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# Temperature and nutrients as drivers of microbially mediated arsenic oxidation and removal from Acid Mine Drainage

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29 **Abstract**

30 Microbial oxidation of iron (Fe) and arsenic (As) followed by their co-precipitation lead to the natural attenuation  
31 of these elements in As-rich Acid Mine Drainage (AMD). The parameters driving the activity and diversity of  
32 bacterial communities responsible for this mitigation remain poorly understood. We conducted batch experiments  
33 to investigate the effect of temperature (20 vs 35°C) and nutrient supply on the rate of Fe and As oxidation and  
34 precipitation, the bacterial diversity (high-throughput sequencing of 16S rRNA gene) and the As oxidation  
35 potential (quantification of *aioA* gene) in AMD from the Carnoulès mine (France). In batch incubated at 20°C, the  
36 dominance of iron-oxidizing bacteria related to *Gallionella* spp. was associated with almost complete iron  
37 oxidation (98%). However, negligible As oxidation led to the formation of As(III)-rich precipitates. Incubation at  
38 35°C and nutrient supply both stimulated As oxidation (71-75%), linked to a higher abundance of *aioA* gene and  
39 the dominance of As-oxidizing bacteria related to *Thiomonas* spp. As a consequence, As(V)-rich precipitates (70-  
40 98% of total As) were produced. Our results highlight strong links between indigenous bacterial community  
41 composition and iron and arsenic removal efficiency within AMD, and provide new insights for the future  
42 development of a biological treatment of As-rich AMD.

43 **Keywords:** Acid Mine Drainage, arsenic and iron oxidation, bacterial community, temperature, nutrient.

## 44 Introduction

45 Arsenic (As) is one of the most toxic pollutants commonly associated with mine tailings and Acid Mine  
46 Drainage (AMD) with concentration in mine waters ranging from  $< 1 \mu\text{g l}^{-1}$  to hundreds of  $\text{mg l}^{-1}$  (Casiot et al.  
47 2003a; Cheng et al. 2009). Because of the severe toxicological effects of As, contaminated waters represent a  
48 serious threat for ecosystems located downstream from mining sites and for public health. Numerous studies have  
49 reported natural attenuation of arsenic pollution in different AMD across the world (Fukushi et al. 2003; Asta et  
50 al. 2010; Egal et al. 2010). The exploitation of the microbially mediated processes involved in this attenuation  
51 represents a promising strategy for the development of treatment of As-rich AMD (Johnson and Hallberg 2005).

52 Natural attenuation involves biological oxidation of ferrous iron (Fe(II)) to ferric iron (Fe(III)) and the  
53 subsequent adsorption of As onto the newly formed Fe(III) precipitates or its co-precipitation (Paikaray 2015;  
54 Ahoranta et al. 2016). Efficiency of arsenic removal from AMD depends on its redox speciation. Indeed, under  
55 acid pH, arsenate (As(V)) is more efficiently trapped onto iron phases than arsenite (As(III)) (Hug and Leupin  
56 2003). In the environment, As(III) oxidation to As(V) is mainly catalyzed by microbial activity, chemical oxidation  
57 being generally very slow (Campbell and Nordstrom, 2014). Therefore, the ability of indigenous bacterial  
58 populations to oxidize As(III) largely contributes, together with the activity of iron-oxidizing bacteria (FeOB), to  
59 a sustainable arsenic pollution mitigation in AMD. FeOB and As(III)-oxidizing bacteria (AsOB) have been  
60 isolated from AMD and their metabolic capacities were investigated (Battaglia-Brunet et al. 2002; Duquesne et al.  
61 2003; Bruneel et al. 2003; Casiot et al. 2003b; Egal et al. 2010). However, the factors driving the activity and  
62 diversity of indigenous complex populations of FeOB and AsOB involved in arsenic mitigation remain poorly  
63 understood.

64 *Acidithiobacillus ferroxydans*, a FeOB associated with attenuation process in AMD, is a strict  
65 chemolithoautotroph bacterium (Duquesne et al. 2003; Johnson and Hallberg 2005; Egal et al. 2009). Conversely,  
66 AsOB can grow heterotrophically or autotrophically (Santini et al. 2000; Battaglia-Brunet et al. 2006; Garcia-  
67 Dominguez et al. 2008). In particular, *Thiomonas* spp. are facultative chemolithoautotrophs that grow optimally  
68 in mixotrophic media containing reduced inorganic sulfur compounds and organic supplements (Kelly et al. 2007;  
69 Bryan et al. 2009; Slyemi et al. 2011). Thus, nutrient supply in AMD is expected to have a contrasted incidence  
70 on pollution mitigation depending on the metabolic feature of the bacterial populations involved. Temperature is  
71 another primary factor governing activity and diversity of bacterial community inhabiting AMD (Méndez-García  
72 et al. 2015). Previous work on FeOB and AsOB bacterial strains isolated from diverse polluted environments  
73 showed that their growth and their oxidation activities were temperature dependent (Battaglia-Brunet et al. 2002;  
74 Dopson et al. 2006; Kim et al. 2008; Ito et al. 2012). Furthermore, temperature variation was suggested as a driving  
75 factor shaping bacterial communities structure in AMD (Volant et al. 2014). Recently, Debiec and colleagues  
76 (2017) showed that both nutrient concentration and temperature were key factors controlling the growth and the  
77 oxidation rate of *Sinorhizobium* sp. M14, an AsOB isolated from neutral gold mine waters. Under batch conditions,  
78 a temperature increase (from 10 to 23 or 30°C) resulted in the stimulation of bacterial growth associated with a  
79 faster As(III) oxidation rate. Under continuous conditions, a supply of yeast extract stimulated both the growth  
80 and the As(III) oxidation activity of *Sinorhizobium* sp. M14 (Debiec et al. 2017). Whether or not such studies  
81 based on a single strain may be extrapolated to a metabolically and taxonomically diverse indigenous community  
82 has not been explored so far. In this context, the aim of the present study was to assess the influence of temperature

83 and nutrients on the diversity of an indigenous AMD bacterial community and on its efficiency for iron and arsenic  
84 oxidation and removal from water.

85 For this purpose, we conducted batch experiments with As-rich AMD water of Carnoulès mine (Southern  
86 France) incubated either at 20°C or 35°C and supplied or not with yeast extract. Iron and arsenic speciation was  
87 monitored in the dissolved phase and in the biogenic precipitates that formed during batch incubation. Diversity  
88 of bacterial community was characterized by high-throughput sequencing of 16S rRNA genes and the genetic  
89 potential for arsenic oxidation was evaluated by the quantification of *aioA* genes.

## 90 **Materials and methods**

### 91 **Water sampling and batch experiment setup**

92 Water was collected in June 2015 from the source of Reigous creek at the abandoned Carnoulès mine  
93 (Southern France: 44°7'2.14"N; 4°0'6.00"E). Its physicochemical characteristics were as follows: pH 4.7, 2.69  
94 mg O<sub>2</sub> l<sup>-1</sup>, 14.9°C, 2.56 mS cm<sup>-1</sup>, 590.6 mV, 511 mg Fe l<sup>-1</sup> (100% Fe(II)), 53.8 mg As l<sup>-1</sup> (14% As(V)).

95 Batch experiment was setup within six hours of water collection by transferring 450 ml of water in 1 liter  
96 Schott Duran® bottles, previously acid-cleaned and autoclaved. Two conditions were tested: biotic (water with  
97 indigenous microbial communities) and abiotic (sterile-filtered water with 0.22 µm cellulose acetate filter). For  
98 each condition, four treatments ("T") were applied: (i) 20°C without nutrient supply ("T20"), (ii) 35°C without  
99 nutrient supply ("T35"), (iii) 20°C with nutrient supply ("T20Y"), and (iv) 35°C with nutrient supply ("T35Y").  
100 Nutrient supply consisted of spiking water with yeast extract at a final concentration of 0.2 g l<sup>-1</sup>. This concentration  
101 is typically used in culture media for the heterotrophic growth of AsOB like *Thiomonas* spp. (Battaglia-Brunet et  
102 al. 2002; 2006). A total of 12 biotic batch experiments (four treatments, three replicates) and eight abiotic batch  
103 experiments (four treatments, two replicates) were set up. All batch were closed with cellulose stoppers to prevent  
104 bacterial contamination from outside and to allow oxygen diffusion inside the batch. Batch were placed under  
105 orbital agitation at 150 rpm in thermo-regulated chambers to assure water aeration and a constant temperature (set  
106 up at 20°C or 35°C) throughout the experiment duration.

### 107 **Batch experiment monitoring**

108 The experiment was conducted for eight days. Water samples (~3 ml) were collected from the bottles for  
109 chemical analysis at days 0, 2, 3, 4, 5, 6, 7 and 8. At day 3, 5 and 8, pH and dissolved oxygen were measured with  
110 a multiparameter analyser (Ultrameter™ Model 6P). Dissolved oxygen remained stable, with an average of 7.8 ±  
111 0.5 mg O<sub>2</sub> l<sup>-1</sup> in all batch (data not shown). pH decreased during incubation from 4.7 to 2.7 ± 0.2 and 3.6 ± 0.1 in  
112 the biotic and abiotic experiments respectively (data not shown), in relation with Fe(II) oxidation and subsequent  
113 Fe(III) hydrolysis (Nordstrom and Alpers 1999). After sampling, water was immediately filtered through  
114 disposable filters (cellulose acetate, pore size 0.22 µm) and the filtrate was analyzed for Fe(II), total Fe, As(III)  
115 and As(V) concentration according to routine procedures described in Fernandez-Rojo et al. (2017).

116 At the end of the experiment, batch were sacrificed. After homogenization of liquid and solid phases  
117 (including biogenic precipitates that formed during the incubation in biotic batch), a subsample (100 ml) was  
118 filtered on sterile 0.22 µm cellulose acetate filters. The filters were stored at -80°C before DNA extraction. Another  
119 subsample (3 ml) was collected for biomass quantification by flow cytometry. At last, the remaining batch content  
120 (~300-350 ml) was filtrated using 0.22 µm cellulose acetate filter for quantification of total iron and arsenic species  
121 (As(III) and (V)) in the particulate fractions. The filter was placed in a vacuum desiccator, dried until constant  
122 weight and a chemical extraction with orthophosphoric acid was performed as described in Resongles *et al.* (2016).

### 123 **Chemical analyses**

124 Total dissolved Fe and Fe(II) concentrations were determined with a spectrophotometer (SECOMAN  
125 S250, detection limit = 88 µg l<sup>-1</sup>, uncertainty = ± 5 %) at 510 nm wavelength (Rodier 1996). For As speciation  
126 analysis in the dissolved phase and precipitate extracts, samples were analyzed with HPLC (High Performance

127 Liquid Chromatography) using an anion exchange column (25 cm x 4.1 mm i.d. Hamilton PRP-X100) coupled to  
128 ICP-MS (PQ2+, X Series, Thermo; Detection limit = 0.2 µg l<sup>-1</sup> for As(III), 0.4 µg l<sup>-1</sup> for As(V), uncertainty = ± 5  
129 %) (Héry et al. 2014; Resongles et al. 2016). The Certified Reference Water NIST1643e was used to check the  
130 analytical accuracy for total As concentration and the RSD was always lower than 5 % with respect to the certified  
131 value.

132 The proportion of arsenic oxidized after the eight-day incubation period was calculated using the  
133 following equation:

$$134 \text{As(III)}_{\text{oxidized}} = \frac{(\text{As(III)}_d t^0 + \text{As(III)}_p t^0) - (\text{As(III)}_d t^{\text{final}} + \text{As(III)}_p t^{\text{final}})}{(\text{As(III)}_d t^0 + \text{As(III)}_p t^0)} \times 100$$

135 where As(III)<sub>d</sub> and As(III)<sub>p</sub> were the concentration of dissolved and particulate As(III) (in mg l<sup>-1</sup>), respectively, at  
136 the beginning of experiment (t<sup>0</sup>) or after 8 days of incubation (t<sup>final</sup>).

### 137 **Biomass quantification**

138 Quantification of bacterial biomass from samples (water and precipitate) was performed by flow  
139 cytometric counting method. Prior analysis, bacterial cells were detached from mineral biogenic precipitates  
140 according to the procedure of Lunau et al. (2005), with some modifications. Briefly, methanol 100% was added to  
141 water sample to reach a final concentration of 10% and incubated 15 min in an ultrasonic bath (42 kHz). Detrital  
142 and inorganic particles were removed by a low centrifugation (1 min at 190 × g) and supernatant was recovered in  
143 nine volumes of sterile Milli-Q® water. Then, bacterial cells were pooled by centrifugation at 6500 × g for 10 min,  
144 washed in Milli-Q® water and stained 15 min in the dark with the LIVE/DEAD BacLight bacterial viability kit  
145 (Invitogen, Carlsbad, CA, USA). Bacterial cells abundance was measured with a Gallios™ flow cytometer  
146 (Beckman Coulter, Brea, CA). Live and dead bacterial cells were detected at 520 nm and 630 nm, respectively.  
147 More than 20,000 analytical events were counted in triplicate for each sample and data analysis was processed  
148 with Kaluza software (Beckman Coulter). Since the numbers of live and dead bacteria were in the same order of  
149 magnitude in all the samples, only the total bacteria cells (*i.e.* live + dead bacteria) are presented.

### 150 **DNA extraction and quantification**

151 For each biotic treatment, DNA was extracted from 100 ml of homogenised and filtered batch content (as  
152 described in 2.2) using the Powerwater DNA Isolation Kit according to the manufacturer's recommendations  
153 (MoBio Laboratories Inc., Carlsbad, CA, USA). DNA was quantified with a fluorometer (Qubit®, Invitrogen) and  
154 stored at -20°C until further analysis.

### 155 **Quantification of *aioA* genes**

156 The abundance *aioA* genes encoding the catalytic subunit of the As(III)-oxidase was determined by  
157 quantitative real-time PCR (qPCR). The reverse primers aoxBM2-1R and forward primer aoxBM4-1F were used  
158 to target a 110 bp fragment of the *aioA* gene (Quéméneur et al. 2010). For each sample, 2 ng of DNA were used  
159 in a 20 µl PCR reaction with 0.3 µM of each primer, 100 ng of T4GP32 (MP Biomedicals) and 1X IQ SYBR  
160 Green Supermix (BioRad). The program was run in a CFX Connect (BioRad) and consisted in an initial  
161 denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 54 °C for 20 s, 72 °C for 10 s, and a data

162 acquisition step at 80 °C for 10 s. At the end, a melting curve analysis was performed through measurement of the  
163 SYBR Green I signal intensities during a 0.5 °C temperature increment every 10 s from 65 °C to 95 °C.

#### 164 **Sequencing of 16S rRNA gene**

165 Bacterial diversity was determined by Illumina high-throughput sequencing of bacterial 16S rRNA genes.  
166 V4-V5 region (about 450 bases) was amplified by PCR using primers PCR1\_515F (Barret et al. 2015) and  
167 PCR1\_928R (Wang and Qian 2009). For each sample, 10 ng of DNA were used in a 50 µl PCR reaction conducted  
168 under the following conditions: 94°C for 2 min, 30 cycles of 1 min at 94°C, 65°C for 40 s and 72°C for 30 sec,  
169 followed by 10 min at 72°C. The PCR products were checked by gel electrophoresis and quantified using a  
170 fluorometer (Qubit®, Invitrogen) and sent to GeT-PlaGe platform (Toulouse, France) for Illumina MiSeq analysis  
171 using a 2×300 bp protocol.

#### 172 **Bioinformatic analyses of 16S rRNA gene sequences**

173 Raw sequence reads were merged into full-length sequences by FLASH v1.2.11 (Magoc and Salzberg  
174 2011). Reads were further processed using the software program MOTHUR version 1.31 (Schloss et al. 2009).  
175 Firstly, raw sequences were selected based on the following criteria: (i) length (between 350 and 460 bp), (ii)  
176 homopolymer lengths (< 7) and (iii) the absence of ambiguous bases. Then, sequences were aligned against the  
177 SILVA reference database (Release 123) and removed when they did not align correctly. Chimeric sequences were  
178 detected and removed using the implementation of Chimera UCHIME (Edgar et al. 2011). A further screening  
179 step (pre-cluster) was applied to reduce sequencing noise by clustering reads differing by only one base every 100  
180 bases. Taxonomic affiliation of 16S rRNA genes was performed with a Bayesian classifier (Wang et al. 2007)  
181 (80% bootstrap confidence score) against the SILVA reference database. In order to efficiently compare the  
182 datasets and avoid biased community comparisons, the sample reads were reduced to the lowest datasets by random  
183 selection (4353 reads). The remaining high quality sequences were used to generate a distance matrix and clustered  
184 into Operational Taxonomic Units (OTUs) defined at 97% cutoff using the average neighbor algorithm. OTU-  
185 based diversity indices, rarefaction curves and Unifrac distance were calculated with MOTHUR at a level of 97%  
186 sequence similarity. The raw datasets are available on the European Nucleotide Archive system under project  
187 accession number PRJEB21683.

#### 188 **Statistical analysis**

189 The statistical significance of the bacterial biomass increase during batch incubations was assessed with  
190 the nonparametric Kruskal-Wallis test. Physicochemical parameters, oxidation rates, diversity metric and *aioA*  
191 genes quantification obtained for the biotic treatments were compared by two-way ANOVA and the differences  
192 between them analyzed with a Fisher test ( $P < 0.05$ ). Differences in bacterial community structure between biotic  
193 treatments were characterized using UniFrac distance (Lozupone and Knight 2005). Non Metric Multidimensional  
194 Scaling (NMDS) was used to graphically depict differences between the bacterial communities. The significance  
195 of the observed clustering of samples on the ordination plot was assessed by an ANalysis Of SIMilarity (ANOSIM,  
196 999 permutations). All these statistical analyses were performed with the R free software ([http://www.r-  
197 project.org/](http://www.r-project.org/)).

198



## 199 **Results**

### 200 **Bacterial biomass**

201 Bacterial biomass initially present in the water collected at the Reigous spring was  $1.4 \pm 0.8 \times 10^4$  bacterial  
202 cells  $\text{ml}^{-1}$ . After 8 days of incubation in biotic batch, bacterial cell concentration increased from 1 to 3 order of  
203 magnitude depending on the treatment applied ( $T20 \approx T35 < T35Y < T20Y$ ) (Fig. 1). In abiotic batch, small  
204 particles that most probably corresponded to Fe colloids induced a background noise ranging from  $2.6 \times 10^3$  to  $1.8$   
205  $\times 10^4$  particles  $\text{ml}^{-1}$ .

### 206 **Evolution of dissolved Fe(II), dissolved As(III) and precipitated Fe concentrations**

207 In the biotic batch experiments, the concentration of dissolved Fe(II) and As(III) decreased substantially  
208 over time (Fig. 2A and 2B). In batch incubated at 20°C without nutrient (T20), Fe(II) concentration decreased  
209 gradually from 516  $\text{mg l}^{-1}$  to 20  $\text{mg l}^{-1}$ . At 35°C without nutrients (T35), Fe(II) concentration decreased more  
210 rapidly during the first 5 days of incubation, and then remained stable (136  $\text{mg l}^{-1}$ ). At 20°C with nutrients (T20Y),  
211 there was only a slight decrease of Fe(II) throughout time, with a final concentration of 393  $\text{mg l}^{-1}$ . At 35°C with  
212 nutrient supply (T35Y), the decrease of Fe(II) concentration was delayed; however, a drastic decrease occurred  
213 between day 5 and day 7, reaching a final concentration of 7  $\text{mg l}^{-1}$  of Fe(II) (Fig. 2A).

214 Nutrient supply and higher temperature (T20Y, T35 and T35Y) induced a stronger decrease of As(III)  
215 concentration compared to the 20°C treatment (T20) in the biotic batch experiments (Fig. 2B). The combination  
216 of higher temperature and nutrient supply (T35Y) resulted in a fast and complete removal of As(III) after only  
217 three days. Complete removal of As(III) was also achieved after 7 days in biotic batch incubated at 35°C without  
218 nutrients (T35) and in biotic batch incubated at 20°C with nutrients (T20Y). In the T20 batch, only 67% of the  
219 As(III) was removed from the dissolved phase in 8 days (Fig. 2B).

220 Under biotic conditions, the decrease of the dissolved Fe(II) concentration was closely related to Fe  
221 precipitation. Consistent trends were observed for the two curves in the different batch experiments (Fig. 2A and  
222 C). Slight differences between dissolved Fe(II) loss and precipitated Fe can be explained by the presence of few  
223 dissolved Fe(III).

224 In abiotic batch experiments, the concentration of dissolved Fe(II) and As(III) decreased no more than  
225 0.6 % and 19 % respectively (Fig. 2A and B).

### 226 **Iron and arsenic species in the particulate phase**

227 The amount of precipitates formed after eight days under biotic conditions was 4- to 11- fold higher than  
228 under abiotic conditions (Table 1). In these biogenic precipitates, the As/Fe and As(III)/As(V) ratios showed wide  
229 variations depending on the treatments applied compared to narrow variations observed in the particles formed  
230 abiotically (Table 1). Nutrient-amended biotic batch incubated at 20°C (T20Y) exhibited a significantly higher  
231 As/Fe ratio (0.60) than the other treatments ( $\text{As/Fe} \leq 0.23$ ), which is consistent with the limited iron oxidation and  
232 precipitation observed in these batch (Fig. 2A and 2C). In T20 batch, the particulate phase was As(III)-rich  
233 ( $\text{As(III)/As(V)}$  ratio = 6.48) suggesting that the decrease of dissolved As(III) (Fig. 2B) was not due to its oxidation.  
234 Conversely in T20Y, T35, T35Y batch experiments, the biogenic precipitates were As(V)-rich (average

235 As(III)/As(V) ratio  $\leq 0.43$ ). For these treatments, the decrease of dissolved As(III) observed (Fig. 2B) may then  
236 be linked to a possible As(III) oxidation. The T35Y treatment resulted in the formation of the biogenic precipitate  
237 the more enriched in As(V) (As(III)/As(V) ratio= 0.02).

### 238 **Iron and arsenic oxidation**

239 The proportion of iron and arsenic oxidized during the incubation was calculated based on the  
240 concentration of dissolved and particulate arsenic and iron species at the beginning and at the end of experiment  
241 (Fig. 3). Under abiotic conditions, iron oxidation was negligible and As(III) oxidation did not exceed 6 %. Under  
242 biotic conditions, the lowest proportion of Fe(II) oxidized was observed for the T20Y treatment (24%). No  
243 biological As(III) oxidation was observed in T20 batch experiments. Conversely, the proportion of As(III) oxidized  
244 exceeded 70% in the others treatments (T20Y, T35 and T35Y) (Fig. 3). The highest proportion of As(III) oxidized  
245 was obtained in nutrient supplied batch incubated at 35°C (98%).

### 246 **Diversity of bacterial communities**

247 High-throughput sequencing yielded a total of 119,065 sequences of 16S-rRNA gene corresponding to  
248 4353 quality sequences per sample which adequately covered the bacterial diversity in all the experiments (Table  
249 2 and Fig. S1). For all the treatments, the bacterial diversity indices (Richness, Evenness and Shannon) decreased  
250 significantly after incubation compared to the initial water collected at the Reigous spring. The batch incubated at  
251 20°C without nutrients (T20) exhibited the lower level of bacterial diversity. The higher richness was observed in  
252 the batch incubated at 35°C (Table 2).

253 NMDS analysis of the full bacterial-sequences datasets (Fig. 4) highlighted the establishment of distinct  
254 bacterial community structures at the end of the batch experiments. ANOSIM test confirmed that the genetic  
255 structures of the communities were significantly different ( $R = 0.988$ ,  $P = 0.001$ ). These differences were  
256 associated with different taxonomic compositions (Fig. 5). In agreement with the diversity indices, bacterial  
257 communities for all the treatments (T20, T20Y, T35 and T35Y) were characterized by the dominance of a small  
258 number of OTUs. In the batch incubated at 20°C (T20), bacterial community was dominated by a single OTU  
259 affiliated to the *Gallionella* genus representing an average of 82% of total sequences. Incubation at 35°C led to the  
260 emergence of a dominant OTU affiliated to *Thiomonas* genus (49 to 79%), and to a lesser extent, to *Ferritrophicum*  
261 genus (5 to 9%). The supply of yeast extract in batch incubated at 20°C mainly favored the development of bacteria  
262 related to genera *Acidocella* (47 to 55 %), *Thiomonas* (7 to 14%) and *Gallionella* (8 to 10%). Finally, bacterial  
263 community in the nutrient amended-batch incubated at 35°C was dominated by OTUs affiliated to *Acidicapsa* (13  
264 to 55%), *Gallionella* (17 to 23%), *Thiomonas* (11 to 14%), *Acidocella* (0 to 24%) and *Ferritrophicum* (3 to 6%).  
265 The bacterial groups that dominated at the end of the batch experiments represented no more than 10 to 20% of  
266 the initial community of the Reigous spring water used in these experiments.

267 Batch incubations resulted in an enrichment in bacteria with the genetic potential for As(III) oxidation as  
268 revealed by the quantification of *aioA* genes (Fig. 6). Nutrient supply or incubation at 35°C resulted in a one or  
269 two order of magnitude higher abundance of *aioA* genes (representing on average  $5 \pm 1 \times 10^5$ ,  $2 \pm 1 \times 10^6$ ,  $4 \pm 5 \times$   
270  $10^5$  genes copies per ng of DNA for T20Y, T35 and T35Y, respectively) compared to the batch incubated at 20°C

- 271 (1.4 ± 0.4 × 10<sup>4</sup> genes copies per ng of DNA) and to the initial water (4 ± 2 × 10<sup>3</sup> genes copies per ng of DNA<sup>-1</sup>).
- 272 The highest number of *aioA* gene copies was obtained as a consequence of the incubation at 35°C.

## 273 **Discussion**

274 We investigated the role of temperature and nutrients as drivers of the microbially mediated removal of  
275 iron and arsenic in an As-rich AMD. We confirmed that indigenous microbial communities through their capacity  
276 to oxidize iron and arsenic are the actors of the mitigation of the pollution in AMD (Casiot et al. 2003b; Egal et al.  
277 2010; Mitsunobu et al. 2013). Abiotic oxidation and removal of iron and arsenic remained very limited without  
278 microbial catalysis and were not influenced by nutrient supply or temperature increase.

### 279 **Bacterial diversity in AMD water and its evolution in batch experiments**

280 The initial bacterial diversity in the Carnoulès AMD water was similar to those previously described by  
281 Volant and colleagues (2014). Batch incubations resulted in the decrease of diversity associated with the  
282 preferential development of specific bacterial taxa. This can be explained by the inability of some microorganisms  
283 to thrive under laboratory conditions (Koskella and Vos 2015), and by the strong competitiveness of other  
284 microorganisms (Hibbing et al., 2010; Puspita et al., 2012). The reduction of diversity was moderated in batch  
285 supplied with nutrients and in batch incubated at 35°C. We can hypothesize that a nutrient supply or a temperature  
286 increase led to a diversification of bacterial niches (Hibbing et al. 2010; Koskella and Vos 2015; Okie et al. 2015),  
287 promoting the co-existence of a greater number of taxa.

288 The incubation of AMD water under contrasted conditions of temperature and nutrient status led to the  
289 establishment of distinct bacterial communities (in terms of diversity, taxonomic composition and functional  
290 potential for As-oxidizing activity). These results confirm the influence of temperature and nutrients (C, N, etc.)  
291 on the diversity and activity of microbial communities in diverse environments (Miller et al. 2009; Lawes et al.  
292 2016), including AMD (Kuang et al. 2013, Volant et al. 2014). The development of these distinct bacterial  
293 communities resulted in difference in terms of pollution removal efficiency and of composition of the biogenic  
294 precipitates formed during incubation.

295 In batch incubated at 20°C, bacterial community was largely dominated by OTUs related to the iron-  
296 oxidizing *Gallionella* (92% of total sequences), widely represented in iron-rich environments including AMD  
297 (Bruneel et al. 2006; Volant et al. 2014). Dominance of this bacterial group was associated with complete Fe(II)  
298 oxidation, and a low As(III) oxidation activity. This is in accordance with the formation of iron- and As(III)-rich  
299 precipitates in the T20 batch.

### 300 **Effect of nutrient supply on bacterial communities and on pollution attenuation**

301 In agreement with other studies (Sipura et al. 2005; Leflaive et al. 2008), bacterial cell concentration  
302 increased in nutrient amended batch. The supply of yeast extract stimulated the development of mixotrophic  
303 *Thiomonas*. As a result, As(III) oxidation was stimulated and arsenic removal from the dissolved phase was  
304 complete, both at 20 and 35°C. Members of the *Thiomonas* genus include *aioA*-carrying AsOB commonly found  
305 in AMD-impacted environments (Bruneel et al. 2003; Battaglia-Brunet et al. 2006; Bryan et al. 2009). Conversely,  
306 the rate of iron oxidation was partially inhibited in nutrient amended batch incubated at 20°C despite the presence  
307 of FeOB like-*Gallionella* (10 % of total sequences). *Gallionella* spp. can grow autotrophically (Emerson et al.  
308 2013) or mixotrophically (Hallbeck and Pedersen 1991). We can hypothesize that supply of yeast extract  
309 (representing a diverse source of carbon and nitrogen) resulted in the inhibition of growth or activity of FeOB

310 *Gallionella* to the advantage of heterotrophic and mixotrophic bacteria such as *Acidocella*, *Thiomonas*,  
311 *Rhodanobacter* and *Arthrobacter*. Among them, *Acidocella*, which is commonly found in iron rich environments  
312 including AMD (Sheng et al. 2016), was predominant (52% of total sequences). *Acidocella* is an iron-reducing  
313 bacteria (FeRB), able to use Fe(III) as the sole electron acceptor under anaerobic or oxygen limiting conditions  
314 (Coupland and Johnson 2008; Lu et al. 2010). Interestingly, the inhibitive effect of yeast extract on iron oxidation  
315 was counterbalanced when the batch were incubated at 35°C. In that case, Fe(II) oxidation was delayed but  
316 complete. This efficient Fe removal in T35Y batch was possibly due the increasing proportion of bacteria related  
317 to the *Ferritrophicum* genus which include iron-oxidizing bacteria (Gonzalez-Toril et al. 2011; Hedrich et al.  
318 2011). The possible involvement of other group favored in T35Y including the moderately acidophilic and obligate  
319 heterotroph *Acidicapsa* genus (Kulichevskaya et al. 2012) may not be excluded and would require further  
320 investigation.

### 321 **Effect of temperature on bacterial communities and on pollution attenuation**

322 Enhanced arsenic oxidation and removal at 35°C compared to 20°C was not associated with an increase  
323 of biomass. This suggests that bacterial activity was boosted rather than bacterial growth. Another possible  
324 explanation is the preferential development at 35°C of populations efficient for arsenic removal. The stimulation  
325 of As(III) oxidation at 35°C was associated with the large dominance of *Thiomonas* genus (61% of total sequences)  
326 and a higher proportion of *aioA* genes. Several *Thiomonas* strains exhibit an optimum growth temperature of 30-  
327 37°C (Kelly et al. 2007; Panda et al. 2009). These findings highlight the importance of temperature on both the  
328 abundance and activity of AsOB like-*Thiomonas*, as shown for other AsOB (Ito et al. 2012; Debiec et al. 2017).  
329 The second more abundant OTU was affiliated to the *Ferritrophicum* genus (8% of total sequences). The co-  
330 occurrence of these two bacterial groups in batch incubated at 35°C can be linked to the formation of iron- and  
331 As(V)-rich precipitates.

### 332 **Environmental significance**

333 The biogenic precipitates formed in batch without nutrient exhibited an As/Fe ratio ranging between 0.1-  
334 0.2. Similar ranges (0.15-0.2) were obtained in a continuous flow reactor treating Carnoulès AMD water  
335 (Fernandez-Rojo et al. 2017). These As/Fe ratios are lower than those observed *in situ* in the Reigous streambed  
336 (0.4-0.7, Morin et al. 2003; Egal et al. 2010; Maillot et al. 2013). In the present batch experiments, conditions that  
337 favored As oxidation while limiting Fe oxidation (T20Y) led to As/Fe ratio in the precipitate similar to field values.  
338 This suggests that As oxidation is probably stimulated in the field, and Fe oxidation slowed down, compared to  
339 laboratory conditions. The reason for such difference might be related to the inability of some microorganisms to  
340 develop or maintain their activity under laboratory conditions.

341 Seasonal variations of As speciation were observed in Carnoulès AMD (Morin et al. 2003; Egal et al.  
342 2010) with the preferential formation of As(III)-rich precipitates during the coldest season, mainly in the form of  
343 tooeleite. Conversely, during summer, As(V) dominates in the amorphous ferric arsenate form. These field  
344 observations are in agreement with our results that clearly revealed a positive effect of higher temperature on  
345 microbially mediated As(III) oxidation. As a consequence, in case of an *in situ* biological treatment, contrasted  
346 performance in term of efficiency of arsenic removal and sludge composition is expected depending on the climate

347 or the season. The formation of stable As(V)-rich biogenic precipitates might be favored at temperatures higher  
348 than 20°C.

349 Nutrient supply favored the formation of As(V) rich precipitates, which are preferred to As(III) solid  
350 phases in AMD treatment process due to their stability upon storage (Palfy et al. 1999). However, the stimulation  
351 of iron-reducing bacteria *Acidocella* may result in the remobilization of arsenic if conditions became reducing or  
352 in anoxic micro niches (Héry et al. 2014). For this reason, possible use of organic matter amendement in aerobic  
353 AMD treatment has to be carefully considered.

354 Our results evidenced clear links between taxonomic composition of bacterial community, abundance of  
355 *aioA* gene, and iron and arsenic oxidation and removal from AMD water. They give new insights into the  
356 regulation by temperature and nutrients of microbially mediated processes involved in natural pollution  
357 attenuation. Studies focusing on single strains did not take into account the metabolic and functional diversity  
358 present in natural ecosystems. On the contrary our study based on a complex indigenous community integrates all  
359 the differential effects the applied treatment may have on the different bacterial populations co-existing among the  
360 community. Then, our approach gives a more representative picture of what could occur *in situ* under temperature  
361 or nutrient status changing conditions than previous studies on single Fe(II)- or As(III)-oxidizing bacterial strains.

362

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368 **Compliance with Ethical Standards**

369 Conflict of Interest: The authors declare they have no conflict of interest.

370 Ethical approval: This article does not contain any studies with human participants or animals performed by any  
371 of the authors.

372

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552

## 553 **List of figure legends**

554 **Fig. 1** Bacterial biomass in abiotic (n=5) and biotic (n=8) batch experiment at the beginning (T0) and at the end  
555 of the experiment (T8). Symbols inside boxplots represent the treatments applied (T20: batch incubated at 20°C;  
556 T35: batch incubated at 35°C; T20Y: batch incubated at 20°C with nutrients; T35Y: batch incubated at 35°C with  
557 nutrients). Different letters in brackets indicate statistically significant differences ( $P < 0.05$ ) according to *Kruskal-*  
558 *Wallis* test.

559 **Fig. 2** Evolution of dissolved Fe(II) (A), dissolved As(III) (B) and precipitated Fe concentrations (C) during  
560 incubation time for abiotic (empty symbols) and biotic (lines with full symbols) batch experiment for all treatments  
561 (T20: batch incubated at 20°C; T35: batch incubated at 35°C; T20Y: batch incubated at 20°C with nutrients; T35Y:  
562 batch incubated at 35°C with nutrients).

563 **Fig. 3** Proportion of Fe(II) (A) and As(III) (B) oxidized after 8 days of incubation relatively to Fe(II) and As(III)  
564 concentration in the Carnoulès AMD at  $t_0$  for both abiotic and biotic treatments (T20: batch incubated at 20°C;  
565 T35: batch incubated at 35°C; T20Y: batch incubated at 20°C with nutrients; T35Y: batch incubated at 35°C with  
566 nutrients). For biotic treatments, values with different letters differ significantly ( $P < 0.05$ ) according to *Fisher*  
567 test.

568 **Fig. 4** Non-metric multi-dimensional scaling (NMDS) ordination plot derived from weighted pairwise Unifrac  
569 distances for bacterial communities for each treatment (S: Source; T20: batch incubated at 20°C; T35: batch  
570 incubated at 35°C; T20Y: batch incubated at 20°C with nutrients; T35Y: batch incubated at 35°C with nutrients).  
571 Stress values for ordination plot were  $< 0.2$  which indicates that these data were well-represented by the two  
572 dimensional representation.

573 **Fig. 5** Relative abundance of bacterial genera in the water collected at the Reigous stream and in the batch at the  
574 end of the incubations. All the analyses were performed in triplicates (S: Source; T20: batch incubated at 20°C;  
575 T35: batch incubated at 35°C; T20Y: batch incubated at 20°C with nutrients; T35Y: batch incubated at 35°C with  
576 nutrients). Cluster tree represent phylogenetic community distance based on the OTU composition. Other groups  
577 represent the phylogenetic groups (genus) with a relative abundance  $< 1\%$  calculated on the whole dataset.  
578 Asterisks represent phylogenetic group affiliated to higher taxonomic levels.

579 **Fig. 6** Quantification of *aioA* genes in the Reigous water and in the biotic batch at the end of the incubations (n=3)  
580 with S: Source corresponding to water collected at the Reigous stream used in batch experiments; T20: batch  
581 incubated at 20°C; T35: batch incubated at 35°C; T20Y: batch incubated at 20°C with nutrients; T35Y: batch  
582 incubated at 35°C with nutrients. Letters in brackets indicate significant differences between treatments, according  
583 to *Kruskal-Wallis* test ( $P < 0.05$ ).

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