Ecological and metabolic thresholds in the bacterial, protist and fungal microbiome of ephemeral saline lakes (Monegros Desert, Spain)

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Abstract

We studied the 16S and 18S rRNA genes of the bacterial, protist and fungal microbiomes of 131 samples collected in 14 ephemeral small inland lakes located in the endorheic area of the Monegros Desert (NE Spain). The sampling covered different temporal flooding/desiccation cycles that created natural salinity gradients between 0.1% (w/v) and salt saturation. We aimed to test the hypothesis of a lack of competitive advantage for microorganisms using the “salt-in” strategy in highly fluctuating hypersaline environments where temperature and salinity transitions widely vary within short time periods, as in ephemeral inland lakes. Overall, 5,653 bacterial zOTUs and 2,658 eukaryal zOTUs were detected heterogeneously distributed with significant variations on taxonomy and general energy-yielding metabolisms and trophic strategies along the gradient. We observed a more diverse bacterial assembly than initially expected at extreme salinities and lack of dominance of a few “salt-in” organisms. Microbial thresholds were unveiled for these highly fluctuating hypersaline environments with high selective pressures. We conclude that the extremely-highly dynamism observed in the ephemeral lakes of Monegros may have given a competitive advantage for more versatile (''salt-out'') organisms as compared to those better adapted to stable high salinities usually more common in solar salterns. Ephemeral inland saline lakes offered a well-suited natural framework for highly detailed evolutionary and ecological studies.

1. Introduction

Life in saline aquatic environments has adapted to cope with high osmotic stress, and salinity has been repeatedly described as one of the main ecological factors affecting microbial community composition at the global scale [1, 2]. The progressive decrease of biodiversity as salinity increases is also usually accompanied by a substantial loss in metabolic functions [3], which shapes important ecological changes along the salt gradient [4]. Osmotic regulation becomes crucial to halophile and halotolerant microorganisms to avoid water deficit and plasmolysis, and a wide array of strategies are adopted to cope with changes in salt concentrations [5, 6]. At salts concentrations > 0.3% freshwaters turn saline [7], and marine-like salinities can be usually found ranging between 3% and 5% salts w/v in inland waters [8, 9]. At higher salts concentrations, water becomes hypersaline [10], and an additional threshold is set at 15% based on the biological differentiation between moderate and more extreme halophiles [9, 11, 12].

One of the strategies used by cells to reach osmotic equilibrium between inside and outside environments is the accumulation of inorganics salts in the cytoplasm, actively “pumped in” from the saline surroundings [13]. This salt-in strategy is adopted by several of the organisms living at the most extreme hypersaline conditions (e.g., *Halobacteria*, *Halanaerobiales*, and *Salinibacter*), and requires of permanent adaptation in the intracellular machinery, which, in turn, limits further adaptation back to low salinities [14]. A second strategy is the accumulation of compatible solutes, small organic molecules with neutral charge and low toxicity at high concentrations, usually combined with active

65 “pumping out” of inorganic salts [15]. In general, salt-out microorganisms are
66 better adapted to wider ranges of salinities but they have limitations to colonize
67 extreme salinities due the high energetic cost to produce compatible solutes [3].
68 Indeed, the bioenergetic requirement for maintaining these necessary
69 osmoadaptation mechanisms is one of the main limitations microbes have for
70 colonizing saline environments, and therefore some energetically limited
71 metabolic pathways cannot be present beyond a certain salinity threshold [3].
72 This strategy is followed by most halophilic bacteria and most of the currently
73 known halophilic microeukaryotes [14, 16, 17].
74
75 Osmoadaptation mechanisms are better known in halophilic prokaryotes than in
76 microeukaryotes. This lack of knowledge remains in the higher methodological
77 complexity and the more recent awareness on the ecological relevance of
78 microeukaryotes in hypersaline environments [18]. Most of the knowledge
79 regarding osmoadaptation in microeukaryotes had been gained from the study
80 of the green algae *Dunaliella* and halophilic fungi, e.g. *Hortaea werneckii* [19],
81 *Wallemia ichthyophaga* [20] and *Eurotium rubrum* [21]. However, more recent
82 works have added additional information from phagotrophic protists based on
83 both transcriptomic analysis (*Halocafeteria seosinensis* [22]) and experimental
84 studies (*Schmidingerothrix salinarum* [23]). Although the accumulation of
85 compatible solutes has been described in all the cases as the main
86 osmoadaptation strategy for microeukaryotes, the wide genetic repertory found
87 related to specific adaptations suggests a larger variety of adaptive strategies
88 [21]. Thus, some of the main differences observed at the genome level include
89 acidic residues in predicted proteins and genes linked to membrane cation

transporters and synthesis/transport of compatible solutes. For instance, an elevated presence of genes linked to cation transporters have been associated to low intracellular concentrations of K^+ and Na^+ in *H. werneckii*. Conversely, a limited number of those genes in *W. ichthyophaga* suggests a higher resistance to high intracellular Na+ concentrations [23]. The expected higher adaptability of "salt-out" organisms may potentially provide competitive advantage in highly dynamic environments where frequent water level fluctuations and high evapotranspiration rates led to abrupt salinity changes, with potential dominance of organisms using the salt-out strategy even at the most extreme salinities [14, 24].

Ephemeral inland shallow lakes in arid and saline areas are very convenient natural model systems to test for selective microbial thresholds in highly fluctuating natural environments with high selective pressures. These ephemeral water masses are very dynamic and extremely responsive to changes in climate, with fluctuations in water volume, temperature, and salinity [25] larger than those reported in more stable hypersaline environments (e.g. solar salterns and large hypersaline lakes). The endorheic saline basin of Monegros is rich in ephemeral shallow lakes with severe spatio-temporal changes in salinity, easily ranging from freshwater to salt saturation and vice versa [26]. We evaluated the composition and ecological changes of the planktonic microbiota (bacteria, protists and fungi) along the salt gradient of these highly dynamic environments with frequent and bidirectional salt fluctuations, and the results were contextualized according to the previous knowledge on the ecology of (hyper)saline environments.

2. Materials and methods

2.1. Study site and sampling

The Monegros Desert area (Central Ebro Basin, NE Spain, 41°42′N, 0°20′W) is one of the most arid regions in Europe with a large set of inland ephemeral saline lakes, and constitutes a unique landscape of great geological, edaphic, mineralogical and hydrological interest [27, 28]. The area is an endorheic platform hosting hypersaline brines of Cl–SO4–Na–(Mg) type, where a rich microbial community develops [26, 29, 30]. In this endorheic area, the water balance between precipitation (mean annual rainfall = 337 mm/year) and evaporation and evapotranspiration rates (mean = 1408 mm/year) is negative for most of the months and for the whole year, producing one of the highest mean annual water deficits in Europe [31]. Field sampling was carried out in 14 ponds in different time periods and covered large salinity fluctuations (Supplementary Table S1). Conductivity, temperature, pH, dissolved oxygen and redox potential were measured in situ using an HQ40d multiparameter meter (Hach, Loveland, CO, USA). For chlorophyll *a* (Chl *a*) analysis, 1 L water was filtered through 47-mm-diameter Whatman GF/F filters (0.7 µm nominal particle retention), stored in the dark, and kept frozen. Chl *a* concentration was determined in acetone extracts by spectrophotometry (UV-2401PC, ultraviolet-visible Spectrometer; Shimadzu) as previously reported [32]. Salinity was measured in situ by a hand salinity refractometer (Atago S-28E, Japan). Five salinity categories were stablished for comparative purposes as follows: Freshwater to slightly-brackish (0.1% - 1.4%, w/v), Brackish (1.5% - 2.9%), Marine (3% - 4.9%), Hypersaline (5% - 14.9%), and Extreme hypersaline (15% -

40%).

2.2. DNA Extraction and sequencing

For DNA analyses, water samples were prefiltered in situ through a 50 micron-pore-size net to retain large zooplankton and algae, and then between 100 and 500 mL (depending on the initial cells concentration) were successively filtered on 5-micron and 0.2-micron pore-size polycarbonate membranes (47 mm diameter). The analyzed eukaryal size range (5-50 µm) mainly covered the nanoplanktonic eukaryotes (2-20 µm) and a fraction of microplanktonic eukaryotes (20-200 µm). The 5 µm (for 18S rRNA gene analyses) and 0.2 µm (for 16S rRNA gene analyses) membranes were separately stored in lysis buffer (40 mM EDTA, 50 mM Tris, pH 8.3, 0.75 M sucrose), enzymatically digested and phenol extracted (phenol–chloroform–isoamyl alcohol; 25:24:1, v/v/v) as previously reported [33]. The purification and concentration steps were carried out with Amicon® Ultra 4 Centrifugal Filter Units – 100 000 NMWL (Millipore). DNA extracts were quantified using Quant-it assays in a QubitTM fluorometer (Invitrogen). PCR amplification of the V4 region of the 16S rRNA gene (primers set 515F-806R [34]) and further sequencing with the Illumina 2x250 MiSeq platform were carried out according to the genomic core facilities protocols and methods of the RTSF-MSU (Michigan State University, USA) (https://rtsf.natsci.msu.edu/). The V9 region of the 18S rRNA gene was PCR amplified with the primers set 1391f-EukrBr [35] and sequenced with the Illumina 2x150 MiSeq platform (RTSF-MSU).

2.3. 16S rRNA and 18S rRNA genes sequence analysis

Raw rRNA gene sequences were processed with UPARSE [36]. The total number of sequences prior to quality filtering was 14,445,263 (mean of 79060 per sample) for the 16S (70.6% had ≥ Q30) and 14,374,602 (mean of 83489 per sample) for the 18S (87.1% had ≥Q30) rRNA genes dataset. After merging of read pairs, each dataset was filtered by setting a minimum sequence length (250 bp for the 16S gene and 150 bp for the 18S gene) and a maximum number of expected errors of 0.25 [37]. After chimera filtering using default parameters, the UNOISE algorithm [38] clustered filtered sequences into Operational Taxonomic Units at 100% identity, i.e., zero-radius OTUs (zOTUs). The taxonomic assignment was carried out with SINA aligner v.1.2.11 [39], using the SILVA 132 reference database [40]. Specific BLAST searches were carried out to obtain a more detailed taxonomic classification of relevant zOTUs when needed. zOTUs were filtered by their alignment quality score (>85%) and 16S rRNA gene sequences classified as archaea, mitochondria, and chloroplasts were excluded. Archaea showed high richness and very low abundances below 20% salinities, and were treated separately in a different study covering temporal colonization-extinction turnover rates along the gradient [30]. Only samples with > 6,800 reads for bacteria and > 8,100 reads for eukaryote were retained. The resulting zOTU tables consisted of 5,653 Bacteria (131 samples) and 2,658 Eukarya (129 samples). Data was normalized by rarefying the reads of all samples to the minimum number of reads in each dataset. For community related analysis, rarefaction at the same depth was repeated 100 times in order to avoid the loss of less abundant zOTUs, and the resulting rarefactions were unified on an average rarefied zOTU table. Phylogenetic diversity was calculated in QIIME [41] using *alpha_diversity.py* after building a maximum-

likelihood phylogenetic tree using *make_phylogeny.py* [42]. General metabolic and trophic traits for bacteria and eukarya were obtained from the closest-match in SILVA database and after literature surveys [43–46]. Energetic metabolism was focused on (i) photoautotrophy, (ii) sulfur chemolithotrophs, (iii) sulfur chemoorganotrophs, (iv) methanotrophs, and (v) ammonia-oxidizing chemolithotrophs. For the trophic traits of protists and fungi, we established five groups as follows: (i) osmotrophs (which includes photosynthetic organisms), (ii) phagotrophs, (iii) mixotrophs, (iv) saprotrophic, and (v) parasitic.

The whole gene sequence datasets were deposited to the NCBI Sequence Read Archive and are available through BioProject record ID PRJNA429605.

2.4. Statistical analysis

Statistical analyses were run in R environment (http://www.r-project.org/). Species richness, and Shannon-Weaver diversity index were calculated using "vegan" package version 2.4-5 [47]. Spearman's Rank order correlation coefficient was carried out to test monotonic associations between numeric variables (environmental data, relative abundances, diversity indexes). Pearson's correlation coefficient was used to test lineal correlation between numeric variables. Differences among group means were tested using Tukey's range test, function *HSD.test* within "agricolae" package version 1.2-8, after ANOVA test. The Dufrene-Legendre Indicator Species Analysis, that identifies indicator taxa based on fidelity and relative abundance, was carried out at the genus level with the *indval* function on the "labdsv" package version 1.8, in order to show the genera significantly associated (p-value < 0.05) to each salinity category. The ten genera with the highest abundance in each salinity category were further analyzed. The *titan2* function from "TITAN2" package

version 2.1 was used to identify significant variations on the taxonomic composition along the environmental gradient [48]. This is done after fractioning the environmental gradient in two at each midpoint of the gradient, and calculating taxa significantly associated to each side of that midpoint, which is expressed as a Z score. Then, Z scores are based on the Indicator Species Analysis at each midpoint of the gradient. High values of negative Z scores (Z-) indicated high association of taxa to the left side of the midpoint, the one with lower salinity, so represent points of the gradient where the environmental variable starts affecting negatively some taxa. Conversely, positive Z scores (Z+) indicated the opposite pattern, where taxa are significantly associated to the most saline side of the midpoint.

3. Results

3.1. Alpha diversity descriptors along the salinity gradient

Bacteria and microbial eukarya showed differences in alpha diversity community descriptors (Richness, Shannon, and Faith's Phylogenetic Diversity) along the gradient (Fig. 1, and see additional information in Supplementary Table S2). Bacterial richness (R) was higher (average 372±116 zOTUs, range 127-793 zOTUs) than microbial eukaryotes R (average 124±69 zOTUs, range 14-351 zOTUs). Shannon-Weaver diversity index (H) also reached higher values for bacteria (from 1.9 to 5.1, mean 3.47) than for eukarya (from 0.09 to 4.15, mean 2.21). The plots of richness and alpha diversity indicators vs. salinity showed a significant and negative trend for eukaryotes (Fig. 1D and 1E, respectively), but was not significant for bacteria (Fig. 1A and 1B). The negative trend was observed for both photosynthetic and heterotrophic protists, and also

for fungi (Supplementary Table S2). At any rate, the phylogenetic diversity of both bacteria and eukarya showed a significant reduction as salinity increased that became stronger for microbial eukaryotes (Fig. 1C and 1F).

3.2. Taxa replacement along the salinity gradient

We identified significant variations on the taxonomic composition along the salinity gradient based on the Z scores (Fig. 2), with significant ecological thresholds detected. Similar values for Z+ and Z- scores were observed in bacteria whereas, interestingly, eukaryal Z+ scores were significantly lower than Z-. The Z- for both bacterial and eukarya followed a similar pattern, with its maximum and most of the contribution to the cumulative frequency observed between 2-3 % salinity. The higher Z+ bacterial scores at salinities 5 to 20%, indicated a partial replacement of the bacterial community by better adapted taxa that was not observed for eukarya.

3.3. Indicator bacterial taxa (IndVal) along the salt gradient

As a whole, bacteria averaged 98% (relative abundances range 54-100%) of the total prokaryotes present in the PCR mixture (Supplementary Table S1). The most abundant phyla were *Gammaproteobacteria* (25% - 44%, mean relative abundances), *Alphaproteobacteria* (10% - 35%), *Bacteroidetes* (18% - 29%), and *Actinobacteria* (c. 5%) (Supplementary Fig. S1, and see a zoom for salinities >35% in Supplementary Fig. S2). *Halanaerobiaeota* (a recently proposed phylum in the SILVA database which includes the single order *Halanaerobiales*) were only noticeable at the most extreme end. Conversely, *Firmicutes* reached higher abundances at low salinities. The phylum *Spirochaetes* showed a positive correlation between relative abundance and

salinity (Spearman's rho 0.60, p-value < 0.001), with a highly diverse composition, and reached the 3% of bacterial reads at 20-25% salinity in the ephemeral lake Salineta (Supplementary Fig. S3).

The *Indval* analysis unveiled the genera with the highest fidelity for each salinity category (Fig. 3). The genera *Polaromonas*, *Polynucleobacter*, *Limnohabitans*, *Hydrogenophaga*, *Bacillus*, *Pedobacter* and *Flavobacterium* reached significant abundances at low salinity, whereas *Salinivibrio*, unclassified *Nitrococcaceae* (94.4% of 16S rRNA gene sequence identity with *Nitrococcaceae* members included in SILVA database), *Halothiobacillus*, and genera belonging to the order *Halanaerobiales* became more important in terms of abundance at high salinities. Interestingly, the high abundance of the unclassified *Nitrococcaceae* at salinities >15% was mostly related to a single population (zOTU 7, NCBI acc. number MT129572, Supplementary Table S3) 100% identical to the recently described *Spiribacter salinus* (*Ectothiorhodospiraceae*) [49]. Conversely, *Salinibacter* sp. was always present at relative abundances <0.3% even at salinities >30%, and maximal abundances reached 3-4% only in two samples for the whole dataset (Salineta, samples MON 88 and 84). Finally, *Psychroflexus* and unclassified *Rhodobacteraceae*, despite being significantly associated to a given salinity category, were substantially abundant along the whole salinity gradient.

3.4. Indicator eukaryal taxa (IndVal) along the salt gradient

Despite the significant decrease of eukaryotic diversity observed along the salinity gradient, the whole eukaryal microbiome showed an outstanding diversity in the ephemeral saline lakes of Monegros, with up to 2658 zOTUs

293 belonging to Amoebozoa, Archaeplastida, Centrohelida, Cryptophyceae,
294 Excavata, Haptophyta, Opisthokonta and SAR (Supplementary Fig. S4).
295 Unclassified Ciliophora and *Cryptomonas* showed the highest affinity for low
296 salinities, whereas *Dunaliella* (Chlorophyta) prevailed at extreme salinities (Fig.
297 3). Most of the reads classified by SINA aligner as unclassified Chlorophyta.
298 belonged to zOTU 3 (100% identity to *Dunaliella salina*, NCBI acc. number
299 MG022673), which reached 80% of the total eukaryote microbiota for most of
300 the extreme-hypersaline samples. It was also remarkable the high abundance
301 of diatoms of the genus *Navicula* (Ochrophyta, Bacillariophyceae) along most of
302 the salinity gradient, reaching > 25% of the relative abundance of eukaryotes in
303 the brackish range. The dinoflagellate *Suessiacea* (unclassified) represented by
304 a single zOTU (zOTU 2), reached high abundances and fidelity at salinities
305 <15% and showed 100% identity to the halotolerant dinoflagellate *Biecheleria*
306 sp. (NCBI acc. number KF463288 isolated from the saline lake Tirez, Spain).
307 Finally, fungi prevailed at the lower salinity end (Fig. 3 and Supplementary Fig.
308 S5) and were less abundant as salt content increased. The most abundant taxa
309 were Chytridiomycota, and two unknown fungi that deserve further research
310 (zOTU 57, up to 23% of the eukaryotic reads in sample MON 2, salinity 1.2%,
311 NCBI acc. number MW040517; and zOTU 102, up to 15.5% of the eukaryotic
312 reads in sample MON 6, salinity 1.1%, NCBI acc. number MW040518). These
313 unknown fungi showed <91% identity to any previously known sequences and
314 were putatively allocated within the Basidiomycota in SINA.
315
316 ***3.5. Metabolic and trophic thresholds along the salt gradient in ephemeral lakes***

Changes in the general energy metabolism processes and trophic strategies were closely linked to shifts in microbial composition. Bacterial photoautotrophy (cyanobacteria and anoxygenic photoautotrophs) was a minor component and with no significant differences along the salt gradient categories (Fig. 4). Conversely, sulfur bacteria both chemolithotrophs (Sulfur Oxidizing Bacteria, SOB) and chemoorganotrophs (Sulfate Reducing Bacteria, SRB) increased along the gradient. Methanotrophs showed low abundances and were undetectable at salinities >15%. Ammonia oxidizers were also poorly represented in the Monegros microbiome, with AOB undetected and AOA (*Thaumarchaeota*) mostly detected at salinities up to 10% [30].

Osmotrophy by photosynthetic microeukaryotes was the dominant eukaryal trophic strategy along the whole salt gradient, reaching almost 100% dominance at salinities >15% (Fig. 5) in agreement with the highest relative abundances of *Dunaliella* and *Navicula.* Conversely, phagotrophic eukaryotes (Ciliophora and Cercozoa) averaged c. 25% of the abundance at salinities <15% and became minor component at salinities > 20%, whereas the abundance of mixotrophic (mostly *Cryptomonas* and Dinoflagellata) reached its maximum at salinities <1.5% and unseen at salinities >3%. Finally, saprotrophic and parasitic microeukaryotes, related to the presence of Chytridiomycota, were observed mostly at <5% salinity.

4. Discussion

The dynamics and succession of planktonic microbial populations in ephemeral saline lakes have been poorly studied [50]. In hypersaline inland environments

of arid regions, it is challenging to collect enough relevant information over temporal and spatial scales to properly study natural microbial processes, and successional studies are scarce [51]. The Monegros desert contains one of the largest sets of inland saline lakes in Europe offering a wide repertoire of accessible heterogeneous and small ephemeral lakes within a short distance, easily covering different interannual dry and wet periods and ecological conditions [26, 30]. In the present study, we observed consistent changes in ecological and metabolic thresholds along the saline gradient that selectively affected different microbial populations according to their expected physiological and metabolic traits. The best known ecological thresholds with clearly segregated species distributions are usually found at the interfaces between land and water, oxic and anoxic environments, and marine waters and freshwaters. However, ecological thresholds may also exist when environmental changes are progressive [52]. As a whole, ecological thresholds are very helpful to understand ecosystems structures, to guide conservation policies, and to establish quantitative predictions for the ecological impact of environmental changes [32, 52]. In the intermittent saline lakes of Monegros, the strong environmental selection pressures (low rain precipitation, high solar irradiation, strong and persistent winds, high evaporation rates, and salts concentration and accumulation) provide an easy to access natural model system with progressive changes in salinity where ecological and metabolic thresholds appeared finely allocated.

Microorganisms inhabiting saline and hypersaline environments have been described in detail in the literature, first using culture dependent approaches

[53, 54], and more extensively after the use of molecular biology techniques. Generally, halophilic bacteria and archaea dominate in saline environments, whith lower contribution of microeukaryotes to the total diversity. Most of the halophile and halotolerant bacteria are considered to be moderate rather than extreme halophiles [55]. The Bacteria domain contains many halophiles from diverse phylogenetic groups such as *Proteobacteria*, *Cyanobacteria*, *Spirocheaetes*, *Actinomycetes* and *Firmicutes*. The genera *Bacillus* [56], *Salinibacter* [57] and members of the order *Halanaerobiales* [57] are some of the most commonly found taxa expected in these environments. Archaea become more abundant as salinity increases [58], mostly with *Nanohaloarchaeota* and *Halobacteria* populations [30, 58] being *Haloquadratum and Halorubrum* predominant at the hightest salinities [59–61]. Microeukaryal diversity tends to be lower at high salinities [24] and are mainly represented by Chlorophyta, Dinophyceae and Bacillariophyta, with the photosynthetic algae *Dunaliella* as one of the most common taxa at extreme salinities [62–64]. Together with other reported halophilic algae [65], both halophilic and halotolerant fungi (e.g. *Hortaea werneckii* and *Wallemia ichthyophaga*) and halophilic heterotrophic protists [14, 62] are also usually reported in saline environments.

Therefore, in principle, a similar assembly should be present in the saline lakes of Monegros. However, the taxonomic composition found, specially at the most saline end, significantly differed from the expectations following previous reports. Although specialist groups as *Salinibacter* and *Halanaerobiales* were detected at extreme salinities, they only represented a very low fraction of the

total bacterial assembly. Conversely, a very rich assembly of *Gammaproteobacteria*, *Bacteroidetes* and *Alphaproteobacteria* using “salt-out” strategies dominated the bacterial community at extreme salinities. Most of the populations found matched *Halomonas* [66, 67], *Salinivibrio* [61], *Spiribacter salinus* [49] and members of the order *Halanaerobiales* [55], among others, with high relative abundances at salinities close to NaCl saturation (Supplementary Fig. S2). This fact led to a higher bacterial diversity at extreme salinities than expected from studies carried out in more stable environments, where usually “salt-in” archaea, *Salinibacter* and *Halanaerobiales* dominate [68, 69]. As previously reported, this strategy is more effective at the most extreme salinities and reduces adaptation to lower salinities [13, 70] and, therefore, should be less competitive for the most dynamic natural environments [14, 71]. Conversely, “salt-out” mechanisms provide higher plasticity at the expense of poorer adaptation at the most saline end [72]. Microeukaryotic assembly was mostly dominated by *Dunaliella* and *Navicula*, both accumulating compatible solutes as the main osmoadaptation strategy [63, 73], with progressively lower contribution of halophilic fungi and heterotrophic protists as salinity increased. Overall, the extremely high dynamism observed in the ephemeral lakes of Monegros selectively enriched more versatile ("salt-out") organisms rather than those better adapted to stable high salinities, usually more common in solar salterns.

Changes in metabolic and physiological performance were also consistent along the salinity gradient. Autotrophic ammonia oxidation, autotrophic nitrite oxidation, and acetate-based methanogenesis, among others, are usually restricted to low salinity environments [3, 43, 74]. The low abundance of

methanotrophs observed in the ephemeral lakes of Monegros may be explained by constraints for aerobic methane oxidation in high-salt environments [43] but also for limitations in methanogenesis. Methanogenic activity based on H_2+CO_2 (group 1) and acetate (group 3) have never been detected above c. 12.5% salinity [43, 75]. Methanogenesis based on methylated amines (group 2) may potentially occur up to 25% of salts (w/v), under anaerobic conditions [43, 58]. However, Monegros is a gypsum-rich area with a rich presence of sulfur bacteria and a potentially active sulfur cycle in most of the sediments [29] that do not favor methanogenesis.

We also observed consistent changes of the trophic strategy for microbial eukaryotes in the ephemeral lakes. The dominance of osmotrophy (which includes photosynthetic organisms) was linked to the high abundance of *Dunaliella* and *Navicula* covering all the gradient. The low abundance of Cyanobacteria at high salinities agrees with previous reports [76] being *Dunaliella* the main primary producer at the most extreme salinities. The progressive decrease of phagotrophic organisms agrees with previous studies in coastal solar salterns that showed lack of bacterivory above 25% salinity [18]. Groups potentially including halophile and halotolerant melanized fungi (‘black yeasts’) usually found in hypersaline environments [77], were detected at low abundances along the gradient in Monegros. Saprotrophic fungi were mostly found at salinities <5%. However, more detailed studies and refined taxonomic assignments are needed for the aquatic fungi found in Monegros. Finally, parasitic microeukaryotes were present all over the gradient suggesting potential key missing pathways in ephemeral lakes dynamics. Taken all

442 together, these unseen groups offered new perspectives for improving the
443 understanding of planktonic food webs [45] and should be more carefully
444 studied in the future.
445
446 Overall, we have reported a very dynamic and rich microbial assemblages in
447 the Monegros wetlands system, even under the most extreme conditions. The
448 particular characteristics and high environmental variability of this area, allow us
449 to observe natural transitions in detail both at the taxonomic and at the
450 functional level. Most of the high saline waters traditionally studied are quite
451 stable environments with rather constant salinities [51, 62, 78] or where salt
452 production practices guides salinity increases unidirectionally [61]. Ephemeral
453 saline lakes with high turnover and frequent salinity transitions follow closely
454 environmental perturbations and flooding and desiccation dynamics, offering a
455 well-suited natural framework for the study of evolutionary and ecological
456 microbial responses [32] under highly dynamic fluctuations and strong selective
457 pressures.
458

Ethics Declarations

Conflict of Interest

The authors declare no conflict of interest

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Author contributions

EOC designed the study, EOC and XTM carried out the field work, MMS carried out the analyses. MMS and XTM wrote a first draft of the manuscript. EOC led the final version of the manuscript.

Data Availability

The whole gene sequence datasets were deposited to the NCBI Sequence Read Archive and are available through BioProject record ID PRJNA429605.

Code availability

Not applicable

Ethics Approval

Not applicable

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Figure legends

Fig 1. Variations on ZOTU richness, Shannon-Weaver diversity index and Faith's phylogenetic diversity along the salinity gradient, both for bacteria (a, b, c) and eukaryote (d, e, f). Pearson's correlation is given when significant p-value: * p-value < 0.05; **p-value < 0.01; NS = non-significant correlation.

Fig 2. Representation of the sums of Z scores (positive and negative) calculated for each midpoint of the salinity gradient using *titan2* function from "TITAN2" R package, both for bacterial and eukaryal communities. Sums of Z scores are based on the *Indval* analysis performed after segregating all samples along the salinity gradient as less/more saline than the tested point. Continuous and dashed lines represents the accumulated frequency of negative and positive Z scores respectively, and its pronounced increase in certain points represents potential thresholds on the community composition variation. As the Indval analysis requires of a minimum number of samples at each side of the gradient midpoint, this could not be performed at the salt gradient tails.

Fig 3. Relative abundances (%) of the genera obtained from the *Indval* analysis along the different salinity ranges. Highlighted abundances represent the indicator species for each salinity range.

Fig 4. Relative abundance of the bacterial taxa involved on diverse metabolic processes. Chemoorganotrophs S: as Sulfate reducing bacteria; Chemolitotrophs S: as Sulfur oxidizing bacteria. Grey dots represent group abundance on each sample. Black dots represent boxplots outliers.

837

838 **Fig 5.** Relative abundance of the eukaryal taxa based on its trophic strategy.

839 Grey dots represent group abundance on each sample. Black dots represent

840 boxplots outliers.

841

Supplementary Material

Tables

Supplementary Table S1. Detailed information on sampling date, sampling site, water height (cm), salinity (%, w/v), conductivity (mS/cm), water temperature (°C), pH, redox conditions (mV), dissolved oxygen (mg/l), Chl *a* (mg/L) and DAPI cells density count (cel/mL) for each sample in Monegros. Community descriptive parameters are also included: bacterial richness and its relative abundance in the 16s rRNA gene pool (including Archaea); total eukaryotic richness and individual richness and relative abundance (in the 18S rRNA gene pool) of photosynthetic and heterotrophic protists and fungi.

Supplementary Table S2. Pearson's correlation and significance for richness and diversity values against salinity (log, % w/v). For eukarya, correlation is given considering the whole eukaryal community and also photosynthetic and heterotrophic protists and fungi, independently.

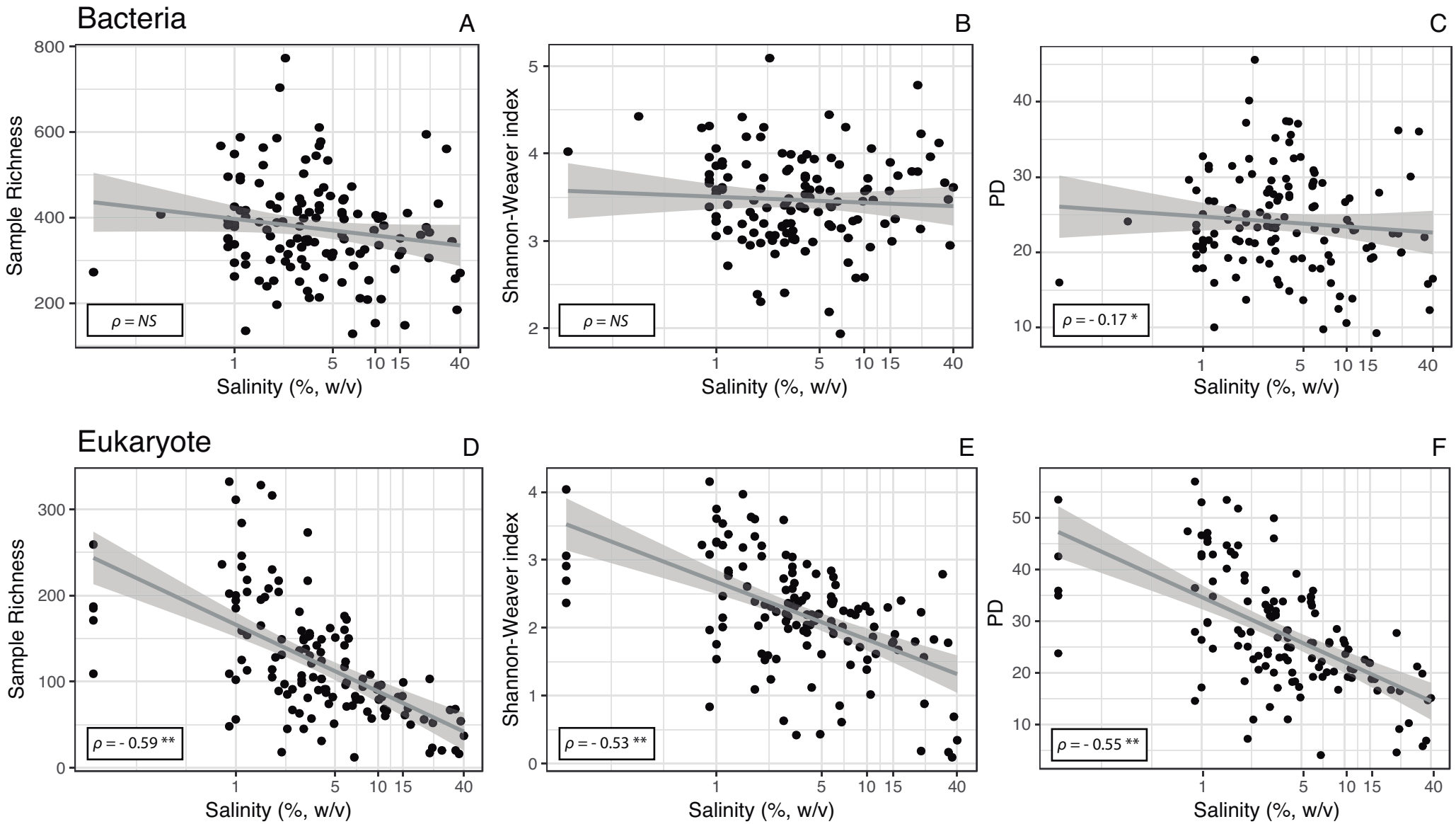
Supplementary Table S3. Most abundant zOTUs within the taxa found along the gradient for the different saline ranges and their individual sequence GenBank accession number.

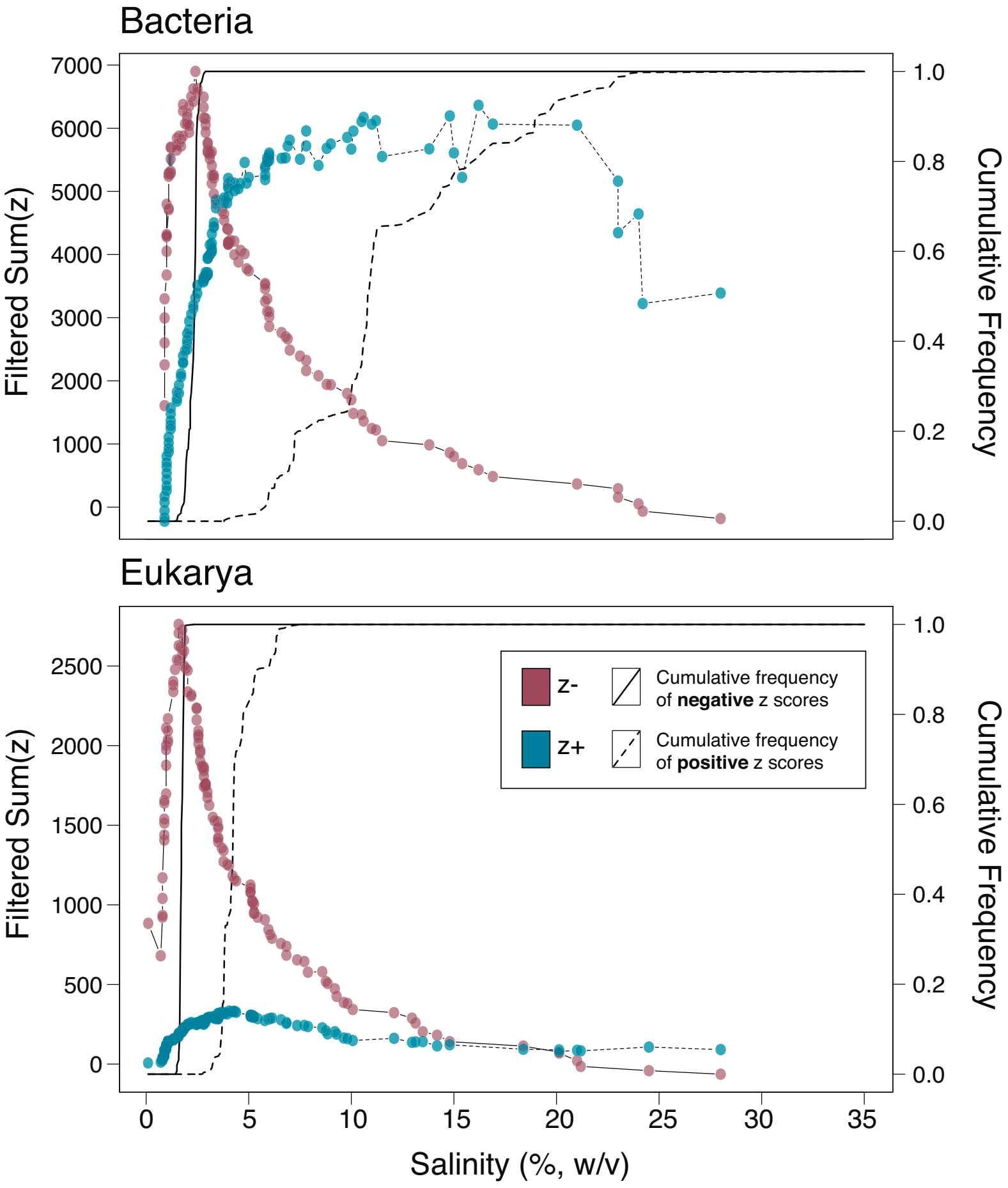
Figures

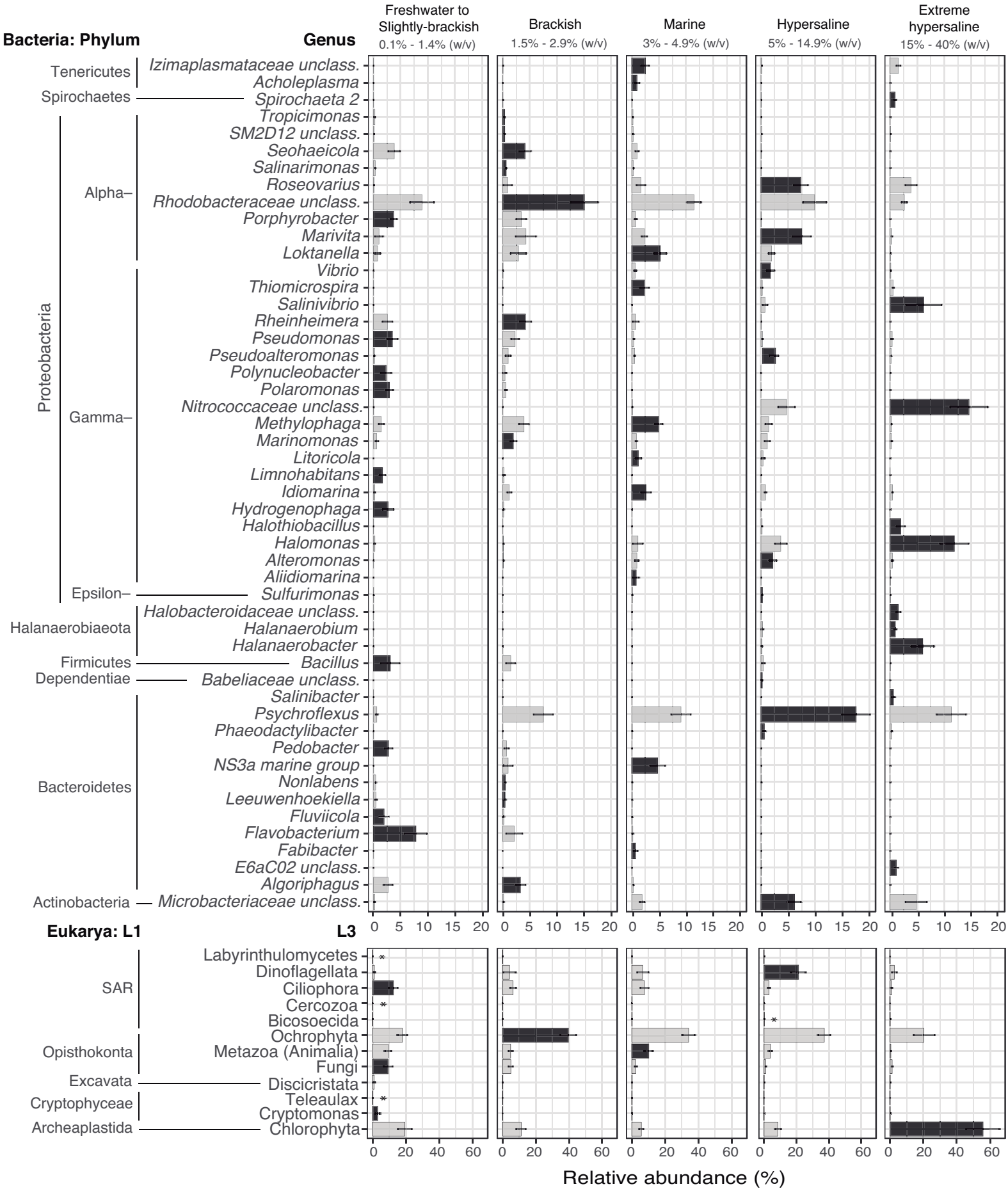
Supplementary Figure S1. Taxonomic profile representing the most abundant bacterial phyla along the salinity ranges.

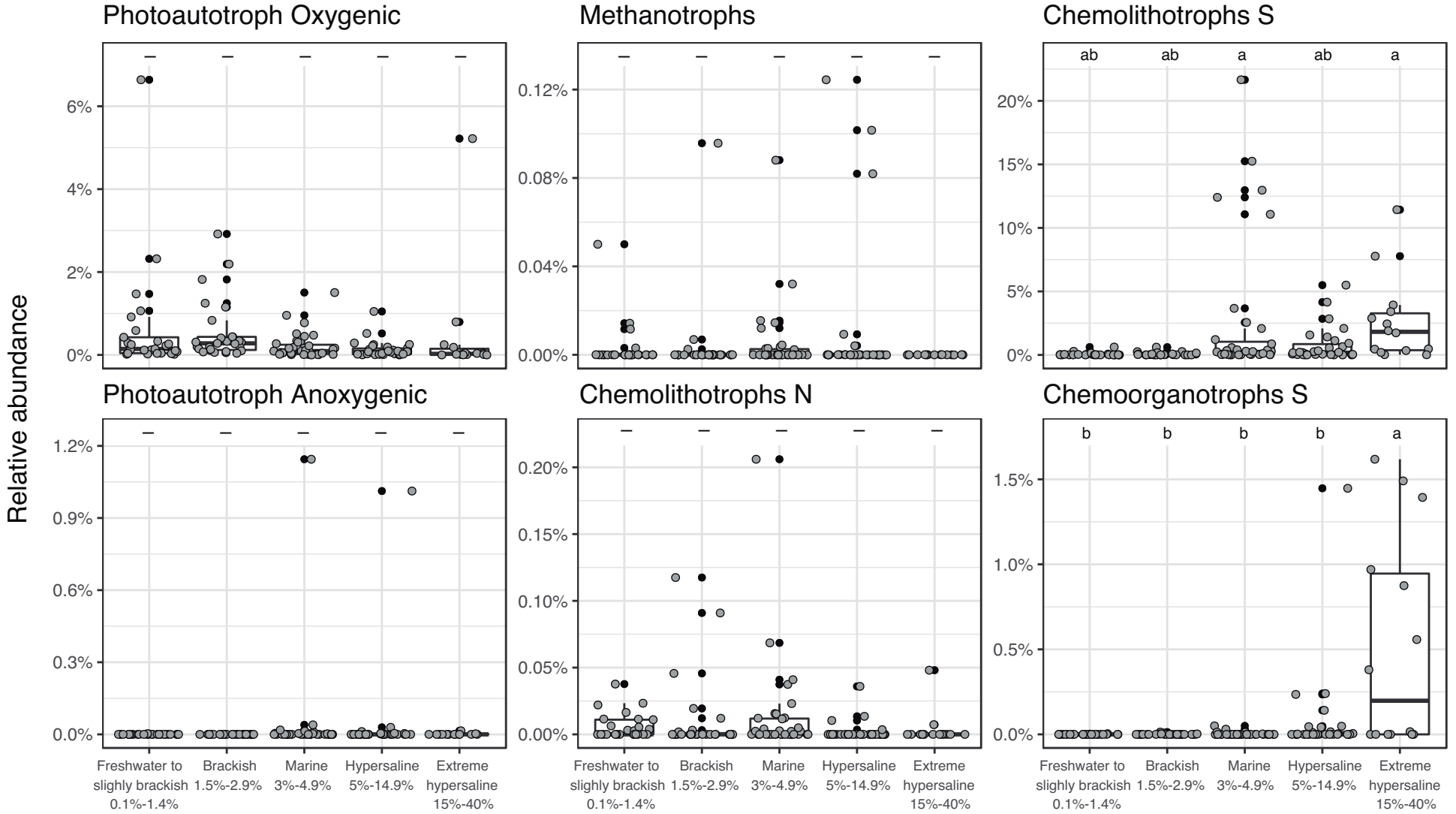
867 **Supplementary Figure S2.** Detailed taxonomic profile for the extreme saline
868 samples (>35% w/v) including the whole 16S rRNA gene pool (archaea and
869 bacteria), at phylum level (a) and at higher taxonomic resolution (b) (genus
870 level, when possible).
871
872 **Supplementary Figure S3.** Relative abundance of *Spirochateae* along the
873 salinity gradient, including detailed information about samples with the highest
874 abundance, and a preliminary phylogenic allocation based on quick add marked
875 partial sequences without optimizing topology in an ARB maximum parsimony
876 optimized tree provided by default (http://help.arb-home.de/pa_quick.html).
877
878 **Supplementary Figure S4.** Taxonomic profile representing the most abundant
879 eukaryotic groups along salinity ranges.
880
881 **Supplementary Figure S5.** Taxonomic profile of the Fungi community along
882 salinity ranges, considering their relative abundance within the whole 18S rRNA
883 gene pool.
884

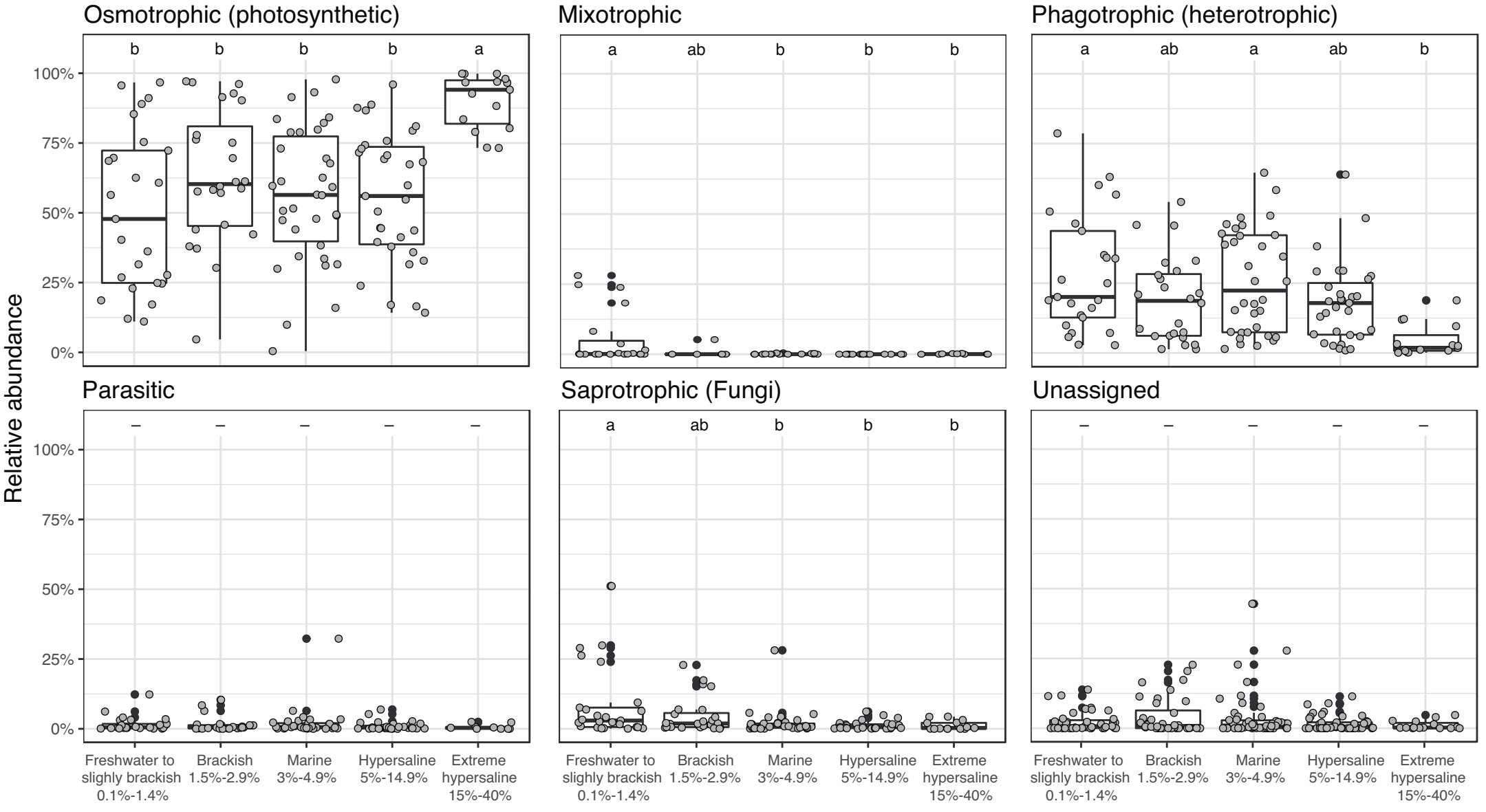
Figures


Bacteria
A
B
C
Sample Richness
Shannon-Weaver index
PD
Salinity (%, w/v)
ρ = NS
ρ = NS
ρ = - 0.17 *
Eukaryote
D
E
F
Sample Richness
Shannon-Weaver index
PD
Salinity (%, w/v)
ρ = - 0.59 **
ρ = - 0.53 **
ρ = - 0.55 **


Bacteria
Filtered Sum(z)
Cumulative Frequency
7000
6000
5000
4000
3000
2000
1000
0
1.0
0.8
0.6
0.4
0.2
0.0
Eukarya
Filtered Sum(z)
Cumulative Frequency
2500
2000
1500
1000
500
0
z-
z+
Cumulative frequency of **negative** z scores
Cumulative frequency of **positive** z scores
0
5
10
15
20
25
30
35
Salinity (%, w/v)


Freshwater to Slightly-brackish
0.1% - 1.4% (w/v)
Brackish
1.5% - 2.9% (w/v)
Marine
3% - 4.9% (w/v)
Hypersaline
5% - 14.9% (w/v)
Extreme hypersaline
15% - 40% (w/v)
Bacteria: Phylum
Genus
Tenericutes
Izimaplasmataceae unclass.
Acholeplasma
Spirochaetes
Spirochaeta 2
Proteobacteria
Alpha–
Tropicimonas
SM2D12 unclass.
Seohaeicola
Salinarimonas
Roseovarius
Rhodobacteraceae unclass.
Porphyrobacter
Marivita
Loktanella
Gamma–
Vibrio
Thiomicrospira
Salinivibrio
Rheinheimera
Pseudomonas
Pseudoalteromonas
Polynucleobacter
Polaromonas
Nitrococcaceae unclass.
Methylophaga
Marinomonas
Litoricola
Limnohabitans
Idiomarina
Hydrogenophaga
Halothiobacillus
Halomonas
Alteromonas
Aliidiomarina
Epsilon–
Sulfurimonas
Halanaerobiaeota
Halobacteroidaceae unclass.
Halanaerobium
Halanaerobacter
Firmicutes
Bacillus
Dependentiae
Babeliaceae unclass.
Bacteroidetes
Salinibacter
Psychroflexus
Phaeodactylibacter
Pedobacter
NS3a marine group
Nonlabens
Leeuwenhoekiella
Fluviicola
Flavobacterium
Fabibacter
E6aC02 unclass.
Algoriphagus
Actinobacteria
Microbacteriaceae unclass.
0 5 10 15 20
Eukarya: L1
L3
SAR
Labyrinthulomycetes
Dinoflagellata
Ciliophora
Cercozoa
Bicosoecida
Opisthokonta
Ochrophyta
Metazoa (Animalia)
Fungi
Excavata
Discicristata
Cryptophyceae
Teleaulax
Cryptomonas
Archeaplastida
Chlorophyta
0 20 40 60
Relative abundance (%)


Photoautotroph Oxygenic
Methanotrophs
Chemolithotrophs S
Photoautotroph Anoxygenic
Chemolithotrophs N
Chemoorganotrophs S
Relative abundance
ab ab a ab a
b b b b a
Freshwater to slighly brackish 0.1%-1.4%
Brackish 1.5%-2.9%
Marine 3%-4.9%
Hypersaline 5%-14.9%
Extreme hypersaline 15%-40%


Osmotrophic (photosynthetic)
Mixotrophic
Phagotrophic (heterotrophic)
Parasitic
Saprotrophic (Fungi)
Unassigned
Relative abundance
100%
75%
50%
25%
0%
b b b b a
a ab b b b
a ab a ab b
– – – – –
a ab b b b
– – – – –
Freshwater to slighly brackish 0.1%-1.4%
Brackish 1.5%-2.9%
Marine 3%-4.9%
Hypersaline 5%-14.9%
Extreme hypersaline 15%-40%