

1 Untapped microbial composition along a horizontal oxygen gradient in a Costa Rican 2 volcanic influenced acid rock drainage system

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31 Abstract

32 Based on the analysis of 16S rRNA gene metabarcoding, here we report the shift in the microbial community
33 structure along a horizontal oxygen gradient (0.40-6.06 mg L⁻¹) in a volcanic influenced acid rock drainage (VARD)
34 environment, known as San Cayetano (Cartago, Costa Rica; pH =2.94-3.06, sulfate ~0.87-1.19 g L⁻¹, iron ~35-61
35 mg L⁻¹). This VARD is dominated by microorganisms involved in the geochemical cycling of iron, sulfur and
36 nitrogen; however, the identity of the species changes with the oxygen gradient along the river course. The anoxic
37 spring of San Cayetano is dominated by a putative anaerobic sulfate-reducing Deltaproteobacterium as well as
38 sulfur-oxidizing bacteria (such as *Acidithiobacillus* or *Sulfobacillus*), which favor the process of dissolution of
39 sulfide minerals and oxidation of H₂S. In oxic conditions, aerobic iron-oxidizers (*Leptospirillum*, *Acidithrix*,
40 *Ferritrophicum*, *Ferrovum*) and heterotrophic bacteria (Burkholderiaceae Betaproteobacterium, *Trichococcus*,
41 *Acidoceella*) were identified among others. Thermoplasmatales archaea closely related to environmental phylotypes
42 found in other ARD/AMD niches were also found throughout the entire ecosystem. This work describes the
43 changes in bacterial diversity, and possible metabolic activities occurring along a horizontal oxygen gradient in a
44 volcanic influenced acid rock drainage system.

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46

47 Introduction

48

49 Water bodies with elevated metal content and very high acidity are found throughout the world as the result of
50 volcanic activity and/or biotic/abiotic processes as in acid rock drainage (ARD) sites. In ARD environments, water
51 emerges from underground having a low pH value and remarkable amounts of chemical species such as iron,
52 sulfate and other metals (Baker and Banfield 2003; Johnson *et al.* 2009; Sánchez-Andrea *et al.* 2011; Arce-
53 Rodríguez *et al.* 2019). Most of the studied ARD ecosystems in the world have been caused by human activity,
54 especially mining processes. The anthropogenic influence modifies the geology, hydrology and biology of the
55 ARD, making difficult to pinpoint the inherent chemical and natural microbiological conditions of these sites. It is
56 therefore of great interest to study ARD ecosystems with no history of anthropogenic influence (*i.e.* no mining).
57 Some works on the chemistry and microbiology of natural ARD systems include the Pastoruri Glacier area in
58 Huascarán National Park (Perú) (González-Toril *et al.* 2015), the Antarctic landmass (Dold *et al.* 2013), the
59 Río Sucio (Braulio Carrillo National Park, Costa Rica) (Arce-Rodríguez *et al.* 2017) and the Río Tinto,
60 located at the Iberian Pyrite Belt (IPB) of Spain (González-Toril *et al.* 2003; López-Archilla *et al.* 2004; García-

61 Moyano *et al.* 2012; Sánchez-Andrea *et al.* 2012). In the latter case, although the river and its source are located
62 on a site that has been mined for thousands of years, it is believed by some authors that the conditions of extreme
63 acidity and heavy metal pollution along the river are due to natural processes that were operative before mining in
64 the region started five thousand years ago (Fernández-Remolar, Rodríguez and Gomez 2003; Amils, Fernández-
65 Remolar and the IPBSL Team 2014). Due to its conditions, it is a good consensus that Rio Tinto should be
66 considered a study model of ARD environments. On the other hand, the extreme acidity environments produced
67 by discharge of the products of volcanic or hydrothermal activity (e.g. H₂S oxidation or SO₂ reaction with water)
68 into water bodies like lakes and rivers, share most of their natural composition with that of ARD sites and are of
69 interest because they can be unequivocally assigned to natural causes (Urbieta *et al.* 2015, Arce-Rodríguez *et al.*
70 2017). Thus, low pH values due to sulfuric acid and high metal content, especially iron, are a common factor to
71 both ARD and volcanic influenced water bodies.

72

73 From a microbiological point of view, the common factor in natural ARD and volcanic environments is the
74 presence of iron- and sulfur-oxidizing bacteria. For example, in the Pastoruri Glacier area the presence of
75 *Acidithiobacillus*, a sulfur- and iron-oxidizing acidophilic bacterium, was detected (González-Toril *et al.* 2015). Dold
76 *et al.* (2013) reported the formation of an ARD system in the Antarctic landmass due to the activity of psychrophilic
77 acid mine drainage microorganisms found in cold climates, specifically *Acidithiobacillus ferrivorans* and
78 *Thiobacillus plumbophilus*. In Costa Rica, a country with an important volcanic influence (Castellón *et al.* 2013)
79 and tropical climatic conditions, Arce-Rodríguez *et al.* (2017) reported that Rio Sucio (a pristine ARD), is
80 dominated by *Gallionella* spp. and other chemolithoautotrophic iron- and sulfur-oxidizing bacteria. Studies in Rio
81 Tinto revealed that eighty percent of the water column's bacterial species corresponds to only three bacterial
82 genera: *Leptospirillum*, *Acidithiobacillus* and *Acidiphilium*, all involved in iron cycling (González-Toril *et al.* 2003,
83 2010). Minor levels of other iron-oxidizing bacteria such as *Ferrimicrobium*, *Acidimicrobium* and *Ferroplasma* have
84 also been detected in Rio Tinto.

85

86 In this work, we investigated the microbiological characteristics of the source and early course of an ARD riverine
87 environment that has a contribution of sulfuric acid by volcanic activity as well as associated chemical species
88 product of rock dissolution processes. This volcanic influenced acid rock drainage (VARD), known as San
89 Cayetano Creek is located in a private farm northeast of Irazu volcano (Cartago, Costa Rica) (Fig. 1). Due to its
90 location within a private property and difficult access this site has seen little human influence. This acid stream has

91 a length of about 3 km from its source to its confluence with the previously described Rio Sucio (Arce-Rodriguez *et*
92 *al.* 2017) but is only a minor tributary. Our results indicate that chemistry of San Cayetano is both the result of
93 volcanic input and biological activity of iron- and sulfur-oxidizing bacteria distributed along a well-established
94 oxygen gradient.

95

96 **Materials and Methods**

97

98 **Sampling and field measurements**

99

100 The San Cayetano acid rock drainage system is located in a private farm in Cartago, Costa Rica. All the needed
101 permits for sampling water and sediments were requested directly to the owners of the property. In August 2016,
102 samples of water (three samples of 1 Liter each) were collected at different points along this stream (see Fig. 2):
103 SC-1 (sampling point located at the origin, 10.032308 N 83.864449 W), SC-2 (10 meters from origin), SC-3 (30
104 meters from origin), SC-4 (600 meters from origin) and SC-5 (900 meters from origin). At all sampling sites (except
105 for the rocky source SC-1, where no sediment material was observed) it was possible to collect sediment samples:
106 specifically three from the second sampling point (SC-2S1, SC-2S2, SC-2S3), three from the third sampling point
107 (SC-3S1, SC-3S2, SC-3S3), one from the fourth sampling point (SC-4S1) and four from the fifth sampling point
108 (SC-5S1, SC-5S2, SC-5S3, SC-5S4). The temperature, pH and dissolved oxygen (DO) amounts were measured
109 with a dissolved oxygen meter Model 50B (Yellow Springs Instrument Company Inc, Ohio, USA). Water samples
110 for chemical analysis were collected in clean glass bottles, chilled on ice, and stored at 4 °C until analysis.
111 Samples for analysis of microbial communities were collected in clean and sterile glass bottles, and processed
112 within less than 24 h.

113

114 **Chemical Analysis**

115

116 The water samples were analysed for major anionic and cationic species with an ion-exchange chromatograph
117 (IC, MIC-II, Metrohm Co., Switzerland) and an inductively-coupled-plasma mass spectrometer (ICP-MS, Agilent
118 7500 instrument, Agilent Technologies, Tokyo, Japan). Samples were filtered with polycarbonate membrane filters
119 (0.45 µm) before analysis. The anions were determined with an IC equipped with an anionic exchange resin
120 (Metrosep A Supp 5 - 100/4.0). Operating conditions were a mobile phase at 33 °C, Na₂CO₃ (3.2 mM) / NaHCO₃

121 (1.0 mM) and flow rate 0.7 mL/min. The anions were identified and quantified relative to certified commercial
122 standards (Certipur® Anion multi-element standard I, II, Merck, Germany). For ICP-MS analysis a certified multi-
123 element stock solution (Perkin-Elmer Pure Plus standard, product number 9300233) was used. All determinations
124 were performed in triplicates.

125

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

127

128 The three water samples from each sample point (1 L each) were pooled and filtered through a vacuum system
129 under sterile conditions using a membrane filter (pore size 0.22 µm; Millipore, GV CAT No GVWP04700). To
130 prevent rupture, another filter membrane (pore size 0.45 µm; Phenex, Nylon Part No AF0-0504) was placed
131 below. The upper filter was collected and stored at -80 °C until processing. The DNA was extracted from
132 aseptically cut pieces of the filter with a DNA isolation kit (PowerSoil®, MoBio, Carlsbad, CA, USA) as described
133 by the manufacturer. Cell lysis was accomplished by two steps of bead beating (FastPrep-24, MP Biomedicals,
134 Santa Ana, CA, USA) for 30 s at 5.5 m s⁻¹. To process the sediments, a homogeneous sample of 500 mg was
135 sampled and DNA was extracted using the same protocol. For the construction of microbial 16S rRNA amplicon
136 libraries, the V5-V6 hypervariable regions were PCR-amplified with universal primers 807F and 1050R (Bohorquez
137 *et al.* 2012). The barcoding of the DNA amplicons and the addition of Illumina adaptors were conducted by PCR
138 as described previously (Camarinha-Silva *et al.* 2014). The PCR-generated amplicon libraries were subjected to
139 250 nt paired-end sequencing on a MiSeq platform (Illumina, San Diego, CA, USA).

140

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

142

143 Bioinformatic processing was performed as previously described (Schulz *et al.* 2018). Raw reads were merged
144 with the Ribosomal Database Project (RDP) assembler (Cole *et al.* 2014), obtaining overall 530,413 paired-end
145 reads. Sequences were aligned within MOTHUR (gotoh algorithm using the SILVA reference database; Schloss *et al.*
146 *et al.* 2009) and subjected to preclustering (diffs=2) yielding the so-called operational taxonomic units (OTUs) or
147 phylotypes that were filtered for an average abundance of ≥0.001% and a sequence length ≥250 bp before
148 analysis. OTUs were taxonomically classified into the SILVA v132 taxonomy (Yilmaz *et al.* 2014) as reported by
149 the SINA classification tool (Pruesse *et al.* 2012). OTUs were assigned to a taxonomic rank only if their best hit in
150 the SILVA database (Pruesse *et al.* 2007) had an identity higher than the threshold established by Yarza *et al.*

151 (2014) for that rank (94.5% for genus, 86.5% for family, 82.0% for order, 78.5% for class and 75.0% for phylum).
152 Moreover, the sequences of some highly abundant OTUs were also manually examined by means of BLASTN
153 (Altschul *et al.* 1997) against the non-redundant and against the bacterial and archaeal 16S rRNA databases. The
154 statistical analyses and visualizations were performed in R (R Core Team, 2017). We used Vegan (Oksanen *et al.*
155 2017) to calculate alpha diversity estimators, non-metric multidimensional scaling analyses, (NMDS), and the
156 permutational analysis of variance (Permanova) on normalized tables of OTUs (Supplementary Table S1).

157

158 **Results and Discussion**

159

160 **Physicochemical analysis of San Cayetano stream**

161

162 The pH along the 0.9 km section of the stream studied in San Cayetano (Fig. 1) was acidic (pH of 3) (Table 1) and
163 the temperature decreased slightly when moving away from the origin (~38° C in SC-1 to ~30° C in SC-5; Fig. 2).
164 Moreover, a prominent gradient in the oxygen concentration was observed throughout the VARD: in SC-1
165 conditions were almost anoxic (0.40 mg L⁻¹ O₂ corresponding to an oxygen saturation of 6.6% at the temperature
166 and pressure of the sampling site), while at the last sampling point SC-5 water has become oxic (6.06 mg L⁻¹ O₂;
167 81% O₂ saturation). As shown in Fig. 2, San Cayetano has its origin in a rocky outcrop within the forest where
168 warm underground water emerges. From there, the water flows for about 3 km to its confluence with the Rio Sucio
169 (Arce-Rodríguez *et al.* 2017). Near the source, very clear water with a depth of about 1.3 m flows smoothly over a
170 clean gravel riverbed, but about 20 m downriver there is a 6 m high waterfall where a light-brown microbial mat is
171 formed. From this point on, water flows faster and it is estimated to discharge at a rate of nearly 100 L/s (Baldoni
172 *et al.* unpublished).

173

174 The chemical analysis of filtered samples (Table 1) revealed the presence of sulfate, iron, manganese, aluminium,
175 chromium, zinc, arsenic and magnesium at concentrations much greater than of typical freshwater rivers; the
176 sulfate (~0.87-1.19 g L⁻¹) and iron (~35-61 mg L⁻¹) levels were particularly high, which is characteristic of volcanic
177 and ARD environments (Arce-Rodríguez *et al.* 2019). The low [iron]/[sulfate] ratio and the absence of precipitated
178 iron minerals (see Figs. 2a,b) as it occurs in Rio Sucio with the iron-oxyhydroxysulfate Schwertmannite (Arce-
179 Rodríguez *et al.* 2017), suggests that much of the sulfate is produced from volcanic sources (*i.e.*, from biotic or
180 abiotic H₂S oxidation and SO₂ water reaction) rather than the oxidation of pyrites or other metal sulfides. The

181 moderately high temperature (~38°C) that occurs at the origin of San Cayetano also indicates volcanic influence.
182 These observations suggest that the extreme conditions observed in San Cayetano might be produced by
183 discharge products of volcanic or hydrothermal activity into the water bodies of the river, a phenomenon well
184 described in geology ((Vaselli *et al.* 2010). When gases SO₂ and H₂S from volcanic origin are intercepted by water
185 bodies on their way to the surface, various chemical reactions might occur: For example, when SO₂ is discharged
186 into deep underground water bodies this can produce sulfate and acidic conditions ($3\text{SO}_2 + 3\text{H}_2\text{O} \rightarrow 2\text{SO}_4^{2-} +$
187 $\text{H}_2\text{O} + \text{S}^0 + 4\text{H}^+$) (Kusakabe *et al.* 2000). On the other hand, if present, H₂S may be subjected to biotic or abiotic
188 oxidation to also generate sulfate (e.g. $\text{H}_2\text{S} + 2\text{O}_2 \rightarrow \text{H}_2\text{SO}_4$). In any case, it is clear that the low [iron]/[sulfate]
189 ratio, reflects that there is a sulfate source other than the dissolution and oxidation of minerals such as pyrite, i.e.
190 from volcanic origin. For example, in Río Tinto ARD system the [Fe]/[SO₄²⁻] molar ratio is 0.58 at the origin and
191 only decreases to 0.29 downstream as expected from the 0.5 ratio from oxidation of pyrite FeS₂; however, in San
192 Cayetano stream, we measure far lower ratios between 0.07 at the origin and 0.07-0.12 downstream. Our ratios
193 compare well to the 0.15 and 0.06 ratios at Copahue Volcano's (Urbieta *et al.* 2015).

194

195 Both processes (ARD or volcanic activity) produce acid that favors the dissolution of minerals (e.g. iron oxides,
196 sulfides and aluminosilicate rocks). This correlates well with the acidic pH of San Cayetano (pH 2.94-3.06) and the
197 presence of dissolved metals such as aluminum, zinc, arsenic, manganese or chromium. In summary, the
198 physicochemical parameters suggest that: (i) the selected sampling points describe a gradient of increasing
199 oxygen concentration starting at the origin with almost anoxic conditions and (ii) San Cayetano has the typical
200 chemical composition of an ARD environment but with an important contribution of sulfuric acid due to volcanic
201 processes. For that reason, we have proposed the acronym VARD (volcanic influenced acid rock drainage) to
202 describe this specific type of ARD ecosystem.

203

204 **The concentrations of dissolved oxygen along the river shapes the microbial community of San Cayetano**

205

206 In spite of the acidic conditions and high concentrations of sulfate, iron and toxic metals, a relatively high diversity
207 of microorganisms was observed in the San Cayetano VARD. We identified in total 1904 OTUs (excluding
208 singletons) from 530,310 16S rRNA gene sequences originating from 36 phyla and 177 families from both bacteria
209 and archaea (Fig. 3 and Supplementary Table 1). According to the relative percentage of sequences, the most
210 abundant phylogenetic groups were Gammaproteobacteria (40.2%), Euryarchaeota (10.0%), Actinobacteria
211 (9.1%), Nitrospirae (6.4%) and Firmicutes (6.2%). However, we detected important variations between the

212 different sampling sites (Fig. 3). These results are consistent with the alpha-diversity estimations showing, for
213 example, that 63% of the samples in San Cayetano presented richness values higher than 500 OTUs, and that
214 88% of the samples presented values of the Shannon index greater than 3 (Supplementary Figure S1).

215

216 Despite the high abundance of microbes found in San Cayetano, the taxonomic composition of this VARD showed
217 a remarkable abundance of unclassified microorganisms amongst the most represented OTUs, both in water and
218 sediment samples. This was specially the case for the water samples taken at the first two sampling points, where
219 the dissolved oxygen concentrations do not exceed 0.50 mg L^{-1} . Nevertheless, we could detect the presence of
220 microorganisms commonly found in ARD environments like iron-, sulfur-, sulfide- and thiosulfate-oxidizing
221 bacteria. Furthermore, we determined significant differences in the community structure at the OTU level
222 (Permanova, $P=0.001$) along the oxygen gradient.

223

224 NMDS and Permanova analyses were consistent at showing the role of oxygen availability in shaping the structure
225 of the microbial communities in the VARD ecosystem (Fig. 4). We observed a clear separation of the samples
226 according to the habitat within each oxygen condition. This difference between microbial communities in the water
227 column respect to those in sediments was significant (Permanova, $P=0.018$). The water samples near the origin
228 (SC-1 and SC-2) that contain very little concentrations of oxygen reflect a very similar microbial community
229 amongst each other. The same situation was observed among the sediments obtained in the second sampling
230 point (SC-2S1, SC-2S2 and SC-2S3). As expected, samples from the third sampling point, containing
231 intermediate levels of oxygen, are not grouped with SC-1 and SC-2 (low oxygen) nor with SC-4 and SC-5 (high
232 oxygen). Again the sediments at this point (SC-3S1, SC-3S2 and SC-3S3) are grouped together. Finally, the water
233 samples of points four and five, as well as their respective sediments are clearly grouped. As already mentioned,
234 the last two sampling points present very similar aerobic conditions, so it is expected that the microbial community
235 in these sites will be grouped as shown by the NMDS analysis.

236

237 As mentioned above, in the first sampling point (SC-1), which corresponds to the spring, the waters contain only
238 $0.40 \text{ mg L}^{-1} \text{ O}_2$. At this site, the water springs from the subsurface between the rocks. The microbial community of
239 the water sample (SC-1 in Fig. 5 and Supplementary Table 1) is dominated by an unclassified
240 Deltaproteobacterium (OTU_SC0012; 14.8% of the total reads in SC-1), with high (97.2%) similarity to
241 environmental OTUs obtained from other AMD/ARD environments such as Río Tinto, Spain (NCBI accession

242 FN862110) or the TongLing acid mine drainage and the Dexing Copper Mine in China (NCBI accessions
243 KC749174 and EF409870, respectively. Interestingly, this Deltaproteobacterium was also similar (96.8%) to the
244 uncultured phylotype K5_62 (NCBI accession EF464599), which has been proposed to represent a novel type of
245 acidophilic sulfate-reducing bacteria (Winch *et al.* 2009). Given the high concentrations of sulfate that exist in the
246 origin of San Cayetano (~1.19 g L⁻¹), the presence of microorganisms capable of using this oxyanion as the
247 terminal electron acceptor is expected. The second most abundant microorganism in the origin of San Cayetano
248 (OTU_SC0019; 14.5%) corresponds to an unclassified bacterium according to our classification methodology. A
249 closer look into its sequence by means of BLAST revealed that the first 183 nucleotides of the DNA fragment
250 share 96.7% of similarity with *Sulfobacillus thermosulfidooxidans*, while the rest of the sequence corresponds to a
251 low complexity, GC-rich DNA region that does not match with any other sequence within the non-redundant
252 nucleotide collection (Supplementary Table S1). We, therefore, resolved to manually curate the sequence of this
253 this OTU and we reassign it within genus *Sulfobacillus* (Fig. 5). These bacteria are Gram-positive, moderately
254 thermophilic, facultative anaerobic (Johnson *et al.* 2008) acidophiles that grow in sulfurous and sulfidic
255 environments. It has been previously reported that they attach to sulfide mineral surfaces, which may promote
256 faster sulfide oxidation (Watling, Perrot and Shiers 2008). *Sulfobacillus* species are also involved in ferrous-iron
257 oxidation (Norris *et al.* 1996; Pina *et al.* 2010). In addition to these two highly represented genera at the origin of
258 San Cayetano, we identified other microorganisms related to acidophilic, sulfur- and iron-oxidizing bacteria, such
259 as *Acidithiobacillus* (OTU_SC0016; 12.5%). This genus includes chemolithotrophic bacteria capable of oxidizing
260 sulfide to sulfate, coupling this reaction to ferric iron (under anoxic conditions) or oxygen (under oxic conditions)
261 reduction (Suzuki *et al.* 1990; Pronk and Johnson 1992). Together with *Acidithiobacillus*, another putative
262 chemolithotrophic sulfur oxidizer from family Hydrogenophilaceae was found in sample SC-1 (OTU_SC0015;
263 8.9%). This organism is closely related to a group of bacteria capable to obtain energy by the oxidation of sulfur
264 compounds with oxygen or nitrate as the terminal electron acceptor, including *Sulfuritortus calidifontis* (93.2% of
265 similarity according to BLAST), *Annwoodia aquaesulis* (92.8% similarity) and *Thiobacillus thioparus* (92.4%
266 similarity)(Kojima, Watanabe and Fukui 2017; Boden, Hutt and Rae 2017).

267

268 Two archaea from the order Thermoplasmatales were also detected amongst the most abundant microorganisms
269 in the water samples from SC-1 (OTU_SC0008; 6.6% and OTU_SC0010; 5.0% of total SC-1 reads). Both
270 phylotypes share high similarity to environmental OTUs detected in other AMD/ARD niches such as the Rio Tinto
271 (NCBI accession FN862291), the hydrothermal ponds in the Copahue region in Argentina (NCBI accession

272 JX989254) or the Kamchatkan hot springs in Russia (NCBI accession JF317816). Moreover, the closest isolates
273 in both cases belong to genus *Cuniculiplasma* (Fig. 5 and Supplementary Table 1). Archaea from the order
274 Thermoplasmatales are considered as extreme thermoacidophiles that generally grow at pH less than 4 (Auernik,
275 Cooper and Kelly 2008). Specifically, *Cuniculiplasma* is classified as a facultative anaerobic, organotrophic and
276 mesophilic whose only isolate (*C. divulgatum*) was obtained from acidic streamers formed on the surfaces of
277 copper-ore-containing sulfidic deposits in south-west Spain and North Wales, UK (Golyshina *et al.* 2016). Other
278 species of order Thermoplasmatales such as *Thermoplasma acidophilum* (Yasuda *et al.* 1995), *Thermoplasma*
279 *volcanium* (Seeger *et al.* 1988), *Thermogymnomonas acidicola* (Itoh, Yoshikawa and Takashina 2007), and
280 *Picrophilus torridus* (Schleper *et al.* 1995; Serour and Antranikian 2002) have been obtained from solfataric
281 hydrothermal areas or acidic streamers containing sulfidic deposits, i.e. sulfur-rich environments. Thus, it is likely
282 that the two Thermoplasmatales OTUs found at the spring waters of San Cayetano are also involved in sulfur
283 metabolism.

284
285 In the second sampling point (SC-2), just 10 meters from the origin, the content of dissolved oxygen only
286 undergoes a slight increase (0.50 mg L⁻¹ O₂, 7.7% O₂) so the microbial community does not change drastically. In
287 this site, the microorganisms found in SC-1, i.e., *Acidithiobacillus* (16.2%), the putative sulfate reducing
288 Deltaproteobacterium (15.9%), both Thermoplasmatales archaea (OTU_SC0008; 10.9% and OTU_SC0010;
289 8.9%), and the *Sulfobacillus* (6.9%), are maintained in a major proportion. At this sampling point, it was possible to
290 assess also the microbiota in the sediments. The most abundant microorganism in all the sediment samples at
291 SC-2 is an unclassified Anaerolineae chloroflexi bacterium (OTU_SC0006) distantly related to genera *Anaerolinea*
292 (89.8% similar to *Anaerolinea thermolimos*), *Ornatilinea* (88.0% similar to *Ornatilinea apprima*) and *Caldilinea*
293 (85.5% similar to *Caldilinea aerophila*). These genera are reported as chemo-organoheterotrophic chloroflexi,
294 which thrive in very diverse habitats such as hot spring aquifers (Grégoire *et al.* 2011) or anaerobic wastewater
295 sludge (Sekiguchi *et al.* 2003; Yamada *et al.* 2005). Interestingly, we could also identify the presence of *Geothrix*
296 in the sediments of sample SC-2 (OTU_SC0021). Only one species from this genus, *Geothrix fermentans*, has
297 been isolated to date. It is a strict anaerobe capable to oxidize a wide variety of organic acids with ferric iron
298 serving as the sole electron acceptor (Coates *et al.* 1999). This organism could play a very important role in the
299 sediments of the San Cayetano ecosystem, not only by the oxidation of organic matter but also in the dissolution
300 of iron(III). By doing so, the iron(II) produced by its respiration becomes available for other iron-oxidizing bacteria
301 along the river course (i.e. *Ferritrophicum* spp., *Leptospirillum* spp., *Acidithrix* spp., *Ferrovum* spp., see below).

302

303 Besides one of the Thermoplasmatales microorganism found also in the water sample (OTU_SC0008), another
304 archaeon of the same order appeared as highly abundant in the sediments of SC-2 (OTU_SC0004; Fig. 5). Albeit
305 it also belongs to an unclassified species, it shows high (99.2%) similarity to environmental OTUs obtained from
306 the Kamchatkan hot springs (NCBI accession JF317816) or the sulfuric ponds of the Tatung Volcano area in
307 Taiwan (NCBI accession FJ797335). The closest isolates from this OTU lie within the *Picrophilus* genus (90.7%
308 similarity to both *P. torridus* and *P. oshimae* (Schleper *et al.* 1995). These microorganisms represent the most
309 thermoacidophilic organisms known, with the ability to survive at a pH around 0 (Schleper *et al.* 1995; Fütterer *et al.*
310 *al.* 2004). Strains of this species were first isolated from a dry solfataric field in Japan (Fütterer *et al.* 2004). Thus,
311 it is possible that both Thermoplasmatales archaea take some part in the metabolism of sulfur in the sediments at
312 this sampling point.

313

314 At the third sampling point (30 meters from the source, after a small waterfall) there is a significant change in the
315 oxygen content (3.77 mg L⁻¹ O₂; 58% O₂) as well as in the microbial community. Interestingly, more than 55% of
316 the microbial community in the water sample (SC-3) is dominated by two Gammaproteobacteria: a bacterium of
317 genus *Ferritrophicum* (OTU_SC0007; 33.2%) characterized for metabolizing iron compounds, and an unclassified
318 Burkholderiaceae (OTU_SC0001; 25.1%)(Fig. 5). Of the genus *Ferritrophicum* only one species has been
319 reported, *F. radicola*, which uses ferrous iron as an energy source for lithotrophic growth. It is a microaerophilic
320 bacterium isolated from a plant growing in an acid mine drainage system (Weiss *et al.* 2007). Thus, it is highly
321 possible that the *Ferritrophicum* from San Cayetano participates in the oxidation of dissolved ferrous iron. On the
322 other hand, representative species of Burkholderiaceae family are very abundant, occupying diverse ecological
323 niches and performing very diverse metabolic functions (e.g. nitrogen fixation, aromatic compounds catabolism,
324 etc) (Compant *et al.* 2008). Therefore, it is difficult to assign a specific role to this generalist Betaproteobacterium
325 in San Cayetano VARD. The *Acidithiobacillus* and the two Thermoplasmatales found in the waters of SC-2 were
326 also present in this sample in lower abundance.

327

328 The sediments of SC-3 also show the presence of the same Burkholderiaceae bacterium (OTU_SC0001) found in
329 the water sample, as well as the Thermoplasmatales archaea (OTU_SC0004 and OTU_SC0008, respectively)
330 found in the sediments of SC-2 (Fig. 5). The presence of the two archaea suggests their capacity to adapt to a
331 wide range of oxygen conditions. In addition, we found a highly abundant Gammaproteobacterium that clusters

332 within the Acidithiobacillaceae group RCP1-48 (OTU_SC0009). Members from this group have been previously
333 detected in the sediments of other AMD habitats like Rio Tinto (Sánchez-Andrea *et al.* 2011) or Los Ruedos
334 mercury underground mine in Spain (Mesa *et al.* 2017), and it has been suggested that they could have an
335 important role in the oxidation of iron and sulfur compounds (Mesa *et al.* 2017). Besides those microorganisms,
336 these sediments host two other highly abundant iron oxidizing-bacteria: A representative species of genus
337 *Leptospirillum* (OTU_SC0003), and the same *Ferritrophicum* sp. found in the water sample. Particularly, the
338 presence of *Leptospirillum* has been widely reported in acid mine drainage (AMD) environments. They catalyze
339 the ferrous iron oxidation, accelerating iron(III)-mediated oxidative dissolution of sulfide minerals and thus the
340 formation of AMD (Goltsman *et al.* 2013). Due to its extraordinary characteristics, *Leptospirillum* species have
341 been used throughout the world in industrial bioleaching operations (Sand *et al.* 1992; Issotta *et al.* 2016). In
342 summary, at sampling point SC-3 we observe an important increase of heterotrophic and iron-oxidizing bacteria,
343 while anaerobic sulfur oxidizers drastically decrease (compared to samples SC-1 and SC-2). This observation also
344 correlates with the increase of oxygen concentration at SC-3 (see table 1 and Fig. 4).

345

346 Finally, the sampling points four and five (600 m and 900 m from the origin, respectively) have similar high oxygen
347 contents and very similar microbial composition (Figs 4 and 5). The oxygen content was 5.85 mg L⁻¹ O₂ (82% O₂)
348 and 6.06 mg L⁻¹ O₂ (81% O₂) respectively. The microbial community in those waters is also dominated by the
349 same Burkholderiaceae bacterium found in SC-3 (36.9% in SC-4 and 30.0% in SC-5), followed by the
350 *Leptospirillum* sp. present in the sediments of SC-3 (18.3% in SC-4 and 11.4% in SC-5). In the third place, we
351 detected large quantities of the heterotrophic acidophile *Acidithrix* (OTU_SC0005; 7.58% in SC-4 and 11.88% in
352 SC-5). To the best of our knowledge, *Acidithrix ferrooxidans* is the unique species from this genus that has been
353 described to date. This bacterium is capable to catalyze the dissimilatory oxidation of ferrous iron under aerobic
354 conditions, and the reduction of ferric iron under micro-aerobic and anaerobic conditions (Jones and Johnson
355 2015). Given the oxic nature of SC-4 and SC-5, it is presumable that the *Acidithrix* detected in both water samples
356 contributes to the oxidation of ferrous iron. These conditions are very appropriate also for the obligate aerobe
357 *Leptospirillum* sp. (see above) and for other iron-oxidizing bacteria present in these samples in minor abundance
358 (i.e *Ferritrophicum* spp. and *Ferrovum* spp.). In addition, other heterotrophic bacteria from the genera
359 *Trichococcus* and *Acidocella* were found in the water from sampling points SC-4 and SC-5, respectively.

360

361 Finally, the sediments obtained from SC-4 and SC-5 showed a similar microbial composition (Fig. 5). The most
362 prominent organism in both samples is a Gammaproteobacterium of the genus *Metallibacterium* (OTU_SC0002).
363 Once again, this genus has only one reported species (*M. scheffleri*) and has been very poorly studied. Among the
364 few known metabolic functions of this species, it is known that it is capable of reducing ferric iron, but does not
365 oxidize ferrous iron (Ziegler *et al.* 2013). We found also a large abundance of some of the microorganisms
366 identified in the water samples from sampling points SC-4 and SC-5, such as the Burkholderiaceae
367 Betaproteobacterium, *Acidithrix* sp., *Leptospirillum* sp. and *Ferrovum* sp. Other microorganisms found in the
368 sediments of SC-5 were a Gammaproteobacterium (OTU_SC0013) from the uncultured group KF-JG30-C25 and
369 another Actinobacterium (OTU_SC0020) whose closest isolate (91.4% similarity according to BLAST) is the
370 heterotrophic iron(III)-reducer *Aciditerrimonas ferrireducens* (Itoh *et al.* 2011). Specifically, members of KF-JG30-
371 C25 group have been detected in AMD habitats such as the uranium mining waste pile at Johanngeorgenstadt in
372 Germany (Selenska-Pobell 2002).

373

374 **Conclusions**

375

376 This work reports the microbiological composition of the volcanic influenced acid rock drainage known as San
377 Cayetano (northeast of Irazu volcano, Costa Rica). The low [iron]/[sulfate] ratio suggest that most of the sulfuric
378 acid in this VARD is of volcanic origin, possibly due to the disproportionation reaction of SO₂ and biotic or abiotic
379 H₂S oxidation in the groundwater. The moderately high temperature of the water at the spring of San Cayetano is
380 also consistent with underground mixing with volcanic water. We analyzed the microbial composition of water and
381 sediment samples along 900 meters from the origin of the river. Interestingly, we found a remarkable abundance
382 of a yet not-described diversity of microorganisms amongst the most represented taxa thriving in San Cayetano
383 VARD. Nevertheless, we could also identify the presence of bacteria and archaea that, along with the chemistry of
384 the site, resembles the composition of typical ARD environments. The structure of the microbial community in San
385 Cayetano undergoes important changes as a function of the oxygen gradient observed in the distinct water
386 samples. At the origin of the river (SC-1 and SC-2), the microbial profile is dominated mainly by putative
387 anaerobic, sulfur-oxidizing microorganisms such as *Sulfobacillus* sp., *Acidithiobacillus* sp., the putative sulfate
388 reducing Deltaproteobacterium and the *Hydrogenophilaceae* Gammaproteobacterium. Their presence at the first
389 two sampling points, suggest that these microorganisms are likely involved in both sulfate reduction as well sulfide
390 oxidation, completing the sulfur cycle. At the third sampling point (SC-3), the oxygen content increases eight-fold

391 (from 0.40 mg L⁻¹ to 3.77 mg L⁻¹), also generating a drastic change in the microbial community. Most of the sulfur-
392 oxidizing taxa found at the origin of the river are substituted by iron-oxidizers (*Ferritrophicum spp.*, *Leptospirillum*
393 *sp.*) and heterotrophic microorganisms (Burkholderiaceae Betaproteobacterium). After 600 meters downstream
394 from the origin of San Cayetano, the river is completely oxygenated. Consequently, the last two samples (SC-4
395 and SC-5) are totally dominated by aerobic microorganisms, mostly related to heterotrophic metabolism
396 (Burkholderiaceae Betaproteobacterium, *Trichococcus sp.* *Acidoceella sp.*) or iron-oxidation (*Leptospirillum sp.*,
397 *Acidithrix sp.*, *Ferritrophicum spp.*, *Ferrovum spp.*). Our data are consistent with the notion that the initial volcanic
398 chemistry of the aquifer is fundamentally modified by the microbial metabolism and by the increase of oxygen
399 along the river course until its confluence with the previously characterized Rio Sucio (Arce-Rodriguez *et al.* 2017).

400

401 Over the 900 meters course of the stream analyzed, we were able to find both aerobic and anaerobic organisms
402 carrying out many different metabolic activities (*i.e.* sulfur- and iron- oxidation/reduction, nitrogen metabolism,
403 carbon oxidation, etc.). Furthermore, these organisms have to cope with the high concentration of heavy metals
404 such as arsenic, chromium or zinc. Taking also into account the large diversity of unclassified microorganisms
405 found along San Cayetano, it is reasonable to presume that this untapped ecosystem is endowed with metabolic
406 functions and enzymatic activities with a biotechnological interest that could be further exploited. This work also
407 contributes to the knowledge of volcanic and ARD environments in which the conditions are created only by
408 natural factors which contrasts with the majority of published works on ARD environments in the world which have
409 been influenced by human activity.

410

411

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413

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419

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421

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424

425

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578 **Tables**

579
580 **Table 1. Physical properties and chemical composition of San Cayetano.**

581

Property/element/ion	SC-1	SC-2	SC-3	SC-4	SC-5
Temperature (°C) / ± 0.1	38.0	38.2	37.8	32.7	29.7
pH / ± 0.1	3.0	2.9	3.0	3.1	3.0
Dissolved oxygen (mg L ⁻¹) / ± 0.01	0.40	0.50	3.77	5.85	6.06
Aluminum (mg L ⁻¹) ± 5	41	33	41	34	45
Arsenic (µg L ⁻¹) ± 0.7	19.2	20.8	20.7	12.9	11.3
Cadmium (µg L ⁻¹)	< 0.11	< 0.11	< 0.11	< 0.11	< 0.11
Calcium (mg L ⁻¹) ± 2	4	4	4	4	3
Chloride (mg L ⁻¹) ± 10	246	203	227	200	222
Copper (mg L ⁻¹)	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
Chromium (µg L ⁻¹) / ± 2	30	33	33	32	29
Fluoride (mg L ⁻¹)	<0.09	<0.09	<0.09	<0.09	<0.09
Iron (mg L ⁻¹) / ± 2	45	51	61	40	35
Magnesium (mg L ⁻¹) ± 3	58	64	64	63	59
Manganese (mg L ⁻¹) ± 0.06	1.98	2.05	1.98	2.00	2.02
Nickel (µg L ⁻¹) / ± 2	6	10	8	10	10
Nitrate (mg L ⁻¹)	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
Lead (µg L ⁻¹)	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
Potassium (mg L ⁻¹) ± 0.6	31.8	34.2	33.4	35.0	33.8
Sodium (mg L ⁻¹) ± 2	73	79	78	78	75
Sulfate (mg L ⁻¹) ± 50	1190	950	883	870	874
Zinc (µg L ⁻¹) ± 10	115	109	141	119	121

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584

585 **Figure legends**

586

587 **Fig. 1 San Cayetano stream in Central Mountain Range, Costa Rica.** The San Cayetano stream is located in
588 the Central Valley, next to the Irazú volcano. This stream has a length of about 3 km to its mouth in the Rio Sucio.

589

590 **Fig. 2 Sampling points from San Cayetano stream.** Water and sediment samples were taken in five places
591 along the river: at the origin (SC-1), ten meters from the origin (SC-2), 30 meters from origin (SC-3), 600 meters
592 from origin (SC-4) and 900 meters from origin (SC-5). As shown in the figure, between sampling points a gradient
593 occurs in the oxygen content.

594

595 **Fig. 3 Taxonomic composition of San Cayetano.** Relative abundance of bacterial and archaeal organisms to
596 the phylum level. The OTUs were taxonomically classified into the RDP taxonomy as reported by the SINA
597 classification tool, as described in Materials and Methods. The water samples at each of the sampling points are
598 identified as SC-1 to SC-5. Sediment samples are identified with legends SC-2S1 to SC-5S4.

599

600 **Fig. 4 Non-metric multidimensional scaling analysis of the prokaryotic communities in San Cayetano**
601 **volcanic influenced acid rock drainage.** A clustering of communities according to the oxygen content of the
602 samples and also by the habitat (water column versus sediment) is shown. The NMDS and the Permanova
603 analyses were performed with the package Vegan.

604

605 **Fig. 5 Heat map representing the most abundant genera in each sample.** The heat map depicts the relative
606 percentage of 16S rRNA gene sequences assigned to each genus (y axis) across the 16 samples analysed (x
607 axis). Square colors shifted towards black indicate higher abundance.

608

Sucio River

Map Location

SC-5
SC-4
SC-1, 2, 3

Landslide

Sucio River
source



0 1 2 3 km

----- Irazú Volcano Road

- - - - - Farm Road

————— Sucio River

⊙ Irazu Volcano crater

● Sampling Point




SC-1




38.0 °C 0.40 mg/L O₂

SC-2 10 m




38.2 °C 0.50 mg/L O₂

SC-3 30 m




37.8 °C 3.77 mg/L O₂

SC-4 600 m



32.7 °C 5.85 mg/L O₂

SC-5 900 m

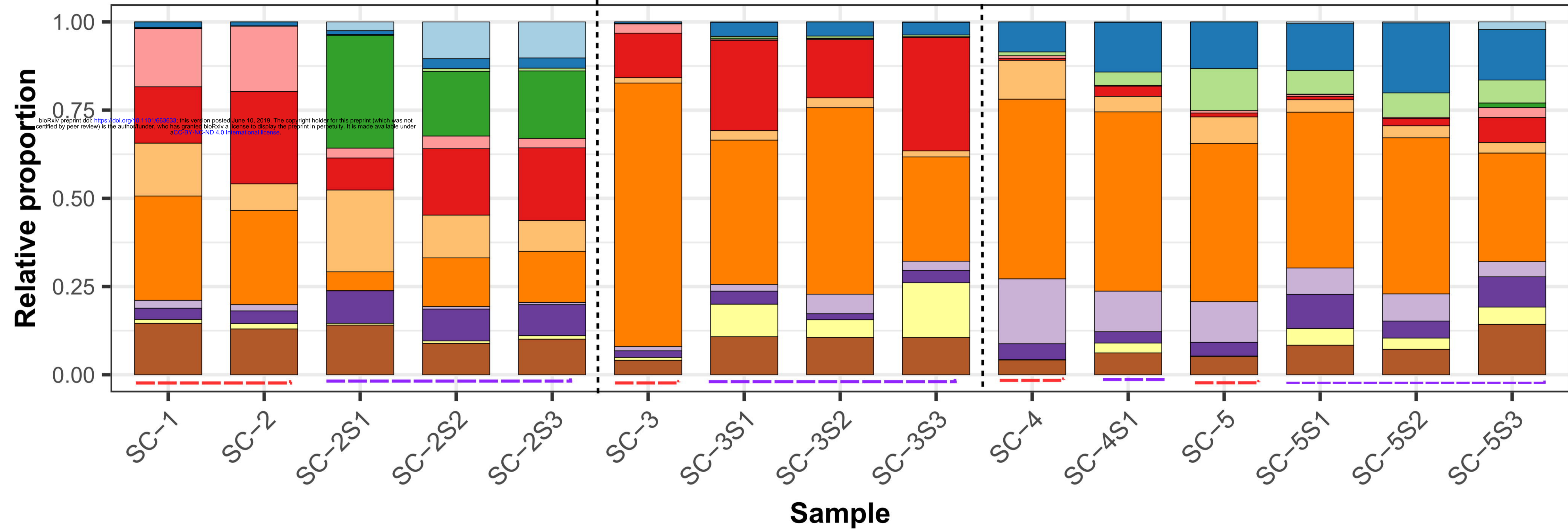


29.7 °C 6.06 mg/L O₂

0.4-0.5 mg/l O₂

3.7 mg/l O₂

5.8-6.1 mg/l O₂



NMDS2

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