Thermoplasmatales and sulfur-oxidizing bacteria dominate the microbial community at the surface water of a CO_2-rich hydrothermal spring located in Tenorio Volcano National Park, Costa Rica

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Abstract

Here we report the chemical and microbial characterization of the surface water of a CO_2-rich hydrothermal vent known in Costa Rica as Borbollones, located at Tenorio Volcano National Park. The Borbollones showed a temperature surrounding 60 ºC, a pH of 2.4 and the gas released has a composition of ~97% CO_2, ~0.07% H_2S, ~2.3% N_2 and ~0.12% CH_4. Other chemical species such as sulfate and iron were found at high levels with respect to typical fresh water bodies. Analysis by 16S rRNA gene metabarcoding revealed that in Borbollones predominates an archaeon from the order Thermoplasmatales and one bacterium from the genus *Sulfurimonas*. Other sulfur- (genera *Thiomonas*, *Acidithiobacillus*, *Sulfuriferula* and *Sulfuricurvum*) and iron-oxidizing bacteria (genera *Sideroxydans*, *Gallionella*, *Ferrovum*) were identified. Our results show that CO_2-influenced surface water of Borbollones contain microorganisms that are usually found in acid rock drainage environments or sulfur-rich hydrothermal vents. To our knowledge, this is the first microbiological characterization of a CO_2-dominated hydrothermal spring from Central America and expands our understanding of those extreme ecosystems.

Introduction

The analysis of microbial communities in extreme environments aims to understand how organisms have adapted to environmental conditions unfavorable for life. One of these extreme environments are the hydrothermal springs: fissures in the surface of the Earth's crust through which gases emerge. These sites are located in areas with volcanic activity and their waters are usually rich in dissolved minerals. Costa Rica is a country with a large volcanic influence. Its territory is traversed by four mountain ranges, with most volcanoes in the Guanacaste and central volcanic ranges (Castellón et al. 2013, Alvarado, 2011). In the complex basaltic-andesitic volcanic massif of Tenorio

59 (volcanic mountain range of Guanacaste) multiple environmental niches of volcanic origin such as
60 thermal waters, acidic rivers or streams rich in sulfur compounds and silicates can be found
61 (Castellón et al. 2013). One of those niches is located in the tourist visitation area of the Tenorio
62 Volcano National Park, popularly known as the Borbollones or Hervideros (see Fig. 1 and the
63 supplementary video S1) (Alvarado, 2011). The Borbollones show a continuous release of gas
64 specifically in the area of the Rio Roble (Roble river) and a geochemical survey conducted in 2011
65 (Capecchiacci et al., 2015) indicates that the gas is composed of 90-95 % vol/vol CO_2, 0.04-0.08
66 % vol/vol H_2S, 0.037-0.039 % vol/vol CH_4 and 1.85-2.32 % vol/vol N_2.
67
68 Worldwide, several ecosystems have been reported in which CO_2 is naturally released at high
69 levels. At these extreme sites termed mofettes CO_2 migrates from the lithosphere to the surface
70 water (wet mofettes) or the soil (dry mofettes) (Kämpf et al. 2013; Krauze et al. 2017). In addition
71 to the high levels of CO_2 these environments are characterized by the presence of other gases
72 such as H_2S, H_2, and CH_4, similar to what was found in the Borbollones (Oppermann et al. 2010;
73 Beulig et al. 2015; Frerichs et al. 2013). From a microbiological point of view, the vast majority of
74 studies have been conducted in dry mofettes (Oppermann et al. 2010; Frerichs et al. 2013; Sáenz
75 de Miera et al. 2014; Beulig et al. 2015) whereas only few studies are available on wet mofettes
76 (Krauze et al. 2017). These studies have indicated the presence of diverse microbial communities
77 mainly composed of anaerobic, microaerophilic, acidophilic and chemolithotrophic bacteria. For
78 example, Oppermann et al. (2010) described CO_2-utilizing methanogenic archaea,
79 Geobacteraceae and sulfate-reducing bacteria in a dry mofette in Latera Caldera in the Vulsinian
80 volcanic district in central Italy and Beulig et al. (2015) reported a microbial community dominated
81 by methanogens and acidobacteria in a wetland mofette in the Czech Republic. Analysis of the
82 microbial composition along a CO_2 gradient in a dry mofette located in Campo de Calatrava, Spain
83 revealed that the relative abundance of members of the phylum Chloroflexi (genera

Thermogemmatispora, *Ktedonobacter* and *Thermomicrobium*) increased with increasing CO_2 flux, whereas the relative abundance of members of phyla Acidobacteria, Verrucomicrobia and Gemmatiomonadetes decreased (Sáenz de Miera et al. 2014). Also, Frerichs et al. (2013) reported on changes in the microbial community as a function of the spatial distribution along the CO_2 vent in a pastured field in Germany where Geobacteraceae decreased and sulfate-reducing prokaryotes increased in relative abundance in the vent centre. Interestingly, Krauze et al. (2017) described the microbial communities in surface and subsurface water in a wet mofette in the Czech Republic. The authors concluded that microbial communities at the surface water from mofettes of central Europe are in large proportions similar to the deep biosphere of geysers and marine thermal vents, such as black smokers, that is, environments rich in microorganisms involved in the sulfur metabolism.

So far, all the microbiological studies on mofettes have been conducted in Europe or North America. In the current study we investigated in detail the chemistry and microbiological composition of the surface water of the Costa Rican mofette known as Borbollones. Our results show that the surface water of this wet mofette have a chemical and microbial composition similar to acid rock drainage (ARD) environments or sulfur-rich hydrothermal vents, where microorganisms belonging to the sulfur cycle are predominant. These results constitute the first report of microbiota of mofettes in Costa Rica and Central America and contribute to our knowledge on microbial communities inhabiting ecosystems with extraordinary levels of CO_2.

Materials and Methods

Sampling and field measurements

All necessary permits for sampling waters were obtained from the National System of Conservation Areas (SINAC) of the Ministry of Environment and Energy (MINAE) of Costa Rica (Resolution No. 097-2014-ACAT). For chemical analysis, samples of water were collected on October 2015, February 2017 and January 2018, directly in the bubbling zone (N10.70585 W84.99415; video S1 in Supplementary Information). The temperature and pH were measured in the field with a dissolved oxygen meter Model 50B (Yellow Springs Instrument Company Inc, Ohio, USA). Gas samples were collected with a submerged inverted funnel connected to an evacuated glass flask (250 mL, "Giggenbach bottle") containing NaOH solution (50 mL, 4 M), following the methods of Giggenbach (1984). For chemical analysis of cations and anions, surface water samples were collected in clean glass bottles, chilled over ice and stored at 4 ºC until analysis. For analysis of microbial communities, three samples (1 L each) of surface water were collected in 2015 January directly at the bubbling zone, into clean and sterile glass bottles and processed within less than 24 h.

Chemical Analysis

Gas samples were analyzed at OVSICORI-UNA following the methods outlined by de Moor et al. (2013). Head space gases (H_2, He, Ar, O_2, N_2, CH_4, CO) were analyzed on a gas chromatograph (Agilent 7890) equipped with two molecular-sieve columns (30 m, HP). Samples were introduced via a vacuum line with Ar or H_2 as carrier gases. Gases were measured with a thermal-conductivity detector (TCD) on the Ar side of the GC, and via a second TCD and a flame-ionization detector (FID; configured in series with the TCD) on the H_2 side of the system. The liquid portion of the sample (*i.e.* components dissolved in NaOH solution) was analyzed for CO_2 content with an alkalinity titration, and for total sulfur (all H_2S) after oxidation to sulfate (using H_2O_2, followed by 10x

dilution and filtration using cartridges (Dionex OnGuard IIH) to remove hydroxyl groups) on an ion chromatograph (Dionex) at OVSICORI-UNA.

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

Water samples were passed through a vacuum filtration system under sterile conditions using a membrane filter (pore size 0.22 µm; Millipore, GV CAT No GVWP04700). To prevent rupture, another filter membrane (pore size 0.45 µm; Phenex, Nylon Part No AF0-0504) was placed below. The upper filter was collected and stored at -80 °C until processing. The DNA was extracted from aseptically cut pieces of the filter with a DNA isolation kit (PowerSoil®, MoBio, Carlsbad, CA, USA). Cell lysis was accomplished in two steps of 30 s bead beating (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA) at 5.5 m s^{-1}. For the construction of microbial 16S rRNA amplicon libraries, the V5-V6 hypervariable regions were PCR-amplified from a pool of DNA extracted from the 3 samples with universal primers 807F and 1050R (Bohorquez et al. 2012). The barcoding of the DNA amplicons and the addition of Illumina adaptors were conducted with PCR as described previously (Camarinha-Silva et al. 2014; Burbach et al. 2016). The PCR-generated amplicon libraries were subjected to 250 nt paired-end sequencing on an Illumina MiSeq (San Diego, CA, USA).

Bioinformatic and phylogenetic analysis of 16S rRNA gene(s) amplicon data

Raw MiSeq sequences were quality-filtered ((moira.py script with default parameters and the --paired flag; Puente-Sánchez et al. 2016). Filtered sequences were subsequently analyzed (mothur version 1.31.2; Schloss et al. 2009), as recommended by Kozich et al. (2013). Briefly, the sequences were aligned to a combination of silva.archaea and silva.bacteria databases (Quast et

158 al. 2013), screened for chimeras (UCHIME; Edgar et al. 2011) and clustered at 97 % similarity with
159 an average-neighbour algorithm. The most abundant taxa (i.e. OTU with relative abundance > 0.05
160 % of total reads sampled) were retrieved and classified against the Ribosomal Database Project
161 (RDP) reference using the Classify tool (version 4.3.3; Wang et al. 2007). The results from this
162 initial classification were individually verified and curated manually using the RDP Seqmatch tool.
163
164 Additionally, a phylogenetic analysis of the two Thermoplasmatales OTUs was carried out as
165 follows. The 16S rRNA gene sequences from validly described type strains and isolates belonging
166 to phylum Euryarchaeota, as well as some uncultured representatives closely related to our OTU
167 candidates were retrieved using the RDP SeqMatch tool and by blastn against the curated 16S
168 ribosomal RNA sequence database of NCBI. The sequences were aligned by means of the SINA
169 web-based tool (Pruesse et al. 2012) and the alignments were used for the reconstruction of the
170 phylogenetic tree with MEGA7 software (Kumar et al. 2016) and the maximum-likelihood method
171 based on the general time-reversible model. In total, 100 bootstrap replications were calculated to
172 ensure the robustness of the results.
173
174 **PCR amplification of *mcrA*.** The *mcrA* gene was amplified using the degenerated primer pair ML-
175 F (5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′) and ML-R (5′-
176 TTCATTGCRTAGTTWGGRTAGTT -3′) (Luton et al. 2002). Each PCR mixture (50 μL) contained
177 the reaction buffer (GoTaq colorless buffer, 10 μL, 5X), DNA polymerase (GoTaq, 0.25 μL, 5
178 U/mL), deoxynucleoside triphosphate mixture (1 μL, 10 mM), primers ML-F and ML-R (1 μL each,
179 1 μM), and DNA sample (3 μL, 7 ng/μL). Due to the degenerate nature of primers, the polymerase
180 chain reaction (PCR) was performed on a Mastercycler ep Gradient S thermal cycler (Eppendorf,
181 Hamburg, Germany) using a slow ramp in temperature (0.1°C s^{-1}) between the annealing and
182 extension cycles, as described by (Luton et al. 2002). As a positive control, we prepared a mixture

of DNA isolated from methanogenic archaea (including *Methanobrevibacter smithii, Methanocella arvoryzae, Methanococcus vannielii, Methanomicrobium mobile, Methanopyrus kandleri, Methanosaeta concilii, Methanomassiliicoccus luminyensis, Methanothermobacter marburgensis and Methanococcoides burtonii*). A negative control was prepared with all components of the PCR reaction except the DNA sample. PCR products were analyzed on 2 % agarose gels using standard protocols.

Results and discussion

Physico-chemical analysis of the Borbollones hydrothermal vent

The Borbollones hydrothermal vent has a temperature surrounding 60 ºC and a pH of 2.4 (Table 1). This temperature is within the range reported for CO_2-rich springs (Pauwels et al. 1997; Giammanco et al. 2007). The Borbollones presents a lower pH than those reported in similar environments in which values between 3.5-4.7 are characteristic (Beaubien et al. 2008; Rennert et al. 2011). The analysis of the released gas reveals a composition of almost exclusively CO_2 (97 % vol/vol) with smaller levels of H_2S (0.07 % vol/vol), N_2 (2.3 % vol/vol) and CH_4 (0.12 % vol/vol). Despite the small levels of H_2S, its presence at the site is evident and readily identifiable as its odour is perceptible from several metres around the mofette. The composition of the gas obtained here is similar to that reported for samples taken in 2011 which indicates a highly stable chemical composition (Cappecchiacci et al 2015). The chemical analysis of filtered samples (Table 1) revealed the presence of sulfate, chloride and iron at concentrations much higher than those typical of freshwater rivers. The sulfate level was particularly high (0.25 g/L), and characteristic of ARD environments (He et al. 2007; Sánchez-Andrea et al. 2012; Jones et al. 2015; Arce-Rodríguez et al. 2017). Sulfate in these environments is produced by the biotic or abiotic oxidation of sulfur

208 compounds (e.g pyrite or H_2S). The high sulfate levels hence indicate that the low pH of Borbollones
209 is mainly a product of the oxidation of H_2S or sulfides to sulfate. Furthermore, considering that the
210 product of the CO_2 dissolution is carbonic acid (a weak acid), it is very reasonable to speculate that
211 its contribution to the acidification of the environment is minimal with respect to that generated by
212 sulfur oxidation.
213
214 In summary, the physico-chemical data confirm that Borbollones is a wet mofette. The high sulfate
215 concentrations, the presence of iron minerals and the acidity indicate that biochemical processes
216 similar to those in the ARD environments or sulfur-rich hydrothermal vents are present in the
217 Borbollones. The resulting chemistry in this type of environments is usually the product of the
218 metabolic activity of microorganisms (Arce-Rodríguez et al. 2017). From the 16S rRNA gene (s)
219 data and the literature, it is possible to identify the microbial community of the Borbollones as well
220 as infer some of the metabolic activities that explain the chemical composition of the ecosystem.
221 Next, based on 16S rRNA gene(s) data, the microbiota of the Borbollones as well as its putative
222 metabolism will be discussed.
223
224 **Analysis of microbial communities in the surface waters of Borbollones**
225
226 A total of 110,424 read pairs were generated and subjected to pair merging and quality filtering.
227 The remaining 76,769 high-quality sequences were clustered into 128 OTUs belonging to thirteen
228 phyla from both bacteria and archaea were identified (Fig. 2A and Supplementary Table S1). The
229 largest number of sequence reads (72.05 %) was assigned to the phylum Proteobacteria and in
230 particular members of the Betaproteobacteria class (34.13 %) were highly abundant, followed by
231 Epsilon- (20.9 %) and Gammaproteobacteria (13.24 %). A significant portion of the total sequence
232 reads belonged to two phylotypes from phylum Euryarchaeota (21.24 %) of order

233 Thermoplasmatales (unknown family). The remaining bacterial and archaeal phyla were present
234 only in relatively low amounts (>1.5% each, see supplementary table S1). The classification by
235 families (Fig. 2B) reveals that the most abundant was the Helicobacteraceae (~19.46 %), followed
236 by families Acidithiobacillaceae (~8.03 %), Burkholderiales *incertae sedis* (~7.59 %),
237 Gallionellaceae (~4.58 %), Comamonadaceae (~3.15 %), Sulfuricellaceae (~3.08 %) and
238 Desulfovibrionaceae (~1.10 %). The detected microbial community is formed almost exclusively by
239 aerobic, microaerophilic or facultatively anaerobic microorganisms.
240
241 The detection of members of the archaea domain with the present method is known as a previous
242 report of primers used in this work for the amplification of V5-V6 regions of 16S rRNA gene was
243 able to amplify >97% of the total archaeal sequences from the RDP database (version of 2008;
244 Bohorquez et al. 2012). Specifically, we found two Thermoplasmatales sequence types, of which
245 one (OTU RCBor_001) constitutes 17.50 % of the total sequence reads. The second archaeal OTU
246 (OTU RCBor_005) represents 3.74 % of the microbial population (Supplementary Table S1).
247 Members of the order Thermoplasmatales are described as facultatively anaerobic,
248 thermoacidophilic, autotrophic or heterotrophic organisms (Huber and Stetter 2006). Members of
249 this kind of microorganisms are also considered as extreme thermoacidophiles that generally grow
250 at optimum temperatures of > 60 ºC and at pH < 4 (Auernik et al. 2008), conditions which match
251 perfectly with the niche observed in Borbollones (Table 1). The presence of Thermoplasmatales
252 was previously reported in another CO_2-rich environment by Oppermann et al. (2010). Other
253 species of the order Thermoplasmatales have been isolated from hydrothermal springs (DeLong
254 1992; Yasuda et al. 1995; Takai and Horikoshi 1999) and soils or sediments within solfatara fields
255 (Segerer et al. 1988). The archaeal OTUs identified in Borbollones (RCBor_001 and RCBor_005)
256 are phylogenetically related to a group of non-cultivated Thermoplasmatales (see Fig. 3) with the
257 closest isolates being *Thermoplasma acidophilum* (Yasuda et al. 1995), *Thermoplasma volcanium*

(Segerer et al. 1988), *Thermogymnomonas acidicola* (Itoh et al. 2007), *Cuniplasma divulgatum* (Golyshina et al. 2016), and *Picrophilus torridus* (Schleper et al. 1996; Serour and Antranikian 2002). All these strains have been obtained from solfataric hydrothermal areas or acidic streamers containing sulfidic deposits, *i.e.* sulfur-rich environments which may indicate that the Thermoplasmatales identified in Borbollones could be involved in sulfur metabolism. Both oxidation and reduction processes of sulfur compounds have been described in archaea. Specifically, anaerobic elemental sulfur reduction has been reported in microorganisms from Thermoplasmatales order (Barton et al. 2014).

As shown in Figure 3 the archaea identified in this study are only distantly related to methanogenic archaea. In accordance with the absence of sequences of methanogenic archaea in our samples, no amplification of the *mcrA* gene was obtained from DNA isolated from Borbollones samples (Fig. S1). This gene codifies for the α-subunit of methyl coenzyme M reductase, which has been established as a molecular marker for methanogenic archaea (Paul et al. 2012; Jiang et al. 2011) This contrasts most other studies on microbial communities in CO_2-rich environments where methanogenic archaea could usually be identified (Beulig et al. 2015; Oppermann et al. 2010). However, the samples analyzed here originate from surface water where oxic conditions predominate. The chemical analysis of Borbollones (i.e. presence of CO_2 and methane) indicates that at anoxic zones of the mofette (i.e. in the maximum depth or the subsoil) methanogens might in fact be present.

In addition to the identified Thermoplasmatales, the microbial composition of Borbollones showed a community with microorganisms usually found in ARD environments or sulfur-rich hydrothermal vents such as sulfur-, sulfide-, thiosulfate- and iron-oxidizing bacteria. As mentioned above, the most abundant family was Helicobacteraceae (~19.46%), in which is found the second most

283 abundant microorganism in Borbollones, a bacterium of genus *Sulfurimonas* (~13.54%, OTU
284 RCBor_002; see Figure 2B and Supplementary Table S1). *Sulfurimonas* is a bacterial genus
285 known for reducing nitrate and oxidizing sulfur and to date consists of four species (Labrenz et al.
286 2013). This genus is extremely versatile and is considered one of the most important organisms in
287 the sulfur cycle. Microorganisms of this genus can be aerobic (Inagaki et al. 2003) aerotolerant, or
288 facultatively anaerobic (Labrenz et al. 2013). The ecological niches of *Sulfurimonas* vary from
289 deep-sea hydrothermal vents (Zhou et al. 2009; Akerman et al. 2013) to transition zones
290 oxic/anoxic with sulfidic environments (Sievert et al. 2008). For *Sulfurimonas* species, optimal
291 growth occurs chemolithoautotrophically with sulfide, S^0, thiosulfate and H_2 as electron donors, and
292 with nitrate, nitrite and O_2 as electron acceptors, using CO_2 as a carbon source (Labrenz et al.
293 2013). As seen in Table 1, the chemical composition of Borbollones has all the necessary nutrients
294 for an adequate growth of *Sulfurimonas* species (i.e. CO_2, sulfide, nitrate, hydrogen, etc.) so its
295 presence in high proportions in this hydrothermal vent is reasonable. Specifically, the taxonomic
296 breadth of OTU RCBor_002 indicates that it is closely affiliated with *S. denitrificans* (Sievert et al.
297 2008), which was isolated by the first time from the Dutch Wadden Sea (Hoor 1975). After that
298 report, the bacterium has been isolated from several hydrothermal and marine habitats
299 (Reysenbach et al. 2000; Huber et al. 2003; Zhang et al. 2009). Consistently to our results, Krauze
300 et al. (2017) reported *Sulfurimonas* and *Sulfuricurvum* species as the main microorganisms in a
301 CO_2-dominated wet mofette in the Czech Republic. Other sulfur-oxidizing bacteria of genera
302 *Acidithiobacillus* (~9.14%), *Thiomonas* (~6.69%), *Sulfuriferula* (~3.08%), *Sulfuricurvum* (~2.47%)
303 and *Sulfurovum* (~1.06%) were identified. In addition to sulfur-oxidizing bacteria, a small portion of
304 sulfur-reducing bacteria were found in the surface waters of Borbollones. Sulfate-reducing bacteria
305 are strict anaerobes, so the small percentage of bacteria in the family Desulfovibrionaceae (~1.10
306 %) possibly originates in the anoxic zone of Borbollones. Sulfate-reducing bacteria obtain energy
307 by coupling the oxidation of organic compounds or H_2 to the reduction of sulfate, generating

hydrogen sulfide (Camacho 2009). Taking together these results, in the Borbollones hydrothermal vent, microorganisms were identified with the metabolic activities necessary to complete the sulfur cycle: sulfur-oxidizing activity is carried out by Proteobacteria (*Sulfurimonas*, *Sulfuricurvum*) and sulfur-reducing activity could be carried out by thermophilic archaea (i.e. Thermoplasmatales) and *Desulfovibrio* bacteria.

On the other hand, within genus *Acidithiobacillus* (family Acidithiobacillaceae) is found the third most abundant OTU in Borbollones (RCBor_003; ~5.56 %), an *Acidithiobacillus ferrooxidans*-like bacterium (Valdés et al. 2008). This chemolithotrophic bacterium is also capable of oxidizing sulfide to sulfate, coupling this reaction to iron (III) (under anaerobic conditions) or oxygen reduction (under aerobic conditions) (Suzuki et al. 1990; Pronk et al. 1992). The low pH of the Borbollones mainly obtained by sulfur oxidation, also favors the presence of this acidophilic bacterium. We consider that *A. ferrooxidans* is a key bacterium in the Borbollones ecosystem as it participates in both iron and sulfur metabolism, which is consistent with the chemistry of the wet mofette. In addition to Thermoplasmatales and sulfur-oxidizing bacteria, we identified the presence of iron-oxidizing (genera *Sideroxydans*, *Gallionella*, *Ferrovum*), ammonia and nitrite-oxidizing bacteria (e.g. genus *Nitrospira*). Bacteria belonging to these genera are autotrophic iron oxidizers, which catalyse the oxidation of iron(II); their presence is reasonable considering the levels of iron (~2.86 mg/L) measured in the Borbollones.

Conclusion

The hydrothermal vent known as Borbollones, located at Volcano Tenorio National Park, releases gas with ~97 % vol/vol CO_2, and its waters present a chemical composition similar to those found in ARD environments, i.e., low pH (2.4) and large levels of sulfate. 16S rRNA gene(s) analysis

reveals a microbial community dominated by aerobic, microaerophilic or facultatively anaerobic microorganisms which is reasonable consideringthe sample corresponds to surface water. The most abundant species in the Borbollones corresponds to a non-methanogenic archaeon of order Thermoplasmatales. Based on its phylogeny, we hypothesize that this archaeon participates in the sulfur cycle in this hydrothermal vent. The chemical composition is also consistent with the notion that reduced sulfur compounds (e.g. H_2S) are transformed to sulfate by sulfur-oxidizing bacteria belonging to genera *Sulfurimonas*, *Acidithiobacillus*, *Thiomonas*, *Sulfuriferula*, *Sulfuricurvum*, and *Sulfurovum*. These sulfur-oxidizing microorganisms could use CO_2 as a carbon source as well as other organic compounds. In addition, iron-oxidizing bacteria (genera *Sideroxydans*, *Gallionella*, *Ferrovum*) were identified. The results obtained in this study suggest that the microbial community of the surface water in Borbollones are dominated by species involved in sulfur cycling and correspond to a mixture of a low proportion of bacteria which originate from the deep subsurface (e.g. *Desulfovibrio*) and a high proportion of species from the surface (most of the identified microorganisms). The continuous vertical flow of CO_2 and the movement that is generated in the water interconnect the microbial communities in the oxic and anoxic zone. We did not have evidence of the presence of methanogenic microorganisms, which can be explained by the microaerophilic or aerobic environment where the samples were taken (surface water). This report provides the first description of the physicochemical and microbiological composition of a Costa Rican and Central American wet mofette. These findings increase our knowledge of microbial communities that thrive in these extreme environments.

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Compliance with ethical standards

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

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

Author Contributions

AA-R, FP-S, MC conceived and designed the experiments. AA-R, RA, MM-C, MdM performed the experiments. AA-R, FP-S, MC analyzed the data. DHP, MC contributed reagents or materials or analysis tools. AA-R, FP-S, DHP, MC wrote the paper. All authors reviewed and approved the final version of the manuscript.

References

Akerman NH, Butterfield DA, Huber JA (2013) Phylogenetic diversity and functional gene patterns of sulfur-oxidizing subseafloor Epsilonproteobacteria in diffuse hydrothermal vent fluids. Front Microbiol 4:1–14. doi:10.3389/fmicb.2013.00185

Arce-Rodríguez A, Puente-Sánchez F, Avendaño R, et al (2017) Pristine but metal-rich Río Sucio (Dirty River) is dominated by *Gallionella* and other iron-sulfur oxidizing microbes. Extremophiles 21:235–243. doi:10.1007/s00792-016-0898-7

Auernik KS, Cooper CR, Kelly RM (2008) life in hot acid: Pathway analyses in extremely thermoacidophilic archaea. Curr Opin Biotechnol 19:445-453. doi:10.1016/j.copbio.2008.08.001

Barton LL, Fardeau ML, Fauque GD (2014) Hydrogen sulfide: a toxic gas produced by dissimilatory sulfate and sulfur reduction and consumed by microbial oxidation. Met Ions Life Sci 14:237-277. doi:10.1007/978-94-017-9269-1_10

Beulig F, Heuer VB, Akob DM, et al (2015) Carbon flow from volcanic CO_2 into soil microbial communities of a wetland mofette. ISME J 9:746–759. doi:10.1038/ismej.2014.148

Bohorquez LC, Delgado-Serrano L, López G, et al (2012) In-depth characterization via complementing culture-independent approaches of the microbial community in an acidic hot spring of the Colombian Andes. Microb Ecol 63:103–115. doi:10.1007/s00248-011-9943-3

Brock TD, Gustafson J (1976) Ferric iron reduction by sulfur- and iron-oxidizing bacteria. Appl Environ Microbiol 32:567–571

Burbach K, Seifert J, Pieper DH, Camarinha-Silva A (2016) Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. Microbiologyopen 5: 70-82. doi:10.1002/mbo3.312

Camacho A (2009) Sulfur bacteria. Encyclopedia of Inland waters. Academic Press, 261-278.

doi:10.1016/B978-012370626-3.00128-9

Camarinha-Silva A, Jáuregui R, Chaves-Moreno D, et al (2014) Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environ Microbiol 16:2939–2952. doi:10.1111/1462-2920.12362.

Capecchiacci F, Tassi F, Liegler A, Fentrees S, Deering C, Vaselli O, Martínez M, Taylor-Castillo W (2015) Geochemistry of water and gas discharges from the Tenorio volcanic system (Costa Rica). Book of abstracts Conference: Il Pianeta Dinamico: sviluppi e prospettive a 100 anni da Wegener. Firenze 2-4 Settembre 2015.

Castellón E, Martínez M, Madrigal-Carballo S, et al (2013) Scattering of light by colloidal aluminosilicate particles produces the unusual sky-blue color of Río Celeste (Tenorio Volcano Complex, Costa Rica). PLoS One 8: e75165. doi: 10.1371/journal.pone.0075165

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685–5689. doi: 10.1073/pnas.89.12.5685

Denman SE, Tomkins NW, McSweeney CS (2007) Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol Ecol 62:313–322. doi: 10.1111/j.1574-6941.2007.00394.x

de Moor, J.M., Fischer, T.P., Sharp, Z.D., Hilton, D.R., Barry, P.H., Mangasini, F. and Ramirez, C., 2013. Gas chemistry and nitrogen isotope compositions of cold mantle gases from Rungwe Volcanic Province, southern Tanzania. Chem Geol 339:30-42. doi:10.1016/j.chemgeo.2012.08.004

Edgar RC, Haas BJ, Clemente JC, et al (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200. doi:10.1093/bioinformatics/btr381

Falteisek L, Čepička I (2012) Microbiology of diverse acidic and non-acidic microhabitats within a sulfidic ore mine. Extremophiles 16:911–922. doi: 10.1007/s00792-012-0488-2

Frerichs J, Oppermann BI, Gwosdz S, et al (2013) Microbial community changes at a terrestrial volcanic CO_2 vent induced by soil acidification and anaerobic microhabitats within the soil column. FEMS Microbiol Ecol 84:60–74. doi:10.1111/1574-6941.12040

Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of Crenarchaeota and Euryarchaeota in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl Environ Microbiol 67:4880–4890. doi:10.1128/AEM.67.10.4880–4890.2001

Giammanco S, Parello F, Gambardella B, et al (2007) Focused and diffuse effluxes of CO_2 from mud volcanoes and mofettes south of Mt. Etna (Italy). J Volcanol Geotherm Res 165:46–63. doi:10.1016/j.jvolgeores.2007.04.010

Giggenbach, WF, Gougel, R. (1989) Method for the collection and analysis of geothermal and volcanic water and gas samples. Chem Div Report No 2387, New Zealand DSIR.

Golyshina OV, Lünsdorf H, Kublanov IV et al (2016) The novel extremely acidophilic, cell-wall-deficient archaeon *Cuniculiplasma divulgatum* gen. nov., sp. nov. represents a new family, *Cuniculiplasmataceae* fam. nov., of the order T*hermoplasmatales*. Int J Syst Evol Microbiol 66:332-340. doi:10.1099/ijsem.0.000725

Hara K, Shinzato N, Seo M, et al (2002) Phylogenetic analysis of symbiotic archaea living in the gut of xylophagous cockroaches. Microbes Environ 17:185–190. doi:10.1264/jsme2.17.185

He Z, Xiao S, Xie X, et al (2007) Molecular diversity of microbial community in acid mine drainages of Yunfu sulfide mine. Extremophiles 11:305–314. doi:10.1007/s00792-006-0044-z

Hoor AT Ten (1975) A new type of thiosulphate oxidizing, nitrate reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Netherlands J Sea Res 9:344–350. doi:10.1016/0077-7579(75)90008-3

Huber H, Stetter KO (2006) Thermoplasmatales. In: The Prokaryotes, eds Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E. Springer New York, New York, NY, 101–112

457 Huber JA, Butter DA, Baross JA (2003) Bacterial diversity in a subsea floor habitat following a
458 deep-sea volcanic eruption. FEMS Microbiol Ecol 43:393-409. doi:10.1111/j.1574-
459 6941.2003.tb01080.x.
460 Inagaki F, Takai K, Kobayashi H et al (2003) Sulfurimonas *autotrophica* gen. nov., sp. nov., a novel
461 sulfur-oxidizing ε-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa
462 Trough. Int J Syst Evol Microbiol 53:1801-1805. doi:10.1099/ijs.0.02682-0
463 Itoh T, Yoshikawa N, Takashina T (2007) *Thermogymnomonas acidicola* gen. nov., sp. nov., a
464 novel thermoacidophilic, cell wall-less archaeon in order *Thermoplasmatales*, isolated from a
465 solfataric soil in Hakone, Japan. Int J Syst Evol Microbiol 57:2557–2561.
466 doi:10.1099/ijs.0.65203-0
467 Jiang L, Zheng Y, Chen J et al (2011) Stratification of archaeal communities in shallow sediments
468 of the Pearl River Estuary, Southern China. Antonie van Leeuwenhoek 99:739-751.
469 doi:10.1007/s10482-011-9548-3
470 Jones DS, Kohl C, Grettenberger C, et al (2015) Geochemical niches of iron-oxidizing acidophiles
471 in acidic coal mine drainage. Appl Environ Microbiol 81:1242–1250. doi:10.1128/AEM.02919-
472 14
473 Kämpf H, Bräuer K, Schumann J, et al (2013) CO_2 discharge in an active, non-volcanic continental
474 rift area (Czech Republic): Characterisation ($\delta^{13}C$,$^{3}He/^{4}He$) and quantification of diffuse and
475 vent CO_2 emissions. Chem Geol 339:71–83. doi:10.1016/j.chemgeo.2012.08.005
476 Kozich JJ, Westcott SL, Baxter NT, et al (2013) Development of a dual-index sequencing strategy
477 and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing
478 platform. Appl Environ Microbiol 79:5112–5120. doi:10.1128/AEM.01043-13
479 Krauze P, Kämpf H, Horn F, et al (2017) Microbiological and geochemical survey of CO_2-dominated
480 mofette and mineral waters of the Cheb Basin, Czech Republic. Front Microbiol 8:2446.
481 doi:10.3389/fmicb.2017.02446

482 Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version
483 7.0 for bigger datasets. Mol Biol Evol 33:1870-1874. doi: 10.1093/molbev/msw054
484 Labrenz M, Grote J, Mammitzsch K et al (2013) *Sulfurimonas gotlandica* sp. nov., a
485 chemoautotrophic and psychrotolerant epsilonproteobacterium isolated from a pelagic
486 redoxcline, and an emended description of the genus *Sulfurimonas*. Int J Syst Evol Microbiol
487 63:4141-4148. doi:10.1099/ijs.0.048827-0
488 Li CL, Liu DL, Jiang YT, et al (2009) Prevalence and molecular diversity of archaea in subgingival
489 pockets of periodontitis patients. Oral Microbiol Immunol 24:343–346. doi:10.1111/j.1399-
490 302X.2009.00514.x
491 Luton PE, Wayne JM, Sharp RJ, Riley PW (2002) The *mcrA* gene as an alternative to 16S rRNA
492 in the phylogenetic analysis of methanogen populations in landfill. Microbiology 148:3521-
493 3530. doi:10.1099/00221287-148-11-3521
494 Mihajlovski A, Doré J, Levenez F, et al (2010) Molecular evaluation of the human gut methanogenic
495 archaeal microbiota reveals an age-associated increase of the diversity. Environ Microbiol
496 Rep 2:272–280. doi:10.1111/j.1758-2229.2009.00116.x
497 Miyata R, Noda N, Tamaki H, Kinjyo K (2007) Phylogenetic relationship of symbiotic archaea in the
498 gut of the higher termite *Nasutitermes takasagoensis* fed with various carbon sources.
499 Microbes 22:157–164. doi:10.1264/jsme2.22.157
500 Mora C, Frazier AG, Longman RJ, et al (2013) The projected timing of climate departure from
501 recent variability. Nature 502:183–187. doi:10.1038/nature12540
502 Oppermann BI, Michaelis W, Blumenberg M, et al (2010) Soil microbial community changes as a
503 result of long-term exposure to a natural CO_2 vent. Geochim Cosmochim Acta 74:2697–2716.
504 doi:10.1016/j.gca.2010.02.006
505 Paul K, Nonoh JO, Mikulski L, Brune A (2012) “Methanoplasmatales” Thermoplasmatales-related
506 archaea in termite guts and other environments, are the seventh order of methanogens. Appl

507 Environ Microbiol 78:8245-8253. doi:10.1128/AEM.02193-12
508 Pauwels H, Fouillac C, Goff F, Vuataz FD (1997) The isotopic and chemical composition of CO_2-
509 rich thermal waters in the mont-dore region (Massif-Central, France). Appl Geochemistry
510 12:411–427. doi:10.1016/S0883-2927(97)00010-3
511 Pronk JT, De Bruyn JC, Bos P, Kuenen JG (1992) Anaerobic growth of *Thiobacillus ferrooxidans*.
512 Appl Environ Microbiol 58:2227–2230.
513 Pronk JT, Liem K, Bos P, Kuenen JG (1991) Energy transduction by anaerobic ferric iron respiration
514 in *Thiobacillus ferrooxidans*. Appl Environ Microbiol 57:2063–2068.
515 Pruesse E, Peplies J, Glöckner FO (2012) SINA: Accurate high-throughput multiple sequence
516 alignment of ribosomal RNA genes. Bioinformatics 28:1823–1829.
517 doi:10.1093/bioinformatics/bts252
518 Puente-Sánchez F, Aguirre J, Parro V (2016) A novel conceptual approach to read-filtering in high-
519 throughput amplicon sequencing studies. Nucleic Acids Res 44:e40.
520 doi:10.1093/nar/gkv1113
521 Quast C, Pruesse E, Yilmaz P, et al (2013) The SILVA ribosomal RNA gene database project:
522 Improved data processing and web-based tools. Nucleic Acids Res 41:D590-D596.
523 doi:10.1093/nar/gks1219
524 Reysenbach A, Longnecker K, Kirshtein J (2000) Novel bacterial and archaeal lineages from an in
525 situ growth chamber deployed at a mid-atlantic ridge hydrothermal vent. Appl Environ
526 Microbiol 66:3798–3806. doi:10.1128/AEM.66.9.3798-3806.2000
527 Rillig MC, Hernandez GY, Newton PCD (2000) Arbuscular mycorrhizae respond to elevated
528 atmospheric CO_2 after long-term exposure: evidence from a CO_2 spring in New Zealand
529 supports the resource balance model. Ecol Lett 3:475–478. doi:10.1046/j.1461-
530 0248.2000.00178.x
531 Sáenz de Miera LE, Arroyo P, de Luis Calabuig E, et al (2014) High-throughput sequencing of 16S

RNA genes of soil bacterial communities from a naturally occurring CO_2 gas vent. Int J Greenh Gas Control 29:176–184. doi:10.1016/J.IJGGC.2014.08.014

Sánchez-Andrea I, Rojas-Ojeda P, Amils R, Sanz JL (2012) Screening of anaerobic activities in sediments of an acidic environment: Tinto River. Extremophiles 16:829–839. doi:10.1007/s00792-012-0478-4

Schleper C, Pühler G, Klenk P, Zillig W (1996) *Picrophilus oshimae* and *Picrophilus tomdus* fam. nov., gen. nov., sp. nov., Two species of hyperacidophilic, thermophilic, heterotrophic, aerobic archaea. Int J Syst Evol Microbiol 46:814-816. doi:10.1099/00207713-46-3-814

Schloss PD, Westcott SL, Ryabin T, et al (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. doi:10.1128/AEM.01541-09

Segerer A, Langworthy TA, Stetter KO (1988) *Thermoplasma acidophilum* and *Thermoplasma volcanium* sp. nov. from solfatara fields. Syst Appl Microbiol 10:161–171. doi:10.1016/S0723-2020(88)80031-6

Serour E, Antranikian G (2002) Novel thermoactive glucoamylases from the thermoacidophilic archaea *Thermoplasma acidophilum*, *Picrophilus torridus* and *Picrophilus oshimae*. Antonie van Leeuwenhoek 81:73-83. doi:10.1023/A:1020525525490

Šibanc N, Dumbrell AJ, Mandić-Mulec I, Maček I (2014) Impacts of naturally elevated soil CO_2 concentrations on communities of soil archaea and bacteria. Soil Biol Biochem 68:348–356. doi:10.1016/j.soilbio.2013.10.018

Sievert SM, Scott KM, Klotz MG, et al (2008) Genome of the epsilonproteobacterial chemolithoautotroph *Sulfurimonas denitrificans*. Appl Environ Microbiol 74:1145–1156. doi:10.1128/AEM.01844-07

Suzuki I, Takeuchi TL, Yuthasastrakosol TD, Oh JK (1990) Ferrous iron and sulfur oxidation and ferric iron reduction activities of *Thiobacillus ferrooxidans* are affected by growth on ferrous

557 iron, sulfur, or a sulfide ore. Appl Environ Microbiol 56:1620–1626
558 Takai K, Horikoshi K (1999) Genetic diversity of archaea in deep-sea hydrothermal vent
559 environments. Genetics 152:1285–1297. doi:10.1016/s0723-2020(87)80053-x
560 Tamura K, Stecher G, Peterson D, et al (2013) MEGA6: Molecular evolutionary genetics analysis
561 version 6.0. Mol Biol Evol 30:2725–2729. doi:10.1093/molbev/mst197
562 Valdés J, Pedroso I, Quatrini R, et al (2008) *Acidithiobacillus ferrooxidans* metabolism: From
563 genome sequence to industrial applications. BMC Genomics 9:597. doi:10.1186/1471-2164-
564 9-597
565 Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of
566 rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267.
567 doi:10.1128/AEM.00062-07
568 Wright AG, Williams AJ, Winder B, et al (2004) Molecular diversity of rumen methanogens from
569 sheep in Western Australia. Appl Environ Microbiol 70:1263–1270.
570 doi:10.1128/AEM.70.3.1263
571 Yanagawa K, Morono Y, de Beer D, et al (2013) Metabolically active microbial communities in
572 marine sediment under high-CO_2 and low-pH extremes. ISME J 7:555–567.
573 doi:10.1038/ismej.2012.124
574 Yasuda M, Oyaizu H, Yamagishi A, Oshima T (1995) Morphological variation of new
575 *Thermoplasma acidophilum* isolates from Japanese hot springs. Appl Environ Microbiol
576 61:3482–3485
577 Zhang M, Zhang T, Shao MF, Fang HHP (2009) Autotrophic denitrification in nitrate-induced marine
578 sediment remediation and *Sulfurimonas denitrificans*-like bacteria. Chemosphere 76:677–
579 682. doi:10.1016/j.chemosphere.2009.03.066
580 Zhou H, Li J, Peng X, et al (2009) Microbial diversity of a sulfide black smoker in main endeavour
581 hydrothermal vent field, Juan de Fuca Ridge. J Microbiol 47:235–247. doi:10.1007/s12275-

582 008-0311-z

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584 ## Tables

585

586 **Table 1. Physical properties and chemical composition of the wet mofette Borbollones.**

Property/Component	Value/concentration	Property/element/ion	Property/Component
Temperature /ºC	60.4 ± 0.1	Flouride (mg/L)	2.70 ± 0.06
pH	2.4 ± 0.1	Chloride (mg/L)	175.4 ± 0.2
CO_2 (% vol/vol)	96.9 ± 0.5	Bromide (mg/L)	ND
H_2S (% vol/vol)	0.07 ± 0.02	Sulfate (mg/L)	253 ± 10
CH_4 (% vol/vol)	0.12 ± 0.02	Nitrate (mg/L)	4.99 ± 0.01
N_2 (% vol/vol)	2.3 ± 0.4	Iron (mg/L)	2.86 ± 0.37
O_2 (% vol/vol)	0.54 ± 0.08	Calcium (mg/L)	75 ± 19
H_2 (% vol/vol)	0.0026 ± 0.0015	Sodium (mg/L)	19.2 ± 0.9
Ar (% vol/vol)	0.05 ± 0.01	Potasium (mg/L)	3 ± 1

587

588 ## Legends of figures

589

590 **Fig. 1** The wet mofette known as Borbollones at Tenorio Volcano National Park, Costa Rica.
591 Borbollones is located in Tenorio Volcano National Park (Guanacaste Mountain Range), on the Rio
592 Roble, beside the touristic path of the park. In the mofette the release of gas is observed and a
593 strong odour of sulfur compounds is perceptible.

594

595 **Fig. 2** Taxonomic composition at phylum and family level of the microbial community of the wet
596 mofette Borbollones. The relative abundance of bacteria and archaea is shown **(A)** at the phylum
597 and **(B)** at the family level.
598
599 **Fig. 3** Phylogenetic tree showing relationships of identified Thermplasmatales (OTU RCBor_001
600 and OTU RCBor_005) and closely related members of the Euryarchaeota phylum. The tree was
601 reconstructed using the maximum-likelihood method based on the general time-reversible model
602 as described in Methods. Solid circles (●) indicate bootstrap values ≥90; open circles (○) denote
603 bootstrap values ≥70.
604
605 **Fig. 4** Cartoon of Borbollones representing the main transformations and microorganisms involved.
606 The surface waters of the Borbollones are acidic, with high temperature, rich in iron and sulfate.
607 The bubbling gas is almost exclusively made of CO_2. The microbiota of Borbollones is rich in
608 species involved in sulfur and iron metabolism.
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