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Taxonomic Structure of Planktonic Protist Communities in Saline and Hypersaline Continental Waters Revealed by Metabarcoding

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Abstract: Saline and hypersaline waters are one of the most peculiar ecosystems of our planet, characterized by extreme life conditions. Despite their worldwide distribution, the diversity and abundance of protist communities in these ecosystems remain poorly studied. Here, we analyze planktonic communities of protists sampled across 38 saline and hypersaline water environments (2–390‰) from arid climatic zones of the South Urals and Crimea in light of environmental data using high-throughput 18S rDNA amplicon sequencing. A total of 9 eukaryotic supergroups, 34 phyla, 104 classes, 184 orders, 315 families and 548 genera have been identified. We revealed significant differences in the taxonomic structure of protist communities depending on salinity, geographic location and pH. The protist communities demonstrated linear regression of richness and diversity and growth of the percentage of unclassified Eukaryota (up to 43%) with the increase in salinity. Centrohelids demonstrated the ability to inhabit a broad range of salinities, up to 320‰, which is four times higher than previously reported. Centrohelid species *Pinjata ruminata* and *Yogsothoth* sp. are assumed to be specifically adapted to salinity of 3–210‰. The obtained results provide insight into the taxonomy and diversity of protists in saline and hypersaline environments and highlight the great potential for the discovery of new taxa due to the large number of unclassified 18S rDNA sequences.

Keywords: 18S rDNA; protist; plankton communities; centrohelids; saline lake; hypersaline lake; extreme environments; NGS

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1. Introduction

Extreme environments are widely distributed around the world and represented by terrestrial hot springs, deep sea hydrothermal vents, glaciers and permafrost, acid mine drainage and hypersaline habitats [1,2]. Despite extreme chemical and physical conditions, they are inhabited by a wide range of organisms from the three domains of life [3–7].

The hypersaline lakes are one of the most peculiar ecosystems with extreme life conditions due to high salinity, low oxygen concentration and unstable hydrology [8–11]. Hypersaline waters (from 40‰ to more than 300‰) have never been clearly classified [12]. In this study, by "hypersaline" we mean waterbodies with a salinity exceeding marine (>40‰) according to the Venice System [13] and classify them according to Por [12]. Hypersaline lakes are geographically widespread and usually originate as inland drainage basins and occur in arid and semi-arid climatic zones [10,14–16]. Most hypersaline lakes are located in South America, Africa, Spain, Australia, the south-western parts of the United States, China and Russia [17,18]. Hypersaline lakes still remain largely understudied despite their wide variety, large surface area, and intense microbial activity. Their detailed

studies have been carried out in different areas of the world including the USA [19,20], Mongolia [21], Iran [22], Australia [23], Portugal [24], the Andean altiplano [25] and Spain [18], among others. Early biodiversity and ecological studies of these environments were mainly focused on the prokaryotic microbial communities [26–33], while much less attention has been paid to microbial eukaryotes [4]. It was found that high salinity causes high osmotic stress in organisms inhabiting hypersaline waters and is one of the main environmental factors influencing the composition of the microbial community [34,35].

High-throughput sequencing (HTS) technology has great potential for biodiversity studies and analysis of the influence of ecological and environmental factors [4,18,36,37]. Environmental DNA metabarcoding overcomes the limitations of traditional surveys [8,38,39] and has proven to be valuable for the study of many ecosystems [40], especially extreme environments [5]. Over the past 20 years, our knowledge of protist communities and taxa inhabiting hypersaline environments has significantly improved through the application of HTS [6,41,42]. Hypersaline environments have been shown to contain unexpectedly large genetic diversity and novelty of protists [6,8,43–45]. Diversity, distribution along salinity gradients and large-scale biogeography of protist communities from hypersaline waters have also been studied using HTS applications [18,44,46].

Crimea, the largest peninsula of the Black Sea, contains more than 50 saline and hypersaline lakes of continental and marine origin [11]. The extreme conditions of Crimean lakes are caused not only by high salinity, but also by unstable hydrology, high air and water temperatures (up to 35–40 °C in summer), and low oxygen concentration [11]. The investigated group of the South Ural hypersaline environments contains seven Sol-Iletsk lakes and a waterlogged area with a salinity gradient along the Tuzlukkol' River. The Sol-Iletsk lakes have a karst-anthropogenic origin [47]. They belong to the national heritage of Russia and are recreation sources of Sol-Iletsk resort and are used for balneological and saline treatment. The lowland Tuzlukkol' River is a small left-bank tributary of the Ural River. We investigated the waterlogged solonchak area within the natural boundary of the Tuzlukkol' River. The solonchak has a geological origin and is associated with the occurrence of salts of the Kungurian stage of the Permian. There are numerous underground hypersaline springs of chloride–sulfate type, which merge with the main water flow and cause a salinity gradient within the natural boundary of the Tuzlukkol' River. The waterlogged solonchak contains small lakes with therapeutic mud that are used for balneological and saline treatment. All studied saline and hypersaline water environments of the South Urals and Crimea have unstable hydrology and belong to a group of high mineralized water bodies of sodium–chloride type.

Here, we present the first study of the planktonic protist's diversity from saline and hypersaline water environments (2–390‰) from the South Urals and Crimea in the light of 18S rDNA metabarcoding data. In addition, we discuss changes in the protist community composition from different locations and its dependence on salinity and pH.

2. Materials and Methods

2.1. Sampling Sites and Collection

Fifty-seven planktonic water samples were collected from saline and hypersaline continental water bodies (predominantly lakes) with a salinity of 2–390‰ from July to August 2021. Samples were obtained from geographically distinct water environments of arid climatic zones of the South Ural (Sol-Iletsk lakes, the Tuzlukkol' River) and Crimea. Most of the studied habitats belong to the recreation zones or are located in specially protected areas but are publicly accessible (see Figure 1 and Table S1 for more detailed information about the sampling sites).

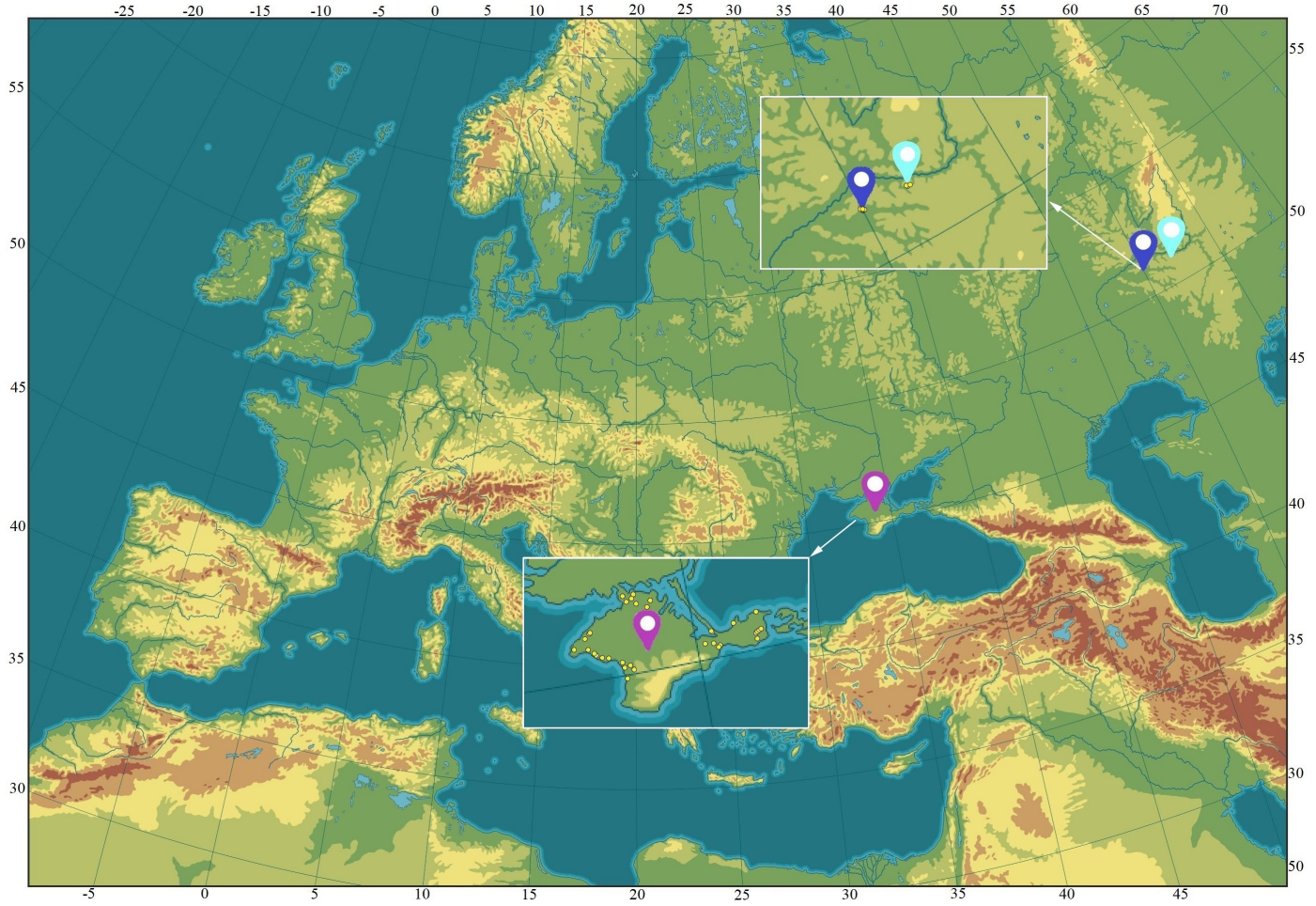

Figure 1. Map of sampling sites of the South Urals and the Crimea.

Planktonic water samples were taken in sterile containers at 1–30 cm depth. Samples were stored in a portable refrigerator at +4 °C. For DNA analysis, water samples were consequentially filtered through 5.0 µm and 0.45 µm pore-size nitrocellulose membranes using sterile reusable 25 mm syringe filter holders (Sartorius, Göttingen, Germany) within a few hours after collection. After filtration, each membrane filter was placed in a 1.5 mL Eppendorf tube and stored in 200 µL of 2× DNA/RNA Shield (Zymo Research, Tustin, CA, USA) at +4 °C (throughout the expedition). We studied a total of 114 environmental water samples collected from the Tuzlukkol' River (14 sampling points, 28 samples), Sol-Iletsk (7 lakes, 14 samples) and Crimea (36 sampling points, 72 samples).

Salinity was measured in situ using the Master-S28a salinity refractometer (Atago, Tokyo, Japan). For salinity measurement exceeding the maximum range of 280‰, the sample was diluted v/v by distilled water. Samples were classified into six salinity categories: mixohaline (0.5–30‰), euhaline (30–40‰), alpha-hypersaline (40–100‰), beta-hypersaline (100–140‰), gamma-hypersaline (140–200‰) and delta-hypersaline (above 200‰).

2.2. eDNA Extraction, Amplification and 18S rDNA Library Preparation

The samples were stored in the laboratory at −80 °C until DNA extraction. For DNA extraction, each membrane was transferred into a Lysing Matrix E tube (MP Biomedicals, LLC, Solon, OH, USA) with the addition of 2× volume of tris-saline buffer (1M Tris-HCl; 0.5M EDTA; 5 M NaCl; MQ) and homogenized for 5 min at 50 Hz (TissueLyser LT, Qiagen, Hilden, Germany). Then, membranes were enzymatically digested (lysozyme, proteinase K, SDS in total conc. 1%) and DNA was phenol-chloroform extracted (phenol-chloroform 1:1 v/v; chloroform-isoamyl alcohol 24:1 v/v) as reported previously [45]. Purification

and desalination of DNA were carried out using a DNA Clean and Concentrator-5 kit (Zymo Research, Tustin, CA, USA). The final quality of the DNA was checked using electrophoresis in 1% agarose gel. The concentration of DNA was quantified with a Qubit 4 fluorometer (Invitrogen, Carlsbad, CA, USA) using a Qubit 1× dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). The nuclease-free water (Qiagen, Hilden, Germany) was taken as a negative control of water sample filtration, and ZymoBIOMICS Spike-in Control I (Zymo Research, Tustin, CA, USA) was used as a positive control of high-throughput sequencing. PCR amplification of the 18S rRNA gene (primers set according to [45]) and further sequencing were performed using the Illumina MiSeq platform and MiSeq Reagent Kit V3 2 × 300 bp (Illumina, San Diego, CA, USA) according to the Illumina workflow (Illumina protocol, Part #15044223, Rev. B).

2.3. eDNA Analysis

The quality of sequenced reads was checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 1 December 2022). Cutadapt v. 4.1 [48] was used for the removal of primer sequences from the reads. The DADA2 workflow v. 1.26 [49] was used for further sequence analysis, including quality filtering (maxEE = 2), reads merging, chimera removal, and amplicon sequence variants (ASVs) generation. The obtained ASVs were taxonomically classified using a pre-trained naive Bayes classifier, which was trained on the PR2 database version 4.14.0 [50]. The macrotaxonomy in the PR2 database does not always correspond to the latest taxonomic revisions of the eukaryotic tree [51,52], in particular for Excavata and Hacrobia, but we used it for consistency of our data with the results of previous metabarcoding analyses. The identity of the ASVs assigned to the centrohelids (Centroplasthelida) was additionally checked using the BLAST algorithm identity against the NCBI nucleotide database.

The raw reads dataset was deposited in the National Center for Biotechnology Information (NCBI) and is available under project accession number PRJNA971617.

2.4. Phylogenetic Analysis of Centrohelids' ASVs

Sequences of centrohelid 18S rDNA from NCBI and ASVs were aligned using the L-INS-i algorithm in MAFFT version 7.475 [53] and trimmed using the '-gappyout' method in TrimAl version 1.2 [54]. Maximum likelihood phylogeny for 311 taxa (255 centrohelids, 45 ASVs and 11 noncentrohelid protist taxa as the outgroup) was inferred using IQ-TREE v1.6.12 [55] with 816 unambiguously aligned positions, 1000 nonparametric bootstraps under the best fit model (TN + F + R5) determined by the in-built ModelFinder.

2.5. Statistical Analysis

All statistical analyses and visualization were performed in the R environment using phyloseq [56], ggplot2 [57], MicrobiotaProcess [58] and microbiome [59] packages. Indexes of alpha diversity were calculated using the alpha function from the microbiome R package. The Bray–Curtis dissimilarity was used for beta diversity comparison in the *get_pcoa* function. Pearson's rank correlation coefficient was used to analyze a possible dependence between alpha diversity indexes and the mineralization or pH of water samples. The statistical differences in community composition by mineralization, pH and geographical location were evaluated with PERMANOVA using the *adonis2* function with 999 permutations.

Rarefaction curves were constructed based on the observed ASVs after filtering of non-eukaryotic and metazoan sequences. The ratio of the total number of detected ASVs to the species richness index (Chao1 index) was used to quantify the coverage of protist communities.

3. Results

3.1. Sequence Data Overview

A total of 5,249,054 paired-end reads of the 18S rRNA gene were obtained, of which 5,163,087 (98.4%) remained after quality filtering. The average depth of pair-end reads was about 46,000 per sample. After the removal of non-specific amplification sequences, filtering by quality, dereplication, removal of chimeric sequences and reads merging at the output of the DADA2 pipeline, an average of 46.1% of the reads were retrieved (Table S2). Non-eukaryotic and metazoan ASVs were removed from further analysis. Among the remaining 7604 ASVs, 3237 (42.6%) were assigned to supergroups, 2783 ASVs (36.6%) to phyla, 2533 ASVs to classes (33.3%), 2338 ASVs to orders (30.7%), 2161 ASVs to families (28.4%) and 1768 ASVs to genera (23.3%).

The highest number of the ASVs were observed in the Tuzlukkol', Kyzyl-Yar and Opuk samples, the smallest ones in delta-hypersaline lakes Krasnoe (Zelenoe), Razval and Novoe. An average of 163 ± 92.7 ASVs per sample were found in the 38 saline and hypersaline water environments. We detected a total of 4367 ASVs (16% of total reads) belonging to eukaryotic supergroups and lineages of uncertain affiliation. Unclassified ASVs averaged 57.4% at the supergroups level, 63.4% at the phyla level, 66.7% at the classes level, 69.3% at the orders level, 71.6% at the families level and 76.6% at the genera levels.

The rarefaction curves reached a plateau after a point of 5000 sequences in the majority of sequencing libraries (Figure 2). The sequencing coverage value for the samples varied from 75.8% in Dunino Lake to 100% in Krugloe Lake (an average of 96.38 ± 0.004% for all samples), indicating that the protist communities in all studied water habitats were analyzed almost completely (Table S3).

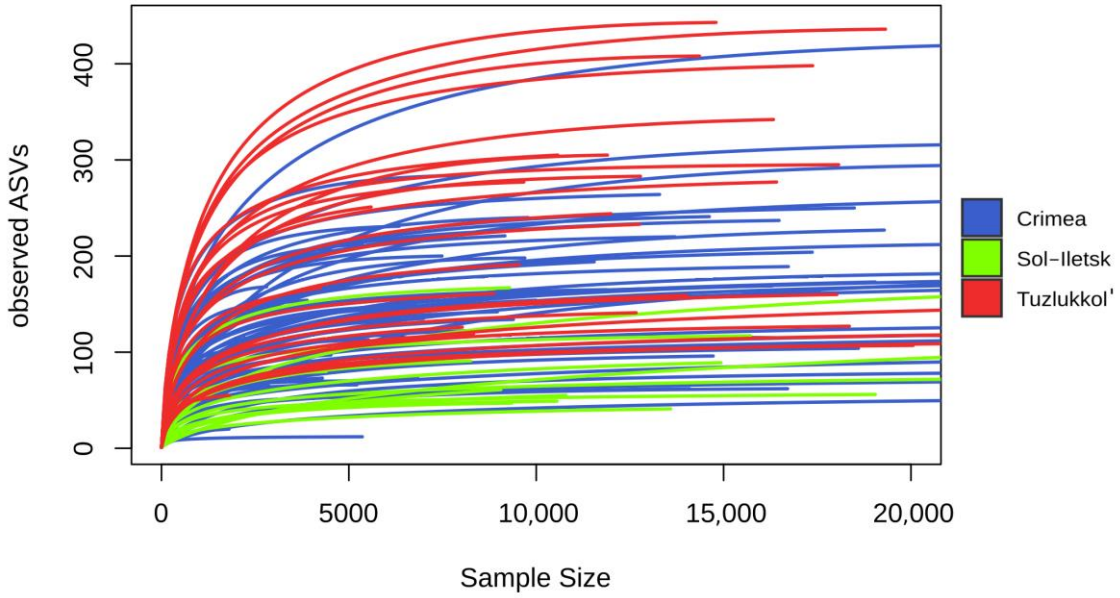

Figure 2. Rarefaction curves of sequenced libraries plotted between the total number of reads (*x*-axis) and the number of amplicon sequence variants (*y*-axis). The color-coded curves correspond to the sampling sites.

3.2. Taxonomic Composition and Distribution of Protists Communities

A total of 9 eukaryotic supergroups, 34 phyla, 104 classes, 184 orders, 315 families and 548 genera have been identified (Table S4). The communities of protists were represented by Alveolata, Amoebozoa, Apusozoa, Archaeplastida, Excavata, Hacrobia, Opisthokonta, Rhizaria and Stramenopiles supergroups (Figure 3). Archaeplastida (30.7%), Alveolata (24.9%) and Stramenopiles (16.6%) were dominant in studied environments (Table S5), whereas the amount of Amoebozoa, Apusozoa and Rhizaria were scarce (<1%). The

relative abundance of unclassified Eukaryota at the supergroups level accounted for 16% of total reads (Table S5). Chlorophyta, Ciliophora, Ochrophyta, Dinoflagellata, Fungi and Discoba were the most abundant phyla of protists in saline and hypersaline environments (Tables S6 and S7). According to sequencing data, Chlorophyta, Ciliophora, Ochrophyta, Dinoflagellata, Fungi, Discoba, Opalozoa and Cercozoa predominate in all samples of the studied ecosystems. The taxonomic composition of the investigated water bodies is shown in Figure 3 and Table S4.

Archaeplastida was clearly the dominant group in the samples from Sol-Iletsk and Crimea, accounting for 36.8% and 22.5% in terms of relative abundance, respectively (Figure 4).

Chlorophyta dominated among Archaeplastida and were especially abundant in the Sol-Iletsk (36.8%) and Crimea (21.7%) samples (Figure 5).

Overall, the top three dominant genera among the Archaeplastida were *Chlamydomonas*, *Dunaliella* and *Sphaeroplea* (Figure 6). *Dunaliella* appeared as the dominating genus of green alga in the Sol-Iletsk and Crimea samples, accounting for 18.2% and 18.1% of the reads, respectively.

Alveolata was the most prevalent group in the samples from Tuzlukkol' and the second dominating supergroup in the Crimea samples (Figure 4). The most common phylum among alveolates was Ciliophora, namely the genera *Platyophryida*, *Fabrea*, *Strombidium*, *Frontonia*, *Ancistrum* and *Euplotes*. The representatives of Ciliophora were one of the dominants in the Tuzlukkol' samples (Figure 5). At the genus level, *Strombidium*, *Fabrea*, *Ancistrum* and *Frontonia* were most prevalent in Tuzlukkol' habitats, followed by unclassified *Choreotrichida* and *Scuticociliatia* (Figure 6). Alveolate phylum Dinoflagellata (Figure 5) was the most represented in Crimea samples and mainly includes genera *Prorocentrum*, *Gyrodinium*, *Diplopsalis* and *Ansanella* (Figure 6).

Stramenopiles was the second dominating supergroup in the Tuzlukkol' samples and third dominating supergroup in the Crimean samples, accounting for 24.9% and 16.1%, respectively (Figure 4). Among them, Diatomea genera *Cyclotella*, *Cylindrotheca*, *Navicula* and *Chaetoceros* were prevalent (Figure 6). In particular, the stramenopiles genus *Halocafeteria* (Opalozoa) was specific for the Crimea samples (Figure 6).

Amoebozoa and Apusozoa were detected at a relative abundance of <1% in the protist communities of saline and hypersaline water bodies of the investigated areas (Figures 3 and 4). Notably, Amoebozoa were absent in the Sol-Iletsk samples (Figure 4).

Hacrobia were mainly found in samples from Crimea and Tuzlukkol', accounting for 2.3% and 1.9% of the reads, respectively, and were one of the less represented groups in the Sol-Iletsk lakes (Figure 4). The most represented phyla of Hacrobia in all samples were Cryptophyta, Haptophyta and Centroheliozoa. The representatives of the Cryptophyta were mainly affiliated with genera *Cryptomonas*, *Guillardia*, *Hemiselmis*, *Rhodomonas* and *Teleaulax* (Tables S6 and S7).

The remaining ASVs belonged to Excavata and Opisthokonta supergroups (Figures 3 and 4). Notably, excavates were mainly represented in the Sol-Iletsk samples, accounting for 10.6% of the reads, whereas opisthokonts were more typical for samples from Crimea (6.4%) and Tuzlukkol' (5.6%) (Figure 4). Excavate phylum Discoba was the third most prevalent group in the Sol-Iletsk samples, accounting for 36.5% of the reads. At the genus level, Discoba was mainly affiliated with *Euplaesiobystra* (Heterolobosea), accounting for 10.5% of the reads in the samples from Sol-Iletsk (Figure 6). Overall, a high percentage of the total reads belonging to Opisthokonta was assigned to Fungi (Figure 5). Choanoflagellida was present in a low percentage, almost entirely belonging to the order Craspedida.

Unclassified eukaryotes were the most represented in Sol-Iletsk and Crimea samples, accounting for 39.5% and 28.4% of the total number of sequenced reads, respectively (Figure 4). At the same time, 17.5% of reads in the samples from Tuzlukkol' could not be precisely assigned to any known eukaryotic taxonomic group (Figure 4).

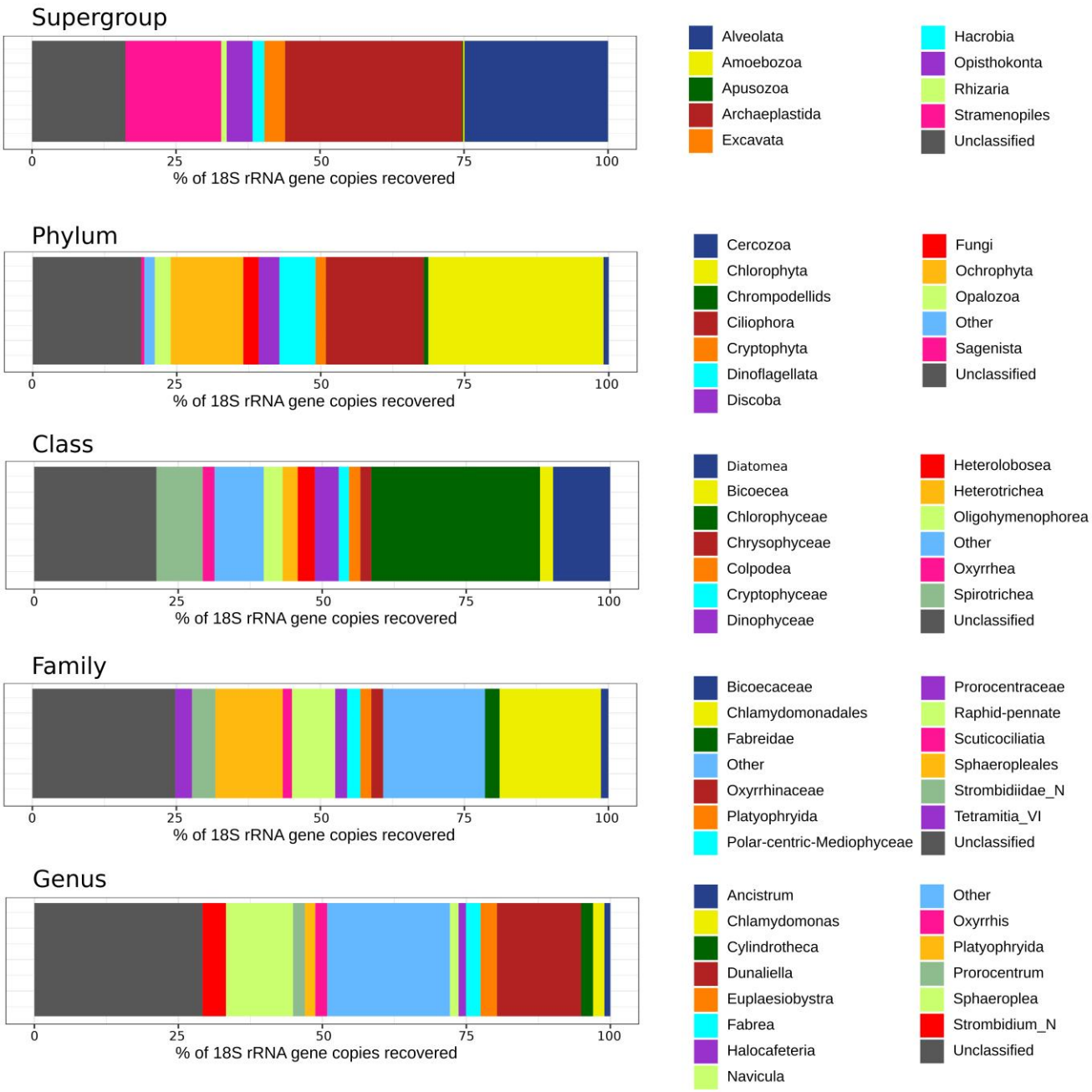

Figure 3. Protist community composition at different taxonomic levels. Only relative abundances greater than 1% are shown. Colors correspond to the taxa from each taxonomic level.

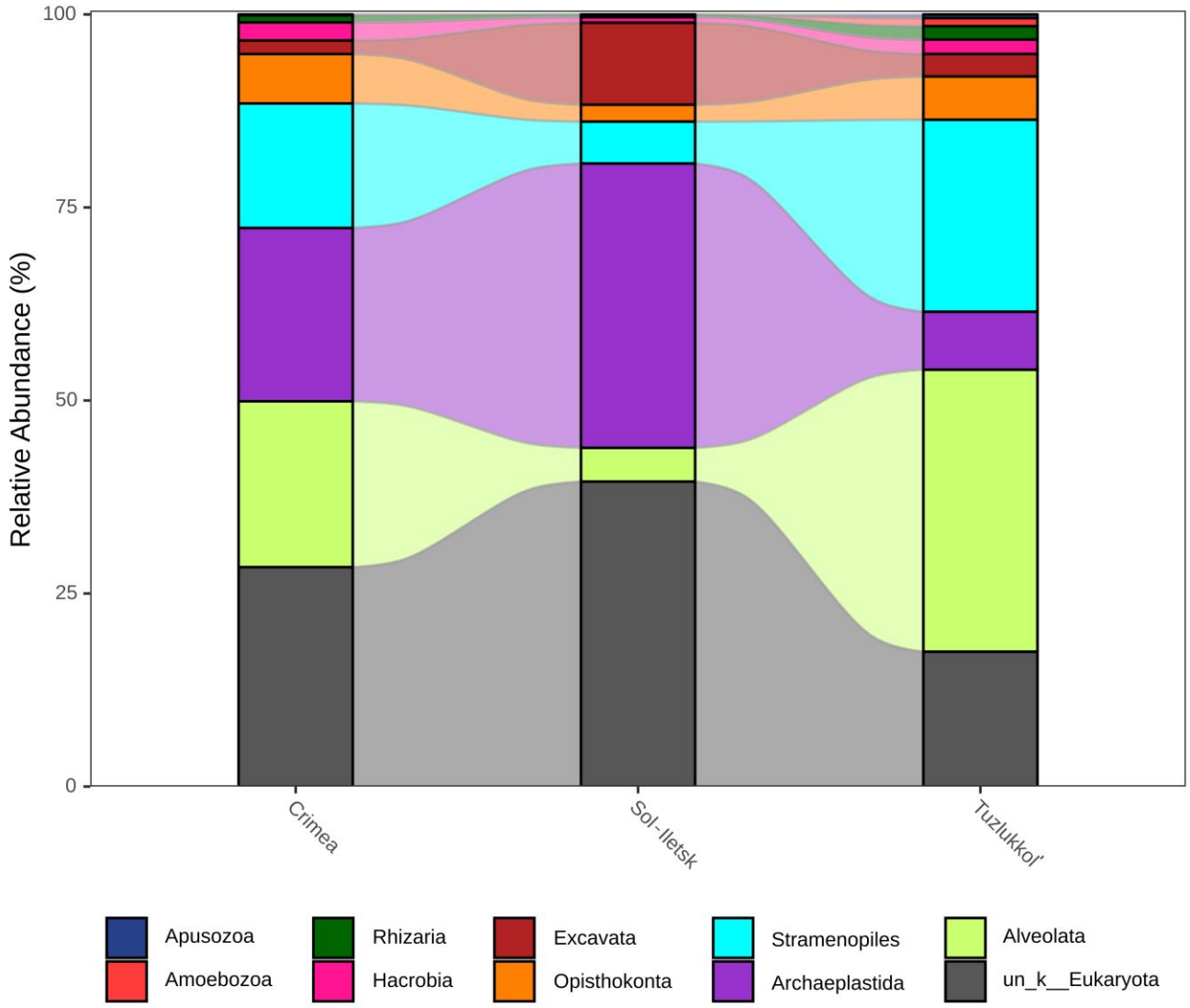

Figure 4. The relative abundance of the protist supergroups of the investigated areas. Colors correspond to the supergroup names.

Considering the taxonomic structure of protist communities in three geographically distinct water environments (Table S6), it is noteworthy that the most represented supergroups in the Crimea samples were Archaeplastida, Alveolata, Stramenopiles and Opisthokonta (Figure 4). The most prevalent genera in the Crimean lakes were *Euplotes*, *Fabrea*, *Platyophryida*, *Strombidium* (Ciliophora, Alveolata), *Oxyrrhis*, *Prorocentrum* (Dinoflagellata, Alveolata), *Dunaliella* (Chlorophyta, Archaeplastida), *Navicula*, *Chaetoceros* (Ochrophyta, Stramenopiles) and *Exobasidiomycetes* (Fungi, Opisthokonta) (Figure 6). The protist communities in the Sol-Iletsk lakes are mostly represented by Archaeplastida, Excavata, Stramenopiles and Alveolata supergroups (Figure 4). Genera *Dunaliella*, *Sphaeroplea*, *Chlamydomonas* and *Carteria* (Chlorophyta, Archaeplastida), *Euplaesiobystra* (Discoba, Excavata), *Cyclotella* (Ochrophyta, Stramenopiles) and *Fabrea* (Ciliophora, Alveolata) were especially abundant in the Sol-Iletsk lakes (Figure 6). Protist communities in the Tuzlukkol' samples were mainly represented by Alveolata, Stramenopiles, Archaeplastida and Opisthokonta (Figure 4). The most abundant genera were *Strombidium*, *Fabrea*, *Ancistrum*, *Frontonia*, *Parallelostrombidium* (Ciliophora, Alveolata), *Chlamydomonas* (Chlorophyta, Archaeplastida) and *Cylindrotheca*, *Navicula*, *Entomoneis* (Ochrophyta, Stramenopiles) (Figure 6).

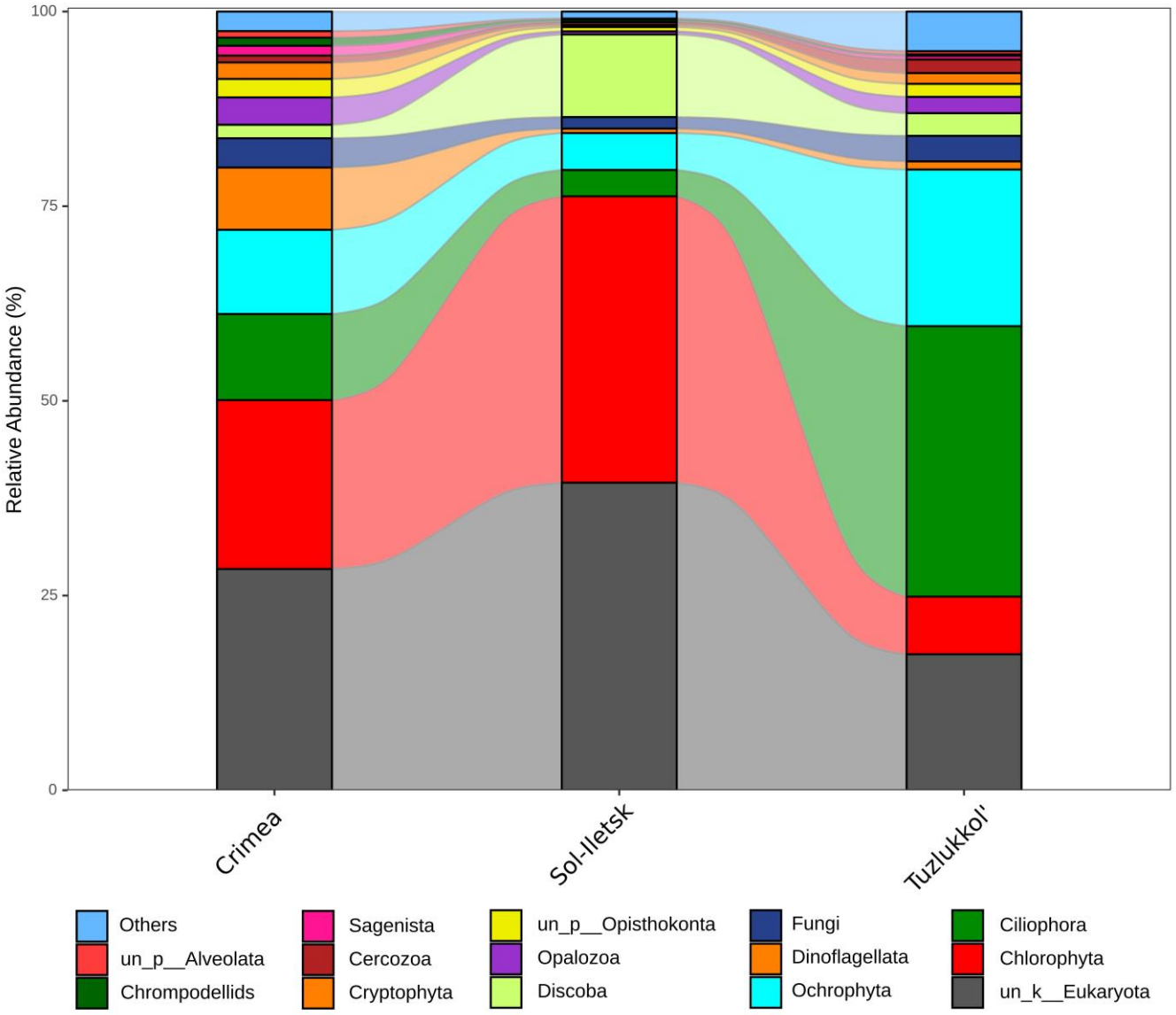

Figure 5. The relative abundance of the 12 most dominant protists phyla of the investigated areas. The category 'Others' represents the sum of all other taxonomic groups. Colors correspond to the phyla names.

3.3. Protist Diversity and Salinity

We analyzed the diversity of the protist community across a broad salinity range (2–390‰). In mixohaline waters, Alveolata (33.6%) and Stramenopiles (28.6%) were the most abundant supergroups, while Amoebozoa and Apusozoa were scarce (<1%) (Figure 7, Table S7).

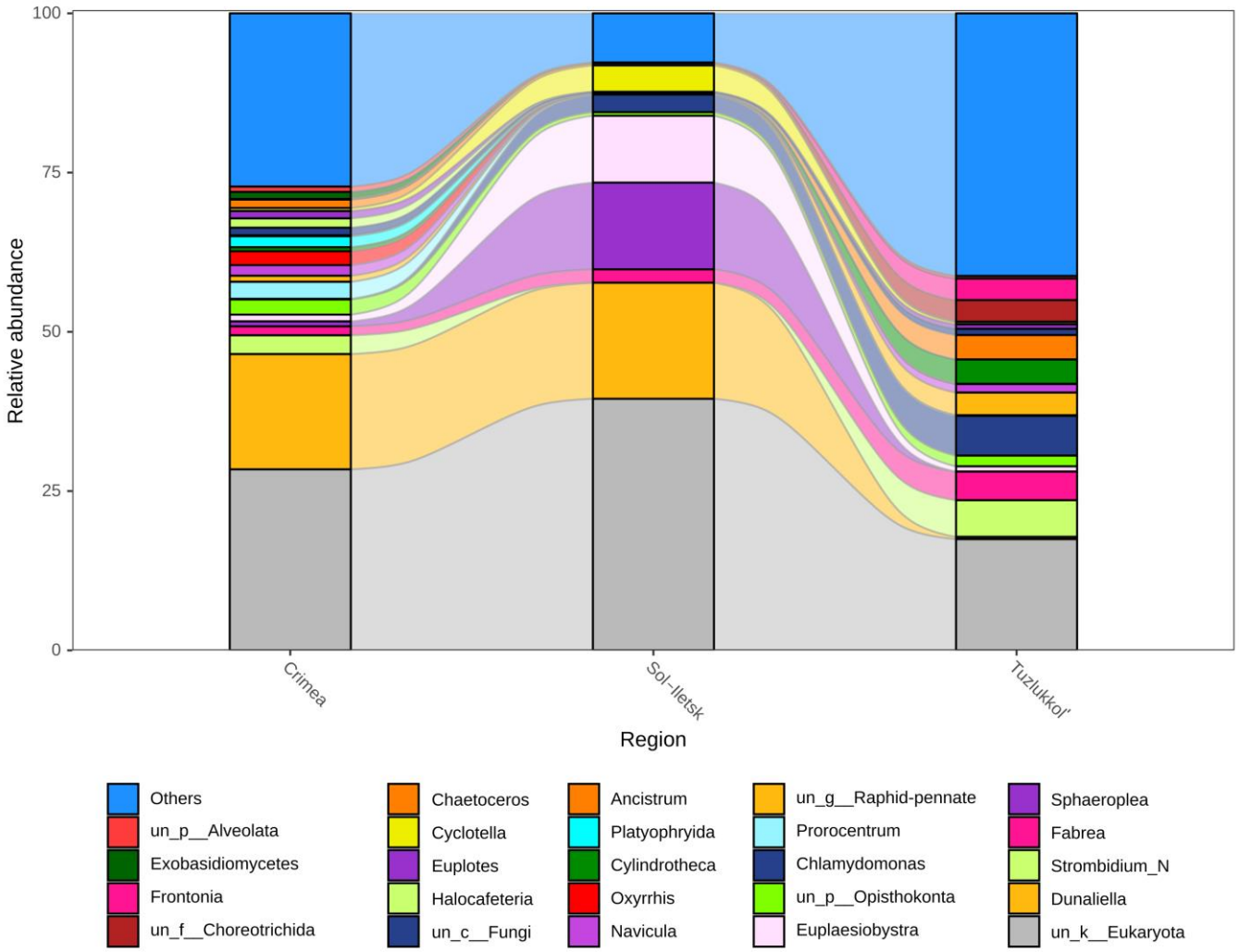

Figure 6. The relative abundance of the 22 most dominant genera of protists of the investigated areas. The category 'Others' represents the sum of all other taxonomic groups. Colors correspond to the genera names.

The abundance of all other supergroups ranged from 1.6% to 5% of the total reads. The phyla Ochrophyta (24.1%) and Ciliophora (23.2%) were clearly dominant in mixohaline waters (Figure 8).

In particular, the most prevalent classes among Ochrophyta were Chrysophyceae and Diatomeae, and Spirotrichea and Oligohymenophorea were prevalent among Ciliophora. A total amount of unclassified eukaryotes in the mixohaline waters was 18.3% at the level of supergroups (Figure 7).

In euhaline waters, Alveolata (42.5%), Stramenopiles (16.7%), Opisthokonta (15.5%) and Archaeplastida (12.6%) were the most represented supergroups (Figure 7). Relative abundance of Alveolata, Archaeplastida and Opisthokonta was higher compared to mixohaline waters, while the abundance of Stramenopiles decreased by almost two times.

In general, Alveolata (44.5%) and Archaeplastida (22.6%) tend to increase in relative abundance in alpha-hypersaline waters compared to mixohaline and euhaline ones, while Stramenopiles (13.3%) show the opposite. The relative abundance of the Ciliophora and Chlorophyta increased compared to mixohaline and euhaline waters, while the abundance of dinoflagellates decreased. In addition, the number of unclassified eukaryotes decreased to 9.3% in alpha-hypersaline waters (Figure 8).

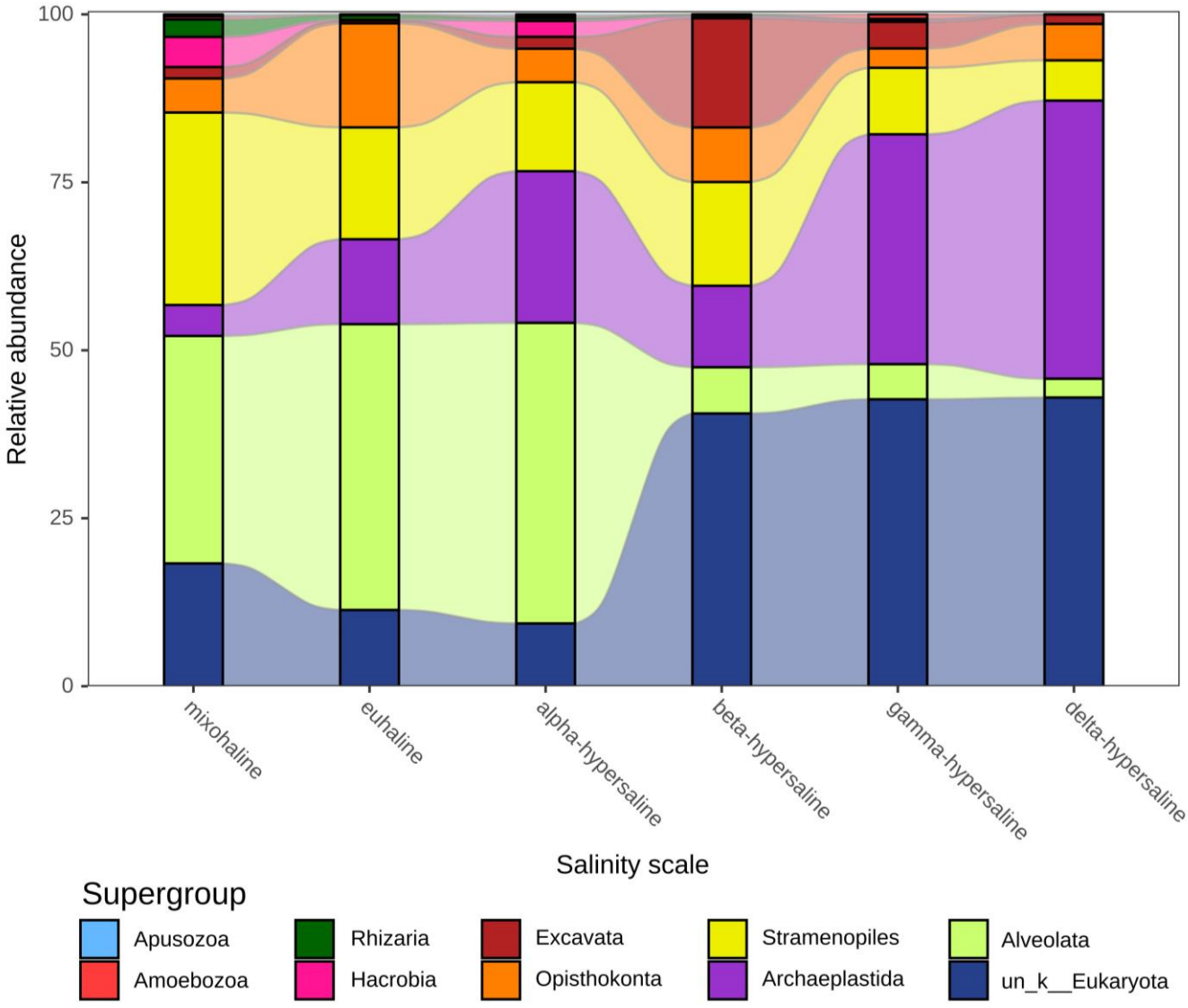

Figure 7. The taxon composition of protist communities within the different salinity classes. Relative abundances of the protist supergroups are presented. Colors correspond to the supergroup names.

In beta-, gamma- and delta-hypersaline waters, a significant increase in the relative abundance of unclassified eukaryotes (to 40.6%, 42.7% and 43%, respectively) and a decrease in the representation of known supergroups were noted (Figure 8). Archaeplastida (especially *Dunaliella*) demonstrates considerable growth in relative abundance in gamma- (34.2%) and delta-hypersaline waters (41.4%), but their abundance drops to 12% in beta-hypersaline waters (Figure 9).

Alveolates and Stramenopiles are characterized by a significant decrease in their abundance in beta-, gamma- and delta-hypersaline waters (Figure 7). The relative abundance of Opisthokonta decreased from beta- to delta-hypersaline water habitats. The abundance of Hacrobia, Amoebozoa and Rhizaria supergroups was insignificant (<1%). Interestingly, despite the insignificant abundance of Excavata (0.4–3.9%) at all other salinities, they reach a significant relative abundance in beta-hypersaline waters (16.3%) (Figure 7).

Considering the distribution of representatives of the main supergroups, it is notable that Alveolata was more represented in alpha-hypersaline, euhaline and mixohaline water bodies. The most common alveolate phylum here was Ciliophora. Chlorophyta (Archaeplastida) were confined mainly to gamma-hypersaline and delta-hypersaline water bodies, accounting for 34% and 39.8% of reads, respectively. *Dunaliella* was one of the most preva-

lent genus of Chlorophyta in the hypersaline water bodies of Sol-Iletsk and Crimea, with a salinity of more than 140‰. The abundance of Stramenopiles and Rhizaria decreased with the increasing salinity. The most represented group of Stramenopiles, Ochrophyta, prevailed at a salinity of less than 40‰. Chrysophyceae were mainly affiliated with mixohaline water bodies. It is noteworthy that members of Opisthokonta and Excavata were present at both the lowest and highest salinity. The largest share of representatives of opisthokonts and excavates (>15% of reads) was associated with euhaline and beta-hypersaline water bodies, respectively. *Euplaesiobystra* (Discoba) was mainly presented in beta-hypersaline Sol-Iletsk water bodies. Amoebozoa, Apusozoa and Hacrobia were less represented in the investigated water bodies and absent at the higher salinities. Unclassified eukaryotes were especially abundant (>40% of reads) in hypersaline waters with salinities over 100‰.

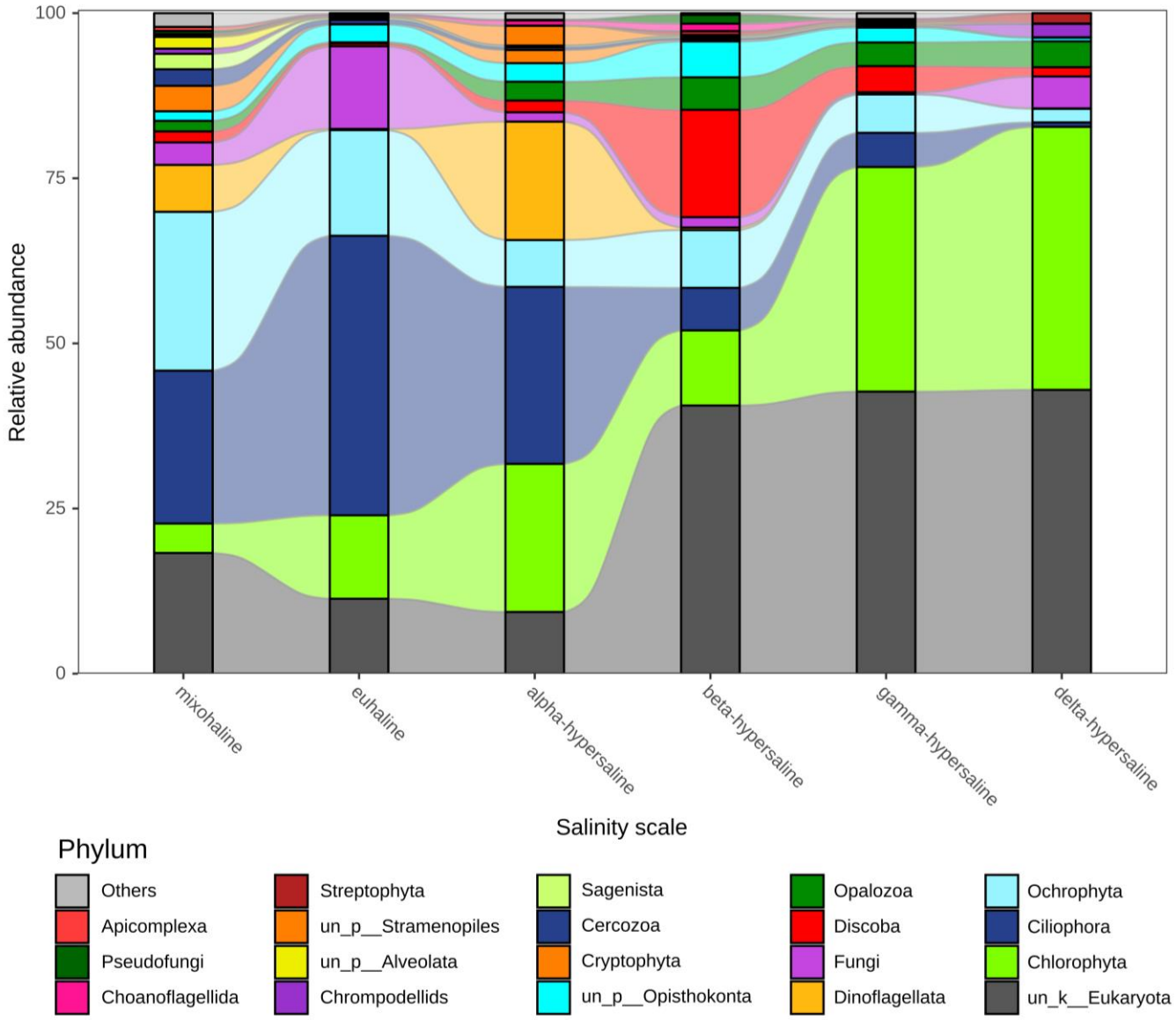

Figure 8. The composition of protist communities within the different salinity classes. Relative abundances of the 18 dominant phyla of protists are presented. The category 'Others' represents the sum of all other taxonomic groups. Colors correspond to the phyla names.

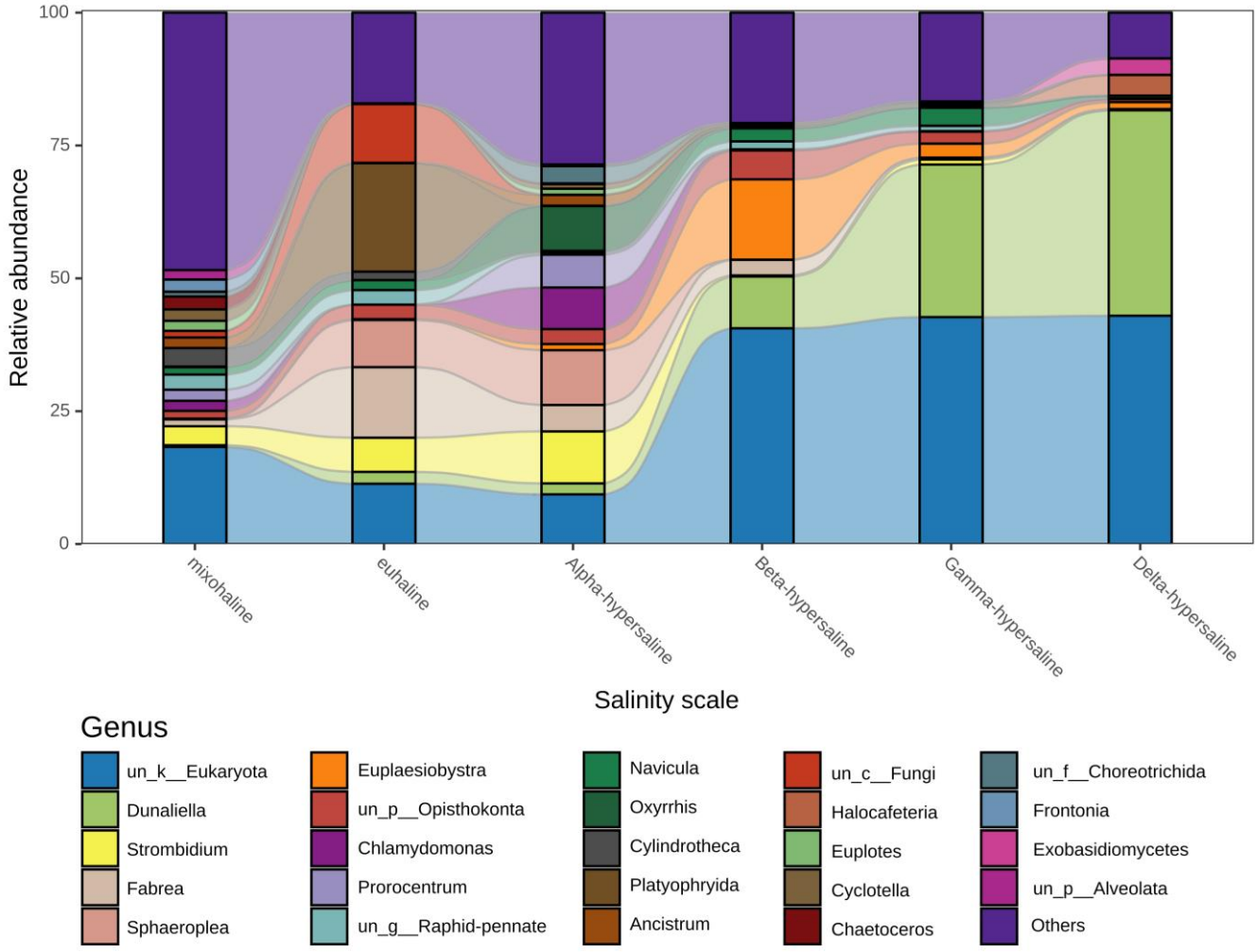

Figure 9. The composition of protist communities within the different salinity classes. Relative abundances of the 22 most dominant genera of protists are presented. The category 'Others' represents the sum of all other taxonomic groups. Colors correspond to the genera names.

3.4. Alpha- and Beta-Diversity Metrics

The protist communities from 38 saline and hypersaline water environments (114 samples) were assessed for alpha-diversity (Chao1 richness and Shannon diversity indexes from the pooled habitat dataset, Table S3) and beta-diversity (Bray–Curtis) metrics. Pearson's rank correlation coefficient, PCoA and NMDS ordination were used to assess the impact of four environmental factors on the microbial eukaryotic community diversity and structure.

The alpha-diversity analyses (Figure 10) showed a strong negative correlation between diversity indexes and salinity ($R = -0.64$ for Chao1 and $R = -0.6$ for Shannon).

Based on the linear regression analysis (explanatory power: $R^2 = 0.3208$, p-value $= 3.072 \times 10^{-11}$ for Chao1 and $R^2 = 0.2612$, p-value $= 3.753 \times 10^{-9}$ for Shannon diversity), a decrease in the richness and diversity of protist communities with an increase in salinity has been shown. Protist communities in Sol-Iletsk and Tuzlukkol' differed most strongly in terms of alpha-diversity metrics (Chao1 $= 2.7 \times 10^{-7}$; Shannon $= 7.8 \times 10^{-6}$). Sol-Iletsk and Crimean communities are characterized by moderate differences (Chao1 $= 1.1 \times 10^{-5}$; Shannon $= 3.2 \times 10^{-5}$), while the Tuzlukkol' and Crimean communities were more similar (Chao1 $= 3.9 \times 10^{-4}$; Shannon $= 1.86 \times 10^{-3}$). The nonmetric multidimensional scaling (NMDS) analysis shows that eukaryotic community composition was ordinated along the salinity gradient with a gradual separation (Figure 11).

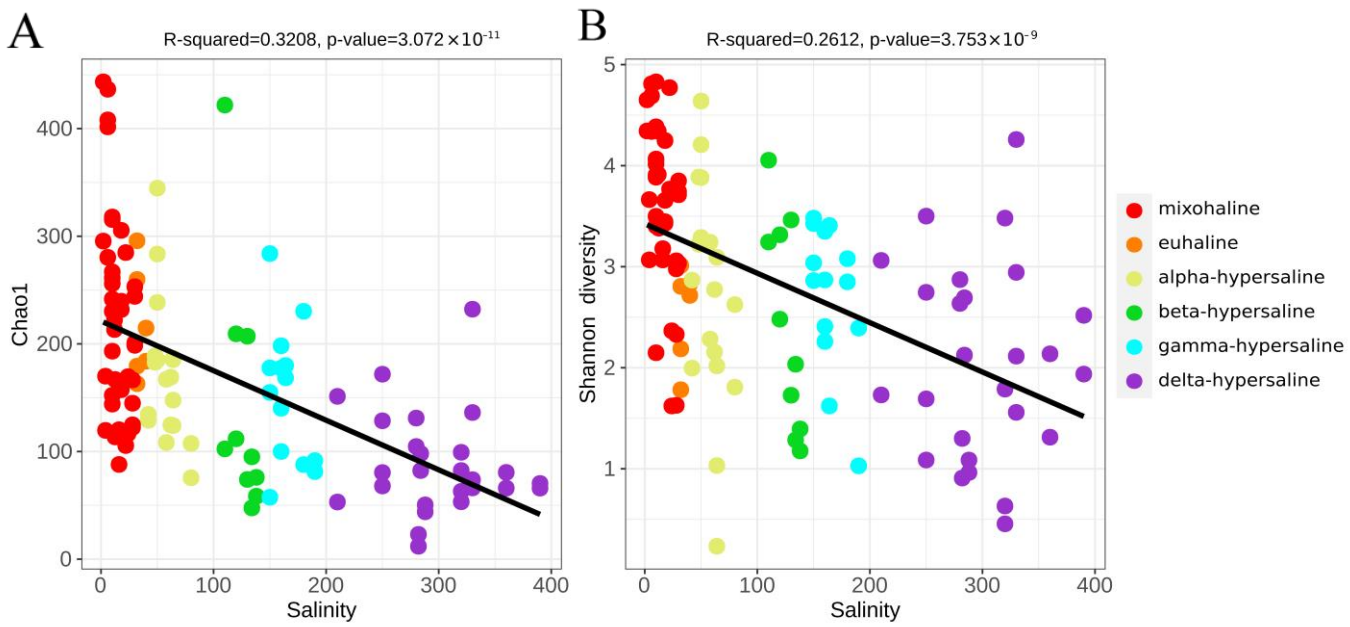

Figure 10. Alpha-diversity of the protist communities. (**A**) Chao Index of protist richness. (**B**) Shannon Index of protist community evenness. Samples were ranked and colored according to salinity and are displayed in ascending order of salinity. The black line shows the regression of species richness and diversity depending on salinity.

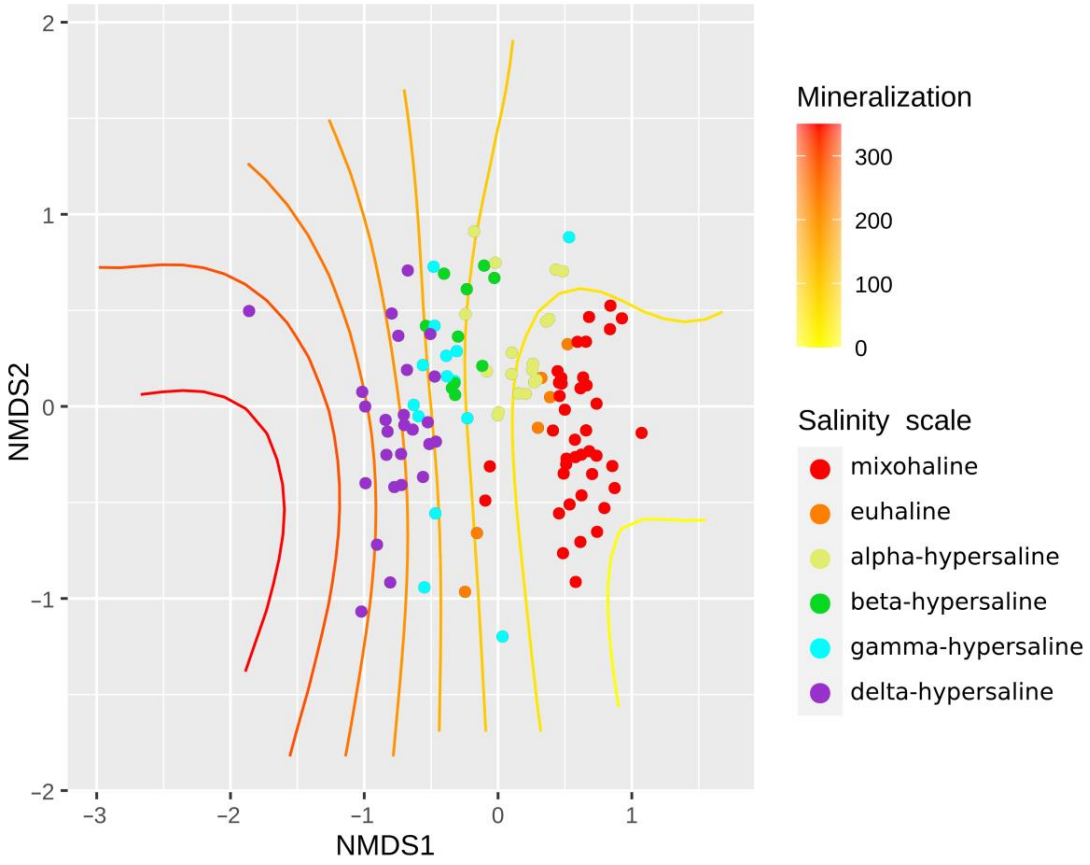

Figure 11. Non-metric multidimensional scaling (NMDS) analysis based on Bray–Curtis dissimilarities of the protist communities composition (ASVs) colored by the salinity. Gradient scale represents a salinity value.

The PCoA analysis based on the Bray–Curtis similarity indices has revealed significant differences between communities (PERMANOVA, $p < 0.05$) depending on the geographical location and pH value (Figure 12). At the same time, the PCoA plot has not revealed clearly defined groups of samples by pH. The analysis of 18S rDNA sequences and the principal coordinates analysis of protist communities found a significant influence of geographic location in shaping the protist community composition (Figure 12).

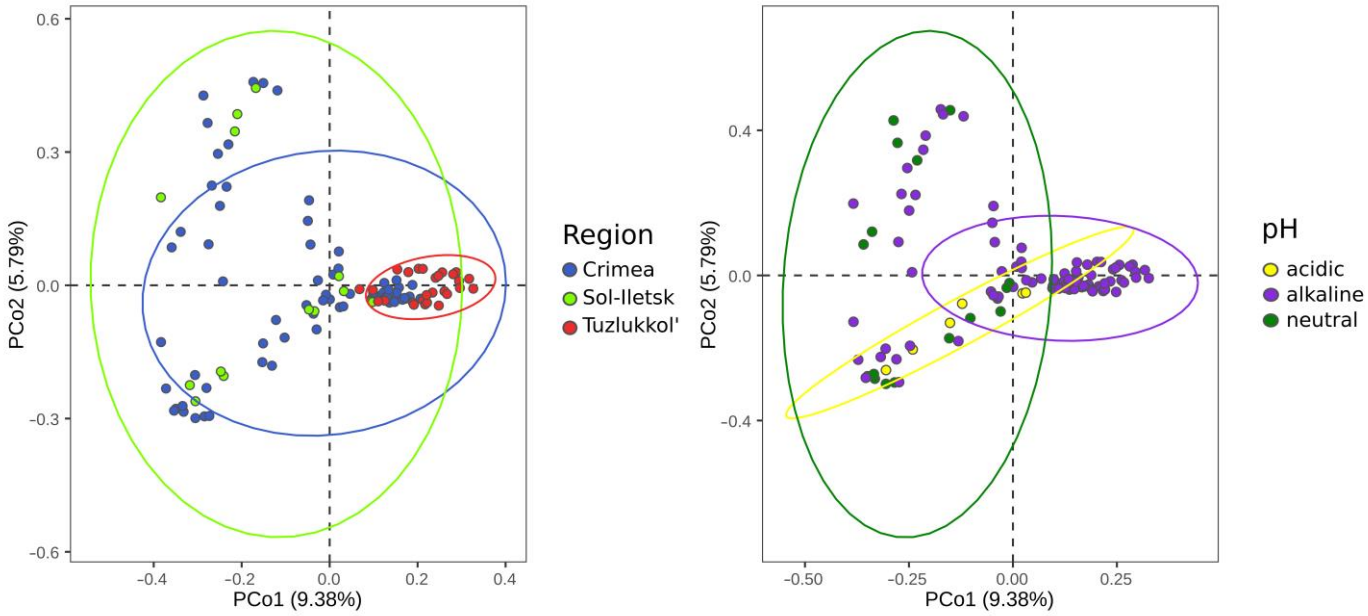

Figure 12. The results of principal coordinates analysis (PCoA) of the protist communities based on the Bray–Curtis dissimilarities, labeled by geographical location (**left**) and pH (**right**). PCo1 (Axis 1) and PCo2 (Axis 2) explained 9.38% and 5.79% of the variance of the protist community at the ASV level, respectively.

Alpha-diversity index metrics also confirm the evidence that community composition depends on geographic location (Figure 13).

In addition, we observed no significant correlation between membrane pore sizes (5.0 µm and 0.45 µm) with alpha-diversity indexes and protists community composition.

3.5. Centohelid Taxonomy and Diversity

In this survey, we especially focused on centrohelid heliozoans, as a target group to estimate its distribution across a broad range of salinities. A total of 1419 reads belonging to 45 ASVs of Centroheliozoa were revealed. Centrohelid diversity includes 14 ASVs belonging to Pterocystida, 1 ASV belonging to Acanthocystida and 30 ASVs of Centroheliozoa with an undetermined affiliation according to the PR2 database. The revealed centrohelid ASVs were additionally checked using the BLAST algorithm to determine their identity against the NCBI nucleotide database. Only 13 centrohelid ASVs had matches with known genera or species (ASV1553, ASV3052 with *Pinjata ruminata* MK641802, 100% identity; ASV2965 with *Marophrys nikolaevi* ON152764, 100%; ASV2977 with *Triangulopteris lacunata* OL739463, 99.6%; ASV3099, ASV4221 and ASV6608 with *Pterocystis* sp. AY749608, 100%, 93% and 96.4%, respectively; ASV3698 with *Pterocystis jongsooparkii* MW298843.1, 99.5%; ASV5804 with *Clypifer cribrifer* MW700077, 96.2%; ASV6064 with *Choanocystis* sp. AY749615, 97.6%; ASV6259 with *Pterocystis* sp. AY749604, 100%; ASV6320 with *Heterophrys marina* AF534710, 96.8%; ASV6379 with *Raphidiophrys drakena* KU178911, 98.9%). At the same time, the majority of centrohelid ASVs were related to the unknown environmental sequences with 93.9–100% identity.

The majority of centrohelid sequences were revealed in the Tuzlukkol' and Crimea samples in mixohaline (38 ASVs) and alpha-hypersaline (8 ASVs) waters. Significantly fewer numbers of centrohelid ASVs were detected at other salinities. The majority of centrohelid sequences were revealed in 5 μm pore size fraction, while the nanoplankton fraction (0.45 μm) was less represented and occurred mostly in the Tuzlukkol' samples.

3.6. Phylogenetic Position of Revealed ASVs and Marine/Freshwater Divergence of Centrohelids

Further analysis was focused on the assessment of the phylogenetic position of centrohelid ASVs and analyzing marine/freshwater divergence in comparison with other sequences on the phylogenetic tree (Figure 14). Centrohelid ASVs were revealed from a broad salinity range (2–320‰) of water samples and attributed to both Pterocystida and Panacanthocystida clades.

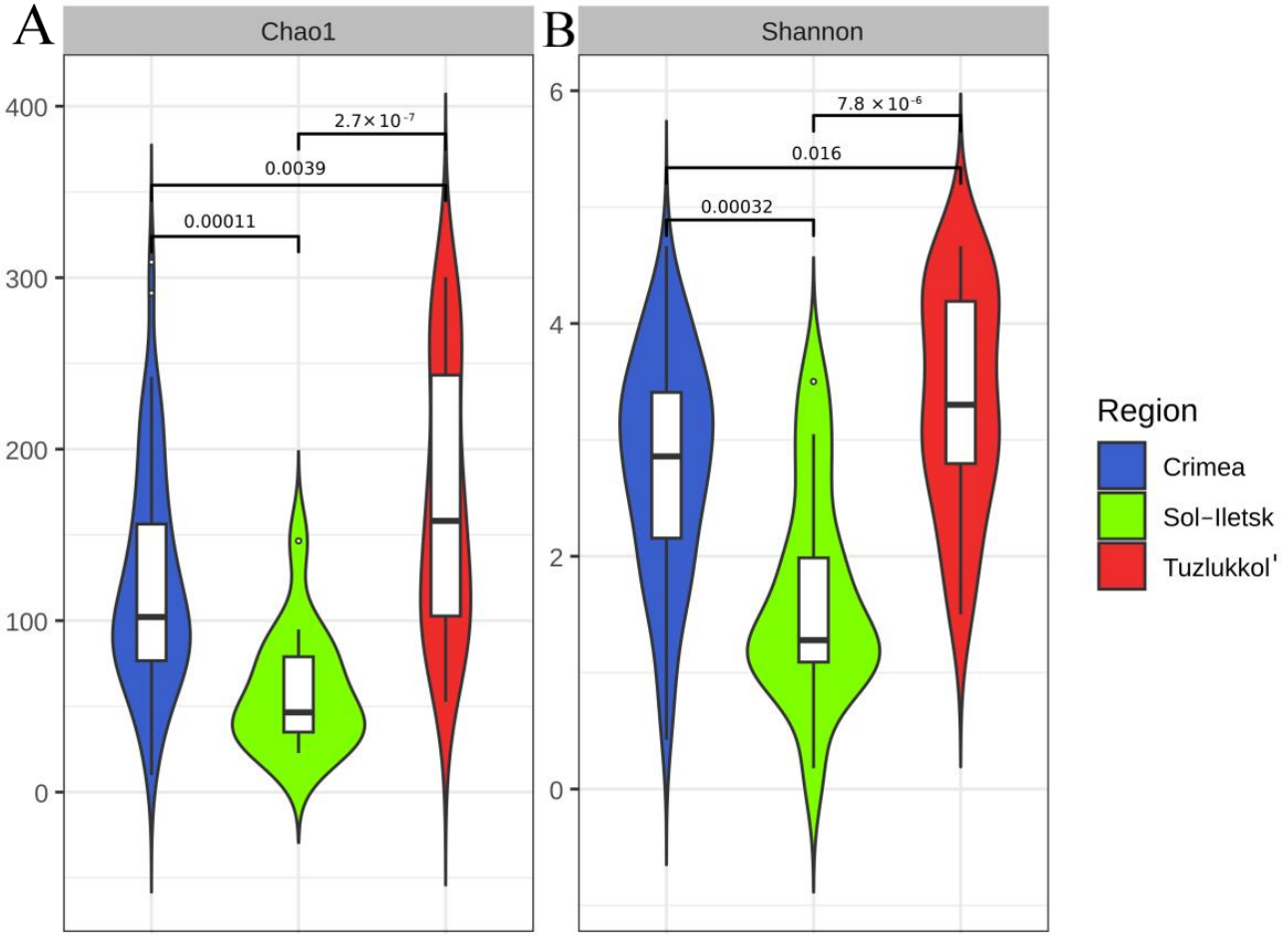

Figure 13. Alpha-diversity indexes of the protist community. (**A**) Chao Index of protist richness. (**B**) Shannon Index of protist community evenness. Samples were ranked and colored according to different salinities and are displayed by salinity increase.

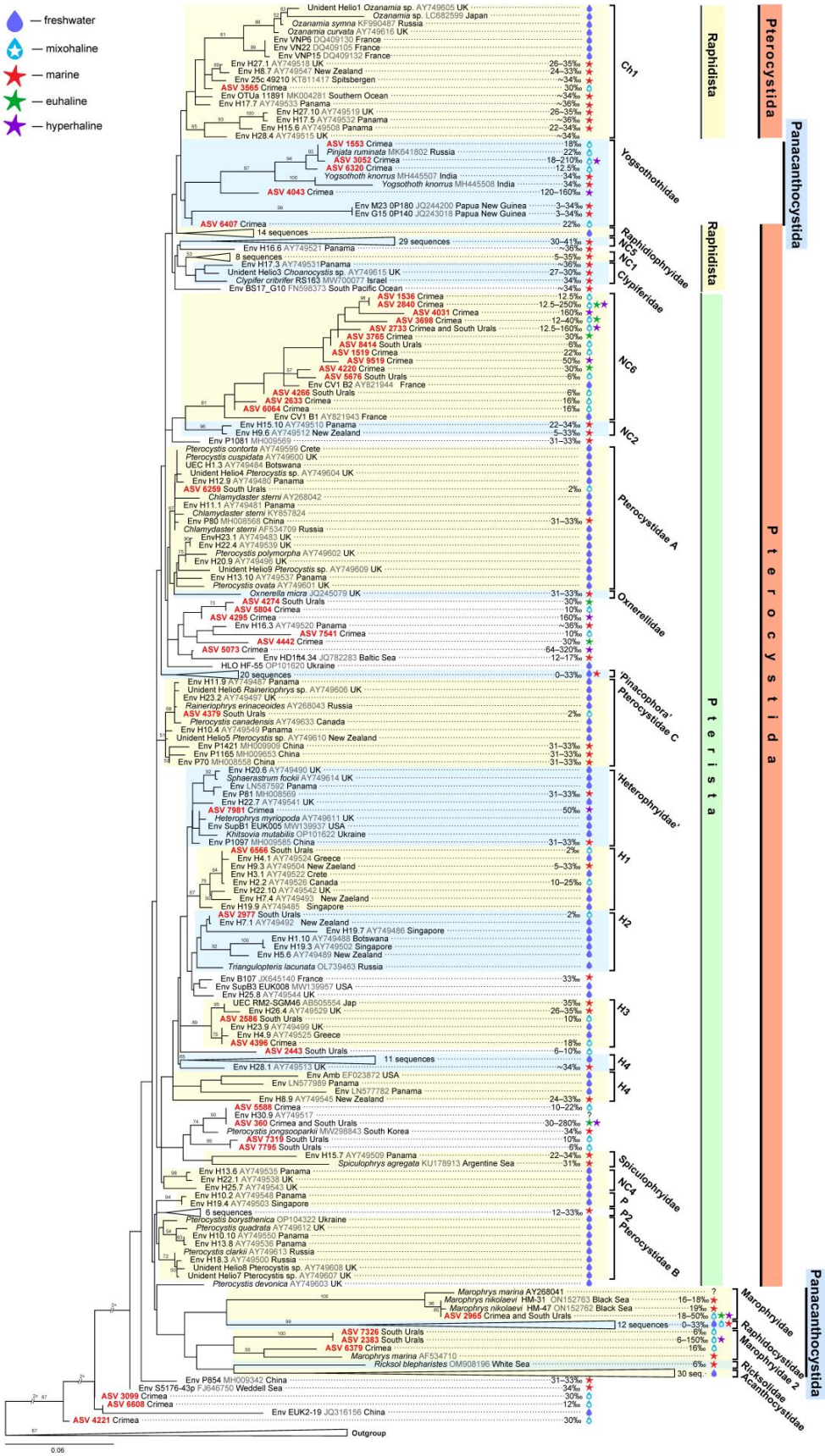

Figure 14. Maximum likelihood tree for 18S rDNA of 300 centrohelids and 11 outgroup sequences (816 sites; TN+F+R5 model; 1000 bootstrap iterations). Bootstrap values $\geq$ 50 are shown. The sequences

obtained in this research are marked in red. The symbols "//" and "2×" indicate that the branch is shortened by two times to improve visualization. Outgroup: AF534711; GQ365770; GQ365840; GQ365798; AJ278035; KM057843; AB847973; AB983344; X77478; AB330051 and GQ365769.

The majority of resulting sequences (33 ASVs) form diverse phylogenetic lineages and are placed within Pterocystida. The ASV3565 from 30‰ Crimean lake clustered in the Ch1 clade at an unresolved phylogenetic position. This clade contains a well-supported freshwater subclade uniting *Ozanamia* sp. (AY749605, LC682599), *Ozanamia symna* KF990487, *Ozanamia curvata* AY749616 and environmental sequences (DQ409130, DQ409105, DQ409132). The majority of revealed ASVs were attributed to the well-supported (81%) environmental NC6 clade, whose representatives demonstrate a considerable level of salinity tolerance from freshwater (AY821944, AY821943) to extremely hypersaline (250‰) habitats. The ASV6259 from mixohaline biotope (2‰) of the South Urals has an uncertain position inside Pterocystidae A. Interestingly, Pterocystidae A mainly contains sequences from freshwater biotopes, except ASV6259 and environmental sequence MH008568 from marine samples (31–33‰). The group of ASVs (4274, 5804, 4295, 7541, 4442, 5073) from the euhaline to gamma-hypersaline samples (10–160‰) and two environmental sequences (AY749520, JQ782283) of marine origin form a new clade of uncertain position within Pterista, albeit without support. The ASV4379 from the 2‰ sample of the South Urals was grouped with freshwater sequences of *Pterocystis canadensis* AY749633, *Raineriophrys erinaceoides* AY268043 and environmental sequences AY749497, AY749606 and AY749487 with low bootstrap support (68%) within Pterocystidae C. Besides this grouping, Pterocystidae C also includes a low-supported subclade (MH009909, MH009653, MH008558) of marine origin and two environmental freshwater sequences (AY749549, AY749610), though with negligible bootstrap support (51%). In general, Pterocystidae C can be characterized by moderate salinity tolerance (0–33‰). The ASV7981 from the 50‰ Crimean sample has an uncertain position within "*Heterophryidae*" without support. The H1 clade (bootstrap support 67%) is another group within Pterocystida with moderate salinity tolerance (0–33‰). Along with marine and freshwater environmental sequences, it contains ASV6566 from the sample with 2‰ salinity.

The unsupported H2 clade consists of environmental sequences and *Triangulopteris lacunata* OL739463 and is characterized by low salinity tolerance due to the freshwater origin of its constituent sequences, including ASV2977 found in a 2‰ sample. The environmental H3 clade was one of the best-supported clades (89%) and was characterized by moderate salinity tolerance. The H3 clade contains two ASVs from 10 to 18‰ Crimean and Southern Urals samples and environmental sequences from freshwater and marine biotopes (26–35‰). ASV5588 (10–22‰) and ASV360 (30–280‰) were grouped with the environmental sequence H30.9 AY749517 of unknown salinity origin with 90% bootstrap support. This grouping is sister to *Pterocystis jongsooparkii* MW298843 from a marine sample of South Korea with moderate support (74%). Mixohaline ASVs 7319 and 7795 form a strongly supported group of uncertain phylogenetic position.

The rest of the revealed ASVs belonged to Panacanthocystida. The Yogsothothidae clade contains five ASVs from Crimean samples and demonstrates a considerable level of salinity tolerance (3–210‰). The ASVs from 18 to 210‰ water samples grouped with *Pinjata ruminata* MK641802 from the brackish environment (22‰) with 93% bootstrap support. The subclade contained *Pinjata ruminata* MK641802 + two ASVs robustly grouped with ASV6320 from a 12.5‰ salinity sample. Together, they grouped (97%) with *Yogsothoth knorrus* MH445507 and MH445508 from marine (34‰) habitats. Overall, the Yogsothothidae clade contains sequences from mixohaline to extremely hypersaline environments and demonstrates a remarkable level of euryhalinity (3–210‰). The ASV2965 from 18 to 50‰ environments was grouped with two marine strains of *Marophrys nikolaevi* (ON152763, ON152762) from the Black Sea (16–19‰) within the Marophryidae clade with 96% bootstrap support. The subclade *Marophrys nikolaevi* + ASV2965 was grouped with maximum support with *Marophrys marina* AY268041 from a biotope of unknown salinity. ASV7326 and ASV2383 revealed a broad range of salinities (6–150‰) in the South Urals and were

grouped together with maximum support. The ASV6379 from the 16‰ Crimean lake is related to *Marophrys marina* AF534710.

Two Crimean ASVs, 3099 and 6608, and 4221 from mixohaline salinity have uncertain positions in the basal part of the phylogenetic tree.

4. Discussion

4.1. High-Throughput Sequencing and Promise in Biodiversity Studies

Hypersaline waters still remain largely understudied despite their worldwide distribution and a wide variety [60]. Protist communities in high-salinity waters of the South Urals and Crimea have been studied mostly in morphological surveys using different microscopic techniques [15,61–69]. The review on the biodiversity of hypersaline waters in the Crimean arid zone [70] reported 190 unicellular eukaryotic organisms. Authors noted that diatoms and ciliates were the most diverse groups [70]. Diatoms have been recorded only from seven lakes of marine origin and not throughout all seasons and their presence in the sulfate lakes on the Kerch peninsula has not been checked [70]. The diatom-related metabarcodes were obtained in 33 investigated environments (68 libraries), including six lakes of the Kerch peninsula. Diatom ASVs were related to 35 genera, with *Cyclotella*, *Cylindrotheca*, *Navicula* and *Chaetoceros* being the most abundant. The review [70] reported that planktonic ciliates have been examined in two lakes of marine origin, and a large number of undetermined species have been revealed in anoxic layers under filamentous algal mats. In our study, planktonic ciliates were the most prevalent phylum among alveolates and account for more than 100 genera. The most abundant were *Platyophryida*, *Fabrea*, *Strombidium*, *Ancistrum* and *Euplotes*. It is possible that ciliates from anoxic layers differ sharply from those in planktonic communities, and the list of genera and species of ciliates identified in hypersaline waters will be significantly expanded in future studies.

In other research, Prokina [68] reported 29 species of heterotrophic flagellates belonging to 20 genera obtained from 10 saline and hypersaline lakes of the Crimean Peninsula. Our data confirm almost all Prokina's findings except for four genera, *Ministeria*, *Ciliophrys*, *Bordnamonas* and *Phyllomitus*. Interestingly, *Pseudobodo tremulans* have been identified only in the Sivash Lake [68], and this finding was confirmed by the observations of related ASVs, again only in this lake. However, the salinity of the water samples was different, 99‰ in Prokina's sample and 64‰ in our sample. In addition to the species identified by Prokina [68], the eDNA analysis reveals more species for the genera *Salpingoeca*, *Caecitellus*, *Cafeteria*, *Goniomonas* and *Ancyromonas*.

The taxonomic composition of microeukaryotes in Sol-Iletsk saline lakes includes about 100 species of protists, mainly belonging to heterotrophic flagellates [61,65], microalgae [71,72] and centrohelid heliozoans [73,74]. The most abundant flagellates in the Sol-Iletsk samples were *Cafeteria roenbergensis*, *Monosiga ovata*, *Spumella* sp., *Paraphysomonas* sp. and *Neobodo* (*Bodo*) *designis* [65]. Our data confirmed the observation of *Cafeteria*, *Monosiga* and *Paraphysomonas* genera. In general, our data revealed only one-third of the taxa previously described from the Sol-Iletsk samples and do not match the majority of microscopically identified heterotrophic flagellates and centrohelid heliozoans. Our data confirm previously reported observations [71,72] related to the microalgal genera *Dunaliella*, *Navicula*, *Chlamydomonas*, *Amphora*, *Cryptomonas* and *Nitzschia*. The weak agreement with previously obtained microscopic observations may be due to the fact that other types of biotopes were investigated in these studies, including bottom sediments [65,68]. In addition, some taxa are characterized by pronounced seasonality, for example, those reported for diatoms [70], ciliophora and foraminifera [75]. A seasonal variation in protist composition and abundance has been reported for many protist taxa [76–79], which have exhibited a clear seasonal pattern. A pronounced effect on the protist community composition has been also noted for meromictic lakes, where fluctuations in temperature, oxygen concentration and salinity play an important role in the formation of the composition of protists [80–84]. The lack of 18S rDNA data for many microscopically identified taxa in the reference databases is another important factor in the observed differences.

The protist community of the Tuzlukkol' River remains almost undescribed, with the exception of centrohelid heliozoans [85–88]. The 18S rDNA metabarcoding approach has been applied to examine centrohelid heliozoan communities in the Sol-Iletsk lakes and the Tuzlukkol' River [45]. Centrohelid OTUs have been grouped within Pterocystidae B, Pterocystidae C, Heterophryidae, Marophryidae, Raphidocystidae and Ch1 clades, as well as in environmental H1, H3, NC1, NC2 and NC9 (=NC6) clades [45]. Additionally, some centrohelid OTUs formed a new clade and belonged to the new phylotypes [45]. The current study expands our understanding of the phylogenetic diversity of the environmental lineages of centrohelids, especially for Yogsothothidae.

In general, NGS amplicon sequencing was shown as superior in detecting rare species in comparison with microscopic surveys [8,39]. Our high-throughput sequencing survey of protist diversity in saline and hypersaline environments revealed 446 protist genera in Crimea and 325 genera in the South Urals and highlighted the gap between the current knowledge of microscopically described protists diversity [61,65,71–74] and 18S rDNA metabarcoding. The taxonomic structure of the protist community in saline and hypersaline environments of the South Urals and Crimea is shown to be poorly understood. A gap between described and undescribed protist taxa is still large and points to the necessity of a comprehensive study of 18S rDNA along with microscopy.

4.2. Protist Communities and Their Relationships with Salinity and Geographic Location

The taxonomic composition of the studied planktonic communities was represented mainly by Archaeplastida, Alveolata and Stramenopiles, while amoebozoans, apusozoans and rhizarians were less represented. A similar composition was noted in other studies of microeukaryotic communities in hypersaline environments [7,24,42,43,89]. In our study, Alveolata showed the highest affinity to mixohaline and euhaline waters, whereas Archaeplastida prevailed in extreme ones. Amoebozoa, Apusozoa, Rhizaria, Excavata and Hacrobia were insufficiently present in all salinities, with a few exceptions. Apusozoa is completely lacking from beta- to delta-hypersaline waters. Stramenopiles were the most represented from mixohaline to beta-hypersaline environments and decreased in their abundance in gamma- and delta-hypersaline environments. It is noteworthy that protist communities demonstrated a gradual separation along the salinity gradient. Alveolata, Archaeplastida, Excavata, Opisthokonta and Stramenopile supergroups have wide transitional boundaries along the salinity gradient and are represented in all salinity classes, but are characterized by gradual decreasing with increasing salinity. Archaeplastida demonstrated considerable growth through the wide transitional boundaries with increasing salinity. Apusozoa and Rhizaria had an affinity to mixohaline, euhaline and alpha-hypersaline waters with a gradual decrease in relative abundance. Rhizaria was scarce in beta- to delta-hypersaline waters, whereas Amoebozoa was absent in the three last salinity classes.

The attempts to identify the transition boundaries of protistan communities across high-salinity gradients are not numerous [44,90–92]. Casamayor and co-authors [90] described salinities of 80‰ and 150‰ as boundaries of an abrupt decrease in protist richness from 30 to 10 taxa. Other research [91] identified 50‰, 150‰ and 250‰ salinities as transitional boundaries for protistan communities from two multipond solar saltern systems. Close results were shown for protistan communities from three different solar saltern ponds in Portugal [24] and salt works of Sfax in Tunisia [92], indicating 120‰ and 150‰ salinities as transition boundaries, respectively. The transitional boundary between alpha- and beta-hypersaline waters was shown as a critical point in a harsh reduction in the representation of the protistan community at the genus level.

The tendency of a reduction in diversity in protist communities with growing salinity was shown almost in all ecological research [7,12,44]. We observed a decrease in the relative abundance for the majority of protist taxa with growing salinity, with some exceptions. *Dunaliella* (Archaeplastida) demonstrates considerable growth in relative abundance with increasing salinity and was the most represented in delta-hypersaline waters with salin-

ity from 200‰ to 390‰. In some similar studies, *Dunaliella* is reported to be found in high-salinity environments, including waters with NaCl saturation [7,89,93]. *Dunaliella* is described as a key component of protist communities in hypersaline waters worldwide and the main or even the sole primary producer in these environments [94,95]. It has been shown that *Dunaliella* is able to live in hypersaline waters due to the presence of photosynthetically produced glycerol as an osmotic stabilizer [95]. We described *Dunaliella* from all salinity classes and as one of the most prevalent genera in Sol-Iletsk and Crimean lakes. Some other genera revealed in hypersaline waters in our study, *Euplaesiobystra* (Heterolobosea, Discoba), *Halocafeteria* (Bicoecea, Opalozoa), *Navicula* (Diatomea, Ochrophyta), *Exobasidiomycetes* (Basidiomycota, Fungi) and *Fabrea* (Heterotrichea, Ciliophora), were also noted in hypersaline environments, but were more rarely abundant [7,89]. Some species of Heterolobosea, Bicosoecida and Ciliophora have been characterized as extreme or borderline extreme halophiles [4]. In our study, ciliates were mainly affiliated with a salinity <100‰, but such genera as *Fabrea*, *Strombidium*, *Chlamydodon*, *Ancistrum* and *Euplotes* were detected in alpha-hypersaline, beta-hypersaline and gamma-hypersaline water bodies.

The assessment of the taxonomic structure of eDNA biodiversity revealed significant differences in protist communities depending on the geographic location of sampling sites, but the salinity was the strongest selection factor, which was also reported in a recent study [44].

4.3. Salinity Tolerance and Marine/Freshwater Divergence in Centrohelids

The vexed question about marine/freshwater divergence of centrohelids arose on the assumption suggested by Mikrjukov [96,97] based on morphological data, which contradicts the suggestion by Cavalier-Smith and Heyden [98] based on molecular phylogenetic data. Mikrjukov [96,97] concluded that centrohelids are a group with remarkable levels of euryhalinity and cosmopolitism. Conversely, sanger sequencing data by Cavalier-Smith and Heyden [98] demonstrated that "almost all clades contain exclusively either marine or freshwater lineages" and suggested that "strong physiological and ecological barriers exist to successful dispersal between these environments, probably generally preventing effective colonization of freshwater . . . species into marine environments and vice versa".

Used in taxonomic research and to test different evolution hypotheses, 18S rRNA is a main marker gene for molecular phylogenetic studies of centrohelids [98,99]. Additionally, the 18S rRNA gene was shown as an efficient barcoding marker for centrohelids in environmental studies, indicating a considerable diversity and novelty of centrohelids in the environmental samples [6,98]. It was suggested by 18S rDNA sequencing that about 90% of centrohelids still remain undescribed [98]. Actually, new species of centrohelids have been described in almost every research focused on this group in marine [100–102], freshwater [103,104], saline [85,87,88] and soil biotopes [105,106]. In addition, the 18S rDNA amplicon sequencing was applied to metabarcoding research and estimation of taxonomic diversity of centrohelids in saline and hypersaline inland waters (2–78‰) and the assessment of marine/freshwater divergence within the group [45]. The phylogenetic analysis focused on centrohelid heliozoans revealed freshwater/low salinity (0–2‰) clades, some presumably marine/brackishwater clades and seven clades, demonstrating broad salinity tolerance (from 1–2‰ to 78‰) [45].

In this study, we estimated taxonomic diversity and analyzed marine/freshwater divergence of centrohelids from continental water environments in a wide salinity range (2–320‰), and have shown the new cases of broad salinity tolerance, demonstrated by separate lineages. Phylogenetic analysis attributed most of the obtained ASVs to previously established clades; however, the topology of the basal branches of the tree was unsupported.

We revealed that ASV297 from a mixohaline sample with low salinity (2‰) was grouped with the H2 clade, previously described as freshwater [45,98]. In the current analysis, we attribute this clade to the freshwater/low salinity group and consider that the distribution of their members will be restricted by high-salinity barriers.

Two ASVs (6259, 4379) from mixohaline samples with low salinity (2‰) were grouped with freshwater subclades within Pterocystidae A and Pterocystidae C, respectively. Due to the fact that Pterocystidae A and Pterocystidae C also contained subclades or sequences of marine origin, we assume that members of these clades can probably overcome freshwater and low-salinity barriers up to marine waters and back again in the evolutionary course. Similar cases were observed in the Ch1 and environmental H3 clade, which include sequences of marine, mixohaline and freshwater origins. The members of both Ch1 and H3 clades have a moderate level of salinity tolerance and perhaps also can overcome freshwater and marine barriers there and back again. However, it is noteworthy that a well-supported subclade containing *Ozanamia* sp. + three environmental sequences within Ch1 has a freshwater origin.

The well-supported NC6 clade unites the majority of revealed ASVs and is also characterized by a considerable level of salinity tolerance (0–250‰) and attributed to a clade with freshwater/marine/hypersaline origin. In the previous analysis [45], the NC6 clade, mistakenly distinguished as the new NC9, also unites the majority of revealed Operational Taxonomic Units with high support and has been shown as a clade with the most significant level of salinity tolerance (2–78‰), among others.

Some clades from our analysis demonstrated a considerable level of salinity tolerance and contained sequences of exclusively mixohaline, marine or hypersaline origin, namely Yogsothothidae (3–210‰), Marophryidae 2 (6–150‰) and an unnamed clade sister to OP101620 (10–320‰). Additionally, Marophryidae (6–50‰) and the unnamed clade containing H30.9 + *Pterocystis jongsooparkii* (6–280‰) also demonstrated a considerable level of salinity tolerance, but due to the uncertain origin of H30.9 and *Marophrys marina* AY268041, we only tentatively assign it to clades with mixohaline/marine/hypersaline origins.

Along with *Raphidocystis contractilis*, which has been described as a species specifically adapted to the waters of 5–15‰ salinity in our previous research [38], here we characterize *Pinjata ruminata* MK641802 and *Yogsothoth* sp. as species with a potentially considerable high level of salinity tolerance (3–210‰) within Yogsothothidae. Initially, *Pinjata ruminata* was described from inland brackish water of 22‰ of Russia [85] and *Yogsothoth knorrus* from a 34‰ marine sample from India [100].

Our understanding of the marine/freshwater divergence of separate clades or species distribution of centrohelids is changing dramatically as data on centrohelid eDNA diversity accumulate. In comparison with the previous research [45], here we show a higher salinity tolerance for the NC6 clade, and significantly higher salinity tolerance for the clade containing H30.9 (up to 280‰ in comparison with 78‰ in the previous study). Additionally, we show a high level of salinity tolerance for the Yogsothothidae clade, whose marine/freshwater divergence has not been previously analyzed due to the absence of related environmental sequences. A significantly higher salinity tolerance was revealed for Marophryidae members, up to 150‰ in comparison with 14‰ in the previous study.

The current survey significantly expanded our understanding of euryhalinity of separate clades (up to four times) and highlighted the novelty of revealed centrohelid ASVs, also demonstrated in previous research [6,45,98], including hypersaline biotopes. Phylogenetic analysis has identified clades with both high and low ranges of salinity tolerance, which does not negate the validity of the assumptions of both Mikrjukov and Cavalier-Smith and Heyden. Mikryukov's assumption about the absence of endemism in centrohelids is confirmed by the presence of the clades uniting lineages of freshwater/marine/hypersaline origin (NC6). Cavalier-Smith's and Heyden's suggestion is also not rejected, since within the euryhaline clades, there are subclades that are exclusively of freshwater (*Ozanamia*) or marine origin (Pterocystidae C, Yogsothothidae).

5. Conclusions

The obtained results provide the first molecular reference into the taxonomy and diversity of protist communities in saline and hypersaline environments of the South Urals and Crimea and highlighted the gap between the current knowledge of microscopically described protist diversity and 18S rDNA metabarcoding data. The taxonomic structure of the protist community was represented by Alveolata, Amoebozoa, Apusozoa, Archaeplastida, Excavata, Hacrobia, Opisthokonta, Rhizaria and Stramenopiles supergroups. Unclassified Eukaryota accounted for 16% of the total reads. The assessment of the taxonomic structure of 18S rDNA biodiversity revealed significant differences in protist communities depending on the geographic location of sampling sites, with salinity being the strongest selection factor. The protist communities showed a tendency to reduce diversity and richness with growing salinity and demonstrated a gradual separation along the salinity gradient. The transitional boundary between alpha- and beta-hypersaline waters was shown as a critical point in a harsh reduction in the representation of the known protist taxa. The taxonomic structure of the protist community of the South Urals and Crimea is shown to be poorly understood and has great potential for the discovery of new taxa in future biomonitoring surveys. A gap between described and undescribed protist taxa in saline and hypersaline waters of the South Urals and Crimea is still large and points to a necessity for a comprehensive study of 18S rDNA along with microscopy.

The current survey highlighted the novelty of revealed centrohelid ASVs and significantly expanded our understanding of the euryhalinity of some clades, presumably capable of overcoming marine salinity barriers for their distribution there and back again. Centrohelids demonstrated the ability to inhabit a broad range of salinities, up to 320‰, which is four times higher than previously reported. A high level of salinity tolerance was suggested for centrohelid species *Pinjata ruminata* and *Yogsothoth* sp. due to the wide salinity ranges of related ASVs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15112008/s1. References [107,108] are cited in the Table S1.

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