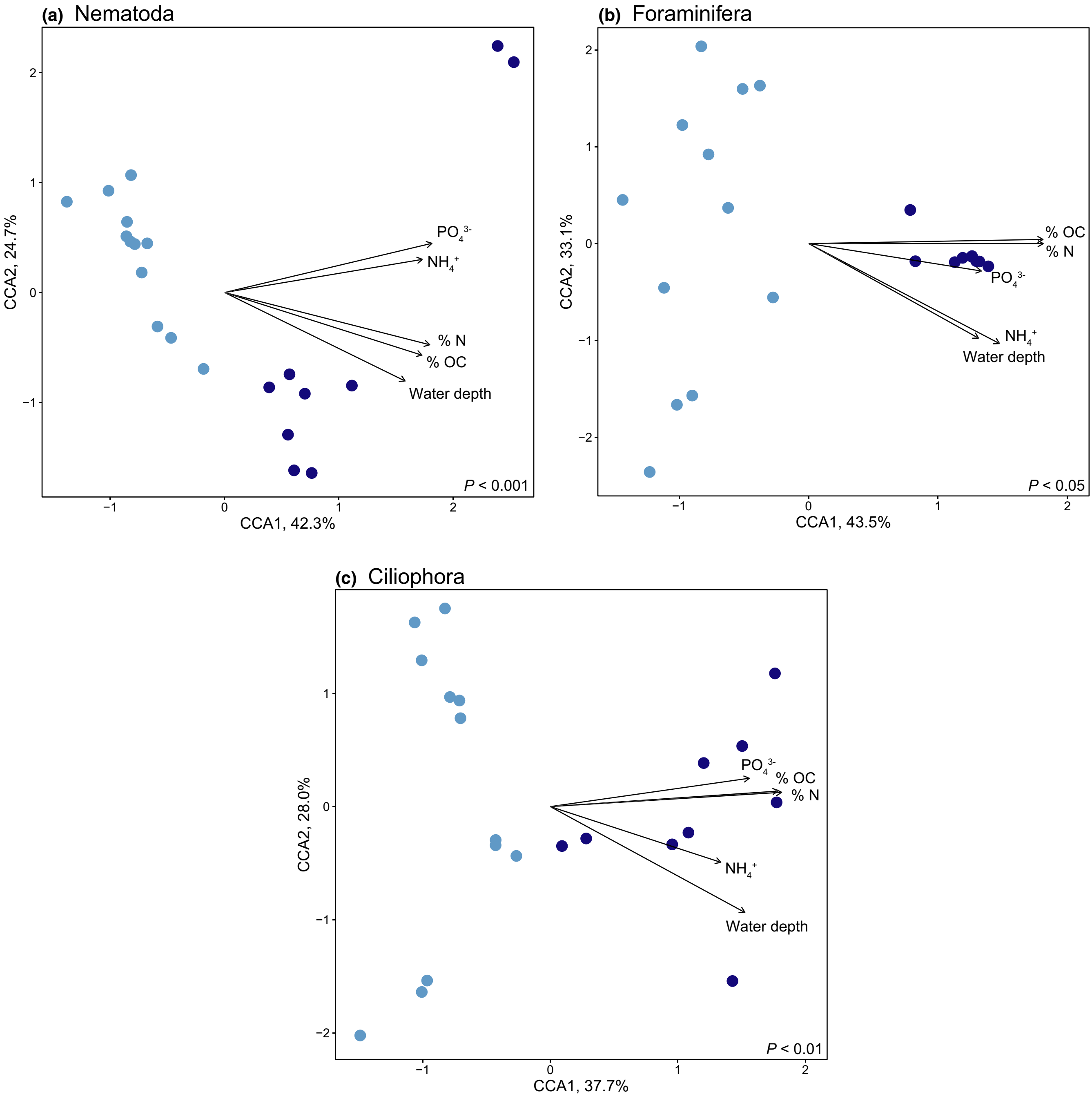

FIGURE 5 CCAs showing the distribution of (a) Nematoda; (b) Foraminifera; and (c) Ciliophora among the Low OC stations (light blue circles) and High OC stations (dark blue circles). The data was based on the relative abundances of genera for each taxonomic group. The grey triplots shows the direction of the measured abiotic variables (water depth, sediment %OC and %N, plus pore water NH_4^+ and PO_4^{3-}) in relation to the community composition. NH_4^+ values were first reported in (Broman, Sun, et al., 2020). Each circle represents one sediment core. The *p*-values shows the statistical significance (PERMANOVA) between the abiotic data and community composition when tested between Low OC and High OC stations

3.6 | Differences in Ciliophora community structure

Examples of Ciliophora genera that were significantly different between the Low OC and High OC stations included *Cryptocaryon* that had a significantly higher relative abundance at the Low OC stations (17.4% ± 1.4% compared to High OC 12.4% ± 1.4% (% denote portion of Ciliophora community), Mann-Whitney U tests, *U* = 21, *p* = .018; Figure 6c). In contrast, the genus *Spirotrachelostyla* had a significantly higher relative abundance at High OC (2.9% ± 0.5%) when compared to Low OC (0.8% ± 0.1%, Mann-Whitney U test, *U* = 9, *p* = .00066; Figure 6c). Ciliophora with low relative abundance that had significant difference, although with high variation, between Low OC and High OC stations included e.g., *Bresslaua and Epiphyllum* having a higher relative abundance at Low OC (2.0% ±

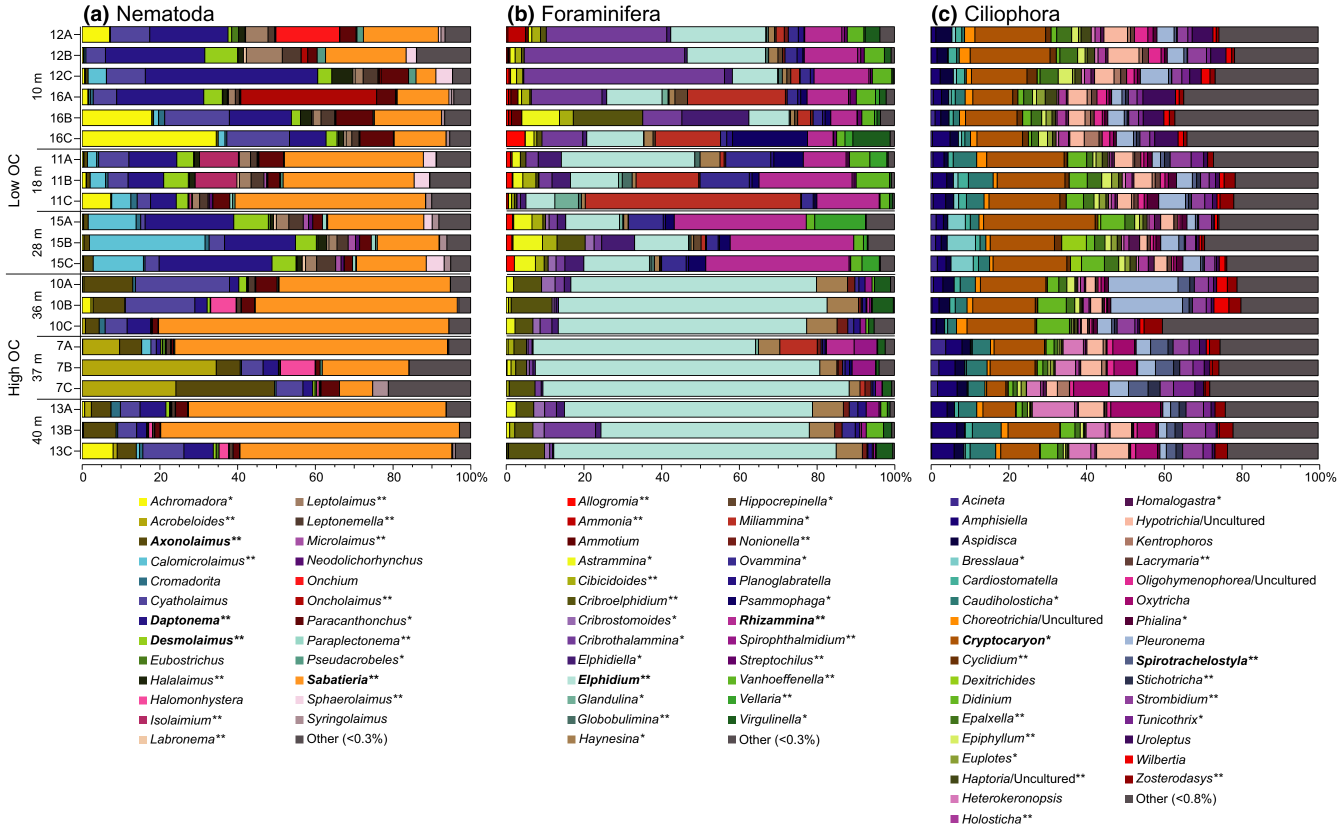

FIGURE 6 The stacked bars show the taxonomic classifications for the groups (a) Nematoda; (b) Foraminifera; and (c) Ciliophora based on 18S rRNA data (nematodes and forams classified against NCBI NT, and ciliates against the SILVA database). The y-axis shows the station names, their water depth (m), and replicates denoted with A, B, C. The x-axes show the relative abundance (%) within each taxonomic group. Taxonomic classifications that are significantly different between Low OC and High OC sites mentioned in the results have been indicated with bold text. The asterisks show genera that were significantly different between Low OC and High OC stations, with * $p < .05$ and ** $p < .01$

2.5% and 1.8% ± 0.8% compared to 0.1% ± 0.1% and 0.9% ± 0.5% at High OC, respectively), and *Zosterodasys* having a higher relative abundance at High OC sites (2.8% ± 1.0% compared to 1.3% ± 1.0% at Low OC; Mann-Whitney U tests, $p < .05$; Figure 6). A full list of taxonomic classifications and sequence counts for all three studied groups are available in Data S6.

3.7 | Nematoda functional ecology

The maturity index calculated from classified Nematoda genera showed no difference between High OC and Low OC stations (1.9 ± 0.1 maturity index for all samples, one-way ANOVA test, Data S7). Considering that values closer to one indicate habitat colonizers (and values closer to five indicate habitat persisters) the nematode communities in this study are considered colonizers. Looking closer at the classified feeding type of the nematodes the Genera classified as nonselective deposit feeders (1b, following the classification systems by Wieser [1953]) had a significantly higher number of reads in the High OC stations when compared to Low OC (log2 fold change 1.79, DESeq2 analysis, false discovery rate (*FDR*) <0.01; Figure 7). In contrast, the Low OC stations had significantly more genera classified as selective deposit feeders (1a, log2 fold change 1.62) and predator/omnivores (2b, log2 fold change 1.40) ($FDR < 0.01$ and $FDR < 0.05$, respectively; Figure 7). A full list of all maturity index and feeding type classifications and their relative abundance per Nematoda genera is available in Data S7.

4 | DISCUSSION

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

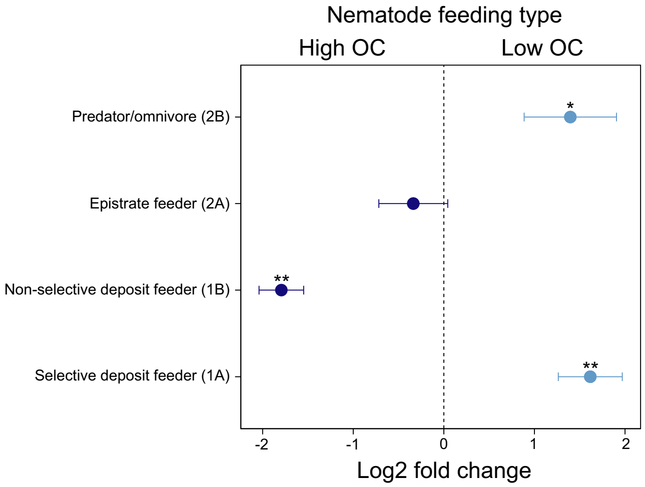

FIGURE 7 Nematoda genera were classified into a feeding type category according to Wieser (1953) and the plot is based on the sum of all classifications between the Low OC and High OC stations. DESeq2 statistical analysing showed significant differences for three of the four feeding types ($FDR < 0.05$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

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

The foram genera *Elphidium* and *Rhizammina* showed contrasting patterns in the data set, with *Elphidium* having higher relative abundance at High OC stations, and *Rhizammina* at the Low OC stations. Both *Elphidium* and *Rhizammina* are known to exist in the south-western 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 probably 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 42% in the High OC stations) never previously detected in the Baltic Sea (Data S6). Almost 100 foram species have been reported from the south-western Baltic Sea, but very few studies investigating forams in the central and north Baltic Sea are available (Frenzel et al., 2005). Hard-shelled (calcitic and agglutinated) forams have low densities in our study area and soft-shelled (organic) forams are not often studied morphologically (thus limiting 18S rRNA databases). The NCBI NT data also reported potential alien species such as *Planoglabratella* previously detected in shallow New Zealand sediment (Hayward, 1999) and this could also be due to database limitations (Figure 6). It is therefore possible that the differences we observed between databases are due to a limited number of 18S rRNA foram sequences in the databases. There are specific foram databases such as the PFR[2] (Morard et al., 2015). However, this database is focusing on oceanic planktonic forams which are absent from the Baltic Sea. Nevertheless, we report good results with the NCBI NT database.

Regarding the ciliate community, Stoeck et al. (2018) used metabarcoding and showed that benthic ciliate communities in the vicinity of fish farms (i.e., areas with high organic matter) had a lower alpha diversity and different community composition compared to nonaffected reference sites. In our study we also observed a significant difference in ciliate community composition between the Low OC and High OC sites, and although there was a decrease in alpha diversity it was not significant. Neither was there a difference between ciliate genera with a high relative abundance (except for *Cryptocaryon*). Therefore, the difference observed in beta diversity is probably due to differences in low abundant genera. The higher variance in ciliate alpha diversity (compared to nematodes and forams) indicates that higher replication and associated statistical power is required to detect differences in ciliate diversity between the stations. The ciliate genus *Cryptocaryon* was more prominent at the Low OC stations. This is a marine genus known to include parasitic species targeting fish (Wright & Colorni, 2002). However, low-saline (5–7 ppt) variants of *Cryptocaryon* have previously been described (Yambot, Song, & Sung, 2003). *Cryptocaryon*-like ciliates have previously been detected phylogenetically in the more saline (~14 ppt) deeper waters of the Baltic Sea (Stock, Jürgens, Bunge, & Stoeck, 2009), and both SILVA and NCBI NT (and manually checking classified sequences via BLAST) confirmed *Cryptocaryon*-like ciliates in our samples. Potentially because the Low OC stations were located in shallow areas closer to the shore, the *Cryptocaryon*-like ciliates detected in this study might be adapted to lower salinities (~7 ppt) and related to host organisms residing in these more diverse and euphotic habitats. In addition, considering that the summer heatwave of 2018 was one of the most intense ever recorded in the study area (Humborg et al., 2019), the warmer waters might attract *Cryptocaryon*-like ciliates which are typically more common at temperate and tropical temperatures (Colorni & Burgess, 1997). As far as we are aware, this is the first time such species have been reported from the Gulf of Finland based on molecular data. Finally, measurement of more geochemistry variables such as iron and sulphate, and sediment microprofiles (e.g., oxygen profiles) could help to further explain differences in nematode, foram, and ciliate diversity and taxonomy between low and high OC stations.

Limitations of this study include the relatively small sample size for RNA extraction (~2 g). A previous study investigating the effect of sample size on diversity estimations of microeukaryotes and metazoans using metabarcoding, found that larger volumes of sediments are necessary to accurately estimate small-scale spatial heterogeneity in biodiversity (Nascimento, Lallias, Bik, & Creer, 2018). Here we used the available commercial extraction kit that could process the largest input of sediment volume. It will be useful for future studies to develop larger kits for eRNA extraction and investigate similar effects of sample size on biodiversity assessment based on eRNA. Nevertheless, even with this sample size we report clear differences in biodiversity of multiplied communities including metazoans along the environmental gradients. We also used a kit to deplete bacterial rRNA in the laboratory, and this might have influenced the eukaryotic rRNA results. However, this influence should be similar among all samples and have a negligible impact in the results here shown. Previous studies focusing on eukaryotic RNA data backed up with microscopy has shown that bacterial rRNA depletion did not change the main findings as determined by microscopy (Broman, Bonaglia, et al., 2020; Broman, Varvara, Dopson, & Hylander, 2017). Organisms contain multiple ribosomes and while this is not an issue when analysing species richness or beta diversity with a presence/absence index, for community composition organisms with a large number of ribosomes could skew the proportions. This issue also exists in DNA metabarcoding studies, with many eukaryotic organisms carrying multiple genome copies per cell (i.e., polyploidy) (Edgar, Zielke, & Gutierrez, 2014) and for prokaryotes that can also be polyploid (Soppa, 2017). With the shotgun sequencing approach there is not a specific region of the 18S rRNA targeted by PCR and instead all regions are sequenced randomly. This could potentially influence the classification for certain taxa such as Foraminifera where some 18S rRNA regions have been shown to be more precise than others (Pawlowski & Lecroq, 2010). However, in this study the goal was to investigate if changes in biodiversity of multiple communities along environmental gradients could be detected using eRNA shotgun sequencing. For this purpose getting a large number of reads covering as much as possible of the 18S rRNA region for different taxa is probably a benefit.

Methods used in this study (and metabarcoding studies as well) relies heavily on the information in the reference databases when classifying taxonomy. In this study we set the lower limit to “genus” during taxonomic classification to compare relative abundances, as species would probably decrease the accuracy. However, we discovered that a large portion of unclassified eukaryotic sequences by Kraken2 had been distributed by Bracken2 to protists groups Malawimonadidae and Hemimastigophora. These two groups have been found in soil, as well as freshwater for Malawimonadidae (Adl et al., 2019; Lax et al., 2018). Considering Kraken2 only detected a few hundred sequences for these taxa, while Bracken2 distributed millions of unclassified eukaryotic sequences to these taxa it is possible that this is an effect of the information available in the databases. For example, Hemimastigophora was recently phylogenetically placed outside all eukaryote supergroups (Lax et al., 2018), and this could explain why Bracken2 would distribute unclassified sequences to such taxa. When databases grow with more reference species for microeukaryotes tools such as Kraken2 are likely to become more accurate for both genus and species level. The cost of shotgun sequencing on the latest platform is still quite high (several thousands of USD per Illumina NovaSeq S4 lane, yielding ~2,000 million read-pairs) compared to metabarcoding of marker genes (a few thousand USD per Illumina MiSeq flowcell, yielding ~18 million read-pairs; yields are based on information from SciLifeLab, Stockholm). However, there has been a large decrease in sequencing cost over the past 20 years (Wetterstrand, 2020) and if this trend continues, alongside streamlined bioinformatic protocols, large scale eRNA shotgun studies could be a future possibility in biomonitoring programmes.

Shotgun sequencing "catches" all organisms in the sample including both prokaryotes and eukaryotes (Zepeda Mendoza, Sicheritz-Pontén, & Gilbert, 2015). rRNA has a relatively short lifespan in the environment (Blazewicz, Barnard, Daly, & Firestone, 2013) and sediment surfaces in shallow water systems are highly active environments. The approach used in this study therefore probably targeted active organisms present in the study area, as well as eRNA derived from other motile organisms that were originally not located in the collected sediment (up to ~13 hr or longer in biofilm, see Wood et al., 2020). However, there have been studies that indicate that rRNA might be stable for long periods, potentially up to several years in deep sea sediments, therefore potentially failing to reflect live communities better than eDNA in such stable environments (Brandt et al., 2019; Orsi, Biddle, & Edgcomb, 2013). It is possible that rRNA is also prevalent for some time in other sediments including shallow systems. However, because coastal ecosystems are highly active environments rRNA degradation is expected to be faster. eRNA sequencing is therefore a potential useful method to study benthic communities, especially considering that a substantial portion of sediment consists of long-lasting dead organic matter (Burdige, 2006). For example, forams are a known microfossil group and with the use of DNA extraction it has been possible to study the ancient DNA of these organism (Lejzerowicz et al., 2013). However, limitations such as rRNA stability, especially below the oxic sediment surface, cannot be ruled out. Nevertheless, for biomonitoring studies changes in biodiversity and taxonomy are studied over time from the same stations and this might be less problematic. Shotgun sequencing of eRNA has been used in a wide variety of marine studies, including investigations of prokaryotic communities (Broman, Sachpazidou, Pinhassi, & Dopson, 2017; Broman, Sjöstedt, Pinhassi, & Dopson, 2017; Klindworth et al., 2014; Urich et al., 2014), marine viruses, (Culley, Lang, & Suttle, 2006), sediment eukaryotic metatranscriptomes (Broman, Varvara, et al., 2017), nematodes in oxygen deficient sediment (Broman, Bonaglia, et al., 2020), old marine groundwaters in the deep terrestrial biosphere (Lopez-Fernandez et al., 2018), and has also been used in similar environments such as soil ecosystems (Urich et al., 2008). Many studies have used eRNA to study prokaryotes (as mentioned above and e.g., Cottier et al., 2018), however there is paucity of studies using eRNA to assess the biodiversity of microeukaryotes in sediment. Although 18S rRNA metabarcoding has gained popularity to investigate such communities (see e.g., Birrer et al., 2018; Comeau, Lagunas, Scarcella, Varela, & Lovejoy, 2019; Rodríguez-Martínez et al., 2020), we have here shown that eRNA shotgun sequencing is also a viable approach to detect differences in diversity and community compositions for multiple communities as a response to different environmental conditions. Even though not directly compared in this study, shotgun sequencing avoids PCR limitations of metabarcoding such as (a) PCR primers only targeting certain species; (b) amplifying certain species more than others; and (c) the amount of cycles and type of polymerase used has been shown to influence diversity and community composition (Kelly et al., 2019; Nichols et al., 2018). In addition, eRNA shotgun studies also provide information on all organisms over a large range of trophic stages in the sediment, and it is also possible to study the RNA transcripts of expressed genes to estimate oxidation and reduction processes from prokaryotic metabolism (see e.g., Broman, Sjöstedt, et al., 2017). Finally, common shotgun sequencing bioinformatic pipelines are intricate and include many different software which increases the complexity of the data analysis. Here, conversely, we followed a protocol with few and straightforward steps, including: (a) Quality trimming (removal of Illumina adapters and phiX control sequences, quality trimming, and verifying final quality); (b) extraction of SSU rRNA sequences from the data set; (c) taxonomic classification of the SSU rRNA sequences using Kraken2 against the NCBI NT and SILVA databases; and (d) estimation of relative abundance at genus level using Bracken2 (for more details on the Kraken2+ Bracken2 combination see Wood et al., 2019). These new bioinformatic tools make it less daunting and possible to classify large data sets containing hundreds of millions of sequences within minutes to hours which would previously have taken several weeks using traditional aligners. As such this approach is closer to current metabarcoding bioinformatic pipelines with relatively straightforward steps (see e.g., the DADA2 pipeline Callahan et al., 2016). Moreover, ongoing developments in machine learning could make eRNA shotgun sequencing a powerful tool for future biomonitoring programmes. Such innovative approaches combined with eRNA shotgun sequencing would allow to bypass some of the database limitations when assigning taxonomy as the data can be incorporated into taxonomy-free models (Cordier et al., 2018; Cordier, Lanzén, Apothéloz-Perret-Gentil, Stoeck, & Pawlowski, 2019).

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

ACKNOWLEDGEMENTS

The authors acknowledge support from the National Genomics Infrastructure in Stockholm funded by Science for Life Laboratory, the Knut and Alice Wallenberg Foundation and the Swedish Research Council. The computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project SNIC 2018-8-246. Research activities were funded by the Stockholm University's strategic funds for Baltic Sea research. SB was supported by the Swedish Research Council Formas (Grant No. 2017-01513). Finally, the authors acknowledge Anders Lanzén and two anonymous reviewers for excellent feedback on the manuscript.

AUTHOR CONTRIBUTIONS

E.B. designed the study, sampled in the field, conducted molecular laboratory work, bioinformatics, analysed molecular data, and drafted the manuscript. S.B. conducted chemistry laboratory work, analysed chemistry data, and gave feedback on the manuscript. A.N. sampled in the field and gave feedback on the manuscript. S.C. provided input on the study and feedback on the manuscript. F.J.A.N. designed the study, helped with data analyses, and gave feedback on the manuscript. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

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

ORCID

Elias Broman https://orcid.org/0000-0001-9005-5168
Stefano Bonaglia https://orcid.org/0000-0003-4366-0677
Francisco J.A. Nascimento https://orcid.org/0000-0003-3722-1360

REFERENCES

Adl, S. M., Bass, D., Lane, C. E., Lukeš, J., Schoch, C. L., Smirnov, A., … Zhang, Q. (2019). Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. *Journal of Eukaryotic Microbiology*, *66*(1), 4–119. https://doi.org/10.1111/jeu.12691

Alve, E., Korsun, S., Schönfeld, J., Dijkstra, N., Golikova, E., Hess, S., … Panieri, G. (2016). Foram-AMBI: A sensitivity index based on benthic foraminiferal faunas from North-East Atlantic and Arctic fjords, continental shelves and slopes. *Marine Micropaleontology*, *122*, 1–12. https://doi.org/10.1016/j.marmicro.2015.11.001

Andersen, J. H., Carstensen, J., Conley, D. J., Dromph, K., Fleming-Lehtinen, V., Gustafsson, B. G., … Murray, C. (2015). Long-term temporal and spatial trends in eutrophication status of the Baltic Sea. *Biological Reviews*, *92*(1), 135–149. https://doi.org/10.1111/brv.12221

Andrews, S. (2010). FastQC: A quality control tool for high throughput sequence data.

Armenteros, M., Ruiz-Abierno, A., Fernández-Garcés, R., Pérez-García, J. A., Díaz-Asencio, L., Vincx, M., & Decraemer, W. (2009). Biodiversity patterns of free-living marine nematodes in a tropical bay: Cienfuegos, Caribbean Sea. *Estuarine, Coastal and Shelf Science*, *85*(2), 179–189. https://doi.org/10.1016/j.ecss.2009.08.002

Birrer, S. C., Dafforn, K. A., Simpson, S. L., Kelaher, B. P., Potts, J., Scanes, P., & Johnston, E. L. (2018). Interactive effects of multiple stressors revealed by sequencing total (DNA) and active (RNA) components of experimental sediment microbial communities. *Science of the Total Environment*, *637–638*, 1383–1394. https://doi.org/10.1016/j.scitotenv.2018.05.065

Blazewicz, S. J., Barnard, R. L., Daly, R. A., & Firestone, M. K. (2013). Evaluating rRNA as an indicator of microbial activity in environmental communities: Limitations and uses. *The ISME Journal*, *7*(11), 2061–2068. https://doi.org/10.1038/ismej.2013.102

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bonaglia, S., Deutsch, B., Bartoli, M., Marchant, H. K., & Brüchert, V. (2014). Seasonal oxygen, nitrogen and phosphorus benthic cycling along an impacted Baltic Sea estuary: Regulation and spatial patterns. *Biogeochemistry*, *119*(1), 139–160. https://doi.org/10.1007/s10533-014-9953-6

Bongers, T., Alkemade, R., & Yeates, G. W. (1991). Interpretation of disturbance-induced maturity decrease in marine nematode assemblages by means of the Maturity Index. *Marine Ecology Progress Series*, *76*, 135–142. https://doi.org/10.3354/meps076135

Bonsdorff, E., Laine, A. O., Hanninen, J., Vuorinen, I., & Norkko, A. (2003). Zoobenthos of the outer archipelago waters (N. Baltic Sea)-the importance of local conditions for spatial distribution patterns. *Boreal Environment Research*, *8*(2), 135–146.

Bower, C. E., & Holm-Hansen, T. (1980). A Salicylate-Hypochlorite Method for Determining Ammonia in Seawater. *Canadian Journal of Fisheries and Aquatic Sciences*, *37*(5), 794–798. https://doi.org/10.1139/f80-106

Brandt, M. I., Trouche, B., Henry, N., Liautard-Haag, C., Maignien, L., de Vargas, C., … Arnaud-Haond, S. (2019). An assessment of environmental metabarcoding protocols aiming at favouring contemporary biodiversity in inventories of deep-sea communities, *bioRxiv*, 836080. https://doi.org/10.1101/836080

Broman, E., Bonaglia, S., Holovachov, O., Marzocchi, U., Hall, P. O. J., & Nascimento, F. J. A. (2020). Uncovering diversity and metabolic spectrum of animals in dead zone sediments. *Communications Biology*, *3*, 1–12. https://doi.org/10.1038/s42003-020-0822-7

Broman, E., Raymond, C., Sommer, C., Gunnarsson, J. S., Creer, S., & Nascimento, F. J. A. (2019). Salinity drives meiofaunal community structure dynamics across the Baltic ecosystem. *Molecular Ecology*, *28*(16), 3813–3829. https://doi.org/10.1111/mec.15179

Broman, E., Sachpazidou, V., Pinhassi, J., & Dopson, M. (2017). Oxygenation of hypoxic coastal Baltic Sea sediments impacts on chemistry, microbial community composition, and metabolism. *Frontiers in Microbiology*, *8*(2453), 1–15. https://doi.org/10.3389/fmicb.2017.02453

Broman, E., Sjöstedt, J., Pinhassi, J., & Dopson, M. (2017). Shifts in coastal sediment oxygenation cause pronounced changes in microbial community composition and associated metabolism. *Microbiome*, *5*(96), 1–18. https://doi.org/10.1186/s40168-017-0311-5

Broman, E., Sun, X., Stranne, C., Salgado, M. G., Bonaglia, S., Geibel, M. C., … Nascimento, F. J. A. (2020). Low abundance of methanotrophs in sediments of shallow boreal coastal zones with high water methane concentrations. *Frontiers in Microbiology*, *11*, 1536.

Broman, E., Varvara, S., Dopson, M., & Hylander, S. (2017). Diatoms dominate the eukaryotic metatranscriptome during spring in coastal 'dead zone' sediments. *Proceedings of the Royal Society of London B: Biological Sciences*, *284*(1864). https://doi.org/10.1098/rspb.2017.1617

Brondizio, E., Settele, J., Díaz, S., & Ngo, H. (2019). *Global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services*. Bonn, Germany: IPBES Secretariat.

Burdige, D. J. (2006). *Geochemistry of marine sediments*. Princeton, NJ: Princeton University Press.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., … Naeem, S. (2012). Biodiversity loss and its impact on humanity. *Nature*, *486*(7401), 59–67. https://doi.org/10.1038/nature11148

Carugati, L., Corinaldesi, C., Dell'Anno, A., & Danovaro, R. (2015). Metagenetic tools for the census of marine meiofaunal biodiversity: An overview. *Marine Genomics*, *24*, 11–20. https://doi.org/10.1016/j.margen.2015.04.010

Charrieau, L. M., Filipsson, H. L., Ljung, K., Chierici, M., Knudsen, K. L., & Kritzberg, E. (2018). The effects of multiple stressors on the distribution of coastal benthic foraminifera: A case study from the Skagerrak-Baltic Sea region. *Marine Micropaleontology*, *139*, 42–56. https://doi.org/10.1016/j.marmicro.2017.11.004

Charrieau, L. M., Filipsson, H. L., Nagai, Y., Kawada, S., Ljung, K., Kritzberg, E., & Toyofuku, T. (2018). Decalcification and survival of benthic foraminifera under the combined impacts of varying pH and salinity. *Marine Environmental Research*, *138*, 36–45. https://doi.org/10.1016/j.marenvres.2018.03.015

Chen, Q.-H., Xu, R.-L., Tam, N. F. Y., Cheung, S. G., & Shin, P. K. S. (2008). Use of ciliates (Protozoa: Ciliophora) as bioindicator to assess sediment quality of two constructed mangrove sewage treatment belts in Southern China. *Marine Pollution Bulletin*, *57*(6), 689–694. https://doi.org/10.1016/j.marpolbul.2008.03.015

Colorni, A., & Burgess, P. (1997). *Cryptocaryon irritans* Brown 1951, the cause of 'white spot disease' in marine fish: An update. *Aquarium Sciences and Conservation*, *1*(4), 217–238. https://doi.org/10.1023/a:1018360323287

Comeau, A. M., Lagunas, M. G., Scarcella, K., Varela, D. E., & Lovejoy, C. (2019). Nitrate Consumers in Arctic Marine Eukaryotic Communities: Comparative Diversities of 18S rRNA, 18S rRNA Genes, and Nitrate Reductase Genes. *Applied and Environmental Microbiology*, *85*(14), e00247. https://doi.org/10.1128/aem.00247-19

Cordier, T., Forster, D., Dufresne, Y., Martins, C. I. M., Stoeck, T., & Pawlowski, J. (2018). Supervised machine learning outperforms taxonomy-based environmental DNA metabarcoding applied to biomonitoring. *Molecular Ecology Resources*, *18*(6), 1381–1391. https://doi.org/10.1111/1755-0998.12926

Cordier, T., Lanzén, A., Apothéloz-Perret-Gentil, L., Stoeck, T., & Pawlowski, J. (2019). Embracing environmental genomics and machine learning for routine biomonitoring. *Trends in Microbiology*, *27*(5), 387–397. https://doi.org/10.1016/j.tim.2018.10.012

Cottier, F., Srinivasan, K. G., Yurieva, M., Liao, W., Poidinger, M., Zolezzi, F., & Pavelka, N. (2018). Advantages of meta-total RNA sequencing (MeTRS) over shotgun metagenomics and amplicon-based sequencing in the profiling of complex microbial communities. *NPJ Biofilms and Microbiomes*, *4*(1), 2. https://doi.org/10.1038/s41522-017-0046-x

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W. K., … Bik, H. M. (2016). The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution*, *7*(9), 1008–1018. https://doi.org/10.1111/2041-210x.12574

Cristescu, M. E. (2019). Can Environmental RNA Revolutionize Biodiversity Science? *Trends in Ecology & Evolution*, *34*, https://doi.org/10.1016/j.tree.2019.05.003

Culley, A. I., Lang, A. S., & Suttle, C. A. (2006). Metagenomic Analysis of Coastal RNA Virus Communities. *Science*, *312*(5781), 1795. https://doi.org/10.1126/science.1127404

Damak, M., Frontalini, F., Elleuch, B., & Kallel, M. (2019). Benthic foraminiferal assemblages as pollution proxies along the coastal fringe of the Monastir Bay (Tunisia). *Journal of African Earth Sciences*, *150*, 379–388. https://doi.org/10.1016/j.jafrearsci.2018.11.013

Dray, S., & Dufour, A.-B. (2007). The ade4 package: Implementing the duality diagram for ecologists. *Journal of Statistical Software*, *22*(4), 120.

Edgar, B. A., Zielke, N., & Gutierrez, C. (2014). Endocycles: A recurrent evolutionary innovation for post-mitotic cell growth. *Nature Reviews Molecular Cell Biology*, *15*(3), 197–210. https://doi.org/10.1038/nrm3756

Ewels, P., Magnusson, M., Käller, M., & Lundin, S. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Foissner, W. (2016). Protists as bioindicators in activated sludge: Identification, ecology and future needs. *European Journal of Protistology*, *55*, 75–94. https://doi.org/10.1016/j.ejop.2016.02.004

Forster, D., Filker, S., Kochems, R., Breiner, H.-W., Cordier, T., Pawlowski, J., & Stoeck, T. (2019). A Comparison of Different Ciliate Metabarcode Genes as Bioindicators for Environmental Impact Assessments of Salmon Aquaculture. *Journal of Eukaryotic Microbiology*, *66*(2), 294–308. https://doi.org/10.1111/jeu.12670

Frenzel, P., Tech, T., & Bartholdy, J. (2005). Checklist and annotated bibliography of Recent Foraminiferida from the German Baltic Sea coast. *Studia Geologica Polonica*, *124*, 67–86.

Frontalini, F., & Coccioni, R. (2011). Benthic foraminifera as bioindicators of pollution: A review of Italian research over the last three decades. *Revue De Micropaléontologie*, *54*(2), 115–127. https://doi.org/10.1016/j.revmic.2011.03.001

Gong, J., Shi, F., Ma, B., Dong, J., Pachiadaki, M., Zhang, X., & Edgcomb, V. P. (2015). Depth shapes α- and β-diversities of microbial eukaryotes in surficial sediments of coastal ecosystems. *Environmental Microbiology*, *17*(10), 3722–3737. https://doi.org/10.1111/1462-2920.12763

Grasshoff, K., Kremling, K., & Ehrhardt, M. (2009). *Methods of Seawater Analysis*. John Wiley & Sons.

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*(1), 9.

Hayward, B. W. (1999). Recent New Zealand shallow-water benthic foraminifera: Taxonomy, ecologic distribution, biogeography, and use in paleoenvironmental assessment. *Institute of Geological Sciences and Nuclear Sciences. Monograph*, *21*, 264.

Holovachov, O., Haenel, Q., Bourlat, S. J., & Jondelius, U. (2017). Taxonomy assignment approach determines the efficiency of identification of OTUs in marine nematodes. *Royal Society Open. Science*, *4*(8), https://doi.org/10.1098/rsos.170315

Humborg, C., Geibel, M. C., Sun, X., McCrackin, M., Mörth, C.-M., Stranne, C., … Norkko, J. (2019). High Emissions of Carbon Dioxide and Methane From the Coastal Baltic Sea at the End of a Summer Heat Wave. *Frontiers in Marine Science*, *6*, 1–14. https://doi.org/10.3389/fmars.2019.00493

Ingels, J., Kiriakoulakis, K., Wolff, G. A., & Vanreusel, A. (2009). Nematode diversity and its relation to the quantity and quality of sedimentary organic matter in the deep Nazaré Canyon, Western Iberian Margin. *Deep Sea Research Part I: Oceanographic Research Papers*, *56*(9), 1521–1539. https://doi.org/10.1016/j.dsr.2009.04.010

Jiang, Y., Xu, H., Hu, X., Warren, A., & Song, W. (2013). Functional groups of marine ciliated protozoa and their relationships to water quality. *Environmental Science and Pollution Research*, *20*(8), 5272–5280. https://doi.org/10.1007/s11356-013-1525-0

Jorissen, F., Nardelli, M. P., Almogi-Labin, A., Barras, C., Bergamin, L., Bicchi, E., … Spezzaferri, S. (2018). Developing Foram-AMBI for biomonitoring in the Mediterranean: Species assignments to ecological categories. *Marine Micropaleontology*, *140*, 33–45. https://doi.org/10.1016/j.marmicro.2017.12.006

Kelly, R. P., Shelton, A. O., & Gallego, R. (2019). Understanding PCR Processes to Draw Meaningful Conclusions from Environmental DNA Studies. *Scientific Reports*, *9*(1), 12133. https://doi.org/10.1038/s41598-019-48546-x

Kennedy, A. D., & Jacoby, C. A. (1999). Biological Indicators of Marine Environmental Health: Meiofauna – A Neglected Benthic

Component? *Environmental Monitoring and Assessment*, *54*(1), 47–68. https://doi.org/10.1023/A:1005854731889

Klindworth, A., Mann, A. J., Huang, S., Wichels, A., Quast, C., Waldmann, J., … Glöckner, F. O. (2014). Diversity and activity of marine bacterioplankton during a diatom bloom in the North Sea assessed by total RNA and pyrotag sequencing. *Marine Genomics*, *18*, 185–192. https://doi.org/10.1016/j.margen.2014.08.007

Kopylova, E., Noé, L., & Touzet, H. (2012). SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics*, *28*(24), 3211–3217. https://doi.org/10.1093/bioinformatics/bts611

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*, 357. https://doi.org/10.1038/nmeth.1923

Lax, G., Eglit, Y., Eme, L., Bertrand, E. M., Roger, A. J., & Simpson, A. G. B. (2018). Hemimastigophora is a novel supra-kingdom-level lineage of eukaryotes. *Nature*, *564*(7736), 410–414. https://doi.org/10.1038/s41586-018-0708-8

Legendre, P., & Gallagher, E. D. (2001). Ecologically meaningful transformations for ordination of species data. *Oecologia*, *129*(2), 271–280. https://doi.org/10.1007/s004420100716

Lejzerowicz, F., Esling, P., Majewski, W., Szczuciński, W., Decelle, J., Obadia, C., … Pawlowski, J. (2013). Ancient DNA complements microfossil record in deep-sea subsurface sediments. *Biology Letters*, *9*(4), 20130283. https://doi.org/10.1098/rsbl.2013.0283

Levin, L. A., Boesch, D. F., Covich, A., Dahm, C., Erséus, C., Ewel, K. C., … Weslawski, J. M. (2001). The Function of Marine Critical Transition Zones and the Importance of Sediment Biodiversity. *Ecosystems*, *4*(5), 430–451. https://doi.org/10.1007/s10021-001-0021-4

Lopez-Fernandez, M., Simone, D., Wu, X., Soler, L., Nilsson, E., Holmfeldt, K., … Dopson, M. (2018). Metatranscriptomes reveal that all three domains of life are active but are dominated by bacteria in the Fennoscandian crystalline granitic continental deep biosphere. *mBio*, *9*(6), e01792. https://doi.org/10.1128/mBio.01792-18

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lu, J., Breitwieser, F. P., Thielen, P., & Salzberg, S. L. (2017). Bracken: Estimating species abundance in metagenomics data. *PeerJ Computer Science*, *3*, e104. https://doi.org/10.7717/peerj-cs.104

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research*, *27*(2 Part 1), 209–220.

Martins, M. V. A., Silva, F., Laut, L. L. M., Frontalini, F., Clemente, I. M. M. M., Miranda, P., … Dias, J. M. A. (2015). Response of Benthic Foraminifera to Organic Matter Quantity and Quality and Bioavailable Concentrations of Metals in Aveiro Lagoon (Portugal). *PLoS One*, *10*(2), e118077. https://doi.org/10.1371/journal.pone.0118077

McCann, K. S. (2000). The diversity–stability debate. *Nature*, *405*, 228. https://doi.org/10.1038/35012234

McDonald, D., Clemente, J. C., Kuczynski, J., Rideout, J. R., Stombaugh, J., Wendel, D., … Meyer, F. (2012). The Biological Observation Matrix (BIOM) format or: How I learned to stop worrying and love the ome-ome. *GigaScience*, *1*(1), 7. https://doi.org/10.1186/2047-217X-1-7

Moens, T., Traunspurger, W., & Bergtold, M. (2006). Feeding ecology of free-living benthic nematodes. *Freshwater Nematodes. Ecology and Taxonomy. CAB International Publishing*, 105–131.

Montagna, P., & Harper, D. E. (1996). Benthic infaunal long-term response to offshore production platforms in the Gulf of Mexico. *Canadian Journal of Fisheries and Aquatic Sciences*, *53*(11), 2567–2588. https://doi.org/10.1139/f96-215

Morard, R., Darling, K. F., Mahé, F., Audic, S., Ujiié, Y., Weiner, A. K. M., … de Vargas, C. (2015). PFR2: A curated database of planktonic foraminifera 18S ribosomal DNA as a resource for studies of plankton ecology, biogeography and evolution. *Molecular Ecology Resources*, *15*(6), 1472–1485. https://doi.org/10.1111/1755-0998.12410

Murray, J. W. (2006). *Ecology andAapplications of Benthic Foraminifera*. Cambridge, MA: Cambridge University Press.

Nascimento, F. J. A., Dahl, M., Deyanova, D., Lyimo, L. D., Bik, H. M., Schuelke, T., … Gullström, M. (2019). Above-below surface interactions mediate effects of seagrass disturbance on meiobenthic diversity, nematode and polychaete trophic structure. *Communications Biology*, *2*(1), 362. https://doi.org/10.1038/s42003-019-0610-4

Nascimento, F. J. A., Karlson, A. M., & Elmgren, R. (2008). Settling blooms of filamentous cyanobacteria as food for meiofauna assemblages. *Limnology and Oceanography*, *53*(6), 2636–2643. https://doi.org/10.4319/lo.2008.53.6.2636

Nascimento, F. J. A., Lallias, D., Bik, H. M., & Creer, S. (2018). Sample size effects on the assessment of eukaryotic diversity and community structure in aquatic sediments using high-throughput sequencing. *Scientific Reports*, *8*, 1–12. https://doi.org/10.1038/s41598-018-30179-1

Nascimento, F. J. A., Näslund, J., & Elmgren, R. (2012). Meiofauna enhances organic matter mineralization in soft sediment ecosystems. *Limnology and Oceanography*, *57*(1), 338–346. https://doi.org/10.4319/lo.2012.57.1.0338

Nichols, R. V., Vollmers, C., Newsom, L. A., Wang, Y., Heintzman, P. D., Leighton, M., … Shapiro, B. (2018). Minimizing polymerase biases in metabarcoding. *Molecular Ecology Resources*, *18*(5), 927–939. https://doi.org/10.1111/1755-0998.12895

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Ohara, R., Simpson, G. L., … Wagner, H. (2018). vegan: Community Ecology Package. R package version 2.5-2.

Orsi, W., Biddle, J. F., & Edgcomb, V. (2013). Deep Sequencing of Subseafloor Eukaryotic rRNA Reveals Active Fungi across Marine Subsurface Provinces. *PLoS One*, *8*(2), e56335. https://doi.org/10.1371/journal.pone.0056335

Pan, J., Marcoval, M. A., Bazzini, S. M., Vallina, M. V., & Marco, S. (2013). Coastal marine biodiversity: Challenges and threats. In A. H. M. Arias, & C. Menéndez (Ed.), *Marine Ecology in a Changing World* (pp. 43–67). Boca Raton, FL: CRC Press.

Pawlowski, J., Esling, P., Lejzerowicz, F., Cedhagen, T., & Wilding, T. A. (2014). Environmental monitoring through protist next-generation sequencing metabarcoding: Assessing the impact of fish farming on benthic foraminifera communities. *Molecular Ecology Resources*, *14*(6), 1129–1140. https://doi.org/10.1111/1755-0998.12261

Pawlowski, J., & Lecroq, B. (2010). Short rDNA barcodes for species identification in foraminifera. *Journal of Eukaryotic Microbiology*, *57*(2), 197–205. https://doi.org/10.1111/j.1550-7408.2009.00468.x

Pawlowski, J., Lejzerowicz, F., Apotheloz-Perret-Gentil, L., Visco, J., & Esling, P. (2016). Protist metabarcoding and environmental biomonitoring: Time for change. *European Journal of Protistology*, *55*, 12–25. https://doi.org/10.1016/j.ejop.2016.02.003

Payne, R. J. (2013). Seven reasons why protists make useful bioindicators. *Acta Protozoologica*, *52*(3), 105–113.

Pinto, R., Patrício, J., Baeta, A., Fath, B. D., Neto, J. M., & Marques, J. C. (2009). Review and evaluation of estuarine biotic indices to assess benthic condition. *Ecological Indicators*, *9*(1), 1–25. https://doi.org/10.1016/j.ecolind.2008.01.005

Pochon, X., Wood, S. A., Keeley, N. B., Lejzerowicz, F., Esling, P., Drew, J., & Pawlowski, J. (2015). Accurate assessment of the impact of salmon farming on benthic sediment enrichment using foraminiferal metabarcoding. *Marine Pollution Bulletin*, *100*(1), 370–382. https://doi.org/10.1016/j.marpolbul.2015.08.022

Pusceddu, A., Gambi, C., Zeppilli, D., Bianchelli, S., & Danovaro, R. (2009). Organic matter composition, metazoan meiofauna and nematode biodiversity in Mediterranean deep-sea sediments. *Deep Sea Research Part II: Topical Studies in Oceanography*, *56*(11), 755–762. https://doi.org/10.1016/j.dsr2.2008.10.012

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(Database issue), D590–D596. https://doi.org/10.1093/nar/gks1219

Rabalais, N. N., Turner, R. E., Díaz, R. J., & Justić, D. (2009). Global change and eutrophication of coastal waters. *ICES Journal of Marine Science*, *66*(7), 1528–1537. https://doi.org/10.1093/icesjms/fsp047

Raposo, D., Clemente, I., Figueiredo, M., Vilar, A., Lorini, M. L., Frontalini, F., … Laut, L. (2018). Benthic foraminiferal and organic matter compounds as proxies of environmental quality in a tropical coastal lagoon: The Itaipu lagoon (Brazil). *Marine Pollution Bulletin*, *129*(1), 114–125. https://doi.org/10.1016/j.marpolbul.2018.02.018

Ristau, K., Spann, N., & Traunspurger, W. (2015). Species and trait compositions of freshwater nematodes as indicative descriptors of lake eutrophication. *Ecological Indicators*, *53*, 196–205. https://doi.org/10.1016/j.ecolind.2015.01.010

Robertson, C. E., Harris, J. K., Wagner, B. D., Granger, D., Browne, K., Tatem, B., … Frank, D. N. (2013). Explicet: Graphical user interface software for metadata-driven management, analysis and visualization of microbiome data. *Bioinformatics*, *29*(23), 3100–3101. https://doi.org/10.1093/bioinformatics/btt526

Rodríguez-Martínez, R., Leonard, G., Milner, D. S., Sudek, S., Conway, M., Moore, K., … Richards, T. A. (2020). Controlled sampling of ribosomally active protistan diversity in sediment-surface layers identifies putative players in the marine carbon sink. *The ISME Journal*, *14*(4), 984–998. https://doi.org/10.1038/s41396-019-0581-y

Schratzberger, M., Warr, K., & Rogers, S. I. (2006). Patterns of nematode populations in the southwestern North Sea and their link to other components of the benthic fauna. *Journal of Sea Research*, *55*(2), 113–127. https://doi.org/10.1016/j.seares.2005.07.002

Schratzberger, M., Warr, K., & Rogers, S. I. (2007). Functional diversity of nematode communities in the southwestern North Sea. *Marine Environmental Research*, *63*(4), 368–389. https://doi.org/10.1016/j.marenvres.2006.10.006

Schweizer, M., Polovodova, I., Nikulina, A., & Schönfeld, J. (2011). Molecular identification of Ammonia and Elphidium species (Foraminifera, Rotaliida) from the Kiel Fjord (SW Baltic Sea) with rDNA sequences. *Helgoland Marine Research*, *65*(1), 1–10. https://doi.org/10.1007/s10152-010-0194-3

Singer, G. A. C., Fahner, N. A., Barnes, J. G., McCarthy, A., & Hajibabaei, M. (2019). Comprehensive biodiversity analysis via ultra-deep patterned flow cell technology: A case study of eDNA metabarcoding seawater. *Scientific Reports*, *9*(1), 5991. https://doi.org/10.1038/s41598-019-42455-9

Snelgrove, P. V. R., Thrush, S. F., Wall, D. H., & Norkko, A. (2014). Real world biodiversity–ecosystem functioning: A seafloor perspective. *Trends in Ecology & Evolution*, *29*(7), 398–405. https://doi.org/10.1016/j.tree.2014.05.002

Soppa, J. (2017). Polyploidy and community structure. *Nature. Microbiology*, *2*, 16261. https://doi.org/10.1038/nmicrobiol.2016.261

St John, J. (2011). SeqPrep. Retrieved from https://github.com/jstjohn/SeqPrep.

Stock, A., Jürgens, K., Bunge, J., & Stoeck, T. (2009). Protistan diversity in suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. *Aquatic Microbial Ecology*, *55*(3), 267–284. https://doi.org/10.3354/ame01301

Stoeck, T., Kochems, R., Forster, D., Lejzerowicz, F., & Pawlowski, J. (2018). Metabarcoding of benthic ciliate communities shows high potential for environmental monitoring in salmon aquaculture. *Ecological Indicators*, *85*, 153–164. https://doi.org/10.1016/j.ecolind.2017.10.041

Takata, H., Takayasu, K., & Hasegawa, S. (2006). Foraminifera in an organic-rich, brackish-water lagoon, Lake Saroma, Kokkaido. *Japan. Journal of Foraminiferal Research*, *36*(1), 44–60. https://doi.org/10.2113/36.1.44

Urich, T., Lanzén, A., Qi, J., Huson, D. H., Schleper, C., & Schuster, S. C. (2008). Simultaneous Assessment of Soil Microbial Community Structure and Function through Analysis of the Meta-Transcriptome. *PLoS One*, *3*(6), e2527. https://doi.org/10.1371/journal.pone.0002527

Urich, T., Lanzén, A., Stokke, R., Pedersen, R. B., Bayer, C., Thorseth, I. H., … Øvreas, L. (2014). Microbial community structure and functioning in marine sediments associated with diffuse hydrothermal venting assessed by integrated meta-omics. *Environmental Microbiology*, *16*(9), 2699–2710. https://doi.org/10.1111/1462-2920.12283

Uthicke, S., & Nobes, K. (2008). Benthic Foraminifera as ecological indicators for water quality on the Great Barrier Reef. *Estuarine, Coastal and Shelf Science*, *78*(4), 763–773. https://doi.org/10.1016/j.ecss.2008.02.014

Villnäs, A., Janas, U., Josefson, A. B., Kendzierska, H., Nygård, H., Norkko, J., & Norkko, A. (2019). Changes in macrofaunal biological traits across estuarine gradients: Implications for the coastal nutrient filter. *Marine Ecology Progress Series*, *622*, 31–48. https://doi.org/10.3354/meps13008

Wetterstrand, K. A. (2020). DNA sequencing costs: Data from the NHGRI Genome Sequencing Program (GSP) (Accessed: January 14, 2020). Retrieved fromwww.genome.gov/sequencingcostsdata

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Berlin, Germany: Springer.

Wieser, W. (1953). Die Beziehung zwischen Mundhohlengestalt, Ernahrungsweise und Vorkommen bei freilebenden marinen Nematoden. *Arkiv for Zoologi*, *4*, 439–484.

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, *20*(1), 257. https://doi.org/10.1186/s13059-019-1891-0

Wood, S. A., Biessy, L., Latchford, J. L., Zaiko, A., von Ammon, U., Audrezet, F., … Pochon, X. (2020). Release and degradation of environmental DNA and RNA in a marine system. *Science of the Total Environment*, *704*, 135314. https://doi.org/10.1016/j.scitotenv.2019.135314

Wright, A.-D.- G., & Colorni, A. (2002). Taxonomic re-assignment of *Cryptocaryon irritans*, a marine fish parasite. *European Journal of Protistology*, *37*(4), 375–378. https://doi.org/10.1078/0932-4739-00858

Yambot, A. V., Song, Y.-L., & Sung, H.-H. (2003). Characterization of *Cryptocaryon irritans*, a parasite isolated from marine fishes in Taiwan. *Diseases of Aquatic Organisms*, *54*(2), 147–156. https://doi.org/10.3354/dao054147

Zepeda Mendoza, M. L., Sicheritz-Pontén, T., & Gilbert, M. T. P. (2015). Environmental genes and genomes: Understanding the differences and challenges in the approaches and software for their analyses. *Briefings in Bioinformatics*, *16*(5), 745–758. https://doi.org/10.1093/bib/bbv001

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Broman E, Bonaglia S, Norkko A, Creer S, Nascimento FJA. High throughput shotgun sequencing of eRNA reveals taxonomic and derived functional shifts across a benthic productivity gradient. *Mol Ecol*. 2020;00:1–17. https://doi.org/10.1111/mec.15561