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ORIGINAL PAPER

Microbial diversity in *Los Azufres* geothermal field (Michoacán, Mexico) and isolation of representative sulfate and sulfur reducers

Elcia M. S. Brito · Norberto Villegas-Negrete · Irene A. Sotelo-González · César A. Caretta · Marisol Goñi-Urriza · Claire Gassie · Florence Hakil · Yannick Colin · Robert Duran · Felix Gutiérrez-Corona · Hilda A. Piñón-Castillo · Germán Cuevas-Rodríguez · Olaf Malm · João P. M. Torres · Anne Fahy · Georgina E. Reyna-López · Rémy Guyoneaud

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Abstract *Los Azufres* spa consists of a hydrothermal spring system in the Mexican Volcanic Axis. Five samples (two microbial mats, two mud pools and one *cenote* water), characterized by high acidity (pH between 1 and 3) and temperatures varying from 27 to 87 °C, were investigated for their microbial diversity by Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and 16S rRNA gene library analyses. These data are the first to describe microbial diversity from *Los Azufres* geothermal belt. The data obtained from both approaches suggested a low bacterial diversity in all five samples. Despite their proximity, the sampling points differed by their physico-chemical conditions (mainly temperature and matrix type) and thus exhibited different dominant bacterial populations: anoxygenic phototrophs related to the genus *Rhodobacter* in the biomats, colorless sulfur oxidizers *Acidithiobacillus* sp. in the warm mud and water samples, and *Lyzobacter* sp.-related populations in the hot mud sample (87 °C). Molecular data also allowed the detection of sulfate and sulfur reducers related to *Thermodesulfobium* and *Desulfurella* genera. Several strains affiliated to both genera were enriched or isolated from the mesophilic mud sample. A feature common to all samples was the dominance of bacteria involved in sulfur and iron biogeochemical cycles (*Rhodobacter*, *Acidithiobacillus*, *Thiomonas*, *Desulfurella* and *Thermodesulfobium* genera).

Keywords Hydrothermal mud · Microbial mats · Acidophile · Biodiversity · *Desulfurella*

Abbreviations

AM1 and AM2	*Los Azufres* mud samples (AM1, 87 °C and AM2, 37 °C)

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E. M. S. Brito (✉) · N. Villegas-Negrete · I. A. Sotelo-González · H. A. Piñón-Castillo · G. Cuevas-Rodríguez
Ing. Ambiental (IA), Dept. Ing. Civil, Div. Ingenierías-CGT, Universidad de Guanajuato (UG), Av. Juárez, #77, Zona Centro, C.P. 36000 Guanajuato, Mexico
e-mail: emsbrito@gmail.com

C. A. Caretta
Dept. Astronomía, Div. Ciencias Naturales y Exactas-CGT, Universidad de Guanajuato (UG), Callejón de Jalisco, S/N, Col. Valenciana, C.P. 36240 Guanajuato, Mexico

M. Goñi-Urriza · C. Gassie · F. Hakil · Y. Colin · R. Duran · A. Fahy · R. Guyoneaud
Environmental Microbiology Group, EEM-UMR IPREM 5254, Université de Pau et des Pays de l'Adour (UPPA), Avenue de l'Université, 64012 Pau, France

F. Gutiérrez-Corona · G. E. Reyna-López
Dept. Biología, Div. Ciencias Naturales y Exactas-CGT, Universidad de Guanajuato (UG), Noria Alta, S/N, C.P. 36050 Guanajuato, Mexico

O. Malm · J. P. M. Torres
Lab. Radioisótopos Eduardo Penna Franca, Inst. Biofísica Carlos Chagas Filho (IBCCF), Universidade Federal do Rio de Janeiro (UFRJ), Av Carlos Chagas Filho, #373, Prédio do CCS-Bloco G, Rio de Janeiro CEP 21941-902, Brazil

AB1 and AB2	*Los Azufres* microbial mat samples (27 and 35 °C, respectively)
AW	*Los Azufres* water sample (36 °C at the surface)
AZLFE3	Isolated strain from AM2 sample, related to *Desulfurella kamchatkensis*

Introduction

Los Azufres geothermal field, located 250 km west of Mexico City in the state of *Michoacán*, is a Pleistocene silicic volcanic area situated 3000 m above the sea level (Aguilar et al. 1987). It is part of the Mexican Volcanic Belt, which crosses Mexico from East to West. This geothermal field is covered by a dense conifer forest, and is part of a national natural reserve. It comprises many natural hydrothermal springs, fumaroles and boiling mud pools, all naturally highly mineralized (Birkle and Merkle 2000).

The roles of microorganisms in geothermal sediments and deposits have been studied in several sites throughout the world (Walter et al. 1972; Schultze-Lam et al. 1995; Jones and Renaut 1996, 1997; Jones et al. 2000; Guidry and Chafetz 2002; Tobler et al. 2008), some of these studies based on isolated strains (Brock et al. 1972; Hugenholtz et al. 1998; Huber et al. 1998; Santos et al. 2007; Hamilton-Brehm et al. 2010). Microbial diversity has been extensively investigated in some sites like the Yellowstone National Park in USA. (Barns et al. 1994; Clingenpeel et al. 2011), some hot springs in Japan (Inagaki et al. 1997), the Great Artesian Basin in Australia (Kimura et al. 2005), the Rupi Basin in Bulgaria (Tomova et al. 2010) or the Uzon volcano in Kamchatka Peninsula, Russia (Gumerov et al. 2011). Of special interest here are the investigations on the links between physico-chemical conditions and microbial diversity and activity in geothermal sites (Blank et al. 2002; Hetzer et al. 2007; Gomez-Alvarez et al. 2007; Hall et al. 2008; Sayeh et al. 2010; Tobler and Benning 2011; Yang et al. 2012; Huang et al. 2011). However, few studies have investigated mud pools from these sites and no data are available on Los Azufres geothermal field.

Undoubtedly, Yellowstone National Park has been the most extensively studied site, and its wide variety of geochemical conditions is reflected in the composition of its microbial communities, the most famous one related to *Thermus aquaticus* (Brock and Freeze 1969). Many microorganisms involved in the sulfur and iron cycles have been detected in such environments. The presence of acidophilic sulfur-oxidizing autotrophic microorganisms (such as *Thiobacillus thiooxidans* and *Sulfolobus* spp.) has been demonstrated in solfatara soils (Fliermans and Brock 1972; Brock et al. 1972), and a thermophilic sulfate reducer from Obsidian Pool, *Thermodesulforhabdus norvegica*, has been described (Hugenholtz et al. 1998).

Most of the scientific studies of *Los Azufres* geothermal field and the Mexican Volcanic Belt in general concerned the geology, and few explored the microbial diversity as a whole. Microorganisms inducing corrosion in the condenser of a geothermal electric power unit were observed in *Los Azufres* (Torres-Sanchez et al. 1996), and Castorena et al. (2006) detected *Desulfotomaculum*, *Desulfovibrio*, *Thermodesulfobacterium* and *Burkholderia* populations within pitting corrosion. Investigating this environment could expand our knowledge of microbial diversity in extreme ecosystems, and may contribute to biotechnological applications. Microorganisms inhabiting such ecosystems can potentially be a source of novel biocatalysts (Grüning et al. 2009), and new remediation bioprocesses for acidic wastes such as acid mine drainage (Johnson and Hallberg 2005) could be developed using the metabolic capacities of populations from such environments. Among them, sulfate and sulfur reducers are promising candidates (Kaksonen and Puhakka 2007). The present work aims to investigate the microbial diversity from different sampling sites at *Los Azufres* spa, using T-RFLP and 16S rRNA gene library analyses. These data are discussed from the point of view of bioprospection (Brito et al. 2007), with a special interest for sulfidogenic microorganisms and their potential biotechnological application for metal removal.

Materials and methods

Sampling sites and procedure

Five samples from the spa resort *Los Azufres* (19°46′51.7″N and 100°39′23.6″W), located within *Los Azufres* geochemical field (Fig. 1), were analyzed. Two mud samples were collected: AM1, taken directly from the main geyser and characterized by a temperature of 87 °C, and AM2, collected 10 m downstream (west) at a temperature of 37 °C (see Fig. 2a, b, respectively). Two biomat samples, AB1 and AB2, were collected on the eastern shore of *Los Azufres*'s mud lake: AB1 formed a thin white microbial mat (27 °C, Fig. 2d), while AB2 (35 °C) was a red gelatinous mat floating at the surface (Fig. 2e, f). The fifth sample (AW) was taken from the water of a *cenote* (36 °C at the surface), which is the only one of the five sites isolated from human contact (Fig. 2c). These five samples were taken as representatives of five typical ecological niches within this geothermal site, varying in terms of temperature and matrix (mud, mat, water). Mud and biomat samples were collected in sterile plastic tubes. For

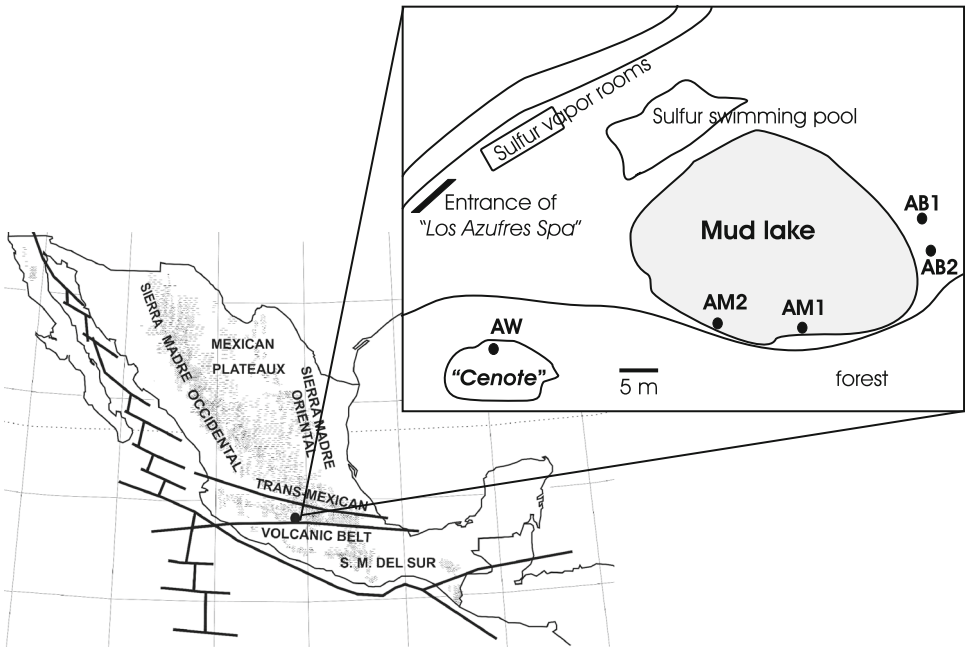

Fig. 1 Location of *Los Azufres* geothermal field (*black circle*) (19°46′51.7″N and 100°39′23.6″W) and map of the sampling area. *Solid lines* represent the tectonic zone

AW, 2 L of water was filtered (0.2 μm sterile filters) and the filters were stored for further analysis. Samples for molecular and chemical analyses were stored at −20 °C, those for microbial enrichments at +4 °C. Mud and water samples (collected in 2008) were processed in the EEM IPREM5254—University of Pau (France), while biomat samples (collected in 2009) were processed in the IA—University of Guanajuato (Mexico). Chemical analyses of all samples were performed in the IBCCF—Federal University of Rio de Janeiro (Brazil).

Physical and chemical characterization

pH, conductivity, temperature and dissolved oxygen were measured on site (Conductronic PC18 pH meter, J.T. Baker® and sensionion 6, Hach®). Metal contents were determined for AM1 mud sample collected in September 2008 and for biomat samples collected in September 2009. Unfortunately, metal content analyses were not done for AM2 and AW samples. The samples were lyophilized, crushed and prepared as described by Malm et al. (1989), briefly by digesting them with concentrated HNO_3 and HFl (Merck P.A.), for 18 h at 120 °C and, after, by evaporating the digested and adding 10 mL of 0.1 N HCl. They were analyzed using a Varian spectrophotometer (AA240FS, Fast Sequential Atomic Absorption Spectrometer, USA). Hg analysis was performed by cold vapor atomic absorption spectrometry with a Flow Injection Mercury System 400, Perkin Elmer USA (Bastos et al. 1998). Analytical grade reagents were used. Blanks were run throughout the analyses to check for any contamination. Triplicate measurements were done for each heavy metal determination. The detection limits are as follows: 0.0001 for Hg, 0.262 for Zn, 0.560 for Mn, 0.010 for Cr, 0.003 for Ni, 0.216 for Pb, 0.005 for Cu, 0.002 for Cd, and 0.017 for Fe (all in mg kg^{-1}). Detection limits were calculated using the formula: $(3 \times S_b)/X_b$ where S_b is the standard deviation of 6 measurements of the blank and X_b is the mean of the angular coefficient of the calibration curve (Silva and Alves 2006).

DNA extraction

DNA was extracted using a protocol adapted from Tsai and Olson (1991). After crushing in liquid N_2, 1 g of sample was mixed with 400 μL Tris–Glucose–EDTA solution containing lysozyme (final concentration 1 mg mL^{-1}) and achromopeptidase (final concentration 1500 U mL^{-1}), and incubated at 37 °C for 10 min. Two μL of proteinase K (20 mg mL^{-1}) and 20 μL of 10 % sodium dodecyl sulfate (SDS) was added followed by incubation for 30 min at 37 °C. A further 20 μL 10 % SDS was added and incubation prolonged for 10 min at 60 °C. After this cellular lysis, a deproteinization step was performed with organic solvents (phenol, phenol–chloroform, chloroform–isoamyl alcohol: 24/24/1 v/v) (Ogram et al. 1987). DNA precipitation was carried out by adding 10 % volume of sodium acetate (3 M, pH 5.2) and 2 volumes of 95 % ethanol, followed by overnight incubation at −20 °C. After centrifugation (14000*g*, 30 min) and washing in 70 % ethanol, the DNA was re-suspended in sterile Milli-Q® water and incubated with 1 μL RNAse (1 mg mL^{-1}) at 37 °C for

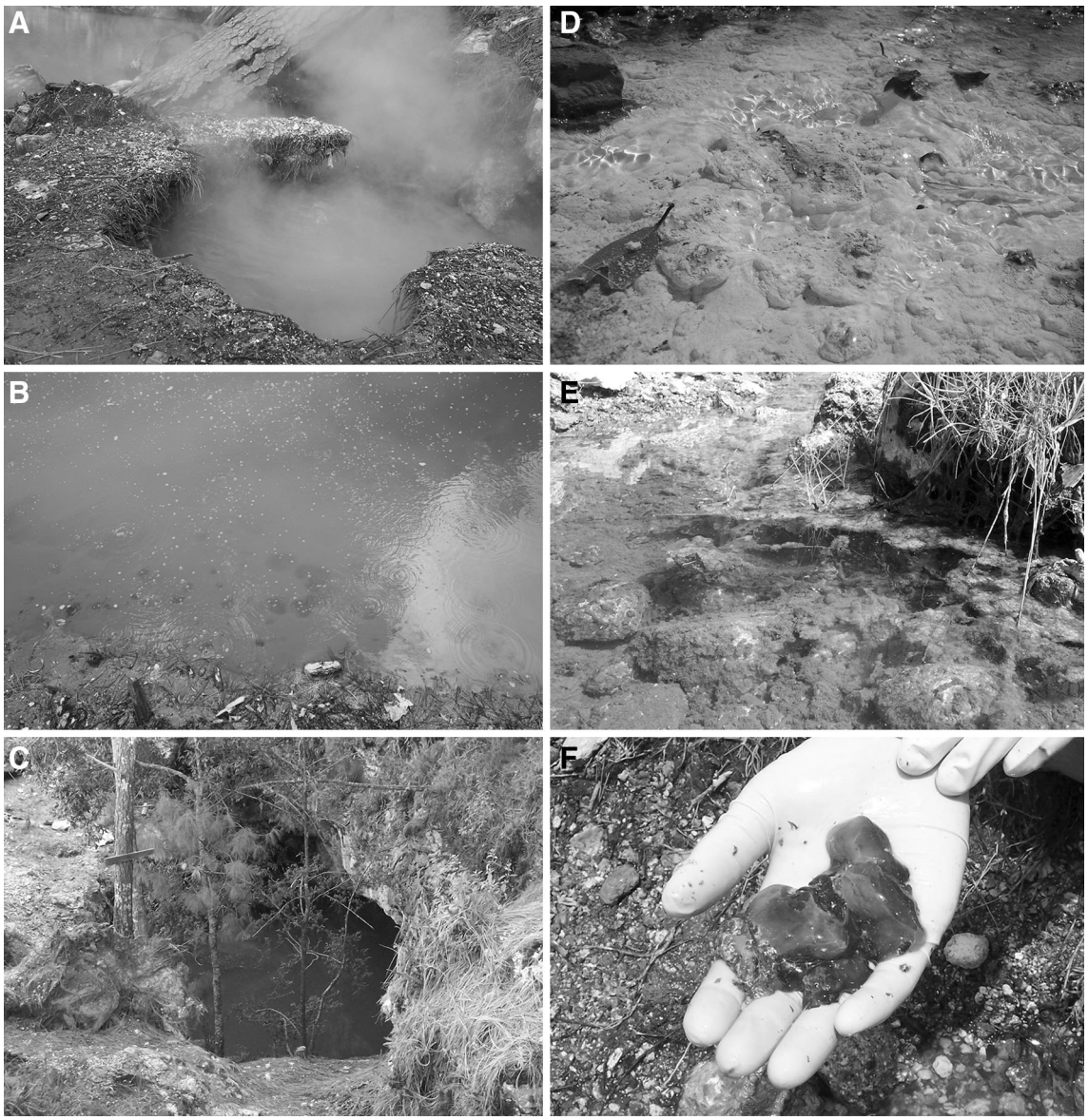

Fig. 2 Photographs of the sampling points: **a** hyperthermal mud AM1, **b** mesothermal mud AM2, **c** cenote AW, **d** white biomat AB1, **e** floating red biomat AB2, and **f** detail of AB2

30 min. DNA quality was verified by electrophoresis in a 1 % agarose gel in Tris-Acetate–EDTA buffer. DNA solutions were stored at −20 °C until further analysis.

PCR amplification of 16S rRNA gene sequences, T-RFLP and 16S rRNA gene libraries analyses

The 16S rRNA gene was amplified using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′; Lane 1991) and 907R (5′-GCCCCCGTCAATTCMTTTRAGTTT-3′; Lane et al. 1985) according to Paissé et al. (2008) in a PTC 200 Thermo-cycler (MJ research). The PCR products were purified using GFX PCR DNA purification kit (Amersham) following manufacturer's instructions.

For T-RFLP analysis the 16S rRNA gene amplification was performed using a carboxifluorescein-labeled (FAM) 8F primer and an unlabeled 907R primer. The purified PCR products were digested with 3 U of restriction enzyme *Hae*III or *Hin*P1I at 37 °C for 3 h. Approximately 50 ng of digested DNA was mixed with 20 µL of deionized formamide and 0.5 µL of TAMRA® ladder (Applied Biosystem®, USA) and denatured (94 °C for 5 min and chilled on ice). The length of the terminal restriction fragments (T-RFs) was determined by capillary electrophoresis on an ABI prism 310 (Applied Biosystem®, USA) according Brito et al. (2006). The T-RFLP profiles were analyzed using GeneScan Software (Applied Biosystem®, USA). Data sets were normalized and T-RFs representing <1 % of

total fluorescence were removed (Hewson and Fuhrman 2006). Statistical analyses were carried out with MVSP software (Multi-Variate Statistical Package 3.1, Kovach Computing Services, UK).

Clone libraries were obtained with unlabeled PCR products using pCR2.1 TOPO® TA cloning kit (Invitrogen, Inc.). The cloned 16S rRNA gene fragments were amplified by PCR using universal M13 primers and sequencing was carried out by Research and Advanced Studies Center of the National Polytechnic Institute (CINVESTAV-Irapuato, Mexico) or GATC Biotech Company (Germany). The sequences obtained were checked for chimeras and compared to those in the database of The National Center for Biotechnology Information (NCBI) website.

(http://www.ncbi.nlm.nih.gov) using BLAST. The cloned sequences, together with reference sequences, were aligned with the ClustalW program and phylogenetic trees were obtained with the Mega software using the neighbor-joining method. The confidence of the phylogenetic trees was assessed by bootstrap using 1000 resampling. The sequences determined in this study have been submitted to EMBL database and assigned accession numbers HF677518 to HF677569.

A population census of species using the 16S rRNA gene, or other molecular marker, provides limited information on the presence or absence of species in an environmental sample; a more robust approach includes statistical analyses that account both for species richness and phylogeny (Schloss and Handelsman 2005; Gomez-Alvarez et al. 2007). A population or operational taxonomic unit (OTU) was defined as a group of sequences with similarity >97 %. We used coverage values (C) to estimate the sampling depth of our 16S rRNA libraries (Good 1953; Chao and Lee 1992): $C = 1(n_i/N)$, where n_i is the number of OTUs in a library and N is the total number of sequences in this library.

Cloned sequences were digested in silico with *Hae*III and *Hin*P1I to generate virtual T-RFs which were subsequently compared to actual T-RFLP data. This procedure is described in detail by Caretta and Brito (2011). Briefly, aligned sequences (including the primer) are searched by a computer program to find the restriction site of an enzyme. At the first appearance of this restriction site, the program calculates the length of the T-RF in nucleotides. These in silico T-RFs and their distribution can be directly compared to actual T-RFLP results, providing additional support when assigning species names to T-RFs in T-RFLP fingerprints, especially those associated with abundant and intermediate populations. For abundant populations the match is usually 100, 64 % for the intermediate and about 52 % for the poorly represented populations (Caretta and Brito 2011).

Culture enrichments and strains isolation

The first enrichment steps were performed using culture media made from filtered (0.2 μm) site water as described by Guyoneaud et al. (1996). The culture medium was supplemented with acetate-Na, lactate-Na, glycerol or ethanol (10 mM each). Sulfate (20 mM) or elemental sulfur was added as electron acceptor. The medium was distributed in sterile flasks closed with a butyl stopper according to Guyoneaud et al. (1996) and reduced by adding $Na_2S_2O_4$ (200 μM) before use. Isolation procedures were performed by deep agar dilution series using the same medium. The identification of isolates was performed by 16S rRNA gene amplification and sequencing, as previously described (Guyoneaud et al. 2002). The biomat and mud samples AB1, AB2, and AM2 were used to isolate endemic microorganisms; however, we only succeeded in isolating organisms from AM2 warm mud fluid.

Results

Physical and chemical characterization

The physicochemical characteristics of the samples are summarized in Table 1. All the samples were found to be highly acid (pH values between 1.0, for AW *cenote* water, and 4.8, for AB1 biomat). Dissolved oxygen (DO) measurements showed that the hot mud sample was anaerobic (below detection limit) while the biomat samples exhibited limited amount of oxygen (about 3 mg L^{-1} DO), the others presenting values between these two (0.5 mg L^{-1} for AM2 and 0.75 mg L^{-1} for AW). The three samples analyzed for metal content (AM1, AB1 and AB2) showed similar levels of Ni, Cu and Cd (although the Ni was slightly higher for AB2 and the Cu for AB1). Indeed, all samples showed very high levels of heavy metal concentrations, in particular Hg, Pb, and Fe (more than 1000 times the reference for water contamination levels). Other studies carried out on *Los Azufres* geothermal site, associated to the geothermal power plants, showed lower values for mud samples (Birkle and Merkel 2001), except for Fe, while analyses for runoff water (Birkle and Merkle 2000) revealed values around the range in which the measurements for our samples are (except for Mn, Cu and Fe, for which their values were higher, respectively, 1109, 44.5 and 42000, and for Pb and Cd, for which their values are lower, respectively, 21.7 and below DL, all values in mg kg^{-1}). As observed at other extreme sites (Tobler and Benning 2011), it is expected that parameters such as pH, temperature and concentration of metals and metalloids strongly influence the microbial diversity in *Los Azufres* muds and waters.

Table 1 Physicochemical characterization of samples AM1, AB1 and AB2

	T	DO	EC	pH	Hg	Zn	Mn	Cr	Ni	Pb	Cu	Cd	Fe
AM1	87–90	<DL	1.0	3.7 ± 0.3	2.90 ± 0.09	214.21 ± 17.36	119.58 ± 18.94	11.55 ± 4.05	10.02[(a)]	54.57 ± 0.03	13.82 ± 4.25	3.04 ± 0.4	1.12 ± 0.13
AB1	27	4.9	0.1	4.8 ± 0.5	22.28[(a)]	20.59 ± 3.50	20.38 ± 4.94	2.83 ± 1.55	11.51 ± 1.60	119.50 ± 14.58	15.77 ± 4.83	2.35 ± 0.06	2.20 ± 0.05
AB2	35	2.6	0.2	2.0 ± 1.6	39.32 ± 2.12	13.58 ± 9.69	18.74[(a)]	19.86 ± 14.05	24.00 ± 3.40	90.28 ± 39.89	13.85 ± 2.76	4.53 ± 0.63	15.32[(a)]

T temperature (°C), *DO* dissolved oxygen (mg L^{-1}), *EC* electric conductivity (μS cm^{-1}), *DL* detection limit. Metal concentrations in mg kg^{-1}, except for Fe (g kg^{-1}); (a) no sampling replicate. The recommended international criteria for water quality of USEPA are (mg L^{-1}): 0.002 for Hg, 7.4 for Zn, 0.1 for Cr, 0.47 for Ni, 0.015 for Pb, 1.3 for Cu, 0.005 for Cd, and 1.0 for Fe (source http://water.epa.gov/drink/contaminants/index.cfm and http://water.epa.gov/scitech/swguidance/standards/current/)

Bacterial diversity

The bacterial diversity analyses for AM1 (mud), and AB1 and AB2 (biomats) were carried out using T-RFLP (Fig. 3). The number of T-RFs detected in the samples was low, suggesting low bacterial diversity. Six, 12 and 15 T-RFs (*Hae*III digest) and 4, 10, and 15 T-RFs (*Hin*P1I digest) were identified for AM1, AB1 and AB2 samples, respectively. Moreover, the bacterial communities showed dominant Operational Taxonomic Units (OTUs). In T-RFLP analysis, each T-RF peak in considered as an OTU; however, different species can produce the same T-RF, thus bacterial diversity can be underestimated with this method. The double dominant (*Hae*III digest), corresponding to T-RF 35 bp and T-RF 79 bp, represented 70 and 30 % in AM1 mud sample and 77 and 23 % in AB1 biomat sample, whereas in AB2 biomat sample the diversity was distributed across four main OTUs corresponding to T-RFs 35, 79, 213 and 219 bp (45, 20, 15 and 20 %, respectively). When using *Hin*P1I restriction enzyme, we also found a double dominant pattern for AM1 and AB1 samples, respectively, T-RFs 79 and 101 bp (78 and 22 %) and 79 and 431 bp (32 and 68 %). Similarly, diversity in

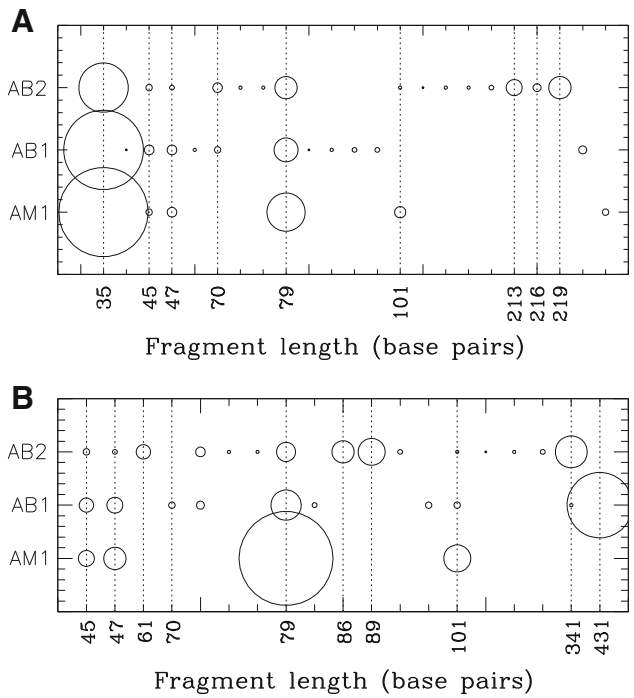

Fig. 3 T-RFLP analysis for bacterial communities from *Los Azufres* samples collected in September 2008 and September 2009: **a** digestion with *Hae*III enzyme and **b** digestion with *Hin*P1I enzyme. AM1: hot spring mud; AB1 and AB2: biomat samples. Circle size is proportional to the relative abundance of T-RFs. Six, 12 and 15 T-RFs (*Hae*III digest) and 4, 10, and 15 T-RFs (*Hin*P1I digest) were identified for AM1, AB1 and AB2 samples, respectively

AB2 was distributed across four main OTUs: T-RFs 79, 86, 89 and 341 bp (19, 22, 27 and 32 %, respectively).

To investigate the effect of environmental parameters (Cu, Pb, Hg, Fe, Ni, Cd, Cr, Zn and Mn and temperature, DO, EC and pH) on bacterial community structure and composition, a canonical correspondence analysis (CCA) was performed using MSVP software with aggregated T-RFLP patterns (i.e. combining both enzymes). This analysis (Fig. 4) suggested that AM1 mud bacterial community was mainly influenced by Zn, Mn, temperature and EC; AB1 biomat bacterial community by Cu and slightly by Pb and pH; and AB2 biomat bacterial community by Ni and Fe (and marginally by Hg, Cd and Cr). Hg was placed between AB1 and AB2 since both samples presented relatively higher levels of this metal. The three samples were relatively separated in this analysis, consistent with the slightly different T-RFLP profiles they exhibited. This does not mean they were completely unrelated but instead that the method could highlight their differences. The method could also detect small differences in chemical compositions, for instance the small overabundance of Cu in sample AB1 (compared to samples AB2 and AM1 which present about the same amount of this element) indicated that its populations may be influenced by this factor.

16S rRNA gene libraries were constructed using DNA from all five samples: 82, 84, 90, 61 and 102 sequences were obtained from AM1 and AM2 mud samples, AW water sample, AB1 and AB2 biomat samples, respectively. Coverage values showed that sufficient sequences were analyzed to be representative of each library ($C = 0.91$, 0.93, 0.91, 0.90 and 0.95 for AM1, AM2, AW, AB1 and AB2, respectively). Low bacterial diversity was observed for all samples with 7, 6, 8, 6 and 5 OTUs for AM1, AM2, AW, AB1 and AB2, respectively, based on 97 % similarity for 16S rRNA gene sequences, with a strong dominance of some populations (Fig. 5). Indeed, with the exception of AM2, one dominant population was observed in all libraries, accounting for 74–84 % of the diversity, between one and three populations were median (6–12 %), the remaining populations being less represented (Fig. 5). In the case of AM2 sample, three codominant populations (related to *Acidithiobacillus* sp., *Desulfurella kamchatkensis*, and an uncultured *Thermotogales*) were observed (Fig. 5). Taking into account all samples, the *Proteobacteria*-affiliated sequences were predominant (366 clones out of 419), the remainder being affiliated to *Cyanobacteria*, *Chlorobi*, *Firmicutes*, *Acidobacteria* and *Thermotogae* (Figs. 6, 7). The sequences were mostly affiliated to strains or uncultured organisms detected in acid and hot environments, such as hydrothermal vents and acid mine drainage.

An in silico T-RFLP approach (Caretta and Brito 2011) led to correlate T-RF 89 and 341 bp (*Hin*P1I restriction enzyme) to *Chlorobaculum* and *Rhodoblastus* genera, while T-RF 92 bp corresponded to a *Sulfurimonas*. The *Hae*III in silico restriction of sequences related to *Rhodoblastus* gave a 34 bp T-RF, the ones related to *Thiomonas* gave a 218 bp T-RF, the ones related to *Thermodesulfobium* a 220 bp T-RF and the ones related to *Sulfurimonas* gave a 221 bp T-RF. Comparing in silico T-RFs with T-RFLP data (*Hae*III restriction) revealed that T-RF 35 corresponds to *Rhodoblastus* and T-RFs 216 and 219 bp correspond to the other three genera. Thus, the in silico analysis of 16S rRNA cloning sequences suggested an identity for most T-RFs from AB1 and AB2 samples: with *Hin*P1I, *Chlorobaculum*, *Rhodoblastus* and *Sulfurimonas*, and with *Hae*III, *Thiomonas, Thermodesulfobium* and *Sulfurimonas*. The sum of these populations represented

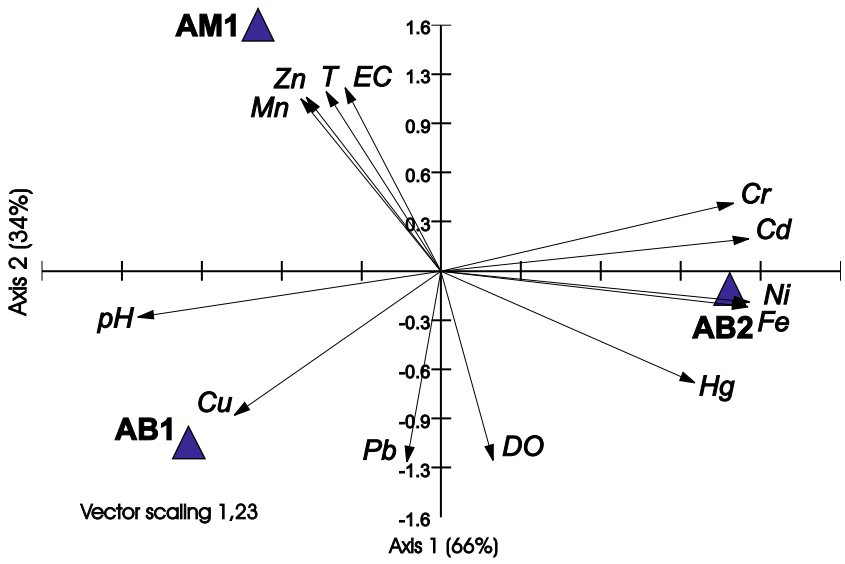

Fig. 4 CCA combining T-RFLP data (from both enzymes *Hae*III and *Hin*P1I) and environmental factors (temperature, metals). AM1: hot spring mud; AB1 and AB2: biomat samples. Axis 1 accounts for 64 % of the variability, and axis 2 for 36 %

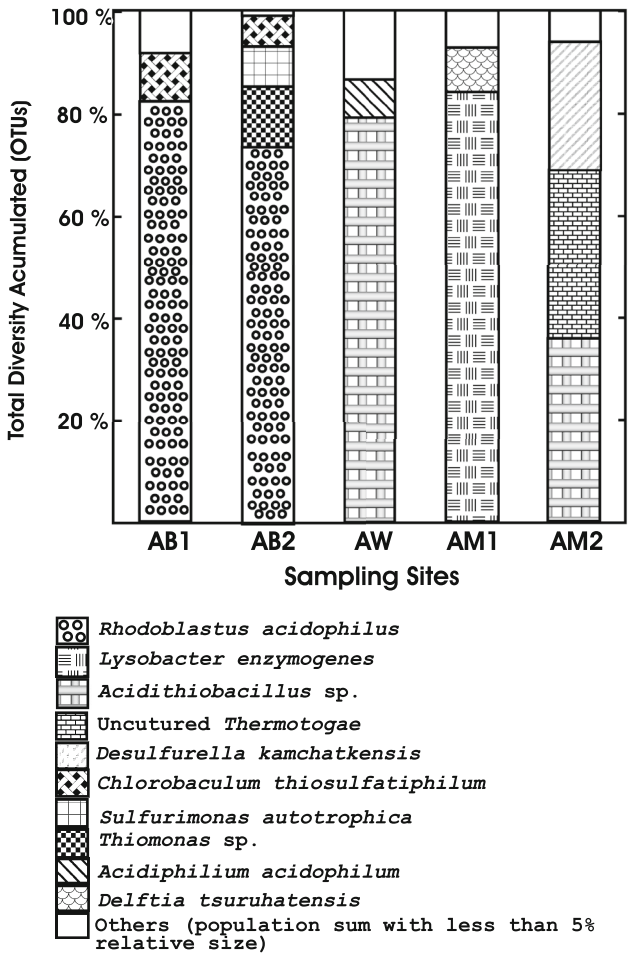

Fig. 5 Total diversity of bacterial 16S rRNA gene libraries obtained from mud (AM1 and AM2), *cenote* water (AW) and biomat (AB1 and AB2) samples

nearly 95 and 99 % of the sequences, respectively, for AB1 and AB2. For AM1 no correlation between in silico T-RFs and T-RFLP data could be highlighted. It is worth to note that the dominant populations in AB1 and AB2 were about the same, although their T-RFLP profiles revealed they were slightly different. This demonstrated that the differences were mostly based on the less dominant populations, detected by the T-RFLP analysis but abundant enough to be detected in the clone library.

Using specific culture media for sulfate and sulfur reducing bacteria, one strain was isolated in pure culture (strain AZLFE3, from sample AM2). This strain was related to the genus *Desulfurella*, representing 25 % of AM2 16S rRNA gene library. Strain AZLFE3 is a moderate thermophilic and acidophilic bacterium able to use S^0 as main electron acceptor. In addition, enrichments of a sulfate reducer, related to *Thermodesulfobium* were obtained and identified according to their bisulfite reductase sequences (data not shown). These *Thermodesulfobium* were obtained in co-cultures with *Desulfurella* representatives.

Discussion

Mud geothermal eruptions may be classified as mud volcanoes (large edifices built up mainly of mud and other sedimentary constituents, periodically or continuously venting liquid mud and other volcanic material, e.g. Hovland et al. 1997), mud cones (small mud volcanoes, shorter than 10 m, produced by the extrusion of viscous gas-charged mud, e.g. Camerlenghi et al. 1995), "gryphons" (steep mud cones, shorter than 3 m, e.g. Yakubov et al. 1971) and "*salses*" (large pools of bubbling clay and water, e.g. Newton et al. 1980). In this context, the *Los Azufres* mud pool studied in the present work must be classified as *salse*.

The geothermal potential of *Los Azufres* site had been exploited since 1982 to produce electrical energy. Environmental studies discussing the impact of the thermoelectric power stations were published by Birkle and Merkle (2000). By investigating several sites outside the thermoelectric area (springs, surface runoff water and river waters), the authors drew attention to the water quality of some small streams whose metal concentrations exceeded EPA and WHO drinking water standards (US EPA 1990; WHO 1993). This was particularly the case for Fe, Mn, As, B, Hg, Pb, F and Se, which can represent a considerable risk to human health and the environment (Birkle and Merkle 2000). In contrast to Birkle and Merkel (2001), in the present study we analyzed mud and biomat samples, thus it is reasonable to expect distinct characteristics and contamination levels. We found that contamination was up to 3 orders of magnitude higher than the WHO directives for drinking water. Our samples were collected within one of the most popular spas of *Los Azufres* geothermal site, where a sulfurous mud pool is considered by the local population to have healing properties. Users only cover their body and never ingest the mud, reducing the risk of metal uptake. However, the whole *Los Azufres* region is used as a recreation area, with thermal swimming pools and hot springs, as well as for trout farming. Thus it is important to check the level of metals and to assess the risk for the environment and human health. For instance, the mercury concentration observed in this work exceeded by 3 orders of magnitude the WHO directives, and by 4 orders of magnitude those found in volcanic aquifers in Italy and the Antilles (Bagnato et al. 2009a, b, c). The concentration of mercury in water depends on many factors, mainly pH (Jenne 1970), mineral Hg adsorption being lower under acidic conditions (Anderson 1979). Furthermore, the high sulfate contents in thermal waters are systematically

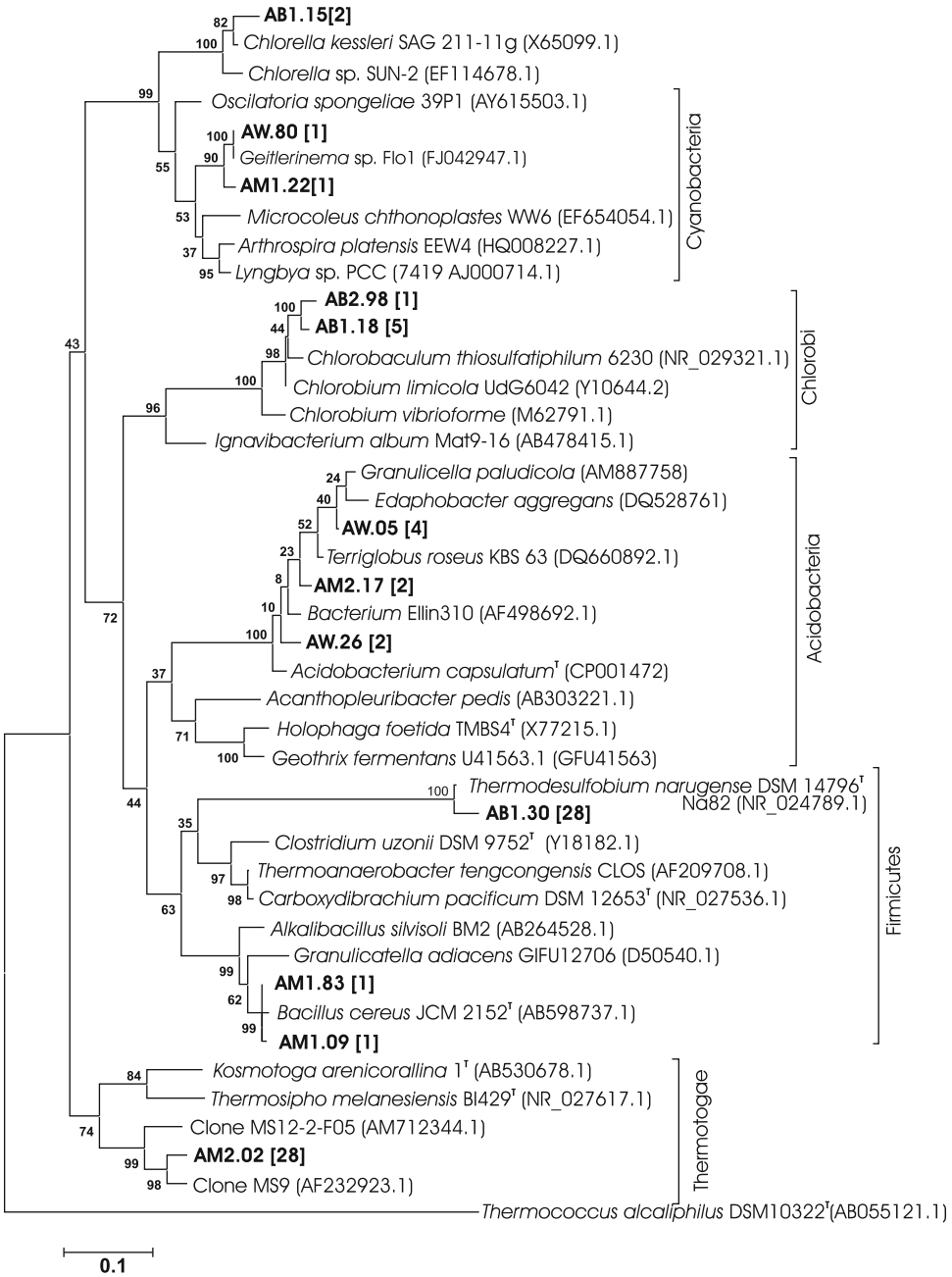

Fig. 6 Phylogenetic relationship between the 16S rRNA gene sequences obtained from mud (AM1 and AM2), water (AW) and biomats (AB1 and AB2) from *Los Azufres* geothermic field. The tree takes into account all phyla with the exception of the *Proteobacteria*. *Numbers inside square brackets* indicate the number of sequences for this OTU. The tree was constructed with related sequences obtained from NCBI database, using neighbor-joining algorithm, with an alignment of 754 bp. The *scale bar* represents the number of substitutions per nucleotide

associated with low pH and Eh, thus favoring the scavenging of Hg adsorbed onto mineral surfaces, which in turn can be emitted into the atmosphere as poorly reactive Hg^0 (Giggenbach 1988). Even if part of Hg is released into the atmosphere from fumaroles, an important amount of Hg persists in the water and mud, probably as mercury-sulfide complexes $Hg(H_2S)_2$ and $Hg(HS)_2$ or Cl-complexes($HgCl_2$) (Varekamp and Buseck 1984; Christenson and Mroczek 2003).

Despite the proximity of the sampling sites and the inter-connection of hydrothermal sources, defining, for example, their acidic conditions, the physicochemical characteristics revealed considerable differences. Temperatures ranged from mesothermal conditions (25–37 °C) for biomats, AM2 and AW samples, to hyperthermal for the mud close to the geyser (84 °C in AM1). The most extreme conditions were those present in AM1 mud since, besides the acidity, hyperthermic and anoxic conditions were detected. AW *cenote* water and AM2 mesophilic mud were also considerably extreme due to their very high acidity. In terms of metal concentrations, mud sample AM1 exhibited higher levels of Zn and Mn, while biomat sample AB1 showed a marginally higher concentration of Cu and AB2 of Ni and Fe, both biomats presenting higher levels of Hg.

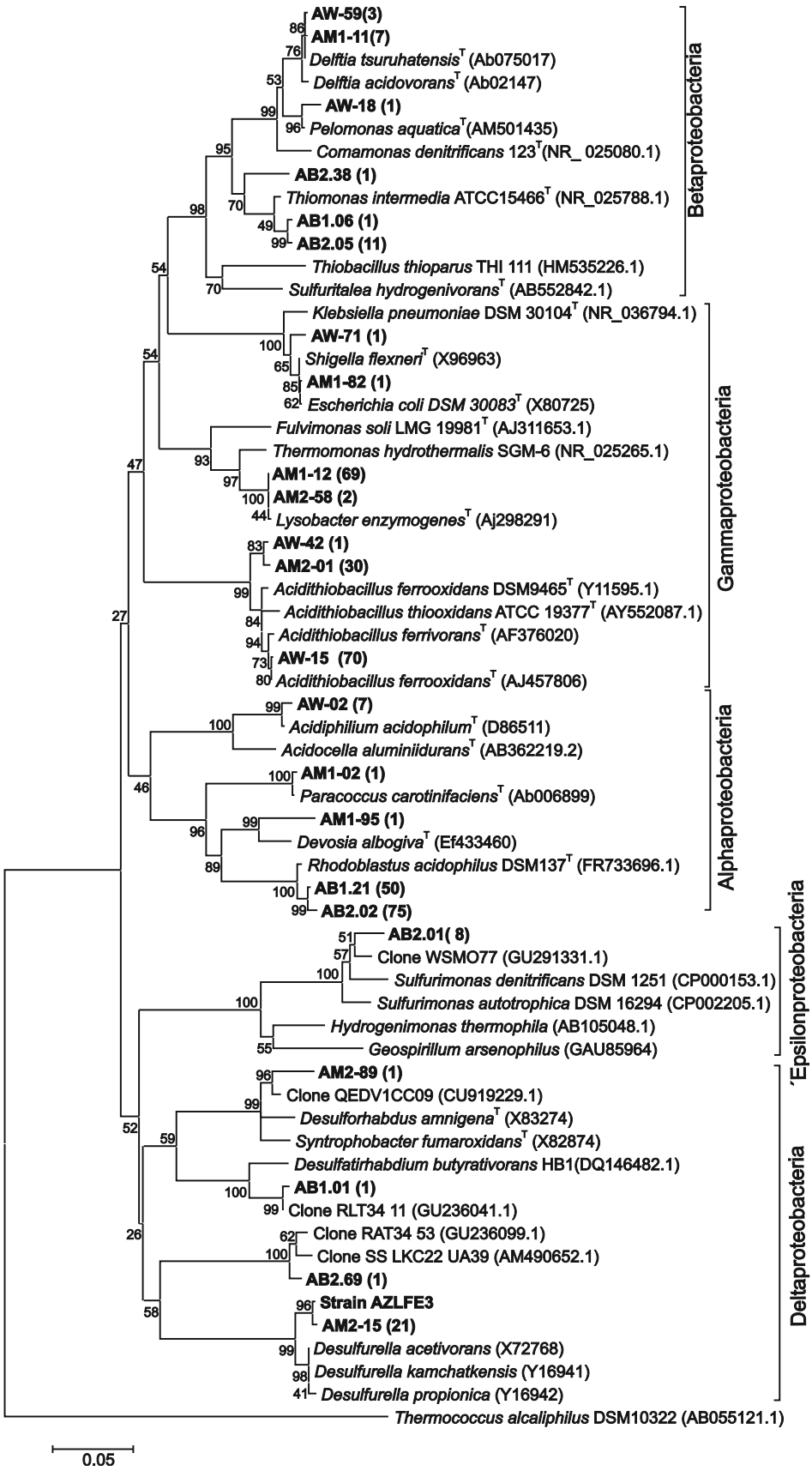

Fig. 7 Phylogenetic relationship between the 16S rRNA gene sequences, within the *Proteobacteria,* obtained from mud (AM1 and AM2), water (AW) and biomats (AB1 and AB2) from *Los Azufres* geothermic field. *Numbers inside square brackets* specify the number of sequences. The tree was constructed with sequences obtained from NCBI database, using neighbor-joining algorithm, with an alignment of 716 bp. The *scale bar* represents the number of substitutions per nucleotide

The DO was higher for both biomats since the streams where they formed were noticeably shallow (favoring the exchange of oxygen with the atmosphere), supporting the growth of biofilms with aerobic or facultative anaerobic organisms. As will be discussed below, these physico-chemical characteristics are directly related to the dominant populations found in the samples:

- a versatile soil microorganism, which was found to inhabit from alkaline to acidic environments (AM1) and to resist to high metal concentrations and high temperatures (*Lysobacter* spp.).
- a known acidophilic microorganism (*Acidithiobacillus* spp.), involved on the iron and sulfur cycles, living in acidic and mesophilic environments (AW and AM2).
- a biomat-developing microorganisms (*Rhodoblastus* spp.) that participate in the sulfur cycle, inhabiting mesophilic environments, rich in metals, specially Hg, and with limited amount of available oxygen (AB1 and AB2).

Regarding specifically the microbiological analyses, both T-RFLP and 16S rRNA gene libraries data suggested low bacterial diversity in the studied samples. The dominant populations detected through 16S rRNA gene libraries were related to microorganisms adapted to acidic environments and/or to functional groups involved in iron and sulfur biogeochemical cycles. Indeed, bacteria involved in the sulfur cycle were found among the bacterial communities in the mats, whereas the *cenote* water was dominated by bacteria involved in the iron cycle. The mesophilic mud sample was colonized by microorganisms related to both cycles.

Rhodoblastus acidophilus-affiliated sequences were dominant in the microbial mat samples (50 and 75 sequences for AB1 and AB2, respectively). This is a purple non-sulfur bacterium adapted to acidic environments. Representatives of this species are metabolically versatile, being able to grow phototrophically under anoxic conditions, or chemotrophically under microoxic to oxic conditions (Imhoff 2001). *Rhodoblastus acidophilus* was already detected on Hawaiian volcanic deposits (Gomez-Alvarez et al. 2007). Some purple non-sulfur bacteria can use hydrogen or reduced sulfur compounds (H_2S, S^0 and $S_2O_3^{2-}$) as electron donors for photolitotrophic metabolism, and thus also participate to the sulfur cycle under not extreme sulfur concentration conditions (Guyoneaud et al. 1996).

Lysobacter enzymogenes-related sequences were exclusively detected in the mud samples, and were dominant (84 %) in the hot mud (AM1). *Lysobacter* spp. are characterized by their strong lipase, protease and hemolytic activities (Christensen and Cook 1978; Lee et al. 2006; Yassin et al. 2007), as well as their antibiotic activity against gram-positive bacteria (Park et al. 2008) and fungi zoospores (Folman et al. 2003). *Lysobacter* sp. was first described as a typical soil bacterium (Christensen and Cook 1978), however, it is now known to be present in other environments, including extreme environments (Romanenko et al. 2008; Liu et al. 2011; Tobler and Benning 2011; Brito et al. 2013). In addition, Liu et al. (2011) isolated a thermo- and alkalitolerant *Lysobacter* sp., resistant to UV light, from an abandoned gold mine. A *Lysobacter* sp. was also detected in a bacterial community from hypersaline and alkaline industrial residues (pH 9–12) highly contaminated with Cr(VI) (Brito et al. 2013), and in Icelandic geothermal waters of pH approaching 10 (Tobler and Benning 2011). As far as we know, this is the first time such microorganisms were found under acidic conditions.

According to the 16S rRNA gene libraries, *Acidithiobacillus* spp. were also highly represented (24 % of total libraries), being dominant in the *cenote* water and in the mesophilic mud AM2, where both acidic and mesophilic conditions were found. These *Proteobacteria* are acidophilic iron and sulfide oxidizers, able to obtain energy from the oxidation of ferrous iron coupled to oxygen reduction. These bacteria are also able to use reduced sulfur compounds or hydrogen to obtain energy. These properties may be of interest for biotechnological applications, in particular for the remediation of acid- and metal-enriched effluents through bioleaching and subsequent metal trapping by other functional groups (Hedrich et al. 2011) such as sulfate reducers or metal reducers. The other iron and/or sulfide oxidizing species detected in *Los Azufres* samples were related to *Thiomonas* and *Paracoccus* (respectively, 13 and 1 sequences). *Thiomonas* species are mixotrophic sulfur-oxidizing Betaproteobacteria, able to precipitate ferric iron. *Thiomonas* sp. were detected in the thermophile (60 °C), neutral pH and sulfur-rich waters from Uzon Caldera (Gumerov et al. 2011). *Paracoccus* is described as a facultative autotrophic Alphaproteobacterium able to grow under both oxic and anoxic conditions. *P. carotinifaciens*, detected in AM1 hot mud, is a soil species able to grow between 10 and 33 °C at pH 6.5–7.5 (Tsubokura et al. 1999); thus in very different environmental conditions to those observed in *Los Azufres*. Nevertheless, *Paracoccus* spp. were detected on a mud volcano in China (Yang et al. 2012) and geothermal springs in New Zealand (Hetzer et al. 2007).

We also detected uncultured bacteria (39 sequences, 28 of which revealed the presence of an uncultured *Thermotogae*, AF232923.1, sequence MS9) detected in water and sediment samples from acidic, geothermal pools in the Caribbean island of Montserrat (Burton and Norris 2000). The closest described species (81 % similarity) is the thermophilic sulfur-reducing *Thermosipho melanesiensis*. Other sulfate or sulfur reducers, such as

Thermodesulfobium and *Desulfurella*-related sequences, were detected through our 16S rRNA gene libraries, demonstrating the abundance of functional groups involved in the sulfur cycle, mainly in the mud and mat samples.

Despite the complexity of isolating both thermophilic and acidophilic microorganisms, the study of isolates is necessary to further investigate their metabolic capacities and thus ecological role. Until now, we achieved the isolation of one strain, AZLFE3, closely related to *Desulfurella kamchatkensis* (98 % similarity). The type strain of *D. kamchatkensis* was originally isolated from a microbial mat developing in a sulfide-rich volcanic hot spring in Kamchatka (Miroshnichenko et al. 1998). It was also found in Yellowstone National Park (Hall et al. 2008) and *Desulfurella multipotens* representatives were detected in the thermophile (60 °C), neutral pH and sulfur-rich waters from Uzon Caldera (Gumerov et al. 2011). The exploration of the diversity and ecological role of the microbial communities from *Los Azufres* communities is just beginning, and further efforts will determine optimal ecophysiological conditions for the growth of such microorganisms.

As concluding remarks, we underline that this work is the first to investigate the microbial diversity within *Los Azufres* geothermal field. Like other volcanic environments presenting extreme physico-chemical conditions, these areas are disappearing due to anthropogenic exploration, so their biodiversity needs to be investigated and preserved. To date, most of the several geothermal sites on Mexican territory remain yet unexplored and unprotected. From a public health point of view, the physico-chemical characterization of *Los Azufres* is also of great concern since this area is used for leisure. The high metal concentrations may expose humans and other species to danger, if not by direct contact with mud or water, but by consumption of trouts. Besides, it is important to be aware whether emanating gases contain high concentrations of toxic metals, such as mercury or volatile alkylated metal species. Moreover, the anoxic conditions and the presence of sulfate-reducing microorganisms may influence mercury speciation, resulting in the production of the toxic form methylmercury. Finally, the detection of bacterial sulfate-reducing populations in *Los Azufres* geothermic site is promising for the search for new biotechnologies involving metal transformation such as bioleaching and bioremediation. To follow the present work, we will investigate these communities further to find isolates capable of transforming metals into less toxic forms, for instance CrVI to CrIII. This application is of great interest, since environmental problems caused by industrial chromium residues worldwide could be greatly alleviated. Other biotechnological applications may also be discovered.

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References

Aguilar Y, Vargas VH, Verma SP (1987) Composición química (elementos mayores) de los magmas en el cinturón volcánico mexicano. Geofis Int 26:195–272

Anderson A (1979) Mercury in soils. In: Nriagu JO (ed) The biogeochemistry of mercury in the environment. Elsevier, Amsterdam, pp 79–112

Bagnato E, Aiuppa A, Parello F, D'Alessandro W, Allard PS (2009a) Calabrese mercury concentration, speciation and budget in volcanic aquifers: Italy and Guadeloupe (Lesser Antilles). J Volcanol Geotherm Res 179:96–106

Bagnato E, Parello F, Valenza M, Caliro S (2009b) Mercury content and speciation in the Phlegrean Fields volcanic complex: evidence from hydrothermal system and fumaroles. J Volcanol Geotherm Res 187:250–260

Bagnato E, Allard P, Parello F, Aiuppa A, Calabrese S, Hammouya G (2009c) Mercury gas emissions from La Soufrière volcano, Guadeloupe island (Lesser Antilles). Chem Geol 266:267–273

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Bastos WR, Malm O, Pfeiffer WC, Cleary D (1998) Establishment and analytical quality control of laboratories for Hg determination in biological and geological samples in the Amazon, Brazil. Ciencia e Cultura 50:255–260

Birkle P, Merkel B (2001) Mineralogical–chemical composition and environmental risk potential of pond sediments at the geothermal field of *Los Azufres*, Mexico. Environ Geol 41:583–592

Birkle P, Merkle B (2000) Environmental impact by spill of geothermal fluids at the geothermal field of Los Azufres, Michoacán, Mexico. Water Air Soil Pollut 124:371–410

Blank CE, Cady SL, Pace NR (2002) Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park. Appl Environ Microbiol 68:5123–5135

Brito EMS, Guyoneaud R, Goñi-Urriza M, Ranchou-Peyrouse A, Verbaere A, Crapez MAC, Wasserman JCA, Duran R (2006) Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments of Guanabara Bay, Brazil. Res Microbiol 157:752–762

Brito EMS, Andrade LH, Caretta CA, Duran R (2007) Microorganisms bioprospection: a new tendency in microbial ecology. In: Pawley LE (ed) Leading-edge environmental biodegradation research, 1st edn. Nova Sc Publ, New York, pp 199–222

Brock TD, Freeze H (1969) *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J Bacteriol 98:289–297

Brito EMS, Piñón-Castillo H, Guyoneaud R, Caretta CA, Gutiérrez-Corona, JF, Duran R, Reyna-López G, Nevárez-Moorillón VG, Fahy A, Goñi-Urriza M (2013) Bacterial biodiversity from anthropogenic extreme environments: a hyper-alkaline and hyper-saline industrial residue contaminated by chromium and iron. Appl Microbiol Biotechnol 97:369–378

Brock TD, Brock KM, Belly RT, Weiss RL (1972) *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch Mikrobiol 84:54–68

Burton NP, Norris PR (2000) Microbiology of acidic, geothermal springs of Montserrat: environmental rDNA analysis. Extremophiles 4:315–320

Camerlenghi A, Cita MB, Della Vedova B, Fusi N, Mirabile L, Pellis G (1995) Geophysical evidence of mud diapirism on the mediterranean ridge accretionary complex. Mar Geophys Res 17:115–141

Caretta CA, Brito EMS (2011) *In silico* restriction for identifying microbial communities in T-RFLP fingerprints. J Comp Interdiscip Sci 2:123–129

Castorena G, Mugica V, Le Borgne S, Acun ME, Bustos-Jaimes I, Aburto J (2006) Carbazole biodegradation in gas oil/water biphasic media by a new isolated bacterium *Burkholderia* sp. strain IMP5GC. J Appl Microbiol 100:739–745

Chao A, Lee SM (1992) Estimating the number of classes via sample coverage. J Am Stat Assoc 87:210–217

Christensen P, Cook FD (1978) *Lysobacter*, a new genus of nonfruiting, gliding bacteria with a high base ratio [soil and water organisms]. Int J Syst Bacteriol 28:367–393

Christenson BW, Mroczek EK (2003) Potential reaction pathways of Hg in some New Zealand hydrothermal environments. Soc Econ Geol 10:111–132

Clingenpeel S, Macur RE, Kan J, Inskeep WP, Lovalvo D, Varley J, Mathur E, Nealson K, Gorby Y, Jiang H, LaFracois T, McDermott TR (2011) Yellowstone Lake: high-energy geochemistry and rich bacterial diversity. Environ Microbiol 13:2172–2185

Fliermans CB, Brock TD (1972) Ecology of sulfur-oxidizing bacteria in hot acid soils. J Bacteriol 111:343–350

Folman LB, Postma J, van Veen JA (2003) Characterisation of *Lysobacter enzymogenes* (Christensen and Cook 1978) strain 3.1T8, a powerful antagonist of fungal diseases of cucumber. Microbiol Res 158:107–115

Giggenbach WF (1988) Geothermal solute equilibria derivation of Na–K–Mg–Ca geoindicators. Geochim Cosmochim Acta 52:2749–2763

Gomez-Alvarez V, King GM, Nusslein K (2007) Comparative bacterial diversity in recent Hawaiian volcanic deposits of different ages. FEMS microbial Ecol 60:60–73

Good IJ (1953) On the population frequencies of species and the estimation of population parameters. Biometrika 40:237–264

Grüning B, Hills G, Veit T, Weitemeyer C, Frave-Bulle O, Nguyen H-K, Ravot G (2009) Thermostable esterases from thermophilic bacteria. US Paten 7,595,181 B2

Guidry SA, Chafetz HS (2002) Factors governing subaqueous siliceous sinter precipitation in hot springs: examples from Yellowstone National Park, USA. Sedimentology 49:1253–1267

Gumerov VM, Mardanov AV, Beletsky AV, Bonch-Osmolovskaya EA, Ravin NV (2011) Molecular analysis of microbial diversity in the Zavarzin spring, Uzon Caldera, Kamchatka. Microbiol 80:244–251

Guyoneaud R, Matheron R, Baulaigue R, Podeur K, Hirschler A, Caumette P (1996) Anoxygenic phototrophic bacteria in eutrophic coastal lagoons of the French Mediterranean and Atlantic coasts (Prévost Lagoon, Arcachon Bay, Certes Fishponds). Hydrobiologia 329:33–43

Guyoneaud R, Mouné S, Eatock C, Bothorel V, Hirschler-Réa A, Willison J, Duran R, Liesack W, Herbert R, Matheron R, Caumette P (2002) Characterization of three spiral-shaped purple nonsulfur bacteria isolated from coastal lagoon sediments, saline sulfur springs, and microbial mats: emended description of the genus *Roseospira* and description of *Roseospira marina* sp. nov., *Roseospira navarrensis* sp. nov., and *Roseospira thiosulfatophila* sp. nov. Arch Microbiol 178:315–324

Hall JR, Mitchell KR, Jackson-Weaver O, Kooser AS, Cron BR, Crossey LJ, Takacs-Vesbach CD (2008) Molecular characterization of the diversity and distribution of a thermal spring microbial community by using rRNA and metabolic genes. Appl Environ Microbiol 74:4910–4922

Hamilton-Brehm SD, Mosher JJ, Vishnivetskaya T, Podar M, Carrol S, Allman S, Phelps TJ, Keller M, Elkins JG (2010) *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from Obsidian Pool, Yellowstone National Park. Appl Environ Microbiol 76:1014–1020

Hedrich S, Schlömann M, Johnson DB (2011) The iron-oxidizing proteobacteria. Microbiol 157:1551–1564

Hetzer A, Morgan HW, McDonald IR, Daughney CJ (2007) Microbial life in Champagne Pool, a geothermal spring in Waiotapu, New Zealand. Extremophiles 11:605–614

Hewson I, Fuhrman JA (2006) Improved strategy for comparing microbial assemblage fingerprints. Microb Ecol 51:147–153

Hovland M, Hill A, Stokes D (1997) The structure and geomorphology of the Dashgil mud volcano, Azerbaijan. Geomorphology 21:1–15

Huang Q, Dong CZ, Dong RM, Jiang H, Wang S, Wang G, Fang B, Ding X, Niu L, Li X, Zhang C, Dong H (2011) Archaeal and bacterial diversity in hot springs on the Tibetan Plateau, China. Extremophiles 15:549–563

Huber R, Eder W, Heldwein S, Wanner G, Huber H, Rachel R, Stetter KO (1998) *Thermocrinisruber* gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. Appl Environ Microbiol 64:3576–3583

Hugenholtz P, Pitulle C, Hershberger KL, Pace NR (1998) Novel division level bacterial diversity in a Yellowstone hot spring. J Bacteriol 180:366–376

Imhoff JF (2001) Transfer of *Rhodopseudomonas acidophila* to the new genus *Rhodoblastus* as *Rhodoblastus acidophilus* gen. nov., comb. nov. Int J Syst Evol Microbiol 51:1863–1866

Inagaki F, Hayashi S, Doi K, Motomura Y, Izawa E, Ogata S (1997) Microbial participation in the formation of siliceous deposits from geothermal water and analysis of the extremely thermophilic bacterial community. FEMS Microbiol Ecol 24:41–48

Jenne EA (1970) Atmospheric and fluvial transport of mercury. Mercury in the environment. Geol Surv Prof 713:40–45

Johnson DB, Hallberg KB (2005) Acid mine drainage remediation options: a review. Sci Tot Environ 338:3–14

Jones B, Renaut RW (1996) Influence of thermophilic bacteria on calcite and silica precipitation in hot springs with water temperatures above 90 °C: evidence from Kenya and New Zealand. Can J Earth Sci 33:72–83

Jones B, Renaut RW (1997) Formation of silica oncoids around geysers and hot springs at El Tatio, northern Chile. Sedimentology 44:287–304

Jones B, Renaut RW, Rosen MR (2000) Stromatolites forming in acidic hot-spring waters, North Island, New Zealand. Palaios 15:450–475

Kaksonen AH, Puhakka JA (2007) Sulfate reduction based bioprocess for the treatment of acid mine drainage and recovery of metals. Eng Life Sci 7:541–564

Kimura H, Sugihara M, Yamamoto H, Patel BK, Kato K, Hanada S (2005) Microbial community in a geothermal aquifer associated with the subsurface of the Great Artesian Basin, Australia. Extremophiles 9:407–414

Lane DJ (1991) rRNA sequencing. In: Stackebrandt GME (ed) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc Natl Acad Sci USA 82:6955–6959

Lee JW, Im W-T, Kim MK, Yang D-C (2006) *Lysobacter koreensis* sp. nov., isolated from a ginseng field. Int J Syst Evol Microbiol 56:231–235

Liu M, Liu Y, Wang Y, Luo X, Dai J, Fang C (2011) *Lysobacter xinjiangensis* sp. nov., a moderately thermotolerant and alkalitolerant bacterium isolated from a gamma-irradiated sand soil sample. Int J Syst Evol Microbiol 61:433–437

Malm O, Pfeiffer WC, Fiszman M, Azcue JMP (1989) Heavy metal concentrations and availability in the bottom sediments of the Paraiba do Sul-Guandu river system, RJ, Brazil. Environ Technol Lett 10:675–680

Miroshnichenko ML, Rainey FA, Hippe H, Chernyh NA, Kostrikina NA, Bonch-Osmolovskaya EA (1998) *Desulfurella karnchatkensis* sp. nov. and *Desulfurella propionica* sp. nov., new sulfur-respiring thermophilic bacteria from Kamchatka thermal environments. Int J Syst Bacteriol 48:475–479

Newton RS, Cunningham RC, Schubert CE (1980) Mud volcanoes and pockmarks: seafloor engineering hazards or geological curiosities? Proc Offshore Technol Conf Pap OTC 3729:425–435

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. J Microbiol Methods 7:57–66

Paissé S, Coulon F, Goñi-Urriza M, Peperzak L, McGenity TJ, Duran R (2008) Structure of bacterial communities along a hydrocarbon contamination gradient in a coastal sediment. FEMS Microbiol Ecol 66:295–305

Park JH, Kim R, Aslam Z, Jeon CO, Chung YR (2008) *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. Int J Syst Evol Microbiol 58:387–392

Romanenko LA, Uchino M, Tanaka N, Frolova GM, Mikhailov VV (2008) *Lysobacter spongiicola* sp. nov., isolated from a deep-sea sponge. Int J Syst Evol Microbiol 58:370–374

Santos R, Fernandes J, Fernandes N, Oliveira F, Cadete M (2007) *Mycobacterium parascrofulaceum* in acidic hot springs in Yellowstone National Park. Appl Environ Microbiol 73:5071–5073

Sayeh R, Birrien JL, Alain K, Barbier G, Hamdi M, Prieur D (2010) Microbial diversity in Tunisian geothermal springs as detected by molecular and culture-based approaches. Extremophiles 14:501–514

Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501–1506

Schultze-Lam S, Ferris FG, Konhauser KO, Wiese RG (1995) *In situ* silicification of an Icelandic hot spring microbial mat: implications for microfossil formation. Can J Earth Sci 32:2021–2026

Silva AP, Alves MCC (2006) Como iniciar a validação de métodos analíticos. ENQUALAB-2006-Congresso e Feira da Qualidade em Metrologia: 8–15

Tobler DJ, Benning LG (2011) Bacterial diversity in five Icelandic geothermal waters: temperature and sinter growth rate effects. Extremophiles 15:473–485

Tobler DJ, Stefansson A, Benning LG (2008) *In situ* grown silica sinters in Icelandic geothermal areas. Geobiology 6:481–502

Tomova I, Stoilova-Disheva M, Lyutskanova D, Pascual J, Petrov P, Kambourova M (2010) Phylogenetic analysis of the bacterial community in a geothermal spring, Rupi Basin, Bulgaria. World J Microbiol Biotechnol 26:2019–2028

Torres-Sanchez R, Magaña-Vazquez A, Sanchez-Yañez JM, Martinez-Gomez L (1996) High temperature microbial corrosion in the condenser of a geothermal electric power unit. CORROSION96 The NACE Int Ann Conf Exp, No. 293, pp 1–14

Tsai YL, Olson BH (1991) Rapid method for direct extraction of DNA from soil and sediments. Appl Environ Microbiol 57:1070–1074

Tsubokura A, Yoneda H, Mizuta H (1999) *Paracoccus carotinifaciens* sp. nov., a new aerobic gram-negative astaxanthin-producing bacterium. Int J Syst Bacteriol 49:277–282

US EPA (1990) National oil and hazardous substances pollution contingency Plan, 40 CRF Part 300. US Environmental Protection Agency, Washington, DC

Varekamp JC, Buseck PR (1984) The speciation of mercury in hydrothermal systems, with applications for ore deposition. Geochim Cosmochim Acta 48:177–186

Walter MR, Bauld J, Brock TD (1972) Siliceous algal and bacterial stromatolites in hot spring and geyser effluents of Yellowstone National Park. Science 178:402–405

WHO (1993) WHO guidelines for drinking water quality. http://www.who.int/water_sanitation_health/GDWQ/index.htlm. Accessed 12 October 2011

Yakubov AA, Ali-Zade AA, Zeinalov MM (1971) Gryazevye vulkany Azerbaidzhanskoi SSR: Atlas (mud volcanoes of the Azerbaijan SSR: Atlas). Azerbaijan Academy of Sciences, Baku

Yang H-M, Lou K, Sun J, Zhang T, Ma XL (2012) Prokaryotic diversity of an active mud volcano in the Usu city of Xinjiang, China. J Basic Microbiol 52:79–85

Yassin AF, Chen WM, Hupfer H, Siering C, Kroppenstedt RM, Arun AB, Lai W-A, Shen F-T, Rekha PD, Young CC (2007) *Lysobacter defluvii* sp. nov., isolated from municipal solid waste. Int J Syst Evol Microbiol 57:1131–1136