

High abundance of heterotrophic prokaryotes in hydrothermal springs of the Azores as revealed by a network of 16S rRNA gene-based methods

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Abstract Two hydrothermal springs (AI: 51 °C, pH 3; AIV: 92 °C, pH 8) were analysed to determine prokaryotic community composition. Using pyrosequencing, 93,576 partial 16S rRNA gene sequences amplified with V2/V3-specific primers for Bacteria and Archaea were investigated and compared to 16S rRNA gene sequences from direct metagenome sequencing without prior amplification. The results were evaluated by fluorescence in situ hybridization (FISH). While in site AIV Bacteria and Archaea were detected in similar relative abundances (Bacteria 40 %, Archaea 35 %), the acidic spring AI was dominated by Bacteria (68 %). In spring AIV the combination of 16S rRNA gene sequence analysis and FISH revealed high abundance (>50 %) of heterotrophic bacterial genera like *Caldicellulosiruptor*, *Dictyoglomus*, and *Fervidobacterium*. In addition, chemolithoautotrophic Aquificales were detected in the bacterial community with *Sulfurihydrogenibium* being the dominant genus. Regarding Archaea, only Crenarchaeota, were detected, dominated by the family Desulfurococcaceae (>50 %). In addition, Thermoproteaceae made up almost 25 %. In the acidic spring (AI) prokaryotic diversity was lower than in the hot, slightly alkaline spring AIV. The bacterial community of site AI

was dominated by organisms related to the chemolithoautotrophic genus *Acidithiobacillus* (43 %), to the heterotrophic *Acidicaldus* (38 %) and to *Anoxybacillus* (7.8 %). This study reveals differences in the relative abundance of heterotrophic versus autotrophic microorganisms as compared to other hydrothermal habitats. Furthermore, it shows how different methods to analyse prokaryotic communities in complex ecosystems can complement each other to obtain an in-depth picture of the taxonomic composition and diversity within these hydrothermal springs.

Keywords Biodiversity · Thermoacidophile ecology · Thermophile ecology · Metagenome

Introduction

With regard to their physicochemical conditions, terrestrial hydrothermal springs are highly diverse habitats, offering a wide range of ecological niches. These niches exhibit extreme conditions, such as high temperature, low or high pH, and the presence of toxic ions. Their extreme features are expected to lead to limited biodiversity, making hydrothermal habitats ideal model systems to study principles of community structure and function. Prokaryotic diversity in hydrothermal ecosystems has been extensively studied, in particular at Yellowstone National Park repeatedly revealing characteristic taxonomic groups like Aquificales and specific groups of Crenarchaeota, in particular (among others Hamamura et al. 2012; Meyer-Dombard et al. 2005; Spear et al. 2005).

The abundant, often predominant occurrence of Aquificales has led to the hypothesis that H₂-based chemolithoautotrophic processes play a dominant role in hot-temperature ecosystems, in which photosynthesis is no longer possible (Spear

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et al. 2005; Inskeep et al. 2010; Hugenholtz et al. 1998). Aquificales often form the basis for chemolithotrophic biofilm or mat structures, e.g. the pink filaments of *Thermocrinis ruber* and the white sausage-shaped filaments found in sulphur-turf mats of Japanese hot springs (Blank et al. 2002; Yamamoto et al. 1998).

Estimates on community composition, however, have been performed mainly on the basis of clone libraries with the known bias of cloning-based technologies and relatively low sample size taken into account (among others Blank et al. 2002; Hugenholtz et al. 1998; Reysenbach et al. 1994; Swingley et al. 2012). With next generation sequencing methods the analysis of larger 16S rRNA gene data sets has become feasible, making it possible to increase the extent of sequence-based biodiversity studies, leading to a higher degree of reliability. Analysis of large sets of short rRNA gene fragments comprising variable regions has been applied to infer community composition and biodiversity estimates in terrestrial and marine habitats (Huse et al. 2008; Miller et al. 2009; Nacke et al. 2011; Roesch et al. 2007; Will et al. 2010; Youssef et al. 2009). However, the analysis is usually based on PCR-amplified DNA fragments, which still suffers from potential primer bias. Direct sequencing of metagenomic DNA can circumvent this bias, reducing, however, the amount of data available for analysis since non-16S rRNA gene sequences are also generated (Inskeep et al. 2010). To obtain an in-depth picture of prokaryotic hydrothermal spring communities, we combined both sequencing of short variable rRNA gene fragments and phylogenetic analysis of the metagenome, evaluating it with fluorescence in situ hybridization (FISH), which allows direct quantification of specific microbial lineages in the sample, directly. Our study was conducted with samples from the Furnas Valley, situated on São Miguel, Azores.

The Azores are a group of geographically isolated islands in the Atlantic Ocean. They are almost exclusively of volcanic origin, with the main hydrothermal area being the Furnas Valley on the Island of Sao Miguel. However, compared to other hydrothermal zones like Yellowstone National Park and Iceland the overall amount of data available is still limited. Brock and Brock (1967) gave a detailed description of the hot springs of Furnas Valley pointing out the thermal and chemical complexity of the Furnas springs and a major difference with regard to other terrestrial hydrothermal areas: in Furnas "... the largest spring is at the highest elevation and is alkaline, whereas some of the lower springs are smaller and more acidic. This is completely opposite to the situation found in Yellowstone National Park and Iceland... (Allen and Day 1935; Barth 1950)". Here the higher springs are small and acidic and the lower ones large and alkaline. The hot springs of

Furnas Valley have been a valuable source for thermophilic microorganisms and thermostable enzymes with useful features for biotechnological applications (among others Albuquerque et al. 2008, 2010, 2012; Franca et al. 2006; Riessen and Antranikian 2001; Friedrich and Antranikian 1996). However, only little data are available on overall diversity (Hamamura et al. 2012).

Applying different 16S rRNA gene-based and metagenomic approaches, we provide an in-depth study of bacterial and archaeal diversity as a quantitative basis for understanding ecological interactions within the prokaryotic community. The study reveals the wealth of biocatalytic potential for enzymes from heterotrophic organisms still waiting to be recovered from these challenging extreme habitats.

Materials and methods

Study site and sampling

Sediment, biofilm, and water samples were collected from various hydrothermal springs at Furnas Valley, São Miguel, Azores, in September 2010. All necessary permits were obtained for the described field studies from the regional government of the Azores (LICENÇA No 107/2010/DRA). A description of the different sampling sites is provided in Table 1. Several samples were collected at a solfataric field (sampling site A) (Fig. 1). Additional samples were collected from the hydrothermal vents Caldeirão (sampling site B) and Caldeira do Asmodeu (sampling site C). With the exception of the solfataric sample AIV (water sample) mainly sediments, muds or biofilms were collected and supplemented with water from the respective hydrothermal spring. Sediments and mud were characterized by brownish, ochre, grey, black or green appearance. The biofilms were black, white or light-grey, and the microbial mats encountered along the effluent stream of one hydrothermal vent exhibited green, orange, yellowish or brownish colours (Fig. 1).

Each sample was transferred to a sterile serum bottle, preventing exposure to air as far as possible. After sampling, the bottles were closed immediately with a butyl rubber stopper and a screw cap and 0.01 % w/v (final concentration) sodium sulphide was added by means of a syringe to counteract potential oxygen intake. Within 4 h after sampling, aliquots of 500 µL per sample were fixed for 1 h with 3 % (final concentration) paraformaldehyde for subsequent FISH analyses as described by Ravenschlag et al. (2000) and stored at -20°C . The original samples were stored at 4°C .

Table 1 Sampling site characteristics

| Site | T (°C) | pH | Biotope | Sample |
|------|--------|-------|---|--------|
| A | 51 | 3 | Small shallow hydrothermal spring at solfataric field near Caldeira do Esguicho | AI |
| | 84 | 2.5–3 | Small shallow hydrothermal spring at solfataric field near Caldeira do Esguicho | AII |
| | 85 | 8 | Small shallow hydrothermal spring at solfataric field near Caldeira do Esguicho | AIII |
| | 92 | 8 | Solfatara (Caldeira do Esguicho), effluent stream | AIV |
| B | 60 | 6 | Hydrothermal spring (Caldeirão) | BI |
| | 65 | 7 | Small stream near Caldeirão (Chalet Quente) | BII |
| | 70 | 6 | Hydrothermal spring (Caldeirão, paved basin) | BIII |
| C | 55 | 8 | Effluent of Caldeira do Asmodeu | CI |
| | 76 | 8 | Hydrothermal spring (Caldeira do Asmodeu), edge zone | CII |



Fig. 1 Solfataric field (study site A) with sulphur deposits and various hydrothermal springs of temperatures up to 97 °C and pH values varying between 2.5 and 8. The rising gases (mainly water vapour and hydrogen sulphide) originate from a solfatara, Caldeira do Esguicho (red arrow, sample AIV). Several sediment and water samples were collected at this site. The green arrow depicts site AI

Determination of chemical parameters

Aliquots of approx. 120 mL of the original samples were centrifuged at $3,800\times g$ for 40 min. The supernatants were filtered (pore size of the filters $<0.45\ \mu\text{m}$) and analysed for chemical parameters applying the following methods/instruments: optical emission spectroscopy with inductively coupled plasma (Optima 7000 DV OES/ICP, PerkinElmer, Inc.) (Na, K, Mg, Ca, Fe, S), ultraviolet–visible spectrophotometry (Lambda 25 UV–VIS, PerkinElmer, Inc.) (NH_4^+ , Fe^{2+} , NO_2^-), ion chromatography (Metrohm AG, Germany) (Cl^- , NO_3^- , SO_3^{2-} , SO_4^{2-} , PO_4^{3-}) and TOC analyser (highTOC, Elementar Analysensysteme GmbH, Germany) (DOC, TIC).

Isolation of DNA

Total community DNA was isolated as described by Zhou et al. (1996), based on lysis with high-salt extraction buffer and extended heating in the presence of sodium dodecyl sulphate (SDS). The samples were centrifuged at $3,939\times g$ for 1 h and 2–3 g of the pellet was resuspended in DNA extraction buffer, containing 1 % hexadecylmethylammonium bromide (CTAB). Prior to DNA isolation, three freeze-and-thaw cycles in liquid nitrogen and a water bath at 65 °C were conducted. The precipitated DNA was dissolved in 120 μL sterile water and analysed spectrophotometrically at 260/280 nm and by agarose gel electrophoresis (0.4 % agarose). DNA solutions derived from samples with high proportions of sediments appeared intensively orange or yellowish, indicating the co-extraction of humic substances which interfere with UV-photometric measurement of DNA concentration. Thus, quantification of DNA extracted from these samples was merely done by agarose gel electrophoresis.

In addition, we applied the PowerMax™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) as an alternative method for isolation of genomic DNA from all samples. Microorganisms were lysed by a combination of SDS and further disruption agents, and mechanical force using beads. Approx. 2 g of sediment was used for DNA extraction. The DNA was eluted in 2 mL sterile water and quantified as described above. Pooled DNA from both methods was used for metagenomic pyrosequencing or PCR amplification.

Denaturing gradient gel electrophoresis

PCR amplification and DGGE were performed as described by Muyzer et al. (1998). We routinely added 3 µg/µL (final concentration) bovine serum albumin to the PCR reaction mixture to prevent inhibition of the DNA polymerase by humic substances. The sequences of the primers used for amplification of 16S rRNA gene-fragments were as follows: Arc334F: 5'-CGCCCGCCGCGCCCCGCGCCCGTC CCGCCGCCCCGCCCCGACGGGGYGCAGCAGGCGC GA-3', Arc915R: 5'-GTGCTCCCCGCCAATTCCT-3' (Archaea); 314F: 5'-CGCCCGCCGCGCCCCGCGCCCGT CCGCCGCCCCGCCCCGCTACGGGAGGCAGCAG-3', 907R: 5'-CCGTCAATTCMTTGTGATTT-3' (Bacteria). *Taq* DNA polymerase (Fermentas GmbH, St. Leon-Rot, Germany) was used in PCR. The touchdown PCR protocol by Muyzer (annealing temperatures ranging from 65 to 55 °C) was applied for both primer pairs. We used the Dcode™ System (Bio-Rad Laboratories GmbH, Munich, Germany) for DGGE. Electrophoresis was carried out using a 6 % polyacrylamide gel with a denaturing gradient of 20–80 %, at 55 °C, 200 V for 5 h (Bacteria-DGGE), and a denaturing gradient of 30–80 % at 60 °C, 150 V for 2 h (Archaea-DGGE). After electrophoresis, the gels were stained in Roti®-Gel Stain (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and photographed on a UV transilluminator.

Amplification of 16S rRNA gene fragments and pyrosequencing

DNA preparations from both isolation approaches were used as template for individual PCR amplification of 16S rRNA gene fragments. The PCR products derived from either DNA preparation were combined for sequencing in equal amounts. The sequences of the bacterial and archaeal V2/V3 region-specific primers used in respective PCRs are as follows: Bacteria, V2f: 5'-AGTGGCGGACGGGTGAGTAA-3'; V3r, 5'-CCGCGGCTGCTGGCAC-3' [(Will et al. 2010) V3r modified]; Archaea, Arc113f: 5'-ACKGCTSAGTAACACG TGG-3'; Arc520r, 5'-TACCGCGGCKGCTGGCA-3' [Arc113f, (Baker and Cowan 2004); Arc520r, this study]. The template-specific primer sequences were complemented with

a GS FLX Titanium primer sequence including the sequence adaptor, a four-base library key sequence, and a MID (multiple identifier) sequence at the 5' end of the specific primer. The Amplicon Fusion Primers were commercially synthesized (Fermentas GmbH, St. Leon-Rot, Germany).

The PCR mixtures (final volume 50 µL) contained 10 µL fivefold Phusion HF buffer (New England Biolabs GmbH, Frankfurt am Main, Germany), 300 µg BSA, 0.2 mM final concentration of each dNTP, 0.4 µM final concentration of each primer, 0.5 U Phusion High-Fidelity DNA polymerase (New England Biolabs GmbH, Frankfurt am Main, Germany), and 30–70 ng DNA. For Archaea-specific PCR the following steps were conducted: initial denaturation at 98 °C for 30 s and 28 cycles of denaturation at 98 °C for 10 s, primer annealing for 20 s using a temperature gradient ranging from 68 °C to 55 °C (1 °C touchdown every two cycles) and extension at 72 °C for 15 s, followed by a final extension period at 72 °C for 10 min. For Bacteria-specific PCR the cycling scheme deviated from the one described above by the annealing temperature gradient which was from 68 to 58 °C over 22 cycles and six additional cycles at a constant annealing temperature of 58 °C. Subsequently, the PCR products were purified by employing the GeneJet™ Gel Extraction Kit (Fermentas GmbH, St. Leon-Rot, Germany) and quantified spectrophotometrically.

The isolated metagenomic DNA was used to create a 454-shotgun library following the GS Rapid library protocol (Roche). The library was sequenced with the Genome Sequencer FLX (Roche, Mannheim, Germany) using Titanium chemistry. Sequencing was performed by the Göttingen Genomics Laboratory. In total, 2,044,797 shotgun reads were generated by two complete sequencing runs.

The partial 16S rRNA gene amplicons were sequenced using the unidirectional sequencing protocol (Roche) and Titanium chemistry. One medium lane of a Titanium Picotiter plate was used for sequencing, resulting in 125,702 shotgun sequences.

The sequences have been deposited at NCBI database under the accession number: SRA059339.

Analysis of pyrosequencing-derived data

All pyrosequencing reads obtained from partial 16S rRNA gene amplicons were reassigned to samples AI and AIV based on the unique MIDs. Different scripts of the QIIME software pipeline (Caporaso et al. 2010) and additional programs were applied to perform pyrosequencing data preprocessing and downstream analyses. Removal of sequences <200 bp, and sequences containing more than two primer mismatches or long homopolymers (>8 bp) was performed by applying the script "split_libraries.py".

Pyrosequencing noise was removed by using the scripts “denoise_wrapper.py” and “inflate_denoiser.py”. Subsequently, the programs cutadapt (Martin 2011) and UCHIME (Edgar et al. 2011) (reference database: Greengenes gold database) were applied to remove remaining primer sequences and chimeras, respectively.

Operational taxonomic units (OTUs) were identified at genetic distances of 3 and 20 %, representing the species and phylum level, respectively, according to Schloss and Handelsman (2005) by applying the QIIME script “pick_otus.py” (Schloss and Handelsman 2005). In addition, taxonomic classification of OTUs as well as calculation of rarefaction curves, the Shannon index and the Chao1 index were performed using QIIME (Shanon and Weaver 1949; Chao and Bunge 2002). With respect to the script “assign_taxonomy.py”, related to taxonomic classification of OTUs, preprocessed sequences were compared to the SILVA ribosomal RNA database (release 108) using BLASTN (Pruesse et al. 2007). Furthermore, a customized script to remove all OTUs from the OTU table that have been classified as chloroplasts was applied.

Metagenomic 16S rRNA gene sequences with a minimum length of 100 bp were analysed in the same way by comparing them to the SILVA ribosomal RNA database (release 108) using BLASTN (Pruesse et al. 2007).

Fluorescence in situ hybridization

An 80 µL aliquot of the fixed sample was diluted 1:10 in PBS/ethanol and treated by sonication using Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, USA) at a setting of 1 min, duty cycle 20 and output control 2, three times with 60 s lapse between each sonication step while cooled on ice. Subsequently, the sample was resuspended in 400 µL PBS/ethanol. In situ hybridization was conducted following the protocol by Snaird et al. (1997). An aliquot of 2.5 µL was applied to a poly-L-lysine-coated microscopic slide. After dehydration, hybridization of the respective 5'-cyanine 3-labelled oligonucleotide probe to the ribosomal sequence in the target cells was carried out at 46 °C for 100–120 min. The slide was washed and stained with 2 µg/mL 4',6'-diamidino-2-phenylindol (DAPI) for 5 min on ice. Cells were counted using a fluorescent microscope (Zeiss Axioplan) and means were calculated by counting 10–20 randomly chosen fields for each analysis, which corresponded to at least 1,000 Dapi-stained cells.

The FISH probes applied in this study were Eub338 (5'-GCTGCCTCCCGTAGGAGT-3', specificity: domain Bacteria, 35 % formamide in hybridization buffer) (Amann et al. 1990), Arc915 (5'-GTGCTCCCCGCCAATTCCT-3', specificity: domain Archaea, 35 % formamide in

hybridization buffer) (Stahl and Amann 1991), Cal450 (5'-CTCCCCGTCCAAAGAGGT-3', specificity: genus *Caldicellulosiruptor*, 30 % formamide in hybridization buffer) (O-Thong et al. 2007), SHyd540 (5'-TCGCGCAAC GTTCGGGACC-3', specificity: genus *Sulfurihydrogenibium*, 60 % formamide in hybridization buffer), and Fer660 (5'-GTTCCGTCTGCCTCTGCC-3', specificity: genus *Fervidobacterium*, 20 % formamide in hybridization buffer). Probes were commercially synthesized as 5'-indocyanine 3-labelled oligonucleotides (Fermentas GmbH, St. Leon-Rot, Germany). Specificity for the newly designed oligonucleotide probes SHyd540 and Fer660 was verified by hybridization to control strains that possess one mismatch position within the ribosomal target sequence (SHyd540: *Rhodothermus marinus* (DSMZ 4252); Fer660: *Fervidobacterium gondwanense* (DSMZ 13020) and *F. nodosum* (DSMZ 5306)). Under the given stringency discrimination of one mismatch was possible.

Results

Sampling site and sample characteristics

Samples were collected in September 2010 in the Valley of Furnas from nine sites with a wide variety of physico-chemical characteristics. In situ temperatures ranged from 51 to 92 °C, whereas pH values varied between 8 and 2.5 (Table 1). Two sampling sites (AI and AIV) were selected for in-depth analysis, including the determination of several chemical parameters (Table 2). Site AI had a temperature of 51 °C and an acidic pH of 3. The greyish muddy hot spring exhibited oxidized conditions with high concentrations of sulphate (12.5 mM) and iron, which was mainly present as Fe³⁺ (1,400 µM). Site AIV, with a temperature of 92 °C and a pH of 8, exhibited reduced conditions, indicated by the proportion of Fe²⁺ to Fe³⁺, which can be taken as a proxy for redox conditions (Mathur et al. 2007). The concentrations of sulphate and iron were low (100 and 20 µM) and iron was mainly present as Fe²⁺. Organic carbon content was high with 0.006 % (60 mg/L) at site AI and 0.03 % (300 mg/L) at site AIV.

Community fingerprinting using DGGE

Prokaryotic diversity was estimated using denaturing gradient gel electrophoresis (DGGE) based on 16S rRNA gene fragments amplified with Bacteria- and Archaea-specific primers. Results are shown in Figs. 2 and 3. Taking the number of bands in the DGGE-profile as diversity indicator, the highest bacterial diversity was detected at sites with temperatures between 55 and 85 °C and pH values between 7 and 8. Analysis of the DGGE-profiles revealed a

Table 2 Chemical analysis of sampling sites AI and AIV

| | Na (mM) | K (mM) | Mg (mM) | Ca (mM) | Fe ²⁺ (μM) | Fe ³⁺ ⁽¹⁾ (μM) | Fe (μM) | NH ₄ ⁺ (μM) | Cl ⁻ (mM) |
|-----|-----------------------------------|-----------------------------------|------------------------------------|------------------------------------|-----------------------|--------------------------------------|---------|-----------------------------------|----------------------|
| AI | 6.0 | 1.5 | 0.7 | 1.1 | 152 | 1391 | 1543 | 388 | 1.0 |
| AIV | 19.7 | 0.7 | 0.1 | 0.3 | 22 | 2 | 25 | 26 | 5.4 |
| | NO ₂ ⁻ (μM) | NO ₃ ⁻ (μM) | SO ₃ ²⁻ (μM) | SO ₄ ²⁻ (mM) | S (mM) | PO ₄ ²⁻ (mM) | TIC (%) | DOC (%) | |
| AI | <200 | <16 | <13 | 12.5 | 12.6 | 0.01 | <0.0005 | 0.006 | |
| AIV | <200 | <16 | <13 | 0.1 | 0.3 | 0.1 | 0.009 | 0.028 | |

Fe³⁺ was calculated as the difference of Fe and Fe²⁺

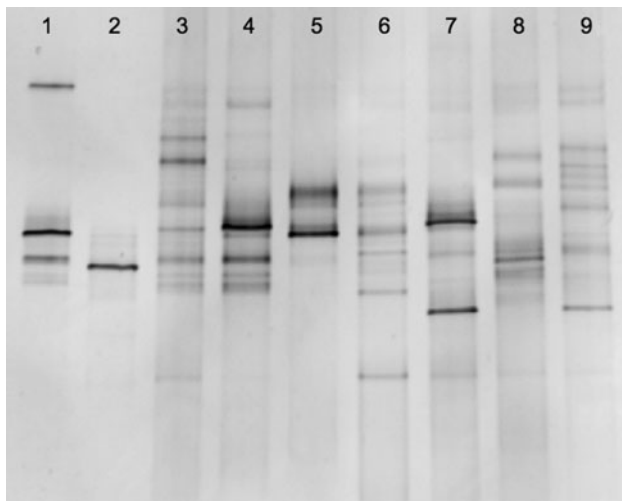


Fig. 2 DGGE analysis of 16S rRNA gene fragments amplified with Bacteria-specific primers. PCR products were analysed on a denaturing gradient from 20 to 80 %. The samples were grouped according to sampling site (A, B, C) and increasing temperature. The lanes can be attributed to the following sites: 1 AI (51 °C, pH 3), 2 AII (84 °C, pH 2.5–3), 3 AIII (85 °C, pH 8), 4 AIV (92 °C, pH 8), 5 BI (60 °C, pH 6), 6 BII (65 °C, pH 7), 7 BIII (70 °C, pH 6), 8 CI (55 °C, pH 8), 9 CII (76 °C, pH 8)

prominent effect of pH. In particular, the combination of acidic pH and high temperature (pH 2.5–3 and 84 °C) led to a very low bacterial diversity (Fig. 2, lane 2) while at moderate temperature of 51 °C and pH 3 more bacterial bands were detected (Fig. 2, lane 1). Analysis of archaeal 16S rRNA genes using DGGE revealed an overall lower number of bands (Fig. 3). Sites AIII and AIV exhibit an almost identical band-pattern despite the difference in temperature (85 vs. 92 °C).

Based on site-specific characteristics and the DGGE results we selected two sites (AI and AIV) for further analysis. Both sites are connected, with the outflow of AIV running into AI. Furthermore, they exhibit medium diversity and particularly challenging conditions with regard to pH (AI: pH 3) or temperature (AIV: 92 °C).

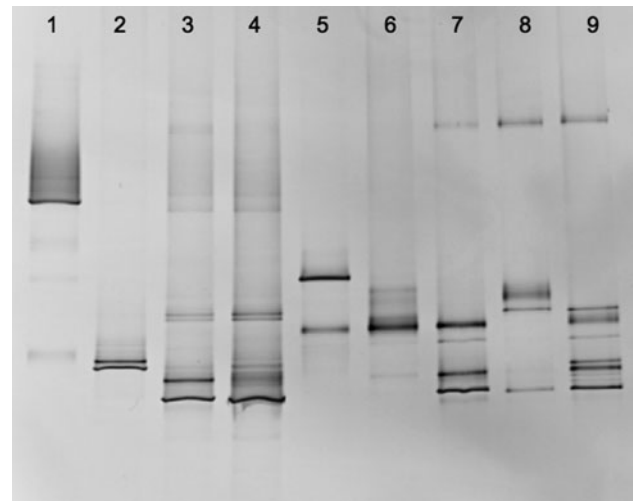
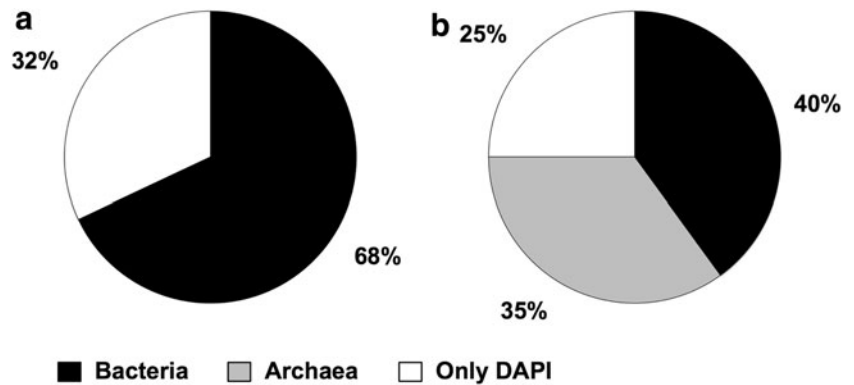


Fig. 3 DGGE analysis of 16S rRNA gene fragments amplified with Archaea-specific primers. PCR products were analysed on a denaturing gradient from 30 to 80 %. The samples were grouped according to sampling site (A, B, C) and increasing temperature. The lanes can be attributed to the following sites: 1 AI (51 °C, pH 3), 2 AII (84 °C, pH 2.5–3), 3 AIII (85 °C, pH 8), 4 AIV (92 °C, pH 8), 5 BI (60 °C, pH 6), 6 BII (65 °C, pH 7), 7 BIII (70 °C, pH 6), 8 CI (55 °C, pH 8), 9 CII (76 °C, pH 8)

Community composition determined by fluorescence in situ hybridization

To quantify the relative contributions of Bacteria and Archaea to the communities of sites AI and AIV, we applied FISH using domain-specific probes (Fig. 4). The acidic hydrothermal spring AI was dominated by Bacteria (68 %). Archaea could not be detected at all and 32 % of the cells could only be stained with DAPI. At the high-temperature site AIV (92 °C) 75 % of all cells could be detected by FISH. The relative contribution of the specific domains was almost even with 35 % Archaea and 40 % Bacteria. Total cell number determined by DAPI-staining was 4.6×10^7 /ml for AI and 2.6×10^8 /ml for AIV.

Fig. 4 Domain-level composition of microbial communities AI and AIV as determined by FISH. For Bacteria EUB333 was used and for Archaea probe Arch915. Relative abundance is based on the total cell number determined by DAPI-staining. **a** Depicts the results for site AI and **b** for site AIV



Phylogenetic analyses based on 16S rRNA gene sequences

To evaluate prokaryotic diversity of the selected sites, partial 16S rRNA genes were amplified from metagenomic DNA and sequenced by pyrosequencing. Based on former studies, we decided to amplify the V2/V3 region for both, Bacteria and Archaea, comprising 435 bp (Nacke et al. 2011; Yu et al. 2008; Will et al. 2010; Kysela et al. 2005). While primers for Bacteria were already available, new primers to amplify the corresponding region in the archaeal 16S rRNA gene had to be designed. The forward primer was based on the data of Baker and Cowan with slight modifications (Baker and Cowan 2004) while the reverse primer was designed de-novo. A total number of 125,702 sequences were generated. After preprocessing including quality filtering and denoising, 93,576 sequences with an average length of 390 bases could be obtained for further analyses. The number of total reads of every sample and remaining numbers of reads after quality filtering and denoising are depicted in Table 3. Amplification was specific, yielding the expected domain-specific sequences apart from archaeal amplification of DNA from site AIV.

Furthermore, metagenomic DNA derived from site AIV was analysed using direct pyrosequencing without prior amplification. Of the data generated 0.1 % represented partial 16S rRNA genes. The 16S rRNA gene fragments $f \geq 100$ bp were extracted from the dataset. The resulting 725 partial 16S rRNA gene sequences were analysed by comparing them to the SILVA ribosomal RNA database using BLASTN.

Comparison of the sequencing approaches

Combined results of the two sequencing approaches are shown in Tables 4, 5 and 6. The acidic spring AI was dominated by Proteobacteria (>80 %) and Firmicutes (10 %) (Table 4) while site AIV was dominated by bacteria belonging to 4 different phyla: Thermotogae,

Table 3 Evaluation of V2/V3 pyrosequencing data

| | Total sequences | Preprocessed sequences | Average read length | Assigned to Bacteria (%) | Assigned to Archaea (%) |
|-----------|-----------------|------------------------|---------------------|--------------------------|-------------------------|
| AI—Bact. | 37,044 | 28,565 | 385 | 97.0 | 3 |
| AI—Arch. | 49,573 | 35,607 | 386 | 1.3 | 98.7 |
| AIV—Bact. | 39,085 | 29,404 | 400 | 99.9 | 0.1 |

Firmicutes, Dictyoglomi, and Aquificae (Table 5). Each of these phylum-groups mainly consisted of one dominating genus. The acidic spring AI was dominated by Proteobacteria known for their acidophilic properties: the heterotrophic genus *Acidicaldus* (38 %) and the chemolithoautotrophic *Acidithiobacillus* (43 %). In addition, almost 8 % of the sequences was related to *Anoxybacillus* (Table 4). Within site AIV (92 °C, pH 8) members of *Dictyoglomus*, *Caldicellulosiruptor*, and *Fervidobacterium* constituted up to 61 % of the bacterial community according to data resulting from non-amplified pyrosequencing of metagenomic DNA, whereas analysis of the amplified V2/V3 16S rRNA gene region indicated an even higher abundance of these heterotrophic genera (88 %) (Table 5). Data from direct metagenome sequencing also revealed high abundance of two genera from the phylum Aquificae, with the chemolithoautotrophic *Sulfurihydrogenibium* being the dominant genus (22 %).

The archaeal community of the hot spring AIV (92 °C) was almost exclusively composed of Crenarchaeota while in the acidic spring AI only Euryarchaeota were detected. Table 6 depicts the detailed composition. The archaeal community of site AI mainly harboured *Thermoplasma* (89 %), while clone group BLSdp215 represented the second largest group (9 % of all sequences). Site AIV was dominated by Desulfurococcaceae (75 %) with the genera *Sulfophobococcus*, *Desulfurococcus* being most abundant within this family. Furthermore, the genus *Pyrobaculum* of the Thermoprotaceae made up 25 % of the sequences.

Table 4 Composition of the bacterial community of AI determined by V2/V3 sequencing

| Phylum | Family | Genus | Rel. abundance (%) AI V2/V3 |
|---|-----------------------------|---------------------------------|--------------------------------|
| Proteobacteria (α) | Acetobacteraceae | <i>Acidicaldus</i> | 38 |
| | | <i>Uncultured</i> | 0.6 |
| Proteobacteria (γ) | Acidithiobacillaceae | <i>Acidithiobacillus</i> | 43 |
| Firmicutes | Bacillaceae | <i>Anoxybacillus</i> | 7.8 |
| | Clostridiaceae | <i>Caloramator</i> | 1.2 |
| | | <i>Clostridium</i> | 0.5 |
| Nitrospirae | Nitrospiraceae | <i>Leptospirillum</i> | 0.9 |
| Actinobacteria | Acidimicrobiaceae | <i>Acidimicrobium</i> | 0.7 |

Only genera that occur at an abundances of above 0.5 % are shown

Dominant taxa are given in bold

Table 5 Detailed comparison of the bacterial community composition of AIV determined by V2/V3 or metagenome sequencing

| Phylum | Family | Genus | Rel. abundance (%) | |
|-----------------------|--------------------------------|------------------------------------|--------------------|----------------|
| | | | AIV V2/V3 | AIV metagenome |
| Aquificae | Hydrogenothermaceae | <i>Sulfurihydrogenibium</i> | 0.4 | 22 |
| | Aquificaceae | <i>Hydrogenobacter</i> | 0.1 | 6 |
| Dictyoglomi | Dictyoglomaceae | <i>Dictyoglomus</i> | 22 | 27 |
| Deinococcus-Thermus | Thermaceae | <i>Thermus</i> | 0.8 | 2 |
| Firmicutes | Thermoanaerobacteraceae | <i>Caldicellulosiruptor</i> | 19 | 21 |
| | Clostridiaceae | <i>Clostridium</i> | 4.9 | 0.6 |
| | Peptococcaceae | <i>Desulfosporosinus</i> | ND | 1 |
| | Ruminococcaceae | <i>Incertae Sedis</i> | 1.63 | ND |
| Thermotogae | Thermotogaceae | <i>Fervidobacterium</i> | 47 | 13 |
| | | <i>Thermotoga</i> | 0.7 | 0.8 |
| Thermodesulfobacteria | Thermodesulfobacteriaceae | <i>Thermodesulfobacterium</i> | ND | 1 |
| Nitrospirae | Nitrospiraceae | <i>Thermodesulfovibrio</i> | 0.72 | 0.4 |

Only genera that occur at an abundances of above 0.5 % in at least one of the two approaches are shown

Dominant taxa are given in bold

ND not detected

Table 6 Detailed comparison of the archaeal community composition of AI and AIV determined by metagenome sequencing and V2/V3 sequencing, respectively

| Phylum | Family | Genus | Rel. abundance of archaea (%) | |
|----------------------|---------------------------|--------------------------------|-------------------------------|------------------|
| | | | AI | AIV ^a |
| Crenarchaeota | Desulfurococcaceae | <i>Sulfophobococcus</i> | ND | 55 |
| | | <i>Desulfurococcus</i> | ND | 19 |
| | | <i>Staphylothermus</i> | ND | 0.5 |
| | | <i>Stetteria</i> | ND | 0.5 |
| | Thermoproteaceae | <i>Pyrobaculum</i> | ND | 25 |
| Euryarchaeota | Thermoplasmataceae | <i>Thermoplasma</i> | 89 | ND |
| | | <i>BSLdp215 uncultured</i> | 9 | ND |
| | | <i>A10 uncultured</i> | 1 | ND |

Only genera that occur at an abundances of above 0.5 % are shown

Dominant taxa are given in bold

ND not detected

^a Data are based on the metagenome analysis

Microbial diversity indices

Hot springs are usually referred to as low-diversity habitats. Diversity indices are well established measures to quantify diversity on a parametric (Shanon) or non-parametric (Chao1) basis. While the Shanon index takes species richness and species evenness into account, the Chao index is based on species richness, with the obtained value giving the number of expected operational taxonomic units (OTUs). To calculate rarefaction curves, richness, and diversity indices, OTUs at genetic distances of 3 and 20 % were determined. With respect to the Bacteria, OTU-based analyses were performed at the same level of surveying effort by using 22,100 randomly selected sequences per sample to enable accurate OTU-based comparisons between site AI and AIV.

Comparison of the rarefaction analyses with the number of OTUs determined by Chao1 richness estimator revealed that 76.2–90.6 % (20 % genetic distance) and 54.5–77.7 % (3 % genetic distance) of the taxonomic richness were covered by the surveying effort (Fig. 5; Table 7). Thus, we did not survey the full extent of taxonomic diversity at these genetic distances, but especially

at the phylum level a substantial fraction of the estimated richness was assessed by the surveying effort. The bacterial diversity at sites AI and AIV exhibits an interesting difference: on the phylum level the acidic spring is more diverse than the slightly alkaline high-temperature site AIV, with 50 versus 30 OTUs (Table 7), whereas on the species level the opposite pattern was discovered (AI: 178 OTUs, AIV: 236 OTUs).

Using Chao1 richness estimator, the maximum number of OTUs for the acidic spring bacterial community predicted at 3 % genetic distance was 229 belonging to 59 predicted phyla. With respect to site AIV (92 °C, pH8), 371 bacterial OTUs on species level and 33 OTUs on the phylum level were predicted by Chao1 richness estimator. The Shanon index (H'), determined for both habitats, indicated a slightly lower diversity in site AI than in site AIV (3 % genetic distance: AI: 1.8, AIV: 2.3; 20 % genetic distance: AI: 0.8, AIV: 1.0).

Archaeal diversity could only be estimated for site AI and was very low (H' : 0.54 at 3 % and 0.09 at 20 % genetic distance) as expected due to the high dominance of *Thermoplasma* (Table 6). At 3 % sequence divergence a maximum number of 99 OTUs were predicted while 21

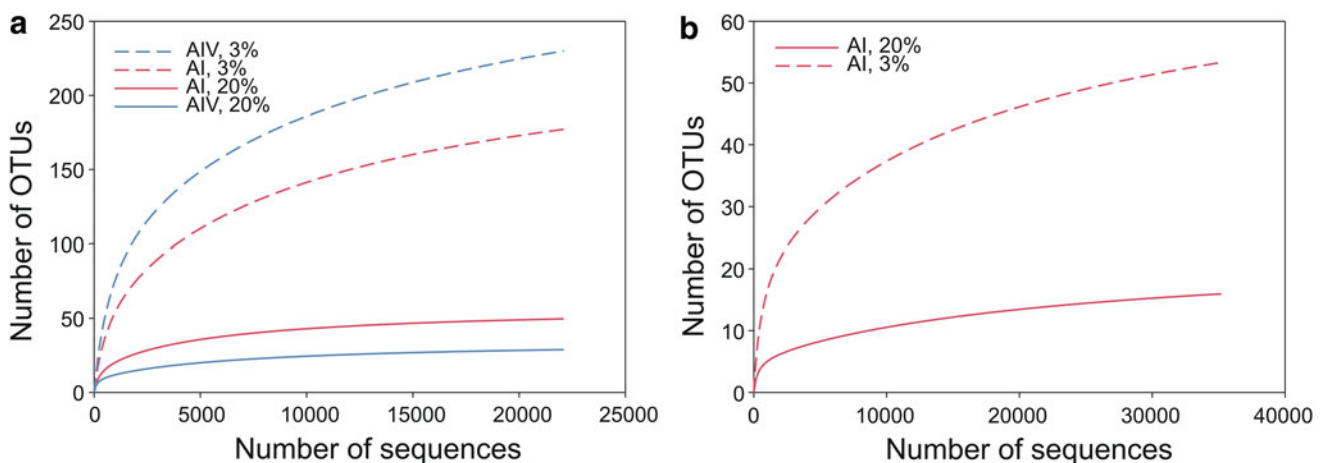


Fig. 5 Rarefaction analysis of V2/V3 sequencing results for Bacteria and Archaea. Rarefaction curves were calculated on the basis of 22,100 sequences for Bacteria (a) and based on 35,200 sequences for

Archaea (b). The curves indicate the observed number of OTUs within the two samples AI (51 °C, pH 3) and AIV (92 °C, pH 8). OTUs are shown at the level of 3 and 20 % genetic distance

Table 7 Diversity estimates based on V2/V3 sequencing

| Distance | Shannon index (H') | | Rarefaction (no. of OTUs) | | Chao1 (no. of OTUs) | | Coverage (%) | |
|---------------------------|------------------------|------|---------------------------|------|---------------------|------|--------------|------|
| | 3 % | 20 % | 3 % | 20 % | 3 % | 20 % | 3 % | 20 % |
| AI Bacteria ^a | 1.80 | 0.83 | 178 | 50 | 229 | 59 | 77.7 | 84.7 |
| AI Archaea ^b | 0.54 | 0.09 | 54 | 16 | 99 | 21 | 54.5 | 76.2 |
| AIV Bacteria ^a | 2.29 | 1.04 | 231 | 29 | 337 | 32 | 68.5 | 90.6 |

^a No. of sequences analysed 22,100

^b No. of sequences analysed 35,200

OTUs were predicted at 20 % sequence divergence using Chao1 richness estimator.

Evaluation of the pyrosequencing-based analyses by group-specific fluorescence in situ hybridization

At site AIV relative abundance of *Sulfurihydrogenibium* and *Fervidobacterium* differed depending on the sequencing approach (Table 5). While sequencing of non-amplified metagenomic DNA indicated high abundance of Aquificae (approx. 28 %), in particular *Sulfurihydrogenibium*, the sequencing of partial 16S rRNA gene amplicons hardly detected any. On the other hand, amplicon sequencing indicated dominance of *Fervidobacterium* (approx. 47 %). We applied FISH to quantify and further analyse these specific groups including also *Caldicellulosiruptor*, which represented approx. 21 % of the bacterial community according to data derived from direct sequencing and pyrosequencing of the V2/V3 16S rRNA gene region. *Dictyoglomus*, the fourth dominant genus in sample AIV could not be evaluated because no specific probe could be designed.

Two probes had to be designed de-novo. The probes specific for *Sulfurihydrogenibium* and *Fervidobacterium* were developed on the basis of signature sequences described by Harmsen et al. (1997), which had been specific for *Hydrogenobacter* and *Thermotoga*. An exchange of one and two bases, respectively, allowed to change the specificity to the genera in question. Primer specificity was confirmed in-silico using ProbeMatch of the RDP Database Project and hybridization conditions were experimentally verified using controls with one mismatch. At the stringency conditions applied, no signal could be detected in cells with 1 mismatch to the probe. A comparison of all three approaches to quantify dominant bacterial genera is shown in Fig. 6. Quantification by FISH underlined the high abundance of *Caldicellulosiruptor*, revealing that this genus represented 28 % of all Bacteria (detected with EUB-specific probe) as compared to 19 and 21 % detected by sequencing of partial 16S rRNA gene amplicons and direct sequencing of metagenomic DNA, respectively. Furthermore, FISH indicated that *Fervidobacterium* accounted for 22 % of the bacterial community and thus had been overestimated by analysis of the V2/V3 16S rRNA region (47 %) and underestimated according to data derived from direct sequencing of metagenomic DNA (14 %). While *Sulfurihydrogenibium* was almost undetected by sequencing of the V2/V3 16S rRNA gene region (0.4 %), the genus was overestimated by direct sequencing of metagenomic DNA (17 %) since FISH revealed it to account for 9 % of the Bacteria. Altogether, almost 60 % of the bacterial cells could be assigned to the three genera

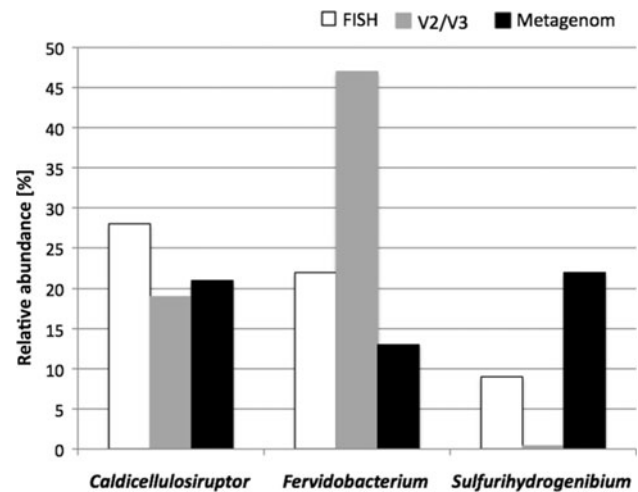


Fig. 6 Comparative quantification using FISH, V2/V3 and metagenome sequencing. The relative abundance relates to % of bacterial cells for FISH, % of bacterial rRNA gene sequences for V2/V3 and metagenome sequences

Caldicellulosiruptor, *Fervidobacterium*, and *Sulfurihydrogenibium* using FISH.

Discussion

Prokaryotic diversity

Hot springs are considered low-diversity habitats due to the high physicochemical constraints and the high degree of geographical isolation (Papke et al. 2003). The effect of temperature on prokaryotic diversity has been studied and results are contradictory. Yim et al. (2006) did not find a linear correlation between prokaryotic diversity and temperature in mats and streamers at temperatures between 52 and 69 °C in alkaline geothermal springs from Tibet. On the other hand, Miller et al. suggested that bacterial diversity along a temperature gradient from 39 to 72 °C in two alkaline hot springs from Yellowstone National Park is primarily controlled by temperature in photosynthetically active microbial mats which has also been shown by Hiraishi et al. in hot springs in Japan (Yim et al. 2006; Miller et al. 2009; Hiraishi et al. 1999). Furthermore, Skirnisdottir et al. (2000) found the lowest bacterial diversity at the highest temperature (80 °C) and highest sulphide concentration when comparing hot springs in Iceland with low and high concentration of sulphide. Based on DGGE analysis of nine sites, we found high bacterial diversity over a range of temperature between 55 and 85 °C as long as the pH was neutral to slightly alkaline. With respect to Archaea, the temperature range was slightly shifted upwards from 70 to 92 °C. In the

hydrothermal springs investigated here, lowest bacterial diversity was detected at acidic pH and high temperature.

If phylogenetic distance is taken into account, sites AI and AIV display a distinctive difference. OTU-based analyses showed that bacterial diversity was more phylum rich in site AI compared to site AIV. However, site AIV was more species rich than site AI. Similar observations have been published previously (Roesch et al. 2007; Yim et al. 2006). Yim et al. suggested that extreme stress might inhibit the survival of large numbers of closely related guild taxa in microbial population from hot spring sediments and streamers leading to higher phylum numbers (Yim et al. 2006). Roesch et al. (2007) found that agricultural soil was phylum poor and species rich, whereas undisturbed forest soil showed the opposite pattern. However, the authors could not find a clear explanation for this. The factors causing the differences between the two habitats AI and AIV investigated here remain elusive. It is possible that coping with low pH is a property more widespread in the phylogenetic tree on phylum level than coping with temperatures above 90 °C. On the other hand it could also mean, that a habitat with temperature above 90 °C allows faster speciation than the low pH habitat. As long as there is no additional data available to show that this is not a coincidental observation, we can only speculate. Further comparative studies will be needed to evaluate this and to establish, whether it also holds for archaeal populations.

Diversity indices help to compare habitats. However, only few data are available on diversity indices from hot springs. For circumneutral springs at temperatures between 73 and 86 °C Costa et al. (2009) calculated Shannon indices between 2.02 and 2.37 at 3 % genetic distance for Bacteria and between 1.18 and 2.7 for Archaea. At a genetic distance of 20 %, the values were between 1.82 and 2.29 for Bacteria and between 0.95 and 2.16 for Archaea. For slightly alkaline springs along a temperature gradient from 52 to 69 °C Lau et al. (2009) calculated slightly higher Shannon indices between 3 and 4 at 3 % genetic distance for Bacteria. In acidic springs at temperatures between 60 and 75 °C Mathur et al. (2007) calculated Shannon indices for bacteria at 3 % genetic distance between 0.83 and 3.28 at springs with high sulphide and low iron concentration and between 2.1 and 3.6 in springs with high iron and low sulphide concentration. Shannon indices for bacteria calculated for sites AI and AIV fall within the same range. The acidic spring AI, which contained high iron and low sulphide concentrations, exhibited a Shannon index of 1.8 for Bacteria and 0.54 for Archaea at 3 % sequence divergence and values of 0.83 and 0.09 at 20 % sequence divergence. The slightly alkaline high-temperature (92 °C) spring AIV shows a Shannon index of 2.29 at 3 % and 1.04 at 20 % sequence divergence for Bacteria. These values are, as

expected, lower than in terrestrial soils, which are considered the most complex microbial ecosystems on Earth, with Shannon indices of 5–7 at 3 % sequence divergence and 2.5–4.5 at 20 % sequence divergence (Will et al. 2010; Nacke et al. 2011). Shannon indices calculated for another extreme environment, glacier ice (3.4 and 1.7 for 3 and 20 % sequence divergence, respectively) (Simon et al. 2009), were in the same range as in our study.

Dominant metabolic pathways in the hot springs of Furnas

Inferring physiology from 16S rRNA gene phylogeny is inherently biased (Jaspers and Overmann 2004), in particular if the closest relatives are sequences from uncultured organisms. Keeping this in mind we suggest putative physiology on the basis of the closest cultivated 16S rRNA gene relatives for our habitat, in particular for AIV because a large proportion of the partial sequences is more than 99 % identical to sequences from cultivated organisms. For *Fervidobacterium* 91 % of the sequences were more than 99 % identical to *F. islandicum*, for *Dictyoglomus* 88 % of the sequences were more than 99 % identical to *D. thermophilum*, and for *Caldicellulosiruptor* 85 % of the sequences were more than 99 % identical to *C. lactoaceticus*. Altogether only the rare members of the sequence pool amounting to 11 % were <97 % identical to cultivated or related to uncultivated species. The situation is different for AI and here in particular for *Acidicaldus*. 94 % of the *Acidicaldus*-specific sequences were related to uncultivated organisms. However, 99 % of the *Acidithiobacillus*-sequences were 99 % identical to *Acidithiobacillus caldus*. This is in contrast to the majority of other environmental studies, in which the largest proportion of sequences does not affiliate with cultivated species (Meyer-Dombard et al. 2005; Hugenholtz et al. 1998; Ivanova et al. 2011; Pagaling et al. 2012).

Although differing for two groups (*Sulfurihydrogenibium* and *Fervidobacterium*), both 16S rRNA gene sequence-based approaches indicated the dominance of heterotrophic bacterial genera in both springs. This is in contrast to many other studies in hot spring-environments where Aquificales have been repeatedly found to dominate 16S rRNA gene-libraries, e.g. from Yellowstone National Park, Iceland, New Zealand, and Japan (Hall et al. 2008; Meyer-Dombard et al. 2005; Blank et al. 2002; Jackson et al. 2001; Skirmisdottir et al. 2000; Hetzer et al. 2007; Yamamoto et al. 1998). These findings led to the hypothesis that chemolithotrophic physiology probably based on the oxidation of H₂ or reduced sulphur compounds is the major metabolic pathway in hot springs. Skirmisdottir et al. compared results from different studies on hot spring microbial mats and sediments and found that, depending on the chemical characteristics of the

spring, different subgroups of Aquificales dominated (Yamamoto et al. 1998; Reysenbach et al. 2000; Hugenholtz et al. 1998). In high-sulphide and sometimes iron-rich habitats groups, J and S were dominating and the related sequences can today be attributed to *Sulfurihydrogenibium*. The high abundance (21 %) of *Sulfurihydrogenibium*-related sequences within the metagenome from site AIV fitted into the picture. However, evaluation with FISH revealed that *Sulfurihydrogenibium* had been overestimated and was only accounting for 9 % of the bacterial cells.

The comparatively low abundance of chemolithoautotrophic organisms at site AIV might be related to the high concentration of dissolved organic carbon (DOC) of 284 mg/L (0.028 %). Only little data are available on the DOC content of hot springs. Yamamoto et al. measured a DOC content of 0.41–0.72 mg/L within the microbial sulphur-turf mat, while Hetzer et al. determined 0.7 mg/L in Champagne Pool, New Zealand, and Hall et al. described a DOC content of 10 mg/L in Coffee Pot hot spring (YNP) (Yamamoto et al. 1998; Hetzer et al. 2007; Hall et al. 2008). All sites showed clear dominance of Aquificales. The more than 20-fold or even 400-fold higher concentration of DOC in the Furnas spring could well be a reason for the abundant occurrence of heterotrophic bacteria. Regarding autotrophic processes at site AIV, however, also Archaea have to be taken into account, since FISH detected 35 % of the cells to be of archaeal origin. Metagenomic rRNA data suggest that the heterotrophic genera *Sulfophobococcus* spp. and *Desulfurococcus* spp. account for approx. 74 % of the sequences while the remaining 26 % may well be related to chemolithotrophic genera. Further studies will show, if high abundance of heterotrophic microorganisms is a common feature for springs with higher organic carbon content.

The dominant genera in site AIV (*Caldicellulosiruptor*, *Dictyoglomus*, and *Fervidobacterium*) have also been detected in in situ enrichment cultures in hot springs from Kamchatka (Kublanov et al. 2009). While *Caldicellulosiruptor* sp. and *Dictyoglomus* sp. grew on polysaccharides like cellulose and chitin, *Fervidobacterium* sp. was enriched on proteinaceous substrates. Although dominated by one OTU, all groups show additional sequence diversity in particular *Dictyoglomus* with 44 OTUs at 3 % divergence. For *Fervidobacterium* and *Caldicellulosiruptor* the number of specific OTUs is 18 and 14, respectively. These results suggest that the hot springs of Furnas are a valuable source to retrieve new polymer-degrading organisms.

Conclusions

The microbial community of two hot springs of Furnas Valley was characterized on the basis of different rRNA gene-based methods. The combination of different primer-

dependent and independent methods revealed major differences, emphasizing the importance of combining different methodological approaches. A large dataset of short rRNA gene sequences and metagenome analysis revealed that the habitats were dominated by three to four genera, both for the bacterial and the archaeal population. Archaeal diversity was particularly limited in the acidic spring AI (pH 3, 51 °C), in which *Thermoplasma* represented almost 90 %. However, overall abundance of Archaea in this acidic environment was low as revealed by FISH, while the hot slightly alkaline spring AIV (pH 8, 92 °C) had almost even abundances of Archaea and Bacteria. Unlike other hydrothermal habitats, the high-temperature slightly alkaline low-sulphate spring AIV was dominated by heterotrophic bacteria, probably due to a high content of dissolved organic carbon, suggesting that not all hydrothermal habitats are dominated by chemolithoautotrophs and that a wide range of chemical characteristics needs to be taken into account when analysing hot springs. This supports the efforts of the Earth Microbiome Project which states the importance of careful and comprehensive metadata collection for each and in particular for metagenomic environmental studies in order to improve comparability, so that general patterns can be identified on the basis of different studies (Knight et al. 2012; Yilmaz et al. 2011).

Our results suggest a natural enrichment of heterotrophic, polymer-degrading genera in the Furnas springs which make them particularly promising for the search of novel thermostable enzymes for application in biotechnology and biorefinery of the second generation.

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