A Preliminary Study on the Eukaryotic Microbial Diversity in Croatian Geothermal Waters

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Abstract: Microbial eukaryotes have essential roles in aquatic ecosystems, yet their diversity and ecological functions in extreme environments remain understudied compared to prokaryotes. This study aims to thoroughly characterize the composition and diversity of microbial eukaryotic communities in 14 geothermal waters across Croatia. Physicochemical analysis revealed significant variations in temperature (36–55 °C), pH (6.5–8.3), and nutrient concentrations, with all sites displaying anoxic conditions except for one. Sequencing of the V9 18S rRNA gene identified 134 taxa, predominantly from the Alveolata, Stramenopiles, and Opisthokonta supergroups. The highest diversity and richness were observed in aquifer groups with moderate temperatures and nutrient levels, while extreme sites exhibited reduced diversity. Among the key environmental factors shaping these communities, temperature, pH, and nitrate concentrations were most significant. Photoautotrophic and mixotrophic taxa, such as Ochrophyta, Dinoflagellata, and Chlorophyta, were prominent, reflecting their roles in primary production and nutrient cycling. Decomposers, including Basidiomycota and Ascomycota, were linked to organic matter degradation. Microeukaryotes showed adaptations to extreme conditions, such as thermotolerance and evolutionary shifts from phototrophy to heterotrophy, highlighting their ecological versatility. These findings underscore the potential of microbial eukaryotes in biotechnological applications, such as bioremediation and biofuel production. Genera like *Tribonema* and *Navicula* demonstrated promising capabilities in nutrient removal and CO_2 fixation. However, further research is necessary to investigate and confirm their suitability for these purposes. To summarize, our research provides new insights into understudied microbial eukaryotes in Croatian hot springs that represent a valuable model for exploring microbial diversity, ecological interactions, and industrial applications in extreme environments.

Keywords: hot springs; microbial eukaryotes; 18S rRNA gene amplicon sequencing; microalgae

1. Introduction

In the early stages of microbial ecology development, research focused on prokaryotic diversity, often overlooking eukaryotic communities, especially microeukaryotes. Most extremophiles were believed to be archaea, dominating various extreme environments [1]. However, subsequent research has shown that bacteria and eukaryotes also thrive in extreme conditions [2]. Consequently, research on eukaryotes has expanded, though they

still receive less attention than prokaryotes. Databases contain more taxonomic data on prokaryotes and significantly less on eukaryotes [3], especially in aquatic and extreme environments like geothermal waters, due to the traditional view that eukaryotes are less adapted to extreme conditions [3,4].

Microeukaryotes are ubiquitous unicellular organisms with diverse roles as primary producers, parasites, predators, and decomposers in terrestrial, marine, and freshwater ecosystems [5], exhibiting various nutritional strategies, including phototrophy, heterotrophy, mixotrophy, and osmotrophy [6]. Evidence suggests that microeukaryotes actively thrive in extreme environments like geothermal waters [[2] and references within]. Therefore, it is crucial to reassess this traditional paradigm and incorporate the study of microeukaryotes to gain a better understanding of their roles within these unique habitats [7].

Microeukaryotes exhibit adaptations to extreme environmental conditions, enabling them to endure thermal stress as well as acidic or alkaline habitats. These adaptations include altering membrane composition to maintain structural integrity [8] and producing specialized proteins that facilitate survival in extreme environments. Many of these proteins are acquired through horizontal gene transfer from bacteria and archaea [9]. Additionally, microeukaryotes produce enzymes with inherent stability at high temperatures and extreme pH levels, while specialized proton pumps and ion transporters allow for precise regulation of intracellular pH [10].

Despite microeukaryotes' adaptability to extreme environmental conditions like high temperatures, studies on eukaryote diversity in geothermal sites remain limited. Early research showed thermophilic microeukaryotes tolerating temperatures of up to 60 °C, findings later confirmed in sites such as Yellowstone National Park and Agano Terme [7]. In Lassen Volcanic National Park, thermophilic microeukaryotes were observed up to 68 °C [11], while in New Zealand, temperatures below 50 °C were shown to limit their presence [12]. Algae and Fungi stand out among microeukaryotes for their adaptability to extreme environments. These organisms thrive in high-temperature settings due to thermostable enzymes and protective extracellular polymeric substances, whose development is fueled by their high rates of primary production [13]. Amoeboid lineages, such as Amoebozoa and Discoba, have also been recognized as inhabitants of extreme habitats, where they act as consumers of archaea and bacteria [14]. Recent studies on hot springs in Yellowstone reported a dominance of groups like Ascomycota, Chlorophyta, Streptophyta, Bacillariophyta, and Euglenida [15]. Similarly, in the hot springs of Odisha, Streptophyta, Basidiomycota, Bacillariophyta, and Ascomycota were prevalent in springs up to 50 °C, while Arthropoda dominated springs reaching 60 °C. Therefore, these findings underscore the value of exploring geothermal habitats to uncover novel protist communities and their ecological roles.

We carried out a comprehensive study to characterize the microbial eukaryotic community in 14 Croatian geothermal waters during autumn. Croatia, located in Southern Europe, is part of a region known for having one of the highest densities of geothermal springs in the world [16]. These geothermal systems within the Croatian part of the Pannonian Basin are characterized by a strong geothermal gradient and a wide range of hydrochemical conditions [17], creating unique niches for microbial communities. Geothermal systems in Croatia are diverse, with varying temperatures, pH levels, and nutrient concentrations [17], providing an opportunity to examine the ecological dynamics of eukaryotic microbes, their adaptations, and their roles in biogeochemical cycles. Given that research on microbial eukaryotes in geothermal habitats remains limited, particularly in Southern Europe, Croatian geothermal waters offer a promising and largely untapped area for global comparative studies.

Our main objective was to gain a deeper understanding of the microbial eukaryote community structure and elucidate the influence of abiotic factors, such as temperature, pH, and nutrient concentrations, on the community composition. While geothermal waters [17] and biofilms [18] in Croatia have been the subject of recent studies focusing on prokaryotic communities and their biotechnological potential, microbial eukaryotes in these environments remain largely unstudied. Previous research in Croatian geothermal waters highlighted the dominance of prokaryotic groups such as Proteobacteria, Campylobacteria, and Crenarchaeota, with bacterial and archaeal communities exhibiting high site specificity [17]. Environmental factors like temperature, pH, nitrogen compounds, and dissolved carbon content were found to significantly shape these communities [17], and similar factors were expected to influence microbial eukaryotes. By employing 18S rRNA gene amplicon sequencing techniques, our study sought to bridge this knowledge gap and provide a thorough characterization of microbial eukaryotes, including microalgae, mixotrophs, decomposers, heterotrophs, and parasites, in Croatian geothermal waters.

2. Materials & Methods

2.1. Research Area and Sample Collection

This study included 14 geothermal water sites across northern, central, and Pannonian Croatia (Figure 1). Sites were categorized into 5 groups based on geographical location, hydrochemical conditions, geological characteristics, and hydrogeological properties of aquifers, as described in [17]. In short, Aquifer Group 1 consisted of 7 hydrothermal sites differing in light exposure and temperature. The enclosed geothermal spring catchments without light exposure were Jezerčica (JEZ), TS Krapina (KRA), and TS Stubica (STU), while TS Sv.Jana (SJA), TS Topusko (TOP), TS Tuhelj (TUH), and TS Varaždin (VAR) were light-exposed open basins, differing in temperature: KRA, STU, and VAR are hyperthermal, while TUH is hypothermal. Geologically, VAR consists of alluvial deposits (gravel and sand), whereas the others have carbonate sediments [19]. The enclosed TS Sv. Martin (SVM) spring belongs to Aquifer Group 2. Aquifer Group 3 included TS Daruvar (enclosed lightless Antunovo vrelo (AVD) and Marijino vrelo (D1D), and an open basin Ivanovo vrelo (IVD)), which are typical dolomite/limestone thermal aquifers [19]. The enclosed TS Bizovac (BIZ) and Bošnjaci (BOS) are part of Aquifer Group 4, characterized by gravelly sand and sandy-clayey gravels [20]. The Krečaves (KRE) site does not belong to any of the previously mentioned groups and represents a unique Na-HCO3 aquifer, characterized by silicate minerals and water-rock interactions [21] (Figure 1).

In autumn 2020, at each geothermal site, water samples were collected in sterile PET bottles for microbial eukaryote diversity and analysis of environmental conditions. Autumn was chosen for sampling to achieve moderate environmental conditions and avoid extreme variations during summer and winter. Sampling in autumn minimized the potential influence of seasonal adaptations of the microbial eukaryotic community to environmental fluctuations in already extreme habitats. As described in [13], water samples were collected from valves at the head of each well and springs directly from underground water outflow. During sampling, disinfected gloves were used, and PET bottles were rinsed with water from the geothermal site before collecting the samples. Water samples of a volume of 5 L were filtered through 0.2 µm pore size polycarbonate filters (Whatman Nuclepore Track-Etch Membrane, diameter 47 mm, Global Life Sciences Solutions Operations UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK), and filters were preserved at −80 °C until further processing. Along with using disinfected gloves, filtration was performed in a clean laboratory environment to prevent contamination of the samples.

Figure 1. Locations of the investigated geothermal waters in Croatia.

2.2. Field and Laboratory Measurements of Physicochemical Parameters

Electrical conductivity (EC), pH values, temperature (T), and dissolved oxygen concentration (O_2) were measured with a WTW multi-probe (WTW, Weilheim, Germany). Concentrations of dissolved cations (Na^+, NH_4^+) and anions (SO_4^{2-}, Cl^-, NO_3^-) in filtered samples were measured on an ion chromatography system Dionex ICS-6000 DC. Hydrogen sulfide (H_2S) levels were determined using Reagent sulfide 1 and Reagent sulfide 2, according to the USEPA Methylene Blue Method using a HACH DR3900 spectrometer. Total nitrogen (TN) was determined with the TNT Persulfate Digestion Method on a HACH DR1900 spectrometer. Silica concentrations in the form of SiO_2 were measured using acid, citric, and molybdate reagents with the HACH DR3900 spectrometer. The dissolved organic carbon (DOC) samples were initially filtered through 0.45 μm membrane filters and analyzed using the HACH QBD1200. All the above analyses were carried out at the Hydrochemical Laboratory of the Croatian Geological Survey.

2.3. DNA Extraction, 18S rRNA Gene Amplification, Sequencing, and Sequence Processing

Total genomic DNA was extracted with the DNeasy PowerWater kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol. The hypervariable V9 region of the gene encoding 18S rRNA was amplified by PCR using a primer pair for eukaryotic identification: 1391F (5′-GTACACACCGCCCGTC-3′) [22] and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) [23]. The V9 region is more conserved than V4, which makes it useful for detecting a broader range of eukaryotic diversity [24]. This is especially beneficial in environmental samples where taxonomic coverage is a priority. The PCR reaction was prepared by adding 6 μL of extracted DNA to a final volume of 50 μL,

which included 10 µL of High Phusion HF buffer (New England BioLabs, UK), 2.50 µL of each 10 µM primer, 1 µL of 10 mM dNTPs (New England BioLabs, UK), and 0.5 µL of Phusion High-Fidelity DNA polymerase (New England BioLabs, UK). The remaining volume was filled with ultrapure PCR-grade water. PCR reactions were performed in triplicate and combined into a single sample (150 µL) before sequencing to minimize potential PCR reaction errors. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 57 °C for 45 s, chain extension at 72 °C for 1 min, and final chain extension at 72 °C for 2 min. This protocol was adapted from the Earth Microbiome Project website with minor modifications (https://earthmicrobiome.org/protocols-and-standards/18s/ (accessed on 21 January 2021)).

Following amplification, all 18S rRNA gene samples were barcoded, purified, normalized, and prepared for sequencing. Paired-end sequencing of purified samples was conducted on an Illumina NextSeq platform at the sequencing facility SeqIT GmbH & Co. KG (Kaiserslautern, Germany).

18S rRNA gene paired-end reads from 14 samples were analyzed using the QIIME2 (v.2019.10). Sequences were cleaned from adapters and demultiplexed using cutadapt (v.2.6). Afterwards, sequences were trimmed to a minimum length of 174 bp based on quality score, with an additional quality-filter threshold (q = 29), de-noised, merged, and used for generating ASVs using dada2 (v.1.10.0). For the taxonomic assignment, the ASVs were aligned with the Protist Ribosomal Reference (PR^2) database (v.4.13.) [25].

2.4. Data Processing and Statistical Analysis

Before statistical analysis, ASVs without taxonomic assignments at the supergroup level were removed. Subsequently, during further investigation, ASVs lacking assignments at other taxonomic levels were also excluded. ASVs classified as Metazoa were removed using manual filtering. Manual filtering was performed using function subset_taxa(), ensuring the removal of all metazoan sequences. ASVs that appeared only once or twice in the entire dataset were retained due to limited sequence count and the generally less well-known taxonomy of microbial eukaryotes. Among the 14 geothermal sites examined, 5 (JEZ, STU, TOP, SVM, and D1D) showed significant microbial compositions and were analyzed comprehensively using statistical methods.

The statistical analyses were performed in R environment v 4.1.1 [26] using the following Bioconductor (v 3.14) packages: vegan v 2.5.7 [27], phyloseq v 1.38 [28], DESeq2 v 3.17 [29], corrplot v 0.92 [30], dplyr [31], plyr v 1.8.8 [32], and tidyr v 1.3.0 [33]. Graphical visualizations were created using ggplot2 package [34].

Alpha diversity was estimated by rarefaction on the dataset, subsampling to the smallest library size, using Shannon [35], Simpson 1-D [36], and Chao1 indices. Rarefaction was performed to standardize sequencing depth using function rarefy_even_depth() function in pyhloseq. The rarefaction depth was selected as the smallest library size across samples; sample TUH had the smallest library size of 14. Beta diversity using Bray–Curtis dissimilarity matrices was assessed for microbial eukaryotic communities via Principal Coordinates Analysis (PCoA). Prior to calculating beta diversity, datasets were normalized to account for differences in sequencing depth across samples. The raw ASV counts were transformed into relative abundances by dividing each count by the total sum of counts in the sample and multiplying by 1 million (per-million scaling). This transformation ensures that observed differences in community composition reflect differences in community structure rather than sequencing depth. Differences among communities across geothermal locations and aquifer groups were tested with PERMANOVA (Permutational Multivariate Analysis of Variance) using the adonis() function in vegan with Bray–Curtis dissimilarity and

999 permutations. Environmental parameters influencing microbial communies ($p < 0.05$) were evaluated using the envfit() function in vegan. Specifically, envfit() was applied to the Bray–Curtis dissimilarity matrix and the environmental dataset with 999 permutations to calculate p-values for the significance of each environmental factor. Significant environmental variables ($p < 0.05$) were projected onto the PCoA plot and visualized with the scores function to assess their influences on microbial community composition. Correlations between physicochemical parameters and identified taxa of interest were analyzed using Pearson correlation after "z-score" normalization.

3. Results

3.1. Physicochemical Water Properties

The physicochemical parameters of five geothermal sites (JEZ, STU, TOP, SVM, and D1D; Table S1) are described because they had the largest presence of microbial eukaryotes. Briefly, the highest temperature was recorded in STU (55 °C), and the lowest in SVM (36 °C). The pH values displayed a range from neutral (7.1 in SVM) to slightly acidic (6.5 in TOP). Only in D1D was the O_2 concentration above 0, while other sites were identified as anoxic. SVM had the highest DIC (1000 mgL^{-1}) and DOC concentrations (28.5 mgL^{-1}), as well as NH_4^+ (20.9 mgL^{-1}), NO_3^- (6.4 mgL^{-1}), and TN (16.4 mgL^{-1}) concentrations. No statistically significant differences were found between the five geothermal sites or aquifer groups (Table S2).

Generally, positive correlations were calculated between EC, HCO_3^-, Na^+, Cl^-, and DIC concentrations (Figure S1). Additionally, these parameters correlated positively with concentrations of NH_4^+, NO_3^-, and TN. Concentrations of H_2S and SO_4^{2-} also correlated positively, along with NO_3^-, NH_4^+, and TN, which showed stronger positive correlations with each other than to sulfur-related compounds.

3.2. Microbial Eukaryotic Community Composition

The sequencing resulted in 41,833 reads. After filtering, the remaining 2915 reads of the V9 18S rRNA gene were affiliated to a total of 134 taxa in 14 samples. The highest number of reads was in 1646 in TOP.

The highest microbial eukaryotic richness (Chao1 index) and diversity (Shannon and Simpson indexes) were measured in wells belonging to Aquifer Group 2, SVM (Figure S2), whereas the lowest were measured in Aquifer Group 4 (BIZ and BOS), alongside KRE. From Aquifer Group 3, IVD showed the lowest richness and diversity. The Kruskal–Wallis rank sum test showed no statistically significant differences among sampling sites for alpha diversity indices (Table S3). The microbial eukaryotic community of all 14 sampled geothermal waters did not differ significantly between aquifer grouping (PERMANOVA: $p = 0.51$, $R^2 = 0.31$; Figure S3), nor did the type of geothermal site significantly affect community composition, whether it was a well or a spring (PERMANOVA: $p = 0.72$, $R^2 = 0.93$).

The predominant taxonomic supergroups across geothermal sites included Alveolata, Opisthokonta, and Stramenopiles, with the highest abundances observed in BIZ (Aquifer Group 4), where Alveolata accounted for 39.5% and Stramenopiles 60.5% of the microbial eukaryotic community. In KRE, the total abundance of microbial eukaryotes from the three supergroups was dominated by Stramenopiles, which represented 83.3% of the community (Figure 2A). Members of the Amoebozoa were exclusively found in Aquifer Group 1 (KRA: 8.3% and TOP: 0.75%) and Aquifer Group 2 (SVM; 0.82%), while representatives of the supergroup Rhizaria were exclusively found in Aquifer Group 1 (JEZ: 1.12%). The main representatives of supergroup Alveolata were divisions Apicomplexa, Ciliophora, and Dinoflagellata (Figure 2B). Dinoflagellata appeared in higher abundances specifically in Aquifer Group 4 (BIZ: 39.5% and BOS: 36%), in two out of three sites in Aquifer Group

3 (IVD: 33.3% and AVD: 26.9%), and in only two sites in Aquifer Group 1 (KRA: 26.9% and STU: 17%). Dinoflagellata alongside Ochrophyta (supergroup Stramenopiles) were the most abundant divisions. In geothermal site KRE, the community was composed of Cryptophyta and Ochrophyta (Figure 2B), particularly classes Cryptophyceae (Figure S4A) and Bacillariophyceae, respectively (Figure S4A). Interestingly, these classes were either completely absent or found in considerably smaller abundances at the other geothermal sites. Furthermore, in Aquifer Group 4, division Conosoa (9.9%) was exclusively detected in KRA with representative genus *Filamoeba* (Figure S4B), Chlorophyta (28.6%) in TUH with representative *Chlorochytrium* (Figure S4C), Cercozoa (2.4%) in JEZ, Mesomycetozoa (3.75%) in SJA, and Choanoflagellida (0.36%) in TOP (Figure 2B).
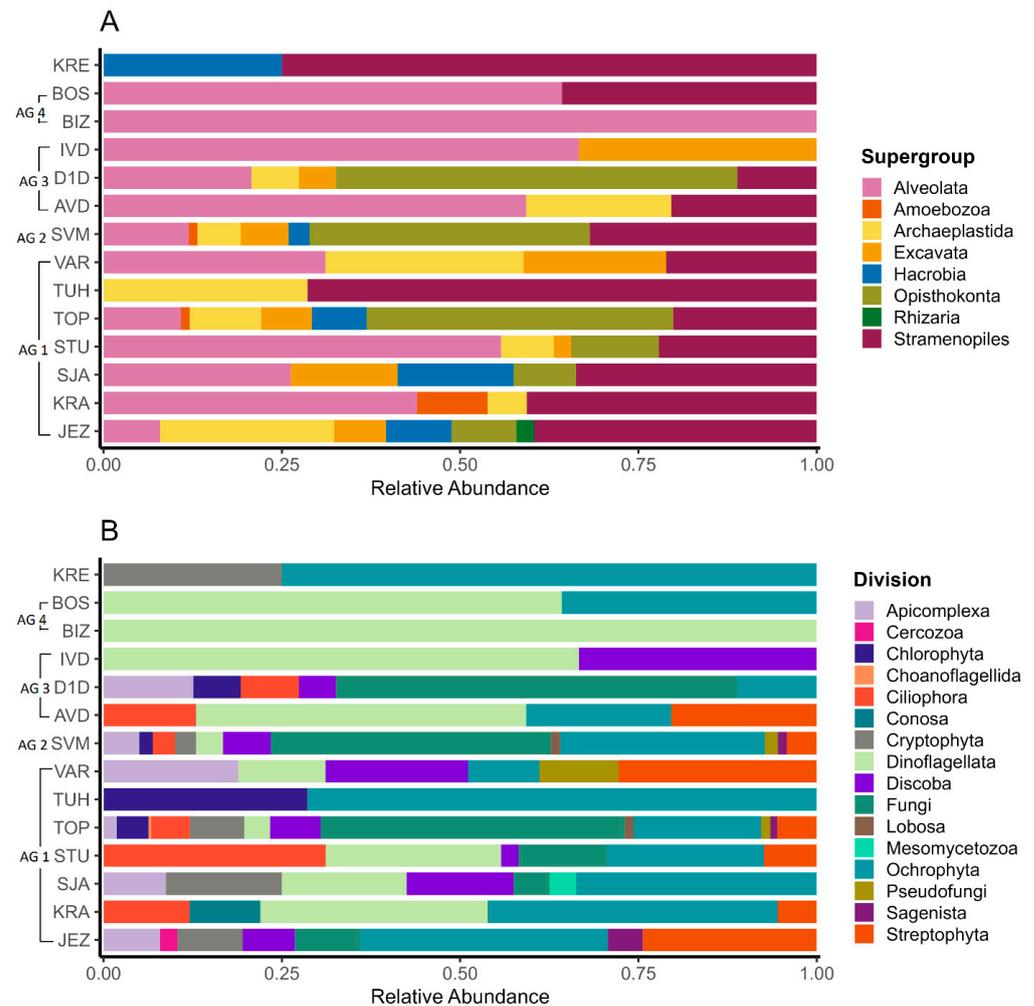
Figure 2. Relative abundance of all eukaryotic communities at the taxonomic level of supergroup (**A**) and division (**B**) identified at 14 geothermal sites in Croatia during the autumn of 2020. Taxa are colored by supergroup and division.

Closer examination of the Ochrophyta divisions at the five geothermal sites (JEZ, STU, TOP, SVM, and D1D) with the highest presence of microbial eukaryotes revealed representatives from the Xanthophyceae and Dictyochophyceae classes (Figure S5). The Chrysophyceae class was dominated by genus *Spumella* (Figure 3A). The mentioned classes, together with Bacillariophyceae, form a community of primary producers, which was dominated by genera *Navicula* and *Cymbella*, *Chromulina*, and *Tribonema* (Figure 3A). On the other side, decomposers, such as Fungi, were most abundant at the D1D (34.7%), SVM (25.6%), and TOP (24.9%) sites (Figure 2B). This division included representatives of Basidiomycota (SVM (16.5%), D1D (15.1%), TOP (14.8%), and Ascomycota (TOP (8.9%)

and SVM (6.9%), Figure S5), featuring prominent genera such as *Malassezia*, *Candida*, and *Rhodosporidium* (Figure 3B).
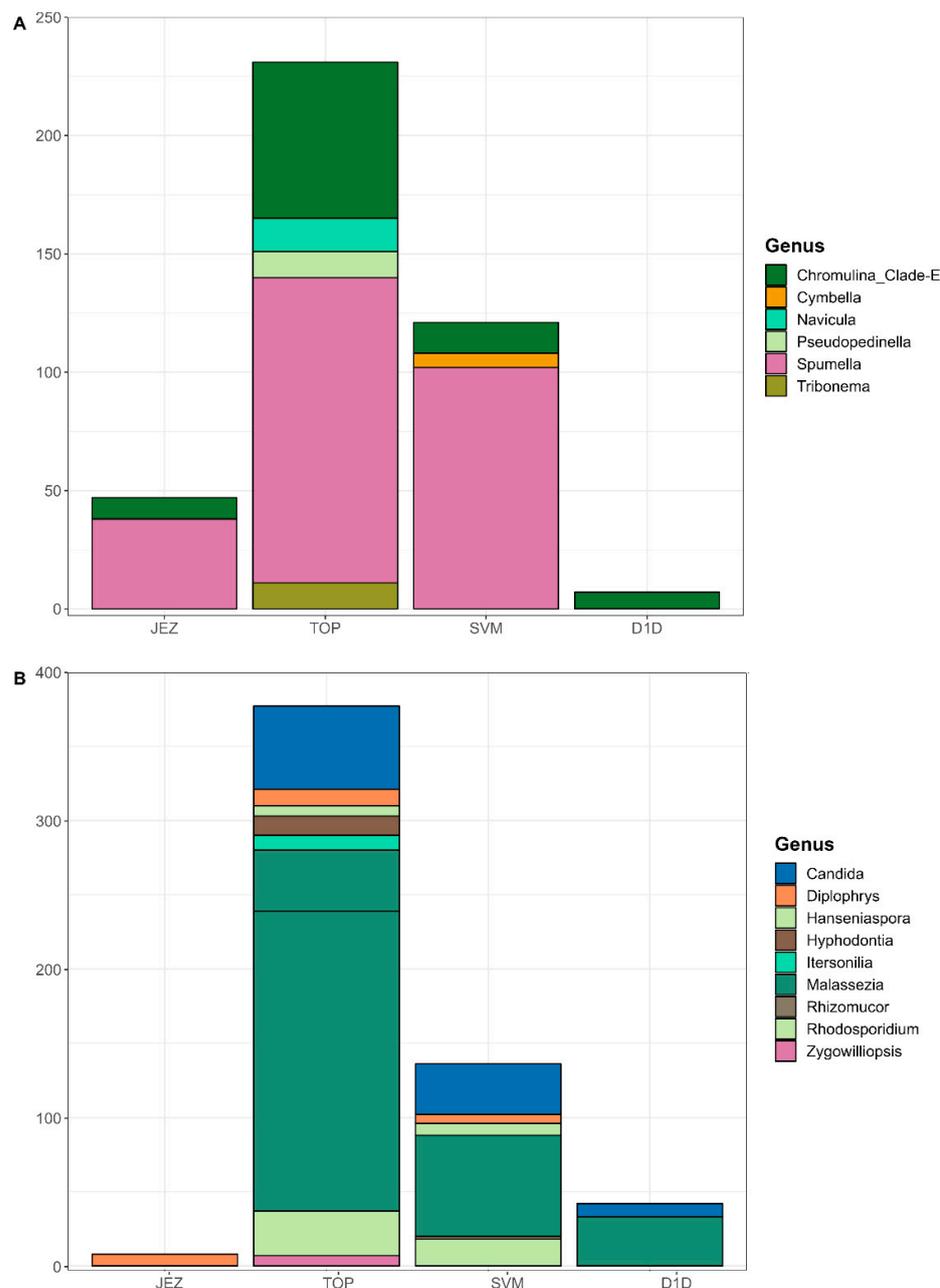

Figure 3. The actual representation of microbial eukaryotes at the taxonomic level of genera at the four geothermal sites in Croatia that displayed the most pronounced quantitative and qualitative composition during the autumn of 2020. The genera associated with the processes of primary production (**A**) and decomposition (**B**) were singled out.

Other highly represented genera of microbial eukaryotes at individual sites included *Naegleria* (Figure S6; division Discoba, (IVD 33.3%; VAR, 14.4%; SJA, 8.8%)), *Cryptomonas* (division Cryptophyta, (SJA, 16.3%; JEZ, 9.1%)), and *Spirogyra* (division Streptophyta, (AVD, 12.9%; STU, 7.4%)), Figure S6.

3.3. The Influence of Environmental Parameters on the Microbial Eukaryotic Community

Physicochemical parameters had no significant influence on microbial eukaryotic community formation across all 14 examined sites (PERMANOVA: $p > 0.05$). Considering

the five geothermal sites (JEZ, STU, TOP, SVM, and D1D) with the largest presence of representatives of microbial eukaryotic communities, remarkable differences in beta diversity were found using PCoA (Figure 4). Across the five geothermal sites, temperature had the most significant impact on community formation (PERMANOVA: $p = 0.02$, $R^2 = 0.27$) with nitrates (PERMANOVA: $p = 0.05$, $R^2 = 0.20$) and pH (PERMANOVA: $p = 0.05$, $R^2 = 0.25$), while other environmental parameters had no statistically significant impact on the microbial eukaryotic community (PERMANOVA: $p > 0.05$).
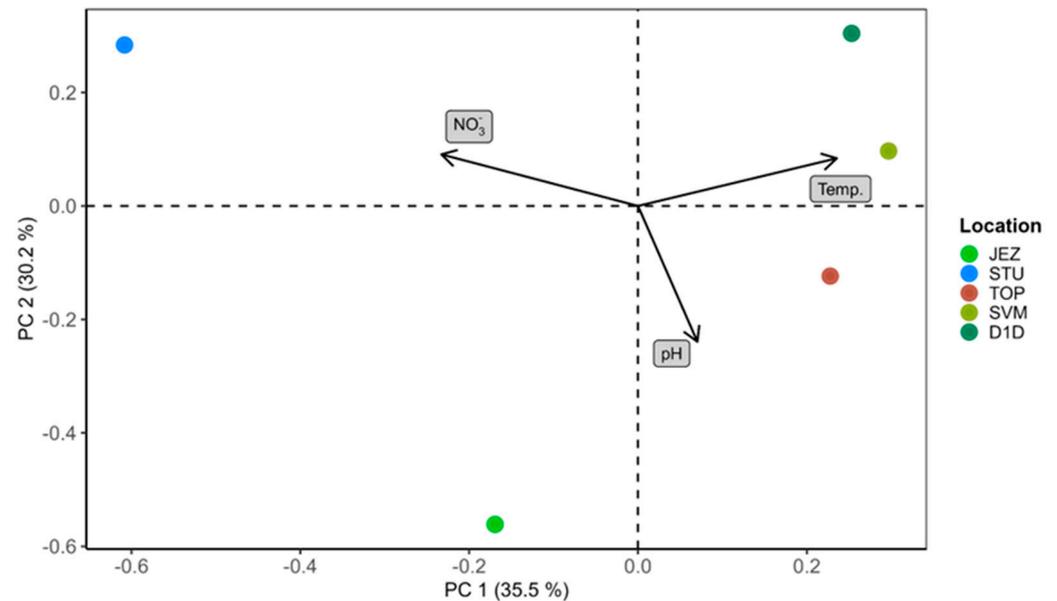
Figure 4. Principal coordinate analysis (PCoA) of microbial eukaryotic community based on Bray–Curtis dissimilarity matrix. Environmental variables that showed a significant influence on the microbial eukaryotic community based on the PERMANOVA test ($p < 0.05$) are represented by vectors: temperature, pH, and NO_3^- concentrations. Points are samples colored by five sampling locations that displayed the most pronounced quantitative and qualitative composition during the autumn of 2020.

MaAslin2 analysis was performed to explore the effect of environmental parameters on the composition of microeukaryotic communities. The analysis revealed a significant negative effect of temperature on Chrysophyceae (Figure S7), with a coefficient of −1.1467 (Figure S7A). The dataset included 14 samples, with 8 samples showing non-zero values for this feature. Temperature was the only environmental parameter that showed a significant effect on Chrysophyceae, while the other variables did not exhibit significant statistical influence (Figure S7B).

Finally, correlation analysis based on Pearson's coefficient revealed a positive correlation between Dinoflagellata and nitrates, and a negative correlation between Ochrophyta, nitrates, and pH values (Figure 5A). In addition, correlation analysis showed positive correlations of Streptophyta with Ochrophyta and Cryptophyta, contrary to negative with Chlorophyta. Contrary to previously mentioned primary producers, decomposers Fungi and Sagenista, as well as Sagenista and temperature, correlated negatively, while Fungi and pH correlated positively (Figure 5B). Moreover, a comprehensive examination of the parameters demonstrated a complete negative correlation between temperature and pH (Figure 5A), a strong negative correlation between temperature and nitrates (Figure 5A,B), and a positive correlation between pH values and nitrates (Figure 5B).
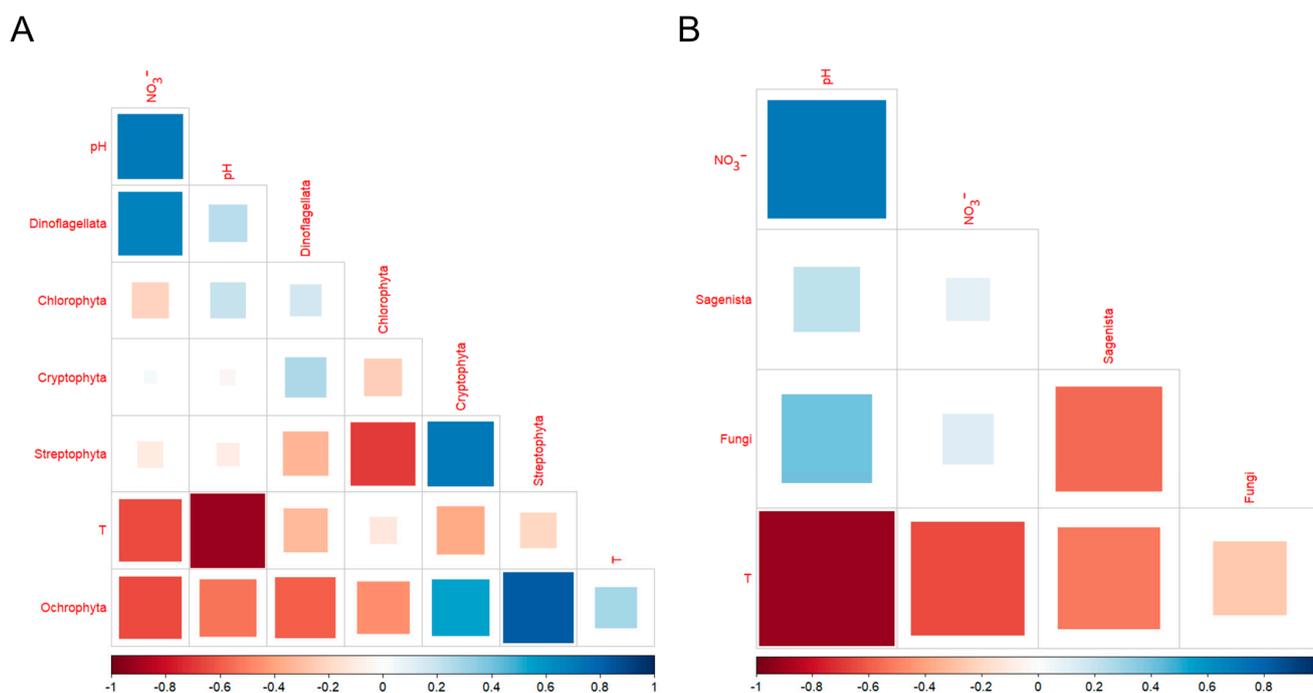
Figure 5. Pearson correlation on the normalized dataset of physicochemical parameters ($p < 0.05$) and at the taxonomic level of the division, which typically contains the majority of representatives associated with the primary production process (**A**) or organic matter decomposition (**B**). The data used for this analysis were gathered from five geothermal locations (JEZ, STU, TOP, SVM, and D1D).

4. Discussion

Assessing the microbial eukaryotic composition over a single season gives an incomplete representation of their seasonal diversity. Nonetheless, it provides a basis for future research in temporal dynamics and the effects of environmental fluctuations on the overall structure of the microbial eukaryotic community thriving in Croatian geothermal waters.

4.1. Comparing Microbial Eukaryotic Structure in Croatian Geothermal Waters with Global Hot Springs

In this study, we provide a detailed characterization of the microbial eukaryotic community in Croatian geothermal waters, shedding light on its composition and ecological interactions. The geothermal springs in Croatia, with their distinct temperature gradients, pH levels, and nutrient availabilities, serve as unique environmental laboratories for understanding microbial life in extreme conditions. These geothermal systems are particularly valuable for studying microbial eukaryotes on a global scale due to their position within the high-density geothermal region of Southern Europe, a largely unexplored area in microbial eukaryotic research [16]. Microbial eukaryotic communities in 14 geothermal waters in Croatia showed low diversity, identifying only 134 taxa (Figure 2). Furthermore, the significant microbial composition was observed in only 5 out of 14 sites, as these samples had the highest number of ASVs per sample; consequently, the majority of the statistical analysis was conducted on these samples due to their greater representation of microbial diversity. The number of detected taxa aligns with previous studies in geothermal waters worldwide, which showed a relatively low representation of microeukaryotic taxa [6]. However, Oliverio et al. [7] observed a much greater taxonomic and phylogenetic diversity of microeukaryotes in the geothermal waters of New Zealand and identified 1088 phylotypes. Still, the community structure of Croatian geothermal waters at the highest taxonomic level reassembled a structure typical for geothermal waters dominated by supergroups Alveolata and Stramenopiles [7,37].

Our study represents the first research of microbial eukaryotic communities in generally understudied Croatian geothermal waters, providing novel insights into their composition and ecological roles. Microbial eukaryotes are key players in biogeochemical cycles, contributing to primary production, organic matter decomposition, and nutrient cycling [38]. Our findings significantly expand current knowledge by revealing unique microbial taxa and ecological interactions in these previously unexplored geothermal habitats.

A substantial portion of the microbial eukaryotic community was composed of photoautotrophs and mixotrophs, including representatives of Chlorophyta, *Tribonema*, *Navicula*, *Cymbella*, *Chromulina*, Streptophyta (*Spirogyra*), Cryptophyta (*Cryptomonas*), and certain Dinoflagellata (Figures 2A and 3A). These photosynthetic microalgae, alongside bacterial primary producers such as Cyanobacteria and Chloroflexi [39], form the foundation of the local microbial food web by generating carbohydrates that sustain mixotrophs and heterotrophs like Ciliophora, Pseudofungi, and Amoebozoa, which metabolize organic matter [40]. The interactions between these primary producers and consumers are not merely competitive, but also indicative of a dynamic, co-evolutionary process involving nutrient exchange and habitat competition. For instance, the positive and negative correlations observed between different groups of primary producers highlight the complexity of nutrient cycling within these ecosystems (Figure 5A).

One particularly interesting finding is the significant presence of *Spumella* (Chrysophyceae) (Figure 3A), a taxon that, while evolutionarily linked to phototrophic relatives, has shifted to a heterotrophic lifestyle [41,42]. This taxonomic shift exemplifies an important adaptation in geothermal ecosystems, where organisms can evolve from photosynthesis to organic matter consumption, enhancing carbon transfer in microbial loops. The abundance of *Spumella* in Croatian geothermal waters (Figure 3A) highlights its crucial role in carbon transfer within the microbial loop and underscores the diversity of survival strategies and adaptive responses in such extreme environments. Additionally, the generally low concentrations of dissolved organic matter (Table S1) at most sites suggest the presence of efficient decomposers. The relatively low abundance of Fungi and Sagenista (Figures 2B and 3B), along with their negative correlation to temperature (Figure 5B), suggest that in these extreme environments, bacterial heterotrophs may play a more dominant role in organic matter degradation [43].

In addition to autotrophic groups, we identified several taxa with notable ecological significance, such as thermophilic species from the *Candida* species (Figure 3B). These organisms exhibit unique survival strategies in high-temperature environments, underscoring their potential relevance for applications in biotechnology, agriculture, and medicine [44]. Additionally, we identified microbial eukaryotic groups with often pathogenic or parasitic lifestyles, such as representatives of Apicomplexa and Mesomycetozoa (Figure 2B) [45,46], highlighting the potential health and ecological implications of these geothermal habitats.

4.2. Ecological Relationships and Community Dynamics Across Sampling Sites

Microbial eukaryotic primary producers in Croatian geothermal waters vary depending on the specific sampling site, probably driven by differences in nutrient availability (carbon and nitrogen) and light exposure, patterns commonly observed in aquatic ecosystems [47]; for instance, the dominance of Dinoflagellata (39.5%) at the BIZ site, which is linked to elevated nitrogen compound concentrations, particularly nitrates [48]. This suggests that, despite their mixotrophic nature, specific Dinoflagellata representatives may be influenced by nitrogen availability, suggesting a competitive advantage in nutrient-rich environments. Their role within the microbial community may extend beyond primary production, influencing trophic interactions by serving as both autotrophic and heterotrophic members of the food web.

The SVM site, characterized by the highest nitrogen concentrations, showed a distinct community composition dominated by producers and mixotrophs such as Chlorophyta, Ochrophyta (*Cymbella*, *Chromulina*), and Streptophyta. This highlights the crucial role of nitrogen, alongside carbon, in sustaining microbial eukaryotes in geothermal waters [47]. In contrast, Ochrophyta was correlated negatively with nitrates, suggesting that the development of genera like *Navicula*, *Tribonema*, and *Chromulina* in nitrogen-limited environments might contribute to nutrient depletion. These findings underscore the complex interplay between microbial primary producers, nutrient dynamics, and competition for resources in geothermal habitats, shaping the ecological structure of these unique environments.

In geothermal springs with sufficient sunlight exposure (TOP, TUH, JEZ, and SJA), we observed a notable presence of Chlorophyta and Cryptophyta (Figure 2B). Additionally, at the KRE site, green biofilms likely formed by Cyanobacteria, Chloroflexi [18], and photosynthetic microalgae were found. The detected positive and negative correlations between different groups of primary producers (Figure 5A) suggest complex interactions involving both coexistence and competition for nutrients and habitat, further indicating their involvement in the microbial loop and organic matter circulation.

4.3. Environmental Influence in Croatian Geothermal Waters

This study specifically focused on autumn, a season when environmental factors, particularly temperature, stabilize following summer fluctuations and before the extremes of winter [49]. This seasonal stability is particularly relevant in geothermal waters, as it minimizes the need for rapid physiological or community-level adaptations, thereby potentially reducing seasonal adaptation effects. The stable environmental dynamics provide microeukaryotes with an extended adaptation period, which may enhance their biological efficiency and resilience. However, additional research is needed to support this, particularly by comparing our findings with previous studies that have analyzed seasonal effects in similar geothermal environments. While some research suggests that seasonal changes do not significantly impact microeukaryotic communities in geothermal waters [15], further comparative analyses are required to fully understand these dynamics. Notably, our previous study on Croatian geothermal waters reported stable environmental conditions across seasons, with no significant effect on prokaryotic communities [17], further suggesting a limited influence of seasonal fluctuations in these extreme habitats.

Microbial eukaryotes in Croatia's geothermal waters, as evidenced by the range of measured temperatures and the demonstrated impact of temperature on community composition, can be recognized as thermotolerant. The highest observed diversity at the TOP, SVM, and D1D sites suggests an optimal growth range for these organisms between 36 °C and 46 °C. The highest recorded temperature (64.8 °C) was found in BIZ, which exhibited only one identified family of microbial eukaryotes (Dinophyceae; Figure S8), in contrast to the study by Oliverio et al. [7], where greater protist diversity was associated with higher temperatures.

Furthermore, neutral to slightly alkaline pH values played a significant role ($p < 0.05$) in shaping the structure of the microbial eukaryotic community in Croatian geothermal waters (Figure 5). For instance, the KRE site had the highest measured pH value (8.3) and the lowest species diversity observed (Figure S8). Here, we observed reduction in protist diversity and spatial dispersion similar to explored extremely alkaline geothermal springs [7,12].

The highest oxygen concentration was associated with the SJA site, which, as expected, had the lowest recorded temperature. However, the abundance and species diversity of microeukaryotes did not increase despite potentially more favorable living conditions (Figure S8). Despite the presence of prokaryotic and some eukaryotic primary producers,

there were sites where anoxia was observed during autumn 2020 (JEZ, KRA, STU, TUH, VAR, and SVM) (Table S1). Such observations may potentially imply the occurrence of anaerobic respiration processes within this habitat, alongside the potential presence of facultative anaerobes or chemosynthetic microorganisms proficient in harnessing oxidized sulfur and nitrogen compounds for energy production, as expounded upon by Oren [50].

4.4. Potential Biotechnological Applications of Eukaryotic Microalgae from Croatian Geothermal Waters

Microalgae are valuable in various biotechnological processes, including bioremediation [47,51]. Microalgae such as *Spirogyra*, certain *Cryptomonas* species, and diatoms such as *Navicula* and *Cymbella* (Figure 3A) play an important ecological role in carbon fixation and oxygen production [52], making them valuable candidates for bioremediation, CO_2 equestration, and biofuel production. Diatoms, in particular, contribute significantly to global oxygen production and have various applications in biofuel production, secondary metabolite synthesis, and the food industry [53]. The thermophilic nature of these organisms makes them particularly well-suited for use in high-temperature industrial processes, providing a natural advantage in biofuel production and wastewater treatment.

Furthermore, microbial eukaryotes such as genus *Tribonema* (Figure 3A) can contribute bioremediation processes by effectively removing nitrates and phosphates from wastewater [54], which is crucial for mitigating nutrient pollution. They can be used in bioremediation [47] to restore water quality in industrial effluents or agricultural runoff.

Although challenges remain because of low culture densities and the microscopic nature of these organisms and could limit their mass utilization in biotechnology, continued research will be needed to address certain issues. This study of Croatian geothermal waters provides a unique opportunity to explore the biotechnological potential of microbial eukaryotes in extreme environments, offering valuable contributions to the fields of sustainable energy and environmental conservation. This research contributes to a broader understanding of microbial eukaryotic diversity in extreme environments and emphasizes the importance of further studies to uncover the functional roles and adaptations of these unique taxa. Given that research on microeukaryotic diversity in geothermal ecosystems remains limited globally [6,7,37,55,56], our findings provide a valuable reference point for future comparative studies.

Future research should focus on identifying environmental factors driving spatial and temporal variations in these ecosystems, particularly through examining interactions between primary producers, mixotrophs, and decomposers. Further studies on the functional roles of these microbial eukaryotes, such as their contributions to biogeochemical cycles and interactions with bacterial communities, would be essential. Experimental approaches like stable isotope tracing and metatranscriptomics could provide deeper insights into nutrient assimilation and energy transfer. Long-term monitoring would also help assess the impact of environmental fluctuations on microbial community structure and ecosystem functioning, deepening our understanding of microbial eukaryotic adaptations in extreme environments.

5. Conclusions

The microbial eukaryote community composition of Croatian geothermal waters in autumn 2020 mirrors global geothermal water communities, encompassing primary producers, mixotrophs, decomposers, heterotrophs, and parasites. Temperature, pH, and nitrate concentrations were identified as the most important parameters shaping microbial eukaryotic communities at five different sites (JEZ, STU, TOP, SVM, and D1D). These sites exhibited the highest microbial eukaryotic compositions among 14 waters studied.

This study highlights potential biotechnological applications of microalgae like *Tribonema* (bioremediation), *Tribonema* and *Chromulina* (biofuel/biodiesel production), and *Cymbella* and *Navicula* (biological indicators). These results provide a basis for studying seasonal and environmental influences on the microbial eukaryotic communities in Croatian geothermal waters, underling their biotechnological potential.

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