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ORIGINAL PAPER

Comparison of the microbial communities of hot springs waters and the microbial biofilms in the acidic geothermal area of Copahue (Neuquén, Argentina)

María Sofía Urbieta · Elena González-Toril · Ángeles Aguilera Bazán · María Alejandra Giaveno · Edgardo Donati

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Abstract Copahue is a natural geothermal field (Neuquén province, Argentina) dominated by the Copahue volcano. As a consequence of the sustained volcanic activity, Copahue presents many acidic pools, hot springs and solfataras with different temperature and pH conditions that influence their microbial diversity. The occurrence of microbial biofilms was observed on the surrounding rocks and the borders of the ponds, where water movements and thermal activity are less intense. Microbial biofilms are particular ecological niches within geothermal environments; they present different geochemical conditions from that found in the water of the ponds and hot springs which is reflected in different microbial community structure. The aim of this study is to compare microbial community diversity in the water of ponds and hot springs and in microbial biofilms in the Copahue geothermal field, with particular emphasis on *Cyanobacteria* and other photosynthetic species that have not been detected before in Copahue. In this study, we report the presence of *Cyanobacteria*, *Chloroflexi* and chloroplasts of eukaryotes in the microbial biofilms not detected in the water of the ponds. On the other hand, acidophilic bacteria, the predominant species in the water of moderate temperature ponds, are almost absent in the microbial biofilms in spite of having in some cases similar temperature conditions. Species affiliated with *Sulfolobales* in the *Archaea* domain are the predominant microorganism in high temperature ponds and were also detected in the microbial biofilms.

Keywords Geothermal field · Acidic environment · Microbial biofilms · Extreme environments diversity

Introduction

The Copahue geothermal area is located in the Cordillera de Los Andes in the North West of Neuquén province (Patagonia) Argentina. The map in Fig. 1 shows the location of the study area in Argentina and the sampling sites. Copahue is crowned by the still active Copahue volcano, located 2965 m above sea level. Approximately 100 m below the crater there are two hydrothermal springs that are the source of Río Agrio, an acidic river that flows down the Copahue–Caviahue valley. Associated with the sustained volcanic activity, the area presents geothermal activity, which is reflected in ponds, pools, hot springs and solfataras with different pH, and temperature conditions. The Copahue geothermal area has very interesting geochemical features; especially attractive for studying prokaryotic biodiversity are the elevated acidity of the aquatic environments (Río Agrio and the springs) and the accumulation of different sulphur and iron compounds and minerals (Mas et al. 1996;

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M. S. Urbieta (✉) · E. Donati
CINDEFI (CCT La Plata-CONICET, UNLP), calle 50 entre 115 y 116 N° 227 La Plata, Buenos Aires, Argentina
e-mail: msurbieta@biol.unlp.edu.ar

E. González-Toril · Á. A. Bazán
Centro de Astrobiología (INTA - CSIC), Carretera de Ajalvir Km. 4, 28850, Torrejón de Ardoz, Madrid, Spain

M. A. Giaveno
Dpto. Química, Facultad de Ingeniería, Universidad Nacional del Comahue. IDEPA (CONICET-UNCo), Buenos Aires 1400, 8300 Neuquén, Argentina

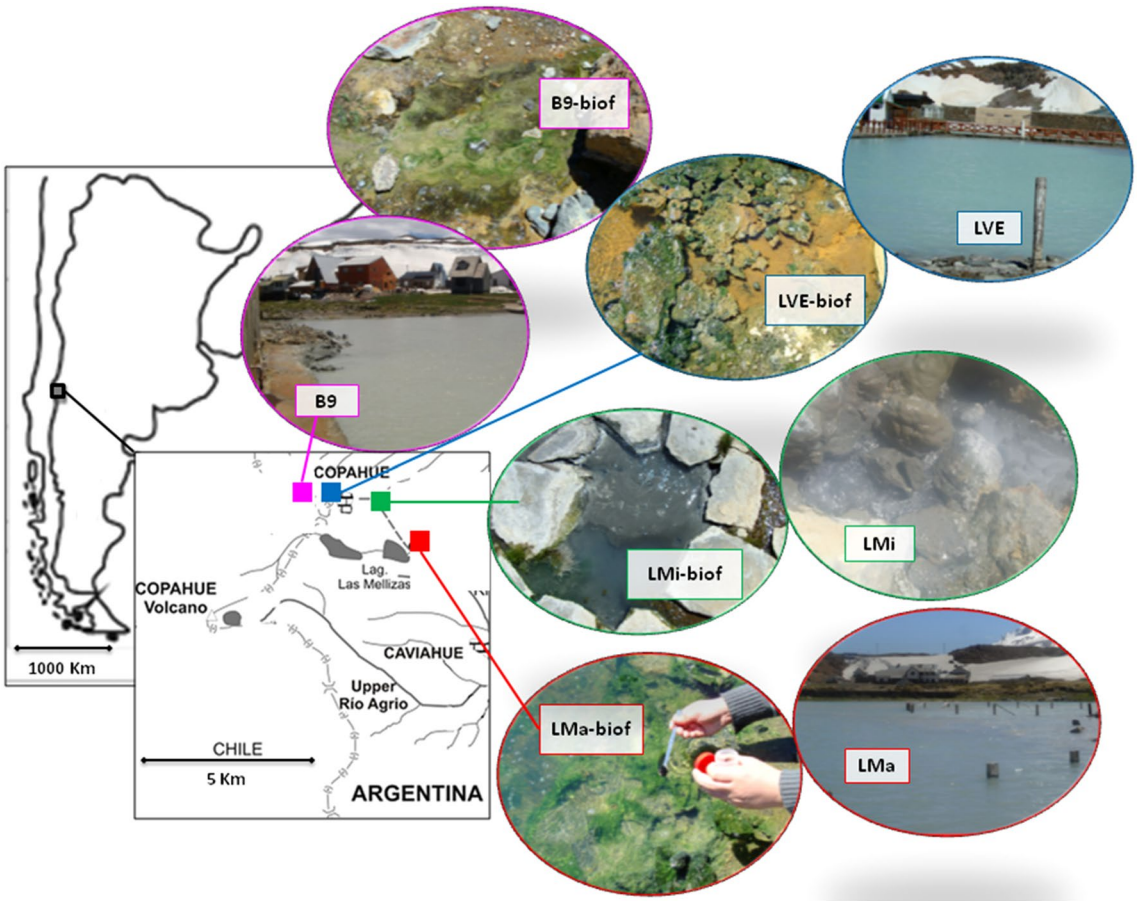

Fig. 1 Location of the Copahue geothermal springs and photographs of the sampling sites

Parker et al. 2008; Varekamp et al. 2009). These extreme conditions impact on the microbial community that colonise the different habitats found in Copahue (Urbieta et al. 2014). In the past few years our research group has been studying the prokaryotic biodiversity in Copahue by multiple approaches, either by using molecular ecology techniques, such as FISH and 16S rRNA gene sequencing, on environmental samples (Urbieta et al. 2014, 2012), or by enrichment cultures searching for acidophilic bacteria (Lavalle et al. 2005; Chiacchiarini et al. 2010) or novel thermoacidophiles, like the recently presented archaea *Acidianus copahuensis* (Giaveno et al. 2013). Our results in previous studies on water samples from Río Agrio and the geothermal ponds showed the presence of different acidophilic bacteria and archaea, most of them related to sulphur and iron cycles. So far, the microbial community that inhabits acidic microbial biofilms in Copahue has not been studied.

Microbial biofilms are common in diverse habitats, from soil, terrestrial and coastal habitats (Cohen and Gurevitz 1992), to others with extreme conditions, such as hypersaline areas, alkaline lakes or hot springs (Ward et al. 1989), although there are not many reports of microbial mats or biofilms from acidic environments. Microbial biofilms and mats in hot springs commonly occur as gelatinous or calcareous mats of several centimetres in thickness and often with various colours. The uppermost layer is generally composed of photosynthetic *Cyanobacteria* and diatoms and the orange, yellow, red or flesh colour layer usually of filamentous bacteria, primarily *Chloroflexi* (Ward et al. 1998). *Cyanobacteria* from geothermal springs can produce extracellular pigments capable to prevent the cells from UV radiation damages and generally are the primary producers in these kind of habitats where grazing is limited (Roeselers et al. 2007). In this way bacteria inhabiting in the deeper layers benefit from this shield and also from the carbon compounds synthesised by *Cyanobacteria* (Stal 2000). Environmental factors such as temperature or water chemistry may affect the species composition and community structure of hot spring microbial biofilms and mats (Skirnisdottir et al. 2000). For instance, *Cyanobacteria* are not usually observed in hot springs with pH below 4.0, and their ability to develop in acidic environments has been questioned by several authors (Brock 1973; Steinberg et al. 1998; González-Toril et al. 2003).

The aim of this study is to compare the prokaryotic biodiversity found in the water of the ponds and hot springs and in microbial biofilms, highlighting the presence of photosynthetic species as important members of the prokaryotic community in microbial biofilms in the Copahue geothermal springs.

Materials and methods

Sample collection and physicochemical determinations

Samples of water and microbial biofilms were collected in December 2009 from four different ponds in the Copahue geothermal area. The four ponds were selected to represent the different physicochemical conditions, mainly temperature and pH, found in the Copahue geothermal area. Temperature and pH were measured in situ with a Hanna HI 8424 NEW portable instrument properly calibrated against calibration standards.

Samples for chemical analysis

The samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS). None of the most relevant elements related with volcanic origin or geothermal activity (Mg, Ca, Mn, Ni Cu, Pb, Cr, Zn, Cd, B, As) were detected in concentrations over 1.0 mg/L. The concentration of sulphate was determined by a turbidimetric method using an excess of barium chloride.

Water samples for DNA extraction

Approximately 1 litre of water was collected in sterile plastic jars and kept on ice. As soon as possible samples were filtered through 0.22 µm Millipore membranes. Filtrates were used for chemical analysis and material retained on membranes for DNA extraction. Filters were washed with pH 2 sterile water and TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) to remove any acidic water containing heavy metals that may cause DNA hydrolysis (Herrera and Cockell 2007). Filters were stored at −20 °C until further processing.

Water samples for fluorescence in situ hybridisation

Samples were fixed on the field. Between 200 and 500 µL of water sample were incubated with the corresponding volume of paraformaldehyde (PFA) to reach a 4 % final concentration. Samples were incubated for 4–12 h, then diluted in approximately 15 mL of pH 2 sterile water and finally filtered through a GTTP 0.25 Millipore filter (0.22 µm) using a filtration column. Filters were washed and neutralised with 20 mL of PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2) and air dried. Fixed samples were stored at −20 °C until hybridisation reaction.

Microbial biofilms samples for DNA extraction

Portions of the microbial biofilms were collected in separate 50 mL sterile polypropylene tubes and kept at 4 °C until further processing.

DNA extraction, amplifications and 16S ribosomal RNA clone library construction

Total genomic DNA was extracted from the water and microbial biofilms samples using the Fast DNA Spin kit for soil (Bio 101, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were disrupted using the mixture of ceramic and silica beads provided in the kit and a laboratory vortex at maximum speed for 10 min. Clone libraries of complete 16S rRNA genes for *Bacteria* and *Archaea* domains were generated from DNA templates. 16S rRNA genes were amplified by PCR using forward primers 8F: 5′-AGAGTTTGATC(A/C)TGGC-3′ for *Bacteria* and 25F: 5′-TCYGGTTGATCCYGCCRG-3′ for *Archaea*; reverse primer for both was 1492R: 5′-TAC CTTGTTACGACTT-3′ (Lane 1991; Achenbach and Woese 1999). Primer numbers correspond to *Escherichia coli* positions. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 38 cycles of denaturation at 95 °C for 1 min, annealing temperature was 46 °C for *Bacteria* domain primers and 50 °C for *Archaea* domain primers and maintained for 1 min, finally extension at 72 °C for 1 min. Amplification reactions contained 20–30 ng of DNA per 50 µL reaction volume, 1× PCR buffer (Promega Biotech), 2.5 mM of each of the deoxynucleotides, 2.5 mM $MgCl_2$, 500 mM of the forward and reverse primers and 0.025 U/mL of Taq DNA polymerase (Promega Biotech). PCR amplification was checked by 1.2 % agarose gel electrophoresis stained with ethidium bromide. Amplified 16S rRNA gene products (>1400 bp) were cloned using the Topo Ta Cloning Kit (Invitrogen CA, USA) and sequenced using a Big-Dye sequencing kit (Applied Biosystem) following the manufacturer's instructions.

Sequences were checked for potential chimeras using Bellerophon Chimera Check program (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) and Mallard software (Ashelford et al. 2006). Sequences detected as chimeras were retrieved from further analysis. A distance matrix generated using Greengenes online tool (http://greengenes.lbl.gov/cgi-bin/nph-distance_matrix.cgi) was used as the input file to distance-based OTU and richness software (Schloss 2005) which assigns sequences to operational

Table 1 Geochemical data of the water and microbial biofilms collected from the Copahue geothermal area

Water samples	*T* (°C)	pH	SO_4^{2-} (mg/L)	Fe (mg/L)	Microbial biofilm samples	*T* (°C)	pH	SO_4^{2-} (mg/L)	Fe (mg/L)
LMa1	36.0	3.2	119.5	7.0	LMa1 biof	36.0	4.8	ND	ND
LVE	31.5	3.0	291.8	3.3	LVE biof	30.0	4.8	ND	ND
B9	40.5	2.7	346.8	7.0	B9 biof	30.0	2.7	ND	2.4
LMi	87.0	2.0	618.7	32.8	LMi biof	35.0	3.5	ND	0.4

ND not detected

taxonomic units (OTUs) for every possible distance (rarefaction curves are presented in Figures S1A and S1B of Supplementary Information). Phylogenetic classification of representative OTUs was done using the Classifier and Taxomatic online tools of the ribosomal database project (RDP) (http://rdp.cme.msu.edu). Nearly full-length (>1400 bp) sequence were imported to a data base of over 50000 prokaryotic 16S rRNA primary structures using the ARB software package aligning tool (http://www.arb-home.de) (Ludwig et al. 2004). OTUs were defined at 97 % sequence similarity using ARB software package. The rRNA alignments were corrected manually and alignment uncertainties were omitted in the phylogenetic analysis. Phylogenetic trees were constructed using ARB tools with Maximum likelihood and PhyML correlation. Filters, which excluded highly variable positions, were used. The retained positions were more than 1000 bp in all the cases. The 16S rRNA sequences representing the OTUs selected were deposited on NCBI data base under the accession numbers JX989227 to JX989264 and KP204487 to KP204546.

FISH and CARD-FISH

Fluorescent in situ hybridisation (FISH) was performed on water samples. Hybridisations were done using fluorescent-labelled probes as described by (Amann 1995). The probes used in this study are listed in Table S1 in the Supplementary Information section. Due to the presence of large amount of autoflorescence material, hybridisations on Las Maquinitas (LMi) water sample were performed using catalysed reported deposition fluorescent in situ hybridisation (CARD-FISH). The protocol reported by Pernthaler et al. (2002) was used except that no overnight treatment with active diethyl pyrocarbonate was done, as the samples did not show high endogenous peroxidase activity. For further cell permeabilisation filters were treated with achromopeptidase (0.6 U/mL final concentration; buffer contained 0.01 M NaCl, 0.01 M Tris–HCl pH 8.0; incubation at 37 °C for 30 min) and then washed with ultra pure water for 1 min. Peroxidases were inhibited by treating the filters with 20 % methanol and 0.015 % H_2O_2 solution for 30 min at room temperature. 4′,6′-Diamidino-2-phenylindole (DAPI) stain was used in all hybridisations to evaluate total cells number. Vectashield Mounting Medium (Vector Laboratories Inc. CA, USA) was added to preparations to avoid fluorescence fading. A Leica DM 2500 epifluorescence microscope was used to visualise hybridisation. Images were taken using a Leica DFC 300 FX camera and its corresponding software (Leica Microscopy Systems Ltd, Heerburgg, Switzerland). Total cell density was calculated as the average of at least 50 DAPI stained fields. Hybridisation percentages for universal probes were calculated as the quotient of the average count of 20 hybridised fields over the average count of those same fields stained with DAPI. Non-specific hybridisation was discarded in all the samples by the use of NON338 probe.

Results and discussion

Sampling sites

Biodiversity and community structure were analysed in microbial biofilms and water samples from Copahue geothermal area. Figure 1 shows photographs of the sampling sites: Las Máquinas (LMa), Baño 9 (B9), Laguna Verde Este (LVE) and LMi. In a geothermal environment like Copahue, microbial biofilms tend to develop in the vicinity of ponds, hot springs and solfataras, where thermal activity is less intense. The four microbial biofilms sampled presented moderate temperature and pH values between 2.7 and 4.8. The water of the ponds were more acidic (pH values between 2.0 and 3.2) and their temperature greatly differed, from moderate to very high values, close to water boiling point at Copahue's altitude. Table 1 presents temperature and pH values, as well as sulphate and iron concentrations measured in the samples analysed in this study.

Biodiversity in microbial biofilms

The OTUs found in microbial biofilm samples, together with the number of clones of each OTU, their closest BLAST hit and their accession number, are listed in Table S2 in the Supplementary Information section. Figure 2 shows the relative abundances of the OTUs found in water

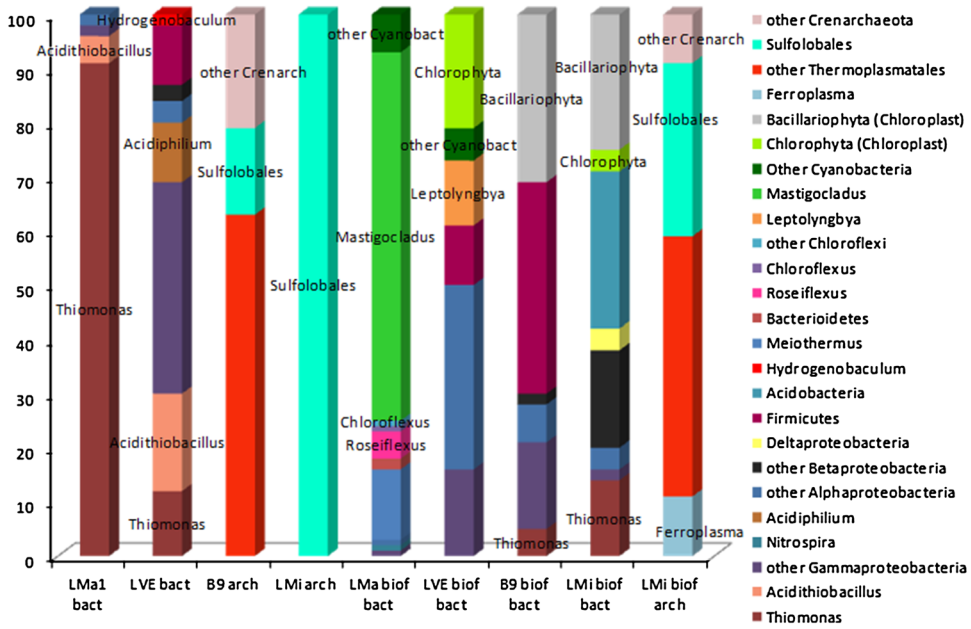

Fig. 2 Relative abundances of bacterial and archaeal OTUs in the microbial biofilms and water samples analysed. The *same colour* indicates the same phylogenetic group but not necessarily the same OTU. Special attention was put on acidophilic and photosynthetic species

and microbial biofilm samples, with special emphasis on acidic and photosynthetic species.

According to RDP Classifier tool the sequences retrieved in the microbial biofilm samples were affiliated with the following taxonomical groups: *Acidobacteria*, *Bacteroidetes*, *Caldilineae*, *Chloroflexi*, *Deinococci*, *Firmicutes*, *Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria*, *Nitrospira*, *Cyanobacteria* and chloroplasts of different photosynthetic species (the Classifier tool of RDP includes the 16SrRNA sequence of chloroplasts in a phylum named *Cyanobacteria*/Chloroplast) and *Thermoplasmatales*, *Sulfolobales*, *Thermoproteales* and *Thermoprotei* in the domain *Archaea*.

Among the microorganisms found in the microbial biofilms the photosynthetic species are particularly interesting as they were not detected at all in the water samples (Fig. 2). The phylogenetic distribution of their representative OTUs can be seen in the tree of Fig. 3 and the taxonomical classification according to RDP and their closest BLASTn match can be found in Table S3 of the Supplementary Information section. Among the photosynthetic species detected, those associated to the phylum *Cyanobacteria*/Chloroplasts (according RDP taxonomical classification tool) were the best represented. The phylogenetically related group taxonomy is the one used by RDP and consequently the one adopted in this work. However, for some authors is believed to be artificial and not reflective of evolutionary relationships, and will likely be revised, or even replaced, as more genetic data become available (Vincent 2009). Recent studies on taxonomy and phylogeny of *Cyanobacteria* agree that classification should include genetic, phylogenetic, ultrastructural and phenotypic data to create a modern system (Howard-Azzeh et al. 2014; Shi and Falkowski 2008; Komárek 2006; Tomitani et al. 2006; Hoffmann et al. 2005).

Continuing with the description of the photosynthetic species found in the microbial biofilms in the Copahue geothermal ponds, six OTUs were affiliated to the *Cyanobacteria* class. Three of them, represented by sequences LMa-biof-bac-h7, LVE-biof-bac-a9 and LVE-biof-bac-b10 (Fig. 3; Table S3) could not be further classified. The first two showed 94 % similarity to sequences of *Synechococcus elongatus* in a BLASTn search. These *Cyanobacteria*, which belong to the *Chroococcales* order, are oxygenic phototrophs that can photolyse either H_2O or H_2S. They are the main source of primary production in oligotrophic aquatic environments and are able to grow at a wide range of light intensities (Scanlan and Nyree 2002). As regards biotechnological applications, *Synechococcus* species have been used in the production of biofuel and different bioactive compounds (Abed et al. 2009; Machado and Atsumi 2012) The OTU represented by sequence LVE-biof-bac-b10 showed 94 % similarity to sequences of *Leptolyngbya antarctica*, a species of *Cyanobacteria* that has been reported in many biodiversity studies done in Antarctica (Taton et al. 2006; Komárek 2007). It is curious to notice in the phylogenetic tree (Fig. 3) that these two sequences clustered together with other *Leptolyngbya* spp. in a separated and robust clade (99 % bootstrap value) distant from the rest of the known *Leptolyngbya* species. These three OTUs might point out Copahue's potential as the habitat of novel uncharacterized *Cyanobacteria* species which might have possible biotechnological applications.

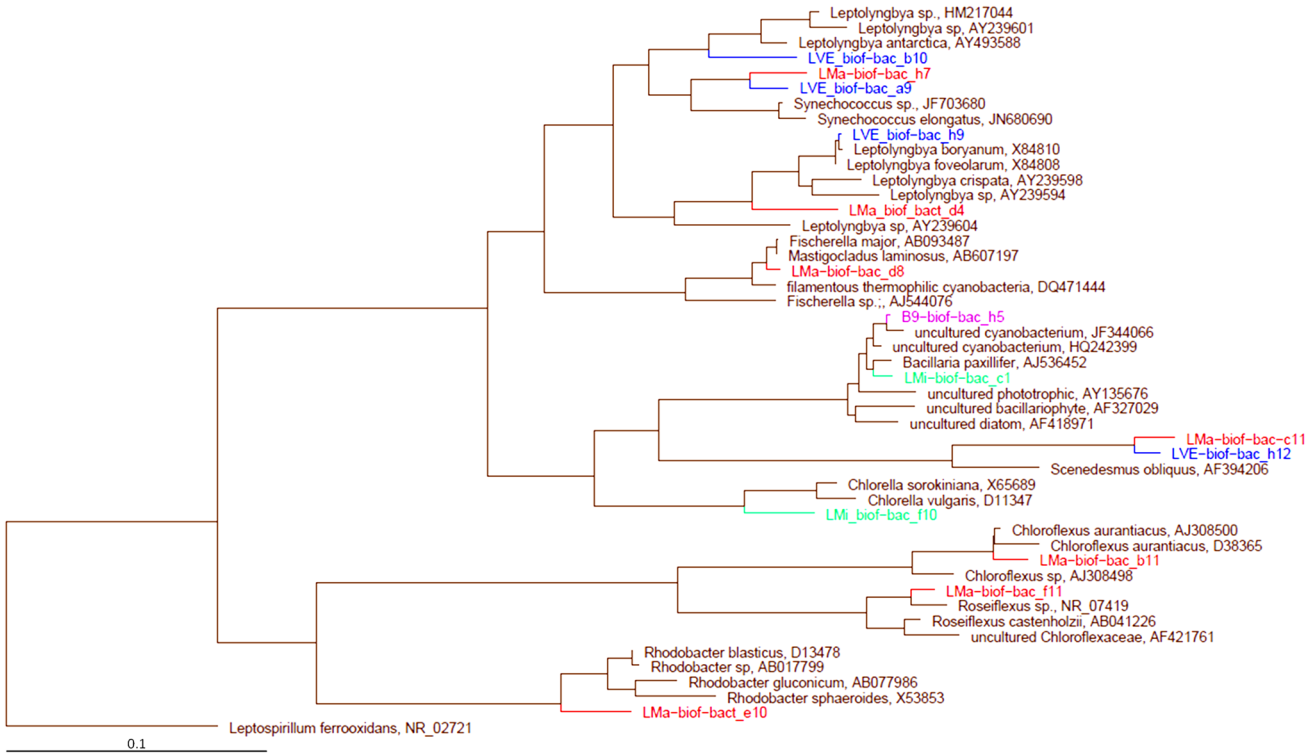

Fig. 3 Phylogenetic tree of the photosynthetic species present in the microbial biofilms in the Copahue geothermal area

One of the most abundant OTUs in the microbial biofilms samples, represented by sequence LMa-biof-bact_d8, was affiliated to *Cyanobacteria* Group I and showed 98–99 % similarity to *Mastigocladus/Fischerella* like species. The genera *Mastigocladus* and *Fischerella* are very similar and have a confused taxonomic history because representatives of both taxa were collected from a hot spring in Czech Republic within 15 years of the description of each other (Kaštovský and Johansen 2008). They belong to the order *Stigonemataceae* and are filamentous thermophilic *Cyanobacteria* typical of hot springs. As thermophilic species, *Mastigocladus/Fischerella* show higher uptake of CO_2 than their mesophilic counterparts; such metabolic feature makes them very interesting biotechnological tools in applications such as bioremediation and biofuel production (Kotelev et al. 2013). Other *Cyanobacteria* detected in the microbial biofilms were classified according to RDP as members of Group V (Table S3) and related by BLASTn search to the genus *Leptolyngbya*. The OTUs are represented by sequences LMa-biof-bact_d4 and LVE-biof-bact_h9. Notice their position in the phylogenetic tree (Fig. 3) close to other well-recognised *Leptolyngbya* species. This genus of filamentous *Cyanobacteria* belongs to the *Oscillatoriales* order and has been reported in high temperature environments (Castenholz 1996, 2001). As many other *Cyanobacteria*, *Leptolyngbya* species have been studied for their potential use in hydrogen and bioactive compound production (Prabaharan et al. 2010; Choi et al. 2010; Thornburg et al. 2011) as well as bioremediation of hydrocarbon contaminated environments (Al-Bader et al. 2013).

Five OTUs were related to the 16S rRNA genes of chloroplast of photosynthetic eukaryotes (class Chloroplast in Table S3 according to the taxonomical classification used by RDP for 16S rRNA gene sequences), two in the genus *Bacillariophyta* and three in *Chlorophyta*. Both genera include thermophilic photosynthetic species. The OTUs of chloroplasts from *Bacillariophyta*, represented by sequences LMi-biof-bact_c1 and B9-biof-bact_h5, were more than 98 % similar to chloroplast sequences found in environments affected by acid mine drainage (González-Toril et al. 2011; unpublished information under NCBI access number AJ536452). The OTUs of chloroplasts of species in the genus *Chlorophyta* showed low similarities to chloroplast sequences of *Scenedesmus obliquus* (90 %) and uncultured *Chlorella* sp. (95 %). The apparent difference to well-characterized species is also reflected in the relative position of the sequences in the phylogenetic tree (Fig. 3). For deeper study of the eukaryotes present in the microbial biofilms we attempted the construction of 18S rRNA gene clone libraries. Unfortunately most of the sequences obtained were of low quality and a comprehensive analysis was not possible. However, we could detect sequences related to *Scenedesmus*, *Chaetophora*, *Pinnularia*,

Semispathidium and *Platyreta* genera (data not shown). It is interesting to notice that no *Cyanobacteria* species were found in the samples B9-biof and LMi-biof that presented pH values lower than 4 (2.7 and 3.5, respectively) in agreement with the reports of other authors (Brock 1973; Steinberg et al. 1998; González-Toril et al. 2003).

In the phylum *Chloroflexi* two OTUs were detected. One was related to the genus *Roseiflexus*, which comprises thermophilic bacteria able to grow photoheterotrophically under anaerobic light conditions and form red mats in natural environments (Hanada et al. 2002). The OTU found in Copahue, represented by sequence LMa-biof-bact_f11 was 98 % similar to a sequence reported in an orange surface microbial mat in El Tatio Geyser Field in Chile (Engel et al. 2013). The other OTU was 98 % similar to *Chloroflexus aurantiacus*, a photoheterotrophic green non-sulphur bacteria also detected in El Tatio Geyser Field (Engel et al. 2013) as well as in other extreme environments microbial mats (Nübel et al. 2001). Species of the phylum *Chloroflexi*, especially *Chloroflexus*, are commonly found in microbial mats, growing under a cyanobacterial layer, taking advantage of the UV light protection and the production of organic compounds (Ruff-Roberts et al. 1994; Boomer et al. 2002); the formation of these photosynthetic multilayer structures has been proven in thermal springs in Yellowstone National Park (Boomer et al. 2009).

Photosynthetic species were also found among *Alphaproteobacteria*. One OTU was classified within the *Rhodospirillaceae*, a family composed mainly of photosynthetic purple non-sulphur bacteria. Sequences related to the genus *Rhodobacter* were also detected in Copahue microbial biofilms. These are purple non-sulphur bacteria that include species with an extensive range of metabolic capabilities, such as lithoautotrophy, photosynthesis, aerobic and anaerobic respiration. As regards microbial diversity, the dominance of *Cyanobacteria*, *Chloroflexus* and eventually *Proteobacteria* has been reported in other microbial mats and biofilms (Coman et al. 2013; Ferris et al. 1996).

Apart from the photosynthetic species described, other bacteria were detected in the microbial biofilms studied. One OTU was classified into genus *Meiothermus* within the phylum *Deinococcus-Thermus* and was 99 % similar to *Meiothermus hypogaeus,* a thermophilic bacterium isolated from a neutrophilic hot spring in Japan (Mori et al. 2012). Another OTU was affiliated to the genus *Geothrix* in the phylum *Acidobacteria* and, according to BLASTn, was 99 % similar to an uncultured bacterium detected in acidic Rio Tinto in Spain (Amaral-Zettler et al. 2011). Sequences related to genus *Thiomonas*, commonly found in natural or related to mining activity acidic environments (Urbieta et al. 2014; Hamamura et al. 2009; Hallberg et al. 2005), were the only sulphur oxidising bacteria detected. The almost absence of sulphur oxidising species is in correlation with the undetection of sulphate ion (the end product of sulphur compounds biooxidation) in the microbial biofilm samples (Table 1). Finally, some sequences were related to species commonly found in soil or aquatic environments, such as *Pseudomonas*, *Aeromonas* and *Clostridium*.

As regards archaea, the 16S rRNA sequences obtained from genomic DNA from the microbial biofilms were affiliated to phyla *Euryarchaeota* and *Crenarchaeota* (Table S4). Within *Euryarchaeota*, the five OTUs detected were classified into the order *Thermoplasmatales* (Fig. 4a). Two of them could not be further classified and showed over 97 % similarity to sequences reported in a copper mine (Xie et al. 2007) and in different hot springs in Yellowstone National Park (unpublished, information available in NCBI under accession number DQ179028). Another OTU was related to *Ferroplasma* species, a genus of acidophilic, lithoautotrophic or mixotrophic archaea capable of iron and pyrite oxidation that are very important members of the microbial community in acidic, heavy metal reach environments (Golyshina and Timmis 2005). The *Ferroplasma*-like sequences found in Copahue microbial biofilms are 99 % similar to sequences detected in Rio Agrio in Copahue and in Rio Tinto in Spain (Urbieta et al. 2012; Amaral-Zettler et al. 2011) (see sequences in purple in Fig. 4a). Other two OTUs were classified in the genus *Thermogymnomonas*; particularly the one represented by sequence LMi-biof-arch d6 was over 99 % similar to sequences detected in Rio Agrio in Copahue (Urbieta et al. 2012) (Fig. 4a).

The sequences in the phylum *Crenarchaeota* were all affiliated to the class *Thermoprotei* (Table S4; Fig. 4b). The OTU represented by sequence LMi-biof-arch-b11 could not be further classified and was distantly related to an archaeal clone from Norris Geyser Basin in Yellowstone National Park (unpublished, information available under NCBI accession number DQ924756). The other crenarchaeal OTUs were related to thermophilic species like *Sulfolobus*, *Pyrobaculum* and *Vulcanisaeta*. The genus *Sulfolobus* has been described long ago by Brock (Brock et al. 1972) as strict aerobic, acidophilic, thermophilic, sulphur oxidizing archaea, typical of geothermal environments. *Pyrobaculum* species are neutrophilic and extreme thermophiles, capable of growing up to 104 °C; the species are either facultative aerobic or strictly anaerobic and they can grow either chemolithoautotrophically by sulphur reduction or organotrophically by sulphur respiration or by fermentation (Volkl et al. 1993). Species from the genus *Vulcanisaeta* are anaerobic, hyperthermophilic and moderately acidophilic, they grow heterotrophically and are able to use thiosulphate as electron acceptor (Itoh et al. 2002). Considering the temperature measured in the microbial biofilms samples probably the 16S rRNA gene sequences of these extreme thermophilic archaea corresponds to species that

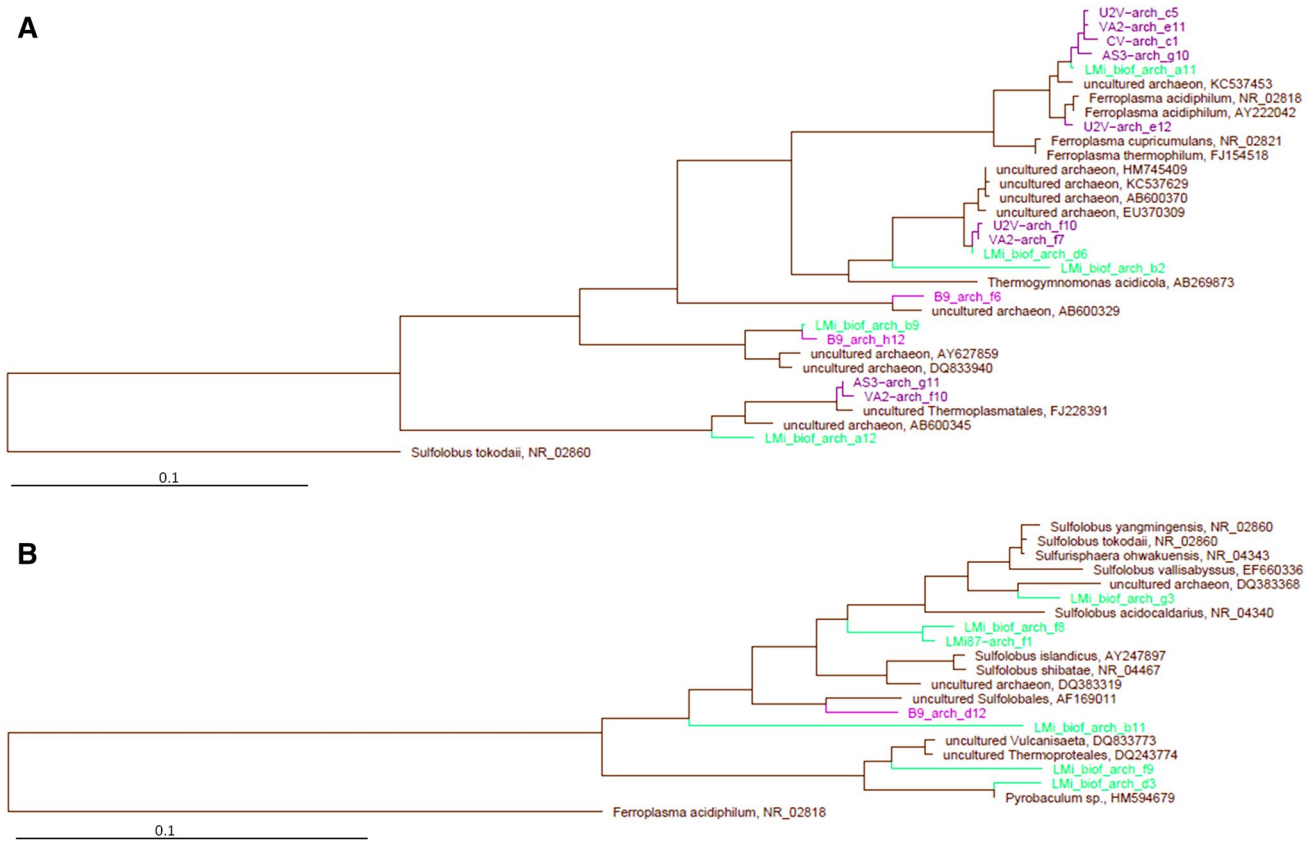

Fig. 4 Phylogenetic representation of the archaea found in the water and microbial biofilms in Copahue geothermal area: **a** *Euryarchaeota*, **b** *Crenarchaeota*. Sequences in purple were detected in Rio Agrio (Urbieta et al. 2012)

are not metabolically active in the microbial biofilms but develop in high temperature environments nearby, such as the hot springs waters (see the following section).

Biodiversity in water of ponds and hot springs

Prokaryotic biodiversity assessment in water samples was approached parallel by two techniques: bacterial and archaeal 16S rRNA cloning and sequencing and in situ hybridisation. The OTUs found in water samples, together with the number of clones of each OTU and their closest BLAST hit and their accession number, are listed in Table S5 in the Supplementary Information section. Table S6 presents absolute counts and hybridisation percentages of the probes used. Figure 5 shows hybridisation percentages of total microorganisms stained with DAPI for probes EUB338 (*Bacteria* domain) and ARQ915 (*Archaea* domain) for LMa, LVE, B9 and LMi water samples. Figure 6 shows epifluorescence microscope images of these hybridisations and their corresponding DAPI stains to provide a quantitative idea of the abundances of the two domains in the different ponds studied. These results show that LMa and LVE were dominated by bacteria, with 84 and

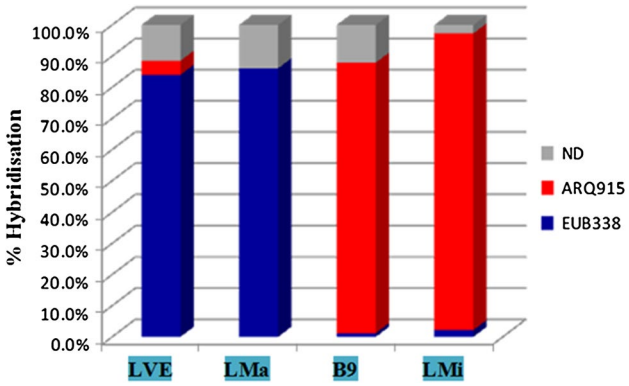

Fig. 5 Hybridisation percentages of total microorganisms (DAPI) stain using bacteria general probe (EUB338) and archaea general probe (ARQ915) on the water samples of the Copahue geothermal ponds

86 % of hybridisation with the bacterial probe EUB338, respectively. On the other hand, B9 and LMi samples were dominated by archaea (88 and 91 % of hybridisation with ARQ915 probe, respectively). Considering that FISH reveals only viable cells, as labile RNAs are the probes

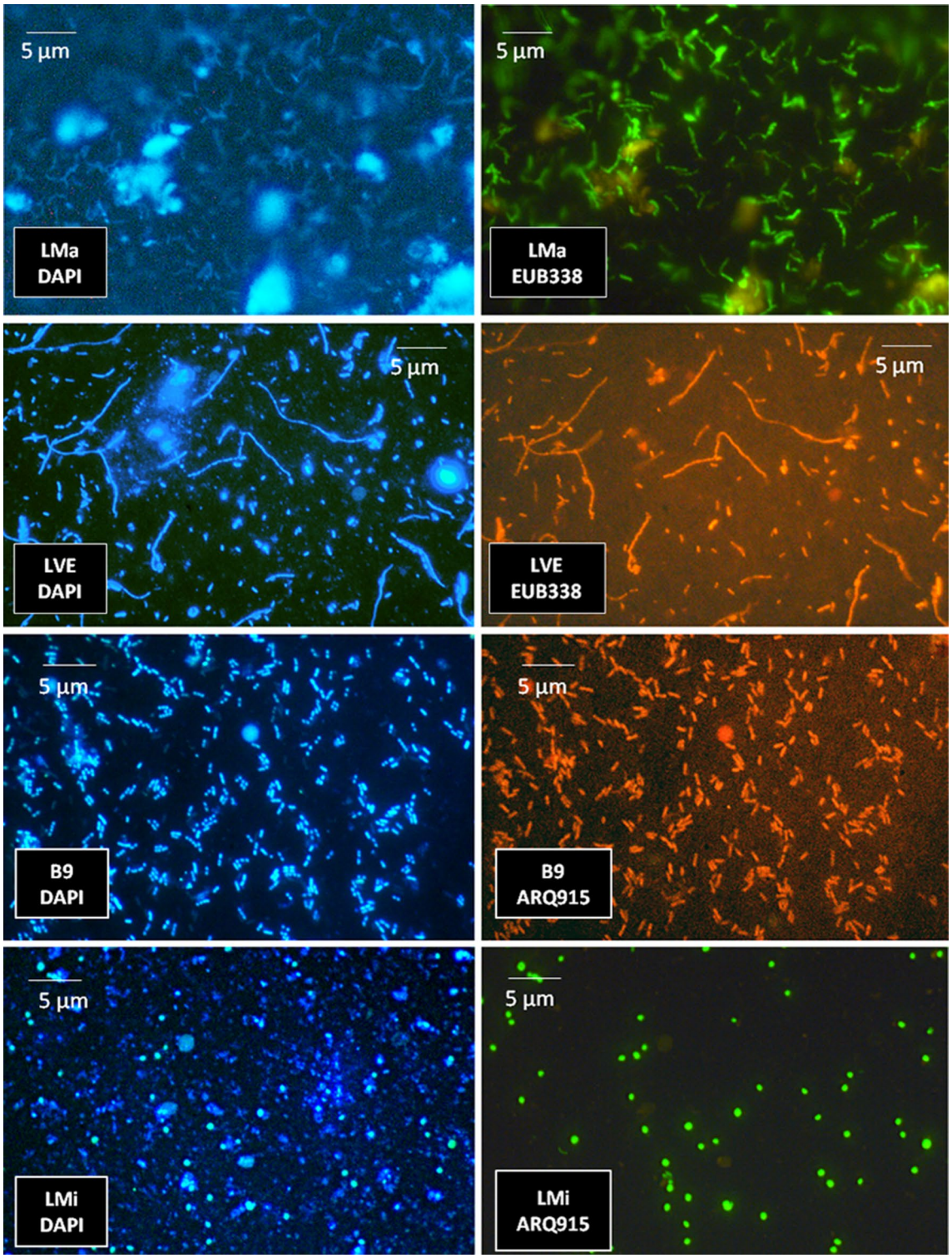

Fig. 6 Epifluorescense microscopic images showing the prevailing domain in water samples of the Copahue geothermal ponds. On the *left side*, DAPI stained all the microorganisms present. On the *right side*, hybridisations with bacteria general probe (EUB338) or archaea general probe (ARQ915). EUB338 probes in LMa FISH hybridisation and in LMi CARD-FISH hybridisation are labelled with the *green* fluorochrome Alexa 488 (*green* emission). The other FISH probes used are labelled with *red* CY3 flourochrome (*red* emission)

target, we used hybridisations counts to guide the biodiversity analysis in the four water ponds studied.

In LMa and LVE, bacterial community was more deeply analysed (Fig. 2; Table S7). In these two acidic, moderate temperature ponds, the bacterial 16S rRNA gene clones libraries were similar. In LMa1, the majority of the clones were approximately 99 % similar to species of *Thiomonas*. These are moderately acidophilic, facultative chemolithotrophic or mixotrophic bacteria, able to grow on some metal sulphides or sulphur (Moreira and Amils 1997) and have been reported in other acidic environments, including those related with mining activity (Hallberg et al. 2005). The second better represented bacteria in LMa waters were related to *Acidithiobacillus* species. This genus is composed of aerobic, obligate acidophilic and lithoautotrophic bacteria. *Acidithiobacillus* species are able to obtain energy from the oxidation of sulphur compounds, and some of them from the oxidation of ferrous iron (Kelly and Wood 2000). In the waters of LVE, *Thiomonas*- and *Acidithiobacillus*-related species were also important members of the pond's bacterial community. Two other species associated with acidic environments appeared in this pond: *Acidiphilium* and *Hydrogenobaculum*. The former are aerobic, acidophilic, obligate chemoorganotrophic bacteria that oxidise sulphur compounds and are generally found accompanying *Acidithiobacillus*, as they metabolise organic compounds which are toxic for lithoautotrophs (Johnson and Hallberg 2003). *Hydrogenobaculum* are aerobic, acidophilic and thermophilic bacteria that obtain carbon from carbon dioxide fixation and energy from oxidising hydrogen or reduced sulphur compounds (Stohr et al. 2001). Conversely to microbial biofilms, in LMa and LVE water samples the detection of a high proportion of sulphur oxidising species is in good correlation with the high concentrations of sulphate ion measured (Table 1). The bacterial clone library in LVE water sample was completed by the detection of some heterotrophic species, such as *Acinetobacter* and *Pseudomonas,* not related with acidic or geothermal environments. Their presence could be explained by the fact that LVE pond is located very near the Copahue thermal health centre, and it has a higher anthropogenic influence.

In the analysis of the prokaryotic community of the other ponds, B9 and LMi, FISH showed that archaea were the dominant species (Figs. 5, 6). The classification of these archaeal OTUs according to RDP is presented in Table S8 in the Supplementary Information section. The water of the pond B9 and chiefly the hot spring LMi presented the highest temperature values and the most acidic pH conditions (Table 1). An interesting fact about the archaea found in the high-temperature ponds in Copahue is that, while the majority of the bacteria sequences were more than 97 % similar to known and cultivated microorganisms, almost all the archaea sequences were less than 97 % similar to any cultivated species, and in some cases even to uncultured clones (Tables S5; S8). According to these results, the Copahue geothermal field could be the habitat of potentially novel, possibly autochthonous, archaea. The archaeal community in B9 waters was dominated by sequences affiliated to the *Thermoplasmatales* family in the *Euryarchaeota* phylum represented by sequences B9-arch-f6 and B9-arch h12 (Fig. 4a; Table S8). A minor fraction, represented by sequence B9-ach-d12, was affiliated to the phylum *Crenarchaeota*, specifically to the genus *Sulfolobus*. It is interesting to notice in the phylogenetic trees of Fig. 4 that the archaeal sequences found in the waters of B9 form long branches within well-established classes, but distant from other reported cultured or uncultured species. In LMi, the extreme physical conditions of this hot spring seem to be reflected in its archaeal population, as the 95 clones analysed belonged to only one OTU affiliated with the *Sulfolobus* genus.

The species related to the sequences found in Copahue water ponds and hot springs are mainly related to the sulphur cycle. The geochemical conditions of the area, particularly the acidity, the reported abundance of sulphur compounds produced by the sustained volcanic activity (Mas et al. 1996; Varekamp et al. 2009; Urbieta et al. 2014) and the measured concentrations of ion sulphate (the final product or sulphur compounds biooxidation) suggest that the sulphur cycle plays a key role in the geochemistry of this environment.

Comparison of microbial species in water and microbial biofilms in Copahue geothermal springs and in other geothermal environments

In spite of some cases presenting similar pH and temperature conditions, microbial biofilms and the water of the ponds and hot springs are different environments within the Copahue geothermal area, which is reflected in their different prokaryotic community composition. The samples B9-biof, LMi-biof, B9 and LMi, have significant difference in temperature and at lesser extent in pH values, with more extreme conditions in the water samples. These marked differences in physicochemical conditions help to explain better their microbial community composition: B9 and LMi water samples are dominated by archaea, with a high proportion of *Sulfolobales*, particularly in LMi; while the biofilm samples show a quite diverse microbial community that includes photosynthetic eukaryotic species such as *Chlorophyta* and *Bacillariophyta*.

In general, the different nature of both environments analysed in this study have a marked impact on their microbial biodiversity. In microbial biofilms, prokaryotic communities are more diverse and photosynthetic species are present while acidophilic sulphur oxidising bacteria, the

main prokaryotes found in the water samples become far less important. That could be related with another important difference in physicochemical parameters between microbial biofilms and water samples; in the former no ion sulphate (the final product of sulphur compounds oxidation) was detected while in water samples the concentration was in all cases (except LMa) higher than the normal values for fresh water (between 10 and 250 mg/L). The sequences related to the genus *Thiomonas* were the only species of acidophilic sulphur oxidising bacteria present in the microbial biofilms. In fact, *Thiomonas* were the only bacterial species detected in both habitats analysed in this work. This result could be considered as an indication of the importance of *Thiomonas* in the microbial community and biogeochemistry of Copahue. On the other hand, no photosynthetic species of any kind were detected in the water of any of the ponds or hot springs studied.

The archaeal community was similar in the water and microbial biofilms samples studied. The species found in both environments are similar to sequences associated with *Thermoplasmatales* and *Sulfolobales* (Fig. 2). It is particularly interesting to notice in the phylogenetic tree of the *Euryarchaeota* phylum (Fig. 4a) that many of the sequences detected in the microbial biofilms clustered together with sequences found in Rio Agrio (coloured in purple), also part of the Copahue geothermal area. Similarly, the *Crenarchaeota* phylum tree (Fig. 4b) shows that the only one archaeal OTU found in the waters of LMi hot spring (LMi87-arch-f1) is highly related to one of the OTUs found in the microbial biofilms (LMi-biof-arch-f8). These findings support the idea that Copahue geothermal area posses a common autochthonous and apparently biogeographically determined archaeal community.

The microbial diversity of the Copahue geothermal system can be compared with that of other similar systems, natural or artificial, around the globe. Rio Tinto, in southern Spain is one of the most studied natural acidic environments. The prokaryotic biodiversity in its water column as well as in the macroscopic filaments found in still water areas is dominated by iron oxidising species such as *At. ferrooxidans*, *L. ferrooxidans* and *Acidiphilium* spp. (García-Moyano et al. 2007; González-Toril et al. 2003). The difference in prokaryotic community composition between Rio Tinto and Copahue is mainly related with the fact that the Spanish river has very high iron concentrations (over 1000 mg/L) compared with the ones measured in Copahue. In algal photosynthetic biofilms in Rio Tinto, species of the eukaryotes *Euglena*, *Pinnularia* and *Chlorella* have been reported but no *Cyanobacteria*, probably due to the low pH of the river (Souza-Egipsy et al. 2008; Amaral-Zettler et al. 2011). As regards archaeal community, all the mentioned studies on Rio Tinto coincide that *Thermoplasmata*, particularly *Ferroplasma* species similar to those detected in Copahue are dominant, but there are no records of *Crenarchaeota* from Rio Tinto. When considering acidic environments of anthropogenic origin, a study done on macroscopic biofilms in acidic, metal-rich waters in an abandoned copper mine and a chalybeate spa in North Wales showed that the biodiversity was more related to the one described in Copahue moderate temperature water samples than in microbial biofilms, although the Welsh biofilms showed a higher presence of iron oxidising species such as *At. ferrooxidans*, *Acidiphilium*, *Ferromicrobium*, *Thiomonas* and no detection of archaea or photosynthetic species (Hallberg et al. 2005). Similar biodiversity, with a higher proportion of *Leptospirillum*, was detected in a biofilm in an acid mine drainage site (Bond et al. 2000). Another very well studied geothermal area is the Uzon Caldera at Kamchatka in Russia; there the microbial community presented some similarities with Copahue, with a marked presence of chemolithotrophic species such as *Hydrogenobaculum* in the most acidic sites (as the area is rich in As) and dominance of *Chloroflexus*, *Deltaproteobacteria* and *Clostridia* in the less acidic pools (Burgess et al. 2012). A study done specifically in microbial mats revealed the presence of many photosynthetic species similar to those found in Copahue (Ward et al. 1994). A similar study done in sulphur and non-sulphur microbial mats in solfataric fields in south-western Iceland showed similar results to those presented in this study, where the sulphur reach samples were dominated by chemolithotrophic sulphur oxidising species and there were no photosynthetic species reported, whereas the non-sulphur mats were dominated by *Chloroflexus*, photoheterotrophic green non-sulphur bacteria, with very little influence of sulphur oxidisers (Skirnisdottir et al. 2000). In a study of bacterial and archaeal biodiversity of microbial mats from a slightly alkaline geothermal region in Romania, similar *Cyanobacteri*a and *Chloroflexi* species were found, however, the other bacteria and chiefly the archaeal community were completely different, more related to methanogenic species such as *Methanomassiliicoccus* and *Methanococcus* (Coman et al. 2013). Recently, a study has been published describing the morphological and phylogenetic diversity of *Cyanobacteria* in Algerian hot springs (temperatures ranging from 39 to 93 °C and pH from 6 to 7) where the hottest springs were dominated by *Leptolyngbya*, *Synechococcus*-like *Cyanobacteria* and *Gloeomargarita*, whereas *Oscillatoriales Chroococcales* and *Stigonematales* dominated lower temperature springs (Amarouche-Yala et al. 2014). It is curious to notice that two of the *Cyanobacteria* species that dominate the very high temperature mats were also present in the moderate temperature microbial mats in Copahue.

Conclusion

The comparison between the prokaryotic biodiversity of the water of ponds and hot springs and the microbial biofilms found in Copahue geothermal area showed two different ecological niches dominated by different species. In the water samples, the dominant prokaryotes were chemolithoautotrophic or mixotrophic, mainly sulphur oxidising, bacteria in the moderate temperature ponds and archaea in the high-temperature hot springs; while in microbial biofilms photosynthetic species, such as *Cyanobacteria* and the eukaryotes *Bacilariophyta* and *Chlorophyta* were the most important primary producers.

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