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High diversity and isolated distribution of aquatic heterotrophic protists in salars of the Atacama Desert at different salinities

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Abstract

The species richness of eukaryotes in the hypersaline environment is generally thought to be low. However, recent studies showed a high degree of phylogenetic novelty at these extreme conditions with variable chemical parameters. These findings call for a more thorough look into the species richness of hypersaline environments. In this study, various hypersaline lakes (salars, 1–348 PSU) as well as further aquatic ecosystems of northern Chile were investigated regarding diversity of heterotrophic protists by metabarcoding studies of surface water samples. Investigations of genotypes of 18S rRNA genes showed a unique community composition in nearly each salar and even among different microhabitats within one salar. The genotype distribution showed no clear connection to the composition of main ions at the sampling sites, but protist communities from similar salinity ranges (either hypersaline, hyposaline or mesosaline) clustered together regarding their OTU composition. Salars appeared to be fairly isolated systems with only little exchange of protist communities where evolutionary lineages could separately evolve.

Keywords: Placididea; Metabarcoding; Hypersaline waters; Evolution; Water chemistry; Heterotrophic flagellates

Introduction

Hypersaline environments are thought to be characterized by a low species richness, especially at extremely high salinities (Pedrós-Alió, 2004). However, various recent studies on microbial organisms have shown a high degree of phylogenetic novelty under these extremely saline conditions (Demergasso et al., 2004; Dorador et al., 2013; Triadó-Margarit and Casamayor, 2013; Eissler et al., 2019; Dorador et al., 2020; Lee et al., 2021), including representatives of all taxonomic groups like archaea, bacteria, viruses, and eukaryotes (Emerson et al., 2013; Oren, 2014). In unicellular eukaryotes, the novelty can be assigned to species that primarily belong to the supergroups of Stramenopiles (Bicosoecida) and Opisthokonta (Choanoflagellata; Triadó-Margarit and Casamayor, 2013). Previous studies have

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identified several new species isolated from hypersaline environments showing a potential specific adaptation to high ranges of salt concentrations (Park and Simpson, 2010; Schiwitza et al., 2018; Rybarski et al., 2021; Heine-Fuster et al., 2021; Schoenle et al., 2022). Furthermore, species from the group of Discoba (Heterolobosea; Tikhonenkov et al., 2019; Carduck et al., 2021) and Alveolata (Ciliophora; Qu et al., 2020) were isolated successfully from these environments. Based on these previous studies, the biodiversity of hypersaline (athallasohaline) water bodies, seems to be broadly underestimated (Harding and Simpson, 2018). In northern Chile, numerous saline lakes and salt flats, so-called salars, sustained by evaporation of groundwater and surface runoff, exist in individual closed basins. Most of them are located on the semi-arid Altiplano Plateau in the Western Andean Cordillera (≥3800 m a.s.l) and some in the western part of the hyperarid Central Depression of the Atacama Desert (~900 m a.s.l.) (Risacher et al., 2003). The spatial separation of the salars from each other and in particular from the ocean by extreme dry areas (Azua-Bustos et al., 2012; Neilson et al., 2012; Valdivia-Silva et al., 2012) assign them as locations with potentially highly adapted organisms and a unique biodiversity (Warren, 2006). Organisms at these locations must not only be adapted to high salinities, but also extreme temperature shifts during day and night, high evaporation rates, altitude, and UV radiation. In addition the presence of as of toxic metalloids (arsenic and lithium) in several salars also affects organisms and may play a role in species composition (Volant et al., 2016) as well as the variation of chemical parameters may influence eukaryotic community structure (Mo et al., 2021). As potentially fast-evolving organisms with a high reproduction rate and the ability to adapt quickly to extreme conditions, protists are predestined for investigations on evolutionary processes in these extreme environments (Arndt et al., 2020). Prior to the geological formation of the Atacama region, placidid Stramenopiles evolved from marine representatives (131 (77–196) Ma ago) and their separation from marine and hypersaline species occurred around 114 (66–175) Ma ago (Arndt et al., 2020). Multiple strains of placidid species were isolated from various hypersaline environments in the Atacama Desert (Rybarski et al., 2021) but high diversity was also found within one salar. This suggests that allopatric as well as sympatric divergence processes with synchronous diversification acted on the established diversity of protists in this region. Placidids seem to be highly flexible to adapt to these extreme habitats and may be used as a model group to characterize these environments (Arndt et al., 2020). The formation of resting stages (cysts) enables a dispersal without the need of water, e.g. via wind or animals like birds (Rogerson and Detwiler, 1999), and to survive phases of non-optimal conditions like desiccation of waterbodies. However, the exchange rate seems to be low since most salars are hundreds of kilometres apart separated by regions of extremely high UV radiation and stepping stones are mostly lacking. It can thus be hypothesized that although there is a small exchange between the different salars, e.g. via birds, this exchange occurs rather seldom, supporting evolutionary processes by isolation. On the other hand, the high diversity of microhabitats in the same salar might allow the coexistence of many genotypes.

The question arises, whether each salar has its unique eukaryotic community which is specifically adapted to the conditions. To get a better overview of the hidden protistan diversity and richness in the Atacama Desert, the V9 region of the SSU rRNA from environmental samples of eleven isolated inland waters (hypersaline salars, lagoons, lakes), located at different altitudes with different salt concentrations and compositions was investigated. To keep sequencing errors and the overestimation of taxa richness to a minimum, a mock community as a reference community was used. These filtered data sets were also compared to the filtering carried out in other metabarcoding studies for comparison (e.g. Schoenle et al., 2021). In addition to the metabarcoding approach, a culture-based approach was accomplished to detect the occurrence of species and to add verified sequences to the V9 region data base using morphological and molecular tools. For this purpose, we used placidid protists as a model group (Rybarski et al., 2021). Where possible, samples from one location including separated pools with different salinities were additionally taken, to identify if each location contains unique eukaryote communities or whether there is a large overlap and potential exchange between sampling sites.

Material and methods

Sampling and DNA isolation of field samples

The inland water samples were classified regarding their salinity according to Hammer (1986). Samples with a salinity of 0.5–3 PSU (practical salinity units) were considered subsaline, 3–20 PSU hyposaline, 20–50 PSU mesosaline and > 50 PSU hypersaline. Surface water was filtered from various inland waters with different salt concentrations from northern Chile during several expeditions (Fig. 1). Water samples were filtered on 0.45 µm polycarbonate filters (Sartorius, diameter 47 mm). The volume of filtered water for each sample varied depending on the salt concentration and the amount of sediment (Table 1). Filters were transferred into 5 ml CryoTubes™ (Thermo Fisher Scientific, Waltham) and covered completely with 2 ml of absolute ethanol (samples from 2017, 2019) or with a DMSO/EDTA/NaCl solution (DESS) for preservation (samples from 2018; Gray et al., 2013; Yoder et al., 2006). At every location, an additional water sample was taken to identify chemical parameters, such as the composition and concentration of different ions. Parameters, like the exact geographic location together with the associated coordinates,

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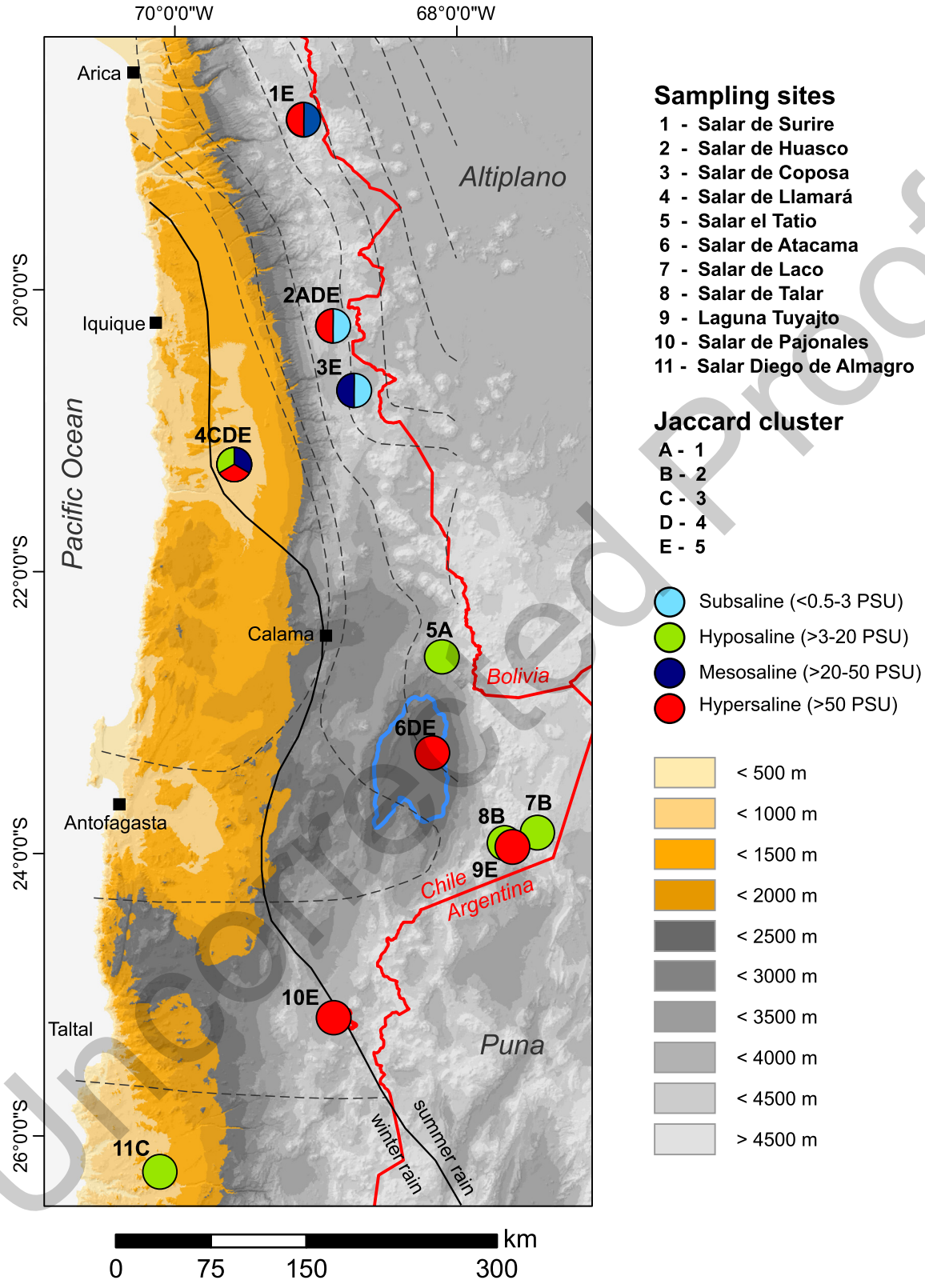

Fig. 1. Map of sampling points in northern Chile. Colours of the positions indicate their salinity, several colours at one position demonstrate different sampling sites with different salinities. Letters indicate the resulting cluster of the Jaccard index calculation. More information on each sampling site can be found in Table 1.

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Table 1. Information of filtered water samples, including the exact geographical location with the associated coordinates, the altitude, the salinity, and the volume of filtered water, together with the type of MID used, and the measured ion concentrations (only those which had detectable concentrations) of each sample.

	Sampling location		Filter	Lat/Long	Salinity [PSU]/ altitude [m]	Sample	Volume [ml]	MID	Na [mmol/l]	K [mmol/l]	Ca [mmol/l]	Mg [mmol/l]	Cl [mmol/l]	S [mmol/l]	As [mmol/l]	B [mmol/l]	Ba [mmol/l]	Li [mmol/l]	P [mmol/l]	Si [mmol/l]	Sr [mmol/l]
1	Salar de Surire	S28	Filter-17_009	18° 47′50.97″S 69° 4′57.05″W	50/4260	Lake	150	UDI_002	617.55	49.49	22.35	25.91	657.05	115.28	0.57	27.58	0	11.3	0	0	0.23
		S19	Filter-17_010	18° 47′42.40″S 69° 5′7.69″W	154/4267	Lagoon	250	UDI_002	3413.84	220.32	15.70	303.63	969.36	1620.93	0.13	90.86	0	68.30	0.05	0.03	0.14
2	Salar de Huasco	S14	Filter-18_029	20° 15′45.1″S 68° 52′33.4″W	1/3790	Pipe	500	UDI_007	3.1	0.14	1.12	0.34	1.23	0.87	0	0.28	0	0	0	0.69	0
		S41	Filter-17_007	20°17′3″S 68°53′15″W	60/3765	Main waterbody	300	UDI_007	607.26	48.01	8.42	20.9	439.06	216.5	0.23	20.85	0	6.39	0	0.58	0.08
		S31	Filter-18_025	20°17′3.6″S 68° 53′15.9″W	97/3770	Main waterbody	100	UDI_005	1427.54	91.50	13.41	357.56	> 6126.57	596.79	0	36.81	0	0	0	0.94	1.71
		S06	Filter-18_030	20° 18′18.3″S 68°53′4.4″W	294/3767	South pool 2	60	UDI_008	> 4388.35	> 481.32	8.78	138.93	3089.36	1074.05	0.21	157.66	0	59.11	0	0.28	0.11
3	Salar de Coposa	S02	Filter-19_018	20° 40′41.088″S 68° 41′34.032″W	2.5/3729	Main waterbody	400	UDI_003	–	–	–	–	–	–	–	–	–	–	–	–	–
		S33	Filter-19_017	20°40′41″S 68°41′36″W	30/3729	Pool	400	UDI_008	248.00	13.34	33.98	40.65	243.54	60.65	0	4.09	0	1.88	0	0.45	0.45
		S42	Filter-19_016	20°40′41″S 68°42′09″W	40/3729	Pool	400	UDI_008	431.07	18.77	61.58	53.68	574.49	54.52	0	2.9	0	3.25	0	0.31	0.31

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4	Salar de Llamará	S29	Filter-18_047	21°16′13.8″S 69° 37′11.1″W	18/743	Pond	250	UDI_003	206.14	3.64	19.2	4.94	205.92	49.30	0	0	0	0	0	1.25	0.05
		S39	Filter-17_004	21°16′13″S 69°37′11″W	41/740	Pool − 6	1000	UDI_004	599.02	10.25	19.2	11.49	569.02	91.92	0.03	1.76	0	0	0	1.63	0.07
		S40	Filter-18_046	21°16′4.5″S 69°37′1.3″W	133/726	Pool	100 + 200 VE	UDI_005	2278.23	39.68	20.08	32.87	2285.93	313.25	0.12	5.97	0	1.40	0	1.16	0.33
		S25	Filter-18_044	21°16′6.8″S 69°37′0.4″W	141/734	Main waterbody	150 + 300 VE	UDI_011	2488.53	40.46	17.28	36.43	2167.45	315.34	0.15	7.28	0	1.66	0	1.26	0.37
5	Salar el Tatio	S21	Filter-18_017	22° 36′35.3″S 68°03′37″W	11/ 3995	–	150	UDI_004	112.49	4.46	0	6.47	110.33	7.99	0.18	5.83	0.01	0	0	0.84	0
6	Salar de Atacama	S05	Filter-18_010	23° 17′16.6″S 68° 10′26.6″W	64/2301	Brine shrimp pool	250	UDI_007	867.20	76.02	11.02	79.85	1249.70	47.64	0.01	17.13	0	36.41	0	0.94	0.08
		S15	Filter-18_012	23° 17′21.1″S 68° 10′40.5″W	97/2301	Main waterbody	250	UDI_008	1287.85	97.97	29.31	119.47	1669.15	92.52	0.03	26.83	0	50.33	0	0.92	0.28
		S17	Filter-18_014	23°17′22″S 68° 10′42.3″W	198/2304	Red pool	250	UDI_012	2610.06	204.74	25.03	244.54	3297.14	168.35	0.10	59.54	0	101.59	0	0.99	0.54
7	Salar de Laco	S03	Filter-18_023	23°51′12.1″S 67° 25′34.2″W	4/4215	–	50	UDI_004	71.6	3.91	4.52	3.69	106.04	6.17	0	1.84	0	0.37	0	1.23	0.1
8	Salar de Talar	S34	Filter-18_019	23° 55′35.9″S 67° 39′40.5″W	4/3930	–	250	UDI_011	35.48	2.40	9.30	4.82	100.05	8.77	0	0.77	0	0.16	0	1.79	0.05
9	Laguna Tuyajto	S35	Filter-18_020	23°57′10″S 67° 35′40.5″W	348/4019	–	35	UDI_012	> 4427.89	337.58	8.09	> 667.62	> 5780.23	303.40	0.02	76.12	0	39.40	0	0.13	0.29
10	Salar de Pajonales	S30	Filter-18_006	25°10′10″S 68°52′24″W	264/3511	Sample 1	250	UDI_004	3193.34	63.98	277.7	295.72	4513.62	12.21	0.05	32.71	0.02	9.79	0	0.71	1.24
11	Salar Diego de Almagro	S11	Filter-18_004	26°15′38″S 70°06′17″W	12/956	Sample 1/ 2	250	UDI_003	85.24	4.04	22.02	2.43	121.42	23.51	0	1.59	0.01	0	0	1.08	0.12

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the altitude and the salinity were recorded (Table 1). Prior to DNA extraction, samples preserved in ethanol were placed in a heating chamber (70–80 °C) to evaporate the total amount of ethanol and cooled down to room temperature. Samples preserved in DESS were vortexed for 2 min, centrifuged (4000 *g*, 4 °C, 20 min) and the supernatant was discarded. DNA extraction was performed directly within CryoTubes™ (Thermo Fisher Scientific, Waltham) using the Quick gDNA ™ Mini Prep Kit (Zymo Research Corporation, CA, USA). Genomic Lysis Buffer was applied (2100 µl) on the filters, vortexed for 2 min and centrifuged (4000 *g*, 4 °C, 15 min). Tubes were vortexed again and incubated at room temperature for 15 min. Further steps were performed applying the manufacturers' protocol except for an additional washing step. The quality of each sample as well as the amount of DNA was measured via a Nanodrop Spectrophotometer ND-1000 (Peqlab, VWR, Erlangen, Germany) and stored afterwards at −20 °C.

DNA isolation and sequencing of the mock community

As an artificial control sample for sequencing (mock community), ten known strains from six various protist supergroups were used: Alveolata (*Protocruzia* sp. MT355146; *Aristerostoma* sp. MT081566), Ancyromonadida (*Fabomonas tropica* MT355148), Rhizaria (*Massisteria* sp. MT355122), Discoba (Rhynchomonadidae sp. MT355133; *Neobodo* sp. MT355124), Stramenopila (*Cafeteria burkhardae* MN315604; Bicosoecida sp. MT355117) and Opisthokonta (*Enibas tolerabilis* MH687869; *Ministeria vibrans* MT355150). All strains originate from the Heterotrophic Flagellate Collection Cologne (HFCC) and sequences were made available on NCBI GenBank (Table S1). Species were selected to cover representatives of the main supergroups. The same mock community is used in our working group to enable comparative studies. 18S rRNA amplification was performed by DNA extraction and standard polymerase chain reaction (PCR). Clonal cultures (30 ml) were transferred into 50 ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuged (4000 *g*, 4 °C, 20 min). The supernatant was discarded and DNA was extracted using the Quick gDNA ™ Mini-Prep Kit (Zymo Research Corporation, CA, USA) applying the manufacturers' protocol, and 3 µl of the isolated DNA was used as a template together with 5 µl of each universal primer with a final concentration of 1 µM as well as a Taq DNA Polymerase Master Mix (2x) (VWR CHEMICALS, Haasrode, Belgium; final volume of 50 µl). The amplification of the 18S rDNA with the primer pairs 18S For (5′ -AACCTGGTTGATCCTGCCAGT- 3′, (Medlin et al., 1988) and 18S Rev (5′ -TGATCCTTCCGCAGGTTCACCTAC- 3′, (Medlin et al., 1988) started with the denaturation step at 96 °C for 2 min, then 34 cycles of 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 2.5 min, then a final elongation step for 7 min at 72 °C. The PCR products were verified by gel electrophoresis using a 1% agarose gel. PCR products were purified afterwards using the Bioscience PCR Purification Kit (Bioscience, Jena, Germany) and sequenced using the 18S rDNA primer sets at GATC Biotech, Germany. Sequences were analysed using NCBI nucleotide BLAST® (Altschul et al., 1990; Wheeler and Bhagwat, 2007).

Amplification of V9 region, library preparation and sequencing

Extracted DNA of the samples as well as strains of the mock community were used for the amplification of the hyper-variable region V9 of the 18S rRNA gene. As a template, 50 ng of the isolated DNA from each sample was used. PCR reaction (50 µl/reaction) consisted of 0.35 µM adapter (MID)-tagged forward and reverse primers 1389F 5′-TTGTACACACCGCCC-3′ and 1510R 5′-CCTTCYG CAGGTTCACCTAC-3′ (Amaral-Zettler et al., 2009) (Table S2), 25 µl Taq DNA Polymerase Master Mix (2x) (VWR CHEMICALS, Haasrode, Belgium) and were stocked to 50 µl by ddH2O. PCR amplification was carried out after de Vargas et al., 2015 (98 °C for 30 sec; 25 cycles of 10 sec at 98 °C, 30 sec at 57 °C, 30 sec at 72 °C; and 72 °C for 10 min) in triplicates to receive enough DNA for Illumina sequencing as well as to smooth intra-sample variance. PCR products were, pooled, and purified afterwards using the Bioscience PCR Purification Kit (Bioscience, Jena, Germany). DNA (20 ng) of each of the ten representative strains of the mock community were pooled to one mock community sample. The DNA of eight filter samples (20 ng DNA each) together with the mock community (20 ng DNA) were pooled to form one sequencing sample for the Illumina run. In total, five such sequencing samples were prepared. The mock community in each sequencing sample was used as the reference sample for filtering at the same sequencing conditions. The length of the amplicons from the pooled sample was checked by gel electrophoresis (1 % agarose gel) again and these five samples were handed in at the Cologne Center for Genomics (CCG) for Next Generation Sequencing (NovaSeq, paired-end 2x150 bp).

Demultiplexing and clustering into operational taxonomic units (OTUs)

The bioinformatic pipeline of Frédéric Mahé (https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline) was used. In brief, the quality and the encoding of all raw files was checked (--fastq_chars), forward and reverse reads were merged (--fastq_mergepairs, default parameters, --fastq_allowmergestagger) with VSEARCH v2.17.0 (Rognes et al., 2016) and only assembled data that contained both adapter (MID)-tagged primers (match: 100 % of the MID, 2/3 of primer length) were used for further

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investigations. Primers and MIDs, as well as sequences containing nonspecific nucleotides (Ns), were removed with Cutadapt v3.4 (Martin, 2011), reads were demultiplexed, dereplicated via VSEARCH v2.17.0 (Rognes et al., 2016), and a quality file was generated. Files from the salars were pooled (22 files), dereplicated and clustered by Swarm v3.1.0 (Mahé et al., 2021) with default settings into operational taxonomic units (OTUs). The five mock files were not pooled but treated separately the same way. OTU representatives were checked for chimera with VSEARCH v2.17.0 (Rognes et al., 2016). V9_DeepSea database (Schoenle et al., 2021a) was updated with additional V9 sequences of protists from the Heterotrophic Flagellate Collection Cologne and used for taxonomic assignment with VSEARCH (global pairwise alignment –iddef 1, matching columns/alignment length). Amplicons were assigned to their best hit or co-best hits in the reference database via Stampa (Mahé, 2016). Chimeric sequences and sequences with a quality value (min. expected error rate/sequence length) higher than 0.0001 were removed, as well as reads shorter than 87 bp and OTUs with a p-identity of < 80 %. OTUs which could not be assigned to a reference sequence, as well as Metazoa, Fungi, Streptophyta and phototrophic organisms, were removed. The OTUs which could not be assigned to a taxonomic group at the taxonomic level were grouped as "Unknown/Uncertain".

All OTUs of salar samples were subsequently filtered by two different filtering procedures: 1) Using a strict filter regarding the mock community that resulted from the same lane in Illumina runs. OTUs were discarded for which the read number was lower than the minimum number of reads obtained for OTUs of the corresponding mock community (Table S4; later called mock filter). 2) Data sets were filtered with a less strict filter: all OTUs for which at least three reads were recorded were taken into account (later called three-read-filter) (e.g. as in Schoenle et al., 2021b).

Data analyses

R-Studio with the R-version 4.0.2 was used to conduct statistical analysis and to illustrate the investigated data regarding community composition (R Core Team, 2020). The V9_DeepSea reference database (Schoenle et al., 2021a) was used for taxonomic assignment. The Jaccard index was calculated to compare the community composition and the Jaccard distance to display beta-diversity using the unweighted pair-group method with arithmetic means (UPGMA) (R package: "vegan"; "vegdist" and "hclust" function, Oksanen et al., 2018). The results were illustrated in a dendrogram using "ggplot2" (Wickham, 2009) and bootstrap values of clusters were calculated with 100,000 bootstrap replicates ("fpc" package; "clusterboot" function) (Hennig, 2007).

A dissimilarity maxtrix of the species communities was calculated with function "vegdist" (with distance = "jaccard" and binary = TRUE) of the R package "vegan". Non-metric multidimensional scaling (NMDS) was performed with this Jaccard distance matrix using the "metaMDS" function (with distance = "jaccard", autotransform = FALSE, k = 3) within R-package "vegan" to show the variation of samples and their communities due to their similarities. We considered the first three NMDS dimensions since they resulted in a good fit according to the stress values and R^2 values calculated with function "nmds" (maxdim = 10, nits = 100) and visualized with "plot.nmds" within the "ecodist" package. Environmental variables were scaled with the "decostand" function, and parameters set to "range" and method="standardize". An environmental fit was performed on the ordinations with „envfit" (permutations = 999) from the package „vegan" to determine the measured abiotic parameters that may influence community composition, after eliminating highly correlated variables analysed by Variance Inflation Factors (VIF) with the "vif.cca" function. Envfit relates the explanatory variables to the site score of the sampling sites in den ordination space and provides R^2 and p-values of the underlying regressions. Vectors indicate the direction of change in environmental factors associated with the distribution of sites in multivariate space. While no variables were significant, no vectors were included in the NMDS plot. Sampling sites were grouped into salinity ranges (such as hypersaline, hyposaline, mesosaline and subsaline) and convex hulls were drawn for this grouping except for the subsaline station due to low sampling size. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences using the "adonis2" function. Data were visualized by using ggplot2.

Venn diagrams that show the number of shared and unique OTUs between all different salinity ranges as well as between different samples at one location were calculated via the R package VennDiagram (Chen and Boutros, 2015). Rarefaction curves and the Shannon-Wiener index were calculated to evaluate the sequencing depth (R package: "vegan"; Oksanen et al., 2018).

Chemical composition

The ion concentrations of water samples were analysed using a Spectro Arcos inductively coupled plasma optical emission spectrometer (ICP-OES, SPECTRO Analytical Instruments, Kleve, Germany) with axial plasma observation. The Spectro Arcos is capable to simultaneously monitor line intensities at wavelengths between 130 and 770 nm using a Paschen-Runge mount equipped with 32 CCD detectors. Sample nebulization and introduction into the plasma is accomplished using a cross-flow nebulizer mounted on a Scott double pass spray chamber. The operating parameters for ICP-OES analyses are given in Table S6. The emission intensities were monitored at the following wave lengths: 167.078 nm (Al), 189.042 nm (As),

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249.773 nm (B), 455.404 nm (Ba), 317.933 nm (Ca), 134.724 nm (Cl), 324.754 nm (Cu), 259.941 nm (Fe), 766.491 nm (K), 670.780 nm (Li), 280.270 nm (Mg), 589.592 nm (Na), 177.495 nm (P), 182.034 nm (S), 251.612 nm (Si), 407.771 nm (Sr), 292.464 nm (V).

Results

High-throughput sequencing of salar samples

The sequencing of 22 salar samples resulted in 221,957,500 raw reads with both adapter-tagged primers, on average with 10.0 ± 5.8 million per sample. After removing reads that were too short, we ended up with 119,032,169 reads (80,237 OTUs), on average with 5.4 ± 3.5 million reads per sample. On average, all unfiltered OTUs could be assigned to a reference sequence with a p-identity of 66 % (Fig. S5). After removing chimera, sequences with a quality value > 0.0001, reads < 87 bp and OTUs p-identity < 80 %, we ended up with 21,848 operational taxonomic units (OTUs) (99,241,596 reads). All 22 samples showed a nearly saturated rarefaction curve with slopes in the range of 0 (S02) to $3{,}13 \times 10^{-4}$ (S34) (Table S3, Fig. S6). The alpha-diversity, calculated via the Shannon index ranged from 0.19 to 4.26, with a mean value of 1.71 (Table S3). Removing Metazoa, Fungi, Streptophyta and phototrophic organisms (OTUs p-identity < 80 %), resulted in 13,918 OTUs (41,370,053 reads) which could be assigned to heterotrophic protists (Table S5). 16.9 % of all filtered OTUs could be assigned with 100 % identity to a reference sequence from the database of which 2.6 % of OTUs were unknown or uncertain. 13.8 % of OTUs could be assigned to sequences with a similarity of 98 %. The average amplicon size of the V9 region of 18S rRNA sequences was 125 bp (±8 bp, standard deviation). Appling the additional filter of the mock community, the salar sample dataset ended up with 461 OTUs (39,716,694 reads), 3,31 % of heterotrophic protists. Most filtered OTUs from 80 to 100 % similarity to reference sequences could be assigned to Alveolata (35.6 %) and Stramenopiles (27.8 %) (Fig. 2, S02).

Community composition

The calculation of the beta-diversity of all 22 samples, collected from salars in the Atacama Desert, formed five separated clusters (Fig. 2, highlighted in green, pink, red, blue, orange). The first cluster (highlighted in green) was represented by one freshwater sample from a groundwater inflow to the Salar de Huasco (1 PSU, 3790 m a.s.l.) and

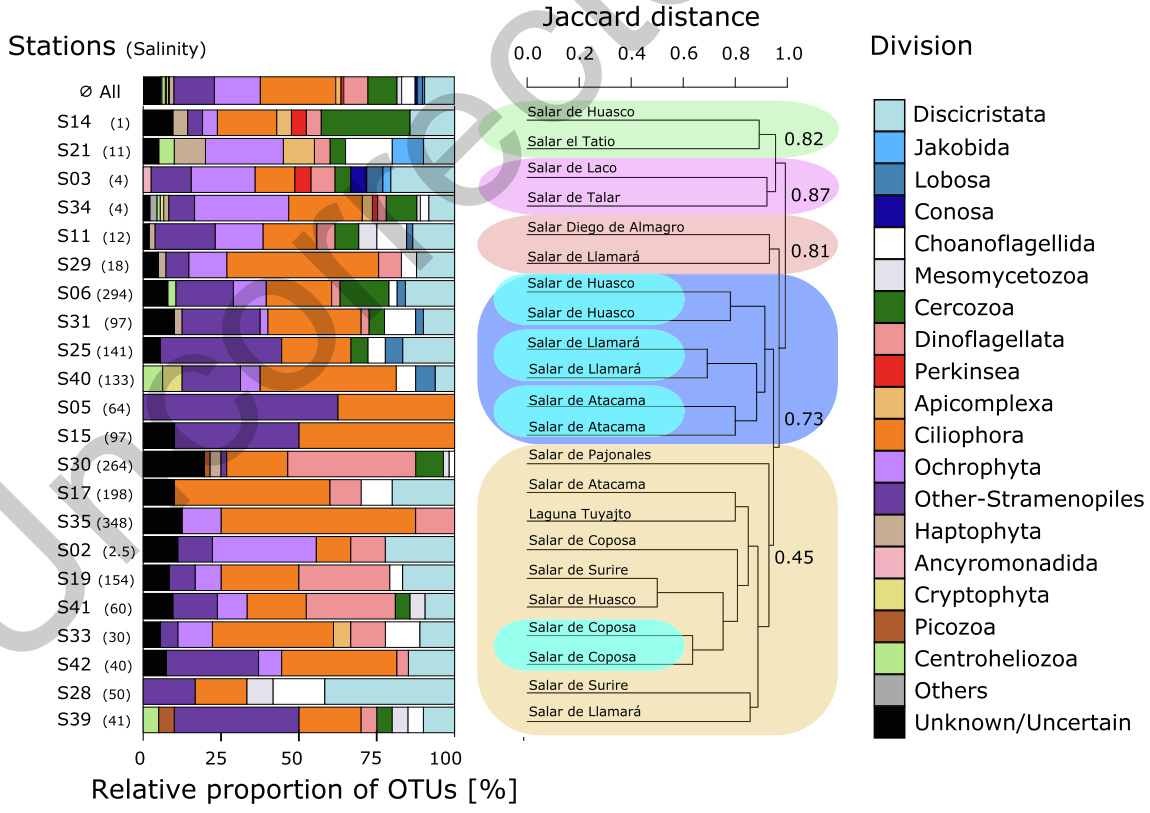

Fig. 2. Community structure and clustering of inland water samples based on OTUs were assigned to taxonomic groups. Others - Relative proportion of OTUs < 1 %; Unknown/Uncertain - OTUs could not be assigned to a taxonomic group. Clusters of the dendrogram, based on the Jaccard index, are highlighted in different colours (s. text) and bootstrap values are given for each cluster.

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a brackish water sample from the Salar el Tatio (11 PSU, 3995 m a.s.l.). The second cluster (highlighted in pink) was formed by two freshwater samples (4 PSU), one from Salar de Laco (4215 m a.s.l.) and one from Salar de Talar (3930 m). The third cluster (highlighted in red) contained two hyposaline samples: a pond sample from Salar de Llamará (18 PSU, 743 a.s.l.) and a sample from Salar Diego de Almagro (12 PSU, 956 a.s.l.) The fourth cluster (highlighted in blue) consists of samples from the Salar de Huasco (n = 2), the Salar de Llamará (n = 2) and the Salar de Atacama (n = 2), where samples from the same location clustered together. The fifth cluster (highlighted in orange) contained all additional samples (n = 10) from locations higher than 2000 m altitude. Within this clade, the samples from the Salar de Coposa clustered together. The first four clusters were supported moderately (bootstrap: 0.73–0.87), the fifth and largest cluster was not well supported (bootstrap: 0.45) (Fig. 2). The samples themselves were mainly dominated by Stramenopiles (27.8 %) and Alveolata (35.6 %; Fig. 2, see left upper panel for average values). Ochrophyta (53.1 % of Stramenopiles) were mainly represented by the class Chrysophyceae (88.2 % of Ochrophyta) and other Stramenopiles (46.9 % of Stramenopiles) of which 38.3 % were Bicosoecida, 20 % Placididea and 16.7 % Oomycota. Ciliophora (68.3 % of Alveolata) were dominated by Oligohymenophorea (31.3 % of Ciliophora), Spirotrichea (25 %), Litostomatea (13.4 %) and Phyllopharyngea (10.7 %). Cercozoa amounted on average for 9.3 % of all filtered OTUs. The samples of the first three clades originated from Andean groundwater supply and were individually very variable in the community structure and were dominated by ochrophytes. Sample S34 (second cluster, Salar de Talar, 4 PSU, 3930 m a.s.l.) showed the highest (29 % of all filtered OTUs) diversity compared to all other sampling locations. The fifth clade contained many samples with unknown or uncertain OTUs, while the fourth clade contained samples where other Stramenopiles as well as Ciliophora were abundant. Sample S05 (fourth cluster, Salar de Atacama, Brine Shrimp Pool, 64 PSU, 2301 m a.s.l.) and sample S35 (fifth cluster, Laguna Tuyajto, 348 PSU, 4019 m a.s.l.) contained the lowest (1.7 % of all filtered OTUs) diversity.

Similarity of heterotrophic protist OTUs at different sites of the same salar

To investigate the community composition within one salar, at several sites, samples were taken at different locations of several salars with different salt concentrations (Salar de Huasco (1x subsaline, 3x hypersaline) and the Salar de Llamará (1x hyposaline, 1x mesosaline, 2x hypersaline), three different samples at Salar de Atacama (3x hypersaline) and the Salar de Coposa (1x subsaline, 2x mesosaline), respectively, and compared to each other (Fig. 3A, A´, B, B´, S1). In all cases, the patterns regarding the relative proportions of OTUs observed using the strict mock filter were very similar to those obtained using the less strict three-read-filter (see also comparisons in Figs. S1 and S2). At both, the Salar de Huasco (Fig. 3A) and the Salar de Llamará (Fig. 3B), there were no (mock filter) or very little (three-read-filter) OTUs shared between the different sites within one salar. Samples with salinities differing from those of the sites of Salar de Atacama (Fig. S1 A) shared 5 % of OTUs, while the samples of the Salar de Coposa shared 14 % (Fig. S1 B) regarding the mock filter. Samples with the lowest and the highest salinity shared no OTUs (mock filter) or had only very little overlap (three-read-filter).

Similarity of heterotrophic protist OTUs between different salars

The comparison of samples from different salars ranging from subsaline to slightly hyposaline conditions showed that no OTUs were shared between all of them (Fig. 3C) or only a low percentage of 2 % (Fig. 3C') and most OTUs were unique to the different salars. Both subsaline samples shared either no or only one OTU (Fig. 3C, C'), the hyposaline samples shared 6 % or even 9 % of OTUs. The lowest subsaline and one hyposaline samples shared only 3 % of OTUs (Fig. 3C, C'). Again, the data filtering options revealed similar results. The comparison of samples from different locations from hyposaline to slightly mesosaline conditions showed also that no OTUs were shared between all of them (Fig. S2A) or only a low percentage of 3 % (Fig. S2A') and most OTUs were unique to the different salars. All three hyposaline samples shared no or only a low percentage (0.3 %) of OTUs.

The comparison of samples from different salars but with approximately the same salinity showed that no or only a small amount of OTUs were shared between all of them and most OTUs were unique to the different salars (Fig. 4A, A´). The sample from the Salar de Coposa contained the highest (22 %) and the sample of the Salar de Atacama the lowest (6 %) amount of unique OTUs. Comparisons of all different salars showed that either no OTUs were shared or at most 2–5 %. Even the three-read-filter approach showed that the Salar de Huasco had the highest percentage of unique OTUs (592 OTUs, 28.8 %) and the Salar de Surire the lowest (77 OTUs, 3.4 %). All samples had about 2 % of OTUs in common (Fig. 4A').

The comparison of the most hypersaline samples from different salars showed that only a small amount of OTUs was shared between all of them (1 % - Fig. 4B, 2 % Fig. 4B') and a low percentage and most OTUs were unique to the different salars. The samples from the Salar de Pajonales contained the highest and the samples from Laguna Tuyajto and the Salar de Atacama the lowest amount of unique OTUs.

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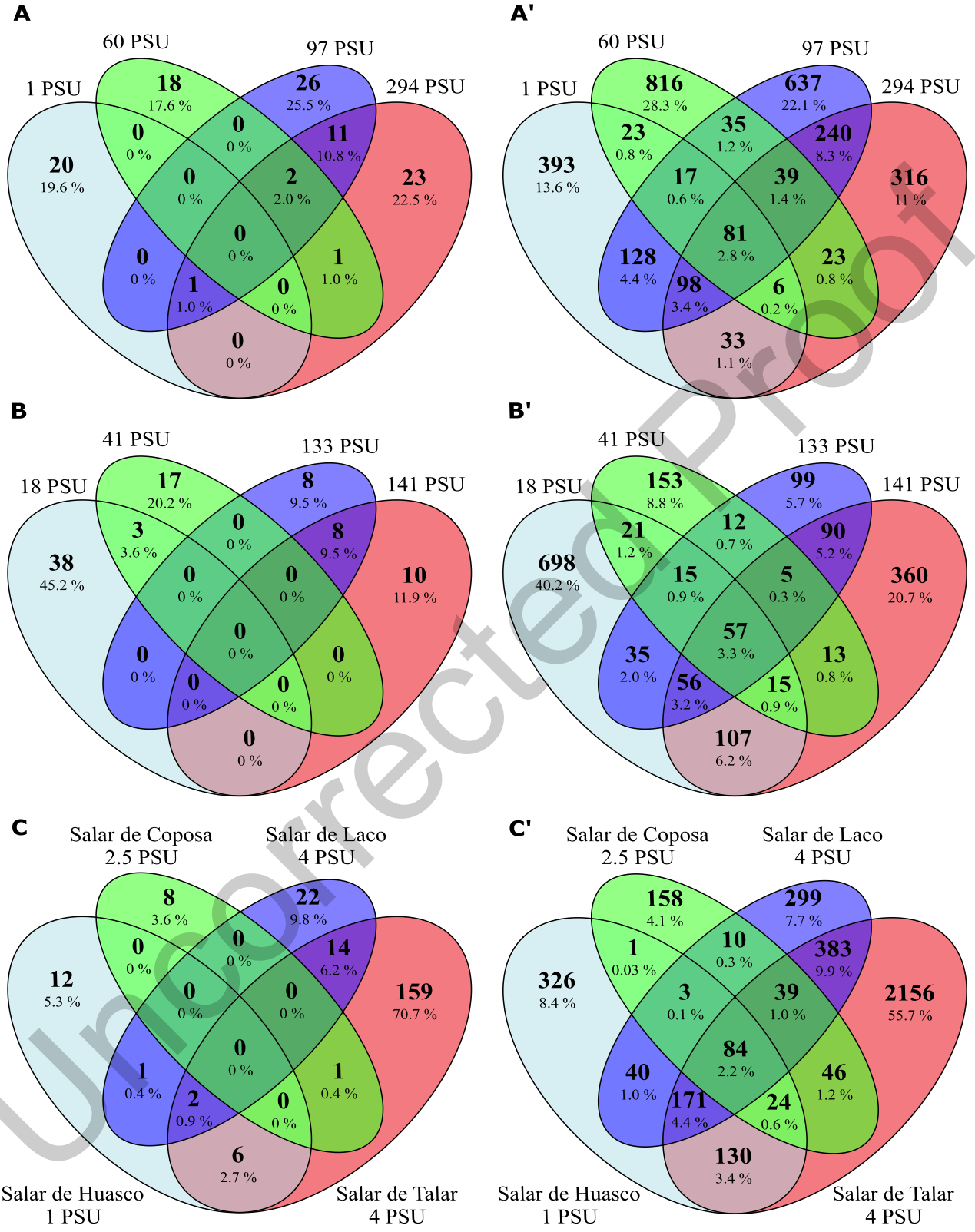

Fig. 3. Comparison of heterotrophic protists' OTUs of samples from the same location (A, A' - Salar de Huasco; B, B' - Salar de Llamará) and from different locations with subsaline to slightly hyposaline conditions (C, C'). Venn diagrams show the number and percentage of unique and shared OTUs. A, B and C were filtered regarding the mock community; A', B' and C' according to Schoenle et al. (2021b).

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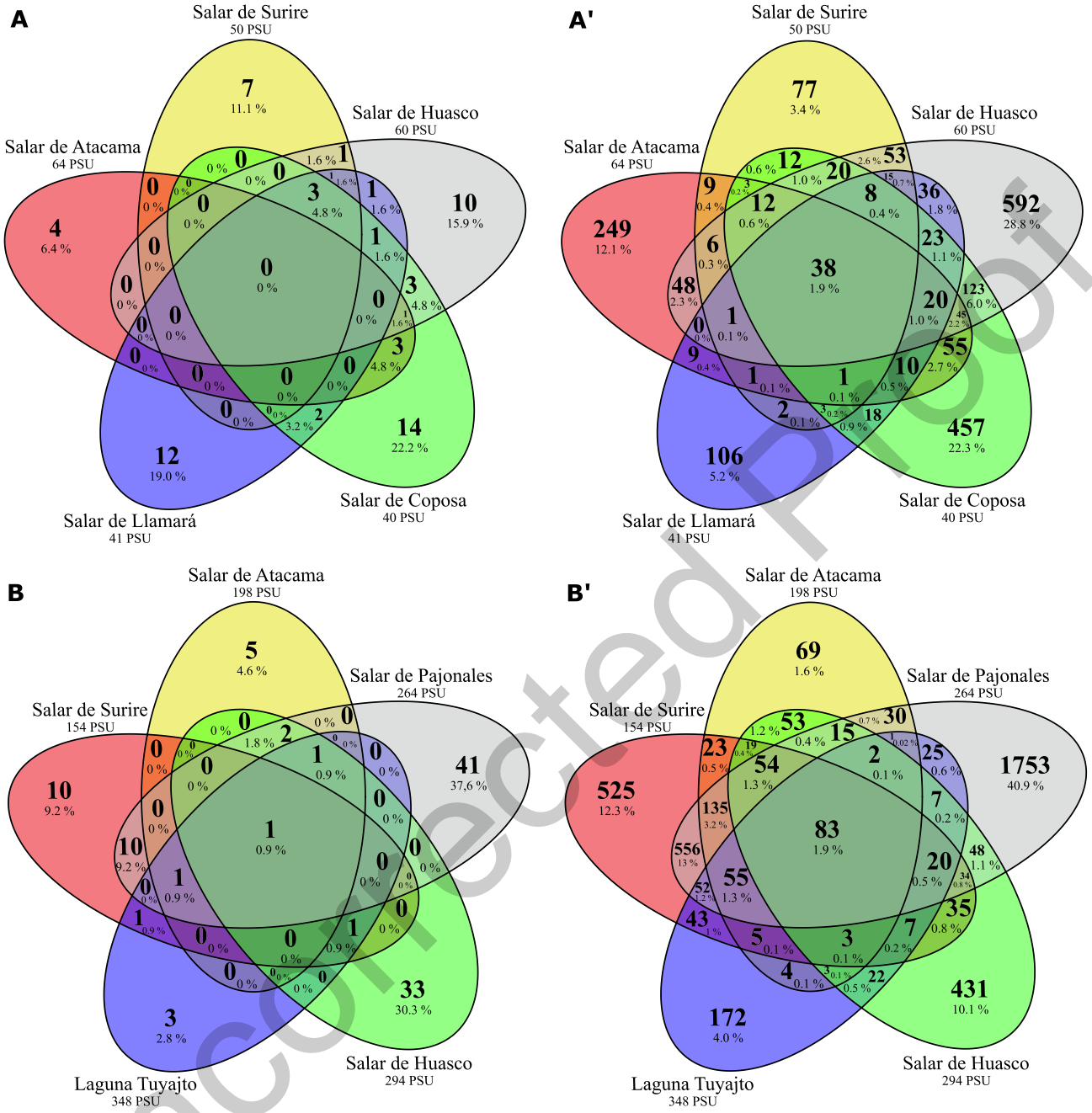

Fig. 4. Comparison of heterotrophic protists' OTUs of samples from different locations with A; A' - nearly the same salinity and B, B' - with extremely high hypersaline conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A and B were filtered regarding the mock community; A' and B' were filtered regarding Schoenle et al. (2021b).

The NMDS plot showed that samples from all sites with similar salinity ranges (hypersaline, hyposaline and mesosaline) clustered together regarding their OTU composition (Fig. 5, $p < 0.001$). For subsaline samples, not enough data were available. The samples from hypersaline and hyposaline ($p = 0.0047$) as well as hypersaline and mesosaline ($p = 0.0146$) were significantly different. In the NMDS ordination of species composition, communities generally occupied non-overlapping areas (slight overlap of meso- and hypersaline sites) in ordination space. For the communities within the different salinity ranges, the stress (0.091) indicates the communities were well represented in two reduced dimensions (see Clarke and Warwick, 2001). PERMANOVA indicated a significant difference between communities within the different salinity ranges. However, only sodium showed a significant ($p = 0.010$) influence on protistan community structure, though with a low coefficient of determination ($R^2 = 0.0624$).

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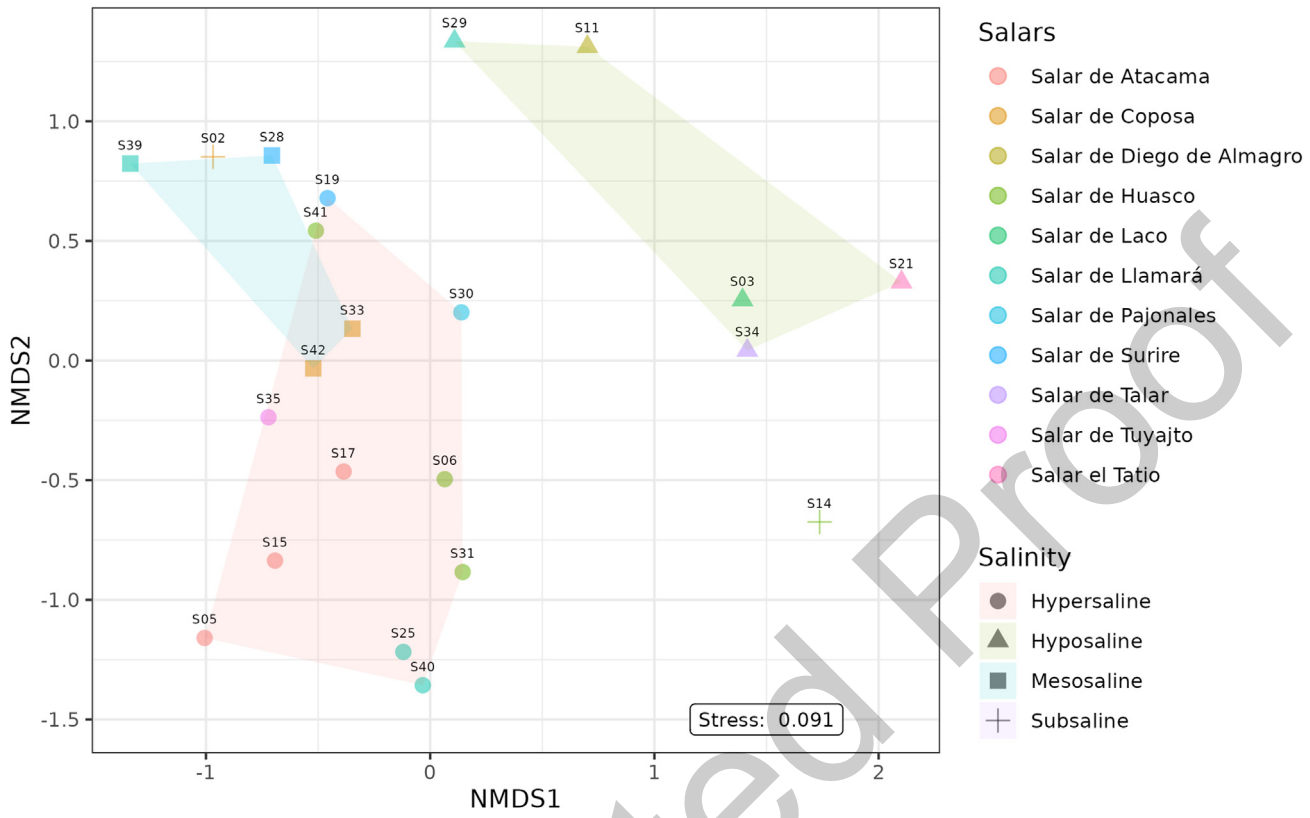

Fig. 5. Non-metric multidimensional scaling (NMDS) plot based on the Jaccard Index and the measured ion concentration. Different symbols indicate the classification regarding the salinity. Ellipses represent groupings of sampling sites regarding the salinity classification with a 95 % c. l. for a multivariate t-distribution.

Chemical composition

To relate the metabarcoding data to the habitat, we investigated the concentration of the main ions contributing to the total salt concentration. The relative contribution of the different ions varied between the different salars, but also between the different sampling sites within one salar (Table 1). In general, the dominant ions were sodium (mean = 31 %) and chloride (mean = 44 %). The highest proportion of sodium occurred in a sample from Salar de Surire (S19, 51 %), highest chlorine concentrations were recorded at a site in the Salar de Huasco (S31, 71 %). Sulphate contributed an average of 7 % to dissolved ions, the highest value occurred in Salar de Surire (S19, 24 %). Besides, calcium and magnesium (each 3 % on av.) and potassium (2 % on av.) contributed significantly to the total salt content. The highest contributions of calcium occurred in Salar de Huasco (S14, 14 %) and the highest values of magnesium occurred in samples from Laguna Tuyajto and the Salar de Coposa (S35 and S33, 6 %). The highest relative potassium value occurred at the Salar de Huasco (S06, 5 %). In addition, all samples contained significant amounts of boron (up to 157.7 mmol/l). Lithium was found in 15 of the 22 samples, and in about one third of the sample set it occurred in significant concentration (10–100 mmol/l). In samples from the Salar de Atacama, the contribution of lithium to total salt content was relatively high (S17, S05, 2 %). Silicon (Si) and strontium (Sr) concentrations were always < 2 mmol/l (Table 1). Some samples showed low concentrations of arsenic (<0.6 mmol/l). Concentrations of barium and phosphor were close to or below the detection limit in all samples. A cluster analysis revealed that a few sites were clearly special regarding their relative ion concentration (Fig. 6 right), including the geyser El Tatio (site 21).

Genotype distribution of placidid flagellates

Since placidid diversity based on cultivated organisms isolated for the Atacama region was studied in detail in a previous study (Rybarski et al., 2021), we used this group as a model group to check for the presence of OTUs belonging to the class of Placididea in the metabarcoding data sets of all samples (Fig. 6). Only OTUs with an identity of 100 % to a known placidid species were included. The species *Allegra hypersalina* and *Allegra dunaii* were detected mainly in hypersaline waters. *Wobblia pacifica* was only found in hyposaline waters of Salar Diego de Almagro and mesosaline waters of the Salar de Llamará. *Haloplacidia cosmopolita* was detected only at two hypersaline

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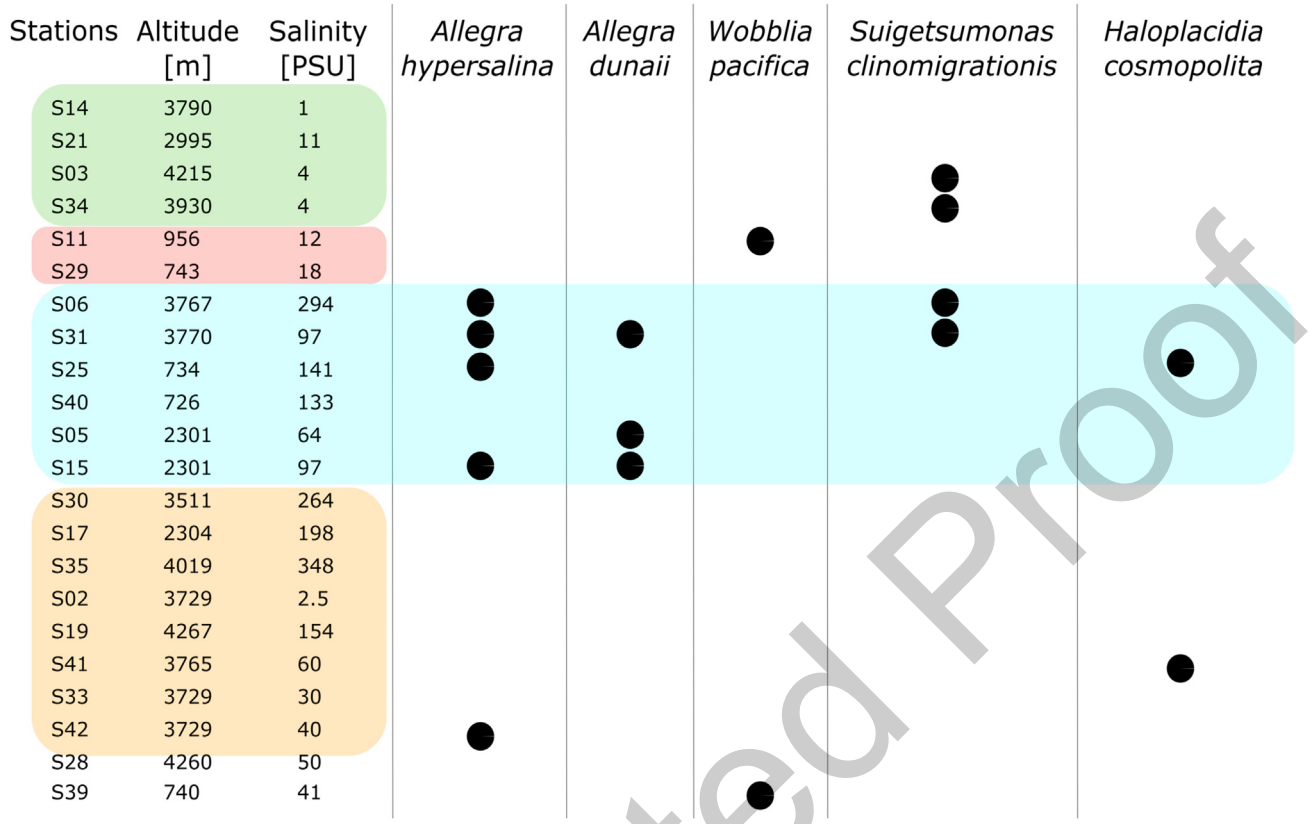

Fig. 6. Genotype identification of Placididea within the different samples. The mock community filtered data was used for this identification. Black dots indicate the presence of this genotype in a sample with 100 % identity. Coloured clusters indicate the clusters that were also obtained by the Jaccard clustering.

locations at the Salar de Huasco and the Salar de Llamará. The species *Suigetsumonas clinomigrationis* occurred in subsaline as well as in hypersaline waters.

Discussion

We investigated 22 different samples from 11 inland waters bodies in the Atacama Desert to get an idea regarding the overlap or distinction of the unicellular eukaryote communities. The V9 region of the 18S rRNA has been identified as a reliable tool to detect the diversity of the microbial community taking the read number into account (Choi and Park, 2020). The bioinformatic pipeline within our metabarcoding studies consisted of two alternative approaches, one strict filtering using the adequate representation of the mock community data as an indicator and one less strict filtering step, as used in previous studies, considering those OTUs occurring at least with three reads. Stimulated by the studies of Fiore-Donno et al. (2018) who advocated the inclusion of mock communities in metabarcoding runs for an objective evaluation of "noisy" OTUs, we also investigated the sequencing result of mock communities. As a consequence of our results (Table S4), we became also advocates for an analysis of the "noisy" OTUs. Like Fiore-Donno et al. (2018), we suggest that always mock communities should be used as a backbone for interpretation of metabarcoding results. Both, the classical filtering and the mock filter have advantages and disadvantages. While the strict mock filtering filters out the rare community, but gives an idea regarding the certainly occurring diversity, the classical filtering considers the rare taxa, but might overemphasize the diversity to a large extent. We propose the use of both filter methods to get more reliable estimates. Nevertheless, as comparisons based on results obtained from both filters (Fig. 3, Fig. 4, Fig. S4) showed comparable results based on relative (percentage) numbers. Previous studies already showed that Stramenopiles (Ochrophyta and other Stramenopiles) and also Alveolata (Ciliophora) are common in hypersaline environments (Park and Simpson, 2010; Qu et al., 2020; Rybarski et al., 2021; Schoenle et al., 2022). Representatives of these two groups also dominated most of the samples from our metabarcoding approach. As hypothesized at the beginning of the study, the high diversity of microhabitats in each salar might allow the coexistence of many genotypes and hence, differences in species composition of protists between the different salars were expected to be low. However, the results of the study revealed the opposite. Not only that

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the different salars showed a unique protist community, but also different sites with a different salinity in one salar were unique to the specific salar. Only a small part of OTUs overlapped between hyposaline, mesosaline and hypersaline sampling sites despite similar salinity ranges. The little overlap of protist communities of the different salars might indicate low exchange rates between the salars. The unhostile environment between the salars (high evaporation, high UV radiation etc.) would support the suggestion of allopatric speciation. Indications for this phenomenon were documented for different groups of protists in salars of the Atacama including placidids, choanoflagellates, percolozoans (Arndt et al., 2020; Carduck et al., 2021; Hohlfeld et al., 2022; Rybarski et al., 2021; Schiwitza et al., 2021).

Besides distance between salars, the chemical composition of the waters could cause differences in the community structure. While sodium and chloride generally dominated in salar waters, the relative contribution of the other ions varied between the different sites (Table 1). In the NMDS plot (Fig. 5) it is obvious that protist communities from nearby sites with a similar chemical composition appeared to be more related to each other indicating that water chemistry might play an important role for protists. However, a clear pattern regarding the protist community structure and the relative ion concentration could not be detected (cf. Fig. 2). Regarding their absolute concentrations, it seems that certain ions like sodium, chloride, magnesium and lithium might have a greater impact on the community than calcium or phosphate. For a better interpretation of the data, detailed laboratory experiments would be necessary in future, not only regarding ion concentrations but also regarding other abiotic factors such as temperature and UV radiation. Very high solar UV irradiance, high evaporation rates and temperature gradients are additional factors limiting even microbial life in the Atacama Desert (Wierzchos et al., 2012; Mörchen et al., 2019; Arndt et al., 2020).

Nevertheless, the results of the present study show that protist communities in the Atacama are able to tolerate a wide range of chemical compositions and salinities that can differ tremendously even within one salar due to different stages of evaporation or groundwater inflow (Table 1). Laboratory studies on protists isolated from different salars of the Atacama have shown a wide range of salinity tolerance. This was shown for many different species isolated from the different salars of the Atacama region and include placidids (Rybarski et al., 2021), bicosoecids (Schoenle et al. 2022), choanoflagellates (Schiwitza et al., 2018, 2019, 2021) and percolomonads (Carduck et al., 2021).

One group of protists (placidids) typically present in the salars was studied more intensively and served here as a representative group to shed further light on the processes behind the differences in the community structure of protists and regarding the high portion of unique genotypes found in the different salars. The pattern observed (Fig. 6), based on the V9 region of the 18S rRNA of different placidid species, which were isolated, cultivated and sequenced from salars in a preceding study (Rybarski et al., 2021), indicated that the ranges of distribution of most genotypes (OTU) did not overlap. Though the V9 region is not always specific on the species level, it was found to be specific for the placidid species found in the Atacama (Rybarski et al., 2021). This was also found for Cafeteriaceae (Schoenle et al., 2022). At least for placidids, we could identify a high degree of species separation and isolation which points to speciation processes due to limited dispersal of genotypes between the different salars. Some salars in the Atacama are potentially connected via groundwater inflow (e.g., Risacher et al., 2003; Surma et al., 2018; Voigt et al., 2021), however, the salars in the Atacama are often in a distance of hundreds of kilometres and are situated in different catchment areas of the desert characterized by an extremely high UV radiation and temperatures (Ritter et al., 2018a,b). The distribution via the air seems to play a minor role for the distribution (Arndt et al., 2020). Similar observations were made for choanoflagellates and other protists in this region (Arndt et al., 2020; Schiwitza et al., 2021).

We analysed the effect of the two different filtering techniques applied in this study which is exemplarily shown in Fig. S7 for the placidids. Our strict filtering based on the results of the mock community underestimates the rare organisms, but it was obvious that all clades were also represented by the strict filter (bluely labelled OTUs in Fig. S7). Regarding the placidid diversity, we found of course more sequences before the bioinformatic step of strict filtering. Sequence variability is known to occur in ciliates with their macronucleus (Zou et al., 2021), but has also been reported for flagellates (Venter et al., 2018). Thus, it is possible that the salars show an even higher diversity of possibly new representatives of protist taxa.

To avoid an overestimation of the degree of uniqueness of the protist communities, we always compared the diversity obtained by strict filtering with that obtained by a much less strict three-read filtering in the metabarcoding output. However, as obvious from the comparisons shown in Fig. 3, Fig. 4 and Fig. S4, the high degree of uniqueness is not affected by the way of filtering. The different separated salars seem to be suitable habitats to identify processes of radiation of different protist lineages. In different analyses, it could be demonstrated that the diversification may have taken place starting from the oldest salar (Salar de Atacama; Schiwitza et al. 2021; Arndt et al., 2020).

Author contributions

Sampling in the Atacama Region was carried out by A.E. R., F.N., and H.A.; DNA isolation was carried out by A.E. R.; A.E.R. and A.S. performed bioinformatical analyses of the data. A.E.R., C.V. and M.S. performed investigations of chemical parameters. The project was launched and

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supervised by H.A.; A.E.R. and H.A. wrote the manuscript. All authors reviewed and revised the manuscript.

Data availability

Data will be made available on request.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2023.125987.

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