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# Eukaryotic microbial diversity of phototrophic microbial mats in two Icelandic geothermal hot springs

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**Summary.** The composition of the eukaryotic community and the three-dimensional structure of diverse phototrophic microbial mats from two hot springs in Iceland (Seltun and Hveradalir geothermal areas) were explored by comparing eukaryotic assemblages from microbial mats. Samples were collected in July 2007 from 15 sampling stations along thermal and pH gradients following both hot springs. Physicochemical data revealed high variability in terms of pH (ranging from 2.8 to 7), with high concentrations of heavy metals, including up to 20 g Fe/l, 80 mg Zn/l, 117 mg Cu/l, and 39 mg Ni/l at the most acidic sampling points. Phylogenetic analysis of 18S rDNA genes revealed a diversity of sequences related to several taxa, including members of the Bacillariophyta, Chlorophyta, Rhodophyta, and Euglenophyta phyla as well as ciliates, amoebae, and stramenopiles. The closest relatives to some of the sequences detected came from acidophilic organisms, even when the samples were collected at circumneutral water locations. Electron microscopy showed that most of the microecosystems analyzed were organized as phototrophic microbial mats in which filamentous cyanobacteria usually appeared as a major component. Deposits of amorphous minerals rich in silica, iron, and aluminium around the filaments were frequently detected. [*Int Microbiol* 2010; 13(1):21-32]

**Keywords:** eukaryotic microbial community · extremophiles · phytobenthos · water chemistry · Icelandic hot springs

## Introduction

The high temperatures associated with geothermal activity frequently result in surface and subsurface geothermal springs, commonly known as “hot springs.” Most of the aquatic habitats related to these areas are considered extreme environments due to the high temperature as well as the low pH of the water.

Acidity is mainly generated by the biological oxidation of elemental sulfur produced as a result of the condensation reactions among sulfur-containing volcanic gases [48]. However, the nature of these geothermal environments is highly dependent on the subsurface geology and on water flow [8]. Particularly important is the mixing percentage between deep volcanically heated water (usually buffered by CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>) and cold shallow ground water, extremely acidified by the microbial and chemical oxidation of sulfur compounds. Thus, geothermal volcanic areas can, in some cases, present an extreme acidic pH (1–3) due to sulfurous liquids and low flow rates, or in other cases a low acidic–neutral–alkaline pH (5–8) due to siliceous liquids and high flow rates [5].

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Although sulfide-rich hot springs with neutral or alkaline pH are relatively rare in most geothermal areas, they frequently occur in Iceland due to high groundwater level and climatic conditions, i.e., melting snow and rain [44]. These hot springs usually contain areas with highly acidic waters (i.e., boiling pots) in close proximity to others characterized by neutral pH. As such, these geothermal areas represent an ideal location for research into extremophilic microbial communities and their relationship to pH and temperature.

Although the microbial ecology of volcanogenic habitats has been widely studied [7,10,25,39], most of the information available about the communities present in these environments has derived from prokaryotic communities of acidic volcanic habitats. Nevertheless, microbial eukaryotes are also present and could play a critical ecological role as well [9,19]. Eukaryotic microbial life actively grows in almost any extreme condition where there is a source of energy to sustain it, except at high temperatures ( $>70^{\circ}\text{C}$ ) [9] and in the deep subsurface biosphere. The development of molecular technologies and their application to microbial ecology has increased our knowledge of eukaryotic diversity in many different environments [11]. This is particularly relevant in extreme environments, which are generally more difficult to replicate in the laboratory.

Recent molecular ecology studies have demonstrated that eukaryotic organisms are exceedingly adaptable and not notably less so than prokaryotes. However, most habitats have not been sufficiently well explored for sound generalizations to be made. In fact, molecular analysis has also revealed novel protist genetic diversity in different extreme environments [4].

Temperature is one of the main factors determining the distribution and abundance of species due to its effect on enzymatic activities [3]. Most extremophiles that survive at high temperatures ( $95\text{--}115^{\circ}\text{C}$ ) are microorganisms from the archaeal or bacterial domains. The highest temperature reported for eukaryotic microorganisms is  $62^{\circ}\text{C}$  [9]. Photosynthetic prokaryotes, such as cyanobacteria, have never been found in hot acidic aquatic systems [8]. Instead, these ecological niches are usually profusely colonized by species of the order Cyanidiales, red unicellular algae [8]. Species from the genera *Galdieria* and *Cyanidium* isolated from hot sulfur springs have an optimal growth temperature of  $45^{\circ}\text{C}$  and a maximum growth temperature of  $57^{\circ}\text{C}$  [50]. These extreme hot springs are usually acidic (pH 0.05–4) and frequently characterized by high concentrations of metals, such as cadmium, nickel, iron, and arsenic, that are highly toxic to almost all known organisms.

Few studies related to eukaryotic biodiversity in Icelandic geothermal areas have been carried out to date, although

there are numerous studies describing prokaryotic, especially archaeal, diversity [22,23,29,30,35] and phototrophic bacteria such as cyanobacteria [12–14,46,47] growing in these environments. To learn more about protist microbial diversity of these extreme geothermal areas, the composition of a eukaryotic microbial community along the thermal and pH gradients of two hot springs in southwestern Iceland was explored. As far as we know, this work is the first description of eukaryotic microbial communities associated with Icelandic hydrothermal areas.

## Materials and methods

**Study sites.** Samples were collected from two different geothermal sites in SW Iceland, the Seltun and Hveradalir hot springs (nine and five sampling locations, respectively), in September 2007. Seltun is one of the four Icelandic volcanic systems that lie along the eruptive fault on Reykjanes peninsula. It comprises a high-temperature geothermal area containing mud pots and steam vents that can reach temperatures above  $200^{\circ}\text{C}$ . [21]. The locations sampled at Seltun are situated along a ca. 300-m stream that originates in the Sveifluhals Highlands (ca. 300 m above sea level). SD1 and SD2 were located along the first 50 m of the stream, and SD3 at the beginning of the geothermal area. At SD4 and SD5, spring water ran off boiling pots and joined the main stream. SD6 was the only bubbling pool sample in this area (ca. 1 m in diameter). SD7 and SD9 were located beyond the hot pools, near the geothermal area exit. SD10 was ca. 2 km from the geothermal area, near Kleifarvatn Lake. For the first 100 m, the stream is of nearly neutral pH, becoming acidic as the waters enter the geothermal field. In this part of the stream, hot acidic water runoffs from numerous boreholes and ponds combine with freshwater flowing down from the hills. The water reaches pH 2–5, with a rise in temperature to  $14\text{--}45^{\circ}\text{C}$ . This geothermal area is characterized by the emission of steam and volcanic gases mainly composed of  $\text{H}_2\text{S}$  and  $\text{H}_2$  [23].

Hveradalir area is also a high-temperature geothermal field, located near the Hengill Mountains (SW Iceland). It is one of Iceland's largest geothermal areas, covering about  $100\text{ km}^2$ , and includes two central volcanoes, Hveragerdi and Hengill [21]. Samples were collected along the 200-m stream that originates in the nearby highlands (ca. 500 m above sea level). Along its first 50 m, the stream is also of nearly neutral pH, with the waters becoming acidic as they enter the geothermal field. As in Seltun, water running off from several mud pots and boreholes combines with the stream, acidifying the water and raising the temperature. Of the five sampling sites, only H1 was outside the geothermal area. H2 and H3 were located at the edge of the geothermal area, and H4 and H5 were streams running off from different boiling pots.

**In situ measurements.** Water variables were measured in situ with a portable multi-meter probe (Multi 197i WTW, Germany). Water samples were collected in 50-ml Falcon sterile tubes, filtered through  $0.45\text{-}\mu\text{m}$  Millipore membranes, and kept at  $4^{\circ}\text{C}$  until their analysis for metal content. Two samples from each cell biofilm were carefully collected by scraping biolayers from the tops of sediments with sterile plastic pipettes and were transported to the laboratory in sterile 5-ml plastic tubes. One of the biofilm subsamples was used for light microscopy and kept at  $4^{\circ}\text{C}$  until the analysis; the other, used for DNA analysis, was kept at  $-20^{\circ}\text{C}$  until needed.

Algae and heterotrophic protists were identified up to the lowest possible taxonomic level by direct microscopy observation using different identification keys [32,41]. The microscope was a Zeiss Axioscope 2 equipped with phase-contrast.

**Water chemical analysis.** The total concentrations of eight recoverable metals were measured for each water sample (Zn, Cu, Fe, Co, Ni, As, Cd, and Cr) using X-ray fluorescence reflection (TXRF) and inductively coupled plasma-mass spectrometry (ICP-MS).  $\text{NO}_3^-$ ,  $\text{PO}_3^{3-}$  and  $\text{SO}_4^{2-}$  concentrations were measured quantitatively at room temperature with a SMART2 colorimeter (LaMotte, USA) using the zinc reduction method (3689-SC), the vanadomolybdophosphoric acid method (3655-SC), and the barium chloride method (3665-SC), respectively. The mineralogy of the solid samples was characterized by powder X-ray diffraction (XRD) using a Philips X'Pert diffractometer (graphite monochromated  $\text{CuK}\alpha$  radiation). XRD patterns were obtained using random powder and air-dried oriented aggregates. The results obtained from physicochemical analysis of the different water samplings were statistically compared by ANOVA using Statistica V.6.0.

**DNA extraction and clone library construction.** The Fast DNA Spin kit for soil extraction (Bio 101, Carlsbad, CA, USA) was used according to the manufacturer's instructions. The cells were disrupted using the mixture of ceramic and silica beads provided in the kit and six 40-s pulses at a speed 5.5 of the FastPrep bead-beating instrument (Bio 101). Biofilm samples were washed five times with TE buffer (10 mM Tris HCl at pH 8, 1 mM EDTA) before DNA extraction in order to remove the natural water.

Clone libraries of complete 18S rDNA genes were generated from the environmental DNA templates. 18S rDNA genes were PCR-amplified using the following general eukaryotic primers: Euk 1a (5'-CTGGTTGATCCTGCCAG-3'), Euk A (5'-AACCTGGTTGATCCTGCCAGT-3') and Euk 20F (5'-GTAGTCATATGCTTGCTC-3') as forward primers, and Euk 1800r (5'-TCCGCAGGTTACCTAGCGA-3') and Euk Br (5'-TGATCCTTCTGCAGGTTACCTAC-3') as reverse primers [1]. The PCR conditions were: initial denaturation at 97°C for 5 min, followed by 29 cycles of denaturation at 94°C for 40 s, annealing at 48°C for 1 min, and extension at 72°C for 1 min. The amplification products were used to construct a clone library with the TOPO 1 Kit (InvitroGen, Carlsbad, CA, USA). Positive transformants were checked for correct insert size by standard agarose gel electrophoresis.

**Sequence analysis.** The PCR products were directly sequenced with a dye terminator cycle sequencing kit (Big-Dye 1.1 sequencing kit, Applied Biosystems) as described by the manufacturer. The sequences were aligned to 18S rRNA sequences obtained from the National Center of Biotechnology Information Database. The sequences were also checked for potential chimeras with the Bellerophon Chimera Check program and subsequently aligned with 18S rRNA reference sequences in the ARB package [<http://www.arb-home.de>]. The rRNA alignment was corrected manually and alignment uncertainties were omitted. Only unambiguously aligned positions were used to construct phylogenetic trees with ARB. Distance analysis using the Jukes-Cantor correction [27] and bootstrap resampling (100 times) were performed, and the distance matrix was used to construct a tree via the neighbor-joining method [43]. Parsimony and maximum-likelihood analysis was carried out using DNAPARS from the PHYLIP package described in [18].

**Scanning electron microscopy in backscattered electron mode (SEM-BSE).** At least two samples (3–4 cm<sup>3</sup>) of characteristic biofilms from each locality were placed in six multiwell plates (Corning, NY, USA). Samples were fixed in the field with 2.5% glutaraldehyde in 1 mM Na-HEPES buffer (pH 7). The samples were kept under cold (5°C) and dark conditions until further processing. In the laboratory, the samples were washed with Na-HEPES buffer and postfixed with 1% osmium tetroxide in distilled water for a minimum of 8 h. Samples were dehydrated using ascending series of ethanol and then infiltrated with LR-white resin for 24 h. The infiltrated samples were polymerized at 65°C for 24 h. The resulting blocks were cut transversally using a diamond saw, fine polished, and carbon-coated following previous protocols [45]. Transverse sections of the polished surfaces of the rocks were examined using a JEOL 5600LV SEM

equipped with a BSE detector and an INCA Oxford microanalytical energy dispersive spectrometer (EDS) system. EDS operating conditions were as follows: 0° tilt angle, 35° take-off angle/EDS, 15 kV acceleration potential, 20 mm working distance.

## Results

**Sample sites environmental conditions.** The main physicochemical variables of the sampling sites are shown in Table 1.

At Seltun locations, the temperature ranged from 8.5°C in the final part of the stream to 90°C in the boiling pools, with the mean temperature of the stream along the geothermal area being ca. 46°C. The pH ranged from ca. 6.5 in the locations outside the geothermal site to ca. 3–5 in the volcanic influence area. At all locations, high conductivity correlated with low pH; thus, the most extreme values were measured at boiling pot SD6 (pH 2.8 and ca. 1800 mS/cm).

Differences in  $\text{NO}_3^-$  concentrations were not statistically significant among sampling points ( $P = 0.073$ ) but were detected for  $\text{PO}_3^{3-}$  and  $\text{SO}_4^{2-}$  ( $P = 0.004$  and  $P = 0.0002$ , respectively). The average  $\text{NO}_3^-$  concentration was  $73.2 \pm 7$  mg/l;  $\text{PO}_3^{3-}$  concentrations ranged from below detection to ca. 8 mg/l, and  $\text{SO}_4^{2-}$  concentrations from 9 to 300 mg/l (Table 1).

At the Hveradalir geothermal site, temperatures ranged from 10 to 55°C, with H5 being the most acidic of the five sites studied (Table 1). The remaining locations had a pH of ca. 6. As in Seltun, the sampling point with the lowest pH (H5) also showed the highest redox and conductivity values. Differences in the  $\text{NO}_3^-$  concentrations among the sampling points were not statistically significant, in contrast to the significant differences in  $\text{PO}_3^{3-}$  and  $\text{SO}_4^{2-}$  ( $P = 0.02$  and  $P = 0.0013$  respectively), which reached values up to ca. 5 and 400 mg/l, respectively.

The concentrations of nine heavy metals and cations were also measured (Table 2) in the water samples. At both geothermal areas, the highest values were recorded in the sampling points located at the boiling pots or near them (i.e., SD6 at Seltun and H4 and H5 at Hveradalir), where the lowest pH occurred. The most abundant elements were Al and Fe, which reached concentrations of ca. 25 g/l at SD6 in Seltun, while As and Cd showed the lowest concentrations, <0.5 g/l in all cases.

**Clone libraries and phylogenetic analysis of the 18S rDNA environmental sequences.** Most of the samples resulted in the amplification of eukaryotic 18S rDNA genes, except for the samples collected from the SD6 boiling pot, the gray filamentous biofilms collected at SD5,

**Table 1.** Means and standard errors of water physical variables as well as a description of the biological samples collected at each sampling point

Site	Sample description	pH	Cond <sup>a</sup>	Redox <sup>a</sup>	Temp <sup>a</sup>	NO <sub>3</sub> <sup>a</sup>	PO <sub>3</sub> <sup>a</sup>	SO <sub>4</sub> <sup>a</sup>
<b>Seltun</b>								
SD1		7.0 ± 0.28	190 ± 12	135 ± 8	15 ± 0.3	61 ± 1.2	2.6 ± 0.5	35 ± 1.4
SD1-1	Green microbial mat							
SD1-2	Green filamentous							
SD1-3	Brown long filamentous							
SD1-4	Green filamentous							
SD1-5	Thin green microbial mat							
SD2		6.2 ± 0.11	288 ± 10	7 ± 0.3	15 ± 0.2	79 ± 2.8	6.2 ± 0.3	200 ± 9.4
SD2-1	Thick green microbial mat							
SD2-2	Thin greenish microbial mat							
SD3		6.1 ± 0.13	358 ± 14	65 ± 4	25 ± 0.4	NA <sup>b</sup>	NA	NA
SD3-1	Thick green microbial mat							
SD3-2	Brown microbial mat							
SD4	Yellow microbial mat	6.4 ± 0.21	660 ± 21	165 ± 9	45 ± 0.5	NA	NA	NA
SD5		6.5 ± 0.09	404 ± 11	86 ± 8	47 ± 0.7	81 ± 2.5	8.2 ± 0.51	40 ± 11.5
SD5-1	Gray filamentous							
SD5-2	Green microbial mat							
SD5-3	Black-green microbial mat							
SD6		2.8 ± 0.03	1825 ± 33	354 ± 11	90 ± 0.6	NA	NA	NA
SD6-1	Bubbling pool water							
SD6-2	Green microbial mat at the edges							
SD7	SD7	5.1 ± 0.11	98 ± 9	330 ± 10	25 ± 0.4	76 ± 3.2	BD <sup>b</sup>	23 ± 5.3
SD7-1	Brown long filamentous							
SD7-2	Thin green microbial mat							
SD9		3.6 ± 0.12	548 ± 17	403 ± 13	26 ± 0.6	71 ± 2.5	BD	300 ± 20.7
SD9-1	Green filamentous							
SD9-2	Thin green biofilm							
SD10	Brown biofilm	7.4 ± 0.90	68 ± 7	168 ± 15	8.5 ± 0.4	71 ± 1.9	BD	9 ± 1.8
<b>Hveradalir</b>								
H1	Green microbial mat	6.6 ± 0.38	136 ± 10	148 ± 9	10 ± 0.7	71 ± 2.2	2.1 ± 0.3	84 ± 9.5
H2	Green-black mat	6.5 ± 0.32	251 ± 11	35 ± 3	20 ± 0.5	76 ± 4.2	BD	23 ± 4.8
H3	Green microbial mat	6.3 ± 0.14	247 ± 9	34 ± 6	20 ± 0.6	74 ± 2.8	BD	53 ± 9.7
H4		6.3 ± 0.21	292 ± 13	90 ± 8	55 ± 0.3	74 ± 3.3	4.6 ± 0.4	150 ± 10.6
H4-1	Gray filamentous							
H4-2	Green long filamentous							
H5	H5	3.4 ± 0.08	676 ± 15	450 ± 17	22 ± 0.7	74 ± 1.9	0.1 ± 0.05	400 ± 18.5
H5-1	Green filamentous							
H5-2	Green microbial mat							

<sup>a</sup> Cond: conductivity in mS cm<sup>-1</sup>. Redox: redox potential in mV. Temp: Temperature in °C. NO<sub>3</sub> and SO<sub>4</sub> in ppm. PO<sub>3</sub> in mg/l.

<sup>b</sup> NA: not analyzed. BD: below detection.

and the samples at H4, despite the use of various PCR conditions and primers (decreased annealing temperature and larger number of cycles). Twenty four clone libraries were constructed with a total of 950 clones that were sequenced and identified with BLASTN searches. Sequences were

manually edited and operational units (OTUs or phylotypes) were created by pairwise similarity calculations using a 98% DNA-DNA similarity. Between one and five representative sequences of each OTU aligned with their 10 closest relatives.

**Table 2.** Mean and standard error of metal concentrations (mg/l) at each sampling site

Location	Cr	Fe	Co	Ni	Cu	Zn	As	Cd	Al
<b>Seltun</b>									
SD1	2.3 ± 0.4	BD <sup>a</sup>	BD	1.87 ± 0.4	12.1 ± 2	13.7 ± 0.9	BD	BD	40.4 ± 9
SD2	3.6 ± 0.3	BD	1.0 ± 0.02	2.50 ± 0.3	0.5 ± 0.01	3.50 ± 0.7	BD	BD	5.90 ± 1.1
SD3	8.7 ± 0.5	313 ± 11	1.0 ± 0.01	2.60 ± 0.2	0.4 ± 0.01	1.40 ± 0.2	0.06 ± 0.01	BD	1.80 ± 0.4
SD4	1.4 ± 0.3	5.9 ± 1.1	0.09 ± 0.01	1.40 ± 0.3	0.20 ± 0.1	2.90 ± 0.6	BD	BD	22.4 ± 2.9
SD5	0.9 ± 0.1	6.8 ± 0.5	0.07 ± 0.01	0.69 ± 0.1	0.2 ± 0.02	3.70 ± 0.3	BD	BD	23.9 ± 3.1
SD6	27.8 ± 2.0	21449 ± 26	34.9 ± 1.50	39.2 ± 2.1	117.9 ± 7	78.2 ± 2.8	BD	0.03 ± 0.01	26632 ± 56
SD7	8.3 ± 1.1	3874.1 ± 33	4.10 ± 0.9	7.70 ± 0.8	5.70 ± 0.7	8.00 ± 1.2	BD	BD	2937 ± 12
SD9	4.9 ± 0.7	7850.2 ± 26	12.7 ± 0.5	23.3 ± 6.1	31.4 ± 1.5	42.4 ± 9.0	0.07 ± 0.01	0.006 ± 0.001	4175 ± 26
SD10	0.86 ± 0.1	143.2 ± 5.8	BD	0.70 ± 0.1	1.0 ± 0.01	0.50 ± 0.01	BD	BD	44.10 ± 3.8
<b>Hveradalir</b>									
H1	3.6 ± 1.1	3.2 ± 0.4	0.01 ± 0.002	1.60 ± 0.2	0.2 ± 0.01	6.9 ± 0.5	0.36 ± 0.01	BD	2.90 ± 0.1
H2	3.7 ± 0.9	BD	1.40 ± 0.30	6.10 ± 0.5	0.2 ± 0.01	16.8 ± 2.0	0.34 ± 0.02	BD	6.60 ± 0.6
H3	4.2 ± 0.4	BD	0.90 ± 0.10	4.10 ± 0.1	0.1 ± 0.02	8.5 ± 2.1	BD	BD	4.80 ± 1.1
H4	0.26 ± 0.1	614 ± 2	3.60 ± 0.10	3.50 ± 0.2	0.7 ± 0.02	25.4 ± 2.0	BD	BD	205 ± 9.2
H5	2.9 ± 0.2	2855 ± 33	4.60 ± 0.20	10.6 ± 0.2	5.50 ± 0.3	29.7 ± 3	0.02 ± 0.01	0.007 ± 0.001	5909 ± 78

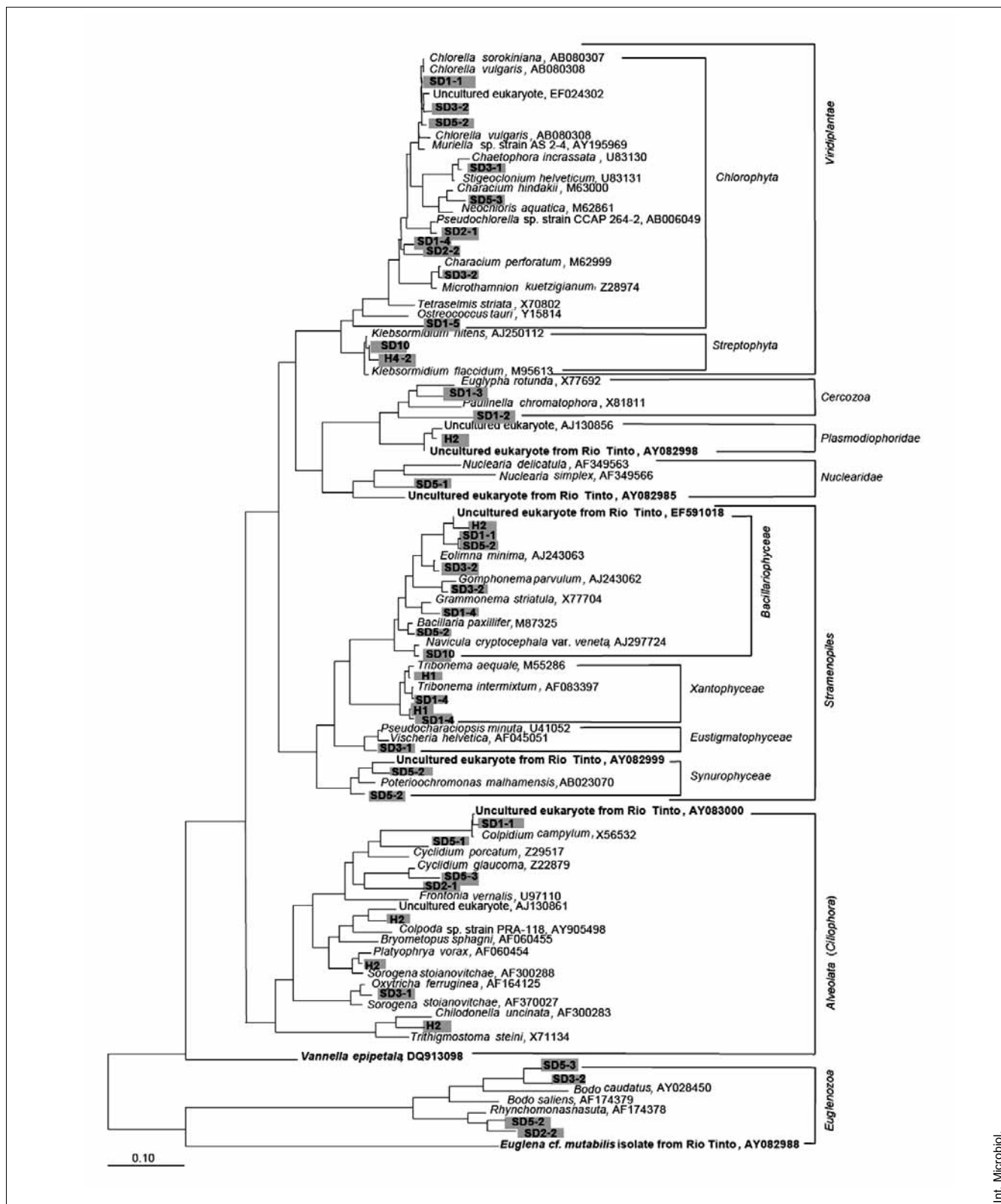
<sup>a</sup>BD: below detection.

Figures 1 and 2 show the results of phylogenetic analysis with representative rDNA gene sequences from the neutral and acidic pH sampling sites, respectively. Analysis of the constructed phylogenetic trees revealed that eukaryotic sequences were widespread and occurred over a wide range of pH and temperature. The sequences branched among 10 eukaryotic families in circumneutral water sampling stations (Fig. 1). Phylotypes related to green algae, red algae, ciliates, amoebas, diatoms, and small flagellates, such as cercozoas or bodos, were identified. In all cases, the obtained sequence affiliations provided results that were consistent with the microscopy observations. Chlorophytes, such as phylotypes related to the genera *Chlorella* and *Chaetophora*, were predominant, but several phylotypes related to diatoms (*Gomphonema*, *Bacillaria*, and *Navicula* genera) and a dozen other phylotypes related to ciliates (*Colpidium*, *Cyclidium*, or *Frontonia* genera), filamentous algae (*Tribonema* genus), or flagellates (*Bodo* and *Rhynchomonas* genera) were detected as well. Some of these sequences were similar to those of eukaryotic species identified in acidic environments. In addition, sequences related to different vascular plants from the surrounding vegetation, not included in the analysis, were found as well.

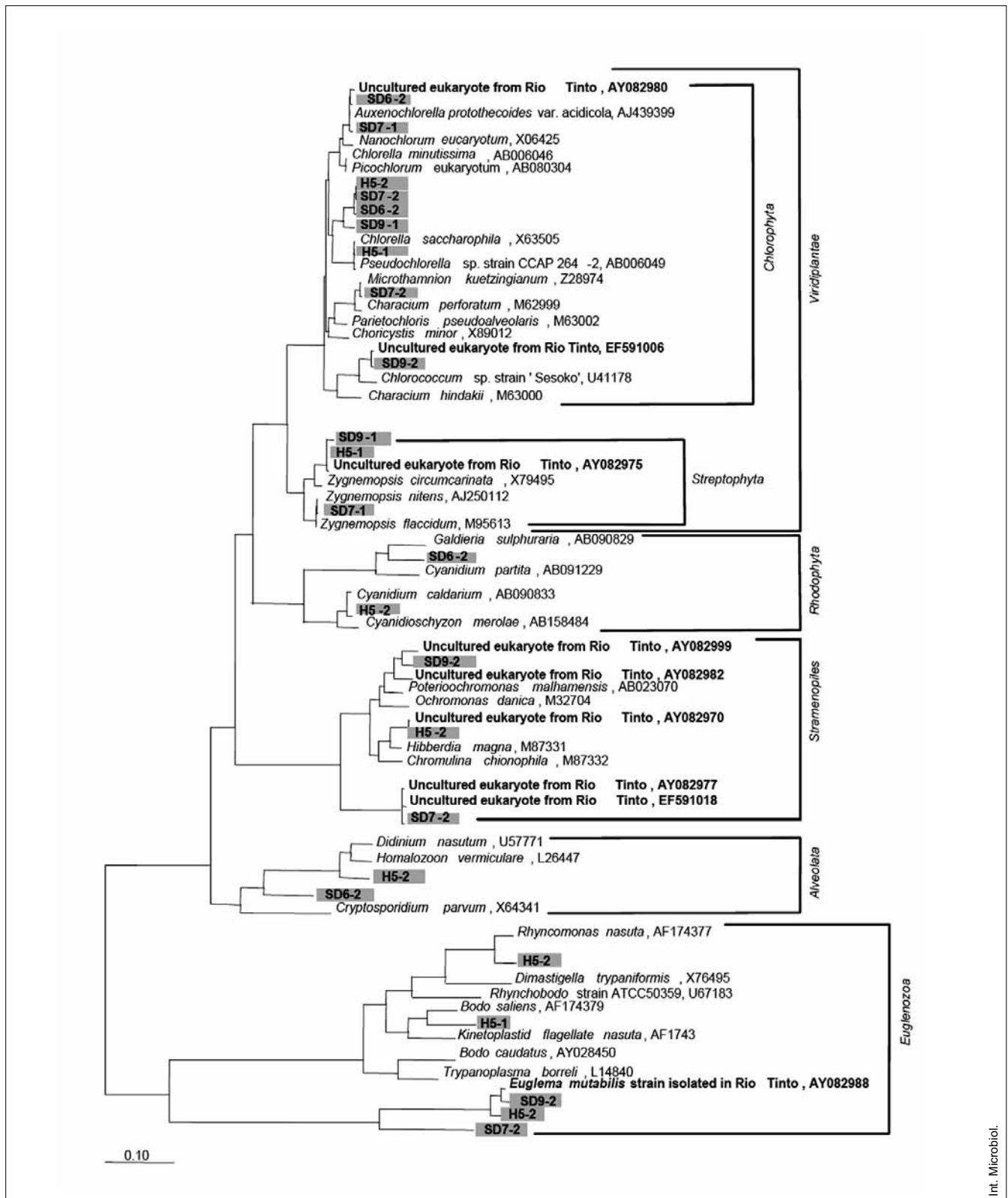
At the acidic sampling points (Fig. 2), the diversity of sequences related to Chlorophytes was higher than that recorded at circumneutral sampling locations. In this case, however, *Chlorella* sequences related to acidophilic species

were also detected. Although only one species similar to *Pinnularia* sp. was identified by microscopy, diatom rRNA genes were not found. Sequences related to autotrophic and heterotrophic flagellates were more varied and included those similar to *Ochromonas* and *Chromulina*, besides the *Bodo* and *Rhynchomonas* species. In addition, sequences related to red algae (genus *Cyanidium*) and *Euglena* were detected.

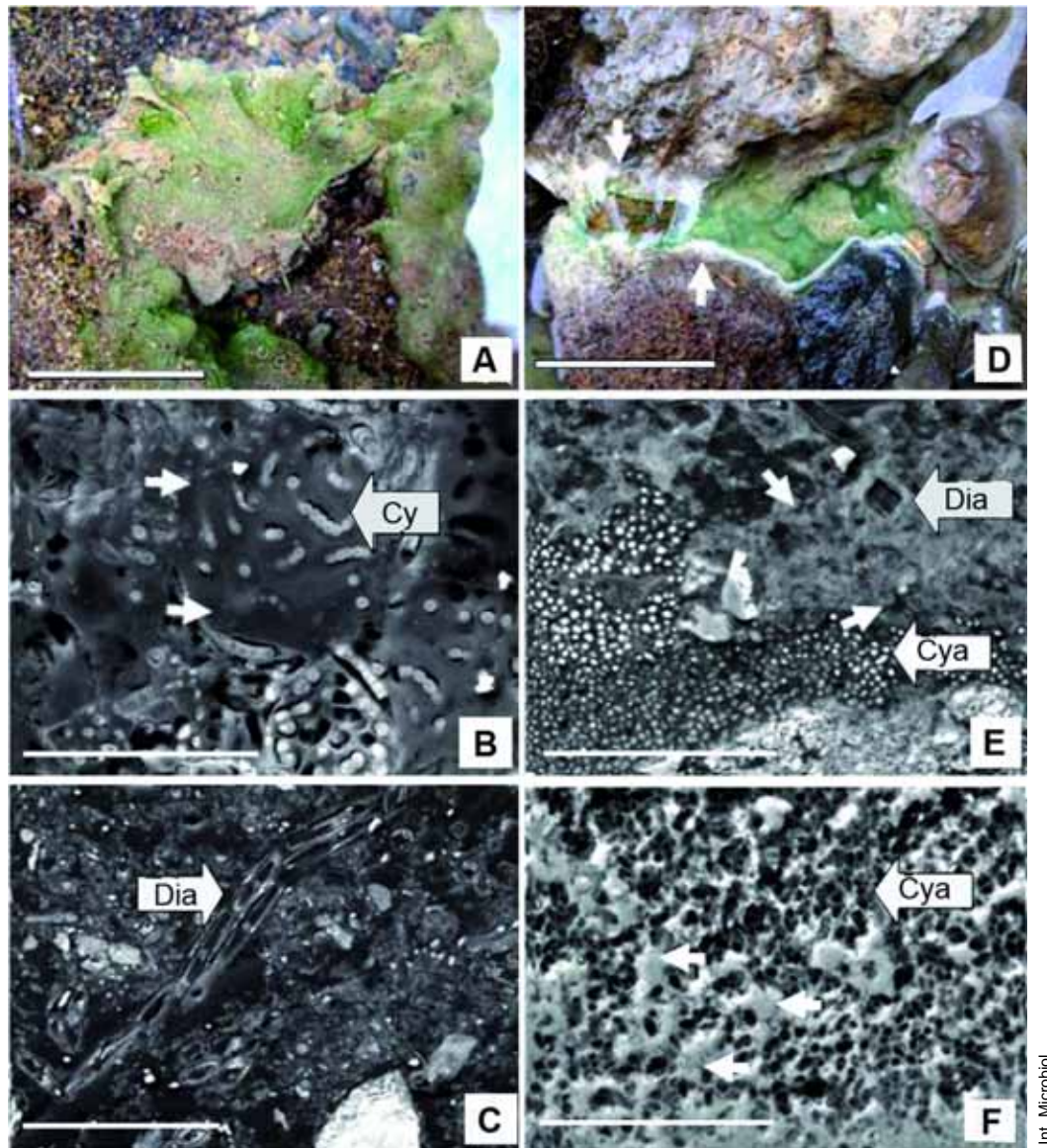
Microscopic structure of phototrophic biofilms. SEM-BSE images of phototrophic biofilms are provided in Figs. 3 and 4. Macroscopically, all selected biofilms clearly differed from each other and could be considered representative of the microbial communities in the area where they were collected. Different types of microbial mats are shown in the figures. The first type was situated outside the geothermal area (SD2 sampling point), where thin green microbial mats mingled with the sediments (Fig. 3A). These mats were mainly dominated by filamentous cyanobacteria similar to *Mastigocladus* spp. communities and were surrounded by a large quantity of extracellular polymeric substances (arrows in Fig. 3B) that aggregated the sediments. Diatoms and some green algae and protists were also recognized within the microbial mat communities (Fig. 3C). The second type of microbial mat was usually located at the beginning of the geothermal area (SD3 sampling point, Fig. 3D). These mats were thin greenish films covered mostly with a



**Fig. 1.** Phylogenetic tree based on 18S rRNA sequences from circumneutral water sampling locations (SD1, SD3, SD5, S10 and H1, H2). The tree was inferred by neighbor-joining analysis of approximately 500 homologous positions of 18S rDNA sequence. New isolates are shaded. Published sequences from organisms isolated in Rio Tinto are indicated in bold.



**Fig. 2.** Phylogenetic tree based on 18S rRNA sequences from acidic water sampling locations (SD6, SD7-8, SD9 and H5). The tree was inferred by neighbor-joining analysis of approximately 500 homologous positions in the 18S rDNA sequence. New isolates are shaded. Published sequences from organisms isolated in Rio Tinto are indicated in bold.



**Fig. 3.** (A) Microbial mat collected at SD2-1, upstream from the geothermal area. Cyanobacterial mats developed intertwined with stream sediments. Bar 5 cm. (B) SEM-BSE image of the typical appearance of the cyanobacteria communities. The filamentous cyanobacteria (Cy) and the amount of EPS (arrows) around the cells contribute to aggregate the sediments. Bar 50  $\mu\text{m}$ . (C) SEM-BSE image of the sediments intertwined with diatoms (Dia). Bar 100  $\mu\text{m}$ . (D) Microbial mat collected at SD3-2, a thin greenish biofilm at the edge of the geothermal area. The mats at the surface of the rocks were covered by a whitish mineral precipitate (arrows). Bar 10 cm. (E) SEM-BSE image of a mat made up mostly of diatoms (Dia) and *Cyanidium* (Cya) surrounded by an amorphous mineral precipitate rich in Fe and Al (arrows). Bar 50  $\mu\text{m}$ . (F) SEM-BSE image of the *Cyanidium* (Cya) cells embedded in the mineral precipitates. Bar 100  $\mu\text{m}$ .

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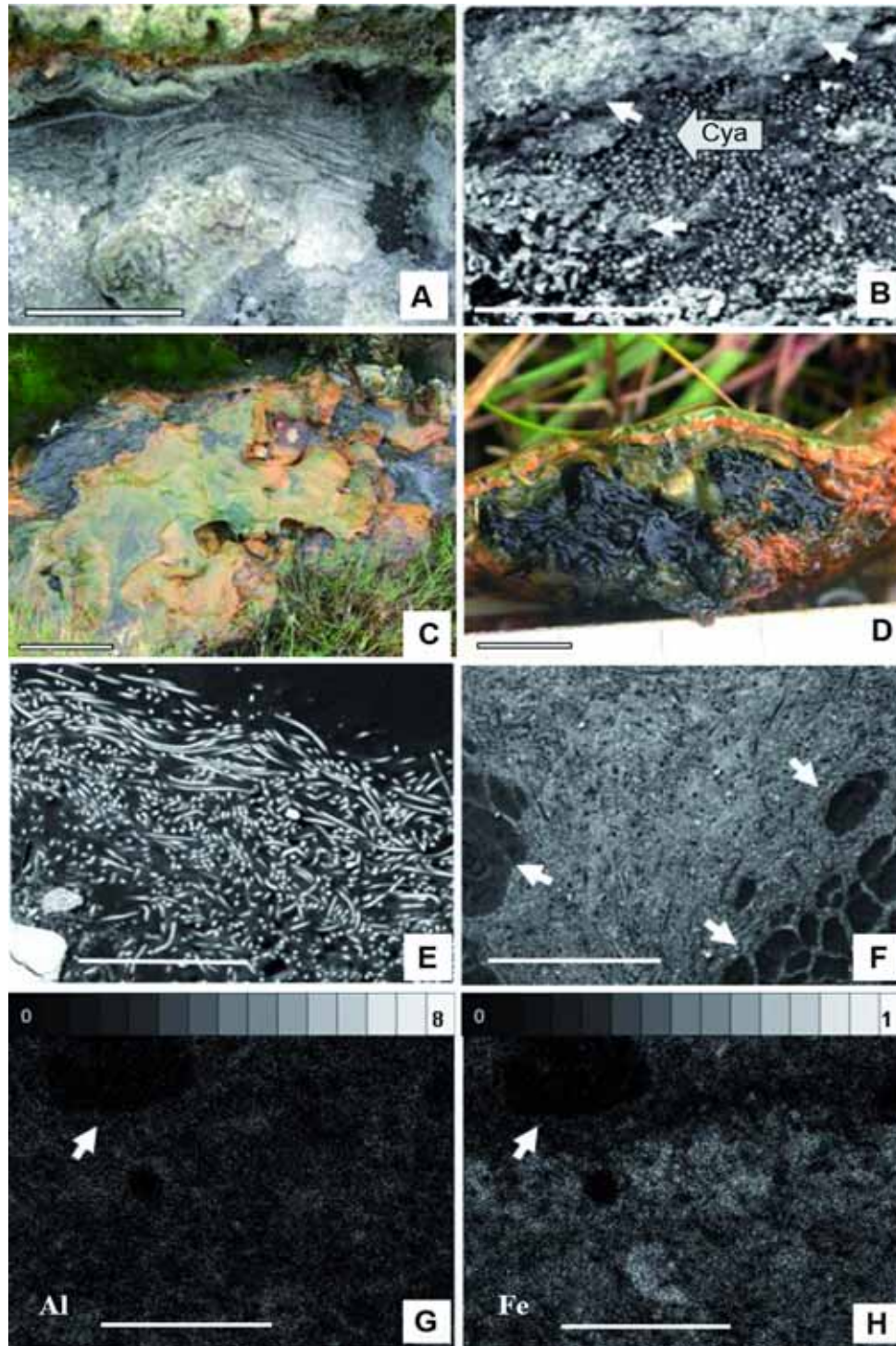
white precipitate (arrows in Fig. 3D). The biomass was mainly formed by diatoms and *Cyanidium* cells with a few protists (Fig. 3E) and was mixed with an amorphous mineral precipitate rich in Fe and Al (arrows in Fig. 3F).

The third type of microbial mat consisted of gray filaments (SD5 sampling point, Fig. 4A) mainly formed by the deposition of minerals on *Cyanidium* cells (Fig. 4B) along the streams running from the hot spots. Protists embedded in

the precipitates were also seen in this type of microbial community.

In all sites, the microbial mats in closest contact with the hot geothermal waters were green-black. At Hveradalir, they covered almost the entire streambed and in some cases were up to several centimeters thick (Fig. 4C,D). In these mats, three different layers could be differentiated: (i) an upper green layer, mainly formed by mat-like dense aggregates of





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**Fig. 4.** Microbial mat collected at SD5 and H2. (A) Microbial mat collected at SD5-1 flowing from a geothermal pot. The microbial communities are covered by a whitish mineral precipitate rich in Si and Al. Bar 5 cm. (B) SEM-BSE image of the *Cyanidium* (Cya) communities present in this SD5 biofilm between precipitated mineral layers (arrows). Bar 50 μm. (C) Cyanobacterial microbial mat along the stream at H2 sampling point. Bar 5 cm. (D) Cross section of the microbial mat, with three different layers, green, brown and black. Bar 1 cm. (E) SEM-BSE image of the upper green layer mainly formed by *Chloroflexus* filaments; several filaments of cyanobacteria related to *Phormidium* genera are also observed under light microscopy. The filaments form a dense layer several-millimeter thick. Bar 100 μm. (F) SEM-BSE image of the brown layer of the microbial mat. An amorphous material rich in Fe and Al surrounds the filaments. Bar 100 μm. (G) EDS aluminum (Al) digital map between the brown and black layer. Concentration range is indicated with scale. Maximum value is 8 weight percent. Bar 100 μm. (H) EDS iron (Fe) digital mapping of the same area shown. Concentration range is indicated with scale. Maximum value is 12 weight percent. Bar 100 μm.

filamentous cyanobacteria and *Chloroflexus*-like filaments (Fig. 4E); (ii) a brown layer consisting of amorphous mineral deposits around the filaments and rich in silica, iron, and aluminum (Fig. 4E); (iii) and the lower black layer, which consisted of iron deposits. The structure of the living filaments could not be recognized in the brown and black layers. In the images of these layers, the degraded organic material appears as dark areas without electron contrast (arrows in Fig. 4G,H).

## Discussion

Icelandic geothermal areas offer a rare opportunity to study microbial diversity under a wide range of physicochemical conditions. They usually contain highly acidic waters in close proximity to waters with neutral pH. Without taking pH into account, the source waters of both studied areas were chemically similar to one another. The main differences corresponded to conductivity, which was slightly higher in Seltun than in Hvelaradalir, probably due to differences in the concentrations of dissolved heavy metals, such as aluminum or iron, which were more abundant in Seltun waters.

The values of most physicochemical variables considered during this study were not in agreement with levels reported for waters from other volcanic areas, most likely due to the lower pH usually characteristic of these environments [5,34,42]. However, heavy metal concentrations were higher than those reported for the other volcanic areas, with average concentrations reaching ca. 15 mg/l for Zn and Cu and ca. 5 mg/l for Cr and Co. In fact, the average concentrations of Ni and Cr were higher than those reported for Río Tinto (SW, Spain), considered one of the most extreme acidic rivers worldwide [2].

The analyses of 18S rRNA gene sequences from mat samples indicated that, although sequences from phototrophic organisms dominated the clone libraries, they were limited mostly to chlorophytes and stramenopiles in the circumneutral sampling locations. Dominant chlorophyte genes included those similar to *Chlorella*, *Chaetophora*, and *Characium* spp. In addition, major stramenopiles sequences were related to pennate diatoms, such as *Gomphonema*, *Navicula*, and *Bacillaria* spp., as well as filamentous algae related to the genera *Tribonema*. The stramenopiles include previously heterotrophic organisms that acquired a chloroplast during their evolution [33].

Among the strictly heterotrophic small eukaryotes, a wide variety of Ciliophora-related sequences were also identified. Sequences related to the genera *Cyclidium*, *Frontonia*, *Platyphrya*, and *Chilodonella* were detected. More ubiquitous

species of ciliates were also found in these areas as well. Phylotypes related to the *Colpidium* or *Oxytricha* recorded in this study also have been found in marine environments and acidic rivers [1,17]. Cercozoa, a complex group of eukaryotes including some of the most abundant non-photosynthetic amoeba and flagellates, were represented with sequences of amoebas related to the genus *Euglypha* or *Nuclearia*. Their morphological, ecological, and genetic diversity is enormous and they are present in a wide variety of environments [28,40].

Some of the sequences detected in these circumneutral areas were closely related to acidophilic eukaryotes found in extremely acidic environments, i.e., amoebas, diatoms, ciliates, and flagellates (Fig.1; accession numbers AY082985, AY082998, AY082999, AY083000). The opposite phenomenon, the discovery of lineages closely related to neutrophilic eukaryotes in acidic environments, was described in two studies [2,4], both of which highlighted the adaptive capacity to variable conditions of pH displayed by these organisms, thus extending the range of these taxa to much higher pH (6.5) conditions.

The eukaryotic microbial diversity in our samples was lower at the acidic locations in these environments. Chlorophytes were well represented, and sequences related to different species of *Chlorella* spp. were found. These species have been described in volcanic acidic areas as well as in low-temperature streams and hydrothermal environments [9,24,31]. Their analysis suggested that acid tolerance among chlorophytes evolved many times over, such that these species are well-adapted to acidic habitats as long as light is present [6]. Other predominant species included the filamentous green algae *Zygnemopsis* spp., the red algae *Cyanidium* spp., and the protist *Euglena mutabilis*, all of them found in abundance in acidic environments [2,36,49]. In addition to ciliates, well known for tolerating acid conditions [37], we also detected sequences from heterotrophic kinetoplastid flagellates similar to *Bodo* or stramenopiles similar to other chrysophyte sequences related to *Chromulina*, *Ochromonas*, and *Poterioochromonas* taxa.

Several well-known acidophilic algae were missing from our clone library, although light microscopy revealed their presence in acidic samples. Not a single sequence of diatoms was detected, although morphological species similar to *Pinnularia* spp., a highly acidophilic species, were observed using light microscopy. Findings based only on an 18S rDNA library and not confirmed by other techniques should be interpreted with caution due to the limitations of molecular techniques when applied to environmental samples [1].

Most of the microecosystems analyzed in this study were organized in phototrophic microbial mats in which filamen-

tous cyanobacteria were usually the major components. Although taxonomic analysis of these organisms lies beyond the scope of this study, the main species identified in our samples belonged to the genera *Phormidium*, *Chloroflexus*, *Oscillatoria*, and *Mastigocladus*, in agreement with previous studies carried out in this geographic area [12,26].

The variety of colors and textures found in hot springs microbial mats is due to the complexity of their microbial components. The use of molecular tools in the ecological study of protists overcomes the difficulty of distinguishing, identifying, and enumerating individual species in aquatic ecosystems. However, they should be used in combination with techniques able to document the presence and amount of protists in relation to other members of microbial communities in natural habitats of ecological importance. Confocal laser scanning microscopy techniques allow detailed observation of protozoa in complex biofilm communities using specific stains [38]. In this study, we tried a different approach, previously applied in other microbial mats [15,51] and biofilms [2]. Using scanning microscopy, we observed that these microbial communities, along with a few heterotrophic protists, formed extremely dense, compact, and complex microbial-mineral assemblages. The biofilms investigated here differed from each other in terms of thickness and number of layers, with the differences probably related to the different species composition. In addition, despite current knowledge that cyanobacterial mats promote sediment accretion by selectively incorporating sediment particles [15], such depositions were not observed.

Extracellular polymeric substances (EPS) closely related to the cells were also detected. Although the role of EPS in microbial mats is not yet completely understood, these substances may be involved in attaching microorganisms to the substrate and in forming a protective matrix, thus enabling microorganisms to survive under severe environmental conditions [2,16,20].

Icelandic geothermal areas are dynamic, challenging environments that offer multiple opportunities for fundamental studies in microbial ecology. In fact, most of these areas contain habitats with very different physicochemical conditions in close proximity. We are only beginning to understand the diversity of the often neglected organisms living in such extreme environments. Accurate *in situ* information regarding the identity and distribution of algae and other eukaryotic organisms provides the basis for realistic descriptions of the ecology of these extreme ecosystems.

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