Rapid shift in microbial community structure in a neutral hydrothermal hot spring from Costa Rica

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Keywords: Hot Spring, Costa Rica, Microbial mat, Thermophiles, Aquificae, *Sulfurihydrogenibium*

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Abstract

In this work, we characterize the geochemistry and microbial community of Bajo las Peñas, a neutral (pH 6.5-7.4), hot spring (T = 62.0-68.0°C) located near Turrialba Volcano, Costa Rica. The microbiota at its two sources belongs mainly to the family Aquificae, comprising OTUs closely related to the genera *Sulfurihydrogenibium*, *Thermosulfidibacter*, *Thermodesulfovibrio* and *Thermocrinis* which is consistent with the presence of moderate levels of sulfate (243-284 mg/L) along the stream. We determined a dramatic shift in the microbial community just a few meters downstream of the sources of the hot spring (15-20 meters), with a change from sulfur related chemoautotrophic (e.g. *Sulfurihydrogenibium* and an OTU closely related to *Thermodesulfovibrio*) to chemoheterotrophic prokaryotes (e.g. *Meiothermus*, *Nitrososphaera*, *Thermoflexus*, *Thermus*). Thus, in this neutral hot spring, the first level of the trophic chain is associated with photosynthesis as well other anaerobic CO₂ fixing bacteria. Then, thermotolerant chemoheterotrophic bacteria colonize the environment to degrade organic matter and use fermentative products from the first level of the trophic chain. Our data demonstrate how quickly the microbial community of an ecosystem can change in response to environmental variables and sheds light on the microbial ecology of less common circumneutral pH hot springs.

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49 Introduction

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Characterization of microbial communities inhabiting hot springs is of great interest for understanding the ecology in extreme environments. Due to their high temperatures, macroscopic organisms are limited in these habitats; as a result, hot spring communities are usually made up exclusively by thermotolerant or thermophilic microorganisms (Akanbi et al. 2019). These conditions define hot springs as “ecologic islands”, where even when foreign microorganisms are constantly inoculated into the water, they are not able to survive the high temperatures. Additionally, due to their geological and chemical properties, a great spectrum of niches exists in this kind of environments, which in consequence allows the coexistence of multiple species (Mitri 2019). The great diversity present in the multiple niches (microbial mat, sediments, surface, and underground water) has been proposed to be a consequence of metabolic heterogeneity that evokes during the community evolution (Mitri 2019). Such a variation of taxa across different niches of the same hot spring has been observed when analyzing the microbial mat and the anoxic region of Mushroom Spring in Yellowstone National Park (Klatt et al. 2011; Thiel et al. 2016; Tank et al 2017). Furthermore, in this hot habitat, it has been shown that specific taxa are associated to particular metabolic functions within the community and cross-feeding may be occurring explaining the high diversity in these habitats (Thiel et al. 2017). The understanding of the rules that govern the ecology of hot springs is relevant because these extreme habitats have also been proposed to be a good model

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67 for understanding the evolution of life, particularly because hot springs resemble hydrothermal vents
68 where life might have emerged (Damer and Deamer 2020).

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70 Hot springs can be found at various locations around the globe and their physicochemical parameters
71 vary widely but they are normally acidic. Previous studies have demonstrated that their acidity levels are
72 highly correlated to the chemical species that are present in the environment, particularly iron and sulfur
73 species (Xu et al. 1998, 2000; Pierson et al. 1999, 2000; Sharma et al. 2001). A particularity shared
74 between the less commonly described neutral pH hot springs with the acidic ones is the significant
75 proportion of ferrous iron produced from mineral dissolution, which can form precipitates (Konhauser
76 1997; Pierson et al. 1999; Yee et al. 2003). The iron minerals in neutral hot springs are usually formed by
77 spontaneous iron oxidation, and the oxides produced consequently nucleate, sometimes in association
78 with silica, and precipitate as ferric hydroxides that include minerals like ferrihydrite, lepidocrocite or
79 goethite (Peng et al. 2013; Liu et al. 2014; Cooper et al. 2017). Due to their mild pH and moderate toxic
80 metals concentrations, the main extreme conditioning factor in neutral hot springs is temperature, limiting
81 the proliferation of multiple microorganisms and directly shaping the community (Ward et al. 1998).

82

83 Photoautotrophic microorganisms play a key role in hot water bodies (Ward and Castenholz 2000; Klatt et
84 al. 2011). Previous reports have found that members of Cyanobacteria and Chloroflexi are common
85 phototrophic groups found in this type of habitats, particularly in microbial mats (Pierson and Castenholz
86 1974; Miller and Castenholz 2000; Alcamán et al. 2015; Prieto-Barajas et al. 2018; Ward et al. 2018). On
87 the other hand, some deep sulfurous hot springs have a low abundance of cyanobacteria but a high
88 concentration of sulfur chemoautotrophic bacteria, particularly from the family Desulfobacteriaceae. In
89 these environments, these microorganisms are the main CO₂-fixers (Nunoura 2014). In addition to the
90 importance of autotrophic microorganisms, multiple taxa classified as heterotroph, such as Deinococcus-
91 Thermus, Nitrospirae, Proteobacteria, Firmicutes and Actinobacteria are also an important part of hot
92 spring communities (Valverde et al. 2012; Wang et al. 2013; Daims 2014; Sahoo et al. 2015; Ghilamical
93 et al. 2017; Rozanov et al. 2017). Complete metagenome studies have also revealed that these microbial
94 communities can have an extremely diverse and complex metabolism, where nutrients can be recycled
95 between phylotypes and multiple cross-feeding processes may be occurring (Mangrola et al. 2015;
96 Saxena et al. 2017; Thiel et al. 2017; Pedron et al. 2019).

97

98 Bajo las Peñas is a natural occurring hot spring located in Cartago province, close to Turrialba and Irazu
99 volcanoes (Fig. 1). This site is a small lotic environment where water from the hot spring source flows

slowly through an extensive bacterial mat for about 20 meters and then joins a cold, mountain stream that eventually becomes a tributary of the Toro Amarillo River. It is characterized for having a reddish gelatinous microbial mat covering the site (Figs 1C and 1D), a neutral pH (6.5-7.4) and a temperature of around 65°C (Uribe-Lorio et al. 2019). Study of circumneutral hot springs like Bajo las Peñas is relevant since they are uncommon in the world (most are acidic) and can potentially become a source of microorganisms and thermostable enzymes of biotechnological interest. After we carried out our sampling in 2016 for this study, a survey of the microbiology of several Costa Rican hot springs reported data of the microbial community inhabiting the microbial mat of Bajo Las Peñas based on samples taken in 2012. (Uribe-Lorio et al. 2019). This last study reported that the microbial community in the mat is formed almost exclusively by Cyanobacteria. Here, we show a more extensive study of the geochemistry and microbial communities that inhabit the water and the sediments in Bajo las Peñas, including samples taken right at the source in order to better understand the microbial diversity and the possible metabolism associated with this close-to-neutral pH hot spring.

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114 **Materials and Methods**

115

116 *Study Site*

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Bajo las Peñas is located in a private farm northeast of Irazu Volcano (Cartago, Costa Rica; Fig. 1A). At the site, we actually identified and sampled two springs (sources) where hot water emerges which are separated by about 15 m (BP1 and BP3; see Fig. 1). The samples were taken along the whole site, which is approximately 20 meters long and included two more sampling sites away from the sources. Along the effluent, a very thick (ca. 30-50 cm) reddish microbial mat covers the site and slows down the flow of water. Interestingly, despite the high temperature in the surroundings of this thermal spring, grass and bushes were found. The BP1 point (Fig. 1; 9.993492 N, 83.801597 W) corresponds to the origin (first spring) of Bajo las Peñas. The second sampling point (BP2; 9.993558 N 83.801639 W) is located 10 m downstream. Five meters further downstream of BP2 (15 m from the first spring) emerges the second spring, which corresponds to our third sampling point (BP3; 9.993603 N, 83.801677 W). Finally, 5 m downstream from BP3 (20 meters downstream from BP1), our fourth sampling point (BP4; 9.993658 N 83.801716 W) was selected, just a few meters before Bajo las Peñas mixes with a cold stream. Permits for sampling were obtained from the Institutional Commission of Biodiversity of the University of Costa Rica (resolution N° 066) and the owners of the property.

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133 *Sampling and field measurements*

134

135 On 18 August 2016, samples of water (three samples of 1 Liter each) were collected at four points (BP1
136 to BP4; see Fig. 1) along this thermal water (i.e. twelve water samples). For this purpose, sterile flasks
137 were attached to a long rod in order to reach down into the water and avoid contamination. At each
138 sampling point it was possible to take one or two sediment samples, for a total of seven sediment
139 samples across the entire spring. Specifically, two sediment samples were taken from sampling site 1
140 (BP1S1 and BP1S2), two from sites 2 (BP2S1 and BP2S2) and 4 (BP4S1 and BP4S2), whereas a single
141 sediment (BP3S1) was retrieved from point 3. For sediment sampling, masses between 10 and 50 g were
142 placed into sterile 50 mL centrifuge tubes. The sediments were taken at depths not exceeding 15 cm and
143 due to the presence of the microbial mat, the sediment samples contain part of it. In the field, temperature
144 was measured with a partial immersion thermometer. To measure dissolved oxygen (DO), bottles (1 L)
145 were filled with water from each sampling point, hermetically sealed and cooled to room temperature (~22
146 °C). At that temperature the dissolved oxygen was measured with a dissolved oxygen meter Model 550A
147 (Yellow Springs Instrument Company Inc, Ohio, USA). pH was measured with a pH meter (Scholar 425
148 pH meter, Corning, Inc., Corning, NY) in the laboratory. Water samples for chemical analysis were
149 collected in clean glass bottles, chilled on ice, and stored at 4 °C until analysis. Water samples for
150 microbial community analysis were collected in clean and sterile bottles, stored at 4 °C and processed
151 within 24 h.

152

153 *Chemical Analysis*

154

155 For chemical analyses, water samples were filtered through polycarbonate membrane filters (0.45 µm;
156 Sartorius 23006-47N) before analysis. Major anionic components (Cl⁻, F⁻, NO₃⁻, SO₄²⁻) in the water
157 samples were analyzed by ion-exchange chromatography (IC, MIC-II, Metrohm Co., Switzerland) using
158 an anionic exchange resin (Metrosep A Supp 5 - 100/4.0). Operating conditions were a mobile phase at
159 33 °C, Na₂CO₃ (3.2 mM) / NaHCO₃ (1.0 mM) and flow rate 0.7 mL/min. The anions were identified and
160 quantified relative to certified commercial standards (Certipur ®Anion multi-element standard I, II, Merck,
161 Germany). On the other hand, elemental components (Al, As, Cd, Ca, Cu, Cr, Fe, Mg, Mn, Ni, Pb, K, Na)
162 were analyzed by inductively-coupled-plasma mass spectrometry (ICP-MS, Agilent 7500 instrument,
163 Agilent Technologies, Tokyo, Japan) using a certified multi-element stock solution (Perkin-Elmer Pure

164 Plus standard, product number 9300233). All determinations were made with three independent samples
165 and the reported results correspond to their average. The uncertainty ($\pm$) was calculated using the
166 standard deviation (s) of the three repetitions (n = 3) with a coverage factor K = 2.

167

168 *X-Ray Diffraction (XRD) analysis*

169

170 Powder X-ray diffraction patterns were recorded with a D8 Advance diffractometer (Bruker AXS, Madison,
171 USA, LynxEye detector) with Cu radiation (λ = 0.154 nm) from 2θ 15° to 80° and step size 0.018°.
172 Identification was made with ICDD PDF-2 2007 Database [ICDD 2007] and comparison with published
173 ferrihydrite diffraction patterns.

174

175 *Scanning Electron Microscope and Electron Dispersive Spectrometer (SEM-EDS).*

176

177 The chemical elements that constitute the reddish mat and sediments were determined with SEM-EDS.
178 For that purpose, samples were analyzed with a scanning electron microscope (Hitachi S-570) with
179 energy-dispersive X-ray spectra (SEM-EDS).

180

181 *Total DNA isolation, construction of 16S rRNA gene libraries and Illumina sequencing*

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183 The three water samples from each sampling point (3 x 1L each) were pooled and filtered through a vac-
184 uum system under sterile conditions using a membrane filter (pore size 0.22 μ m; Millipore, GV CAT No
185 GVWP04700). To prevent rupture, another filter membrane (pore size 0.45 μ m; Phenex, Nylon Part No
186 AF0-0504) was placed below. The upper filter was collected and stored at -80 °C until processing. The
187 DNA was extracted from aseptically cut pieces of the filter with a DNA isolation kit (PowerSoil®, MoBio,
188 Carlsbad, CA, USA) as described by the manufacturer. Cell lysis was accomplished by two steps of bead
189 beating (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA) for 30 s at 5.5 m s⁻¹. To process the sedi-
190 ments, a homogeneous sample of 500 mg was collected and DNA was extracted using the same proto-
191 col. For the construction of microbial 16S rRNA amplicon libraries, the V5-V6 hypervariable regions were
192 PCR-amplified with universal primers 807F (5'-GGATTAGATACCCBRGTAGTC-3') and 1050R (5'-
193 AGYTGDCGACRRCCRTGCA-3') (Bohorquez et al. 2012). The barcoding of the DNA amplicons and the
194 addition of Illumina adaptors were conducted by PCR from approximately 1-10 ng of total DNA as de-
195 scribed previously (Burbach et al. 2016). The PCR-amplified amplicons were verified by 1% agarose gel

196 electrophoresis, purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and quantified
197 with the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, Darmstadt, Germany). The individual
198 amplicons were pooled to equimolar ratios (200 ng of each sample) and purified with the QIAquick PCR
199 Purification Kit (Qiagen, Hilden, Germany). Finally, PCR-generated amplicon libraries were subjected to
200 250 nt paired-end sequencing on a MiSeq platform (Illumina, San Diego, CA, USA).

201

202 *Bioinformatic and phylogenetic analysis of 16S rDNA amplicon data*

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204 The sequence data were deposited in the sequence-read archive (SRA) of GenBank under the BioProject
205 PRJNA678689. Bioinformatic processing was performed as previously described (Schulz et al. 2018).
206 Raw reads were merged with the Ribosomal Database Project (RDP) assembler (Cole et al. 2014).
207 Sequences were aligned within MOTHUR (gtoth algorithm using the SILVA reference database; (Schloss
208 et al. 2009) and subjected to preclustering (diffs=2) yielding the so-called operational taxonomic units
209 (OTUs) that were filtered for an average abundance of $\geq 0.001\%$ and a sequence length ≥ 250 bp before
210 analysis. OTUs were taxonomically classified based on the SILVA v138 database with the RDP taxonomy
211 (Yilmaz et al. 2014) as reported by the SINA classification tool (Pruesse et al. 2012). OTUs were
212 assigned to a taxonomic rank only if their best hit in the SILVA database (Pruesse et al. 2007) had an
213 identity higher than the threshold established by Yarza et al. (2014) for that rank (94.5% for genus, 86.5%
214 for family, 82.0% for order, 78.5% for class and 75.0% for phylum). Moreover, the sequences of some
215 highly abundant OTUs were also manually examined by means of BLASTN (Altschul et al. 1997) against
216 the non-redundant and against the bacterial and archaeal 16S rRNA databases. Sequences that weren't
217 assigned to at least a phylum were excluded for further analysis. The statistical analyses and their
218 visualization were performed with the R statistical program (R-Core-Team 2019) and Rstudio interface.
219 Package Vegan v2.5-6 (Oksanen et al. 2019) was used to calculate alpha diversity estimators (Observed
220 richness, Shannon index, Simpson index). Data tables with the OTUs abundances were normalized into
221 relative abundances and then converted into a Bray–Curtis similarity matrix. The effects of factors on the
222 bacterial community composition were evaluated with non-parametric permutational analysis of variance
223 (PERMANOVA) (adonis2 function with 999 permutations) and using multivariate analysis such as
224 Principal Coordinate Analysis (PcoA). Significant differences between a priori predefined groups of
225 samples were evaluated using Permutational Multivariate Analysis of Variance (PERMANOVA), allowing
226 for type III (partial) sums of squares, fixed effects sum to zero for mixed terms. Monte Carlo p-values

(p_{MC}) were generated using unrestricted permutation of raw data (Anderson 2001) IN PRIMER (v.7.0.11, PRIMER-E, Plymouth Marine Laboratory, UK). We used the Kruskal Wallis test to determine significant differences in alpha diversity estimators (Simpson and Shannon-index).

Results and Discussion

Chemical Analysis

The major physicochemical properties of Bajo las Peñas are shown in Table 1. This habitat corresponds to a hot spring with moderately high temperature, ranging from 62.0-68.0°C, and pH nearly neutral, ranging from 6.5 to 7.4. Despite the small differences in pH and temperature throughout the site, we observed small but consistent differences between BP1 and BP3 (the two sources) and the stream sites BP2 and BP4 being the sources slightly more acidic and hotter. The oxygen levels (2.01-2.53 mg O₂/L measured at 22°C) in all the samples indicate that Bajo las Peñas corresponds to a dysoxic environment. (Spietz et al. 2015). Physicochemical analysis of water samples showed the presence of moderate levels of sulfate (243-284 mg/L), magnesium, manganese, sodium, aluminum, calcium, iron and potassium. Our data (samples taken in 2016) agree with the chemical composition reported by Uribe-Lorio et al (2019) of samples taken in 2012, suggesting that the inorganic chemistry of Bajo Las Peñas has remained relatively stable. Examination of the dried sediment samples revealed considerable heterogeneity and organic matter content. Even a few *ca.* 0.2 mm sized magnetite and sand grains were present and were removed prior to the X-Ray measurements. This heterogeneity is expected as the sediments do not consist of a pure mineral. EDS data showed sediments to be iron-rich though extremely variable (50-70% Fe) and we assign them to ferric hydroxide precipitates. The samples were very amorphous, required several hours of Powder X-Ray data collection and only one did diffract strongly enough to identify a mineral phase. The XRD pattern (Fig. S1) of the reddish sediment sample from site BP1 was assigned to 6-line ferrihydrite rather than the less crystalline 2-line ferrihydrite. The spacings for the diffraction peaks at 2.53, 2.22, 1.96, 1.72 1.50 and 1.46 Å compare very well to the literature (Fig. S1; Drits et al. 1993). Only an unassigned peak at 2.44 Å remained. Interestingly, the conversion from 2-line ferrihydrite into 6-line ferrihydrite has been reported to occur under biotic conditions (Kukkadapu et al. 2003). Ferrihydrite is normally very amorphous and the presence of organic matter made collecting Powder X-Ray data difficult. We could not obtain a diffraction pattern from other sediment samples, and we include as an example the essentially absent diffraction of the sample from the BP2 site as reference (Fig. S1). Additionally, previous reports of similar environments indicate that the mixture of the sediments and the

microbial mat with the water flow produces the characteristic red color of these habitats (Casanova et al. 1999; Bishop and Murad 2002; Naren et al. 2012), as is the case in Bajo las Peñas (see Fig. 2).

In spite that Bajo las Peñas is located close to other acidic rivers (e.g. Rio Sucio and Toro Amarillo rivers) likely associated with the area's sulfate and chloride-rich hydrothermal water, Bajo las Peñas physicochemical properties are particularly different: it is endowed with moderate sulfate concentrations in the water column, low chloride, neutral pH and precipitation of ferrihydrite rather than ferric-oxide sulfate minerals. All these observations suggest that the sulfate source in Bajo las Peñas is different from Acid Rock Drainage (ARD) and Volcanic influenced Acid Rock Drainage (VARD) environments. We have described VARD ecosystems associated with nearby Irazu Volcano's hydrothermal system (Arce-Rodríguez et al. 2020) in which the sulfate and the acidity come from the volcano hydrothermal system rather than from biological oxidation of pyrite as in ARD systems (Amils et al. 2002; Arce-Rodríguez et al. 2017, 2019). However, they do not seem to influence Bajo las Peñas and instead, the likely sources of sulfate are anhydrite/gypsum dissolution perhaps aided by some acid rain as the area is downwind from active Turrialba Volcano (Porowski et al. 2019). The hydrothermal system at Bajo las Peñas contributes therefore mostly heat and ferrous iron to the aquifer rather than acid-forming magmatic gases.

Analysis of microbial communities

We determined the presence of 916 OTUs in all samples, which were taxonomically assigned to 35 phyla of Bacteria and Archaea (Supplementary Table 1). The most-abundant phyla were Actinobacteria (0.8-40.0%), Proteobacteria (4.9-39.7%), Chloroflexi (0.8-29.1%), Nitrospirae (2.6-28.6%), Firmicutes (0.4-25.9%), Thaumarchaeota (0.1-25.8%), Crenarchaeota (0.1-24.0%), Deinococcus-Thermus (0.4-19.6%), Aquificae (0-16.0%), Ignavibacteriae (0-14.0%), Acetothermia (0-5.6%), Dictyoglomi (0-5.1%), Acidobacteria (0.8-4.7%), Cyanobacteria (0-4.4%), and Planctomycetes (0-4.0%). Nonetheless, we observed large variations across samples (Fig. 3).

Comparisons between the global bacterial assemblages in the different sites showed the presence of three well-defined clusters: (i) water springs (BP1, BP3), (ii) sediments in the springs (BP1S1, BPS2, BP1S3), (iii) waters and sediments downstream from the springs (BP2, BP2S1, BP2S2, BP4, BP4S1, BP4S2) (Fig. 4) (PERMANOVA $p = 0.001$). All groups of samples were significantly different from one another as indicated by pairwise tests (source water vs downstream samples $p_{MC} = 0.05$, source sediments vs downstream samples, $p_{MC} = 0.01$ and source water vs source sediment, $p_{MC} = 0.04$). This clustering pattern indicates a fast community turnover right after communities emerge from underground

294 to the surface, possibly in response to new environmental conditions such as increase in oxygen and
295 carbon input.

296

297 Alpha diversity estimations (Fig. S2) showed that all samples present a Shannon index > 2.8. Kruskal-
298 Wallis test showed no significant differences in the diversity indices across the clusters described.
299 Previous analyses of the microbial mats in Bajo las Peñas (Uribe-Lorío et al. 2019) showed a high
300 abundance of photoautotrophic prokaryotes, where almost the entire the community was dominated by
301 few OTUs. However, our analyses, including samples in the water column and sediments showed that
302 this environment holds a very variable microbial community (See Figs 3 and 4). The high dynamism that
303 exists in this extreme environment, where the community fluctuates according to minor changes in the
304 water conditions and external factors, such as the climate (Ferris and Ward 1997; Fraterrigo et al. 2006;
305 Li et al. 2014; Narayan et al. 2018) is also a factor to consider when comparing results. Interestingly, the
306 absence of iron-oxidizing bacteria (see below) can be related to the neutral pH which favors its abiotic
307 oxidation, thus reducing the availability of electron donors for this type of bacteria (Earhart 2009; Johnson
308 et al. 2012). This is one of the clearest differences in Bajo las Peñas compared to other hot springs that
309 we have characterized in Costa Rica (Arce-Rodríguez et al. 2019, 2020), whose physicochemical
310 characteristics are typical of ARD or VARD environments (with acidic pH), where the presence of iron
311 oxidizers is very common.

312

313 The most abundant microorganisms in the origins of Bajo las Peñas (water samples BP1 and BP3) were
314 phylotypes from the genus *Sulfurihydrogenibium*, particularly OTU_439 (10.3-14.8%) (Fig. 5) and some
315 putative members of the family Nitrospiraceae (OTU_67, 10.0-26.8%), which closest relatives were
316 bacteria of the genus *Thermodesulfovibrio* (92.2% identity). The genus *Sulfurihydrogenibium* has to our
317 knowledge five reported species: *S. azorensis* (Aguilar et al. 2004), *S. kristjanssonii* (Flores et al. 2008), *S.*
318 *rodmanii* (O'Neill et al. 2008), *S. subterraneum* (Takai et al. 2003), and *S. yellowstonense* (Nakagawa et
319 al. 2005). These microorganisms are reported as aerobic, thermophilic, and sulfur-oxidizing bacteria.
320 These bacteria isolated from hot springs grow at temperatures between 40-80°C and can use S⁰,
321 thiosulfate and sulfite as electron donors. Some species from this genus have reported the ability to use
322 CO₂ as carbon source. Moreover, these microorganisms are known to be important organic carbon
323 producers in the environments where they were isolated (Nunoura 2014). Thus, we suggest that
324 members of the genus *Sulfurihydrogenibium* could act as CO₂-fixers in Bajo Las Peñas.

325

326 On the other hand, the relatively low sequence identity of OTU_67 with members of the genus
327 *Thermodesulfovibrio*, does not allow us to reliably affirm that they belong to this genus. However,

328 considering that (i) they belong to the family Nitrospiraceae, (ii) the moderate levels of available sulfate,
329 and (iii) the temperature in Bajo las Peñas, presumably members of OTU_67 could participate in sulfate
330 reduction in a similar way as species of *Thermodesulfovibrio* genus do. Bacteria from the
331 *Thermodesulfovibrio* genus have typically been isolated from hot springs and are generally known as
332 anaerobic sulfate reducers that grow at temperatures between 40-70°C. *T. yellowstonii* has the ability to
333 use sulfate, thiosulfate, sulfite and nitrate as terminal electron acceptors and oxidizes organic compounds
334 like pyruvate and lactate to acetate. Autotrophic growth using hydrogen have also been reported in
335 members of this genus (Daims 2014).

336

337 In the sediments taken from Bajo las Peñas sources it was possible to find the same OTU from the genus
338 *Sulfurihydrogenibium* (OTU_439, 1.6-5.2%) and the OTU_67 (2.2-2.8%) found mostly in the waters.
339 However, many other OTUs closely affiliated to *Thermoflexus* (OTU_320, 1.9-2.9%) and *Thermus*
340 (OTU_5, 8.9-18.9%), to the families Dictyoglomaceae, Thermomicrobiaceae and Aquificaceae
341 respectively, (OTU_480, 1.9-5.1%; OTU_492, 2.7-3.8% and OTU_64, 2.5-4.6 %) or to the Clostridia
342 (OTU_128, 7.5-18.9%) were also detected with a high abundance (Figure 5).

343

344 Bacteria from the genera *Thermoflexus* and *Thermus* have been reported to be chemoheterotrophic
345 microorganisms, that may consume byproducts of other microorganism or substrates deposited in the
346 sediments of Bajo las Peñas. Bacteria of the phylum Chloroflexi were also detected in some of these
347 sediment samples (6.1-10.6%) (Fig. 5), which is in accordance with a report by Uribe-Lorio et al. (2019)
348 on the microbial mat. However, our data suggest that in Bajo las Peñas the first level of the trophic chain
349 is not only associated with photosynthesis by Cyanobacteria and Chloroflexi but also by CO₂ fixation
350 linked to sulfur metabolism by autotrophic bacteria which derives probably from the subdeposits of water
351 in Bajo las Peñas.

352

353 A few meters downstream of the origin (BP2 and BP4) the organics produced in the first trophic level
354 (described above) as well as the organic matter input may generate the conditions necessary for
355 thermotolerant chemoheterotrophic bacteria to colonize the place. This would a drastic shift in the
356 microbial community structure with respect to the samples obtained at the origin, although spatially the
357 samples are separated by only 5-20 meters. In the water samples BP2 and BP4, bacteria of the family
358 Nitrososphaera (8.2-25.8%), Ignavibacteriaceae (7.3-7.9%) and of the genus *Nitrospira* (6.2-9.7%) and
359 *Meiothermus* (5.6-10.6%) were dominating. Most of these phylotypes were also dominating in the
360 sediment samples from sampling points 2 and 4 (see Fig. 5). We propose that many of these
361 microorganisms found downstream the origins (sampling points 2 and 4), use fermentation products

generated by other microorganisms found at the origin (sampling points 1 and 3). For instance, fermentation products like acetate, have been reported to be produced by members of the genera *Thermodesulfobacterium* and *Ignavibacterium* (Lino et al. 2009) which were closely related to the microorganism identified in our samples. These byproducts could be used as a carbon source by some acetate-oxidizing bacteria. These observations suggest a continuous cross-feeding between the different niches of this hot spring.

Besides the possible carbon cross-feeding, a well-characterized relationship between ammonium-oxidizing and nitrite-oxidizing microorganisms may be occurring. Members of the family Nitrososphaera (2.1-25.7%), may oxidize ammonium to nitrite, which is subsequently utilized by nitrite-oxidizing Nitrospira (6.2-14.0%), for growth (Hovanec et al. 1998; Stieglmeier et al. 2014; Daims et al. 2015; Lehtovirta-Morley 2018). Such a nitrification process has been reported in other hot springs and is known to be an essential process for the maintenance of the community (Reigstad et al. 2008). Thus, our data are aligned with the existence of multiple cross-feeding processes, which are probably the product of microbial adaptation to the low abundance of organic carbon producers in the environment.

As mentioned above, Bajo las Peñas hot spring is characterized for having a red-gelatinous microbial tappet (Fig. 1C) which, we propose, is made of a complex bacterial community in association with ferrihydrite minerals. Uribe-Lorio et al. (2019) reported that the microbial mat in Bajo las Peñas is composed mainly by Cyanobacteria (*Limnothrix*, *Leptolyngbya*, *Phormidium* and *Fisherella*) and to a lower extent by *Meiothermus*. We were also able to detect *Meiothermus* which is considered a key microorganism in hot springs due to its ability to form a strong biofilm by specialized structures (Spanevello and Patel 2004; Masurat et al. 2005; Wu et al. 2009; Mori et al. 2012).

Conclusions

This study sheds light on the ecology of microbial communities of a rare type of hot springs with neutral conditions located in Bajo las Peñas, Costa Rica. In this hot spring, abiotic precipitation of ferric oxyhydroxides in an extensive bacterial mat contribute to the red-colored appearance of the habitat. The analyses of 16S rRNA sequences revealed that Bajo las Peñas harbors a very complex environment where the microbial community changes along the water flow from a chemoautotrophic to a chemolithotrophic metabolism. CO₂ fixation occurs through photosynthetic microorganisms (Cyanobacteria and *Chlorella*) but also bacteria that fix CO₂ linked to sulfur metabolism (i.e. *Sulfurihydrogenibium*). In

395 this first autotrophic level, organic molecules are produced that then can be used by heterotrophic
 396 microorganisms such as those closely related to *Thermodesulfovibrio* and *Ignavibacterium*. We propose
 397 that the microbial community in Bajo Las Peñas may have a complex metabolism due to niche
 398 specialization where cross-feeding processes between phylotypes plays an essential role. Our data
 399 demonstrate the quick change in the microbial community of an ecosystem in response to environmental
 400 variables such as organic matter and oxygen availability. In Bajo las Peñas it was possible to observe
 401 how in just ~5 meters (e.g. BP1 vs BP2) the global microbial community and the dominant species
 402 change from autotrophic to heterotrophic microorganisms. This work also helps to understand
 403 extremophile communities present in neutral hot springs, which are considerably less explored compared
 404 to acidic hot springs, and shows that they are an interesting environment for the bioprospecting of
 405 microorganisms and extremozymes with promising biotechnological applications.

406

407 **Acknowledgements**

408

409 We thank Carlos Rodriguez of Centro de Investigación en Contaminación Ambiental (CICA) for the help
 410 in the chemical analysis. We also are grateful to Solange Voysest and Jose Jimenez for help in the
 411 design of some figures.

412

413 **Author contributions**

414

415 AAR, FPS, EL, MC conceived and designed the experiments; AAR, RA, RMA, DRG, PF, GCB performed
 416 the experiments; AAR, FPS, DRG, KR, MC analyzed the data; PF, MC, DHP contributed reagents or
 417 materials or analytical tools; DRG, EL, KR, DHP, MC wrote the paper. All authors reviewed and approved
 418 the final version of the manuscript.

419

420 **Funding**

421

422 This work was supported by the Vice-rectory of Research of Universidad de Costa Rica (VI 809-B6-524),
 423 the Costa Rican Ministry of Science, Technology and Telecommunication (MICITT) and Federal Ministry
 424 of Education and Research (BMBF) (project VolcanZyme contract No FI-255B-17) and by the ERC grant
 425 IPBSL (ERC250350-IPBSL). F.P-S. is supported by the Spanish Economy and Competitiveness Ministry
 426 (MINECO) grant CTM2016-80095-C2-1-R.

427

428 **Compliance with ethical standards**

429

430 **Conflict of interest** The authors declare that there are no conflicts of interest.

431 **Ethical approval** This study does not describe any experimental work related to human.

432

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731

732 **Tables**

733

734 **Table 1. Physical properties and chemical composition of Bajo las Peñas hot spring.**

Property/element/ion	BP-1	BP-2	BP-3	BP-4
Coordinates	9.993492 N 83.801597 W	9.993558 N 83.801639 W	9.993603 N 83.801677 W	9.993658 N 83.801716 W
Distance from the first origin (m)	0	10	15	20
Temperature (°C) / ± 0.1	68.0	65.6	67.0	62.0
pH / ± 0.1	6.5	7.4	6.6	7.3
Dissolved oxygen (measured to 22°C)	2.01	2.05	2.23	2.53
Aluminium ($\mu\text{g L}^{-1}$) ± 3	15	25	13	14
Arsenic ($\mu\text{g L}^{-1}$)	<2	<2	<2	<3
Cadmium ($\mu\text{g L}^{-1}$)	< 0.11	< 0.17	< 0.12	< 0.11
Calcium (mg L^{-1}) ± 2	15	11	20	11
Chloride (mg L^{-1}) ± 1	< 0.20	1	1	1
Cooper (mg L^{-1})	< 0.10	< 0.10	< 0.10	< 0.10
Chromium ($\mu\text{g L}^{-1}$)	< 1.2	< 1.2	< 1.2	< 1.2
Fluoride (mg L^{-1})	<0.09	<0.09	<0.09	<0.09
Iron (mg L^{-1}) / ± 0.06	0.60	0.16	0.16	<0.08
Magnesium (mg L^{-1}) ± 0.7	11.6	12.2	12.6	13.0
Manganese (mg L^{-1}) ± 0.06	0.36	0.36	0.41	0.40
Nickel ($\mu\text{g L}^{-1}$)	< 1	< 1	< 1	< 1
Nitrate (mg L^{-1})	< 0.10	< 0.10	< 0.10	< 0.10
Lead ($\mu\text{g L}^{-1}$)	< 1.2	< 1.2	< 1.2	< 1.2
Potassium (mg L^{-1}) ± 0.3	9.8	10.0	10.6	10.6
Sodium (mg L^{-1}) ± 0.8	38.5	39.3	38.1	38.5
Sulfate (mg L^{-1}) ± 20	264	281	284	243
Zinc ($\mu\text{g L}^{-1}$)	< 25	< 25	< 25	< 25

735

736 **Legends of figures**

737

738 **Fig. 1 Bajo las Peñas hot spring** A) Bajo las Peñas hot spring is located in Cartago Costa Rica near
739 Turrialba Volcano and Toro Amarillo River. B) It is a small lotic hot spring, with two springs (BP1 and BP3)
740 where water emerges from underground deposits. C) The water flow around Bajo las Peñas is very slow
741 and it flows for about 20 meters. D) The entire hot spring is covered by a dense reddish gelatinous
742 microbial mat where ferrihydrite precipitate constantly.

743

744 **Fig. 2 SEM-EDS analysis of Bajo las Peñas sediments.** A) SEM micrographs to 2 μm (15.0 kV 7.6 mm
745 $\times$ 60) of the reddish gelatinous sediments at the bottom of Bajo las Peñas. Micrographs indicate that the
746 sediment consists of lumps of mainly amorphous material of nanometer size. B) EDS analysis of the
747 sediments showed that it is composed of mainly iron and oxygen. Other species such as silicon,
748 phosphate and chlorine were found in the sediment in minor proportions.

749

750 **Fig. 3 Taxonomic composition of Bajo las Peñas microbial communities.** Relative abundance of
751 bacterial and archaeal organisms to phylum. The OTUs were taxonomically classified using RDP
752 database, as described in Materials and Methods. Waters of Bajo las Peñas are termed BP1 to BP4 and
753 sediments are termed BP1S1 to BP4S2.

754

755 **Fig. 4 Principal Coordinate Analysis (PCoA) of the prokaryotic communities in Bajo las Peñas.** A
756 clustering of communities according to the sampling point is shown. The PCoA and the PERMANOVA
757 analyses were performed with the package Vegan.

758

759 **Fig. 5 Heat map of the most abundant OTU in each sample.** The heat map depicts the relative
760 percentage of 16S rRNA gene sequences assigned to each OTU (y axis) across the 11 samples analyzed
761 (x axis) with an abundance higher than 2%.

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