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Archaeal and bacterial diversity in five different hydrothermal ponds in the Copahue region in Argentina

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ABSTRACT

Copahue is an acidic geothermal volcanic region in the northwest corner of Neuquén Province, Argentina. In the area, there are various ponds, pools and hot springs with different temperatures, pH values and levels of anthropogenic influence. In this study, the prokaryotic biodiversity of five representative ponds was studied by using two complementary molecular ecology techniques: phylogenetic analysis of 16S rRNA bacterial and archaeal genes and FISH (or CARD-FISH) for quantitative estimation of biodiversity. The results, supported by multivariate statistical analysis, showed that the biodiversity in Copahue ponds seemed to be determined by temperature. High temperature ponds were dominated by archaea, mainly apparently novel representatives from the orders *Sulfolobales* and *Thermoplasmatales* that had no close cultivated relatives. By contrast, moderate temperature ponds were colonised by well-characterised sulphur-oxidising bacteria related to acidic environments, such as other geothermal sites or acid mine drainage, and archaea were absent. By combining the biodiversity results from this study and the reported physicochemical features of Copahue, a preliminary model of the possible biogeochemical interaction was outlined for moderate and high temperature ponds.

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Introduction

The study of biodiversity in extreme environments is a popular subject for modern science. This area of microbiology deals with much more than the mere description of the microorganisms inhabiting a particular habitat. It offers the possibility to investigate species interrelations, their impact on geochemical cycles and the influence of physicochemical variables on community structure. This last point has been addressed for extreme habitats using biostatistics software [4,7,28,46] which has proved to be a very useful tool.

Microbial diversity in acidic geothermal environments has been assessed in different locations around the world, with the most studied being Yellowstone National Park. Community structure generally varies with physicochemical factors, such as temperature, pH and chemical composition. Many of the bacterial species found in these environments are related to the sulphur, hydrogen, iron or arsenic cycles, such as *Hydrogenobaculum*, *Hydrogenobacter* or *Sulfurihydrogenibium* from the *Aquificae* phylum, or *Alicyclobacillus* or *Sulfobacillus* from *Firmicutes*. Species from *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Thermodesulfobacteria* and *Thermotogales* have also been detected in geothermal environments. As regards archaea, the species most frequently found are probably members of the *Sulfolobales* order from the *Crenarchaeota* domain, such as *Sulfolobus*, *Acidianus* and *Metallosphaera*. Depending on the temperature and pH conditions, species affiliated with *Thermoplasmatales* and *Thermoproteales* from the *Euryarchaeota* domain can also be found [10,27,33,34,41].

The Copahue geothermal region is located in the Cordillera Norpatagónica in the north west of Neuquén Province, Argentina and it has been studied very little (Fig. 1). The area is crowned by the Copahue volcano (37°51′ S, 71°10′ W; 2965 m a.s.l.), which is a predominantly andesitic stratovolcano containing a small, very acidic, lake in its crater (pH 0.2–1.1). Approximately 100 m below the lake there are two hydrothermal springs that are the source of the acidic Río Agrio, which flows down into the Caviahue-Copahue region. Its biodiversity consists mainly of acidophilic bacteria related to

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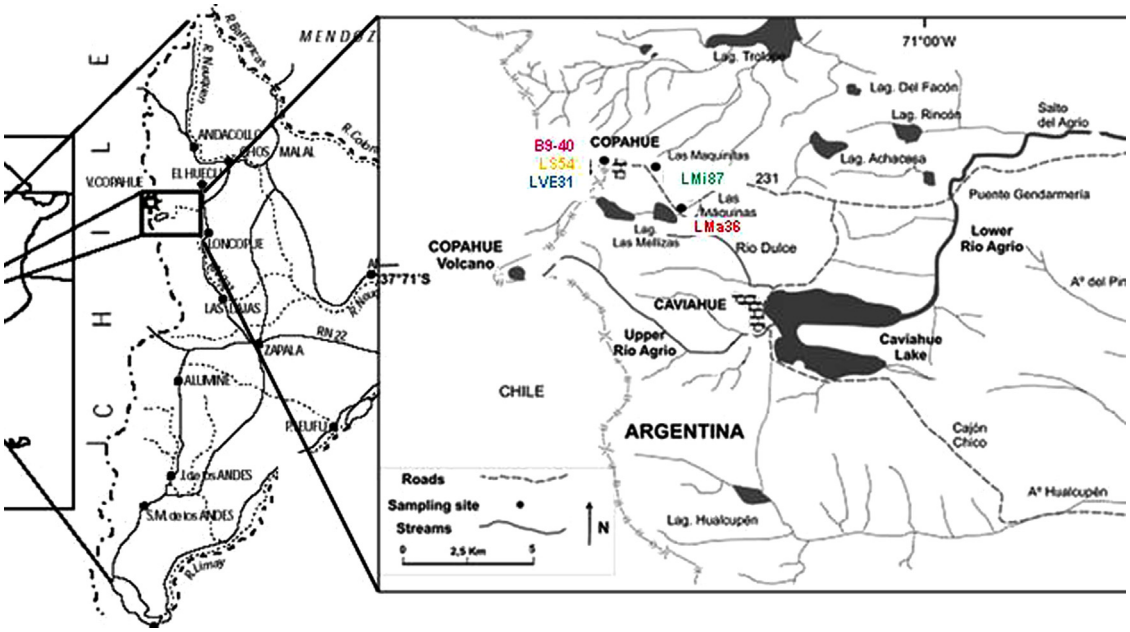

Fig. 1. Location of the Copahue geothermal region in Neuquén Province, Argentina. The five ponds sampled, as well as Copahue volcano and Río Agrio, are marked.

the genera *Acidithiobacillus*, *Acidiphilium* and, in a minor fraction, *Leptospirillum*, as well as archaea associated with the genus *Ferroplasma* [52]. The area is characterised by many fumaroles, pools, ponds, and hot springs associated with the geological origin of Copahue volcano and its constant activity.

As in other extreme environments, the Copahue geothermal system offers the possibility of detecting sequences from as yet unidentified species and, in this particular case, with metabolisms probably related to the sulphur cycle. In this regard, one of the goals of the present biodiversity study was to provide a molecular assessment of biodiversity that would provide guidance on the enrichment and isolation of possible novel species that could be potentially useful in biotechnological applications related to biomining and heavy metal bioremediation [18]. Our group has already obtained one interesting result out of this strategy, since one of the archaeal OTUs detected in this study has been isolated, characterised and reported as *Candidatus* "Acidianus copahuensis" [19]. This thermoacidophilic archaea seems to be autochthonous to Copahue and its wide-ranging flexible metabolism makes it a good candidate for biomining processes.

In this context, the main objectives of this study were to assess the prokaryotic communities of five different ponds in the Copahue geothermal system by means of cloning and sequencing of 16S rRNA genes and fluorescence *in situ* hybridisation (FISH) using specific probes against the major species detected in the samples. This approach was used to evaluate the influence of physicochemical characteristics on the biodiversity found and, finally, to develop a possible preliminary geomicrobiological model of the ponds.

Materials and methods

Study site description

Five acidic geothermal ponds from the Copahue area were selected in order to study their prokaryotic biodiversity based on the temperature conditions and the anthropogenic influence. A brief description of the most relevant characteristics of the sites studied is given in this section. More information on the metrics and geological features of the ponds can be found in studies already published on the subject [40,53]. Their locations are indicated in Fig. 1, and the general appearance of the sites sampled is shown by the photographs in Fig. S1. Physicochemical data measured for this study is presented in Table 1. The acronyms used throughout in order to identify the study sites are a combination of the historical names of the places and the pond temperature, which was the most significant feature of this study. Three of the pools sampled, Laguna Verde Este (LVE31), Baño 9 (B9-40) and Laguna Sulfurosa (LS54) were situated in the Copahue thermal centre, close to a health care facility in Copahue village, where the hydrothermal water is used for medical purposes. The three pools are artificial and have been constructed to exploit naturally acidic hydrothermal springs. LVE31 received its original name from the microalgae mats that cover its surface and borders (Laguna Verde means green lagoon). The appearance of LS54 water was cloudy due to colloidal sulphur and gas that emerges in some places and was visible by bubbling at the water surface. The study site named Las Máquinas was located in the homonymous geothermal manifestation area. The water samples were taken from a moderate temperature hydrothermal pool (LMa36) chosen because it was the least affected by human activity. Geological studies carried out in this area have indicated that the carbon found has a biological origin, as no carbonated mineral or magmatic emissions have been detected [53]. Pyrite, crystallised sulphur, hematite and jarosite were also found in ponds and fumarole walls. The final location sampled, Las Maquinitas, was the smallest of the Copahue geothermal manifestations and it was chosen to represent the most extreme conditions found in the area. The water samples were collected from a small hot spring coming directly out of the rocks (see Fig. S1; LMi87), where overheated vapours of approximately 130 °C hit the cooler rocks leaving traces of crystallised sulphur deposits. In addition, other sulphur minerals, such as pyrite, have also been reported [39]. Recently, this area began to be used for thermal baths and, consequently, human influence in the area has increased, although it preserves its autochthonous features.

Sample collection and in situ determinations

The temperature, electrical conductivity and pH of the water samples were measured *in situ* with a Hanna HI 8424 NEW portable instrument accurately calibrated against calibration standards.

Table 1
Physicochemical data of the Copahue thermal ponds studied. Concentrations of total soluble metals are expressed in mg L^{-1}. The latter columns indicate percentages of iron as Fe(II) and Fe(III).

	T (°C)	pH	Conductivity (μS cm^{-1})	Ca	Na	Mg	Mn	K	SO_4^{2-}	Cl^-
LMa36	36.0	3.20	663	0.98	ND	0.70	ND	0.48	119.50	5.30
B9-40	40.5	2.70	3720	3.78	52.55	1.65	ND	4.34	346.80	2.10
LMi87	87.0	2.00	4250	0.54	40.48	1.25	0.48	9.96	618.70	1.80
LVE31	31.5	3.00	1317	4.68	7.28	1.07	ND	4.34	291.80	35.70
LS354	54.3	3.00	1133	6.67	20.54	3.05	ND	10.97	381.80	56.70

	Fe (total)	Fe(II)	% Fe(II)	% Fe (III)
LMa36	6.96	0.91	13.01	86.99
B9-40	7.02	–	–	–
LMi87	32.85	32.42	98.68	1.32
LVE31	3.34	2.96	88.66	11.34
LS54	3.72	1.45	38.91	61.09

ND, not detected; –, not measured.

Water samples were collected in 1-L sterile plastic jars and kept on ice until further processing. As soon as possible, samples were filtered through 0.22 μm Millipore membranes. Filtrates were used for chemical analysis and the material retained on the membranes for DNA extraction. The filters were washed with pH 2 sterile water and TE buffer (10 mM Tris HCl pH 8, 1 mM EDTA) in order to remove any acidic water containing heavy metals that may have caused DNA hydrolysis. Quantification of Fe(II) was performed immediately by a spectrophotometric method using 1,10-phenanthroline and a NanoColor® UV/VIS spectrophotometer (Macherey-Nagel GmbH & Co., KG, Germany).

Water samples were fixed for fluorescence *in situ* hybridisation in the field. Between 200 and 500 μL of each water sample were incubated with the corresponding volume of paraformaldehyde (PFA) to achieve a 4% final concentration. Samples were incubated for 4–12 h, then diluted in approximately 15 mL pH 2 sterile water and were finally filtered through a GTTP 0.25 Millipore filter (0.22 μm) using a filtration column. Filters were washed and neutralised with 20 mL 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 the hybridisation reaction was performed.

Chemical analysis

The soluble forms of Ca, Cd, Zn, Fe, Cu, Na, Ni, Pb, Co, Mg, Mn, K and Cr were determined on filtered water samples by atomic absorption spectrophotometry using a Shimadzu AA-6650 spectrophotometer. The concentration of sulphate was determined by a turbidimetric method using an excess of barium chloride. The concentration of chloride was determined by titration with mercuric nitrate solution in the presence of diphenylcarbazone bromophenol blue indicator and was pH controlled [48].

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

The total genomic DNA of communities was extracted 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 common laboratory vortex at maximum speed for 10 min.

Clone libraries of complete 16S rRNA genes for the *Bacteria* and *Archaea* domains were generated from environmental 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*, whereas the reverse primer for both was 1492r: 5′-TACCTTGTTACGACTT-3′ [1,35]. Primer numbers corresponded 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, an annealing temperature of 46 °C for *Bacteria* domain primers and 50 °C for *Archaea* domain primers maintained for 1 min, and final extension at 72 °C for 1 min. Amplification reactions contained 20–30 ng DNA per 50 mL 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^{-1} 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 BigDye® Sequencing Kit (Applied Biosystem) following the manufacturer's instructions.

Clone library analysis

Sequences were checked for potential chimaeras using the Bellerophon Chimera Check programme (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) and Mallard software [5]. Sequences detected as chimaeras were removed from further analysis. OTUs were defined at 0.03 average distance between sequences (equivalent to 97% similarity) using the ARB software package [37] (http://www.arb-home.de). A distance matrix generated using the Greengenes on-line tool (http://greengenes.lbl.gov/cgi-bin/nph-distance_matrix.cgi) was used as the input file to distance-based OTU and richness (DOTUR) software [47] which assigns sequences to operational taxonomic units (OTUs) for every possible distance. Rarefaction analysis and the Chao1 non-parametric diversity estimator [11] were applied to the clone library in order to estimate how completely the library had been sampled and to extrapolate to total sequence diversity. Further analysis of one representative of each OTU was carried out using the Classifier and Taxomatic on-line tools of the Ribosomal Database Project (http://rdp.cme.msu.edu). Phylogenetic trees were constructed using ARB tools on a database constructed with over 53,000 16S rRNA sequences updated with BLAST closest matches for Copahue sequences. Neighbour-joining and Jukes-Cantor correlation were used. The rRNA alignments were corrected manually, and bootstrap values were calculated for 1000 replications to give statistical support to the results. Biodiversity indices were calculated using PAST software (version 2.14) [24]. The 16S rRNA sequences representing the OTUs selected were deposited in the NCBI database under the accession numbers JX989227–JX989264.

FISH and CARD-FISH

Fluorescent *in situ* hybridisation (FISH) was performed on PFA-fixed samples from B9-40, LMa36, LVE31 and LS54 with universal and specific CY3-labelled probes as described by Amann [2]. The

Table 2
Oligonucleotide probes used for FISH and CARD-FISH. Abv: abbreviations used in the figures.

Probe	Abv	Target	Target sequence (5′–3′)	(%) FM[a]	Specificity	Ref.
EUB338I	EUB	16S	GCTGCCTCCCGTAGGAGT	0–35	*Bacteria* domain	[3]
EUB338 II		16S	GCAGCCACCCGTAGGTGT	0–35	*Planctomyces*	[13]
EUB338III		16S	GCTGCCACCCGTAGGTGT	0–35	*Verrumicrobia* (and others)	[13]
ALF968	ALF	16S	GGTAAGGTTCTGCGCGTT	20	*Alphaproteobacteria*	[42]
BET42a[b]	BET	23S	GCCTTCCCACTTCGTTT	35	*Betaproteobacteria*	[38]
GAM42a[c]	GAM	23S	GCCTTCCCACATCGTTT	35	*Gammaproteobacteria*[f]	[38]
NTR712[d,e]	NTR	16S	CGCCTTCGCCACCGGCCTTCC	35	*Nitrospirae* group	[14]
ACD840	ACD	16S	CGACACTGAAGTGCTAAGC	10	*Acidiphilium* genus	[9]
TM1G0138	TM	16S	GCAGTTATCCCCCATCAAT	40	*Thiomonas* group 1	[22]
TM2G0138		16S	GTAGTTATCCCCCATCACA	40	*Thiomonas* group 2	[22]
THIO1	THIO	16S	GCGCTTTCTGGGGTCTGC	35	*Acidithiobacillus* spp.	Stoffels, unpb.
Aqui-1197		16S	GCATAAAGGGCATAMTGAYC	30	*Aquificales*	[45]
Arch915	ARCH	16S	GTGCTCCCCCGCCAATTCCT	20	*Archaea* domain	[50]
NON338		–	ACTCCTACGGGAGGCAGC	35	Negative control	[3]

[a] Formamide percentage (v/v) in the hybridisation buffer.
[b] Used in conjunction with a competitor probe, GAM42a (5′-GCCTTCCCACATCGTTT-3′) [38].
[c] Used in conjunction with a competitor probe, BET42a (5′-GCCTTCCCACTT CGTTT-3′) [38].
[d] Used in conjunction with a competitor probe, NTR712c (5′-CGCCTTCGCCACCGGTGTTCC-3′) [14].
[e] The complete name of this probe in the work of Daims et al. [14] is S-*-tspa-0712-a-A-21.
[f] Includes current class *Acidithiobacillia*.

probes used in this study are listed in Table 2. Due to the presence of large quantities of autofluorescent material, hybridisations on the LMi87 sample were performed using catalysed reporter deposition (CARD)-FISH instead of FISH. The protocol reported by Pernthaler et al. [43] was used except that no overnight treatment with active diethyl pyrocarbonate was carried out, as the samples did not show high endogenous peroxidase activity. For further permeabilisation, filters were treated with achromopeptidase (0.6 U mL^{-1} final concentration; buffer containing 0.01 M NaCl, 0.01 M Tris–HCl pH 8.0; incubation at 37 °C for 30 min) and then washed with ultrapure water for 1 min. Peroxidases were inhibited by treating the filters with 20% methanol 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 cell numbers. Vectashield mounting medium (Vector Laboratories Inc., CA, USA) was added to preparations in order 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., Heerbrugg, 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 recount of 20 hybridised fields divided by the average recount of those same fields with DAPI stain. Hybridisation percentages for specific probes were calculated considering EUB338-based cell counts as 100%.

Canonical correspondence analysis (CCA)

In order to evaluate the relationships between physicochemical parameters and biodiversity, two different CCA analyses were performed using CANOCO for Windows 4.5 software (Microcomputer Power, Ithaca, NY, USA) [51]. CCA is an ordination technique that seeks the most prominent linear gradients in multivariable data sets under the constraint that gradients are a linear combination of a set of explanatory variables. A preliminary correspondence analysis was performed and length of gradient values indicated the use of a unimodal approach for both ordination analyses. As many of the environmental parameters were strongly correlated, the number of environmental variables was reduced further in order to simplify interpretation of the graphical results. CANOCO provides an automatic selection option to ensure the inclusion of parameters with low cross-correlation that explain the most variance. This tool showed temperature, pH and conductivity as the environmental variables that explained the system variance. For species data, DAPI recounts were used, as well as FISH and CARD-FISH hybridisation percentages in one analysis and the presence/absence of acidophilic bacteria-associated OTUs and all archaeal OTUs in the other. The significance of the first CCA axis and all CCA axes was tested using Monte Carlo permutation tests [51]. Correlation between species and samples was measured by the distance of their symbols.

Results

Characteristics and physicochemical analysis of Copahue geothermal ponds

Four of the five stations sampled, B9-40, LMa36, LVE31 and LS54 were natural pools with still water. The fifth, LMi87, was a small hot spring from where very hot water (87 °C) and vapour were continuously emitted. The main physicochemical characteristics of the water samples, as well as the concentrations of the soluble forms of the metallic elements detected, are listed in Table 2. Total soluble Cd, As, Zn, Cu, Ni, Pb, Co, and Cr were measured but their concentrations were below atomic absorption spectrophotometry detection limits. The five ponds were acidic, and LMi87 was the most acidic with a pH value of 2. When considering the temperature values, the ponds sampled were representative of the variety of conditions found in Copahue. Iron was the only soluble metal present in the samples in detectable concentrations, and LMi87 was the pool with the highest total soluble concentration (32.85 mg L^{-1}; 99% as Fe(II)). The five water samples showed high conductivity values, with sulphate as the predominant anion (Table 1). LMi87 presented the highest values for conductivity and sulphate concentration.

Clone library analyses

PCR amplifications of the 16S rRNA gene were performed using bacterial and archaeal primers. Genomic DNA from the water of the five ponds sampled was amplified with bacterial primers, thus allowing for clone library construction and posterior sequencing. When using archaeal primers, only DNAs from ponds B9-40, LMi87 and LS54 were amplified. Primers specific for the archaeal domain *Korarchaeota*, Kor236F and Kor1236R [6], were tested, but no amplification was obtained with any of the genomic DNAs. After a quality check and discarding chimaera sequences, identity and phylogenetic studies were carried out on 181 bacterial and 204 archaeal sequences with a length of greater than 800

nucleotides. Tables S1 and S2 (supplementary material) present some features of the bacterial and archaeal clone libraries, respectively. The bacterial libraries had high coverage percentages and two of the three archaeal libraries were 100% covered according to Good's index [20], meaning that all the phylotypes present in the sample were represented in the library. Rarefaction curves for bacterial and archaeal clone libraries are presented in Fig. S2. The numbers of sequences analysed for observed OTUs and OTUs represented by only one sequence (singletons) for both bacterial and archaeal libraries (Tables S1 and S2, respectively) showed a low diversity of species, which was particularly marked for the latter.

Biodiversity in Copahue geothermal ponds

The phylogenetic distribution of the bacteria found in the Copahue geothermal ponds is shown by the tree in Fig. 2A. Table S3 presents the phylogenetic affiliation of bacterial clones according to the Ribosomal Database Project (RDP) together with their closest BLAST match and the sources of the closest relatives, highlighting sites similar to Copahue. Fig. 3A shows the relative abundances of bacterial OTUs.

The bacteria present in the five geothermal ponds studied were grouped into three phyla: *Aquificae*, *Firmicutes* and *Proteobacteria*. In the first two phyla, only two genera were represented: *Hydrogenobaculum* and *Anoxybacillus*, respectively. In the phylum *Proteobacteria*, biodiversity was much higher, with fourteen genera present that affiliated with the classes *Alphaproteobacteri*a, *Betaproteobacteria*, *Gammaproteobacteria* and the recently proposed class *Acidithiobacillia* [55], which contains the type order *Acidithiobacillales* and the type genus *Acidithiobacillus*, formerly included in the class *Gammaproteobacteria* [32]. Figs. 2A and 3A show that no species from any bacterial genera were detected in the five ponds.

Members of genera reported in acidic environments, natural or related to mining activity, such as *Thiomonas*, *Acidithiobacillus* and *Acidiphilium*, were only present in LMa36 and LVE31, the ponds with moderate temperature conditions similar to the optimal growth temperatures of these genera. Ubiquitous bacteria from the genus *Stenotrophomonas* were detected in LMa36, B9-40 and LMi87, whereas *Pseudomonas*-related species were present in LVE31 and LS54. The ponds with the higher temperatures, LMi87 and LS54, did not show thermophilic related species in their bacterial clone libraries. The bacterial sequences from these two ponds were related to species detected in the rhizosphere, in different contaminated environments or were simply related to species ubiquitous in all terrestrial environments (Table S3).

The OTUs represented by sequences B9-40_bact_d3 and LVE31_bact_c8 were affiliated to the genus *Hydrogenobaculum* according to RDP, although they were distantly related to other members and clustered in a separate branch in the bacterial phylogenetic tree (Fig. 2A).

Archaea were detected in the ponds with higher temperature conditions: B9-40, LS54 and LMi87, and Fig. 3B shows the relative abundances of archaeal OTUs. The phylogenetic tree in Fig. 2B illustrates that the archaea found in Copahue formed separate and distant branches from cultivated species, and in some cases even from uncultivated archaeal clones, which indicated the uniqueness of many of these archaeal phylotypes. Table S4 presents the phylogenetic association of the archaeal OTUs according to RDP, together with the sources of their closest relatives. On the contrary to that found for bacterial OTUs, all the archaeal sequences retrieved from Copahue ponds were related to species detected in thermophilic or acidic geothermal environments. OTU LS3-arch_a7, in particular, was 99% similar to *Candidatus* "Acidianus copahuensis" isolated from the Copahue geothermal system [19]. When considering RDP phylogenetic classification, sequences from LS54 were the best distributed over the *Archaea* domain, with sequences affiliated to the acidophilic, thermophilic genera *Thermogymnomonas* (from phylum *Euryarchaeota*), *Acidianus*, *Sulfolobus*, *Thermocladium* and *Vulcanisaeta* (from phylum *Crenarchaeota*) but distantly related to cultivated species. B9-40 archaeal sequences were limited to unclassified *Thermoplasmatales*, unclassified *Thermoprotei* and the genus *Sulfolobus*. In LMi87, the 95 clones analysed only belonged to one OTU affiliated to the genus *Sulfolobus* that was not related to any of the cultivated members, although it was 99% similar to one of the *Sulfolobus* related OTUs found in LS54 (Fig. 3B).

Hybridisation study: FISH and CARD-FISH

A quantitative study of biodiversity in the Copahue thermal ponds was undertaken using the DAPI stain and the FISH (or CARD-FISH) hybridisation technique with universal and specific probes (Table 2). The total cell number was calculated by recounts under the microscope using the DAPI stain, and cell densities were in the order of $2.5–6.6 \times 10^8$ cells mL^{-1}. Although cell densities were similar in all the ponds sampled, the distribution of microorganisms between bacteria and archaea was different for each pond. Fig. 4A shows the hybridisation percentages with the EUB338 and Arch915 probes, specific for the *Bacteria* and *Archaea* domains, respectively. In LMa36, only bacteria were detected by FISH, whereas LVE31, the other pond with moderate temperature conditions, showed low hybridisation (4%) with the Arch915 probe. On the other hand, B9-40, LS54 and LMi87 had high hybridisation percentages with the Arch915 probe, although LMi87 had the highest archaeal proportion (95%).

Hybridisations were performed with probes specific for the taxa *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Nitrospira* and *Aquificae*. The probe NITR712, specific for the *Nitrospira* group, did not hybridise with any of the five samples. The probe specific for the phylum *Aquificae* (Aqui-1197) yielded less than 1% hybridisation from the LVE31 and B9-40 samples and no hybridisation from the others. As expected from the results shown in Fig. 4A, only LMa36 and LVE31 samples hybridised with the probes specific for the different bacterial taxonomic groups. Fig. 4B represents pie charts of these results. LMa36 and LVE31 showed a similar distribution of species into bacterial classes, with a clear prevalence of *Betaproteobacteria* and a smaller percentage of *Gammaproteobacteria* (2.7% of the bacteria for LMa36 and 9.3% for LVE31). In LMa36, 1.9% of the bacteria detected by FISH hybridised with the THIO1 probe, specific for the genus *Acidithiobacillus*, while in LVE31 all cells that were positive with the GAM42a probe were also positive with the THIO1 probe. A total of 2.4% of the bacteria from LVE31 hybridised with the *Alphaproteobacteria* probe (ALF968), and most of them (2%) also hybridised with the probe specific for the genus *Acidiphilium* (ACD840), while 0.4% remained as unidentified *Alphaproteobacteria*. Hybridisation with the probe specific for the class *Betaproteobacteria* (BET42a) was 89.3% for LMa36 and 64.7% for LVE31. Within this class, *Thiomonas* specific probes TM1G0138 + TM1G0238 (TM) developed by Hallberg et al. [22] were used and LVE31 showed 54.6% hybridisation (leaving 10.1% of unclassified *Betaproteobacteria*). The LMa36 fluorescent signal with TM probes was very poor and negative hybridisation was assumed. Fig. 5 shows epifluorescence microscope images of LVE31 water samples hybridised with probes specific for domain *Bacteria*, class *Betaproteobacteria* and genus *Thiomonas*. DAPI stain images depicted cells with different morphologies: long and thin chains, shorter bacilli displayed one after the other and at least two kinds of single bacilli. Many of the different cells hybridised with the BET42a probe. The thin chains and some of the single bacilli (probably cells that had detached from the chains) also hybridised with *Thiomonas*-specific probes. The LMi87 sample showed a small

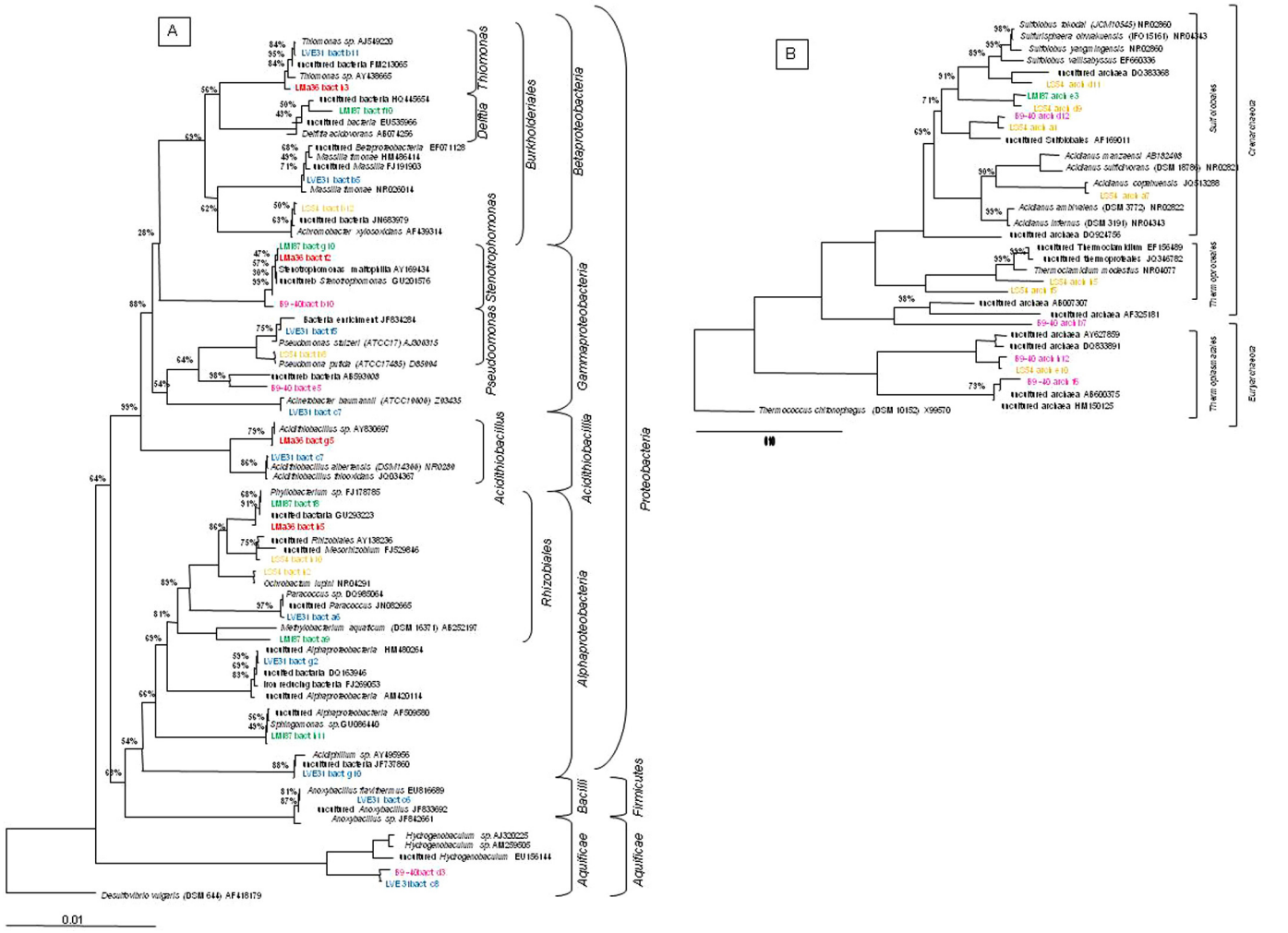

Fig. 2. Representation of the phylogenetic trees showing affiliation of bacteria (A) and archaea (B) found in the water samples of Copahue thermal ponds. The trees were constructed using 16S rRNA sequences of the closest matches from cultured and uncultured species. Organism names are followed by GenBank accession numbers. Sequences from Copahue ponds are shown in colour. The trees were calculated by ARB software using neighbour-joining and Jukes-Cantor correlations. The numbers at the branch nodes are percentage bootstrap values based on 1000 calculations. The scale bar represents the estimated sequence divergence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

percentage of hybridisation with an *Alphaproteobacteria*-specific probe, similar to that obtained with the EUB338 probe.

Discussion

The importance of physicochemical characteristics

The main characteristic of the Copahue geothermal area is its acidity, with the ponds studied having pH values between 2 and 3.2. Soluble forms of heavy metals were not detected in any of the ponds, although many of the sequences retrieved were similar to metal- (and also hydrocarbon-) tolerant species (Table S3). Iron is an important component of many geothermal or acidic environments, and it generally has an influence on biodiversity [4,8]. In Copahue, iron did not appear to play an important role related to microbial communities in the water of the ponds, since it was present at a low concentration.

The five ponds studied presented a narrow range of pH values and a wide range of temperature values. However, since hybridisation with probes specific for the *Bacteria* and *Archaea* domains showed a dramatic shift of community composition when temperature increased over 40 °C, it seems likely that temperature might have been the factor with the strongest influence on microbial community structure. This idea was supported by the results of the correspondence canonical analysis (CCA) correlating the environmental variables temperature, pH and conductivity with FISH hybridisation percentages (Fig. S3) and the presence/absence of acidophilic bacterial and archaeal OTUS (Fig. S4). In the representations of both CCA, the ponds studied were separated along the temperature gradient. In moderate temperature ponds (LMa36 and LVE31), bacteria, predominantly acidophilic, sulphur oxidising genera such as *Thiomonas* and *Acidithiobacillus*, were the dominant prokaryotes. When temperature increased only from 36 °C to 40.5 °C, the microbial community shifted to archaeal dominance (Fig. 4A). In a study carried out to determine how abiotic factors shape acidophile microbial communities in the acidic thermal springs of Yellowstone National Park the authors also found significant differences in phylogenetic composition between samples that differed even by only 5 °C [42].

It is interesting to note that although bacteria were poorly represented in B9-40, the 16S rRNA sequence analysis revealed an important contribution of *Hydrogenobaculum*-related sequences (Fig. 3A). Members of this genus are acidophilic, thermophilic (55–80 °C), sulphur and hydrogen oxidisers that have been found in other acidic geothermal environments sometimes related to the arsenic cycle [10,15,23]. In our study, arsenic was not detected,

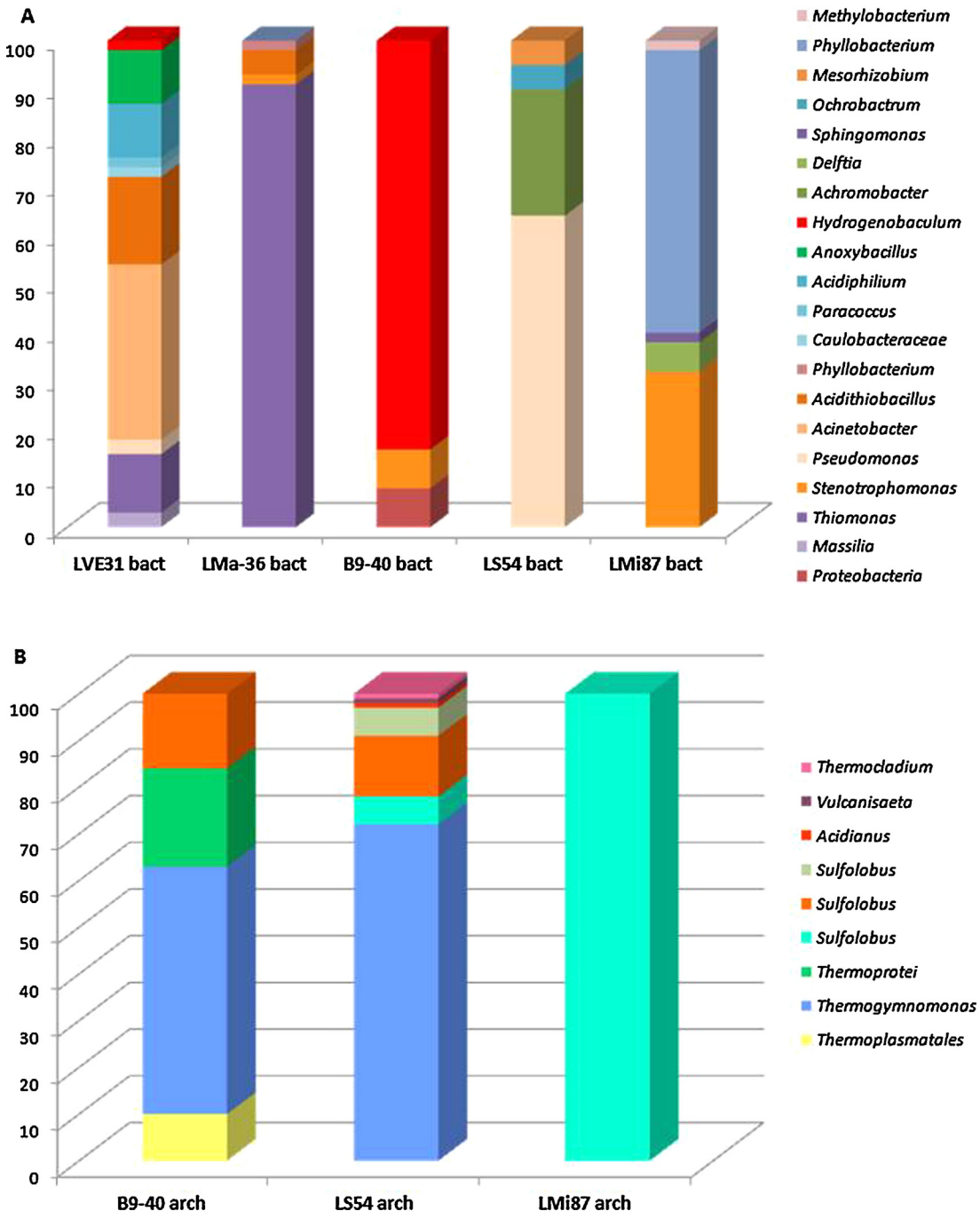

Fig. 3. Relative abundances of bacterial (A) and archaeal (B) OTUs in the five ponds studied. In A, the same colour indicates the same phylogenetic group but not necessarily the same OTU. In B, the same colour indicates the same OTU (less than 97% 16S rRNA sequence similarity). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

although it has been found in very low concentrations in geochemistry studies undertaken in the area [16]. The prevalence of sulphur over arsenic compounds and the low temperature of B9-40 might be probable explanations why *Hydrogenobaculum*-related species were outcompeted by archaea.

Ponds LS54 and LMi87 were at the highest temperature extreme of the gradient. The latter pond was in fact a hot spring and presented the strongest positive correlations with temperature and conductivity, and a negative correlation with pH. These extreme characteristics were reflected in the LMi87 archaeal population, which was composed of only one OTU related to the *Sulfolobus* genus.

Prokaryotic biodiversity in Copahue geothermal ponds

In the following discussion of the biodiversity found in the Copahue geothermal ponds, FISH and CARD-FISH data were used to quantify the different prokaryotic populations. OTU relative abundances were only used as semi-quantitative estimations, mainly for the *Archaea* domain, as no further information could be obtained by hybridising with probes specific for the archaeal groups found. As regards 16S rRNA gene frequencies, a relatively high number of cycles were used for PCR amplifications and this might have introduced biases in the results, masking the biodiversity of the ponds. However, bacterial OTU abundances were quite comparable

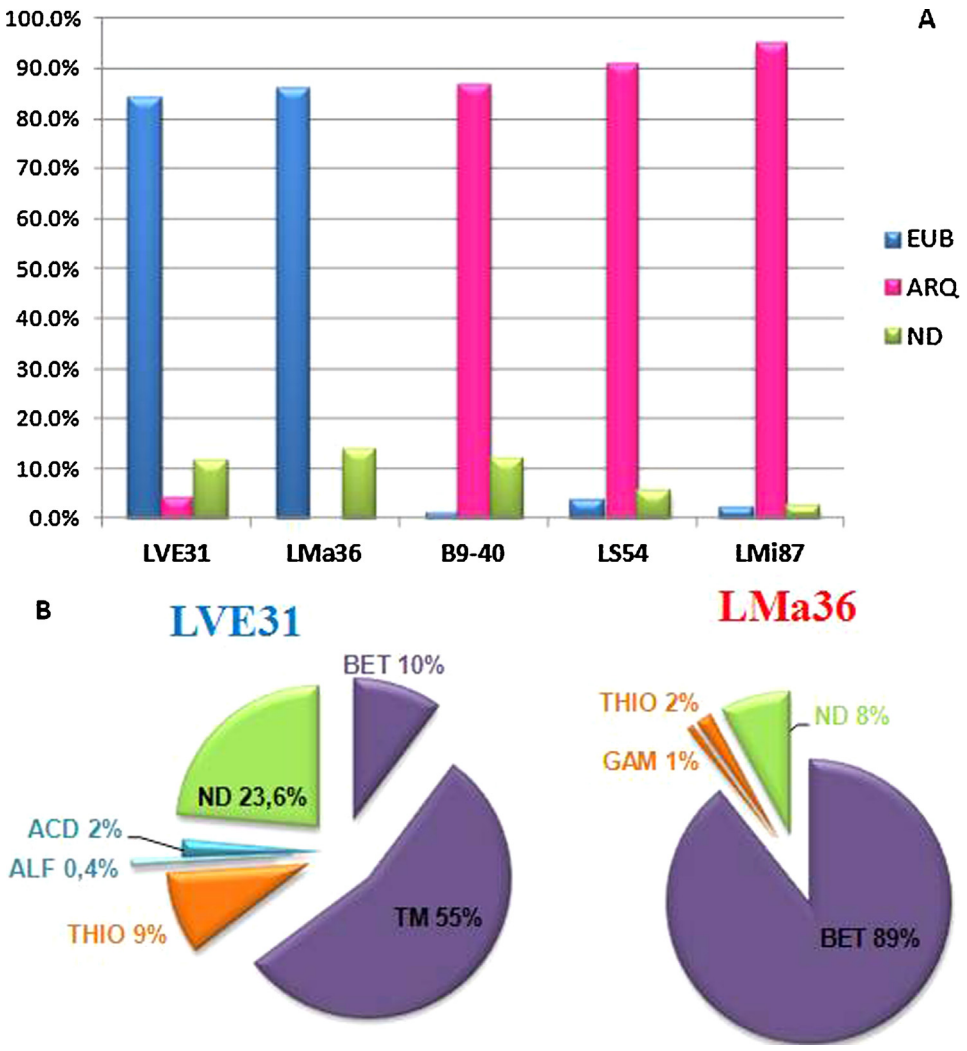

Fig. 4. (A) Hybridisation percentages with bacteria (EUB338)- and archaea (Arch915)-specific probes. ND represents the fraction of microorganisms stained with DAPI and not detected with any of the domain probes. (B) Hybridisation percentages with specific bacterial probes. ND represents the fraction of bacteria that hybridised with the EUB338 probe and did not hybridise with any of the specific probes used. The abbreviations of the probes are listed in Table 2.

to FISH hybridisation percentages when using probes specific for bacterial taxonomic groups.

Bacteria played a significant role only in moderate temperature ponds (LMa36 and LVE31). Despite presenting similar pH and temperature conditions, the bacterial community structure in LMa36 and LVE31 seemed to be different. LMa36 diversity was limited to four OTUs, and was dominated by *Thiomonas*- and *Acidithiobacillus*-related sequences, with a minor presence of chemoheterotrophs. LVE31 was also dominated by *Thiomonas*, according to hybridisations with a TM-specific probe, but presented a higher diversity with twelve OTUs, many of which were related to ubiquitous chemoheterotrophic species associated with animal or human presence (see Tables S1 and S3). One possible explanation for the different composition of these two physicochemically similar ponds could be related to the fact that LVE31 was located close to Copahue health centre which is used for therapeutic baths, while LMa36 was several kilometres away in an area with not much human or animal interaction. LVE31 showed approximately 4% hybridisation with the Arch915 probe, although it was not possible to amplify any archaeal 16S rRNA genes, despite using a wide combination of primers described in the literature.

The detection of *Thiomonas*-related species in LVE31 and LMa36 deserves a separate comment. To quantify their presence, hybridisation was attempted on water samples with TM1G0138 + TM1G0238 probes (TM, as developed by Hallberg et al. [22] specifically for this genus); however, clear hybridisation was only achieved in the LVE31 sample. Due to this unexpected finding, *Thiomonas*-related 16S rRNA sequences were checked from both samples and they were matched with TM1G0138 + TM1G0238 probe sequences using ARB software. Firstly, LMa36 and LVE31 *Thiomonas*-related sequences were found to be 99% similar to each other (distance matrix results not shown). Secondly, none of the *Thiomonas*-related sequences retrieved from Copahue had the target for probe TM1G0138. Despite being very similar, LVE31 sequences had the exact target for the TM1G0238 probe, and LMa36 sequences showed one central mismatch, which was probably responsible for the lack of a clear hybridisation signal. To solve this problem, we are in the process of developing a new *Thiomonas* probe using a 16S rRNA sequence database that includes the sequences retrieved from Copahue.

Hydrogenobaculum were the only thermophilic sulphur-oxidising bacteria reported in this study. Sequences with similarities of approximately 92% to *Hydrogenobaculum* sp. were found only in two of the ponds, however, they did not occur in significant amounts according to FISH hybridisations. No other species of the phylum *Aquificae*, typical of thermal environments [10,28,42], were found in Copahue.

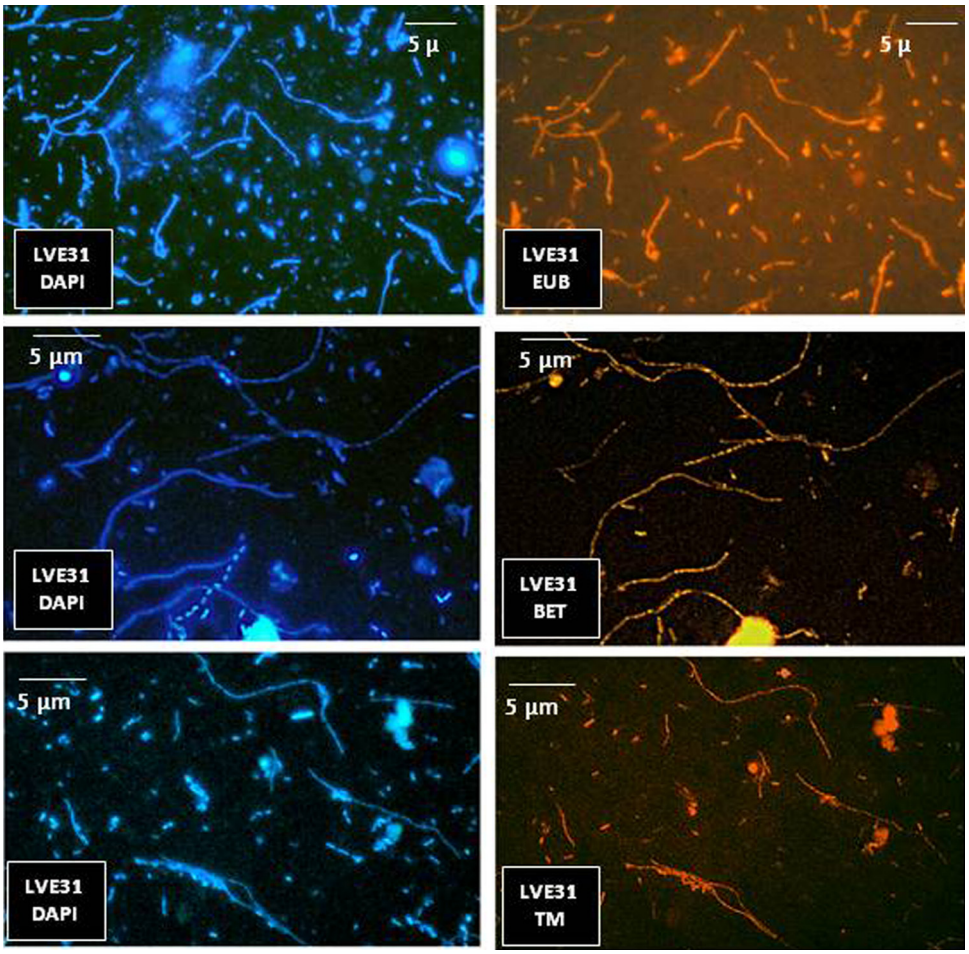

Fig. 5. Epifluorescence microscope images of LVE31 microorganisms stained with DAPI on the left and *Bacteria* (EUB)-, *Betaproteobacteria* (BET)- or *Thiomonas* (TM1G0138 + TM1G0238 probes, in the image abbreviated to TM)-specific probes on the right. Scale bar corresponds to 5 µm.

Archaea were dominant in the three ponds with higher temperature values. When comparing the sequence identities, the ponds with less extreme temperature values, B9-40 and LS54, presented a similar community structure, with prevalence of *Thermoplasmatales* over *Sulfolobales*, most of which were associated with a particular OTU affiliated to the genus *Thermogymnomonas* (Fig. 3B), although they were distantly related to cultivated members. As already mentioned, the archaeal community of LMi87 was dominated by a *Sulfolobus*-related sequence, distantly related to other cultivated or even uncultivated species of the genus, which was also detected in LS54.

While approximately 96% of the bacterial sequences were more than 97% similar to known and cultivated microorganisms, almost all archaea sequences were less than 97% similar to any cultivated species. The 16S rRNA sequences of the archaea found in Copahue geothermal ponds formed separate clades in the phylogenetic tree (Fig. 2B). The archaeal 16S rRNA sequences distantly related to uncultured clones make the Copahue thermal system a very interesting source of novel archaea. One example of this potential is represented by OTU LS54-arch_a7, a sequence affiliated to the *Acidianus* genus, but distantly related to cultivated members of the genus. In a parallel study, we were able to isolate and characterise these species and they have been reported as *Candidatus* "Acidianus copahuensis" [19]. This thermoacidophilic archaea is capable of growing aerobically or anaerobically, using different sulphur compounds, iron or organic carbon as energy sources, which makes it a suitable microorganism for bioleaching and biomining applications.

Besides showing the possible presence of novel species, the fact that the archaeal 16S rRNA sequences detected in Copahue presented low similarity percentages with sequences retrieved from other geothermal environments might reinforce the idea of a biogeographic barrier isolating endemic populations of thermophilic species. Similar to that found when comparing the biodiversity of *Sulfolobus* in different geothermal areas around the world [54], we also found identical *Sulfolobus*-related OTUs in different Copahue ponds (Fig. 3B), supporting the hypothesis that gene flow can occur between ponds separated by short distances, although the ponds in Copahue are much further apart (see the km scale on the map in Fig. 1) than the springs in the study mentioned (less than 50 m). Biogeography for the study of organisms through space and time was beyond the aim of this current study, and would require an in-depth analysis using additional genetic diversity markers and statistical tests. Even so, our results, which showed the presence of ubiquitous bacterial taxa and restricted archaeal taxa in different ponds within a restricted zone, make the Copahue geothermal area a suitable environment for completing studies concerning the identification of the mechanisms that shape biogeographic patterns [25].

Geothermal environments have been deeply studied in the past few years. The resultant large body of literature thus allowed us to compare the prokaryotic biodiversity found in Copahue and highlight its particular characteristics. For example, archaea were found to be more diverse but numerically inferior to bacteria in the very well-studied high temperature environment of Calcite Springs in Yellowstone National Park [44], which was contrary

to that found in the Copahue high temperature ponds. In the Hawaii Volcanoes National Park, the archaeal community is similar to the one described in Copahue, where *Crenarchaeota*, particularly *Sulfolobus*-related species, and uncultured representatives of this phylum are dominant [8]. Benson and co-workers reported that archaeal diversity in the Hawaii Volcanoes National Park decreased as temperature increased [8]. The same behaviour was found in the Copahue thermal ponds, with the extreme case of the LMi87 hot spring where only one archaeal OTU was found (Fig. 3B). The archaea detected in the thermoacidic spring fields in Ohwakudani (Hakone, Japan) [31] were similar to those found in Copahue, although the distribution of species in the different temperature sites was different. In the hot water samples (78 °C), *Crenarchaeota* were also dominant, however, the better represented genera were anaerobic hyperthermophilic *Vulcanisaeta* and *Caldivirga*, whereas *Sulfolobus* only represented a minor fraction. In the moderate temperature samples (28 °C), the prokaryotic community was dominated by thermophilic archaea, such as *Metallosphaera* and *Acidianus*. This was completely different from that found in Copahue moderate temperature ponds, where no archaea were detected. Some phylotypes found in the 28 °C samples were distantly related to the genus *Thermogymnomonas*, which was similar to that found in the high temperature ponds B9-40 and LS54. The most relevant difference between the two archaeal libraries was that the majority of the clones in the Japanese springs were 96–99% similar to cultured species, while in Copahue most of the archaea clones had no close cultivated relatives and seemed to belong to novel, still unreported species. In high temperature, less acidic geothermal environments, such as Champagne Pool in New Zealand (75 °C and pH 5.5) thermophilic bacteria related to the hydrogen and sulphur cycles, such as *Sulfurihydrogenibium* and *Thermoanaerobacter*, respectively, were detected [26].

Moderate temperature ponds in Copahue seemed to have a prokaryotic community similar to those reported for acid mine drainage (AMD) environments [22,29,30] with the prevalence of acidophilic bacteria such as *Acidithiobacillus*, *Acidiphilium* and *Thiomonas*, but with almost no presence of archaea. Species from *Thiomonas* have been detected in geothermal environments, chiefly related to arsenic metabolisms [23]. A substantial difference between LMa36 and LVE31 in Copahue and other well-studied moderate temperature acidic environments, such as AMD, the Frasissi caves in Italy or Río Tinto in Spain, is that in these environments iron oxidising microorganisms, such as *Acidithiobacillus* (*At.*) *ferrooxidans*, *Leptospirillum*, '*Ferrovum*' and *Ferroplasma* are important members of the microbial community [21,30]. The fact that iron was detected in very low concentrations in the five ponds studied here might explain the absence of iron oxidising species. However, strains of *At. ferrooxidans* and *Leptospirillum* have been isolated from the area [12,36].

Outline of preliminary geomicrobiological models in Copahue geothermal ponds

In order to provide a preliminary approach to part of the geomicrobiological model that might be operating in the Copahue geothermal ponds, the main physiological characteristics of the closest cultivated species of the OTUs detected were collected (Table S5). Cultivated species having up to 0.900 similarities with Copahue OTUs were considered in a RDP search focused particularly on acidophilic sulphur oxidising species. In LMa36, *Thiomonas*-related species were dominant (91% of the clone library and presumably an important fraction of the 89% *Betaproteobacteria* detected by FISH hybridisation) and, therefore, it can be assumed that mixotrophic metabolism with oxidation of sulphur compounds prevailed. The second better represented species, *Acidithiobacillus*, is an autotrophic sulphur oxidiser that can act as a primary producer. For B9-40, it is difficult to speculate on which metabolisms could be dominant, as 84% of the archaeal clones did not have close cultured matches. However, aerobic sulphur oxidation could be important in B9-40, since *Hydrogenobacullum*- and *Sulfolobus*-related species were detected. In LMi87, bacteria were a small fraction of the community, thus their metabolism probably had little impact. The unique archaeal OTU detected was affiliated to *Sulfolobus*, a genus comprised of aerobic, thermoacidophilic, facultative chemoheterotrophic species capable of oxidising sulphur compounds. Considering that archaea were dominant, the described metabolisms must have had a main role in LMi87. In LVE31, the species related to the OTUs detected showed wider physiological characteristics, since a greater variety of sequences related to sulphur compound oxidising species were detected in this pool (*Hydrogenobaculum* sp., *Thiomonas* sp., *Acidithiobacillus thiooxidans* and *Acidiphilium* sp.) and, according to FISH, they represented 64% of the viable bacteria. *Hydrogenobaculum* and *Acidithiobacillus* are lithoautotrophic genera; therefore, it can be assumed that they represented part of the primary producers of the LVE31 water ecosystem. A total of 3% of the clones were more than 99% related to *Pseudomonas stutzeri*, and it has been shown that some environmental isolates of this species were able to anaerobically oxidise thiosulphate to tetrathionate [49]. The LS54 bacterial library had 5% of the clones related to 16S rRNA sequences of *Mesorhizobium*, and it is worth mentioning that some isolated species of this genus have shown sulphur oxidising capability [17]. All LS54 archaea-related sequences were similar to thermophilic or hyperthermophilic, acidophilic species capable of oxidising organic and sulphur compounds, and *Candidatus* "Acidianus copahuensis" that also oxidises iron. This pool had the peculiarity of having two sequences related to strict anaerobic species: *Vulcanisaeta distributa* and *Thermocladium modesties*, whereas *Candidatus* "Acidianus copahuensis" can also develop under anaerobic conditions. It is important to note that 72% of the archaea clones in LS54 had no close cultured relatives, so their physiological characteristics and role in the pond's geochemistry remain unknown. As mentioned at the beginning, this discussion of the metabolisms present in the Copahue ponds is a preliminary approach based on the physiological characteristics of the species found and the geochemical information available. To confirm our suppositions, genomic and transcriptomic studies will be required, together with *in situ* determinations of sulphur and carbon compounds.

Considering the information presented, we aimed to elaborate a preliminary model of the interactions between prokaryotic biodiversity and Copahue's geophysicochemical conditions. Due to continuous volcanic activity, many sulphur compounds have accumulated in the area and they serve as energy sources for many of the microbial species of the ecosystem. Oxidation of these sulphur compounds releases protons that contribute to maintaining the acidity of hot springs and pools and this probably helps explain the high concentration of sulphate detected in the water of the ponds studied. The biodiversity results and the multivariate analysis undertaken allowed the prokaryotic community structure in the Copahue ponds to be classified into two groups according to temperature. In both of them, the geomicrobial model seemed to be very similar, although it was represented by different species. In moderate temperature ponds, mesophilic bacteria, such as *Acidithiobacillus* and *Thiomonas*, were responsible for sulphur oxidations. When the temperature increased some thermophilic bacteria appeared, such as *Hydrogenobaculum*, although sulphur metabolism seemed to be mostly carried out by archaea from the genus *Sulfolobus* and *Candidatus* "Acidianus copahuensis". Fig. 6 outlines the models described for moderate temperature (A) and high temperature (B) ponds focusing on sulphur compound metabolism. Finally, for the sulphur cycles proposed, the presence of sulphate reducing microorganisms in the anaerobic sediments

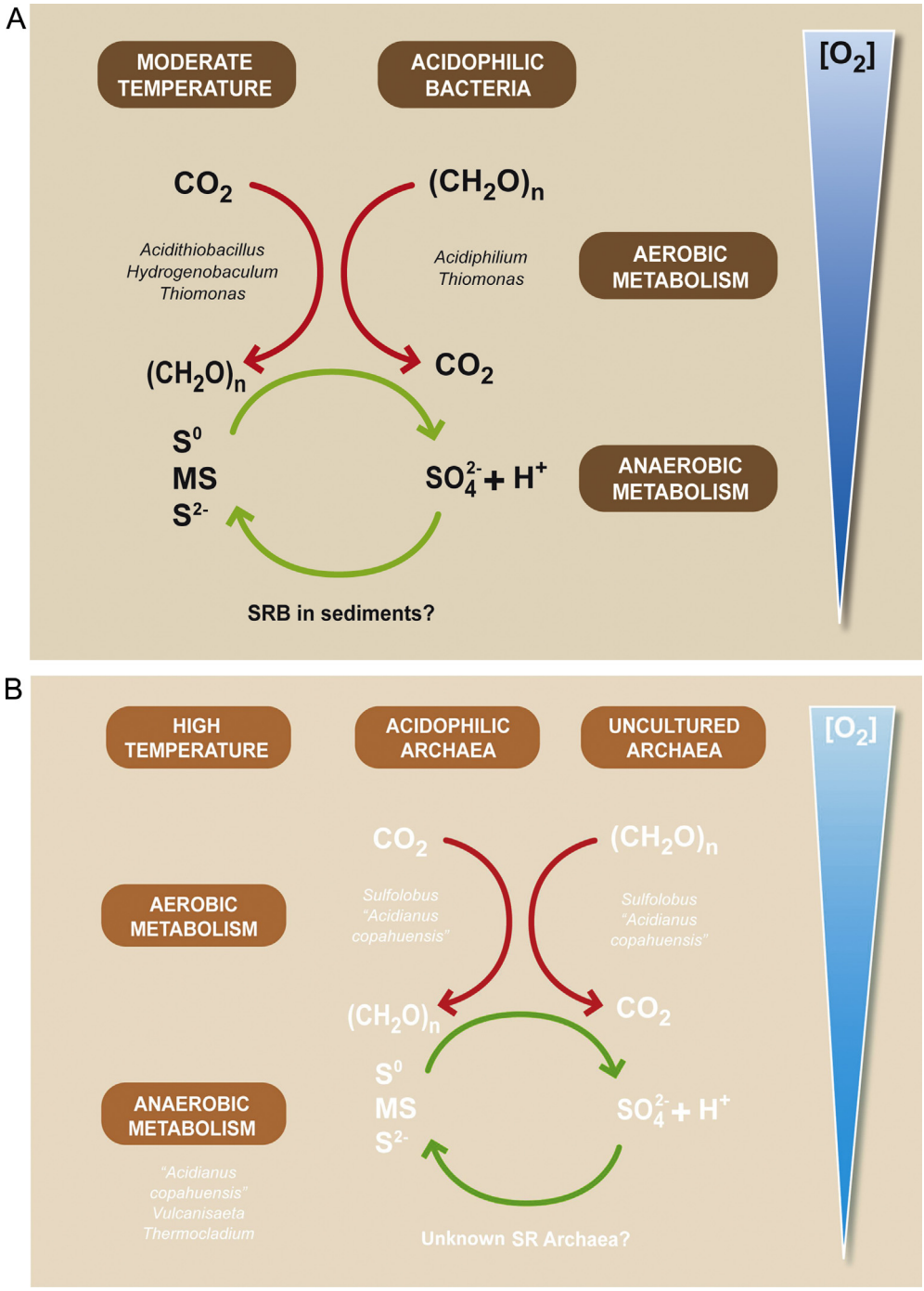

Fig. 6. Outlines of the possible geomicrobiological models of the sulphur cycle that might be operating in moderate temperature (A) and high temperature (B) ponds. MS: metal sulphide. $(CH_2O)_n$: organic compound.

of the ponds is suggested. Even though anaerobic sediments were not analysed in this study, our research group has isolated several sulphate reducing microorganisms from diverse Copahue pond sediments, including those studied in this work [56].

Conclusion

The Copahue geothermal system is different in terms of prokaryotic biodiversity from other similar environments where more thermophilic bacteria have been reported. According to our results, temperature was the main factor that divided the five ponds studied into two groups: LMa36 and LVE31 (moderate temperature ponds), which showed the predominance of bacteria (particularly acidophilic species related to AMD), and B9-40, LS54 and LMi87 (high temperature ponds) that were dominated by archaea related to thermal environments. Considering that most of the archaeal sequences detected in Copahue were distantly related to cultivated species and formed separate clades in the phylogenetic tree, we propose that the Copahue geothermal region is a habitat for potential novel species, probably related to the sulphur cycle. We consider this study as the first step for enrichment, isolation and characterisation of these possible new species.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2014.05.012.

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