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**Title:** Spatial and temporal dynamics of coastal benthic microbial communities along a salinity gradient

**Year:** 2023

**Version:** Accepted version (Final draft)



**Please cite the original version:**

Petersen, H. C., Sapkota., R., Hiillos, A.-L., Hansen, B. W., Banta, G. T., & Knott, K. E. (2023). Spatial and temporal dynamics of coastal benthic microbial communities along a salinity gradient. Aquatic Microbial Ecology, 89, 127-142. https://doi.org/10.3354/ame02002

# Spatial and temporal dynamics of coastal benthic microbial communities along a salinity gradient

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**Abstract**

The Baltic Sea is a unique brackish water ecosystem studied for decades; however, knowledge about the diversity of the benthic communities of bacteria and microbial eukaryotes within this system is sparse. Using an amplicon sequencing approach, we evaluated the diversity of shallow water coastal microbial sediment communities and their relation to several environmental factors, on both a large spatial scale in the Baltic Sea and the adjacent North Sea, as well as on a temporal scale at selected sites along the salinity gradient in the Baltic Sea. We found salinity to be among the strongest drivers of both bacterial and eukaryote communities' species diversity, and that community network structure appeared to change between sites of different salinity. However, for the communities in the poly- to mesohaline sections of the study area, diversity seems affected to a higher degree by temperature, nutrient, and sediment characteristics.

**Keywords**

Baltic Sea, benthic bacteria, benthic protists, brackish microbiology, benthic microbial diversity, microbial networks

## Introduction

Interactions between bacteria and microbial eukaryotes in aquatic environments have been reported in pelagic systems over the last decades (e.g., Miki & Jacquet 2008), and as a result of these interactions the communities of these two taxonomic groups may co-vary. A positive correlation may indicate both non-causal relations such as similar preferred conditions, or interactions through commensalism or mutualism. They may however also result in negative correlations reflecting effects of parasitism (Anderson & Harvey 2020), grazing or competition for common resources (Chow et al. 2014). These interactions between bacteria and eukaryotes may shape diversity for both groups, by applying selection pressure and contribute to the evolution and maintenance of high diversity of microbes (Hiltunen & Becks 2014, Ramanan et al. 2016), and thus shape the community structure of each other besides other ecosystem components e.g., viruses (Chow et al. 2014).

Besides biotic interactions, microbial communities are also affected by environmental factors. In marine environments especially salinity is a known driver of changes in diversity (e.g. Herlemann et al. 2011; Campbell & Kirchman 2013). The Baltic Sea is one of the largest brackish water seas in the world, resembling a large estuary with its characteristic mixing of marine and freshwater (Leppäkoski et al. 2009). Due to the physio-chemical properties of the Baltic Sea, its microbial communities are suspected to be uniquely adapted to the brackish water ecosystem (Ininbergs et al. 2015). The salinity gradient in the Baltic Sea has shown to be a strong driver of community structure for both phytoplankton (Olli et al. 2019), planktonic bacteria (Herlemann et al. 2011, Lindh & Pinhassi 2018, Camarena-Gómez et al. 2021) and microbial eukaryotes (Telesh et al. 2011, Hu et al. 2016). Although the first studies of the microbial communities in the Baltic Sea were conducted more than a decade ago (Riemann et al. 2008), and despite the few studies examining the benthic microbial communities (Klier et al. 2018, Salonen et al. 2018), little is known of the diversity patterns of the benthic bacteria and eukaryote communities of the littoral zone. Salinity is nevertheless known to affect bacterial diversity in the sublittoral zone (Pavloudi et al. 2016, Klier et al. 2018, Li et al. 2021). Benthic bacterial communities have even shown contradictory trends in diversity over salinity gradients, compared to bacterioplankton communities (Campbell & Kirchman 2013, Pavloudi et al. 2016, Klier et al. 2018, Vidal-Durà et al. 2018). However, other physico-chemical driving factors, including seasonal changes in temperature and nutrient availability, could also have a significant influence on the composition of benthic microbial communities, both for bacteria (Vetterli et al. 2015, Lv et al. 2016), and microbial eukaryotes (Massana et al. 2015, Salonen et al. 2018, Anderson & Harvey 2020).

Microbial communities, including bacteria and eukaryotes, are important components of decomposition and nutrient recycling, and are fundamental for all ecosystems. The use of genetic tools, such as metabarcoding and next generation sequencing for documenting uncultured microbes has widened our knowledge of the composition of these communities particularly in the marine environment. However, the focus of such studies has been primarily on marine pelagic microbial communities, and only within the last decade has widened to include microbial communities in sediments (Edgcomb et al. 2011, Bik et al. 2012, Forster et al. 2016), especially in estuaries and brackish water systems (Chariton et al. 2010, Campbell & Kirchman 2013, Klier et al. 2018).

In this study, we monitored changes in diversity and community structure in shallow coastal benthic bacteria and microbial eukaryote communities along a salinity gradient in the Baltic Sea and adjacent North Sea, covering both a large spatial and local temporal scale. These communities may be affected by the species composition and their interactions, which may also be affected by external factors. By assessing associations between bacteria and microbial eukaryotes along the Baltic Sea, we aim to provide a first insight to the interactions, and potential keystone species in the benthic microbial communities of this brackish water sea. Furthermore, we assessed whether and to what extent selected abiotic factors besides salinity (sediment mean grain size, sorting, sediment water content, porosity, C/N ratio, organic content, and sediment temperature) influenced the observed diversity patterns. We hypothesised that the microbial community composition is strongly driven by salinity, and that the microbial diversity changes between sites along the Baltic Sea salinity gradient. Thus, other environmental factors are of less importance in shaping the communities. Because of the expected decrease in diversity along the Balitc Sea we also expected to see a decrease in complexity of interactions between microbial species in these communities as salinity falls. Testing this hypothesis is realised by presenting networks at three selected sites covering a part of the salinity gradient. We expected larger differences among the microbial communities (sites) than among temporal sampling points within the same site.

## Materials and methods

*Sediment sampling*

Samples for assessing spatial variation in microbial community diversity were collected during August 2018, at seven study sites representing a decrease in salinity (List, Saltö, Herslev, Gollwitz, Öland, Tvärminne, Pori) in the North Sea and Baltic Sea (Fig. 1). At three of the sampling sites in the

89 Baltic Sea with profound differences in salinity (Saltö, Herslev, Öland), samples for assessing
90 temporal variation were collected at four time points (August 2018, November 2018, April 2019,
91 August 2019). All sites were sampled from the coast at water depth 0-0.80 m, except Tvärminne
92 where sampling was performed by SCUBA at 3.8-5.0 m depth. Sediment was collected in three
93 replicate cores (5 cm diameter, min. 15 cm depth). After draining, two technical replicates were
94 collected from each core. Samples were taken from the central region of the core (avoiding the core
95 liner walls) by scraping off the top 5 mm of sediment (approx. 1.5 g), using sterile razor blades and
96 placed in sterile 1.5 ml microcentrifuge tubes. The sediment was preserved in 99% EtOH (Harry et
97 al. 2000) and kept frozen at -18°C until DNA extraction.

98 *Analysis of environmental factors*

99 Temperature was measured using handheld field thermometer (Frederiksen Scientific), salinity was
100 measured using ATACO handheld refractometer (resolution of 0.5 salinity units) and sediment
101 characteristics were recorded for each sampling point. Sediment characteristics (including water
102 content, porosity, organic content, carbon and nitrogen content, grain size and sorting) were
103 determined from three replicate cores per sampling station and analysed following Petersen et al., in
104 review.

105 *DNA extraction and amplification*

106 In this study three sampling replicates represent each sampling site/time, except for samples
107 Tvärminne, Gollwitz, Saltö August 2019, Herslev August 2018, and Öland November 2018, these
108 samples are represented by two sampling replicates and one technical replicate.

109 DNA from 250 mg of sediment per sample was extracted with DNeasy Powerlyzer PowerSoil Kit
110 (Qiagen) according to the manufacturer's protocol. Samples were lysed in Bead Ruptor Elite (OMNI
111 International) at 2500 rpm for 45 s with 750 µl of PowerBead solution (Qiagen). DNA concentration
112 was quantified using a Qubit 4.0 fluorometer with 1X dsDNA HS Assay Kit (Thermo Fisher,
113 Cambridge, UK). Bacterial 16S rRNA gene V1-V2 hypervariable regions were amplified using the
114 universal primers 27F (Ludwig et al. 1993) and 338R (Suzuki & Giovannoni 1996). To assess
115 diversity of the microbial eukaryotic community V4 hypervariable regions was chosen to amplify the
116 eukaryotic 18S rRNA gene. For this we used primer set UNonMetF and UNonMetR (Bower et al.
117 2004), combined with primers E572 and E1009R (Comeau et al. 2011). For primer details see
118 Supplementary Table 1. Reactions were performed in 25 µl volumes containing 12.5 µl of iQ™
119 SYBR® Green Supermix (2X) (Bio-Rad), 200 nM of each primer, 5.5-9.5 µl of nuclease-free water

120 and 3 ng of template DNA or PCR product from UNonMet primer pair amplification for E572 forward
121 and E1009R reverse primers. All amplifications were performed in a CFX96 Touch™ Real-Time
122 PCR Detection System (Bio-Rad). Thermocycling protocols are shown in Supplementary Table 2.

123 *Library preparation*

124 A fusion PCR was performed to add the Ion Torrent PGM sequencing adapters: barcoded M13-tailed
125 IonA adapter, target specific P1 adapter and a target specific M13- linker primer (following Mäki et
126 al. 2016) (see Supplementary Table 3). Amplification was achieved with the following conditions:
127 95°C/5 min, (94°C/45 s, 53°C/1 min, 72°C/1 min)*13, followed by final elongation of 72°C/5 min.
128 Reactions (25 µl) consisted of 12.5 µl iQ™ SYBR® Green Supermix (2X) (Bio-Rad), 400 nM of
129 fusion primers (IonA forward and P1 reverse), 40 nM of target specific M13- linker primer, 8.5 µl of
130 nuclease-free water and 1 µl of PCR product from the 16S or 18S rRNA amplification step. Libraries
131 were purified with Quanta SparQ PureMag Beads system (Quantabio). Quality and molarity were
132 determined with Agilent 2200 TapeStation before pooling in equimolar concentration (22 pM). The
133 library pool was sequenced with Ion PGM system using an Ion 318 Chip Kit version 2 (Ion Torrent,
134 Life Technologies) at the University of Jyväskylä.

135 *Analysis of sequencing data*

136 Single-end raw sequence reads were sorted by barcode prior to exporting from the Ion Torrent Suite™
137 Software and trimmed to remove sequencing adapters using Cutadapt (version 1.18, Martin 2011).
138 Length filtering was performed using Cutadapt as follows: 16S: min. 150 bp, max. 400 bp; 18S: min.
139 32 bp max. 400 bp. Quality filtering was performed using FASTX toolkit (Gordon & Hannon 2010),
140 with a minimum quality score of 20 for a minimum of 80% of the bases in each read. Dereplication,
141 singleton and chimera filtering were performed using VSEARCH (version 2.15.1, Rognes et al.
142 2016). All datasets were aligned to the SILVA database (version 138, Yilmaz et al. 2014) using
143 Mothur (version 1.44.3, Schloss et al. 2009) before clustering using greedy 97% similarity threshold
144 in VSEARCH. Amplicon sequence variants (ASVs) were mapped using VSEARCH, at a similarity
145 of 95-97.5%. 16S datasets were classified to the SILVA SEED database (version 138, Yilmaz et al.
146 2014) using the Wang method (Wang et al. 2007) with 8 kmer and 80% similarity in Mothur. 18S
147 dataset was classified to the PR2 database (version 4.12.0, Guillou et al. 2013; del Campo et al. 2018)
148 using the BLAST method with 80% similarity in Mothur.

149 We used universal primers, standardised protocols and well curated public databases in order to
150 minimize potential technical biases such as PCR error, sequencing error, sequencing depth,

bioinformatics analysis, and unassigned classification of the organisms (Guillou et al. 2013, Yilmaz et al. 2014, del Campo et al. 2018). Nevertheless, the 16S spatial dataset generated 7.7% unclassified OTUs, 16S temporal dataset generated 9.0% unclassified OTUs, 18S spatial dataset generated 27.1% unclassified OTUs, 18S temporal dataset generated 34.1% unclassified OTUs at phylum/division level. All unclassified OTUs at domain/kingdom were removed prior to further analyses. In addition, in the 16S dataset, all OTUs assigned to "Chloroplast" at the order level, and in the 18S dataset, all OTUs classified to phylum Metazoa, Rhodophyta and class Embryophyceae were removed.

*Statistical analysis*

All statistical analyses were performed in R version 3.6.3 (2020.02.29). To compensate for large differences in read depth between the samples, all datasets were transformed by rarefaction to an even read depth by repeatable random subsampling (read depth after rarefying in datasets: 16S spatial: 9773, 16S temporal: 15497, 18S spatial: 10957, 18S temporal: 7588), using the package 'phyloseq' (version 1.30.0, McMurdie and Holmes 2013). OTU table plots were made using 'phyloseq' package. Alpha diversity (based on OTU richness and Shannon index) and beta diversity (based on Bray-Curtis dissimilarity) measures, dbRDA and ENVFIT analyses based environmental variables were performed in R using 'vegan' package (version 2.5-7, Oksanen et al. 2017). Redundant (i.e., highly correlated) variables (water content and mean grain size) were removed on basis of ENVFIT analysis. All plots were made using 'ggplot2' (version 3.3.0, Wickham 2016). Significant differentially diversity matrices were detected using the Kruskal-Wallis test followed by Tukey HSD test for pair wise comparison. Salinity gradient test on community structure was performed by subjecting Bray-Curtis dissimilarity matrices in bacterial and microbial eukaryotic communities using the 'adonis' test from the 'vegan' package.

Network analysis was performed to show bacterial-eukaryote correlations and network properties were computed using the "igraph" package (Csardi & Tamas Nepusz 2006) as described earlier (Sapkota et al. 2020). We used only temporal datasets with high salinity (all temporal datasets from Saltö), medium salinity (all temporal datasets from Herslev) and low salinity (all temporal datasets from Öland). Spatial datasets representing lower number of replicates per salinity level (high, medium, and low) were excluded. The bacterial and eukaryote OTU table was trimmed for low abundant OTUs (<50 reads) and normalized as relative abundance counts per million using the "edgeR" package (Robinson et al. 2010). Correlations on all OTU pairs were computed using rcorr function from 'Hmisc' package and only highly significant ($p < 0.001$) correlated OTUs with spearman's rank correlations > 0.7 for positive correlations and < − 0.7 for negative correlations, were

183 used for network graphics using a Fruchterman-Reingold layout with 999 permutations. In the
184 network, OTUs were set as nodes and the correlation as edges. Nodes (OTUs) with the highest number
185 of connections were used as keystone species in the network analysis. For the figures the 10 most
186 connected OTUs represent keystone species, and tables show the 5% if OTUs with most connections.

187 **Results**

188 *Environmental characteristics*

189 Salinity ranged between 33 and 6 among sites in the spatial study, highest at List and lowest at Pori.
190 For the temporal dataset, salinity ranged between 26 and 7, highest at Saltö and lowest at Öland. In
191 addition to salinity, most variation among spatial sites was seen in sediment temperature, water
192 content, and C/N variables; whereas temperature and C/N variables showed most variation among
193 the temporal samples. For details of environmental characteristics for the spatial and temporal
194 samplings see Supplementary table 4.

195 *Bacterial diversity*

196 In the spatial study, the observed number of OTUs per sample for the bacterial communities (16S)
197 ranged between 1300 and 735, highest at Saltö and lowest at List (Fig. 2A), and Shannon index for
198 each sampling site ranged between 6.2 and 4.3, highest at Saltö and lowest in Öland (Fig. 2A,
199 Supplementary Table 5a). Proteobacteria was the most dominant phylum at all sites, followed by
200 Cyanobacteria and Bacteroidota (Fig. 2C) except for the euhaline site List. At List, Patescibacteria
201 was the second relative abundant phylum, and for both List and Saltö this group was generally more
202 relatively abundant than at the remaining sites (Fig. 2C). The relative abundance of Cyanobacteria
203 was also high at all sites, however lowest at Saltö, and it was the second most relative abundant
204 phylum at Herslev, Gollwitz and Öland (Fig. 2C). Chloroflexi had highest relative abundance at
205 Öland, Tvärminne and Pori (Fig. 2C), compared with other sites. Relative abundance of
206 Gemmatimonadota was generally very low at all sites except for Pori (Fig. 2C).

207 Considering alpha diversity for the spatial dataset, only Shannon diversity was related to salinity,
208 according to both an ANOVA test (Supplementary table 6) and Pearson correlation (Supplementary
209 figure 1). Beta diversity was correlated with all environmental factors (Table 1), however when
210 represented by dbRDA (Fig. 3A), List and Saltö clustered closest together with a positive relation to
211 sorting, salinity and C/N ratio, and negative relation to water content explaining their placement.
212 Herslev and Gollwitz formed a discrete cluster, and Öland and one replicate of Tvärminne clustered
213 together but not as closely, while the remaining replicates of Tvärminne and Pori were placed within

proximity to each other along the same axis. Herslev and Gollwitz were grouped by high organic matter and temperature, and the Öland and Tvärminne cluster was grouped by high water content, well sorted sediment, and low salinity and C/N ratio (Fig. 3A). Pori and the two replicates of Tvärminne were characterised by low organic matter and low temperature at the time of sampling (Fig. 3A). Analysis of distance-decay, which tests if communities become more dissimilar with increasing distance, for bacterial communities along the extent of the Baltic Sea covered in the spatial part of the study showed a strong relationship (Supplementary figure 2A, B & C).

In the temporal study, observed OTUs in bacterial communities (16S) ranged between 2122 and 1058 (Fig. 4A). Shannon index ranged between 6.5 and 4.5 and Öland showed significant lower diversity compare to Saltö and Herslev (Fig. 4A, Supplementary table 5B). As seen in the spatial part of the study, Proteobacteria was the most dominant phylum at all sampling sites, followed by Cynanobacteria and Bacteroidota, except for Saltö where Patescibacteria had a higher relative abundance (Fig. 4C). Generally, the distribution and relative abundance of phyla followed the same patterns at each sampling time for each sampling site, respectively (Fig. 4C). There were, however, some fluctuations in the relative abundance following the seasonal cycle; Proteobacteria showed a peak in relative abundance in the April 2019 sampling at both Saltö (S3) and Herslev (H3), and Actinobacteria was highest in November 2018 at both Herslev (H2) and Öland (Ö2) (Fig. 4C). Additionally, Actinobacteriota was relatively more abundant at Herslev and Öland compared to Saltö at all sampling times. Desulfobacterota had a lower relative abundance in April 2019 at Saltö (S3) compared to the other sampling dates and had lowest relative abundance at Öland at all times compared to the other sites.

Considering alpha diversity of the temporal dataset, ANOVA test showed that only Shannon diversity was related to salinity (Supplementary table 6), however Pearson correlation showed that both observed, and Shannon diversity were correlated to salinity (Supplementary figure 1). In the beta diversity analysis, each site formed its own cluster in the dbRDA plot, but temporal samples from Herslev clustered more closely together than did the temporal samples from the other sites (Fig. 3C). Though the beta-diversity of bacterial dataset was correlated to all environmental factors (Table 1), Saltö August 2018 (S1) and 2019 (S4) were best characterised by a positive relation to salinity and sorting, and November 2018 (S2) and April 2019 (S3) were better characterised by higher C/N ratio, water content and lower temperature in the dbRDA (Fig. 3C). Variation between samples at Herslev was related to sediment temperature, water content and C/N ratio, and variation between samples at Öland was related to organic matter (Fig. 3C). Analysis of distance-decay for bacterial communities

along the extent of the Baltic Sea included in the temporal dataset showed a strong relationship (Supplementary figure 2A & B).

Analysis of correlation between salinity and bacterial OTUs showed both positive and negative relationships (Supplementary table 7). Especially several OTUs assigned to genus Candidatus Kaiserbacteria, showed positive correlation to the salinity gradient.

*Microbial eukaryote diversity*

In the spatial study of microbial eukaryote diversity (18S), observed number of OTUs per sample ranged between 619 and 146 (Fig. 2B), and Shannon index ranged between 4.6 and 1.9 (Fig. 2B). Ochrophyta had high average relative abundance and was the most abundant phylum/division at all sites except Herslev and Gollwitz (Fig. 2D). Ciliophora was the highest relative abundant phylum/division at Gollwitz, and in Herslev, Dinoflagellata had highest relative abundance (Fig. 2D). Alveolata, Apicomplexa, and Fungi had highest relative abundance in the mesohaline sites Gollwitz, Öland, Tvärminne and Pori, and these sites, together with Saltö, also had high relative abundance of Ciliophora (Fig. 2D). Abundance of unclassified eukaryotes was high in the datasets from List and Gollwitz. Neither observed richness nor Shannon diversity of the spatial dataset were related to salinity in both ANOVA test and Pearson correlation (Supplementary table 6, Supplementary figure 1 & 3). However, beta diversity was correlated to salinity, together with C/N ratio and water content (Table 1). The dbRDA (Fig. 3B) showed List to cluster together with Öland and one replicate from Saltö and was somewhat close to the Herslev samples also, best described by high C/N ratio, water content and organic matter. The remaining samples from Saltö and Gollwitz were placed somewhat close together, characterised by high organic matter, C/N ratio and poor sorting (Fig. 3B). Tvärminne and Pori formed a distinct cluster mainly characterised by low salinity and temperature, and well sorted sediment (Fig. 3B).

For the temporal study of eukaryotes (18S), between 711 and 136 OTUs were captured (Fig. 4B), and Shannon index ranged between 5.1 and 1.6 (Fig. 4B, Supplementary table 5B). Ochrophyta was the most relatively abundant phylum/division in the April 2019 and August 2019 samplings for all sites, except for Öland August 2019, where it was the second most relative abundant, exceeded by Dinoflagellata (Fig. 4D). Ochrophyta was also most relative abundant in August 2018 for both Saltö and Öland (S1 and Ö1) (Fig. 4D). Dinoflagellata was the most relatively abundant phylum/division in the samples from Herslev August 2018 (H1) and November 2018 (H2), as well as Saltö November

2018 (S2) (Fig. 4D). The most relatively abundant phylum/division at Öland in November 2018 (Ö2) was Fungi (Fig. 4D). Abundance of unclassified eukaryotes was especially high in the datasets from Saltö. Both alpha diversity measures were significantly correlated to salinity (see Supplementary table 6). Community structure was influenced by salinity, as well as temperature, C/N ratio, mean grain size and sediment sorting (Table 1). In the dbRDA plot samples were in general scattered with only little clustering of sites, especially for Herslev and Öland (Fig. 3D), indicating more temporal changes than seen in the bacteria communities (Fig. 3C). For most locations, samples from August for two consecutive years were somewhat similar. For example, Saltö August 2018 (S1) and 2019 (S4) samples clustered together characterised by high temperature, organic matter, and poor sorting. Similarly, August 2018 and 2019 for Herslev (H1 and H4) and Öland (Ö1 and Ö4) were placed closely together, somewhat more like the samples from November or April from those stations compared to Saltö (Fig. 3D). For Saltö, samples from November and April formed a distinct cluster described by different environmental factors than those from August samples, specifically high water content and temperature (Fig. 3D). Temperature was an obvious explanatory variable for the observed community patterns, but also salinity, C/N ratio and sediment sorting were important for the differences in eukaryote communities seen in the dbRDA plots. However, analysis of distance-decay for eukaryote communities along the extent of the Baltic Sea covered in this study only showed a weak correlation for the temporal dataset (Supplementary figure 2C).

Analysis of correlations between salinity and eukaryote OTUs only revealed five positive correlations and one negative correlation (Supplementary table 7). In our eukaryote datasets, a large proportion of OTUs could not be classified to phylum/division level at 80% similarity. This is most likely due to incomplete microbial eukaryote coverage in the database; since the benthic microbes historically have received less scientific attention than the pelagic ones, a large proportion of benthic microscopic eukaryotes are expected to be missing from the databases (Forster et al. 2016, Zhang et al. 2018).

*Microbe-Microbe interactions shown as networks*

To explore bacteria-eukaryote interactions in different salinity environments, we used the temporal dataset and visualised co-occurrence at three different salinity levels as network graphs (Fig. 5). Interestingly, we found distinct co-occurrence patterns in networks from the three different salinity levels. At highest salinity level, co-occurrence networks were strongest, having the more connections compared to networks for the other two salinity levels. Similarly, the lowest salinity level showed the least connections revealing fewer eukaryote-bacteria interactions, and co-occurrence network for the medium salinity level was in between the other two. Several bacterial and eukaryote OTUs were

identified as the keystone species based on the highest number of connections that they showed in the co-occurrence networks (for details see Supplementary table 8A, B & C). In particular, Raphid-pennate diatoms (Stramenopiles) appeared several times as keystone eukaryotic species in all three networks. Also, bOTU607 (a member of Rhodobacteraceae) appeared as a keystone species in two networks.

## Discussion

Using amplicon sequencing, we surveyed microbial communities of shallow coastal sediments on both a large spatial scale in the Baltic Sea and the adjacent North Sea, and a temporal scale in a subset of these communities. We examined diversity patterns of bacterial communities as well as among microbial eukaryotes, and the correlations between the two kingdoms. Because the two taxonomic groups could respond differently to environmental factors, we evaluated the relationship of diversity patterns to selected environmental factors, particularly salinity, temperature, and sediment characteristics for both groups. These analyses suggested that salinity is a strong driver of diversity of microbial communities, however, salinity is not the only driver, as sediment characteristics, nutrients, and temperature also were important. In addition, our results revealed significant interactions and relationships among several bacterial and eukaryote OTUs. This study provides a broader description of coastal benthic microbial communities and their interactions at shallow sites in the Baltic.

*Baltic Sea salinity gradient affects bacterial and microbial eukaryote communities*

Though the ANOVA test showed that OTU richness for bacteria was not affected by salinity for either dataset, Shannon diversity of these communities was significantly affected by salinity. Moreover, we did see a significant positive correlation for both diversity measures using Pearson's correlation on the temporal dataset (Supplementary figure 3). This suggests that the missing correlation between salinity and observed OTUs for the spatial dataset could be due to too few data points. Likewise, distance-decay showed beta-diversity of both bacterial datasets were positively correlated with distance between communities, hence when distance increases the communities become more dissimilar along the gradient (Supplementary figure 1A, B & C). While salinity was a significant driver of beta diversity for bacteria, it might not be an equally strong driver of diversity patterns at all sites. As reflected in the dbRDA (Fig. 3A and C), the communities at the sites with highest salinity (List and Saltö) were described to a higher degree by salinity, however the

communities at lower salinities were not to a high degree described by salinity, except for Öland which was to some degree described by lower salinity. Despite the coarse sampling, we did observe patterns across the salinity gradient: highest diversity was found at the eu- and polyhaline sites (List and Saltö), a diversity minimum at the mesohaline sites (Öland and Tvärminne), followed by a slight increase in diversity at the site with the mesohaline site of lowest salinity (6) (Pori). This suggests that bacterial communities at the sites of highest salinity are more diverse and evenly distributed than communities in the mesohaline. This pattern suggests that estuarine bacterial communities could be adapted to lower salinity. The pattern we observed supported previous findings in the Baltic Sea, where bacterial communities in the mesohaline consisted mainly of bacterial lineages with a broad salinity tolerance (Klier et al. 2018).

Although the bacterial taxonomic composition was dominated by the same phyla at all samplings (both spatial and temporal), there were some differences between the salinity extremes. Patescibacteria had highest relative abundance at the eu- and polyhaline sites, List and Saltö, especially genus Candidatus Kaiserbacteria, showed several OTUs positively correlated to salinity, indicating better conditions for this group at higher salinities. However, a few OTUs assigned to Candidatus Kaiserbacteria also showed a negative correlation to salinity, highlighting the complexity in bacterial diversity. Cyanobacteria generally were abundant at all sites except at the eu- and polyhaline ones (List and Saltö), indicating better conditions for these taxa at lower salinities. However, individual OTUs of Cyanobacteria also showed both positive and negative correlation to salinity, except most OTUs of Synechococcales showed a negative correlation, indicating that this group might favour low salinities. The same argument could be made for phylum Chloroflexi, which had highest relative abundance at the low salinity sites (Öland, Tvärminne and Pori), a trend also previously observed for sublittoral bacterial communities in the Baltic Sea (Klier et al. 2018). Several OTUs of Cloroflexi was negatively correlated to salinity, thus favouring low salinity, especially OTUs of the obligate anaerobe genus Anaerolinea, indicating that the low salinity sites are also affected by low oxygen. Gemmatimonadota had low abundance at all sites except the one with lowest salinity (Pori), and three OTUs of family Gemmatimonadacea (member of phylum Gemmatimonadota) were negatively correlated to salinity, indicating a negative affliction to salinity. However, other local factors, such as lack of associated phytoplankton (Mujakić et al. 2021) may also have an influence on this particular phylum.

The spatial patterns we found for microbial eukaryotes were like those for bacterial communities: salinity played an important role, but it was not the only driver of benthic marine microbial eukaryote

372 community composition and diversity. We observed no clear trends in alpha diversity related to
373 salinity for either eukaryote dataset, but both OTU richness and Shannon diversity of the temporal
374 dataset was significantly affected by salinity (Table 1) in the ANOVA test, although we did not see a
375 correlation for either eukaryote dataset using Pearson's correlation. This suggests the changes were
376 not continuous, but rather in steps. Neither did we see a relation to distance, which could have been
377 expected to follow somewhat the same diversity trend as salinity. We did however observe a weak
378 correlation along the extent of the Baltic Sea when using the temporal samples. There were also some
379 systematic changes in taxonomic composition along the Baltic Sea and its salinity gradient, e.g., while
380 Alveolata, Apicomplexa and Fungi were present at all sites, the three phyla were more abundant in
381 sites with lower salinity. This indicates that lower salinities are a more favourable condition for these
382 taxa, and, for example, supports earlier findings that fungal communities are adversely influenced by
383 salinity (Mohamed & Martiny 2011, Tisthammer et al. 2016). Nevertheless, only one OTU
384 (unclassified Cercozoa) was significantly negatively correlated to salinity. Additionally, only a few
385 OTUs showed a positive correlation to salinity, representing the orders Cercozoa, Bacillariophyta and
386 Apusomonadidae.

387 *Temporal patterns influence microbial assembly*

388 Seasonal fluctuations in the bacterial communities' OTU richness and Shannon diversity were
389 observed at all sampling sites, but with no shared patterns between sites. In the summer samples
390 (August 2018 and 2019), communities were more similar in comparison to other times of the year
391 (Fig. 3C), except for Herslev. Changes in the abundance of several phyla during the seasonal cycle
392 were also described, however, no clear trends were observed common to all sites. These cyclic
393 changes support previous assumptions of seasonal effects on both benthic and planktonic bacterial
394 communities in the Baltic Sea (Vetterli et al. 2015, Herlemann et al. 2016). The inconsistency
395 between sites suggests that the seasonal changes are shaped locally, rather than on a larger regional
396 climatic scale, or that local factors modify a possible common seasonal pattern.

397 In contrast to the bacterial communities, eukaryote communities seem to have more variation in
398 taxonomic composition on a temporal scale, with each site having their own distinctive pattern,
399 supporting previous observations of microbial eukaryote communities on a smaller geographic scale
400 in the Baltic Sea (Salonen et al. 2018). Additionally, fluctuations in richness were observed at all sites
401 over the season. The eukaryote communities potentially cover more functional groups compared with
402 bacteria (Reynolds 2006), some of which could be favoured at different environmental conditions.

The bacterial community may therefore be more resilient to environmental fluctuations, which could explain the differences in patterns, especially the variation in species composition, between bacteria and microbial eukaryotes observed here. However, changes in diversity could be present on a lower taxonomic level e.g., genus level, which will not be reflected in our overview at phylum level. Furthermore, some of the organisms may be represented by dormant stages, especially among microbial eukaryotes (Marcus & Boero 1998), or inactive or even dead organisms added from surrounding water masses (Stoeck et al. 2007) which could be evident in the two organismal groups differently. Although temporal fluctuations were observed in the benthic microbial eukaryote communities, there were no consistent seasonal trends in the species composition, such as signal of spring bloom of either pelagic diatoms or dinoflagellates as earlier observed in benthic microscopic eukaryote communities in the north-eastern Baltic Sea (Salonen et al. 2018). However, the eukaryote community at Herslev in August 2018 had lower alpha diversity than other temporal samples, and was dominated by Dinoflagellata and Chlorophyta, which was distinctly different from the other samples that were largely dominated by Ochrophyta, suggesting that the August 2018 community may have been influenced by blooming dinoflagellates in Herslev.

*Inter-kingdom networks contributing to community structure*

The diversity of communities is likely not only influenced by environmental factors, but also by competition and predation within the communities. These interactions and the structure of the community, in turn, may be affected by changes in community composition and diversity. We found both positive and negative correlations among bacterial and eukaryote OTUs. The most connected OTUs, putatively regarded as the keystone species, belong to both bacteria and eukaryotes. A few keystone eukaryotes did signify their role in microbe-microbe interactions by having several or only negative correlations. Furthermore, the communities of the low salinity site (Öland) were dominated by a few keystone eukaryotes with only negative correlations, which could indicate a top-down regulated system. The communities of high (Saltö) and medium salinity (Herslev) sites exhibited more complex networks (more OTUs) with higher connectivity; also, keystone species of these communities had more connections, compared to those in the site of low salinity (Öland). This suggest that the communities were functionally affected by either salinity, or by distance from the entrance of the Baltic. This network analyses provides a first insight into the bacteria-eukaryote interactions in benthic sediment communities in the Baltic Sea, however, much remains unknown about these community interactions, not least of which the mechanisms and biological nature of these interactions.

*Association with environmental factors other than salinity*

Apart from salinity, other environmental factors also affected the diversity of microbial benthic communities. Sediment nutrient composition had a strong influence on the bacterial community diversity, with organic matter being an important driver on the spatial scale, together with temperature (at the mesohaline sites Herslev, Gollwitz, Pori and two replicates of Tvärminne) (Fig. 3A). On the temporal scale, seasonal change in temperature was also an important driver between summer (August) communities and communities from colder winter and spring periods (November and April) (Fig. 3C). Changes in sediment characteristics were also important in explaining temporal changes. C/N ratio and sediment characteristics were mainly related to changes in community at the polyhaline site (Saltö), while changes at the mesohaline site Herslev were better characterised by changes in temperature together with C/N ratio. At the other mesohaline site, Öland, the community was more driven by changes in organic matter and sediment characteristics. For eukaryote communities the roles of environmental variables other than salinity have been described previously (Salonen et al. 2018, Zhang et al. 2018). Similar patterns were seen in the temporal communities in the present study, where composition over the year was driven to a higher degree by change in temperature, C/N ratio, organic matter and sorting, than it was by temporal changes in salinity. On a spatial scale, we found no clear patterns common to both eukaryote datasets for any of the sites.

## Conclusion

This study provides an insight into the diversity of coastal sediment bacterial and microbial eukaryote communities in the Baltic Sea, on a broad spatial scale as well as over a seasonal cycle along the natural salinity gradient. We found salinity to be among the strongest drivers of both bacterial and eukaryote communities; however, not an equally strong factor for all communities; beta-diversity of mesohaline communities was better described by temperature, nutrients, and sediment characteristics. Bacteria and eukaryote communities also differed from each other, and thus, their species diversity respond differently to the same environmental conditions. The communities may however not only be influenced by external environmental factors, but also be affected by competition and predation within the communities. Analysis of bacteria-eukaryote networks provide a first insight into the microbial interactions in benthic sediment communities in the Baltic Sea. We found network complexity and connectivity to be higher in the communities of high salinity, but were functionally affected by salinity, showing distance decay. A more in-depth look at the community composition

465 patterns at genus rather than phylum level could further elucidate bacterial and microbial eukaryote
466 interactions in Baltic Sea sediments.

467

468 **Acknowledgements**

469 This work was supported by Roskilde University (Department of Science and Environment),
470 University of Jyväskylä (Department of Biological and Environmental Science), Elite Research travel
471 grant, Ministry of Higher Education and Science, Denmark [to HCP], Faxe Fonden [to HCP], H. C.
472 Wegges Mindelegat for Zoologer [to HCP], and Emil Aaltonen Foundation [grant number a6a412 to
473 ALH].

474 We gratefully thank Professor Karsten Reise for help with field sampling and Alfred Wegener
475 Institute Helmholtz Centre for Polar and Marine Research in List, Germany, for use of facilities.
476 Assistant professor and research scientist Stefan Forster for field assistance and Rostock University,
477 Germany, for use of facilities. Professor Kerstin Johannesson and Professor Per Jonsson for help and
478 guidance with collecting field samples, and Tjärnö field station, Gothenburg University, Sweden, for
479 use of facilities. Research coordinator Laura Kauppi for assistance in field sampling, and Tvärminne
480 field station, University of Helsinki, Finland, for use of facilities. Master student Theophilus Alale
481 for sampling help in Tvärminne and Pori. We thank Laboratory assistant Anne Busk Faarborg for
482 assisting with the field sampling and performing the grain size analysis, and Laboratory assistant G.
483 Katrine Bøg for performing CHN analysis. We also thank Master student Aleksi Kolehmainen for
484 assistance with the DNA extraction and amplicon sequencing, and Chief Laboratory Technician Elina
485 Virtanen for assisting with sequencing. We also thank CSC–IT Center for Science, Finland for
486 providing computing facilities.

487

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640

641

**Figure 1.**

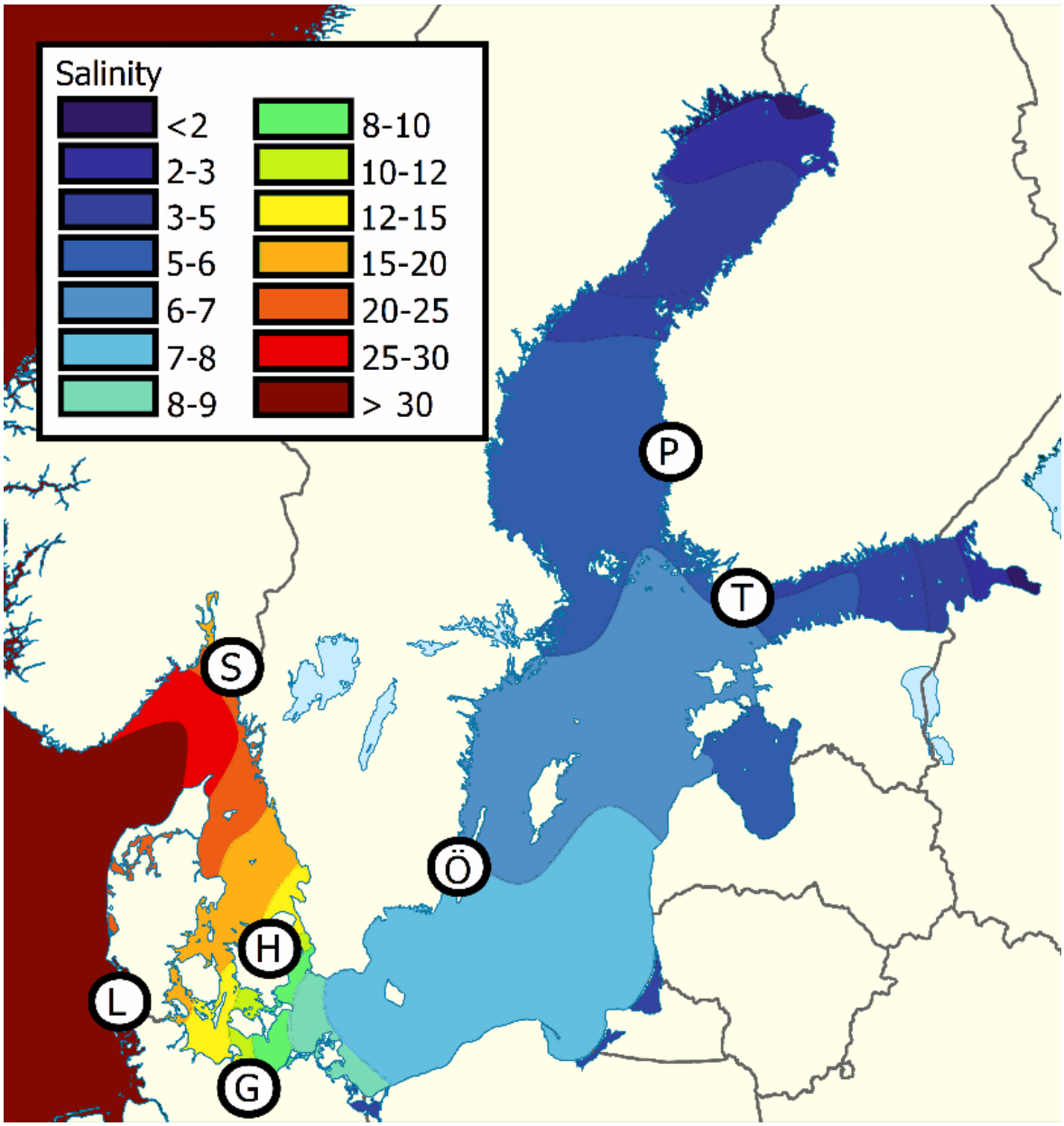

Salinity
<2
2-3
3-5
5-6
6-7
7-8
8-9
8-10
10-12
12-15
15-20
20-25
25-30
> 30
P
T
S
Ö
H
L
G

**Figure 2.**

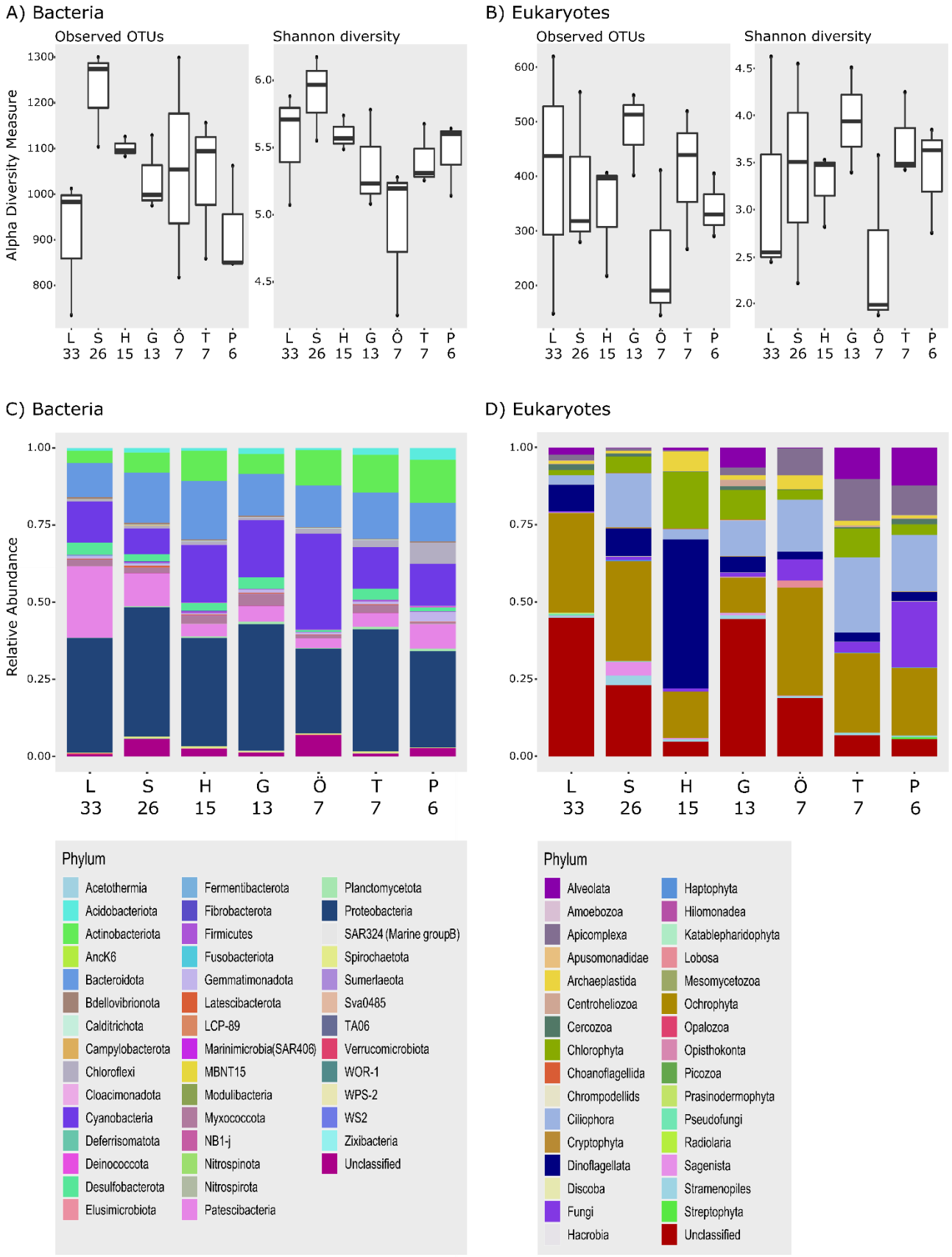
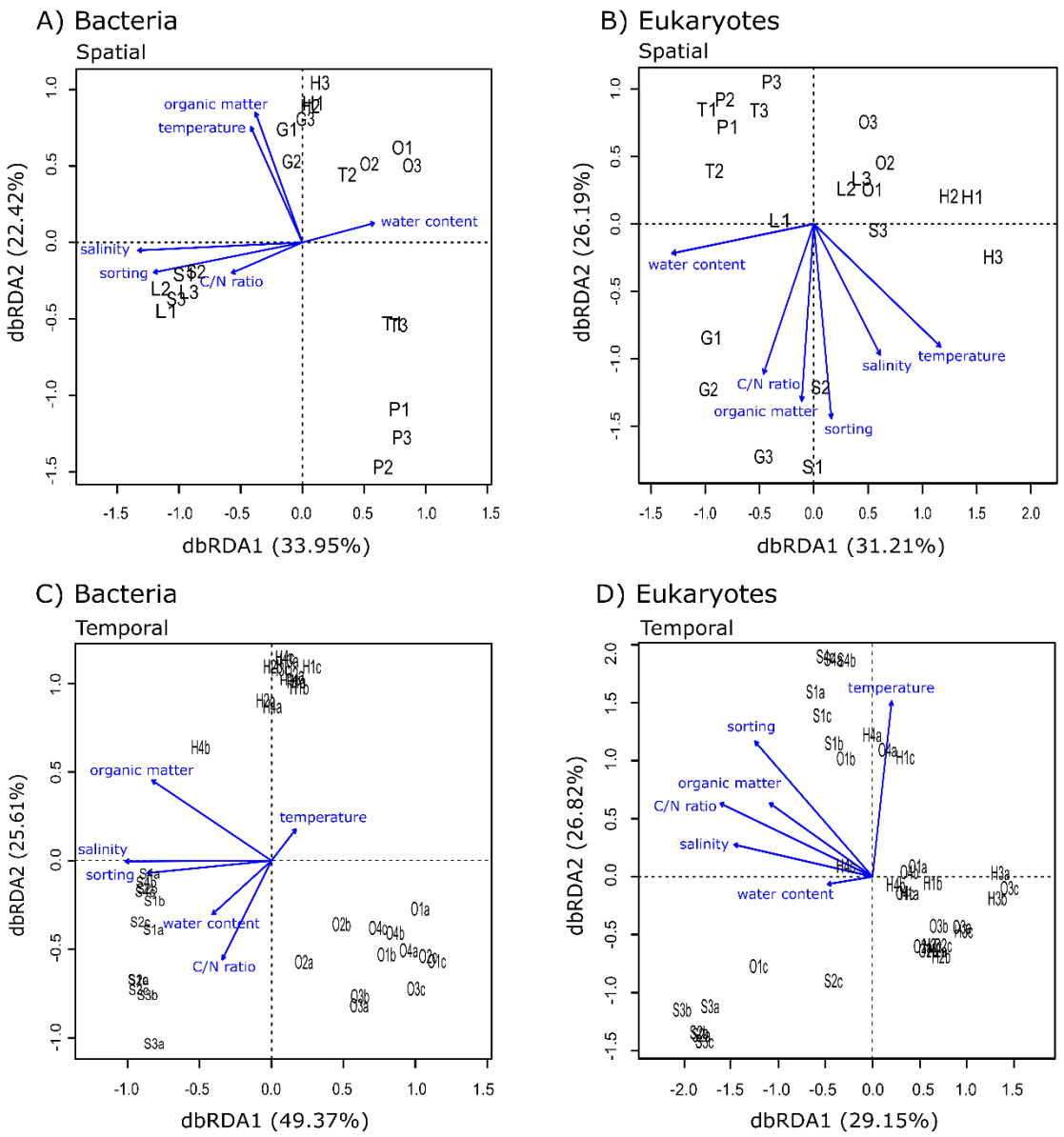
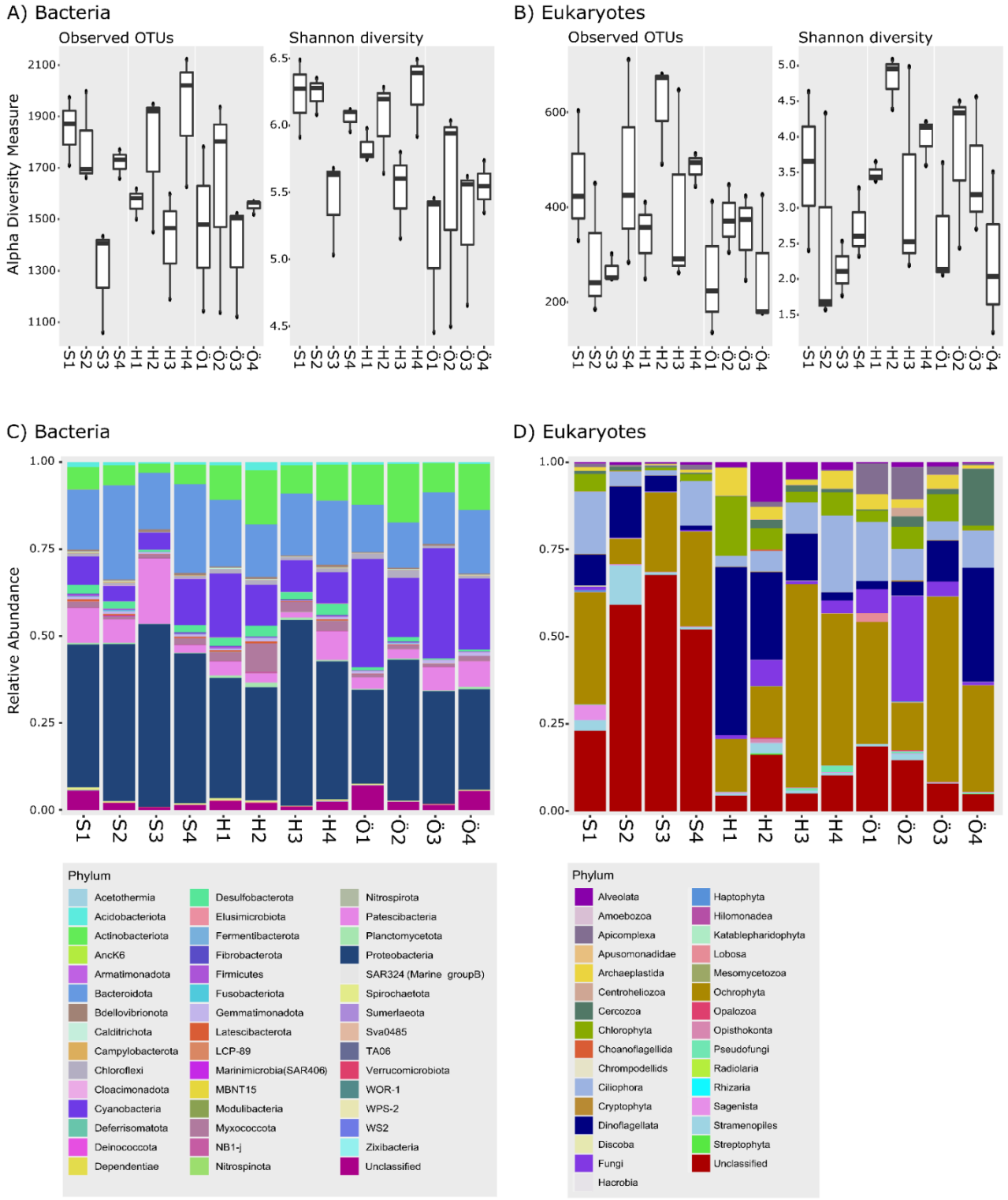

A) Bacteria
Observed OTUs
Shannon diversity
Alpha Diversity Measure
B) Eukaryotes
Observed OTUs
Shannon diversity
L S H G Ö T P
33 26 15 13 7 7 6
C) Bacteria
D) Eukaryotes
Relative Abundance
Phylum
Acetothermia
Acidobacteriota
Actinobacteriota
AncK6
Bacteroidota
Bdellovibrionota
Calditrichota
Campylobacterota
Chloroflexi
Cloacimonadota
Cyanobacteria
Deferrisomatota
Deinococcota
Desulfobacterota
Elusimicrobiota
Fermentibacterota
Fibrobacterota
Firmicutes
Fusobacteriota
Gemmatimonadota
Latescibacterota
LCP-89
Marinimicrobia(SAR406)
MBNT15
Modulibacteria
Myxococcota
NB1-j
Nitrospinota
Nitrospirota
Patescibacteria
Planctomycetota
Proteobacteria
SAR324 (Marine groupB)
Spirochaetota
Sumerlaeota
Sva0485
TA06
Verrucomicrobiota
WOR-1
WPS-2
WS2
Zixibacteria
Unclassified
Phylum
Alveolata
Amoebozoa
Apicomplexa
Apusomonadidae
Archaeplastida
Centroheliozoa
Cercozoa
Chlorophyta
Choanoflagellida
Chrompodellids
Ciliophora
Cryptophyta
Dinoflagellata
Discoba
Fungi
Hacrobia
Haptophyta
Hilomonadea
Katablepharidophyta
Lobosa
Mesomycetozoa
Ochrophyta
Opalozoa
Opisthokonta
Picozoa
Prasinodermophyta
Pseudofungi
Radiolaria
Sagenista
Stramenopiles
Streptophyta
Unclassified

**Figure 3.**


A) Bacteria
Spatial
organic matter
temperature
water content
salinity
sorting
C/N ratio
dbRDA2 (22.42%)
dbRDA1 (33.95%)
B) Eukaryotes
Spatial
water content
temperature
salinity
C/N ratio
organic matter
sorting
dbRDA2 (26.19%)
dbRDA1 (31.21%)
C) Bacteria
Temporal
organic matter
temperature
salinity
sorting
water content
C/N ratio
dbRDA2 (25.61%)
dbRDA1 (49.37%)
D) Eukaryotes
Temporal
temperature
sorting
organic matter
C/N ratio
salinity
water content
dbRDA2 (26.82%)
dbRDA1 (29.15%)

649 **Figure 4.**

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651

**Figure 5a.**

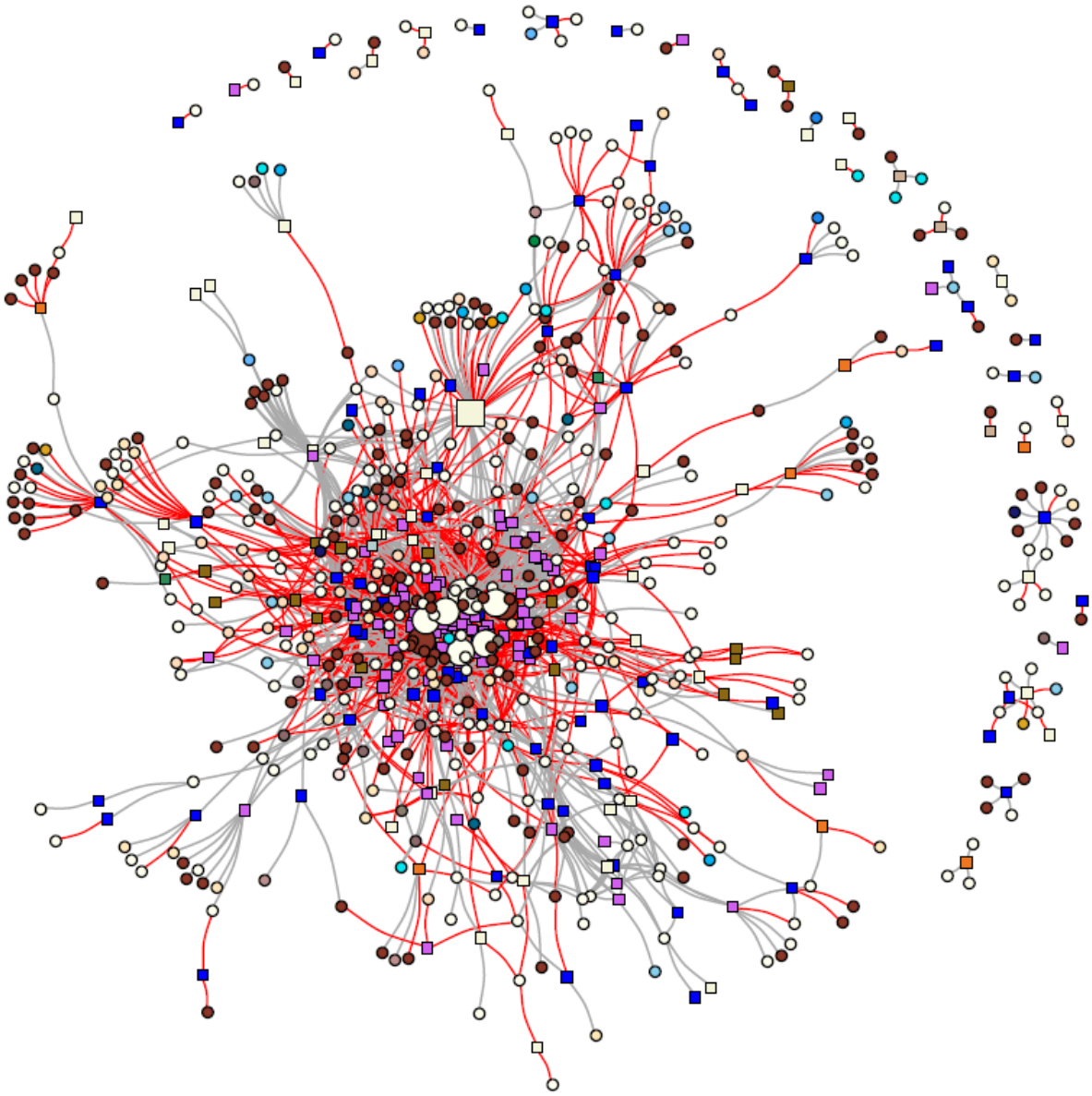


**Figure 5b.**

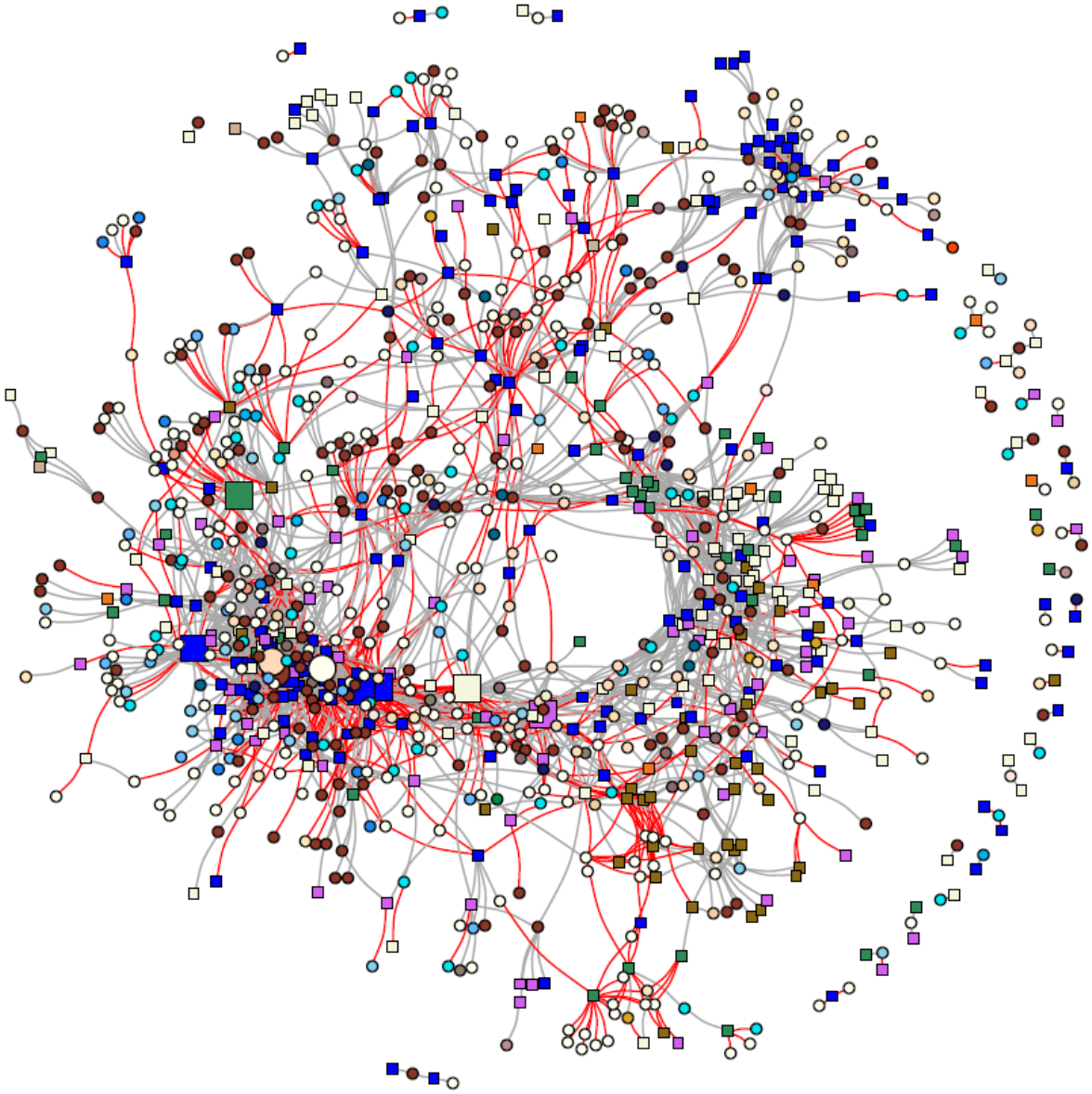
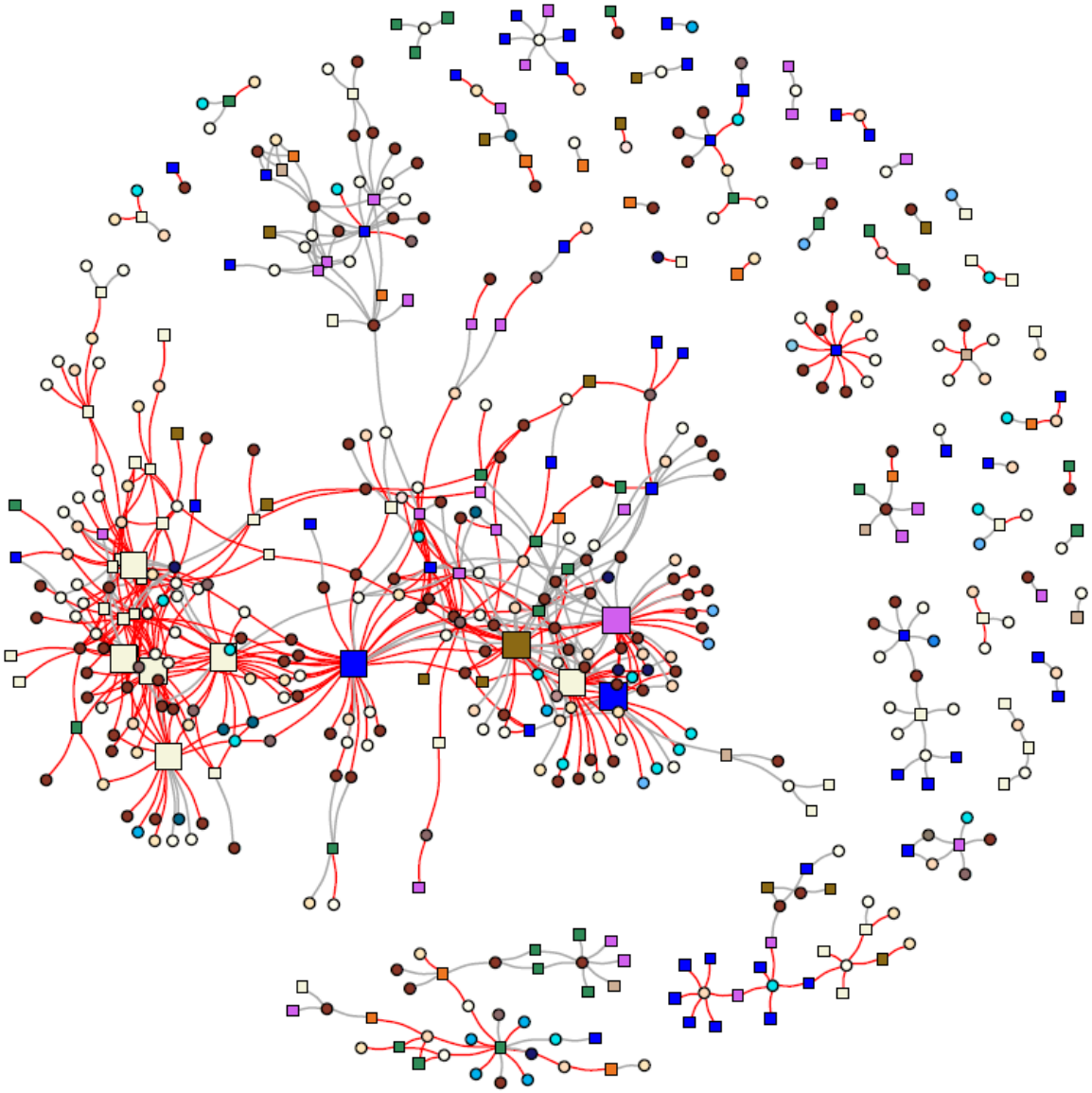


**Figure 5c.**



**Eukaryotes**

- Eukaryota_unclassified(0)
- Alveolata
- Rhizaria
- Archaeplastida
- Stramenopiles
- Opisthokonta
- Hacrobia
- Amoebozoa

**Bacteria**

- Proteobacteria
- Bacteroidota
- Cyanobacteria
- Latescibacterota
- Patescibacteria
- Bdellovibrionota
- Actinobacteriota
- Spirochaetota
- ZUnclassified
- Fibrobacterota
- Desulfobacterota
- Gemmatimonadota
- Acidobacteriota
- AncK6
- Campylobacterota
- Chloroflexi
- Planctomycetota
- Myxococcota
- Sva0485
- Marinimicrobia_(SAR406_clade)

662 **Table 1.**

| **Factors** | **Bacteria spatial** | **Eukaryotes spatial** | **Bacteria temporal** | **Eukaryotes temporal** |
|---|---|---|---|---|
| Salinity | 0.22*** | 0.08* | 0.29*** | 0.07*** |
| Temperature | 0.09*** | ns | 0.05** | 0.06** |
| C/N ratio | 0.07** | 0.08* | 0.09*** | 0.05** |
| Org. matter | 0.12*** | ns | 0.07*** | ns |
| Water content | 0.09*** | 0.08** | 0.05** | ns |
| Porosity | 0.07** | ns | 0.07** | ns |
| Mean grain size | 0.10859** | ns | 0.09814*** | 0.05437** |
| Sediment sorting | 0.17089*** | ns | 0.19869*** | 0.06962** |

663

664

**Figure legends**

**Figure 1.** Map of the Baltic Sea and the adjacent North Sea, including sampling sites: L (List), S (Saltö), H (Herslev), G (Gollwitz), Ö (Öland), T (Tvärminne), P (Pori). Salinity gradient based on Snoeijs-Leijonmalm and Andrén (2017).

**Figure 2**. Spatial patterns of microbial diversity of A, C) bacteria (16S), and B, D) microbial eukaryotes (18S), per site in: L (List), S (Saltö), H (Herslev), G (Gollwitz), Ö (Öland), T (Tvärminne), P (Pori). A) observed number of OTUs and alpha diversity index, Shannon Diversity, in bacteria (16S) and B) observed number of OTUs and Shannon Diversity, in eukaryotes (18S), note y-axes are not equal. C) average relative abundance of phyla/divisions in bacteria (16S) and D) eukaryotes (18S).

**Figure 3**. Distance based Redundancy Analysis (dbRDA) based on Bray-Curtis dissimilarity of bacteria (16S) on spatial (A) and temporal scale (C) and microbial eukaryotes (18S) on spatial (B) and temporal scale (D). A) Sediment characteristics represented by vectors sorting and porosity, B) with sediment characteristics represented by vectors water content and sorting, C) and D) with all sediment characteristics represented; note axes are not equal.

**Figure 4.** Temporal patterns of microbial diversity of A, C) bacteria (16S), and B, D) microbial eukaryotes (18S), per site and collection time. Abbreviations of sample site and time: S1 (Saltö August 2018), S2 (Saltö November 2018), S3 (Saltö April 2019), S4 (Saltö August 2019), H1 (Herslev August 2018), H2 (Herslev November 2018), H3 (Herslev April 2019), H4 (Herslev August 2019), Ö1 (Öland August 2018), Ö2 (Öland November 2018), Ö3 (Öland April 2019), Ö4 (Öland August 2019). A) Observed number of OTUs and alpha diversity index, Shannon Diversity, in bacteria (16S) and B) observed number of OTUs and Shannon Diversity, in eukaryotes (18S); note y-axes are not equal. C) Average relative abundance of phyla/divisions in bacteria (16S) and D) eukaryotes (18S).

**Figure 5.** Networks of microbial communities estimated from temporal samples; bacterial OTUs represented by circles, microbial eukaryote OTUs represented by squares, assignment to specific OTUs indicated in legends. Keystone species (OTUs with highest number of connections) indicated by larger symbols (see supplementary Tables 8A-C). Negative correlations indicated by red connections (lines) and positive correlations indicated by grey connections (lines). **Figure 5a** Communities at the high salinity site Saltö. **Figure 5b** Communities at the medium salinity site Herslev. **Figure 5c** Communities at the low salinity site Öland.

**Table 1.** Correlation of community diversity with measured environmental variables for the spatial and temporal datasets calculated with Adonis on Bray-Curtis distance matrices for bacterial and microbial eukaryote community dissimilarity assessment using 1000 permutations on environmental parameters. Values indicate R2, asterisks indicate significance level, *=p<0.001, **p=0.01, ***p=0.001, ns=not significant.

705 **Supplementary table 1**. Table of primers.

| Primer pair | | Sequences | Amplicon size | Reference |
|---|---|---|---|---|
| **16S V1-V2** | 27F + 338R | F: 5'-AGA GTT TGA TCM TGG CTC AG-3'<br>R: 5'-ATT ACC GCG GCT GCT GG-3' | 311 bp | Ludwig et al. 1993, Suzuki and Giovannoni 1996 |
| **18S V4** | UNonMetF+ UNonMetR | F: 5´-GTG CCA GCA GCC GCG-3´<br>R: 5´-TTT AAG TTT CAG CCT TGC G-3´ | 600 bp | Bower et al. 2004 |
| | E572 + E1009R | F: 5´-CYG CGG TAA TTC CAG CTC-3´<br>R: 5´-CRA AGA YGA TYA GAT ACC RT-3´ | 400 bp | Comeau et al. 2011 |

706

707 **Supplementary table 2.** Thermocycling protocols for each target.

| | **UNonMetF/ UNonMetR** | **E572/E1009R** |
|---|---|---|
| PCR conditions | 1. 94°C, 2 min | 1. 94°C, 2 min |
| | 2. 94°C, 10 s | 2. 94°C, 30 s |
| | 3. 51,1 °C, 30 s } 35x | 3. 55 °C, 30 s } 30x |
| | 4. 72°C, 1 min | 4. 72°C, 1 min |
| | 5. 72°C, 5 min | 5. 72°C, 5 min |

708

709 **Supplementary table 3.** Target specific M13-linker primer and fusion primer sequences. Each
710 sample was barcoded with 10-12 bp long unique barcode (marked with N) attached to IonA-forward
711 fusion primer.

| Primer | Sequence |
|---|---|
| **M13**_27F | **TGTAAAACGACGGCCAGT**AGAGTTTGATCMTGGCTCAG |
| **M13**_E572F | **TGTAAAACGACGGCCAGT**CYGCGGTAATTCCAGCTC |
| **IonP1**_338R | **CCTCTCTATGGGCAGTCGGTGAT**TGCTGCCTCCCGTAGGAGT |
| **IonP1**_E1009R | **CCTCTCTATGGGCAGTCGGTGAT**CRAAGAYGATYAGATACCRT |
| **IonA** _key_**bc**_ M13 | **CCATCTCATCCCTGCGTGTCTCCGA**CTCAG**NNNNNNNNNN**TGTAAAACGACGGCCAGT |

712

713 **Supplementary table 4.** Environmental variables measured at all samplings.

| Site | Sample time | Salinity | Temp. | C/N ratio | Organic matter | Water-content | Porosity | M. grain size | Sorting |
|---|---|---|---|---|---|---|---|---|---|
| List | August 2018 | 33 | 22 | 9.48 | 0.940 | 17.59 | 0.34 | 1.41 | 1.27 |
| Saltö | August 2018 | 26 | 18 | 10.46 | 1.16 | 24.03 | 0.43 | 2.24 | 1.76 |
| | November 2018 | 25 | 3 | 8.86 | 1.24 | 27.22 | 0.50 | 2.82 | 0.82 |
| | April 2019 | 23 | 11 | 8.57 | 1.07 | 21.20 | 0.40 | 2.35 | 1.53 |
| | August 2019 | 22 | 18 | 7.77 | 1.34 | 25.17 | 0.47 | 2.16 | 1.90 |
| Herslev | August 2018 | 15 | 23 | 6.31 | 1.06 | 21.56 | 0.40 | 1.37 | 0.70 |
| | November 2018 | 16 | 5 | 4.40 | 1.16 | 23.35 | 0.48 | 2.15 | 0.85 |
| | April 2019 | 15 | 15 | 1.94 | 1.02 | 22.46 | 0.40 | 2.35 | 0.85 |
| | August 2019 | 15 | 17 | 8.30 | 1.23 | 20.37 | 0.37 | 2.06 | 1.05 |
| Gollwitz | August 2018 | 13 | 23 | 9.83 | 1.42 | 27.63 | 0.49 | 2.78 | 0.89 |
| Öland | August 2018 | 7 | 21 | 9.89 | 0.89 | 22.69 | 0.43 | 2.40 | 0.63 |
| | November 2018 | 8 | 1 | 3.83 | 0.61 | 24.15 | 0.44 | 2.70 | 0.41 |
| | April 2019 | 9 | 14 | 7.06 | 0.55 | 23.42 | 0.44 | 2.50 | 0.46 |
| | August 2019 | 8 | 18 | 6.90 | 0.70 | 20.75 | 0.37 | 2.48 | 0.49 |
| Tvärminne | August 2018 | 7 | 13 | 6.82 | 1.40 | 32.41 | 0.56 | 2.60 | 0.67 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pori | August 2018 | 6 | 18 | 8.64 | 0.24 | 22.42 | 0.40 | 2.49 | 0.63 |

714

715 **Supplementary table 5a**. Differences in Shannon index between sites in spatial study by TukeyHSD
716 test. Different letters indicate significantly different sites. No significant differences were found by
717 Tukey HSD test for richness of bacteria and eukaryotes.

| Spatial Dataset | Shannon_Bacteria |
|---|---|
| Herslev | ab |
| List | ab |
| Pori | ab |
| Saltö | a |
| Tvärminne | ab |
| Öland | b |
| Gollwitz | ab |

718

719 **Supplementary table 5b**. Differences in Shannon index and richness (observed diversity) between
720 sites in temporal study tested by TukeyHSD test. Different letters for each site indicate significantly
721 different sites.

| Temporal Dataset | Bacteria | | Eukaryote | |
|---|---|---|---|---|
| Diversity | Shannon | Observed | Shannon | Observed |
| Saltö | a | a | a | ab |
| Öland | b | a | ab | a |
| Herslev | a | a | b | b |

722

723 **Supplementary table 6.** ANOVA test of alpha diversity measured by richness (observed) and
724 Shannon against salinity. Note salinity is grouped to three categories as low (<10), medium (>10-20)
725 and high (>20). Values indicate p value, asterisk indicate significance level, *=p<0.001, **p=0.01,
726 ns=not significant.

| **Dataset** | **Observed** | **Shannon** |
|---|---|---|
| **Spatial Bacteria** | ns | 0.06 |
| **Temporal Bacteria** | ns | 0.00 ** |
| **Spatial Eukaryotes** | ns | ns |

| | | |
|---|---|---|
| **Temporal Eukaryotes** | 0.04 * | 0.03 * |

727

728 **Supplementary table 7.** See attached excel-sheet. Correlation between OTUs and salinity.

729 **Supplementary table 8a.** Keystone species of high salinity site Saltö, temporal samples. OTU
730 identity listed as botu = bacterial OTU, eotu = eukaryote OTU.

| OTU | Connections | Phylum | Order | Genus |
|---|---|---|---|---|
| botu515 | 60 | Gammaproteobacteria | Halieaceae | Proteobacteria |
| botu607 | 55 | Alphaproteobacteria | Rhodobacteraceae | Proteobacteria |
| botu331 | 53 | Gammaproteobacteria | BD2-7 | Proteobacteria |
| botu613 | 52 | Gammaproteobacteria | BD7-8_fa | Proteobacteria |
| botu700 | 50 | Gammaproteobacteria | Saccharospirillaceae | Proteobacteria |
| botu239 | 47 | Alphaproteobacteria | uncultured | Proteobacteria |
| eotu228 | 45 | Ochrophyta | Bacillariophyta | Stramenopiles |
| botu1484 | 44 | Bacteroidia | Crocinitomicaceae | Bacteroidota |
| botu3889 | 43 | Bacteroidia | Flavobacteriaceae | Bacteroidota |
| botu183 | 42 | Gammaproteobacteria | Alteromonadaceae | Proteobacteria |
| eotu520 | 41 | Unclassified | Unclassified Eukaryota | Unclassified Eukaryota |
| botu2817 | 41 | Bacteroidia | Flavobacteriaceae | Bacteroidota |
| botu929 | 41 | Alphaproteobacteria | Rhodobacteraceae | Proteobacteria |
| eotu1227 | 40 | Unclassified | Unclassified Eukaryota | Unclassified Eukaryota |
| eotu356 | 40 | Ochrophyta | Phaeophyceae | Stramenopiles |

731

732 **Supplementary table 8b.** Keystone species of medium salinity site Herslev, temporal samples.
733 OTU identity listed as botu = bacterial OTU, eotu = eukaryote OTU.

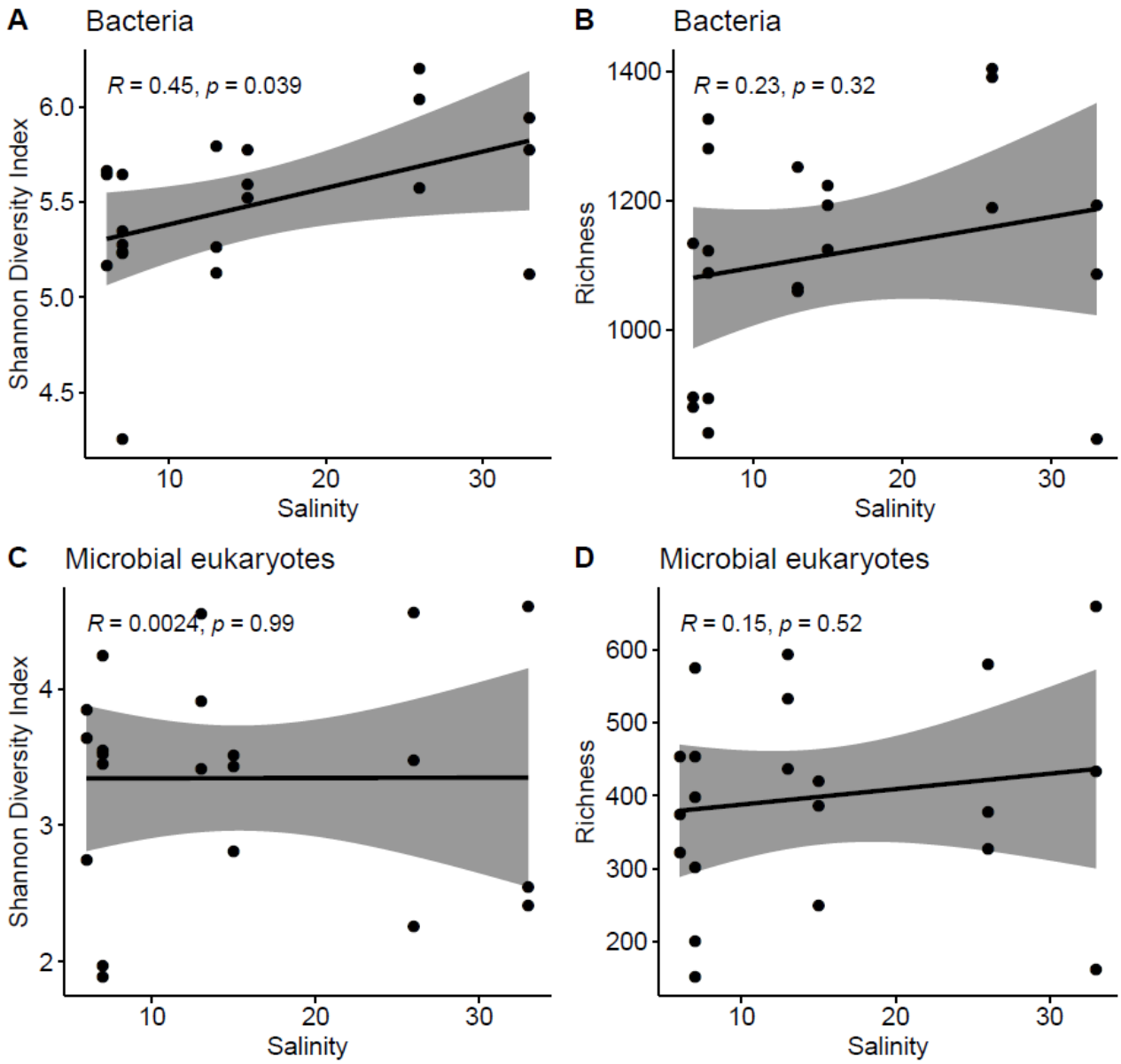
| OTU | Connections | Phylum | Order | Genus |
|---|---|---|---|---|
| botu1673 | 51 | Bacteroidota | Flavobacteriales | Unclassified Flavobacteriaceae |
| eotu933 | 45 | Alveolata | Dinophyceae | Dinophyceae |
| eotu3734 | 40 | Alveolata | Dinophyceae | Unclassified Dinophyceae |
| eotu34 | 37 | Stramenopiles | Bacillariophyta | Unclassified Raphid pennate |
| eotu1174 | 36 | Opisthokonta | Ascomycota | Unclassified Pezizomycotina |
| botu607 | 35 | Proteobacteria | Rhodobacterales | Unclassified Rhodobacteraceae |
| eotu113 | 30 | Unclassified Eukaryota | Unclassified Eukaryota | Unclassified Eukaryota |
| eotu3124 | 28 | Alveolata | Dinophyceae | Unclassified Dinophyceae |
| eotu4284 | 28 | Alveolata | Dinophyceae | Unclassified Dinophyceae |
| botu370 | 28 | Patescibacteria | Candidatus Campbellbacteria | Candidatus Campbellbacteria_ge |
| eotu363 | 27 | Unclassified Eukaryota | Unclassified Eukaryota | Unclassified Eukaryota |
| eotu681 | 27 | Stramenopiles | Unclassified Stramenopiles | Unclassified Stramenopiles |
| botu1222 | 27 | Bacteroidota | Chitinophagales | uncultured |
| botu290 | 27 | Bacteroidota | Flavobacteriales | Lutibacter |

| | | | | |
|---|---|---|---|---|
| botu4620 | 27 | Bacteroidota | Flavobacteriales | Aquibacter |
| eotu852 | 26 | Alveolata | Unclassified Alveolata | Unclassified Alveolata |

**Supplementary table 8c.** Keystone species of low salinity site Öland, temporal samples. OTU identity listed as botu = bacterial OTU, eotu = eukaryote OTU.

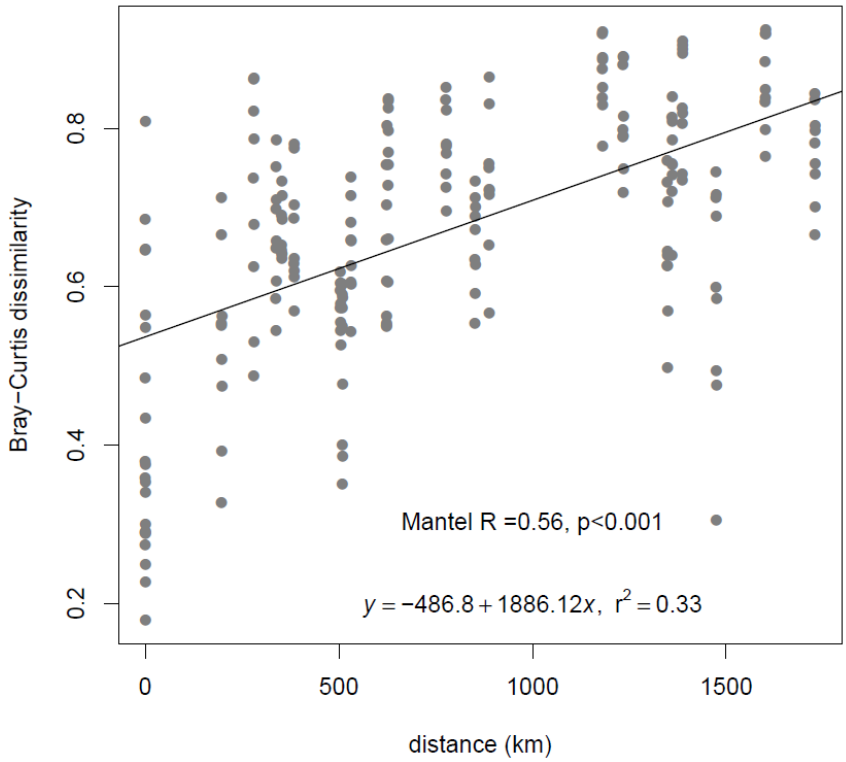
| OTU | Connections | Phylum | Order | Genus |
|---|---|---|---|---|
| eotu368 | 34 | Alveolata | Dinophyceae | Unclassified Gymnodiniaceae |
| eotu338 | 32 | Unclassified Eukaryota | Unclassified Eukaryota | Unclassified Eukaryota |
| eotu67 | 30 | Archaeplastida | Chlorophyceae | Unclassfied Chlamydomonadales |
| eotu3341 | 29 | Stramenopiles | Phaeophyceae | Unclassfied Phaeophyceae |
| eotu231 | 26 | Stramenopiles | Bacillariophyta | Unclassfied Raphid-pennate |
| eotu135 | 22 | Alveolata | Unclassfied Alveolata | Unclassfied Alveolata |
| eotu176 | 22 | Stramenopiles | Bacillariophyta | Unclassfied Raphid-pennate |
| eotu497 | 21 | Stramenopiles | Bacillariophyta | Unclassfied Raphid-pennate |
| eotu393 | 20 | Stramenopiles | Unclassified Ochrophyta | Unclassified Ochrophyta |
| eotu863 | 20 | Stramenopiles | Bacillariophyta | Unclassfied Raphid-pennate |
| eotu730 | 17 | Stramenopiles | Unclassified Sagenista | Unclassified Sagenista |

739 **Supplementary figure 1.** Pearson correlation of alpha diversity and salinity for spatial samples.



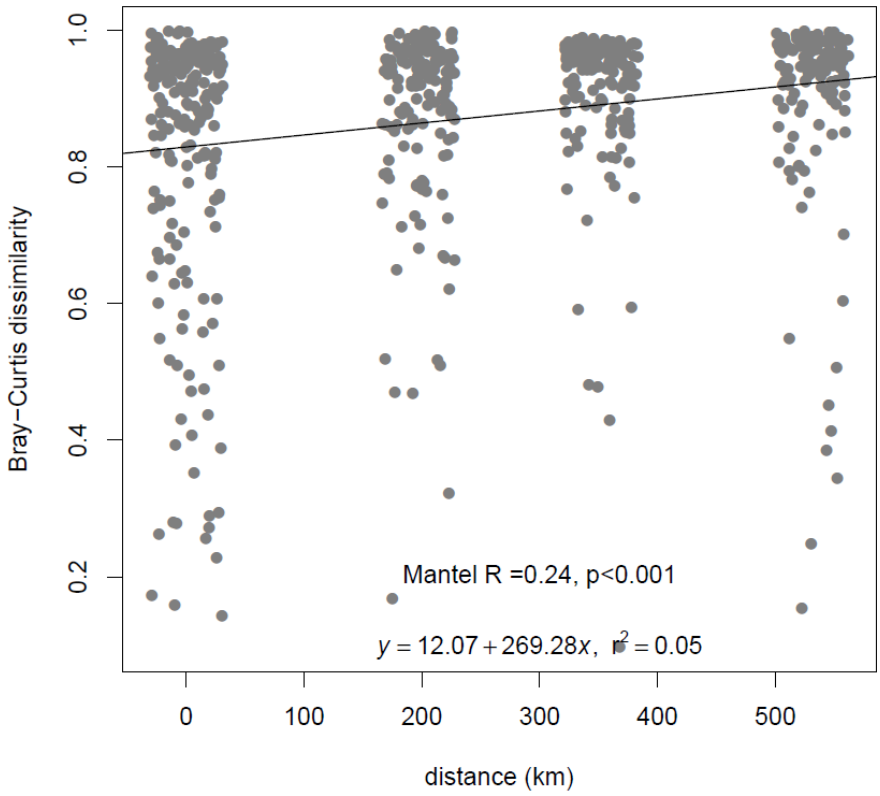

740

741 **Supplementary figure 2a.** Analysis of distance-decay for spatial bacterial dataset.



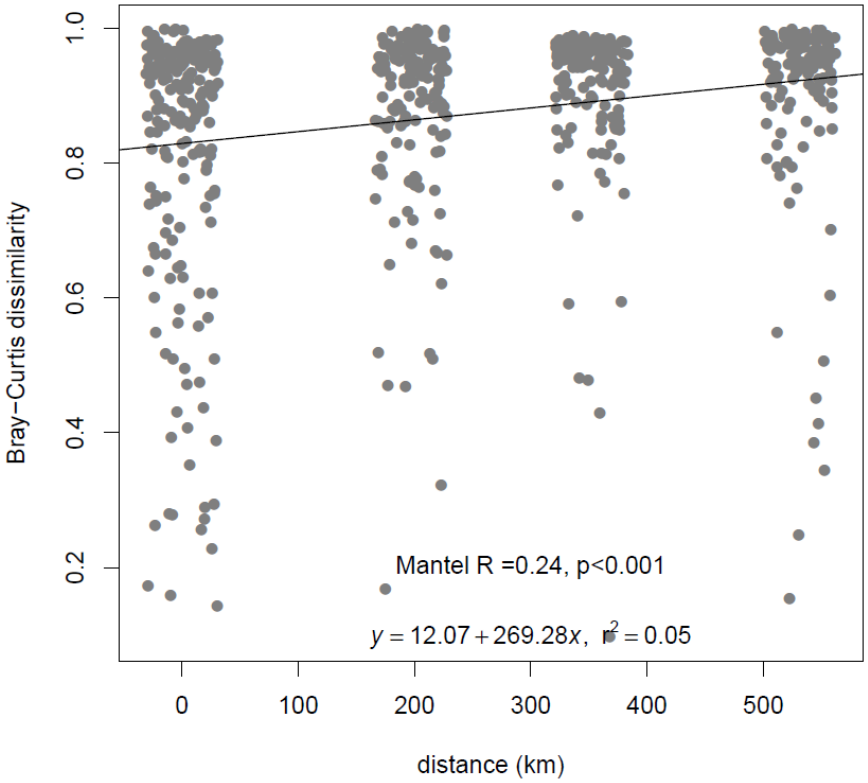

742

743 **Supplementary figure 2b.** Analysis of distance-decay for temporal bacterial dataset.




744

745 **Supplementary figure 1c.** Analysis of distance-decay for temporal microbial eukaryote dataset.

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747

**Supplementary figure 3.** Pearson correlation of alpha diversity and salinity for temporal samples.