**Environmental changes associated with drying climate are expected to affect functional groups of pro- and microeukaryotes differently in temporary saline waters**

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## Highlights

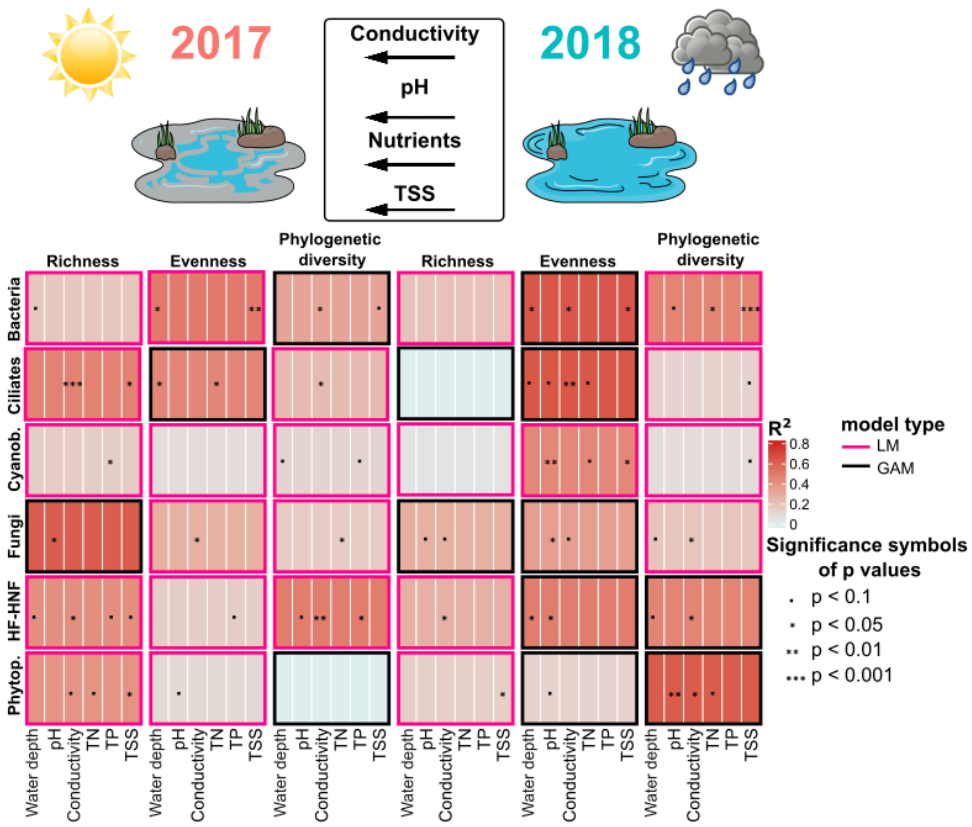
- Microbial diversity of temporary saline ponds is still largely unknown
- We studied six functional groups in a habitat network applying amplicon sequencing
- We compared environmental drivers of diversity during a dry and wet spring
- Conductivity was the most important environmental gradient shaping diversity patterns
- Environmental drivers overall played a stronger role in a dry spring


## Abstract

Temporary ponds are one of the most sensitive aquatic habitats to climate change. Their microbial communities have crucial roles in food webs and biogeochemical cycling, yet how their communities are assembled along environmental gradients is still understudied. This study aimed to reveal the environmental drivers of the microbial diversity and phylogenetic diversity patterns from a network of saline temporary ponds (soda pans in Austria) in two consecutive spring seasons characterized by contrasting weather conditions (one dry, one wetter). These habitats exhibit multiple environmental stress gradients and are therefore a highly suitable model system to understand the responses of microbial communities. We used DNA-based molecular methods (16S rRNA, 18S rRNA) to investigate the microbial community composition. We tested the effect of environmental variables on the diversity of prokaryotic and microeukaryotic functional groups (Bacteria, Cyanobacteria, Ciliates, heterotrophic flagellates and nanoflagellates, Fungi, phytoplankton) within and across the years. Conductivity and the concentration of total suspended solids and phosphorus were the most important environmental variables affecting all six functional groups. Environmental conditions were harsher and they also had a stronger impact on community composition in the dry spring. Our results imply that harsher environmental conditions, which are becoming more frequent with climate change, have a negative effect on most levels of microbial diversity in

temporary saline ponds. At the same time, not all microbial functional groups responded the same way to harsher environmental conditions. This eventually might translate into community-level shifts across trophic groups with changing local conditions with implications for ecosystem functioning. This is the first regional-level study to properly assess how microbial diversity of these rare and threatened ecosystems is shaped by environmental gradients based on amplicon sequencing. We encourage further long-term monitoring studies of these ponds, in order to specifically link changes in the local conditions to the changing climatic parameters.

**Graphical abstract**




**Key words**

Temporary saline ponds, environmental drivers, drying climate, microbial community, diversity patterns

## 1. Introduction

Temporary ponds are a globally widespread habitat type with diverse aquatic communities and a wide variety of ecological functions (Céréghino *et al.*, 2007; Olmo *et al.*, 2022). Despite their small size, their importance in terms of biodiversity is well-known for multiple groups including amphibians (Griffiths, 1997; Boix *et al.*, 2020; Fritz and Whiles, 2021), macrophytes (Jeffries, 2008; Hassall, 2014; Lukács *et al.*, 2015), and macroinvertebrates (Florencio *et al.*, 2014; Meland *et al.*, 2020), and they also serve as a refuge for several rare or endangered species (Céréghino *et al.*, 2007; Boix *et al.*, 2020). At the same time, less is known about their microbial communities (Hahn, 2006). Microorganisms not only provide the foundations of local food webs, but their contribution to trophic relationships and biogeochemical processes are essential in temporary aquatic ecosystems (Felföldi, 2020; Grossart *et al.*, 2020). Even so, biodiversity studies on microorganisms in temporary ponds are still much rarer than those on macroorganisms (Marrone, Fontaneto and Naselli-Flores, 2022). As microbial biodiversity is linked to key ecosystem functions, such as nutrient cycling, mineralization (Ducklow, 2008; Bodelier, 2011) and ecosystem services (Bell *et al.*, 2005; Trivedi *et al.*, 2019), identifying its drivers is of high importance to understand the functioning of temporary waters.

Environmental harshness is generally higher in temporary ponds than permanent ones due to periodic droughts and rapid seasonal changes in environmental conditions (Wellborn, Skelly and Werner, 1996; Chase, 2007; Tweed *et al.*, 2011). As temporary aquatic habitats are usually very shallow, local species often need to withstand high UV radiation (Aguilar *et al.*, 2016) and extreme daily fluctuations in temperature (Boros *et al.*, 2017). Saline temporary waters represent even more extreme habitats, where the overall high salinity levels and their seasonal fluctuation represent additional environmental stress (Lengyel *et al.*, 2019). Microorganisms are known to be able to sustain large local population sizes even in such extreme conditions

due to their high physiological and evolutionary adaptation potential linked to their small size and short generation times (Hahn, 2006; Vieira-Silva and Rocha, 2010). Even so, saline inland waters have so far received little attention when it comes to the diversity of microbial communities (Cunillera-Montcusí *et al.*, 2022) compared to estuaries (Šolić *et al.*, 2015; Traving *et al.*, 2017; Zhang *et al.*, 2021) and man-made salterns (Tkavc *et al.*, 2011; Ali *et al.*, 2016; Paul *et al.*, 2020). Consequently, less is known about temporary saline waters and existing studies are generally limited to specific groups like diatoms (Stenger-Kovács *et al.*, 2014, 2016; Szabó *et al.*, 2018) or bacteria (Szabó *et al.*, 2017, 2020), based on small spatial scales and a limited number of habitats (Cunillera-Montcusí *et al.*, 2022). Although salinity has been a focal point of studies (Benlloch *et al.*, 2002; Horváth *et al.*, 2014; Mo *et al.*, 2021), we still have little information about the phylogenetic diversity of microorganisms along salinity gradients in temporary ponds and how community composition or the relative share of multiple functional groups change along these gradients. Regional-scale studies could deliver important insights into these patterns and processes, but they are still largely missing (Cunillera-Montcusí *et al.*, 2022).

Temporary ponds are especially vulnerable to effects of climate change like warming, changes in seasonal precipitation patterns, and increased duration and intensity of extreme events such as droughts, which leads to shrinking water surface and eventually the disappearance of these habitats (Gómez-Rodríguez, Bustamante and Díaz-Paniagua, 2010; Finger Higgens *et al.*, 2019; Boix *et al.*, 2020). Besides, there is a global trend of naturally permanent ponds becoming temporary (Pekel *et al.*, 2016). This is happening at an increasing rate (Grillas *et al.*, 2021; Xi *et al.*, 2021) and several regions with naturally abundant temporary ponds and wetlands have already suffered significant habitat losses (Zacharias and Zamparas, 2010; Finger Higgens *et al.*, 2019; Horváth *et al.*, 2019). The shortening hydroperiod of temporary ponds under climate change is expected to result in loss of biodiversity (Grillas *et al.*, 2021; Xi *et al.*, 2021; Zhong

*et al.*, 2022), especially when coupled with other stressors, such as an increased rate of salinization (Atkinson *et al.*, 2021). Increasing salinity has been shown to alter the microbial community structure of ponds (Mo *et al.*, 2021; Cunillera-Montcusí *et al.*, 2022), which has a cascading effect on ecosystem functions, e.g. the balance between greenhouse gas emission and carbon sequestration (Whiting and Chanton, 2001; Mitsch *et al.*, 2013; Ardón, Helton and Bernhardt, 2018). At the same time, studies on the responses of microbial communities to elevated salinities were mostly done in the context of salinization of freshwaters (Jeppesen *et al.*, 2020). Therefore, our knowledge of how microbial communities of naturally saline habitats will respond to such changes is still limited.

Here, we present a study based on a network of natural saline temporary ponds to investigate diversity, phylogenetic diversity, as well as community composition of pro- and eukaryotes along local environmental gradients in two consecutive spring seasons. Our study area is a network of temporary soda pans in eastern Austria, with salinities usually ranging from sub- to hyposaline values (Boros *et al.*, 2014). Due to the close proximity of the ponds, they are under the same climatic and geographical conditions, making them a suitable regional case study for the environmental drivers of microbial communities. The long-term future of these vulnerable and geographically restricted inland saline ecosystems is threatened by the intensifying effects of climate change in the form of decreasing precipitation and increasing temperature in the region (Figure 1). Specifically, our first aim is to explore the main environmental drivers of taxonomic and phylogenetic richness and composition of microorganism communities (pro- and microeukaryotes). Second, we test whether the identity and strength of main drivers change with contrasting climatic conditions (by comparing the dry spring of 2017 and the wetter 2018 and comparing these conditions to earlier reference data from 2009-2010), which can have important implications for the future of these vulnerable ecosystems.

## 2. Methods

### 2.1. Sampling and sample processing

Soda lakes are a special type of saline lakes, with carbonate ($CO_3^{2-}$), bicarbonate ($HCO_3^-$) and sodium ($Na^{2+}$) as the dominant ions and a stable alkaline pH (Boros *et al.*, 2014; Felföldi, 2020). They can be found worldwide but are less frequent than other saline aquatic habitats (Sorokin *et al.*, 2014). In Europe, they mainly occur as temporary soda pans on the Pannonian Plain (Austria, Hungary, and Serbia) (Boros *et al.*, 2017).

In the present study, water samples were collected from 26 soda pans from the same region (National Park Neusiedlersee-Seewinkel, Austria) in the same spring period of 2017 (3-6 of April) and 2018 (2-4 of April) (Figure 1, Table S1). These two years had very contrasting weather conditions and hydroregimes. For the spring filling of soda pans, precipitation during the winter months is crucial (Lengyel *et al.*, 2019). During the four months prior to our 2017 survey, the amount of average monthly precipitation (26 mm) was almost half of what was measured in 2018 (47 mm; Supplementary material, Table S2). Additionally, an increasing trend in annual temperature can be observed in the region (Figure 1), which likely contributes to higher evaporation rates. Altogether, there was an overall difference in the hydrological conditions, with one of the soda pans (Kirchsee) even being completely dry in April 2017.

Water depth and Secchi depth were measured in the open water of each soda pan. A total of 20 L of water was collected from at least 20 different points of the soda pans using a one-liter beaker and filtered through a plankton net (mesh size of 100 μm) to remove larger zooplankton and filamentous algae, to prevent sequencing bias in community composition assessments. 1 L of the filtered composite water sample was delivered to the laboratory in a glass bottle in a cool box for measuring total suspended solids (TSS) and processing molecular samples. We collected a similar composite water sample, however unfiltered, to measure conductivity and

pH with a multimeter and to take samples for determining total nitrogen (TN) and total phosphorus (TP) concentration, which were subsequently frozen until further processing.

To measure the amount of TSS, water was filtered through a pre-weighted GF/F filter until it was clogged. The filters were dried overnight at 60 °C and they were weighed again to calculate TSS concentration. TN and TP from the unfiltered water samples were measured according to Clesceri *et al.*, (1999) and Hansen and Koroleff (1999).

1-50 mL of the composite water samples (depending on turbidity) were filtered through a nitrocellulose membrane filter (Ø 47 mm, 0.22 µm pore size) for molecular biology analysis. Filters were stored at -20 °C until DNA isolation.

## 2.2. DNA extraction and sequencing

DNA was extracted from the filters by using PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted DNA was stored at -20 °C until shipping to LGC Genomics (Berlin, Germany) for amplicon sequencing.

For the determination of prokaryotic and eukaryotic community composition, the V4 region of the 16S rRNA gene and the V7 region of the 18S rRNA gene were amplified, using prokaryotic primers EMBf 515F (GTGYCAGCMGCCGCGGTAA, Parada, Needham and Fuhrman, 2016) – EMBr 806R (GGACTACNVGGGTWTCTAAT, Apprill *et al.*, 2015) and eukaryotic primers UnivF-1183mod (AATTTGACTCAACRCGGG) – UnivR-1443mod (GRGCATCACAGACCTG, Ray *et al.*, 2016). All polymerase chain reactions and amplicon sequencing (Illumina MiSeq platform) were carried out by LGC Genomics (Berlin, Germany) following the steps described in Szabó *et al.*, 2022 (Table S3).

## 2.3. Amplicon sequence analysis, taxonomic assignment, and community data

Bioinformatic analysis of the sequences were carried out with mothur v1.43.0 (Schloss *et al.*, 2009) using the MiSeq SOP http://www.mothur.org/wiki/MiSeq_SOP (Kozich *et al.*, 2013), downloaded on 12th November 2020, with the modification of adjusting the deltaq parameter to 10 in the "make.contigs" command. Primers were removed from both ends of the sequences and singletons were excluded from the dataset according to Kunin *et al.*, 2010. Read alignments and taxonomic assignments were carried out using the ARB-SILVA SSU Ref NR 138 reference database with a minimum bootstrap confidence score of 80 (Quast *et al.*, 2012). Reads assigned to non-target specific lineages (e.g. Archaea, Chloroplast, Mitochondria, unknown) were removed from the dataset. Operational taxonomic units (OTUs) were picked at 99% similarity threshold levels. For the 16S rRNA OTUs, TaxAss software (Rohwer *et al.*, 2018) was used with default parameters based on the FreshTrain (15 June 2020 release) and ARB-SILVA SSU Ref NR 138 databases, the taxonomic assignment of 18S rRNA OTUs was performed with the PR2 v4.12.0 reference database (Guillou *et al.*, 2012). OTUs belonging to taxa Streptophyta, Metazoa, Ascomycota and Basidiomycota were excluded from the eukaryotic dataset. Both the 16S rRNA gene and 18S rRNA gene datasets have been subsampled (rarified) to the lowest read count (8620 reads per sample for the 16S rRNA dataset and 2432 reads per sample for the 18S rRNA dataset). Hereinafter, the resulting dataset is referred to as relative abundance data.

The rarefied prokaryotic and microeukaryotic data were further split into six larger groups based on taxonomy and function. This included two groups of prokaryotes: Bacteria and Cyanobacteria, as well as four groups of eukaryotes: Ciliates, Aquatic fungi, Heterotrophic flagellates and Heterotrophic nanoflagellates (HF-HNF), and Phytoplankton. The functional groups were created with the subset_taxa function in the "phyloseq" package (McMurdie and

Holmes, 2013). Data are openly available in the NCBI SRA database as part of the BioProject accession PRJNA748202 (Szabó *et al.*, 2022).

## 2.4. Statistical analysis

To assess the overall environmental variability across the two years, we first applied non-metric multidimensional scaling (NMDS). To normalize the distribution of all environmental parameters, log transformation was used for conductivity, TP, TN, and TSS, and square root transformation was applied for water depth data prior to all analyses. We standardized all data to unit variance and calculated a Euclidean distance matrix of all normalized environmental data with the "vegan" package (Oksanen et al., 2020). We then tested for the effect of the year with a permutational multivariate analysis of variance (PERMANOVA) with the adonis function of "vegan", using the same distance matrix. To test for differences between the two years, we applied paired t-tests for each environmental variable using the package "ggpubr" (Kassambara, 2020). For the paired t-tests, data from only those habitats were included which were sampled both in 2017 and 2018.

To compare environmental conditions over multiple years, environmental variables were compared to earlier reference dates. A set of environmental variables were available from spring of 2010 and summer of 2009 (Boros *et al.*, 2017) for 16 pans which we also sampled during our study (in 2017 and 2018), hence we used this subset for the comparison. Data transformation and NMDS analysis were performed the same way as described previously for 2017 and 2018. To test the differences between the four years, we applied pairwise comparisons with a PERMANOVA using the "pairwiseAdonis" R package by adjusting for multiple comparisons (Arbizu, 2017).

Three diversity measures, richness (number of OTUs observed), Pielou's evenness (referred as evenness later on), and phylogenetic diversity were used to compare the prokaryotic and

microeukaryotic community composition in the two sampling years. Richness and evenness were used to measure the structure of the communities and phylogenetic diversity (PD) was used as a measure of functional diversity. Richness and evenness were calculated using the "phyloseq" package (McMurdie and Holmes, 2013). Phylogenetic trees were created with the FastTree software (Qiime2 plugin) (Burian et al., 2021). Phylogenetic diversity was quantified through Rao's quadratic entropy from the R package "picante" (Kembel et al., 2010). The three measures of diversity were compared in the two sampling years with paired t-tests on a subset of samples that were sampled in both sampling years.

Canonical correspondence analysis (CCA) was performed to examine the relationship between environmental variables and community composition. For prokaryotes, microeukaryotes, CCAs were created based on phyla, while relative abundances of OTUs were used for the six major functional groups (Bacteria, Ciliates, Cyanobacteria, Fungi, HF-HNF, Phytoplankton) separately (using the package "vegan", Oksanen et al., 2020). Relative abundance data of prokaryotes and microeukaryotes were log(x+1) transformed prior to analysis. To reduce the complexity of the datasets, only those prokaryotic and microeukaryotic phyla were included in the analysis that had more than three occurrences and reached at least five percent relative abundance each year. For the six functional groups, OTU tables were used for the analysis (rare OTUs with less than four occurrences were excluded). Significant environmental variables were identified based on both backward and forward stepwise model selection using permutation tests (n = 199). In order to determine what proportion of total variation was explained by significant variables, CCAs for each group and year were rerun as partial CCAs, in which only the significant ones were kept as constraining variables, while all the others were partialled out as conditioning variables. The ratio of constrained and total inertia yielded the proportion of variation explained.

To analyze which environmental parameters had the strongest effect on local diversity (richness, evenness, and PD), we compared the fit of multiple linear regression models (with the “MASS” package of R, Venables and Ripley, 2002) and generalized additive models with smooth terms (using the “mgcv” package of R, Wood, 2017). Model selection was based on the Akaike’s Information Criteria (AIC). Separate models were fitted for the local diversity indices of the six taxonomic groups and three time periods: data of the two sampling years (2017, 2018) separately or pooled, resulting in a total of 36 multiple regression models. We created a summary table of the best fitted ML or GAM models for the six functional groups in the two years with the significant environmental variables using the “ComplexHeatmap" package (Gu, Eils and Schlesner, 2016). For each group and each time period (2017, 2018 separately and 2017-2018 together), GAM model predictions were used to show the direction of responses of richness, evenness, and PD to environmental variables if their relationship was significant in at least one time period (using the “mgcv” package of R, Wood, 2017).

Additional multiple linear regression models with the same selection method were run for the changes in local richness (subtracting 2018 values from 2017 values per habitat) and changes in each environmental parameter among the two years to link differences in local richness to potential environmental drivers.

All of the statistical analysis and illustrations were made with the R program (R Core Team (2022).

## 3. Results

The two sampling years differed considerably based on the local environmental variables. Water level was significantly lower in 2017 than in 2018, which coincided with significantly higher values of conductivity, TN, TP, TSS, and pH (Figure S1). The overall local environment

of soda pans differed significantly between the two years based on the NMDS (Figure 2), which was confirmed by a PERMANOVA (significant effect of sampling year, $R^2$=0.33, p=0.001).

Based on the comparison of environmental variables with reference dates (according to a pairwise PERMANOVA and NMDS; Supplementary material Table S4 and Figure S2), the wetter spring of 2018 was comparable to spring conditions from a decade ago (2010), while it differed significantly both from the dry spring (2017) and the reference summer conditions (2009). On the other hand, dry spring conditions (2017) statistically did not differ from summer conditions (2009).

Richness of Bacteria and Cyanobacteria was higher in 2017 than in 2018, which was also reflected by the significantly higher PD in these two groups in 2017 (Figure S3). Bacteria also showed significantly higher evenness values in 2017 along with Ciliates. Evenness of phytoplankton communities were also higher in 2017 (Figure S3). Phytoplankton showed different patterns, with higher evenness and PD, but lower richness in 2017. The rest of the indices (Ciliate richness and PD and all diversity indices of Fungi and HF-HNF) showed no difference between the two years.

Prokaryotic phylum composition was significantly influenced by water depth and TP in both years, with conductivity being additionally significant in 2018 (Figure 3a, Table S5). Among the prokaryotic phyla, photosynthetic Cyanobacteria were more abundant in more saline and deeper pans which was more explicit in 2018(Table S6). For microeukaryotic phyla, conductivity was a significant predictor in both years according to the CCAs (Figure 3b), together with either TP (2017) or pH (2018) (Table S7). A separation of taxa along the conductivity gradient was conspicuous in both years, with mixotrophic (Dinoflagellata and/or Cryptophyta) and heterotrophic groups (Centroheliozoa, Alveolata, Ciliophora) being more

abundant in less saline (i.e., lower conductivity) waters, while the relative abundances of Haptophyta and Discoba increased with conductivity (Table S8).

Water depth, conductivity and TP were similarly important predictors of OTU composition in the separate CCA analyses of the major functional groups (Table 1). Conductivity was selected as a significant predictor in almost all models. There was a general trend that the environmental predictors explained a higher variation in OTU composition in 2017 (explained variation ranging between 0.12 - 0.25) than in 2018 (0 - 0.15).

Local species richness of the six major groups (Bacteria, Cyanobacteria, ciliates, Fungi, HF-HNF, Phytoplankton) generally responded linearly to environmental gradients. In case of evenness, several non-linear relationships were observed (e.g., GAM predictions provided a better fit over linear models for five out of the six models for evenness in 2018; Figure 4). We also found U-shaped patterns in a few cases, including Bacteria (PD with TSS) (Figure S6), ciliates (richness with TSS, evenness with water depth) (Figure S8), Fungi (richness with pH) (Figure S9), HF-HNF (evenness with TP) (Figure S10), and phytoplankton (PD with conductivity and pH) (Figure S11).

Prokaryote richness (Bacteria and especially Cyanobacteria) was in general poorly explained by the environment ($R^2$ of the LMs of Bacteria: 0.175 in 2017 and 0.189 in 2018, $R^2$ of LM of Cyanobacteria: 0.141 in 2017 and 0.05 in 2018), and no significant predictor was identified for either group in 2018. In general, environment explained a higher variation in richness in 2017 than in 2018 in all eukaryotic groups and in Cyanobacteria (based on $R^2$ values of the models, Figure 4).

Conductivity and TSS were the most frequent significant predictors for richness and PD across the taxonomic groups (Figure 4). Among all the functional groups, HF-HNF was the only one with a positive relationship with conductivity, which was significant both for richness and PD

in both years (Figure S10). Bacteria and Cyanobacteria generally showed negative trends with conductivity (or negative after a threshold) within the two years, which changed to an overall positive trend when the two years were analysed together (Figure S6-7).

The most frequent significant predictor of evenness was pH across the different groups, while water depth and conductivity was also found to be significant for some of the functional groups (Figure 4).

We found that the relationship between changes in local richness and environmental variables were significant in three out of the six functional groups (Table S9). Local changes in Bacteria and Ciliates richness corresponded significantly to changes in conductivity, while for Fungi, the main driver was the change in pH between the two years. These responses were all negative, indicating a drop in richness with increasing conductivity and pH (Figure 5).

## 4. Discussion

We analysed diversity patterns of microbial communities along environmental gradients in two consecutive spring seasons with contrasting weather and hydroperiod conditions. While environmental conditions in the wetter spring (2018) were comparable to the reference spring data from a decade ago, spring conditions in the dry year (2017) were more similar to the reference summer data, indicating a seasonal shift in the aquatic environment. A general trend was found that environmental parameters had stronger effects on the community composition in the dry spring. Conductivity, TSS, and TP were the most important environmental predictors of the diversity and community patterns across six major functional groups of microbes. At the same time, the response of prokaryotes (Bacteria and Cyanobacteria) to the environmental gradients generally differed from the microeukaryotic functional groups. With the ongoing climate change, dryer wheather conditions and stronger environmental stress gradients can be expected in the region, much like in our 2018 dataset. Therefore, climate change might drive

shifts in community composition of microbial communities in temporary saline ponds, where eukaryotic plankton may be more adversely affected by the increasing environmental stress.

According to our results, conductivity was overall the most important environmental factor shaping microbial diversity patterns. Even though the salinity gradient of the studied habitats was relatively short (ranging from 0.6 to 11 mS/cm conductivity, which equals to 0.4-8.8 g/L salinity based on the conversion factor of Boros *et al.*, 2014), studies indicate that the major changes in saline lake communities are rather expected to happen at these lower salinity levels, with major shift between 3-10 g/L (Williams, 1987; Hammer, 1990; Schallenberg, Hall and Burns, 2003). A former study encompassing all soda pans in the Pannonian ecoregion found a threshold of 3.9 g/L, above which a more pronounced drop in zooplankton species richness was observed (Horváth et al 2013 Oikos). Our data was in agreement with this for most microbial groups, as above 3.2 mS/cm conductivity (2.6 g/L salinity), we observed negative trends in richness in five of the six groups (except for HF-HNF which showed a strikingly different pattern, with a clear increase in richness with conductivity). As most conductivity values were higher than 3 mS/cm in 2017, this can also explain the relatively lower biodiversity values for most groups in 2017 compared to 2018.

Based on earlier studies of Hungarian soda pans, phytoplankton communities are generally dominated by pico-sized algae (Felföldi *et al.*, 2009; Somogyi *et al.*, 2009; Pálffy *et al.*, 2014). Their small size and the high inorganic turbidity of the pans, however, makes their traditional microscopic identification challenging, which altogether might explain the general lack of community-level studies on phytoplankton from these systems. Our study is the first survey based on molecular data of the 18S rRNA gene, which resulted in the first high resolution regional dataset on phytoplankton communities. A previous review based on microscopic data available from soda pans concluded that species number in phytoplankton is rarely above 10 (Padisák and Naselli-Flores, 2021). Compared to this, we found an average phytoplankton

OTU richness of 47 in 2017 and 49 in 2018, with the lowest value of 16 (Runde Lacke in 2017) and the highest value of 105 (Obere Höllacke in 2017). This finding shows that these habitats harbour a much higher phytoplankton diversity than previously assumed, in line with molecular results on the picocyanobacteria diversity of soda pans in Hungary and Austria (Felföldi *et al.*, 2009).

Phytoplankton was the only group with a U-shaped pattern in phylogenetic diversity along the salinity gradient in 2018. Intermediate salinity (2.9 g/L) had the lowest phylogenetic diversity, beyond which phylogenetic diversity increased again with salinity. Similar U-shaped species richness patterns of phytoplankton along salinity gradients are recorded from transitional gradients from freshwater through brackish to marine habitats (Olli *et al.*, 2019; Olli, Tamminen and Ptacnik, 2022). In soda pans, however, the increase in phylogenetic diversity was not coupled with a parallel increase in OTU richness, which suggests that this pattern rather emerged due to the disappearance of closely related taxa rather than the emergence of salt tolerant OTUs.

HF-HNF richness and phylogenetic diversity clearly showed an increasing trend with salinity. Heterotrophic nanoflagellates are the main protozoan grazers of bacterio- and picophytoplankton (Tikhonenkov, Burkovsky and Mazei, 2015). However, their ientification and enumeration with classical microscopic techniques is still challenging, and it can be especially difficult from turbid habitats like soda pans, characterized by a high amount of suspended sediment (Hartmut Arndt *et al.*, 2000). Hence, there were so far no explicit studies available on their occurrences in these systems. Previous studies showed that heterotrophic flagellate diversity is mainly driven by salinity and temperature (Je Lee and Patterson, 1998; Azovsky, Tikhonenkov and Mazei, 2016) and follows the “rule of critical salinity”, which means species richness has its minimum in brackish waters (~5-8 g/L of salinity) (Tikhonenkov, Mazei and Mylnikov, 2006). In our study, HF-HNF richness and phylogenetic

diversity showed an exponential increase with increasing salinity, which is a pattern previously not reported from other saline systems. Our findings prove that molecular methods are needed for a more reliable identification and enumeration of phytoplankton and heterotrophic flagellates and nanoflagellates of soda pans.

Even though the environmental variables did not constrain all taxa the same way in the microbial communities, our results indicated that environment explained more community variation in the dry spring (2017), which is in agreement with species sorting becoming stronger with harsher environmental conditions (Van der Gucht *et al.*, 2007; Vanschoenwinkel *et al.*, 2010; Datry *et al.*, 2016). The response of biodiversity indices to changes in environmental conditions between the two years indicated that elevated levels of salinity and other parameters can be associated with lower levels of biodiversity for most of the microbial functional groups. The limitation of our study is that we cannot compare our recent data to earlier community datasets as this is the first regional survey on the microbial communities of these ponds based on amplicon sequencing. Although we hold no long-term data, our results indicate that climate change can be expected to have a serious negative impact on the biodiversity of these aquatic habitats. We promote that longer term monitoring studies would be necessary to follow the long-term changes in these unique aquatic habitats, to explore the direct links between the changing climatic conditions and their biota.

## 5. Conclusions

Our results demonstrated that soda pans host diverse microbial communities, which were so far largely unexplored due to the lack of regional studies and the limited application of molecular methods. Even though these unique and regionally restricted ecosystems serve as important refuges for a number of rare or endangered species (Horváth *et al.*, 2013; Stenger-Kovács and Lengyel, 2015), and serve as important feeding sites for waterbirds (Boros *et al.*,

2017; Szabó *et al.*, 2022), they are critically endangered by climate change (Horváth *et al.*, 2019). The ongoing environmental changes will most likely lead to biodiversity loss in multiple microbial groups according to our results, which might induce critical ecosystem-level consequences. Therefore, longer term biodiversity studies and targeted research for better understanding the functional role of the most sensitive microbial groups would be crucial, not only in this type of ecosystem, but also in other temporary saline pond networks.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

Zsuzsanna Márton: Sample collection, Laboratory work, Data analysis, Visualization, Writing original draft, Editing

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Csaba F. Vad: Conceptualization, Sample collection, Methodology, Editing

Károly Pálffy: Data analysis, Visualization, Editing

Zsófia Horváth: Conceptualization, Sample collection, Methodology, Editing, Supervision

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**Data availability**

Raw sequence reads will be deposited in the NCBI SRA database upon acceptance of the article.

Table 1 Significant environmental variables ($p < 0.05$) of the OTU composition of prokaryotic and microeukaryotic functional groups based on separate CCAs (presented in the supplementary material, Figure S4-S5)

| | | **Water depth** | **pH** | **Conductivity** | **TN** | **TP** | **TSS** | **Explained variation** |
|---|---|---|---|---|---|---|---|---|
| | | | | **2017** | | | | |
| **Prokaryotes** | **Bacteria** | ● | | ● | | ● | | **0.16** |
| | **Cyanobacteria** | ● | | ● | | ● | | **0.15** |
| **Microeukaryotes** | **Ciliates** | | | | | ● | ● | **0.16** |
| | **Fungi** | ● | | ● | | ● | | **0.14** |
| | **HF-HNF** | | | ● | | | | **0.12** |
| | **Phytoplankton** | ● | | ● | ● | ● | | **0.25** |
| | | | | **2018** | | | | |
| **Prokaryotes** | **Bacteria** | ● | | ● | | ● | | **0.15** |
| | **Cyanobacteria** | | | | | | | **NA** |
| **Microeukaryotes** | **Ciliates** | | | ● | | | | **0.06** |
| | **Fungi** | | | ● | | | | **0.04** |
| | **HF-HNF** | | | ● | | | | **0.07** |
| | **Phytoplankton** | ● | | ● | | | | **0.12** |

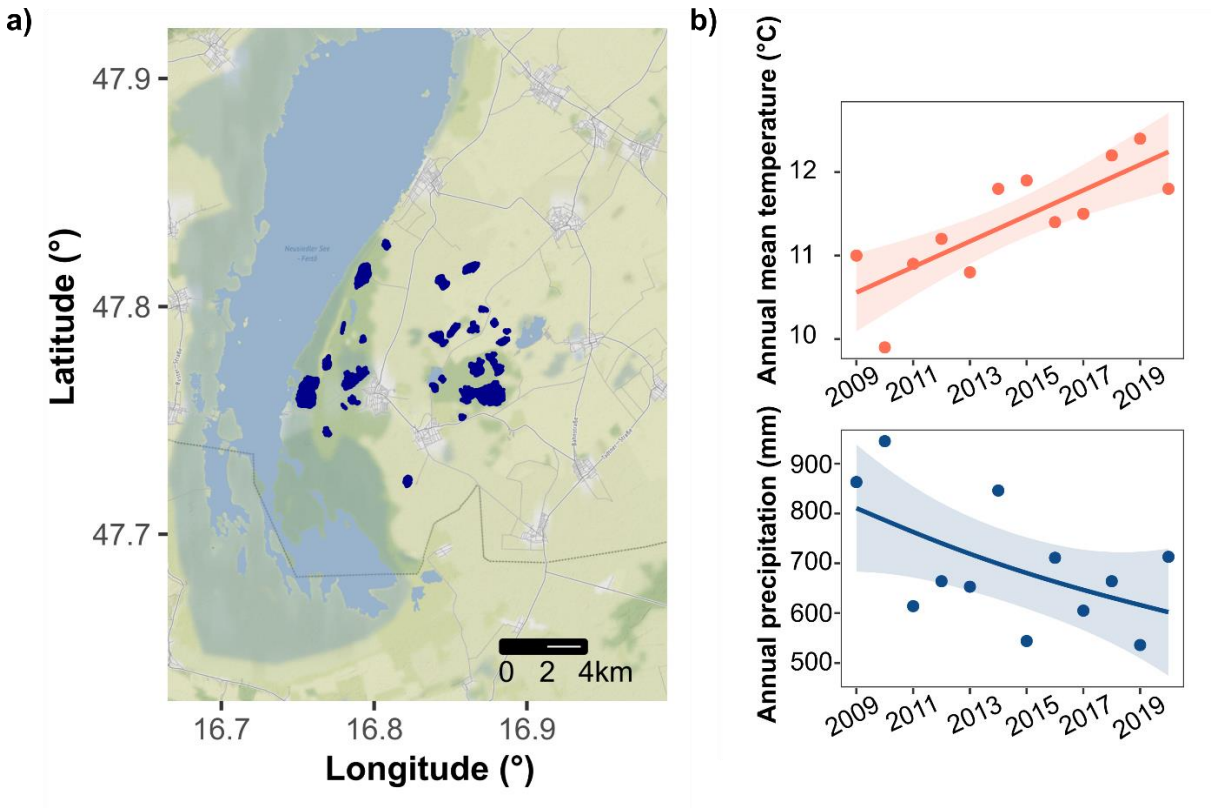


Figure 1 a) Location of the sampled soda pans (dark blue) in the National Park Neusiedlersee-Seewinkel in eastern Austria b) Temporal trends in annual mean temperature and precipitation since 2009 based on meteorological data from the Seewinkel region (Station Eisenstadt; trendlines and confidence intervals are based on Generalized Additive Models, see also in Supplementary material Text S1)

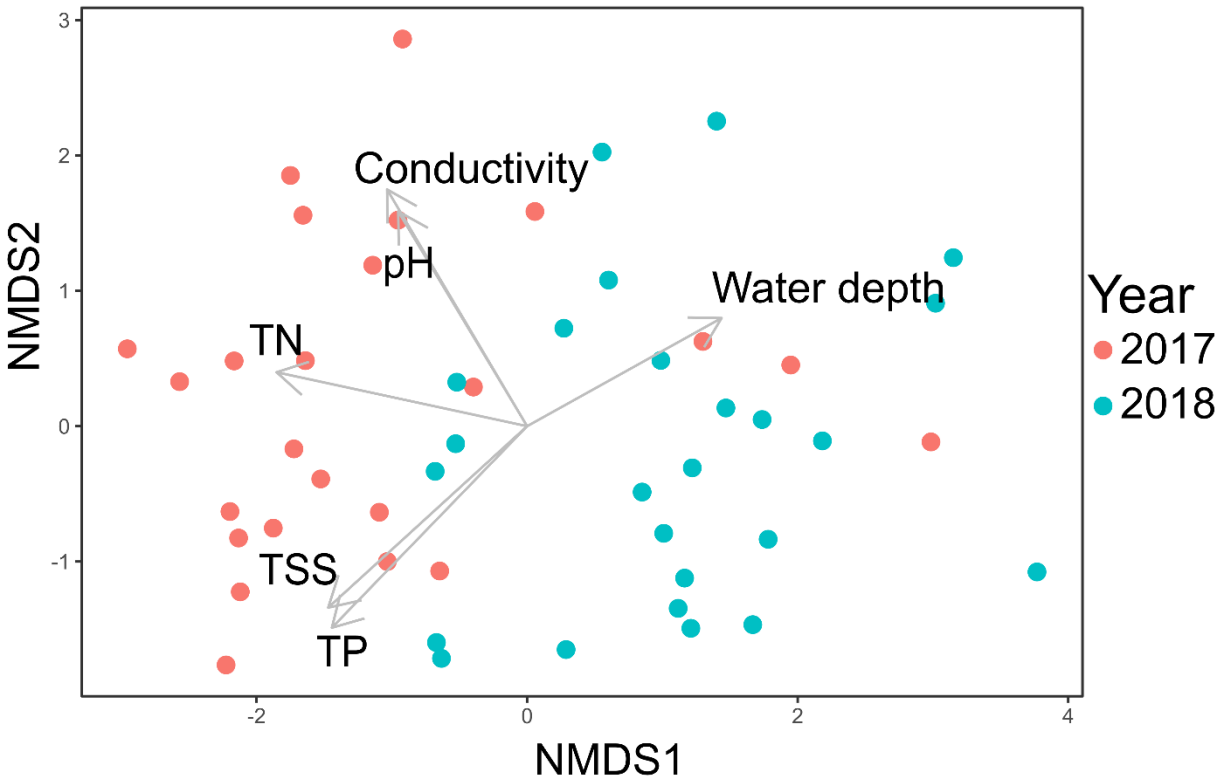


Figure 2 NMDS plot illustrating the separation of samples (each soda pan is represented by one filled circle per year) based on the local environmental variables in the two sampling years

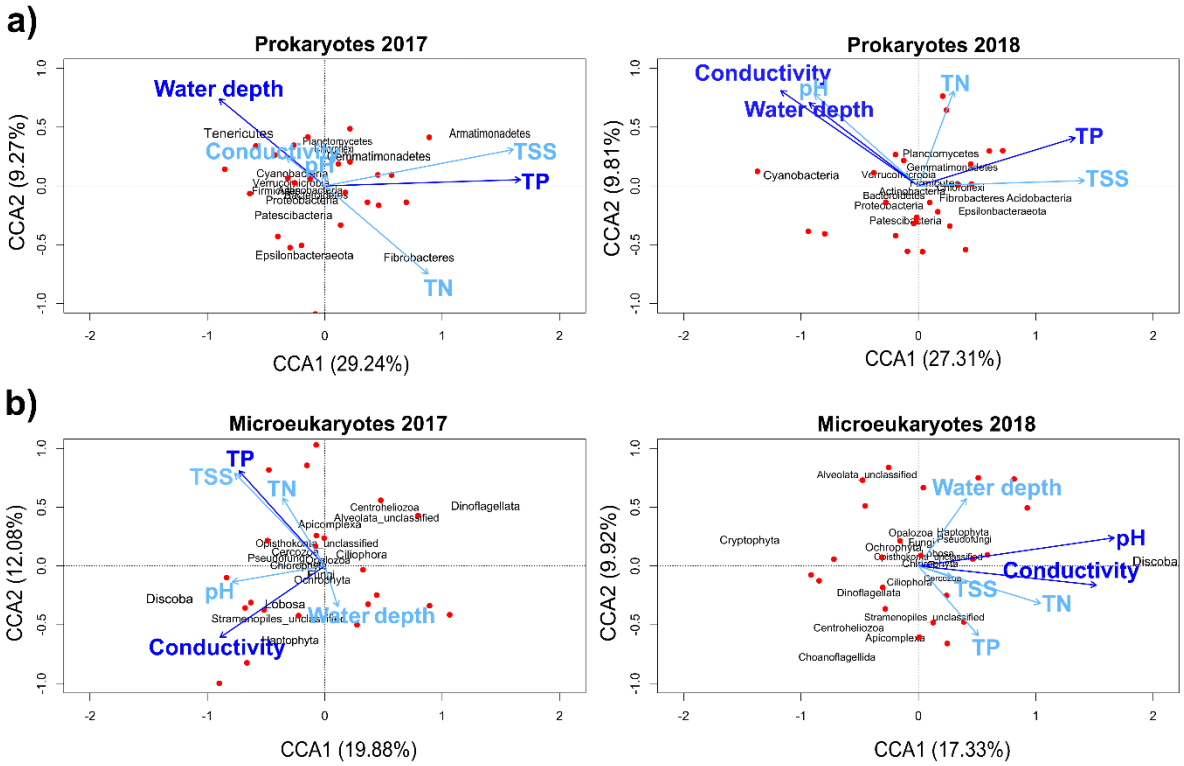


Figure 3 Canonical correspondence analysis (CCA) biplots based on the phylacomposition of prokaryotes (a) and microeukaryotes (b) in the two years (red points: habitat scores, dark blue arrows: significant environmental variables, light blue arrows: all the other environmental variables)

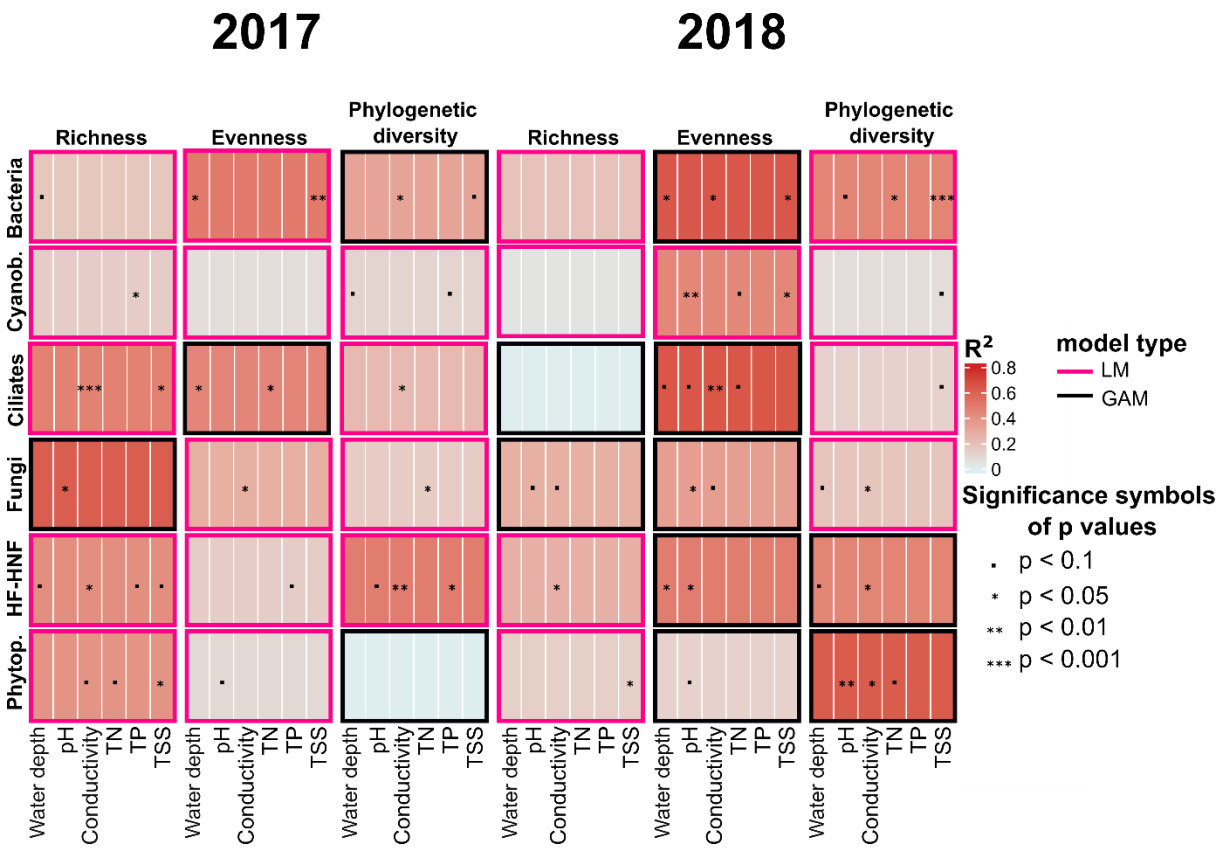


Figure 4 Heatmap of the best-fit models with the significant environmental variables for the six major taxonomic groups (in rows) and their local diversity indices (richness, evenness, phylogenetic diversity) in the two sampling years (in columns). One model is represented by one box, where the shading indicates the overall $R^2$ of each multiple regression model (either linear or GAM-based, indicated by the color of the frame of each box), while the significance symbols belong to individual predictors in the final model. Abbreviation of the group names: Cyanob. = Cyanobacteria, Phytop. = Phytoplankton, HF-HNF = Heterotrophic flagellates and nanoflagellates

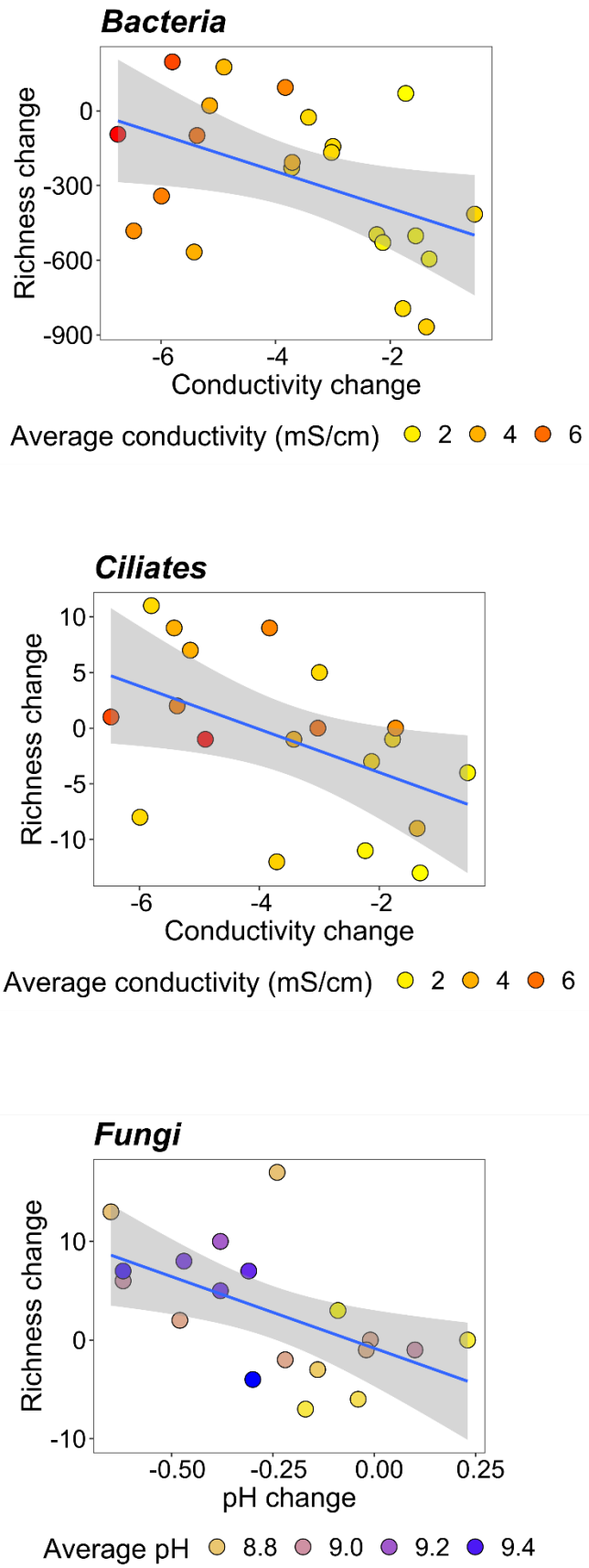


Figure 5 The relationship between Richness changes and significant environmental parameter changes ($p < 0.01$)

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