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Dominance of *Arcobacter* in the white filaments from the thermal sulfidic spring of Fetida Cave (Apulia, southern Italy)

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1 Dominance of *Arcobacter* in the white filaments from the thermal sulfidic spring  
2 of Fetida Cave (Apulia, southern Italy)

3

4 Valme Jurado <sup>a</sup>, Ilenia D'Angeli <sup>b</sup>, Tamara Martin-Pozas <sup>c</sup>, Martina Cappelletti <sup>d</sup>,  
5 Daniele Ghezzi <sup>d,e</sup>, Jose Luis Gonzalez-Pimentel <sup>f</sup>, Soledad Cuezva <sup>g</sup>, Ana Zelia  
6 Miller <sup>a</sup>, Angel Fernandez-Cortes <sup>h</sup>, Jo De Waele <sup>b</sup>, Sergio Sanchez-Moral <sup>c</sup>,  
7 Cesareo Saiz-Jimenez <sup>a\*</sup>

8

9 <sup>a</sup> *Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, 41012 Sevilla, Spain*

10 <sup>b</sup> *Department of Biological, Geological and Environmental Sciences, University of*  
11 *Bologna, 40126 Bologna, Italy*

12 <sup>c</sup> *Museo Nacional de Ciencias Naturales, MNCN-CSIC, 28006 Madrid, Spain*

13 <sup>d</sup> *Department of Pharmacy and Biotechnology (FaBit), University of Bologna, 40126*  
14 *Bologna, Italy*

15 <sup>e</sup> *Laboratory of NanoBiotechnology, IRCCS Istituto Ortopedico Rizzoli, 40136, Bologna,*  
16 *Italy*

17 <sup>f</sup> *Laboratorio Hercules, Universidade de Evora, 7000-809 Evora, Portugal*

18 <sup>g</sup> *Departamento de Geología, Geografía y Medio Ambiente, Universidad de Alcalá de*  
19 *Henares, 28801 Alcalá de Henares, Spain*

20 <sup>h</sup> *Departamento de Biología y Geología, Universidad de Almería, 04120 Almería, Spain*

21

## 22 **Abstract**

23 The thermal spring of Fetida Cave, a still active sulfuric acid cave opening  
24 at the sea level and located in Santa Cesarea Terme, southeastern Salento  
25 (Apulia region, Southern Italy) hosts abundant floating white filaments. The  
26 white filaments were mainly composed of sulfur crystals surrounded by  
27 microbial mass of the phyla *Epsilonbacteraeota*, *Proteobacteria*, *Bacteroidetes*,  
28 and *Patescibacteria*. The most abundant genus in the white filaments collected

29 from the waters in the innermost part of the cave dominated by sulfidic  
30 exhalations was *Arcobacter*. This abundance can be related to the higher  
31 concentration of sulfide dissolved in water, and low oxygen and pH values.  
32 Conversely, lower *Arcobacter* abundances were obtained in the filaments  
33 collected in the entrance and middle part of the cave, where sulfidic water mixes  
34 with seawater, as the cave is subjected to tides and the mixing of fresh  
35 (continental) with marine water. The geochemical analysis of water  
36 geochemistry and atmospheric gases confirmed these environmental  
37 constraints. In fact, higher concentrations of H<sub>2</sub>S in the air and water were  
38 recorded closest to the spring upwelling in the innermost part of the cave, and  
39 the lower ones near the cave entrance. The metabolic versatility of *Arcobacter*  
40 might provide a competitive advantage in the colonization of water bodies  
41 characterized by high sulfide, low oxygen, and dynamic fluid movement.

42

43 Keywords: Microbial diversity, sulfuric acid speleogenesis, microbial filaments,  
44 sulfur, cave atmosphere, water geochemistry

45

## 46 **1. Introduction**

47 Hypogene sulfuric acid speleogenesis (SAS) caves are underground karst  
48 systems formed in carbonate areas where acidic fluids, derived from the  
49 interactions with deep-seated sulfates and/or sulfides, rise through deeply  
50 rooted geological structures (Audra et al., 2009). In particular, SAS caves form  
51 when host rock dissolution is mainly related to the interaction with the sulfuric  
52 acid produced by the oxidation of hydrogen sulfide. Around 25% of the known  
53 worldwide SAS systems are located in Italy, especially along the Italian  
54 Apennine Chain, and some of them are still in active conditions (D'Angeli et al.,  
55 2019a).

56 Within SAS caves, hydrogen sulfide provides a rich energy source for  
57 chemolithotrophic microorganisms, which support chemosynthetic primary  
58 production for the growth of heterotrophic organisms (Jones et al., 2008; Bizic  
59 et al., 2020). In association with this, SAS caves typically host conspicuous  
60 microbial biofilms and mats that are visible on the walls, ceilings, and in the

61 water. Those covering the cave walls and ceilings have variable morphologies  
62 and colors in the form of viscous snottites and vermiculations (Jones et al.,  
63 2010; D'Angeli et al., 2019b). In the water, that has a milky appearance (due to  
64 elemental sulfur), the biofilms are in the form of white filaments that are typically  
65 visible as either rock-attached streamers or sediment surface biofilms. These  
66 two morphologies were first referred to as feathery biofilms and cotton biofilms,  
67 respectively, by Macalady et al. (2007) in Frasassi Cave. Analogous white  
68 filaments were observed and described in a series of other SAS caves in  
69 Acquasanta Terme, Capo Palinuro, Monte Sellaro, Cassano allo Ionio, and  
70 Santa Cesarea Terme (D'Angeli et al., 2019a,b) and also in Romania (Bizic et  
71 al., 2020).

72 The microbiology and composition of white filaments was previously  
73 studied, but limited to a few caves and springs. The caves with major research  
74 efforts were Frasassi (Macalady et al., 2006, 2008; Engel, 2007), Movile  
75 (Hutchens et al., 2004; Chen et al., 2009; Kumaresan et al., 2014; Bizic et al.,  
76 2020), and Lower Kane (Engel et al., 2003, 2004, 2010). A few individual  
77 reports on other caves and springs can be found in the literature (Mattison et  
78 al., 1998; Engel et al., 2001; Elishahed et al., 2003; Barton and Luiszer, 2005;  
79 Reigstad et al., 2011; Rossmassler et al., 2012). Among these, the microbiology  
80 of the water streamers has been the most extensively studied through  
81 molecular methods (16S rRNA clone library) but also through microscopy and  
82 culture-based experiments (Hose and Pisarowicz, 1999; Engel et al., 2004;  
83 Hamilton et al., 2015). Sulfur-oxidizing microorganisms belonging to *Gamma*-,  
84 *Beta*- and *Epsilonproteobacteria*, reclassified as *Epsilonbacteraeota* (Waite et  
85 al., 2017) dominate the water streamer microbial communities. Additionally,  
86 members of *Deltaproteobacteria*, associated with sulfur-reduction processes  
87 were identified in lower abundance (Macalady et al., 2006). Among the different  
88 environmental factors possibly affecting these biofilms, the water flow (shear  
89 stress) and the ratio sulfide/oxygen were reported to be the major ones  
90 influencing white filament morphologies (i.e. long rock-attached streamers or  
91 shorter sediment biofilm) and microbial diversity (Macalady et al., 2008).

92 The present work is focused on the analysis of white filaments from Fetida  
93 Cave, a still active sulfuric acid cave opening at the sea level and located in  
94 Santa Cesarea Terme, southeastern Salento (Apulia region, Southern Italy)

95 (D'Angeli et al., 2019b; 2021) (Fig. 1). The cave hosts abundant microbial  
96 biofilms on cave walls and ceiling as vermiculations and gypsum moonmilk. A  
97 previous study described the mineralogy, geochemistry, and microbial diversity  
98 associated with the different biofilms found in Fetida Cave (D'Angeli et al.,  
99 2019b).

100 The microbial communities featuring Fetida Cave water are characterized  
101 by the presence of abundant floating white filaments and are related with the  
102 constant mixing between the thermal sulfidic fluids, rising from below, and  
103 seawater entering from outside that leads to variable contents of dissolved H<sub>2</sub>S  
104 along the cave (D'Angeli et al., 2021). The purpose of the present work is to  
105 extend the knowledge on the microbial communities composing white filaments  
106 in this unique SAS cave environment. In this regard, gaseous composition of  
107 the cave atmosphere and water geochemistry as well as morphological (through  
108 FESEM) and microbiological (using Illumina sequencing of 16S rRNA gene)  
109 analyses were performed to get deep into the diversity of the white floating  
110 filaments in Fetida Cave (Fig. 1) and the possible predicted metabolic functions  
111 associated with their development.

112

## 113 **2. Methods**

### 114 *2.1. Field sampling*

115 Microbiological and air sampling was performed in different sites inside the  
116 cave (Fig. 1). In particular, four microbiological samples were collected in sites  
117 FC1, FC4, FC6, and FC7, moving from the innermost part of the cave towards  
118 the coastline. D'Angeli et al. (2019b) described three different typologies of  
119 water filaments based on their location, *i.e.*, floating on the water surface  
120 (named F-float), sedimented on the bottom of the water (F-sed), and attached to  
121 the cave rocks (F-stream). The four samples analyzed in this study are different  
122 from those described by D'Angeli et al. (2019) but belong to the F-float category  
123 described in this previous paper. Microbial sampling for this paper was done  
124 during a field trip in June 2017 (summer conditions with calm sea), while the  
125 samples analyzed in the study by D'Angeli et al. (2019) were collected in  
126 October 2015 and December 2017 (during winter, when marine conditions were

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127 rougher). The samples were collected using sterile scalpels, stored in sterile  
128 tubes at 4°C until arrival at the laboratory. Five replicas from each sampling  
129 point were taken for molecular biology as well as for field emission scanning  
130 electron microscopy (FESEM). The samples for molecular biology were  
131 preserved in Lifeguard preservation solution (Qiagen, Hilden, Germany) and  
132 then held at -80°C until analysis, whereas samples for microscopy were fixed in-  
133 situ with 2.5% glutaraldehyde in 0.1M cacodylate-buffer (pH 7.4).

134 Gas samples were taken into the cave in sites P1, P2, P3, P4 (Fig. 1, Table  
135 A1 in Appendix A - Supplementary data), and outside to take external air and  
136 soil in several locations, both in June 2017 and in May 2018. They were  
137 collected using a handheld pump and 1 L Tedlar bags (Fig. 1). For each sample  
138 two replicas were taken, filling only 2/3 of the entire bag to avoid bag damage  
139 and/or explosion during transport. Immediately after the collection, 12 Tedlar  
140 bags (for the two fieldworks in June 2017 and May 2018) were stored in a rigid  
141 luggage and analyzed within 48 hours at the stable isotopes laboratories of  
142 Museo Nacional de Ciencias Naturales in Madrid and University of Almeria.  
143 CO<sub>2</sub> and CH<sub>4</sub> molar fractions and δ<sup>13</sup>C in both gases were measured with a  
144 CRDS spectrometer (G2201-i analyser, Picarro Inc., USA) with a precision of  
145 200 ppb (±0.05 of reading) and 10 ppb (±0.05 of reading) for <sup>12</sup>CO<sub>2</sub> and <sup>13</sup>CO<sub>2</sub>,  
146 respectively, resulting in a precision better than 0.16 ‰ for δ<sup>13</sup>C-CO<sub>2</sub> after 5 min  
147 of analysis. The measurements of methane isotopologues (<sup>12</sup>CH<sub>4</sub> and <sup>13</sup>CH<sub>4</sub>)  
148 reached a precision of 5 ppb (±0.05 of reading) and 1 ppb (±0.05 of reading) for  
149 <sup>12</sup>CH<sub>4</sub> and <sup>13</sup>CH<sub>4</sub>, respectively. The precision for δ<sup>13</sup>C-CH<sub>4</sub> was better than 1.15  
150 ‰ after 5 min of analysis. δ<sup>13</sup>C-isotope values were referenced to the Vienna  
151 PeeDee Belemnite (V-PDB). Three in-house standards with certified CO<sub>2</sub> and  
152 CH<sub>4</sub> concentrations and known δ<sup>13</sup>C values for each gas were processed to  
153 verify the proper functioning of the CRDS analyzer. Fernández-Cortès et al.  
154 (2018) reported further details on the methodological procedures and quality  
155 results. Spot measurements of CO<sub>2</sub> concentration and levels of other key gases  
156 (H<sub>2</sub>S and O<sub>2</sub>) were also taken in the same locations for air sampling using  
157 handheld devices (XP200, Lufft) and a multigas monitor (MX6 iBrid, Industrial  
158 Scientific), respectively.

159 In addition, during June 2017 and May 2018 field trips, water samples  
160 (SCC1, SCB1, SCAfen, SCA1) were collected in the same location of

161 microbiological samplings (Fig. 1, Table 1A in Appendix A - Supplementary  
162 data). The results of a comprehensive water monitoring campaign that began in  
163 October 2015 and ended in November 2018 have been published in the  
164 framework of a hydrogeological-geochemical study (D'Angeli et al., 2021).  
165 Nevertheless, in this work, we will consider only the results obtained during the  
166 microbiological and gas sampling (i.e. June 2017 and May 2018). Water  
167 parameters such as temperature (T °C), pH, electrical conductivity (EC), and  
168 total dissolved solids (TDS) were measured using a multiparametric probe  
169 Hanna HI991001 (relative accuracies at 25 °C:  $\pm 0.5$  °C,  $\pm 0.02$  pH,  $\pm 2\%$   
170 EC/TDS). Two replicas of water were collected in each sampling site in 250-ml  
171 HDPE bottles, and one was acidified with 65% HNO<sub>3</sub>, and stored at 4°C until  
172 analysis at the laboratory of Politecnico di Torino.

173 The  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  in a third replica of water collected during May 2018 were  
174 measured simultaneously at University of Almeria by cavity ringdown  
175 spectroscopy (CRDS) by a L2140-i Picarro water isotope analyzer interfaced  
176 with an A0211 high-precision vaporizer (Picarro Inc., USA), coupled with a  
177 Picarro micro-combustion module (MCM®) to remove combustible organic  
178 compounds from water samples. Each sample was injected 10 times into the  
179 vaporizer, which was heated to 110 °C. Memory effects from previous samples  
180 were avoided by rejecting the first three analyses, so values for the final 7  
181 injections were averaged with a typical in-sample precision ( $\pm 1\sigma$ ) of  $\pm 0.04\text{‰}$  for  
182  $\delta^{18}\text{O}$  and  $\pm 0.18\text{‰}$  for  $\delta^2\text{H}$ . The results were normalized against V-SMOW by  
183 analyzing internal standards before and after each set of twenty samples and  
184 are given as per mil (‰). <sup>13</sup>C measurements of the dissolved inorganic carbon  
185 in water samples ( $\delta^{13}\text{C-DIC}$ ) were also obtained by using the same G2201-i  
186 analyzer coupled with an Automate FX sample preparation device. NBS-18  
187 (Carbonatite) and IAEA-603 (calcite) were used to calibrate the AutoMate-  
188 CRDS system and referring the results to the Vienna Pee Dee Belemnite (V-  
189 PDB). Three replicates per sample were analyzed together with two internal  
190 standards run before and after the water sample set, reaching an average  
191 precision ( $\pm 1\sigma$ ) of  $\pm 0.10$  ‰.

192

193 *2.2. Field emission scanning electron microscopy with Energy Dispersive X-ray*  
194 *Spectroscopy (FESEM-EDS)*

195 The morphology of the white filaments was studied by FESEM using a FEI  
196 Teneo FESEM (FEI Company, Eindhoven, The Netherlands) with secondary  
197 electron detection mode and an acceleration voltage of 5 kV for high resolution  
198 images and 10 kV for elemental microanalysis. Before FESEM observations,  
199 the fixed samples were washed in cacodylate-buffer, post-fixed in 1% osmium  
200 tetroxide and dehydrated by serial dilutions in ethanol and acetone.  
201 Subsequently, samples were dried in a Leica EM CPD300 critical point dryer at  
202 34.5 °C and then sputter coated with a gold thin film, as described by De la  
203 Torre Noetzel et al. (2018).

204

### 205 2.3. DNA extraction, sequencing and functional prediction

206 Genomic DNA extraction was carried out using the FastDNA SPIN Kit for  
207 Soil (MP Biomedicals, Illkirch, France). DNA quantification was measured by  
208 means of a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) and the DNA  
209 concentration values of FC1, FC4, FC6, and FC7 samples were 22.1, 217.6,  
210 22.0 and 31.5 ng/μL, respectively.

211 High-throughput sequencing of extracted DNA was performed by Macrogen  
212 (Seoul, Korea). We targeted the V3-V4 hypervariable regions of the 16S rRNA  
213 gene using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-  
214 GACTACHVGGGTATCTAATCC-3'). PCR amplification reaction per sample  
215 consisted of 5 μL of each primer (1μM), 12.5 μL of 2x KAPA HiFi HotStart  
216 ReadyMix (Roche) and 2.5 μL of DNA template (5 ng/μL), for a total of 25  
217 μL/sample. The PCR program was run as follows: primary denaturation at 95°C  
218 for 3 min, followed of 30 cycles beginning with a denaturalization step at 95°C  
219 for 30 s, a second step of annealing at 56°C for 30 s and a third step of  
220 elongation at 72°C for 1 min. The PCR program concluded with an elongation  
221 step at 72°C for 5 min. Illumina MiSeq platform was used for 2 × 250 paired-end  
222 sequencing, following the Illumina protocol for library Nextera XT Index Kit  
223 preparation.

224 Raw data were checked for quality using FastQC  
225 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Amplicon  
226 Sequence Variant (ASV)-based analyses were conducted with the QIIME2  
227 platform (Bolyen et al., 2019). First, DADA2 (Callahan et al., 2016) filtered the



228 raw data according to the quality, generating an amplicon sequence variant  
229 (ASV) table. Afterwards, taxonomic assignment was implemented using the  
230 feature-classifier *classify-sklearn* (Bokulich et al., 2018) and the SILVA  
231 database 132 version (Quast et al., 2013). Finally, alpha diversity analysis was  
232 carried out for evenness and richness measurement of microbial communities  
233 using the following metrics: Chao1, Shannon and Simpson indices, as well as  
234 Pielou's evenness measure (Chao, 1984; Shannon and Weaver, 1949;  
235 Simpson, 1949; Pielou, 1966).

236 Taxonomic distribution and relative abundance of the microbial community  
237 were depicted in heat-maps using R package *gplots* (Warnes et al., 2015).  
238 Samples were arranged through dendrograms based on the taxonomic  
239 abundance and representativeness. Prediction of functionality in the microbial  
240 community based on 16S rRNA gene data was carried out using FAPROTAX  
241 (Louca et al., 2016).

242

### 243 **3. Results and discussion**

#### 244 *3.1. Gaseous composition of the cave atmosphere and water geochemistry*

245 The values of measured CO<sub>2</sub> and CH<sub>4</sub> concentrations and their stable  
246 isotopic compositions ( $\delta^{13}\text{C-CO}_2$  and  $\delta^{13}\text{C-CH}_4$ ) in Fetida Cave environment,  
247 above-cave soil, and local exterior atmosphere are shown in Table 1. The mean  
248 concentrations of CO<sub>2</sub> and CH<sub>4</sub> in the local external atmosphere were  $437.4 \pm$   
249  $17.3$  ppmv and  $2.00 \pm 0.02$  ppmv, respectively. CO<sub>2</sub> concentration of cave air  
250 varied within a narrow range between 460 and 650 ppmv, i.e. it usually ranges  
251 between 100 and 200 ppmv higher than the local atmospheric background  
252 outside. CH<sub>4</sub> concentration of cave air ( $2.13 \pm 0.09$  ppmv, on average) is slightly  
253 higher than those recorded for the local external atmosphere, but with maximum  
254 values around 2 - 2.2 ppmv in the inner locations closer to the acid springs. H<sub>2</sub>S  
255 concentrations in the air of Fetida Cave ranged from 0.8 to 2.4 ppmv, with the  
256 highest concentrations closest to the acid springs (P2 and P1) and the lower  
257 concentrations at the entrance and middle cave locations (P3 and P4), due to  
258 an efficient exchange and mixing with H<sub>2</sub>S-free outdoor atmosphere.

259 At these cave-air levels of H<sub>2</sub>S, the spectral lines of the CRDS spectrometer  
260 avoid the strongly absorbing H<sub>2</sub>S spectral lines and, therefore, the measurable

261 effect on the reported CO<sub>2</sub> concentrations can be considered negligible. The  
262 changes in the <sup>12</sup>CO<sub>2</sub> and <sup>13</sup>CO<sub>2</sub> with an addition of H<sub>2</sub>S ranging from 0.8 to 2.4  
263 ppm would be 0.5–0.75 % and less than 7%, respectively, according to  
264 Malowany et al. (2015). In the case of a well-ventilated atmosphere as Fetida  
265 Cave, with CO<sub>2</sub> contents slightly higher than local exterior atmosphere and  
266 <sup>13</sup>CO<sub>2</sub> roughly being 1% of the <sup>12</sup>CO<sub>2</sub> molar fraction, these percentages entail a  
267 total variation for CO<sub>2</sub> concentration of only 4.1 ppm, on average (±3.7 ppm for  
268 <sup>12</sup>CO<sub>2</sub> and ±0.4 ppm for <sup>13</sup>CO<sub>2</sub>). The small interferences of H<sub>2</sub>S on CO<sub>2</sub>  
269 measurements by CRDS were confirmed through the field CO<sub>2</sub> measurements  
270 obtained with the handheld XP200 logger equipped with a NDIR probe.

271 However, the H<sub>2</sub>S spectral lines, that partially overlap with some spectral  
272 features of the CO<sub>2</sub> used in the CRDS system, turns into some relative changes  
273 on <sup>12</sup>CO<sub>2</sub> with respect to <sup>13</sup>CO<sub>2</sub> that provokes a remarkable interference on the  
274 CO<sub>2</sub> isotopic measurements (Malowany et al., 2015). Thus, H<sub>2</sub>S in air samples  
275 from Fetida Cave is enough to influence measurements by an unreal increase  
276 in the <sup>12</sup>CO<sub>2</sub> concentration and a more significant decrease in the <sup>13</sup>CO<sub>2</sub>  
277 concentration, resulting in quite depleted δ<sup>13</sup>C-CO<sub>2</sub> values. Consequently, the  
278 carbon isotopic values for CO<sub>2</sub> of cave air were not reported in Table 1.

279 Fortunately, the H<sub>2</sub>S levels of Fetida Cave have a negligible interference on  
280 the CRDS-reported δ<sup>13</sup>C-CH<sub>4</sub> for cave air samples, similar to what reported in  
281 Malowany et al. (2015) described for standard gases in laboratory  
282 measurements, allowing for comparison of results of both concentration and  
283 carbon isotopic signal for this gas. Despite the fact that CH<sub>4</sub> concentrations of  
284 cave air are slightly higher than those recorded for the local atmosphere, the  
285 δ<sup>13</sup>C-CH<sub>4</sub> varies within a narrow range between -45.1 ‰ and -47.5 ‰ and is not  
286 linearly related to the inverse CH<sub>4</sub> concentration. These δ<sup>13</sup>C-CH<sub>4</sub> values  
287 evidence that CH<sub>4</sub> in cave air is not locally generated by biogenic processes  
288 since if this would be the case it should be significantly enriched in <sup>12</sup>C relative  
289 to the external atmosphere. Methane present in cave air, however, is mainly  
290 sourced by the atmospheric CH<sub>4</sub> from ventilation through the sole entrance, i.e.  
291 bypassing the soil zone. This suggests that the subterranean atmosphere is  
292 greatly diluted by inputs from the outside atmosphere, which is also  
293 corroborated by low CO<sub>2</sub> concentrations and O<sub>2</sub> levels close to 21%.

294 Soil-CO<sub>2</sub> concentrations show a remarkable difference between the two  
295 field surveys (Table 1) that depending on the prevailing soil microbial respiration  
296 rate each time, which, in turn, is mainly controlled by soil temperature and  
297 moisture. However, in all samples nearly constant  $\delta^{13}\text{C-CO}_2$  values ( $-19.73 \pm$   
298  $0.98$  ‰, on average) prevail being relatively heavier than the expected  
299 composition range for CO<sub>2</sub> derived from C3 organic matter. These  $\delta^{13}\text{C-CO}_2$   
300 values of soil air likely the results of the upward diffusion of CO<sub>2</sub> to the open  
301 atmosphere and the effects of a kinetic fractionation on the residual CO<sub>2</sub> from  
302 the soil zone. Contrary to what has been observed in the cave-air CH<sub>4</sub>, an  
303 intense microbial oxidation of CH<sub>4</sub> in soil provokes the residual methane to  
304 become isotopically depleted in <sup>12</sup>C, a fact that was particularly evident in the  
305 first air sampling campaign.

306 Overall, the gaseous composition of the external soil denotes its evident  
307 disconnection from the cave atmosphere. Consequently, the isotopic  
308 composition of CO<sub>2</sub> and CH<sub>4</sub> in cave air confirms a prevailing gas exchange  
309 pathway with the atmospheric background source, ruling out both the biogenic  
310 CH<sub>4</sub> and soil-derived CO<sub>2</sub> sources or a remarkable deep-sourced input for both  
311 gases.

312 In general, the collected waters show mean temperature higher than 20°C,  
313 and pH ranges between 6.89 (in the innermost portion -SCC1 and SCB1- of the  
314 cave which is influenced by rising sulfuric acid fluids) and 7.38 (in the sampling  
315 sites close to the entrance of the cave). The higher concentration of [HS<sup>-</sup>]  
316 dissolved in water was observed in the innermost sampling sites SCB1 and  
317 SCC1 (Table 2). In addition, SCB1 showed the most mineralized waters with  
318 higher values of temperature and lower value of pH. Detailed information on the  
319 water geochemistry can be found in D'Angeli et al. (2021).

320 The stable isotope analyses of water from Fetida Cave revealed  $\delta^2\text{H}$  values  
321 that ranged from  $-4$  to  $+7$  ‰, approximately, with an average of  $+2.85$  ‰, and  
322  $\delta^{18}\text{O}$  values from  $-0.5$  to  $+1.1$ ‰, with an average of  $+0.39$  ‰ (Table 3). The  
323 isotopic composition of water samples from the thermal spring of Fetida Cave,  
324 reported by Santaloia et al. (2016), was  $0.60$  and  $0.05$  ‰ for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ,  
325 respectively. This single previous data practically coincides with the average  
326 isotopic composition of the water sampled in the end passage of the cave

327 (SCC1-FC1 and SCB1-FC4 sampling locations), where the main inputs of deep  
328 acid water are observed.

329 The isotopic data pairs of thermal water from Fetida Cave define a Local  
330 Water Line (LWL) (Fig. 2), which is also aligned with the only isotopic data for  
331 local meteoric water, as the ones reported by Santaloia et al. (2016) for a cold  
332 borehole located in the cave's water recharge area. The isotopic data for local  
333 meteoric water roughly matches the average isotopic composition of  
334 precipitation in the area, according to data for St. Maria di Leuca reported by  
335 Longinelli and Selmo (2003). Here, the LWL is fitted with a slope of 5.83 and  
336 deuterium excess of 0.29. This linear function is almost equal to the LWL fitted  
337 by Santaloia et al. (2016) (slope: 5.68 and deuterium excess: -0.69), but which  
338 tends to positive  $\delta^{18}\text{O}$  with respect to the GMWL (Clark and Fritz, 1997) and the  
339 "regional" MWL for Southern Italy (Longinelli and Selmo, 2003).

340 Both the thermal water of Fetida Cave and the previous spring water  
341 reported by Santaloia et al. (2016) have  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values along a line  
342 between the meteoric samples (cold well) and the seawater, suggesting a  
343 mixing of fresh, thermal and marine waters. The low deuterium excess of  
344 samples depends on the contribution of seawater, similar to what has been  
345 described for other sulfide-bearing waters due to the variable contribution of  
346 saline formation waters (Toscani et al., 2001). The positive  $\delta^{18}\text{O}$ -shift with  
347 respect to the GMWL also indicates the prevailing influence of a mixing process  
348 with seawater. Similar precipitation-seawater mixing lines have been isotopically  
349 defined to assess some other coastal processes as groundwater discharge in a  
350 hypersaline lagoon (Rocha et al., 2015) or the precise identification of river  
351 plumes within the Great Barrier Reef (Munksgaard et al., 2012).

352 There is a spatial gradient in the mixing process with seawater, in this way  
353 the  $\delta^{18}\text{O}$ - $\delta^2\text{H}$  data pairs of cave water are closer to those of meteoric water as  
354 we move away from the coastline (Fig. 2 and Table 3). As a reference,  
355 Santaloia et al. (2016) reported a mean isotopic composition of water from  
356 nearby non-thermal water boreholes as  $-32.34 \pm 1.86$ ,  $-5.57 \pm 0.15$  and  $-$   
357  $12.43 \pm 1.27$  for  $\delta^2\text{H}$ ,  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ -DIC, respectively (Table 3). The relative  
358 position of the data pairs on the precipitation-seawater mixing lines will vary  
359 over time and this will be determined by the tides controlling the sea level in  
360 each specific sampling period, as well as the degree of meteoric water recharge

361 at that time (previous rainfall). The  $\delta^{13}\text{C}$ -DIC values also show a gradient with  
362 distance from the shoreline, with more negative values in the innermost areas of  
363 the cave ( $< -2.5\text{‰}$  in SCC1-FC1 and SCB1-FC4 locations - Table 3) indicating  
364 an input of seepage water that is, in any case, distinguishable from seawater.  
365 The more negative  $\delta^{13}\text{C}$ -DIC values coincide with areas with more abundance  
366 of bacteria suggesting processes of biomediated  $\text{CO}_2$ -fixation.

367 An enrichment in hydrogen isotopes based on the  $^2\text{H}$  exchange between H-  
368 bearing species ( $\text{H}_2\text{O}$ – $\text{H}_2\text{S}$ ) has been previously reported in shallow  
369 groundwater mixed with  $\text{H}_2\text{S}$  enriched gases under volcanic and hydrothermal  
370 settings (Chiodini et al., 2000; Hsu and Yeh, 2020). In contrast, our current data  
371 set for Fetida Cave waters shows no evidence of an isotopic exchange of water  
372 with the hydrogen in  $\text{H}_2\text{S}$  linked to rising sulfuric acid fluids. This effect would  
373 lead to an increase in the  $\delta^2\text{H}$  content of the water and, graphically, the isotopic  
374 data pairs would lie along straight lines parallel to the ordinate axis ( $\delta^2\text{H}$ -axis), a  
375 behavior that is not observed in Figure 2.

376

### 377 3.2. Morphology of Fetida Cave white filaments

378 Filamentous morphologies were observed in the four samples of Fetida  
379 Cave, as depicted in Figure 3. A dense net of partially corroded filaments  
380 embedded in extracellular polymeric substances is shown in Figure 3A. Cells  
381 resembling *Arcobacter* morphology were evidenced in this figure. The structures  
382 and morphologies are similar to those previously reported by D'Angeli et al.  
383 (2019b) for filaments collected in this cave. These authors reported that the  
384 damaged structures can be associated with the constant exposure to rising  
385 acidic sulfidic water. Mineral grains were attached and/or entrapped by the  
386 filaments (Figs. 3B and D). D'Angeli et al. (2019b) concluded that the grains  
387 were sulfur crystals based on EDS spectra. The detailed morphology of the  
388 dense tangled masses of septate filaments observed in Figure 3C closely  
389 resemble those reported by Bauermeister et al. (2012) and Flot et al. (2014) for  
390 *Thiothrix* filaments.

391

### 392 3.3. Microbial community composition

393 Sequence quality control and construction of feature table using QIIME2  
394 and DADA2 resulted in a total 180,528 features or sequences for the four  
395 samples, clustering in a total of 5,209 amplicon sequence variants (ASVs). The  
396 mean length of sequences was 453.6 bp, with a range oscillating between 274–  
397 468 bp length. The most representative sample was FC6, with 62,628 features,  
398 whereas FC4 presented only 30,826. Besides, the number of ASVs found in the  
399 four samples varied in the 704–1,902 interval, where FC6 was observed to have  
400 the higher value and FC4 the sample with the lower number. Samples FC6 and  
401 FC1 presented 1,662 and 941 ASVs, respectively.

402 Summarizing the structure of the bacterial communities, assessing the  
403 number of taxonomic groups (richness), and the distribution of abundances of  
404 these groups (evenness) leads to a better understanding of the bacterial  
405 ecology. Bacterial community composition was measured through Shannon,  
406 Simpson and Chao indices, and Simpson's and Pielou's evenness. Thus,  
407 resulted values from alpha-diversity analysis (Table A2 in Appendix A -  
408 Supplementary data) showed the sample FC6 both more diverse and even,  
409 followed by FC7, since the values in every analyzed metric were differentially  
410 higher in these samples than in FC1 and FC4.

411 Table A3 (Appendix A - Supplementary data) shows that the microbial  
412 communities of the white filaments from Fetida Cave were almost totally  
413 composed of *Bacteria*, ranging from 97.9% (in FC6) up to 99.8% (in FC1).  
414 *Archaea* exhibited a low percentage ranging from 0.2% (in FC1) to 2.1% (in  
415 FC6) and were composed exclusively of *Woesearchaeia*. This distribution is  
416 similar to those reported for other caves (Itcus et al., 2018; Jurado et al., 2020).

417 Engel (2007) compared the 16S rRNA gene sequences retrieved from  
418 microbial mats from six active sulfidic caves (including Frasassi, Movile and  
419 Lower Kane caves) and revealed a diverse range of microorganisms among  
420 which the phyla *Epsilonbacteraeota*, *Proteobacteria*, and *Bacteroidetes*, were  
421 identified in all the caves. This agrees with our data on Fetida Cave (Table A4 in  
422 Appendix A - Supplementary data), in which the microbial mats resulted to be  
423 mainly composed of these three phyla with total relative abundances varying  
424 between 57.2 and 82.7%. Moreover, if the phylum *Patescibacteria* is also  
425 considered, the four samples under analysis reached between 72.6 and 88.2%  
426 of the total microbial community composition. This indicates that the microbial

427 mats populating the acidic sulfidic waters have a remarkable homogeneity in  
428 phyla distribution in different geographical settings. Less abundant phyla were  
429 *Planctomycetes*, *Spirochaetes*, and *Chloroflexi*, which were also detected in the  
430 caves studied by Engel (2007).

431 The phyla distribution presented in Table A4 (Appendix A - Supplementary  
432 data) is similar to Engel's (2007) findings. In fact, five additional bacterial phyla  
433 rarely exceed 1% of relative abundance in at least one of the white filaments  
434 investigated: *Actinobacteria*, *Calditrichaeota*, *Deferribacteres*, *Firmicutes*, and  
435 *Lentisphaerae*.

436 In the sulfidic caves studied by Engel (2007) the archaeal phylum  
437 *Euryarchaeota* (class *Methanomicrobia*) was identified, however, in the  
438 filaments from Fetida Cave *Nanoarchaeaeota* (class *Woesearchaeia*) was  
439 present, although the relative abundance of *Archaea* in Fetida Cave was  
440 scarce. The very low contribution of *Archaea* must be related with unfavorable  
441 environmental conditions. Patin et al. (2014) sampled a West Florida Shelf blue  
442 hole and found that *Woesearchaeia* comprised up to 40% of the water column  
443 community below the oxycline around 100 m, which also featured elevated  
444 sulfide levels. The metabolic pathways and potential biogeochemical roles of  
445 *Woesearchaeia* likely include a strict anaerobic lifestyle and possible syntrophy  
446 with a sulfate-reducing gammaproteobacterial clade (Castelle and Banfield,  
447 2018).

448 Figure 4 shows the heat-map of the bacterial classes occurring in the white  
449 filaments. These data were roughly in accordance with those of D'Angeli et al.  
450 (2019b) on Fetida Cave microbial communities, in which a high abundance of  
451 members related to sulfur metabolism and belonging to *Gammaproteobacteria*,  
452 *Deltaproteobacteria*, and *Epsilonbacteraeota* were found in white filaments.  
453 D'Angeli et al. (2019b) investigated floating and sedimented white filaments that  
454 were generally dominated by *Gammaproteobacteria* (12–34%), followed by  
455 *Deltaproteobacteria* (8–15%), *Alphaproteobacteria* (4–10%), and  
456 *Epsilonbacteraeota* (3–12%). Archaeal sequences accounted for a maximum of  
457 6% and were mainly affiliated with the phylum *Woesearchaeota*.

458 Conversely, in our samples, the phylum *Epsilonbacteraeota* (12.9–53.7)  
459 dominated the filaments, followed by *Proteobacteria* (18.0–39.4%). Among  
460 *Proteobacteria*, *Gammaproteobacteria* (10.0–16.4%), *Deltaproteobacteria* (5.9–

461 15.9), and *Alphaproteobacteria* (2.2–7.7%) were the most abundant classes.  
462 *Woesearchaeota* ranged between 0.2 and 2.1%. The different phyla and  
463 classes distribution can be attributed, among other reasons, to the diverse  
464 environmental conditions and the period of sampling (2015 vs 2017) as well as  
465 to different methodological procedures, including the use of different primer  
466 pairs in D'Angeli et al. (2019b) that might provide slightly different but  
467 complementary results (Wasimuddin et al., 2020).

468 The most abundant class was *Campylobacteria*, with relative abundances  
469 of 44.0 and 53.7% in samples FC1 and FC4, respectively, that decreased (12.9  
470 and 15.3%) in FC6 and FC7. With relative abundances above 15% appeared  
471 *Gammaproteobacteria* (F1 and F7) and *Deltaproteobacteria* (FC6 and FC7).  
472 The abundances of *Bacteroidia* ranged between 10.4 and 12.3% in FC4, FC6,  
473 and FC7. *Gracilibacteria* was the class with abundances above 5% (FC6 and  
474 FC7). Other classes reached abundances below 5%.

475 Figure 5 depicts the heat-map of the genera found in the microbial mats.  
476 The genus *Arcobacter* attained 35.3% of relative abundance in FC1 and 45.8%  
477 in FC4. Relative abundances above 5% were found for the genera *Arcobacter*  
478 in FC6, *Sulfurimonas* in FC7, and *Halothiobacillus* in FC1, whereas the genera  
479 *Arcobacter* in FC7, *Saprospira* in FC4, *Sulfurimonas* in FC1, FC4, and FC6,  
480 *Sulfurovum* in FC1, FC4, and FC7, *Thioflexothrix* in FC4, *Candidatus Thiobios*  
481 in FC7, and *Spirochaeta* in FC4 were identified between 5 and 2%. In the same  
482 range, unidentified members of the family *Rhodobacteraceae* and order  
483 *Campylobacteriales* were found in FC1, of the class WS6 (*Dojkabacteria*) in FC6  
484 and a gammaproteobacterium in FC7. Genera with relative abundances around  
485 1% were *Thermomarinilinea* in FC1, FC4, and FC6, *Sulfurovum* in FC6,  
486 *Desulfocapsa* in FC6 and FC7, MSBL7 in FC6, *Peredibacter* in FC7, and  
487 *Hydrogenovibrio* in FC1. In addition, a number of uncultured genera and  
488 families were unevenly distributed among the four samples. It must be noticed  
489 that the genus *Beggiatoa* was only retrieved in sample FC7, with a low relative  
490 abundance (0.7%) and *Thiothrix* reached even lower abundances (0.1-0.2%) in  
491 samples FC1, FC6 and FC7.

492 The high abundance of *Arcobacter* (45.8% in FC4 and 35.3% in FC1) is a  
493 novelty respect to previous studies on SAS caves (Macalady et al. 2006;  
494 D'Auria et al., 2018). This abundance in the two samples taken in the innermost



495 part of the cave can be related to the higher concentration of [HS<sup>-</sup>] dissolved in  
496 water and lower pH values.

497 Recently, Talà et al. (2021) studied the prokaryotic communities in  
498 Zinzulùsa, a submerged coastal cave at 6 km from Fetida Cave. In samples  
499 from submerged black crusts on the walls and bottom sediments (depths 1.9 m  
500 to 2.4 m) *Arcobacter* were lower than 0.01% and 0.07%, respectively. No  
501 filaments were reported. Anaerobic, sulfate-reducing bacteria (mainly  
502 *Thermodesulfovibrio* and *Fervidobacterium*) dominated the black crusts, but  
503 they were absent in Fetida Cave. This seems to indicate that Zinzulùsa  
504 anchialine waters were not favorable for the growth of *Arcobacter*.

505 Sievert et al. (2007) stated that *Candidatus Arcobacter sulfidicus* tolerates  
506 higher concentrations of H<sub>2</sub>S and grows at very low oxygen concentrations,  
507 which allows an efficient competition with other sulfur-oxidizing bacteria. This  
508 bacterium produces sulfur filament mats in high sulfidic waters (Wirsen et al.  
509 2002). The sulfur-oxidizer genus *Arcobacter* includes free-living species with the  
510 ability to fix CO<sub>2</sub>, and grow chemolithotrophically via sulfur-oxidation linked to  
511 denitrification. He et al. (2020) reported that *Arcobacter* showed a relative  
512 abundance of 24.1% in the oligotrophic, anoxic, and sulfidic bottom layer (100  
513 m) of Sansha Yongle Blue Hole, China. Therefore, the metabolic versatility of  
514 *Arcobacter* might provide a competitive advantage in the colonization of  
515 oligotrophic environments characterized by high sulfide, low oxygen, and  
516 dynamic fluid movement.

517 In a 60 m deep sulfide-rich groundwater, Deja-Sikora et al. (2019) found  
518 that the representations of *Beta-* and *Deltaproteobacteria* were small, while the  
519 *Epsilonbacteraeota* genera *Sulfurimonas*, *Sulfurovum*, and *Arcobacter* were  
520 very abundant (nearly 77%). Conversely, in waters collected from greater  
521 depths (148–300 m), the dominance of *Betaproteobacteria* and sulfate/sulfur-  
522 reducing *Deltaproteobacteria* was evident. The authors correlated the shift in  
523 microbial communities to depth, and changing nitrogen and oxygen contents.  
524 Macalady et al. (2008) indicated that high sulfide to oxygen ratio (> 150)  
525 promoted the intensive growth of *Epsilonbacteraeota* (e.g., *Sulfurovum* and  
526 *Arcobacter* among others) in the sulfidic water of the Frasassi Cave. Hotaling et  
527 al. (2019) found that the microbiome of H<sub>2</sub>S-rich stream waters in southern  
528 Mexico was composed of *Acidithiobacillus*, *Sulfuricurvum*, *Sulfurimonas*,

529 *Thiomonas*, and *Arcobacter*, where *Arcobacter* reached a relative abundance of  
530 2.1%.

531 According to Macalady et al. (2006) in Frasassi Cave white filaments are  
532 dominated by filamentous *Gammaproteobacteria* with *Beggiatoa*-like (cottony)  
533 or *Thiothrix*-like (feathery) cell morphologies and abundant sulfur inclusions.  
534 *Beggiatoa*-related clones were present in both biofilm types (cottony and  
535 feathery) and formed a monophyletic clade within the *Thiotrichaceae*. This clade  
536 accounted for almost half of the total sequences retrieved from the cottony  
537 biofilm. *Betaproteobacteria* and *Epsilonbacteraeota* related to *Thiobacillus*,  
538 *Arcobacter*, and other sulfur-oxidizing groups were retrieved in clone libraries  
539 but constituted a small fraction of the biomass in both biofilm types. In Fetida  
540 Cave, *Beggiatoa* was only found in sample FC7 with a minority abundance  
541 (0.7%) and *Thiothrix* was even lower (0.1–0.2%) in three samples (FC1, FC6,  
542 and FC7).

543 D'Angeli et al. (2019b) found *Arcobacter* in different samples of white  
544 filaments inside the cave, either floating or sedimented, but the higher relative  
545 abundance was 2.1% in a white filament at the bottom of the water stream close  
546 to the location of the rising H<sub>2</sub>S-rich fluids. Outside the cave, the presence of  
547 *Arcobacter* in white filaments is missing or insignificant (relative abundance  
548 0.2%). The authors suggested that this can be due to the selection imposed on  
549 the microbial diversity by the peculiar physico-chemical characteristics of the  
550 water inside the cave with a higher concentration of H<sub>2</sub>S, slower water flow and  
551 absence of light.

552 *Arcobacter* was also found in Monte Conca, a Sicilian gypsum cave with an  
553 active sulfidic spring in the inner part of the cave. Other important genera were  
554 *Sulfurovum*, *Sulfurimonas*, and *Thiovirga*. The spring generates a small pool  
555 where in summer, sulfur-oxidizing bacteria reached up to 95%, and in winter  
556 represented 13.6% of the total population. *Arcobacter* relative abundances were  
557 2.5 to 4.6% in summer and absent or insignificant in winter (0–0.2%) (Davis et  
558 al., 2020). These changes in abundances denote seasonal variations.

559 Apart from *Arcobacter*, other sulfur-oxidizing genera identified in the white  
560 filaments were *Sulfurimonas*, *Halothiobacillus*, *Thiothrix*, *Thioflexothrix*,  
561 *Thiomicrospira*, *Sulfurovum*, *Hydrogenovibrio*, *Candidatus Thiobios*, and  
562 *Beggiatoa*. These have been found in many of the investigated sulfide waters

563 (Brigmon et al., 2003; Engel, 2007; Boden et al., 2012; Rossmassler et al.,  
564 2012; Han and Perner, 2015; Fomenkov et al., 2017; Jiang et al., 2017; Deja-  
565 Sikora et al., 2019; Davis et al., 2020).

566 Villanueva Alvarez (2005) studied the occurrence and bacterial succession  
567 in sulfur-rich blooms in the Ebro delta river. She reported that the bacterial  
568 succession started with a dominance of *Beggiatoa*, followed by a *Spirillum*-  
569 bloom composed of a high population of *Arcobacter* and after the dominance of  
570 *Spirillum*-like cells a spirochaetal bloom was observed in which *Halothiobacillus*  
571 and *Thiomicrospira* were detected. The succession was related to the  
572 occupation of microniches at different oxygen and sulfide concentrations. These  
573 could be the factors inducing the abundant population of *Arcobacter* in Fetida  
574 Cave.

575 Sulfur-reducing bacteria, such as *Desulfocapsa* were also present in the  
576 filaments from Fetida Cave, which indicates that microorganisms mediate both  
577 sulfur oxidation and sulfur reduction within the white filaments and stream.  
578 *Desulfocapsa* has been previously found in Lower Kane and Frasassi caves  
579 (Engel, 2007). Other sulfur-reducing bacteria were the lineage MSBL7 (Häusler  
580 et al., 2014; Nigro et al., 2020) and *Spirochaetes* (Berlanga et al., 2008;  
581 Dubinina et al., 2011).

582 Within the *Patescibacteria*, Wrighton et al. (2016) identified RubisCO genes,  
583 with a central role in CO<sub>2</sub> fixation in members of the *Dojkabacteria* (WS6) and  
584 *Parcubacteria* (*Candidatus* Moranbacteria). *Candidatus* Moranbacteria are  
585 relatively abundant in groundwaters (Probst et al., 2018). Other *Patescibacteria*  
586 (*Gracilibacteria*) have limited metabolism and were predicted to be symbionts,  
587 possibly episymbionts (Sieber et al., 2019).

588 The anaerobic ammonium-oxidating SM1A02 lineage (*Planctomycetes*) was  
589 found in an anaerobic biological reactor, using ammonium and sulfate as the  
590 substrate to start sulfate-reducing ammonium oxidation (Zhang et al., 2019).  
591 Other anaerobic bacteria include *Thermomarinilinea* (*Chloroflexi*) first isolated  
592 from a submarine hot spring (Nunoura et al., 2013).

593 The identification of the genus *Peredibacter* and an uncultured bacterium  
594 from the family *Micavibrionaceae* is remarkable, which points to a well-  
595 established trophic chain in the cave. These bacteria belong to the *Bdellovibrio*-  
596 and-like organisms (BALOs), composed of *Bdellovibrionaceae*, *Bacteriovorax*,

597 *Peredibacter*, and *Micavibrio*, and are widespread obligatory predators of other  
598 Gram-negative bacteria, including cyanobacteria (Davidov et al., 2006; Cai et  
599 al., 2014). In addition, *Saprospira*-like organisms have been reported to behave  
600 as predators of bacteria and algae (McIlroy and Nielsen, 2014).

601

#### 602 3.4. 16S rRNA gene-based metabolic inference

603 The most relevant ecological roles of microbial communities in Fetida Cave  
604 were analyzed by FAPROTAX. Almost 25% of ASVs were assigned to at least  
605 one group of the 43 found in the analysis; the remaining percentage could not  
606 be assigned to any group (Fig. 6). This database is not exhaustive; therefore,  
607 only small proportions of ASVs were assigned.

608 The major predicted ecological functions were chemoheterotrophy (7.6–  
609 26.6%), respiration of sulfur compounds (8.5–18.8%), dark oxidation of sulfur  
610 compounds (5.8–15.8%), and fermentation (3.3–14.7%). Chemoheterotrophy  
611 was predicted to be greater in FC4, in which *Proteobacteria* (*Delta*- and  
612 *Gammaproteobacteria*), and *Bacteroidetes* were the most abundant involved  
613 phyla. In addition, the abundance of sequences assigned to the respiration of  
614 sulfur compounds and sulfate respiration was higher in FC6 and, slightly, in  
615 FC7. However, FC6 was the sample with the fewest predicted sequences within  
616 the dark oxidation of sulfur compounds group. Respiration of sulfur compounds  
617 was mainly assigned to the order *Desulfobacterales* while dark oxidation of  
618 sulfur compounds was related to the genera *Sulfurimonas*, *Halothiobacillus*, and  
619 *Beggiatoa*. In a similar way, the abundance of *Desulfobacterales* was related to  
620 the higher abundance of sulfite respiration and thiosulfate respiration predicted  
621 functions in FC6 and FC7, but there was no linkage with the sulfur respiration.  
622 FAPROTAX predictions also identified *Halothiobacillus* as the main taxon  
623 assigned to dark thiosulfate oxidation, dark sulfide oxidation and dark sulfur  
624 oxidation. Dark oxidation of sulfur compounds was not predicted by the  
625 FAPROTAX analysis for the genus *Arcobacter*, probably due to the absence of  
626 metabolic information on *Arcobacter* in the database.

627 Wirsén et al. (2002) related *Candidatus* *Arcobacter sulfidicus*, a  
628 chemolithoautotrophic sulfur oxidizer, to CO<sub>2</sub> fixation and filamentous sulfur  
629 formation. This bacterium has the capacity to fix carbon via the reductive

630 tricarboxylic acid cycle (Hügler et al., 2005). More recently, Nogueroles et al.  
631 (2015) reported that a member of the genus *Arcobacter* fixes CO<sub>2</sub> in the dark in  
632 the sulfidic redoxcline of a meromictic karstic lake, via the same cycle, and  
633 Evans et al. (2018) revealed that *Arcobacter*, associated with filamentous sulfur  
634 material, have the ability to fix CO<sub>2</sub>.

635 Some previous studies on laboratory-grown strains of sulfur-oxidizing  
636 bacteria described a <sup>13</sup>C depletion in the fixed medium with respect to the  
637 ambient source of CO<sub>2</sub> and bicarbonates (Ruby et al., 1987). Turning the  
638 spotlight once more on our δ<sup>13</sup>C-DIC dataset (Table 3), an important difference  
639 in δ<sup>13</sup>C-DIC (around +8 ‰) is noticeable between the meteoric water and  
640 thermal water from Fetida Cave, particularly at the cave locations with greater  
641 evidence of water recharge from the upper vadose zone. These locations  
642 correspond to the innermost points of the cave, where the mixing with seawater  
643 is lower and a greater abundance of white filaments was also detected.  
644 Therefore, the *Arcobacter* from Fetida Cave might likely be involved in the <sup>12</sup>C  
645 fixation from the bicarbonates dissolved in the mixture of meteoric water  
646 (seepage water) and seawater, and this would cause a carbon isotopic  
647 fractionation in the bicarbonates resulting in higher δ<sup>13</sup>C-DIC values in relation  
648 to the typical isotopic values of bicarbonates in the seepage water.

649 Fermentation was predicted in FC4 associated to the genus *Spirochaeta*.  
650 The implication of *Spirochaeta* in the sulfur cycle was not predicted by  
651 FAPROTAX; however, the existence of sulfide-oxidizing and sulfur-reducing  
652 bacteria within the genus *Spirochaeta* has been described by Dubinina et al.  
653 (2011) in sulfide-rich water from a saline spring. Deja-Sikora et al. (2019)  
654 reported the coexistence of sulfur-oxidizing and sulfur-reducing bacteria in  
655 Polish sulfidic waters. In addition, many of the genera they found were the same  
656 as those retrieved from Fetida Cave. A similar coexistence was found in marine  
657 sediments (Ihara et al., 2019).

658 In light of the data shown, *Arcobacter* appears as a sulfur-oxidizing  
659 microorganism in coastal seawater that produces filaments trapping sulfur  
660 crystals and exhibits nitrogen fixation in concurrence with carbon dioxide  
661 fixation, as suggested by the water carbon isotope values.

662 The high variability in the composition of the white filaments, at the lower  
663 taxonomic levels, even in the same cave in different sampling periods, is due to  
664 changes in the water hydrochemistry and hydrodynamics, subjected to tidal  
665 water level fluctuation and the variable mixing of fresh continental with marine  
666 water.

667 Similar fluctuations were observed in Polish sulfide-rich mineral waters,  
668 since the composition of the bacterial communities strongly varied across the  
669 samples. However, most of the bacteria participating in the sulfur cycle were  
670 common in all sulfidic waters (Deja-Sikora et al., 2019). This was also found in  
671 Fetida Cave, where a certain homogeneity in phyla distribution and abundance  
672 can occur at higher taxonomic levels, due to the selection imposed by well-  
673 defined environmental conditions: the sulfide-rich water, and the bacterial  
674 groups participating in the sulfur cycle that were common to sulfidic waters.

675

#### 676 **CRedit authorship contribution statement**

677 Valme Jurado, Ilenia D'Angeli, Tamara Martin-Pozas, Martina Cappelletti,  
678 Daniele Ghezzi, Jose Luis Gonzalez-Pimentel, Soledad Cuezva and Ana Zelia  
679 Miller: Investigation. Angel Fernández-Cortès: Investigation, writing - original  
680 draft. Jo De Waele: Conceptualization, review & editing, supervision. Sergio  
681 Sanchez-Moral: Conceptualization, Writing - original draft, Funding acquisition.  
682 Cesareo Saiz-Jimenez: Conceptualization, Supervision, Project administration,  
683 Funding acquisition, Writing – original draft, Writing - review & editing.

684

#### 685 **Declaration of competing interest**

686 The authors declare that they have no known competing financial interests or  
687 personal relationships that could have appeared to influence the work reported  
688 in this paper.

689

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700

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1059

1060 FIGURE LEGENDS

1061

1062 Figure 1. The planimetry of Fetida Cave and the sampling locations (Image G)  
1063 are shown on the right. Microbial (FC1, FC4, FC6, FC7) and water (SCA1,  
1064 SCAfen, SCB1, SCC1) samples are reported in yellow, whereas gas samples  
1065 are shown in red. The H<sub>2</sub>S spring is indicated with a black line and corresponds  
1066 to the SCB1-FC4 sampling location. The collected white floating filaments and  
1067 their respective habitats (Images from A to F) are visible on the left. AE means  
1068 anthropic entrance, whereas NE natural entrance.

1069

1070 Figure 2. Binary  $\delta^2\text{H}-\delta^{18}\text{O}$  diagram for thermal water samples from Fetida  
1071 Cave, compared with the isotopic composition of meteoric and thermal spring,  
1072 in accordance to Santaloia et al. (2016), and local rainfall. The local water  
1073 meteoric line (black dashed line) is plotted in relation to the water meteoric line  
1074 for southern Italy (Longinelli and Selmo, 2003) and the GMWL (Clark and Fritz,  
1075 1997).

1076

1077 Figure 3. White filaments from Fetida Cave. A: Sample FC1. Damaged  
1078 filaments, extracellular polymeric substances and *Arcobacter*-like cells (in the  
1079 center). B and C: Net of filaments from sample FC6. Magnification of C shows  
1080 *Thiothrix*-like filaments. D: Filaments from sample FC7 and associated mineral  
1081 grains of sulfur.

1082

1083 Figure 4. Heat-map analysis of Fetida Cave samples with taxonomic  
1084 identifications of *Bacteria* at class level. The classes are described in the right  
1085 column and their respective abundances included in the boxes. Colored left bar  
1086 groups the classification at phylum level.

1087

1088 Figure 5. Heat-map analysis of Fetida Cave samples with taxonomic  
1089 identifications of *Bacteria* at family/genus level. The families/genera are  
1090 described in the right column and their respective abundances included in the  
1091 boxes. Colored left bar groups the classification at order level.

1092

1093 Figure 6. FAPROTAX analysis of white filaments from Fetida Cave with the  
1094 predicted ecological functions based on 16S rRNA genes (Y axis). The size of  
1095 the cycles indicates the relative abundance of the assigned ASVs

