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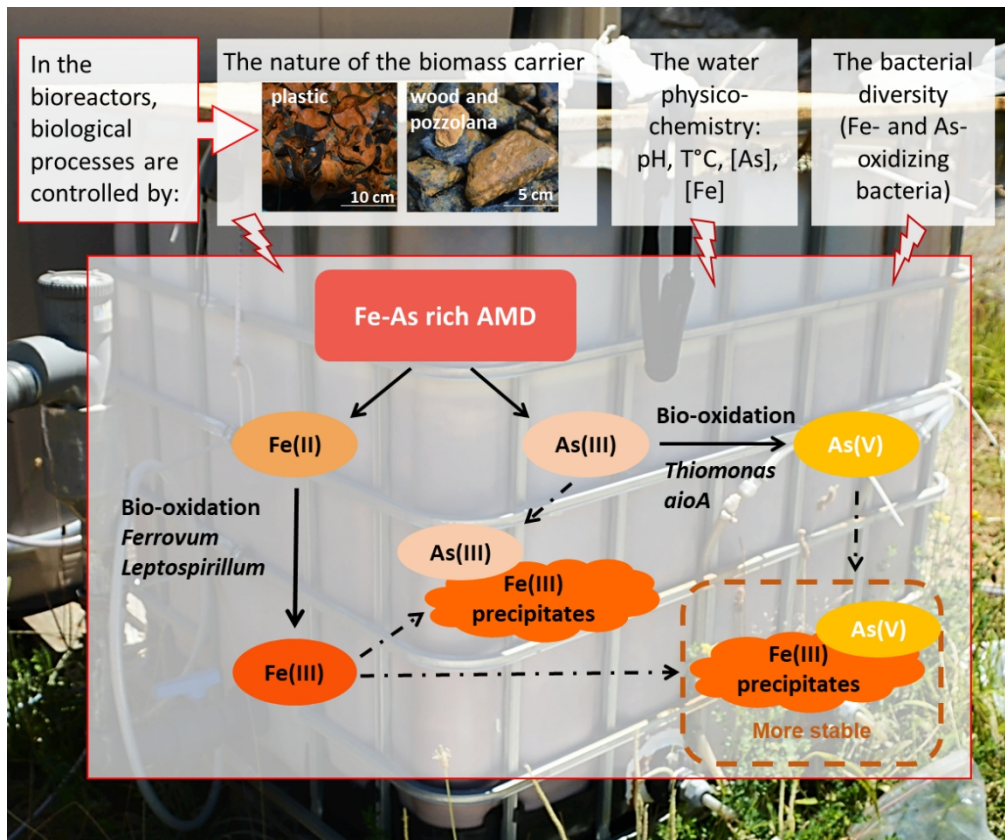
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**Towards an understanding of the factors controlling
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drainage**

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Towards an understanding of the factors controlling bacterial diversity and activity in semi-passive Fe- and As-oxidizing bioreactors treating arsenic-rich acid mine drainage

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Abstract

Semi-passive bioreactors based on iron and arsenic oxidation and co-precipitation are promising for the treatment of As-rich acid mine drainages. However, their performance in the field remains variable and unpredictable. Two bioreactors filled with distinct biomass carriers (plastic or a mix of wood and pozzolana) were monitored during one year. We characterized the dynamic of the bacterial communities in these bioreactors, and explored the influence of environmental and operational drivers on their diversity and activity. Bacterial diversity was analyzed by 16S rRNA gene metabarcoding. The *aioA* genes and transcripts were quantified by qPCR and RT-qPCR. Bacterial communities were dominated by several iron-oxidizing genera. Shifts in the communities were attributed to operational and physiochemical parameters including the nature of the biomass carrier, the water pH, temperature, arsenic and iron concentrations. The bioreactor filled with wood and pozzolana showed a better resilience to disturbances, related to a higher bacterial alpha diversity. We evidenced for the first time *aioA* expression in a treatment system, associated with the presence of active *Thiomonas* spp. This confirmed the contribution of biological arsenite oxidation to arsenic removal. The resilience and the functional redundancy of the communities developed in the bioreactors conferred robustness and stability to the treatment systems.

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2
3 28 **Keywords:** acid mine water, microbial ecotoxicology, biological arsenic oxidation, biological
4 29 iron oxidation, bioremediation, *aioA* gene
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9 31 **Introduction**
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12 32 Acid mine drainages (AMD) are undesired products of the weathering of sulfide minerals
13 33 present in mining wastes. They contribute to decreasing the pH and releasing metals and
14 34 metalloids into aquatic environments. Arsenic is associated with more than 200 minerals
15 35 (Alam and Mchhedran, 2019) such as arsenopyrite (FeAsS). Their oxidation releases this
16 36 toxic metalloid, which is mobilized across the aquatic bodies downstream of the mine tailings
17 37 (Paikaray 2015). The chronic toxicity of arsenic is a threat to public and environmental health
18 38 (Huq *et al.*, 2020).
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26 40 Closed and abandoned mines are the main source of AMD (Rezaie and Anderson 2020).
27 41 Because the cost of remediation is often covered by limited public funds, the development of
28 42 cost-effective and low-maintenance treatments is necessary. Autochthonous microbial
29 43 communities thriving in AMD containing arsenic are the key to treat this pollution. Indeed,
30 44 bacterially mediated iron (Fe) and arsenic (As) oxidation, followed by their co-precipitation is
31 45 a natural process responsible for the partial removal of the arsenic present in AMD (Casiot *et*
32 46 *al.*, 2003; Asta *et al.*, 2010). Arsenic is usually present in AMD under the arsenite As(III)
33 47 form. Biological oxidation of As(III) to As(V) contributes to reduce its mobility, due to the
34 48 higher affinity of As(V) with the Fe-rich solid particles present in mine wastes and streams
35 49 (Maillot *et al.*, 2013). Thus, the combined action of Fe- and As-oxidizing bacteria contributes
36 50 to arsenic and iron removal from polluted waters.
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46 51
47 52 The Reigous creek, near the former Carnoulès mine (Southern France), is an exceptional
48 53 example of an arsenic impacted AMD that has been extensively studied during more than 20
49 54 years (Leblanc *et al.*, 1996, 2002; Casiot *et al.*, 2003; Egal *et al.*, 2010; Volant *et al.*, 2014).
50 55 Seasonal variations of the physico-chemistry of the AMD waters were evidenced (Egal *et al.*,
51 56 2010) and were associated with temporal dynamics of the bacterial diversity (Volant *et al.*,
52 57 2014). On site, natural attenuation mediated by Fe(II)- and As(III)-oxidizing bacteria is
53 58 responsible for the removal of about 30% of the soluble As in the first 40 m of the creek,
54 59 through the formation of biogenic precipitates such as tooleite, schwertmannite and
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3 60 amorphous As(V)- and As(III)-Fe(III) phases (Casiot *et al.*, 2003; Morin *et al.*, 2003; Egal *et*
4 *al.*, 2010). However, natural attenuation is not sufficient to reach the water quality objective
5 61 (0.83 $\mu\text{g L}^{-1}$ over the local geochemical background value) set by European Water Directive
6 62 (2000/60/EC) for dissolved As concentration in freshwater. Furthermore, the accumulation of
7 63 (2000/60/EC) for dissolved As concentration in freshwater. Furthermore, the accumulation of
8 64 As-rich biogenic precipitates in the creek bed is of concern due to the possible remobilization
9 65 of As during extreme rainfall events affecting the region (Resongles *et al.*, 2014, 2015).
10 66

11 67 Previous studies have attempted to develop a treatment system for As-rich AMD based on
12 68 natural attenuation process (Hedrich and Johnson, 2012; Macías *et al.*, 2012; Ahoranta *et al.*,
13 69 2016; Fernandez-Rojo *et al.*, 2017, 2018, 2019). Fernandez-Rojo *et al.*, (2017, 2018) obtained
14 70 nearly 80% As removal in a laboratory-scale continuous flow bioreactor, fed with the
15 71 Carnoulès AMD. Later, a first attempt made with an *in situ* pilot suffered important
16 72 performance variations and highlighted the importance to optimize oxygenation and support
17 73 for bacterial attachment (Fernandez-Rojo *et al.*, 2019). The dynamic of the bacterial
18 74 communities inside this field pilot was described by Laroche *et al.*, (2018). Arsenate (As(V))
19 75 enrichment in the biogenic precipitates was associated with an increase in the abundance of
20 76 As(III)-oxidizing bacteria. However, to the best of our knowledge, the expression of the
21 77 As(III) oxidase, which catalyzes a key process in such treatment, has not been evidenced yet
22 78 in a treatment system. Only the genetic potential for As(III) oxidation has been revealed by
23 79 the detection of the *ainA* gene (Fernandez-Rojo *et al.*, 2017; 2019). Furthermore, there are still
24 80 gaps to be filled about the stability of the bacterial diversity and activity facing seasonal and
25 81 operational fluctuations, and the implications on treatment efficiency and robustness
26 82 (Fernandez-Rojo *et al.*, 2019). Recently, we obtained a relatively stable As removal efficiency
27 83 (67%) in two optimized semi-passive field bioreactors using air injection and biomass
28 84 carriers. These semi-passive treatment units, monitored during one year, showed improved
29 85 and more stable performances compared to previous field study and are promising for the
30 86 treatment of arsenic-rich AMD. Understanding the dynamic of the bacterial communities in
31 87 such systems is of prime importance since Fe oxidation only relies on bacterial activity at acid
32 88 pH and As oxidation is bacterially catalyzed.
33 89

34 90 Hence, the present work aims to characterize the bacterial diversity and activity in the two
35 91 optimized semi-passive field bioreactors described by Diaz-Vanegas *et al.*, (2022) under
36 92 varying on-site operating and seasonal conditions. Two types of biomass carriers (plastic
37 93 support and a mix of wood chips and pozzolana) were compared. We focused on active

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3 94 bacterial communities through the analysis of environmental RNA and distinguished the
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5 95 communities attached to the supports to those in suspension. Bacterial diversity was
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7 96 determined by metabarcoding of the 16S rRNA gene and the presence and the expression of
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9 97 the As(III) oxidase gene quantified by quantitative PCR (qPCR). Then, we related the
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11 98 microbiological parameters to bioreactors performances and discussed the links between the
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13 99 dynamic of the bacterial communities, the variations of the physico-chemistry of the AMD
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15 100 water and the system treatment stability.
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17 101

18 102 **Materials and methods**

20 103 ***Bioreactor setup, operation and sampling***

21 104 Two bioreactors of 1 m³ each were installed downstream the Carnoulès tailing dam as
22
23 105 described in Diaz-Vanegas *et al.*, (2022). Briefly, the bioreactors were continuously fed with
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25 106 the Carnoulès' AMD and the flow rate was controlled by pumps. Each bioreactor was
26
27 107 equipped with air diffusers to avoid oxygen limitation. One bioreactor was filled with a
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29 108 plastic biomass carrier ("PS") (Biofill, Type A), the other with a mixture of coarse wood
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31 109 chips and pozzolana ("WP" for Wood-Pozzolana). The two biomass carriers showed different
32
33 110 properties: the plastic carrier reactor had higher porosity (96%) but lower specific surface area
34
35 111 (>160 m² m⁻³) than the wood-pozzolana mixture (porosity: 64% and specific surface area
36
37 112 estimated to 333 m² m⁻³).
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39 113

40 114 The bioreactors were monitored during 218 days. Monitoring covered all seasons and was
41
42 115 divided in seven periods A, B, C, D, E, F and G from July 2019 to September 2020. The
43
44 116 periods were defined based on the flow rate and some undesired interruptions due to technical
45
46 117 and operational issues and sanitary crisis. Water circulation was stopped between period A
47
48 118 and B due to an electrical supply problem (September-November), so during three months the
49
50 119 bioreactors were operated in batch conditions. During period F and beginning of period G, the
51
52 120 accumulation of biogenic precipitates led to clogging of the air diffusers in both bioreactors.
53
54 121 The air diffusers started working properly again after a maintenance later in period G
55
56 122 (September), 15 days before the last sampling.

57 123 Occasional sampling of the inlet water (i.e. the AMD water feeding the two bioreactors) was
58
59 124 carried out once during period B (19th of December 2019) and once during period F (27th of
60
125 July 2020). Outlet waters and biogenic precipitates formed inside the reactors were collected

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3 126 periodically for bacterial community characterization. Sample collection began in period B
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5 127 (due to the time required to form sufficient quantity of precipitates), and was carried out once
6
7 128 per period for each bioreactor.

8 129
9
10 130 The attached communities correspond to the bacterial communities living in the biogenic
11
12 131 precipitates accumulated in the biomass carriers. These biogenic precipitates were collected
13
14 132 by scrapping the biomass carrier in each bioreactor. In the WP bioreactor, they were sampled
15
16 133 only from the wood chips (due to the difficult access to the pozzolana at the bottom of the
17
18 134 tank). Samples were collected in Falcon tubes (50 mL) and left some minutes for decantation.
19
20 135 The supernatant was discarded and the precipitates were distributed into six cryo-tubes (2
21
22 136 mL). The suspended communities correspond to the bacteria living in the aqueous phase and
23
24 137 associated with the suspended particulate matter (SPM) recovered from filters (0.22 μm). For
25
26 138 each point of water sampling (outlet PS, outlet WP, and inlet water), six replicates of outlet
27
28 139 water (300 mL each one) were filtered on sterile 0.22 μm cellulose acetate filters.
29
30 140 All the solid samples and filters were flash-frozen in dry ice in the field, transported to the
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32 141 laboratory and stored at $-80\text{ }^{\circ}\text{C}$ until DNA and RNA extractions.
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34 142

35 143 ***Geochemistry of the waters and the biogenic precipitates and performance of*** 36 144 ***the two bioreactors***

37 145 The chemical characterization of the inlet water, outlet water and biogenic precipitates of each
38
39 146 bioreactor is detailed in our previous study (Diaz-Vanegas *et al.*, 2022). The data for inlet and
40
41 147 outlet waters corresponding to the sampling performed for microbiological analyses are
42
43 148 summarized in Table 1 and Table SI 1. These data were used for the correlation analyses. In
44
45 149 the inlet water, As concentration varied between 50 and 100 mg L^{-1} and Fe concentration
46
47 150 between 500 and 1000 mg L^{-1} . The AMD was more concentrated in pollutants during the
48
49 151 driest and hottest seasons (periods E, F and G) as described before (Egal *et al.*, 2010). The pH
50
51 152 of the AMD varied between 3.7 and 4.8, with the highest values during period B. The AMD
52
53 153 showed a temperature between 8 and 22°C with the highest value during period E. The
54
55 154 coldest temperature was measured during periods B and C, which also correspond to the
56
57 155 periods with the lowest concentration of total Fe (449-501 mg L^{-1}) and As (49-54 mg L^{-1}).
58
59 156 The hydraulic retention time (HRT) was slightly different between the two devices, with
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157 approximately one hour more in the WP device. Two HRT were tested ($\sim 9\text{h}$ and $\sim 18\text{h}$), but
158 the difference in the performances (rates of Fe and As oxidation and precipitation) was less

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3 159 than 10%. Occasional measurements of dissolved oxygen concentration inside the bioreactors
4 showed dysfunction of the air diffusers in period F.
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6 161

7
8 162 Both bioreactors showed similar Fe oxidation and As removal rates, represented respectively
9 by the first-order kinetic constant of Fe oxidation K_{OFe} and the As precipitation rate
10 163 determined in Diaz-Vanegas *et al.*, (2022). The highest performances were obtained during
11 164 period E (Fe oxidation) and F (As precipitation) as shown in Figure 1. In the biogenic
12 165 precipitates, more than 60 % of total As was under the As(V) oxidation state during the entire
13 166 monitoring (Table SI 2).
14 167
15 168

16 169 The performance indicators used for the correlations include the K_{OFe} (min^{-1}), Fe
17 170 precipitation rate ($\text{mol L}^{-1}\text{s}^{-1}$), As precipitation rate ($\text{mol L}^{-1}\text{s}^{-1}$) and percentage of As(V)
18 171 released in the outlet water (As(V)% outlet).
19 172

20 173 ***Environmental DNA and RNA extraction and cDNA synthesis***

21 174 The molecular characterization of the total and active bacterial communities was based on the
22 175 analyses of environmental DNA and RNA (cDNA) respectively. For each bioreactor, DNA
23 176 was extracted from biogenic precipitates and water filters using DNeasy PowerSoil kit and
24 177 DNeasy PowerWater kit (Qiagen) respectively, according to the manufacturer's
25 178 recommendations. The RNA extractions were performed from biogenic precipitates and outlet
26 179 water filters using FastRNA Pro Soil-Direct kit (MP Biomedicals) and RNeasy Power Water
27 180 Kit (Qiagen) respectively, according to the manufacturer's recommendations. Residual DNA
28 181 was removed from the extracted RNA with an additional Turbo DNase treatment (Turbo
29 182 DNA-free kit, Ambion). The absence of DNA was confirmed by the absence of amplification,
30 183 from RNA extracts, of 16S rRNA gene with 8F (5'-AGAGTTTGATCCTGGCTCAG-3', Lane
31 184 1991) and 1489R (5'-TACCTTGT TACGACTTCA-3', Weisburg *et al.*, 1991) primers.
32 185 Reverse transcription was immediately performed on DNA-free RNA extracts using the
33 186 iScript™ cDNA Synthesis Kit (Biorad).

34 187 The quality of the cDNA was confirmed by performing a PCR targeting 16S rRNA gene
35 188 using 8F and 1489R primers or *aioA* gene using aoxBM1-2F (5-
36 189 CCACTTCTGCATCGTGGGNTGGGNT A-3) and aoxBM3-2R (5-
37 190 TGTCGTTGCCCCAGATGADNCCYTTYT-3) primers (degenerate primers designed to
38 191 target multiple bacterial and archaeal taxa, Quéméneur *et al.*, 2008). DNA and RNA extracts

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3 192 were quantified with a fluorometer (Qubit [®], Invitrogen) and stored at -80°C until further
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5 193 analysis.

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9 195 ***Sequencing of 16S rRNA gene and bioinformatics***

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11 196 For metabarcoding, the V3-V4 region of the bacterial 16S rRNA gene was targeted using
12
13 197 primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-
14
15 198 GACTACHVGGGTATCTAATCC-3'). Libraries of amplicons and Illumina MiSeq
16
17 199 sequencing were performed by MetaHealth metagenomic-based services (CIRAD, PHIM,
18 200 Eco&Sols, Montpellier, France) for biogenic precipitates and outlet waters.

19 201 Illumina sequencing, base calling and demultiplexing were carried out using RTA v1.18.54,
20 202 MCS 2.6 and bcl2fastq 2.17. Paired Illumina MiSeq reads were assembled with vsearch
21
22 203 v2.18.0 (Rognes *et al.*, 2016) using the command `fastq_mergepairs` and the option
23
24 204 `fastq_allowmergestagger`. Demultiplexing and primer clipping were performed with `cutadapt`
25
26 205 v3.4 (Martin 2013) forcing a full-length match for sample tags and allowing a 2/3-length
27
28 206 partial match for forward and reverse primers. Only reads containing both primers were
29
30 207 retained. For each trimmed read, the expected error was estimated with `vsearch`'s command
31
32 208 `fastq_filter` and the option `eeout`. Each sample was then dereplicated, i.e. strictly identical
33
34 209 reads were merged, using `vsearch`'s command `derep_fulllength`, and converted to FASTA
35
36 210 format.

37 211 To prepare for clustering, the samples were pooled and processed by another round of
38
39 212 dereplication with `vsearch`. Files containing expected error estimates were also dereplicated to
40
41 213 retain only the lowest expected error for each unique sequence. Clustering was performed
42
43 214 with `swarm` v3.1.0 (Mahé *et al.*, 2021), using a local threshold of one difference and the
44
45 215 `fastidious` option. Operational taxonomic unit (OTU) representative sequences were then
46
47 216 searched for chimeras with `vsearch`'s command `uchime_denovo` (Edgar *et al.*, 2011). In
48
49 217 parallel, representative sequences were assigned using the `stampa` pipeline
50
51 218 (<https://github.com/frederic-mahe/stampa/>) and a trimmed version of the reference database
52
53 219 SILVA SSURef NR99 v138.1 (Quast *et al.*, 2013).

54 220

55 221 Clustering results, expected error values, taxonomic assignments and chimera detection
56
57 222 results were used to build a raw OTU table. Up to that point, reads that could not be merged,
58
59 223 reads without tags or primers, reads shorter than 32 nucleotides and reads with uncalled bases
60
224 ("N") had been eliminated. To create the "cleaned" OTU table, additional filters were applied

225 to keep only non-chimeric OTUs, OTUs with an expected error per nucleotide below 0.0002,
226 OTUs containing more than three reads or seen in at least two samples. The raw data are
227 available under the bioproject (<https://www.ebi.ac.uk/ena/data/view/PRJEB63466>).

229 The OTU table was processed with R 3.5.2 (R Core Team, 2018) for additional filtering steps
230 and statistical analysis with the packages tidyverse 1.3.0 (Wickham 2017) and vegan 2.5.6
231 (Oksanen *et al.*, 2018). These steps include removing the controls and the samples with less
232 than 1000 reads, and the rarefaction of the remaining samples.

233 ***aioA* and 16S rRNA genes and transcripts quantification**

234 Abundance of the *aioA* gene (encoding the catalytic subunit of the arsenite oxidase) and of the
235 bacterial 16S rRNA genes and their transcripts were quantified by a real-time quantitative
236 PCR (qPCR) from the DNA extracts and from the cDNA, respectively. For 16S, we used
237 universal primers 341F (5'CCTACGGGAGGCAGCAG-3') and 515R
238 (5'ATTACCGCGGCTGCTGGCA-3'). For *aioA*, we used primers m4-1F
239 (5'GCCGGCGGGGGNTWYGARRAYA-3') and m2-1R (5'GGAGTTGTAGGCGGGCCK
240 RTTRTGDAT-3) (degenerate primers designed to target multiple bacterial and archaeal taxa,
241 Quéméneur 2008). The qPCR was performed with the CFX Real-Time PCR Detection system
242 (Bio-Rad), using SSO Advanced SYBR® Green Supermix and each primer at 0.4 µM (16S
243 rRNA gene) or 0.3 µM (*aioA* gene). Calculation of copy numbers was done using a linear
244 calibration curve ($r^2 > 0.9$) obtained over 7 orders of magnitude, ranging from 10^2 to 10^8 gene
245 copies of a linearized plasmid carrying the target gene (gene copies/mL of water or mg of
246 biogenic precipitates).

248 **Biostatistical analysis**

249 All statistical analyses were performed with R version 3. Chao1 and Shannon indices were
250 calculated using the vegan and ggplot2 packages for biological diversity statistics in R
251 (Wickham 2017; Oksanen *et al.*, 2018). Bray Curtis was used as diversity metric to compare
252 the bacterial communities from each bioreactor (R packages phyloseq and dplyr (McMurdie
253 and Holmes 2013; Wickham *et al.*, 2022).

254 The analysis of similarity (ANOSYM, 999 permutations) was performed to compare the
255 statistical differences between pre-defined groups. It allowed us to compare the results
256 obtained for the structure of the bacterial communities based on the following criteria:

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2
3 257 biomass carrier WP versus PS, the different periods of monitoring, the total (DNA) versus the
4
5 258 active (RNA) communities, and the suspended versus the attached bacterial communities (R
6
7 259 package *vegan*, Oksanen *et al.*, 2018).

8 260
9
10 261 A non-metric multidimensional scaling (NMDS) ordination was used to visualize bacterial
11
12 262 community variations among samples, the differences among the structure of the communities
13
14 263 are represented by the distance between the points. The Indicators Species Analysis was
15
16 264 performed to identify the main species responsible for the differences in bacterial community
17
18 265 composition between defined groups (R package *indicspecies*, De Caceres and Legendre,
19 266 2009).

20 267 Significant differences between the different groups were detected with one-way ANOVA (p-
21
22 268 value 0.05) followed by Tukey test. The data that did not follow a normal distribution were
23
24 269 analyzed by Kruskal-Wallis test. Finally, the correlations between biological and
25
26 270 physicochemical variables were performed by the Spearman test, Spearman was used instead
27
28 271 of Pearson's correlation due to non-normal distribution of the data (R package *heatmaply*,
29 272 Galili *et al.*, 2017).

31 32 273 **Results**

33 34 35 274 ***General description of the bacterial diversity***

36
37 275 Illumina sequencing yielded a total of 5 119 340 sequences of bacterial 16S rRNA gene
38
39 276 corresponding to 116 samples. Considering all periods together, based on Chao 1 (richness)
40
41 277 and Shannon (evenness and richness) indices, the alpha diversity was higher (Kruskal-Wallis,
42
43 278 p-value < 0.001) in the bioreactor filled with the wood and pozzolana (WP) than in the
44
45 279 bioreactor with the plastic carrier (PS) (Figure 2.a). For both bioreactors, the richness (Chao1)
46
47 280 of the suspended community sampled from the outlet water was higher than the one of the
48
49 281 attached communities sampled from the biogenic precipitates (Kruskal-Wallis, p-value <
50
51 282 0.05), but the Shannon index (which takes into account evenness) followed the opposite trend
52
53 283 (p-value < 0.01) (Figure SI 1.a). Alpha diversity indices measured for the overall active and
54
55 284 total bacterial communities were similar (Figure SI 2.a; Kruskal-Wallis, p-value > 0.05).

56 285
56 286 The results obtained for the beta diversity (reflecting the taxa turnover) did not follow the
57
58 287 same trend as the one obtained for the alpha diversity. Indeed, we observed a higher beta
59
60 288 diversity associated with the PS bioreactor compared to the WP bioreactor (Mann-Whitney;

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2
3 289 p-value <0.0001) (Figure 2.b). Also, the suspended community showed higher beta diversity
4
5 290 than the attached communities (Figure SI 1.b; Mann-Whitney; p-value <0.0001). Finally, the
6
7 291 total bacterial community presented higher beta diversity than the active one (Figure SI 2.b;
8
9 292 Mann-Whitney; p-value <0.0001).

10 293

11 12 294 ***Dynamic of the bacterial community structures in the PS and WP bioreactors***

13
14 295 The dynamic of the bacterial communities developed in both bioreactors was represented by
15
16 296 non-metric multidimensional scaling (NMDS) (Figure 3). In complement, an ANOSIM test
17
18 297 was used to determine significant differences in the bacterial community composition of
19
20 298 groups of samples. Groups were defined according to the sampling period, the nature of the
21
22 299 biomass carrier, the lifestyle (attached versus suspended communities) and the metabolic
23
24 300 status (total versus active communities). The most important differences were observed
25
26 301 between the different periods of monitoring (represented by the different colors in the NMDS)
27
28 302 (ANOSIM test, R 0.4463, p-value = 0.001). There were marked variations in the bacterial
29
30 303 community structure particularly between the first periods of monitoring (B and C), and the
31
32 304 next ones (D, E, F and G). These variations were more pronounced for the PS bioreactor than
33
34 305 for the WP, reflected by the wider ellipse shown in Figure 3, and in agreement with the higher
35
36 306 beta diversity measured in PS (Figure 2.b).

37 307

38 308 The influence of both lifestyle and biomass carrier was moderate compared to the temporal
39
40 309 dynamic (ANOSIM test, R 0.4463, p-value = 0.001). The lifestyle (suspended or attached in
41
42 310 the biogenic precipitates) was the second criteria driving the bacterial communities (ANOSIM
43
44 311 test, R 0.1935, p-value = 0.001) and the third criteria was the biomass carrier (plastic or
45
46 312 wood/pozzolana) (ANOSIM test, R 0.1535, p-value = 0.001). Finally, active communities
47
48 313 were relatively distinct from the total ones during the first stage of the monitoring (period B
49
50 314 and C). During the other periods, the structure of the active and the total communities
51
52 315 appeared more similar. Overall, the status (total versus metabolically active) of the bacterial
53
54 316 communities was the factor explaining the less the differences between the samples
55
56 317 (ANOSIM test, R 0.05191, p-value = 0.002).

57 318

58 319 Indicators species analysis revealed the bacterial taxa that contributed the most to the shifts in
59
60 320 the bacterial communities. For instance, *Gallionella* and *Gallionellaceae*-related sequences
321 dominated the PS bioreactor during periods B and C. *Acidicapsa*, *Acidiphium*, *Acidibacter*,

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2
3 322 *Acidibrevibacterium*, *Granulicella* and *Tepidsphareales*-related sequences were
4
5 323 representative of samples collected in the WP bioreactor particularly during the first periods B
6
7 324 and *C. Acidithiobacillus* was representative of samples collected in the PS bioreactor at the
8
9 325 end of the monitoring (periods F and G). *Thiomonas* was representative of the total and the
10 326 metabolically active bacterial communities in both bioreactors particularly during period E.
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12 327

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14 328 ***Taxonomic composition of the bacterial communities thriving in the two***
15
16 329 ***bioreactors***

17
18 330 The most abundant phyla identified in the outlet waters and the biogenic precipitates sampled
19
20 331 in both bioreactors were *Proteobacteria* (62%) and *Nitrospirota* (35%). *Acidobacteria* was
21
22 332 the third more abundant phylum, representing only 1% of the total number of sequences. On
23
24 333 the whole data set, the dominant genera were the iron-oxidizing (FeOB) bacteria
25 334 *Leptospirillum*, *Ferrovum*, *Acidithiobacillus* and an undetermined genus of the
26
27 335 *Gallionellaceae* family. *Leptospirillum* was abundant and active from the beginning to the
28
29 336 end of the monitoring, mainly in the biogenic precipitates. *Ferrovum* was more abundant and
30 337 active in the suspended community (outlet water), particularly during the D period of the
31
32 338 monitoring.
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34 339
35 340 The dynamic of the FeOB populations was different in the two bioreactors (Figure 4). In the
36
37 341 PS bioreactor, the first two periods B and C (December and February) were dominated by
38
39 342 *Leptospirillum*, *Gallionella*, and an undetermined genus from *Gallionellaceae* family, while
40
41 343 in the WP bioreactor, *Leptospirillum* was the only dominant FeOB. Then, the FeOB
42
43 344 populations evolved towards a dominance of *Ferrovum* and *Leptospirillum* in both
44
45 345 bioreactors. At the end of the monitoring (period F and G), the communities of the PS
46
47 346 bioreactor were enriched with *Acidithiobacillus*. *Gallionella* was mainly present and active
48
49 347 during the first stage of the monitoring only in the suspended community of the WP
50
51 348 bioreactor, but present in both suspended and attached communities in the PS bioreactor.

52
53 349 The OTUs affiliated to the *Gallionellaceae* family were dominant in PS bioreactor in the
54
55 350 suspended and attached communities during period B and C, but they relative abundance
56
57 351 dramatically decreased from period D. In a lesser extent, the same trend was observed for
58
59 352 *Acidocella* and *Granulicella*.
60

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2
3 354 *Thiomonas* was the sixth most dominant genus identified in the metabarcoding data (Figure
4 355 4). It was always detected in both the total and active communities, although its distribution
5 356 showed a different pattern in the two bioreactors. In the WP bioreactor, *Thiomonas* was more
6 357 abundant in the suspended community during period E, with a similar trend between total and
7 358 active communities. On the contrary, in the PS bioreactor, *Thiomonas* was more represented
8 359 in the attached community, with a stable abundance over time. *Acidiphilium* was present in
9 360 both bioreactors, for most of the monitoring period, but was more represented in the attached
10 361 community than in the suspended community. *Metallibacterium* was present in relatively low
11 362 proportions in both systems, and RNA-based analysis showed that this genus was probably
12 363 not metabolically active.
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21
22 365 Indicator Species Analysis supported our previous observations about the bacterial genera
23 366 representative of a particular group; in the attached communities (biogenic precipitates) the
24 367 representative genera were *Acidiphilium*, *Leptospirillum*, *Acidibacter*, *Acidicapsa* for both
25 368 bioreactors, and *Acidocella*, *Acidibrevibacterium* and *Granulicella* for WP only. On the
26 369 contrary, *Ferrovum* and *Sideroxydans* were representative of the suspended community in
27 370 both bioreactors. *Legionella* and an undetermined genus of *Acidobacteriaceae* family were
28 371 representative of the suspended community only in the WP bioreactor.
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37 ***Abundance and expression of 16S rRNA and aioA genes***

38 374 Based on environmental DNA analysis, the 16S rRNA gene copy number varied between 10^2
39 375 and 10^6 copies mg^{-1} of biogenic precipitate and between 10^4 to 10^7 copies mL^{-1} of water in
40 376 both bioreactors (Figure 5.a and 5.b). The average bacterial biomass of the biogenic
41 377 precipitates community was similar in the two bioreactors (Figure 5.a). The average biomass
42 378 of suspended communities in water remained stable between the inlet and the outlet water of
43 379 the PS and WP bioreactors (Figure 5.b). The biomass varied over time without any clear trend
44 380 (Figure SI 3).
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53 382 Results based on cDNA showed between 10^3 to 10^6 16S rRNA transcripts mg^{-1} of biogenic
54 383 precipitate and between 10^3 to 10^5 16S rRNA transcripts mL^{-1} of outlet water, without
55 384 difference between the two bioreactors (Figure 5.a and 5.b).
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3 386 Based on DNA analysis, the number of *aioA* genes in both bioreactors varied between 10^1 and
4
5 387 10^5 copies mg^{-1} of biogenic precipitate and between 10^2 and 10^6 copies mL^{-1} of water (Fig 5.c
6
7 388 and 5.d). The number of genes and the number of transcripts of the arsenite oxidase were
8
9 389 similar in both bioreactors (Figure 5.c and 5.d), except for the attached community of WP in
10 390 which *aioA* transcripts remained below the detection limit (Figure 5.c). The expression of the
11 391 arsenite oxidase gene was evidenced (by detection of *aioA* transcripts) in 14 of a total of 60
12 392 samples, and showed up to 150 transcripts mL^{-1} of water in the WP bioreactor during period E
13 393 and up to 421 transcripts copies mg^{-1} of biogenic precipitate in the PS bioreactor in period C
14 394 (Figure SI 3). In the other samples, recovery of *aioA* transcripts was below the detection limit.
15 395 The results suggest that the As(III)-oxidizing activity might be more expressed during period
16 396 E and C, compared to other periods. However, technical limitations associated with mRNA
17 397 extraction yield and cDNA synthesis could have interfered with the analysis.

18 398 For DNA-based analyses, the relative abundance of bacteria carrying *aioA* gene in the
19 399 bacterial community and its dynamic in the bioreactors were assessed based on *aioA*/16S
20 400 rRNA genes ratio (Figure 6). In the WP bioreactor, the suspended bacterial communities
21 401 sampled at period E was the most enriched in As(III)-oxidizing bacteria (0.6 copies of
22 402 *aioA*/16S rRNA ratio). On the contrary, in the PS bioreactor, the attached communities were
23 403 the most enriched in As(III)-oxidizing bacteria. The highest enrichment was observed for
24 404 periods C, D and E (average of 0.35 copies of *aioA*/16S rRNA ratio).

25 405
26 406 The relative abundance of *aioA* gene varied widely according to the period, the bioreactor and
27 407 the lifestyle, but without any clear trend. For instance, the average of the relative abundances
28 408 of arsenite oxidase gene was similar in the outlet waters of both bioreactors (0.09 ± 0.15
29 409 copies of *aioA*/16S rRNA ratio) and the feed water (Carnoulès AMD) (0.07 ± 0.02 copies of
30 410 *aioA*/16S rRNA ratio, data not shown).

31 411
32 412 ***Links between the bacterial communities and the physicochemical***
33 413 ***characteristics of the AMD or the treatment performances.***

34 414 Correlations between the microbiology (abundance of the 13 most dominant bacterial taxa
35 415 developed in the bioreactors) and the physico-chemistry of the AMD were assessed by
36 416 calculating Spearman's coefficients (significant when the coefficient was ≥ 0.5 or ≤ -0.5).
37 417 Correlations were also determined between the microbiology and the variables reflecting the
38 418 performance of the treatment system (kinetic constant value K_{OFe} , Fe precipitation rate, As

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3 419 precipitation rate, As(V) % in the outlet, pH outlet) or the operating condition (HRT) (Figure
4 420 7).

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7 422 The relative abundance of sequences related to *Ferrovum*, *Acidithiobacillus* and
8 423 *Acidobacteriaceae* were positively correlated with dissolved As concentration, temperature
9 424 and Fe(II) concentration in the inlet water (correlation > 0.5). In a lesser extent, *Thiomonas*
10 425 also showed a positive correlation with those parameters. On the contrary, taxa *Gallionella*
11 426 and *Gallionellaceae*-undetermined genus showed a negative correlation with those parameters
12 427 (correlation -0.5). The relative abundance of As(III)-oxidizing bacteria (*aioA*/16S rRNA ratio)
13 428 was positively correlated with the temperature, the total dissolved As concentration and the
14 429 percentage of As(III) in the inlet water. The rates of Fe precipitation and As precipitation
15 430 were strongly positively correlated with the relative abundance of *Ferrovum* (correlation 0.6
16 431 and 0.7 respectively). The percentage of As(V) in the outlet water was positively correlated
17 432 with the relative abundance of the As(III)-oxidizing genus *Thiomonas* (correlation 0.6) and in
18 433 lesser extent with *Acidobacteraceae*-undetermined genus and *Acidiphilium* (correlation 0.5).
19 434 On the contrary, this parameter was negatively correlated with *Gallionella* and
20 435 *Gallionellaceae*-undetermined genus (correlation -0.5). The pH of the outlet water was
21 436 positively correlated with the abundance of *Gallionella* and *Gallionellaceae*-undetermined
22 437 genus (correlation 0.5) and negatively correlated with *Ferrovum* (correlation -0.7).
23 438

24 439 The relative abundance of the As(III)-oxidizing bacteria (*aioA*/16S rRNA genes ratio) was
25 440 strongly positively correlated with *Thiomonas* (correlation 0.8). Two HRT were applied in
26 441 both bioreactors (~9h and ~18h). There was a positive correlation between the relative
27 442 abundance of *Acidibrevibacterium*, *Acidiphilium* and *Acidobacteriaceae* relative sequences
28 443 and HRT. However, no significant correlation between the FeOB and the HRT was
29 444 evidenced.
30 445

31 446 **Discussion**

32 447 ***Characterization of the total and active bacterial communities***

33 448 Total and active bacterial communities were dominated by Fe-oxidizing bacteria (FeOB)
34 449 (*Leptospirillum*, *Ferrovum*, *Acidithiobacillus*, an undetermined genus of *Gallionellaceae*,
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3 451 and *Gallionella*). *Ferrovum* was over-represented in the active community (37% and 21% in
4 the whole RNA and DNA data sets respectively). This finding is in line with the probable
5 452 important role played by this Fe-oxidizing bacteria in the treatment of the Carnoulès AMD, as
6 453 reported in other studies (Heinzel *et al.*, 2009; Sheng *et al.*, 2016; Wang *et al.*, 2016;
7 454 Grettenberger *et al.*, 2020). *Leptospirillum* followed the same trend but in a lesser extent (38
8 455 and 36% respectively). On the contrary, *Acidithiobacillus*, undetermined genus of
9 456 *Gallionellaceae* and *Gallionella* were more abundant in the total than in the active
10 457 community, suggesting their possible limited contribution to the treatment process. In
11 458 particular, *Acidithiobacillus* represented 9 % of the total DNA sequences and only 5 % of the
12 459 cDNA sequences reinforcing the idea of its possible lower efficiency in remediation systems
13 460 compared to other FeOB (Heinzel *et al.*, 2009; Laroche *et al.*, 2018). Ebrahimi *et al.*, (2005)
14 461 observed that the short dominance of *Acidithiobacillus ferroxidans* was accompanied by a
15 462 treatment efficiency limited to 90% while a shift towards *Leptospirillum* dominance probably
16 463 contributed to an increase of the performances up to 98%.

17 464
18 465 *Ferrovum* was more represented in the suspended community than in the attached one. Its
19 466 capacity to release extracellular polymeric substances (EPS) (Johnson *et al.*, 2014; Plewniak
20 467 *et al.*, 2020) could promote its development in the small iron flocs suspended in the outlet
21 468 water (Florence *et al.*, 2016). *Ferrovum* has been previously associated with “acid streamers”,
22 469 which are macroscopic biofilms formed by acidophilic microorganisms (Hedrich and Johnson
23 470 2012; Kay *et al.*, 2013).

24 471 *Thiomonas* was the fourth most abundant genus active in both bioreactors (4% of the whole
25 472 cDNA sequences and 2% of the whole DNA sequences). This genus was present in a similar
26 473 abundance in a laboratory bioreactor treating the Carnoulès AMD (2% of DNA sequences,
27 474 Fernandez-Rojo *et al.*, 2018). These proportions are higher (but of the same order of
28 475 magnitude) as those found in the Regious Creek sediments (1.1% of DNA sequences, Laroche
29 476 *et al.*, 2018).

30 477 Heterotrophic Fe(III)-reducing bacteria were also detected: genera *Acidiphillum* (3% and
31 478 2.5% of the total and the active community, respectively), *Acidibacter* (0.6 % of both
32 479 communities), *Acidocella* (1.7% and 0.3% of the total and the active community,
33 480 respectively), *Metallibacterium* (1.3 % and 0.3 % of the total and the active community,
34 481 respectively). They were previously reported in acidic mine waters (Afzal Ghauri *et al.*, 2007;
35 482 Johnson *et al.*, 2014; Gavrilov *et al.*, 2019; Li *et al.*, 2020) and in Fe-oxidizing bioreactors
36 483 (Sheng *et al.*, 2016; Jin *et al.*, 2020). Most of these Fe(III)-reducing bacteria are characterized
37 484 by a metabolic versatility including Fe(III) reduction, carbon fixation and sulfur oxidation

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3 485 capacities (Hao *et al.*, 2010; Bartsch *et al.*, 2017; Gavrilov *et al.*, 2019; Li *et al.*, 2020). These
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5 486 bacteria were globally poorly represented in the active community. However, oxygenation
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7 487 should be carefully controlled to avoid suitable conditions for ferric iron reduction, which
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9 488 would contribute to As remobilization into the aqueous phase (Tufano and Fendorf, 2008).
10 489 Arsenic remobilization has indeed been evidenced from river sediments impacted by the
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12 490 Carnoulès AMD during incubation under anoxic conditions (Héry *et al.*, 2014).
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492 ***Influence of the biomass carrier on the bacterial diversity***

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18 493 Biomass carriers provide a physical support for bacterial growth, extend their survival rate,
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20 494 and enhance oxygen transfer (Tyagi *et al.*, 2011). Al-Amshawee *et al.*, (2021) reported that
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22 495 the biomass attached to the carriers can be up to 500 times more resistant than suspended
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24 496 bacteria to sudden changes. In a membrane bioreactor, the surface of a biomass carrier
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26 497 provided a diversified environment, promoting the growth of diverse types of microorganisms
27
28 498 (Li *et al.*, 2021). On the contrary, diversity was higher in suspended than in attached
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30 499 communities in a waste water treatment plant, possibly due to oxygen limitation inside the
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32 500 biofilm (Kwon *et al.*, 2010). As recently reported (Zhu *et al.*, 2022), the type of biomass
33
34 501 carrier can also influence the diversity and composition of the bacterial communities
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36 502 developing inside the bioreactors.

37 503
38 504 Bioprecipitates were sampled from the surface of the plastic carriers or from the wood chips
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40 505 (no sampling on pozzolana due to the difficult access to the pozzolana at the bottom of the
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42 506 tank). During the early-stage of the monitoring, bacterial community developed on the PS
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44 507 biomass carrier was dominated by chemoautotrophs (i.e. *Galionella* and *Leptospirillum*),
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46 508 while the one associated with WP carrier was composed by both chemoautotrophs (i.e.
47
48 509 *Leptospirillum*, *Acidiphillum*) and chemoheterotrophs (i.e. *Acidobacteriaceae*, *Acidocella*,
49
50 510 *Acidiphillum*, *Acidicapsa*, *Acidibacter* and *Acidibrevibacterium*).
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52 511
53 512 Our first assumption to explain these differences was that organic carbon provided by the
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55 513 wood chips could promote the development of heterotrophic bacteria. The wood chips are
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57 514 often used in bioreactors as biofilm support as well as a source of organic carbon or electron
58
59 515 donors (Lopez-Ponnada *et al.*, 2017; Zhao *et al.*, 2019). This concurs with occasional
60 516 measurements of total organic carbon in the biogenic precipitates, indicating a much higher
61 517 concentrations in the WP (71 ± 47 g kg⁻¹) than in the PS (3 ± 0.7 g kg⁻¹ (dry wt.)). However,

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3 518 the difference was minor in the treated waters (outlet water WP: 5 ± 2 mg L⁻¹; PS: 3 ± 1 mg L⁻¹). Thus, it cannot be excluded that the organic carbon from the WP carrier corresponded to
4 519 ¹). Thus, it cannot be excluded that the organic carbon from the WP carrier corresponded to
5 519 ¹). Thus, it cannot be excluded that the organic carbon from the WP carrier corresponded to
6 520 refractory organic matter. Furthermore, no known cellulolytic bacteria were evidenced in the
7 520 refractory organic matter. Furthermore, no known cellulolytic bacteria were evidenced in the
8 521 WP bioreactor. Consequently, the metabolic pathways involved in organic carbon cycling in
9 521 WP bioreactor. Consequently, the metabolic pathways involved in organic carbon cycling in
10 522 the WP bioreactor would require further investigations. *Gallionella* produces a variety of
11 522 the WP bioreactor would require further investigations. *Gallionella* produces a variety of
12 523 exopolymers that could be an advantage for its attachment on the plastic biocarrier (Emerson
13 523 exopolymers that could be an advantage for its attachment on the plastic biocarrier (Emerson
14 524 *et al.*, 2013). Furthermore, the organic carbon rich environment provided by the wood chips
15 524 *et al.*, 2013). Furthermore, the organic carbon rich environment provided by the wood chips
16 525 may have inhibited the proliferation of *Gallionella*, in the WP bioreactor. According to
17 525 may have inhibited the proliferation of *Gallionella*, in the WP bioreactor. According to
18 526 Fleming *et al.*, (2014), *Gallionella* negatively co-occurred with high concentrations of organic
19 526 Fleming *et al.*, (2014), *Gallionella* negatively co-occurred with high concentrations of organic
20 527 carbon.
21 527 carbon.

22 528
23 529 Our second assumption to explain the differences of bacterial communities thriving on the
24 530 two types of biomass carriers is related to their surface properties (i.e shape, porosity, surface
25 530 two types of biomass carriers is related to their surface properties (i.e shape, porosity, surface
26 531 roughness). According to Pereira *et al.*, (2000) and Campos-Quevedo *et al.*, (2021), the
27 531 roughness). According to Pereira *et al.*, (2000) and Campos-Quevedo *et al.*, (2021), the
28 532 surface roughness is the most important parameter for bacterial colonization, mainly because
29 532 surface roughness is the most important parameter for bacterial colonization, mainly because
30 533 the surface irregularities promote initial colonization and protect microorganisms from
31 533 the surface irregularities promote initial colonization and protect microorganisms from
32 534 detachment. Thus, crevices, cracks and grooves of the wood chips could allow more diverse
33 534 detachment. Thus, crevices, cracks and grooves of the wood chips could allow more diverse
34 535 micro-habitats and for consequence a higher alpha diversity in the WP bioreactor. Feng *et al.*,
35 535 micro-habitats and for consequence a higher alpha diversity in the WP bioreactor. Feng *et al.*,
36 536 (2017) reported that higher alpha diversity can be associated with improved resilience to
37 536 (2017) reported that higher alpha diversity can be associated with improved resilience to
38 537 disturbances. This is in line with the better performance recovery of the WP bioreactor (in
39 537 disturbances. This is in line with the better performance recovery of the WP bioreactor (in
40 538 term of Fe oxidation and precipitation, and of As removal) after operational disturbances
41 538 term of Fe oxidation and precipitation, and of As removal) after operational disturbances
42 539 (interruptions, changes in the HRT and clogging of the air diffusers) (Diaz-Vanegas *et al.*,
43 539 (interruptions, changes in the HRT and clogging of the air diffusers) (Diaz-Vanegas *et al.*,
44 540 2022). Finally, lower beta diversity in the WP bioreactor reflects lower variability and thus
45 540 2022). Finally, lower beta diversity in the WP bioreactor reflects lower variability and thus
46 541 higher stability of bacterial communities.
47 541 higher stability of bacterial communities.

48 542 Despite bacterial community composition differences observed during the early stages, WP
49 542 Despite bacterial community composition differences observed during the early stages, WP
50 543 and PS bioreactors showed similar performances for all the periods of the monitoring. Thus
51 543 and PS bioreactors showed similar performances for all the periods of the monitoring. Thus
52 544 the bacterial groups that differ between the two supports may not play an important role in the
53 544 the bacterial groups that differ between the two supports may not play an important role in the
54 545 process. In the next stages, both carriers were covered by the same mineral phases: jarosite,
55 545 process. In the next stages, both carriers were covered by the same mineral phases: jarosite,
56 546 amorphous schwertmannite and amorphous ferric arsenate (Diaz-Vanegas *et al.*, 2022), which
57 546 amorphous schwertmannite and amorphous ferric arsenate (Diaz-Vanegas *et al.*, 2022), which
58 547 replaced the wood chips and plastic carriers as support for biomass attachment. Such
59 547 replaced the wood chips and plastic carriers as support for biomass attachment. Such
60 548 replacement of biomass carrier by precipitated minerals was described by Ebrahimi *et al.*,
61 548 replacement of biomass carrier by precipitated minerals was described by Ebrahimi *et al.*,
62 549 (2005). Kinnunen and Puhakka (2004) and Wang and Zhou (2012) also showed the role of
63 549 (2005). Kinnunen and Puhakka (2004) and Wang and Zhou (2012) also showed the role of
64 550 jarosite on the attachment of FeOB in a fluidized-bed reactor and its efficiency to maintain a
65 550 jarosite on the attachment of FeOB in a fluidized-bed reactor and its efficiency to maintain a

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3 551 stable bacterial concentration that enhance iron oxidation and promote Fe(III) precipitation by
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5 552 acting as jarosite seed.

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9 554 ***Arsenic oxidation***

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11 555 The role of *Thiomonas* spp. in As oxidation in Carnoulès ecosystem has been demonstrated
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13 556 (Bruneel *et al.*, 2003; Morin *et al.*, 2003). The activity of arsenite oxidase was evidenced *in*
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15 557 *situ* in the Reigous by a proteomic approach (Hovasse *et al.*, 2016). The detection of
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17 558 *Thiomonas* by CARD FISH and metabarcoding recently suggested that As(III) oxidation
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19 559 occurred in a pilot treating arsenic-rich AMD *in situ* and was probably mediated by
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21 560 *Thiomonas* (Laroche *et al.*, 2018). Here, we evidenced for the first time the expression of the
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23 561 arsenite oxidase gene (*aioA*) concomitant with the presence of metabolically active
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25 562 *Thiomonas* in a bioreactor. Furthermore, the relative abundances of *aioA* genes and
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27 563 *Thiomonas*-related 16S rRNA sequences (Figure 6) were positively correlated. These result
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29 564 confirmed the contribution of As(III) oxidation mediated by *Thiomonas* spp. to the treatment
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31 565 process.

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35 567 The detection of *aioA* gene in all the biogenic precipitates and the outlet waters confirmed the
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37 568 stable presence of As(III)-oxidizing bacteria in both bioreactors as observed in a previous
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39 569 field pilot (Laroche *et al.*, 2018). The relative abundance of As(III)-oxidizing bacteria
40
41 570 strongly varied depending on the nature of the samples and the period, with a particular
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43 571 enrichment in the suspended community of the WP bioreactor during period E ($56 \pm 11\%$)
44
45 572 and in the PS bioreactor during period C, D and E (12-34%). Thus As(III)-oxidizing bacteria
46
47 573 may represent higher proportions of the total community compared to what was described in
48
49 574 other systems. For instance, the relative abundance of *aioA* in geothermal samples ranged
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51 575 from 0.1 to 19.5% (Sonthiphand *et al.*, 2021). In groundwater systems of an old tin mine
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53 576 (containing a maximum of $10 \mu\text{g L}^{-1}$ Arsenic), the relative abundance of *aioA* gene ranged
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55 577 from 0.85 to 37.13% (Sonthiphand *et al.*, 2021). In the present study, high arsenic
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57 578 concentrations (50 to 100 mg L^{-1}) mainly in the As(III) form might explain the elevated
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59 579 concentration of As(III)-oxidizing populations quantified in the Carnoulès AMD (inlet water)
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580 that fed the PS and WP bioreactors. The conditions inside the bioreactors did not lead to a
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582 clear increase in As(III)-oxidizing bacteria abundance inside the treatment units compared to
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584 the density measured in the AMD (Figure 5.d).

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3 584 The highest abundances of *aioA* genes and *aioA* transcripts, observed during period E, were
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5 585 concomitant with an increase of *Thiomonas*-related sequences. Period E was characterized by
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7 586 an increase of temperature by more than 5°C, an increase of the total Fe concentration (~ 100
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9 587 mg L⁻¹), and total As concentration (~ 15 mg L⁻¹) (Table 1 and Diaz-Vanegas *et al.*, 2022).
10
11 588 Similarly, Tardy *et al.*, (2018) showed, in batch experiment, that high temperature stimulated
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13 589 As(III) oxidation associated with an increase of both the abundance of *aioA* genes and of
14
15 590 *Thiomonas* spp. Quéméneur *et al.*, (2010) found high *aioA* gene densities associated with high
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17 591 concentration of As in surface water.

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21 593 As(III)-oxidizing bacteria distribution was different in the two bioreactors: they were more
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23 594 represented in the attached community in the PS bioreactor, and in the suspended one in the
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25 595 WP bioreactor. In opposition to the common idea that the formation of a carrier-attached
26
27 596 biofilm enhances bacterial activity, Michel *et al.*, (2007) showed that As(III) oxidation
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29 597 activity of some *Thiomonas* strains was higher for the suspended cells than for the attached
30
31 598 ones. This is in agreement with our results for the WP bioreactor, but contradictory with the
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33 599 results for the PS bioreactor. Consequently, in the present study, As(III) oxidation by
34
35 600 *Thiomonas* did not seem to be conditioned by their lifestyle. It is also possible that the
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37 601 *Thiomonas* strains developed in the PS and WP bioreactors were able to switch from attached
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39 602 to suspended mode as reported by Farasin *et al.*, (2017). There is growing evidence that
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41 603 environmental bacteria are capable of alternating between the free living and the attached
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43 604 fractions (Grossart, 2010) and that the two fractions are connected (Tang *et al.*, 2017).

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47 606 Arsenic oxidation led to the enrichment into As(V) in the biogenic precipitates (80 ± 11 %)
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49 607 and in the outlet water (41 ± 26 %, compared with 25 ± 5 % in the inlet water) (Diaz-Vanegas
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51 608 *et al.*, 2022). Because of the higher affinity of As(V) for iron minerals (Paikaray 2015), As
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53 609 removal under its oxidized form is more efficient and stable.

610 611 ***Geochemical and environmental factors influencing bacterial diversity and*** 612 ***activity***

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614 In AMD affected environments, bacterial diversity is under the control of geochemical and
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616 environmental parameters (Méndez-García *et al.*, 2014, 2015; Volant *et al.*, 2014). As
supported by Spearman correlations, pH, dissolved Fe(II) and As concentrations as well as
water temperature were drivers determining the dynamic of FeOB and As(III) oxidizers

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3 617 populations in the bioreactors. pH has a strong direct and indirect influence on AMD
4 618 microbial communities due to its relationships with geochemical variables such as minerals
5 619 solubility. In particular, pH controls dissolved As and Fe concentrations in the AMD (Jones *et*
6 620 *al.*, 2015; Méndez-García *et al.*, 2015).
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10 621
11 622 In the present study, *Gallionella* and *Gallionellaceae*-related sequences showed a positive
12 623 correlation with pH of both inlet and outlet waters. The decrease of their relative abundance in
13 624 the PS bioreactor coincided with the decrease of the pH. From period D, the bacterial
14 625 communities inside the bioreactors were enriched with *Ferrovum*, known for its capacity to
15 626 grow in more acidic conditions than *Gallionella* (Ziegler *et al.*, 2013). Accordingly, *Ferrovum*
16 627 showed a negative correlation with pH. All these findings are consistent with previous studies
17 628 showing that *Gallionella* is favored at pH > 3 while *Ferrovum* are favored at pH < 3 (Jones *et*
18 629 *al.*, 2015; Sheng *et al.*, 2017).
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27 631 In AMD, dissolved ferrous iron (Fe(II)) is one of the main energy source for bacteria; thus, its
28 632 concentration may be another important factor determining geochemical niches of FeOB
29 633 (Jones *et al.*, 2015). Here, the relative abundance of *Gallionella* and *Gallionellaceae*-related
30 634 sequences were negatively correlated with Fe(II) concentration as described elsewhere
31 635 (Fleming *et al.*, 2014). The abundance of *Ferrovum* increased during period D and E in both
32 636 bioreactors concomitantly to the increase of the Fe(II) concentration in the inlet water.
33 637 Assignment of *Ferrovum* to high Fe concentration was also reported by Jones et al 2015.
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41 639 In the PS bioreactor, *Acidithiobacillus* abundance increased when the highest Fe(II)
42 640 concentrations (712-1049 mg L⁻¹) were measured in the inlet water (periods F and G). This is
43 641 in contradiction with other study (Jones *et al.*, 2015) where this genus was restricted to lower
44 642 Fe(II) concentration (< 224 mg L⁻¹). Then, another factor should be responsible for the
45 643 dynamic of *Acidithiobacillus* in the PS bioreactor at the end of the monitoring. During periods
46 644 F and G, oxygen limitation, due to the clogging of the aeration system, could be detrimental
47 645 to *Ferrovum* and *Leptospirillum* (Ziegler *et al.*, 2013; Johnson *et al.*, 2014). Conversely, the
48 646 capacity of *Acidithiobacillus* to live in microaerophilic conditions has been widely described
49 647 (Dave *et al.*, 2008). Thus, the dynamic of *Acidithiobacillus* in the bioreactors may be
50 648 attributed to the synergy of the lack of oxygenation, the different biomass carrier used or
51 649 another undetermined factor.
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3 651 Additional parameters that influence bacterial diversity in the bioreactors were dissolved As
4 652 concentration and water temperature, as reported by Laroche *et al.*, (2018) and Tardy *et al.*,
5 653 (2018). As discussed previously, the present study supports the positive influence of
6 654 temperature on the raise of the relative abundance of *Thiomonas* / arsenite-oxidizing bacterial
7 655 populations. In contrast, *Gallionella* and *Gallionellaceae*-related sequences were negatively
8 656 correlated with As concentration and temperature of the inlet water, as observed by Fleming
9 657 *et al.*, (2014). The positive correlations between *Ferrovum* abundance and temperature and
10 658 arsenic concentration in the inlet water (Figure 7), have not been described before. They
11 659 revealed the adaptation of *Ferrovum* to high As concentrations, which makes it a good
12 660 candidate for the bioremediation of highly As-rich effluents. On the contrary, the negative
13 661 correlation of *Gallionella* and *Gallionellaceae*-related sequences with As concentration,
14 662 suggest their possible poor adaptability for the treatment of As rich AMD.
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664 ***Links between treatment performance and microbiology***

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29 666 The integrated analysis of performance indicators (As and Fe precipitation rates and Fe(II)
30 667 oxidation kinetic constant KOFe) and microbiological variables provides new insights about
31 668 the relationships between total and active bacterial diversity and treatment efficiency during
32 669 periods of optimal operation (B-E). The abundance of total and active *Leptospirillum* and
33 670 *Ferrovum* were positively correlated with performance indicators. Both genera are well
34 671 known for their highly efficient Fe-oxidation activity (Chen *et al.*, 2013; Johnson *et al.*,
35 672 2014). The maintenance of their activity even at high As concentration (50 – 100 mg L⁻¹) is an
36 673 important point to consider for the treatment of high-As AMD.
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45 675 Despite the slight differences observed in the microbiology of the two bioreactors, the general
46 676 performances (in terms of As and Fe oxidation and removal) were similar. The global stability
47 677 of the system performances, (including both the temporal stability and the capacity to recover
48 678 the performances after disturbances) may rely on functional redundancy of the bacterial
49 679 communities. Indeed, different taxonomic groups sharing similar functions allowed the
50 680 performance of a system to be maintained even when the community structure changes
51 681 (Allison and Martiny, 2008). Thus, the robustness of the treatment system may rely both on
52 682 the resilience capacity and the functional redundancy of the bacterial communities. This is in
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3 683 line with the uncoupling between the resilience in function and the resilience in bacterial
4 684 diversity described by Mills *et al.*, (2003).
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686 **Conclusion**

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688 Inside the bioreactors, FeOB were controlled by pH, dissolved Fe and As concentrations and
689 temperature. The over-representation of *Ferrovum* in the active community, together with the
690 positive correlation between its abundance and the highest treatment performances, confirmed
691 its central role in the treatment efficiency. Furthermore, based on the positive correlation
692 between its relative abundance and dissolved arsenic concentration, *Ferrovum* appeared
693 particularly well adapted for the treatment of As rich effluents. *Leptospirillum* was another
694 permanent member of the active community, which suggests its contribution to the stable
695 performances. The bioreactor filled with wood and pozzolana (WP) showed a faster recovery
696 of its performances after interruptions that may be attributed to a higher bacterial alpha
697 diversity.

698 Our work provided the first evidence of the expression of the arsenite oxidase gene (*aioA*) in
699 treatment bioreactors under field-conditions. This is an important function since As oxidation
700 contributes to a more efficient removal of As, mainly under its more stable form (As(V)),
701 which is a clear advantage for future application in bioremediation. More effort is required to
702 optimize the quantification of *aioA* gene expression to better identify the factors regulating
703 the expression of this key function.

704 The resilience of bacterial communities to operational disruptions and variability of the
705 chemistry of the AMD of on-site treatment is another essential point in the perspective of a
706 large-scale AMD treatment.

707 The validation of our process in real field conditions provided achievement of Technology
708 Readiness Level 5. This technology, which did not use chemical reagent, could be
709 competitive with lime treatment, provided that additional treatment step such as sulfate-
710 reducing bioreactor is used to meet discharge standards.

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3 715 **Acknowledgments**
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For Peer Review

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For Peer Review

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3 1 **Figure 1.** Evolution of the bioreactor performances (represented by KOFe and As precipitation rate).
4 2 Solid lines represent the evolution of the kinetic constant value (KOFe) for the WP and PS bioreactors.
5 3 Dashed lines represent the evolution of the arsenic precipitation rates ($\text{mol L}^{-1} \text{s}^{-1}$) calculated for each
6 4 pilot. The triangles and the squares represent the data from the PS and WP bioreactor respectively
7 5 (Diaz-Vanegas et al., 2022). The stars represent the periods with the clogging of the air diffusers. The
8 6 values used for this graph correspond to the average of the data obtained for each period.

7
8 **Figure 2.** Diversity indices for the total bacterial communities developed in PS and WP bioreactors;
9 alpha diversity: Chao1 and Shannon (a), beta diversity (Bray-Curtis) (b). ("****" =p-value < 0.0001,
10 "****" p-value <0.001). These analyses include total and active communities.

11
12 **Figure 3.** NMDS with the main taxa (genus level) explaining the communities' differences (p-value <
13 0.05). When genus identification was not possible, classification was made at the family or at the order
14 level. The symbols represent the structure of the bacterial communities of each sample. The shape of
15 the symbol represents the lifestyle (suspended or attached) and the status of the community (total
16 community based on DNA analysis or active community based on RNA analysis). The colors
17 represent the periods of monitoring (B, C, D, E, F and G). The two ellipses differentiate the samples
18 from each bioreactor (dashed line for the WP samples and solid line for the PS samples). Stress value=
19 0.2088535.

20
21 **Figure 4.** Evolution of the relative abundances of the main bacterial at genus level (relative abundance
22 > 0.05 % of total number of sequences) over the monitoring periods. The results are presented for the
23 PS (plastic biomass carrier) and WP (wood and pozzolana as biomass carrier) bioreactors according to
24 the different lifestyle (attached versus suspended) and to the status of the communities (total or active).
25 When genus identification was not possible, classification was made at the family level (*) or at the
26 order level (**).

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2
3 **Figure 5.** Abundance of genes and transcripts in the biogenic precipitates and in the water collected at the
4 inlet and at the outlet of the two bioreactors (average of all the periods). 16S rRNA genes and transcripts
5 in the attached community (a), and in the suspended community (b), *aioA* genes and transcripts in the
6 attached community (c) and in the suspended community (d). Analyses were performed on DNA
7 (presence of the genetic potential) and cDNA (gene expression). Values annotated with the same letter are
8 not significantly different (Tukey's multiple range test with $p = 0.05$). The quantification of the *aioA*
9 transcripts from cDNA in the WP biogenic precipitates was below the detection limit.
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15 **Figure 6.** *aioA* / 16S rRNA genes ratio in the suspended (outlet water) and attached communities
16 (biogenic precipitates) in the two bioreactors (based on DNA analyses only). Data are mean values ($n= 3$).
17 *** the three asterisks correspond to period F (bioreactor PS and WP) and period G (only bioreactor PS),
18 for which data are missing due to technical limitations.
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23 **Figure 7.** Heat map of the Spearman correlation coefficients between relative abundance of the most
24 dominant taxa (vertical axis) and physicochemical parameters of the inlet water, operational parameters or
25 performances indicators (represented in the horizontal axis). All the parameters used are presented in
26 **Error! Reference source not found.**, supplementary Table S1 and S2; for the performance indicators
27 only the periods with optimal performances B- E were considered. Correlation analyses were performed
28 with the sum of the sequences data from the total and active communities (based on DNA and RNA
29 analyses). The last group corresponds to the relative abundance of the As(III)-oxidizing bacteria
30 (correlation performed only with the abundance of the total communities (based on DNA analyses). When
31 genus identification was not possible, classification was made at the family level (*) or at the order level
32 (**).
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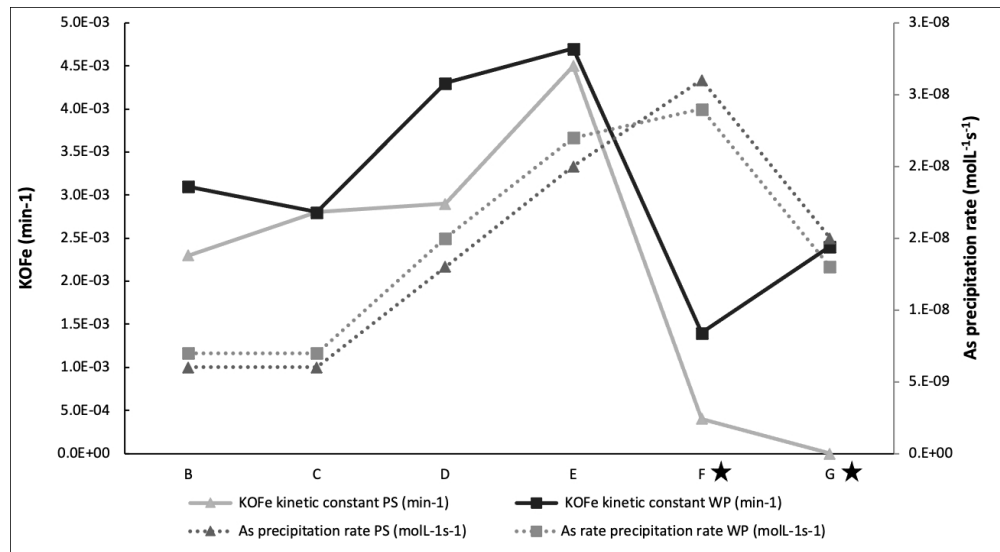


Figure 1. Evolution of the bioreactor performances (represented by KOFe and As precipitation rate). Solid lines represent the evolution of the kinetic constant value (KOFe) for the WP and PS bioreactors. Dashed lines represent the evolution of the arsenic precipitation rates (mol L⁻¹ s⁻¹) calculated for each pilot. The triangles and the squares represent the data from the PS and WP bioreactor respectively (Diaz-Vanegas et al., 2022). The stars represent the periods with the clogging of the air diffusers. The values used for this graph correspond to the average of the data obtained for each period

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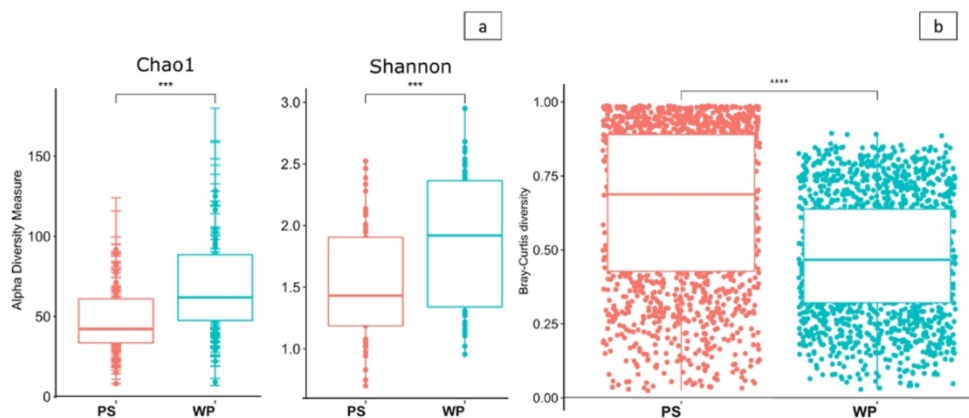


Figure 2. Diversity indices for the total bacterial communities developed in PS and WP bioreactors; alpha diversity: Chao1 and Shannon (a), beta diversity (Bray-Curtis) (b). ("****" =p-value < 0.0001, "****" p-value <0.001). These analyses include total and active communities.

260x107mm (150 x 150 DPI)

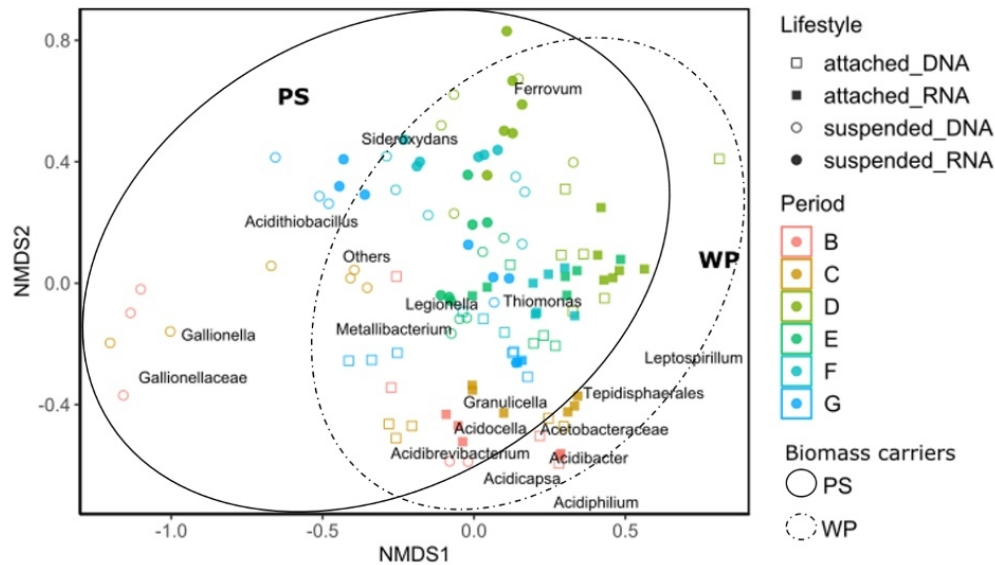


Figure 3. NMDS with the main taxa (genus level) explaining the communities' differences (p -value < 0.05). When genus identification was not possible, classification was made at the family or at the order level. The symbols represent the structure of the bacterial communities of each sample. The shape of the symbol represents the lifestyle (suspended or attached) and the status of the community (total community based on DNA analysis or active community based on RNA analysis). The colors represent the periods of monitoring (B, C, D, E, F and G). The two ellipses differentiate the samples from each bioreactor (dashed line for the WP samples and solid line for the PS samples). Stress value= 0.2088535.

160x90mm (150 x 150 DPI)

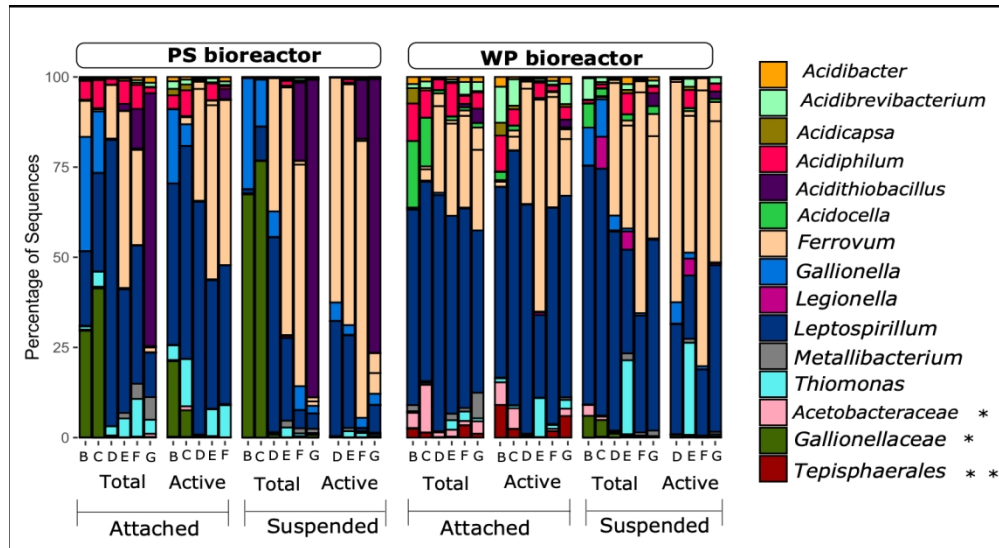


Figure 4. Evolution of the relative abundances of the main bacterial at genus level (relative abundance > 0.05 % of total number of sequences) over the monitoring periods. The results are presented for the PS (plastic biomass carrier) and WP (wood and pozzolana as biomass carrier) bioreactors according to the different lifestyle (attached versus suspended) and to the status of the communities (total or active). When genus identification was not possible, classification was made at the family level (*) or at the order level (**).

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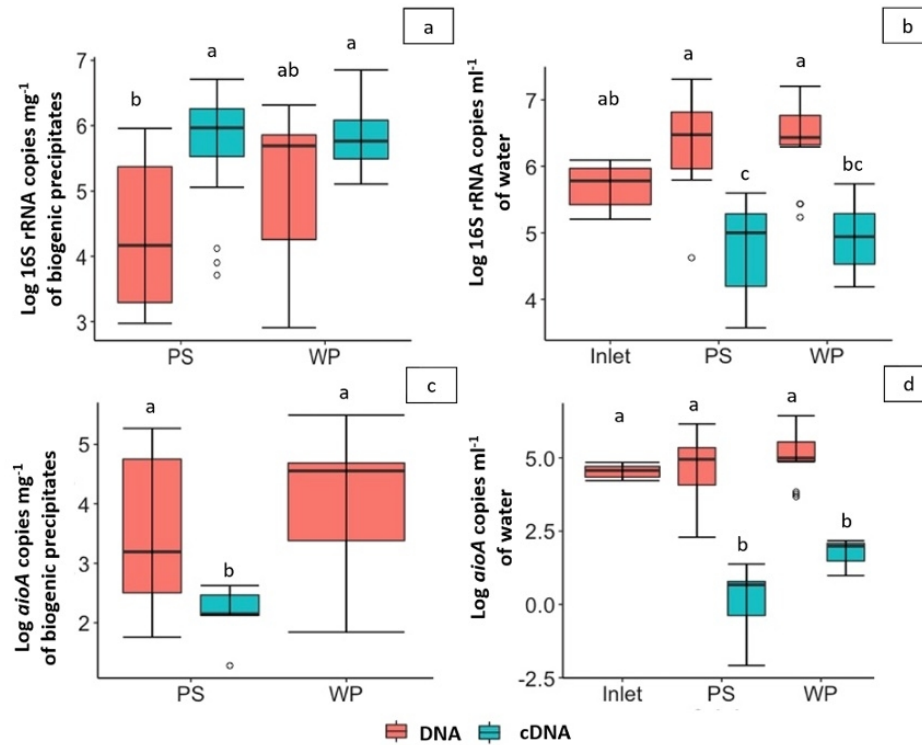


Figure 5. Abundance of genes and transcripts in the biogenic precipitates and in the water collected at the inlet and at the outlet of the two bioreactors (average of all the periods). 16S rRNA genes and transcripts in the attached community (a), and in the suspended community (b), *aioA* genes and transcripts in the attached community (c) and in the suspended community (d). Analyses were performed on DNA (presence of the genetic potential) and cDNA (gene expression). Values annotated with the same letter are not significantly different (Tukey's multiple range test with $p = 0.05$). The quantification of the *aioA* transcripts from cDNA in the WP biogenic precipitates was below the detection limit.

154x121mm (150 x 150 DPI)

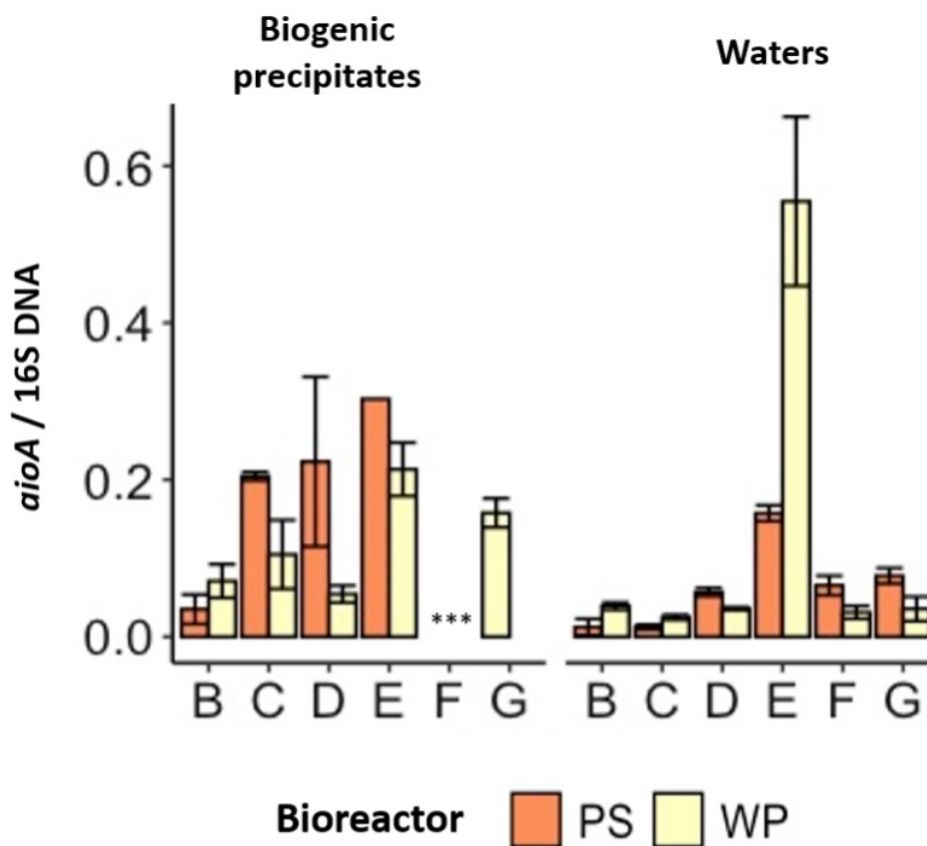


Figure 6. *aioA* / 16S rRNA genes ratio in the suspended (outlet water) and attached communities (biogenic precipitates) in the two bioreactors (based on DNA analyses only). Data are mean values (n= 3). *** the three asterisks correspond to period F (bioreactor PS and WP) and period G (only bioreactor PS), for which data are missing due to technical limitations.

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7	<i>Thiomonas</i>	0.2	0.2	0.4	0.3	-0.3	-0.2	-0.1	-0.2	0.6	-0.3	0.3	0.8
8	<i>Leptospirillum</i>	-0.3	-0.4	-0.3	-0.4	-0.2	0.3	0.1	0.2	-0.2	0.2	-0.3	-0.1
9	<i>Ferroplasma</i>	0.6	0.6	0.5	0.3	-0.5	0.3	0.7	0.6	0.3	-0.7	-0.3	0.4
10	<i>Gallionella</i>	-0.5	-0.4	-0.5	-0.4	0.1	-0.5	-0.3	-0.4	-0.5	0.5	-0.1	-0.4
11	* <i>Gallionellaceae</i>	-0.6	-0.5	-0.6	-0.5	0.2	-0.4	-0.3	-0.4	-0.5	0.6	-0.1	-0.3
12	<i>Acidithiobacillus</i>	0.6	0.6	0.4	0.6	0.4	-0.2	-0.2	-0.2	0.2	-0.3	0.2	0.6
13	<i>Sideroxydans</i>	-0.1	-0.1	0.0	-0.2	-0.2	0.1	0.2	0.2	-0.1	-0.1	-0.2	-0.3
14	<i>Acidicapsa</i>	-0.2	-0.3	0.1	-0.1	-0.1	-0.4	0.0	-0.3	0.3	0.1	0.4	0.1
15	<i>Acidiphilium</i>	-0.2	-0.2	0.0	0.1	-0.1	-0.1	-0.5	-0.4	0.5	0.1	0.5	0.6
16	<i>Acidibrevibacterium</i>	0.0	0.0	0.1	0.2	0.3	0.0	-0.4	-0.4	0.4	0.0	0.7	0.0
17	* <i>Acidobacteriaceae</i>	0.5	0.5	0.4	0.5	0.1	-0.1	-0.2	-0.2	0.5	-0.2	0.5	0.6
18	* <i>Acetobacteraceae</i>	-0.3	-0.4	-0.2	-0.1	0.1	0.2	-0.3	-0.3	0.1	0.2	0.4	-0.1
19	** <i>Tepisphaerales</i>	0.0	0.0	0.0	0.1	0.1	0.1	-0.3	-0.2	0.3	0.1	0.4	0.0
20	<i>aioA/16S ratio</i>	0.6	0.5	0.6	0.7	-0.4	-0.1	0.4	0.1	0.6	-0.3	0.2	1.0
21		As T inlet (mg L ⁻¹)	Fe(II) inlet (mg L ⁻¹)	T (°C) inlet	As(III) % inlet	pH inlet	KOFe (min ⁻¹)	Fe precipitation rate (mol L ⁻¹ s ⁻¹)	As precipitation rate (mol L ⁻¹ s ⁻¹)	As(V) % outlet	pH outlet	HRT (h)	<i>aioA/16S ratio</i>

Figure 7. Heat map of the Spearman correlation coefficients between relative abundance of the most dominant taxa (vertical axis) and physicochemical parameters of the inlet water, operational parameters or performances indicators (represented in the horizontal axis). All the parameters used are presented in Table 1, supplementary Table S1 and S2; for the performance indicators only the periods with optimal performances B- E were considered. Correlation analyses were performed with the sum of the sequences data from the total and active communities (based on DNA and RNA analyses). The last group corresponds to the relative abundance of the As(III)-oxidizing bacteria (correlation performed only with the abundance of the total communities (based on DNA analyses). When genus identification was not possible, classification was made at the family level (*) or at the order level (**).

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Table 1 Inlet and outlet water main characteristics (DO was measured inside the bioreactor)

Date (d.m.y)	Period	T (°C)	pH inlet	As T* inlet (mg L ⁻¹)	Fe (II) inlet (mg L ⁻¹)	HRT (h)		TOC (mg L ⁻¹)		DO (mg L ⁻¹)**	
						PS	WP	PS	WP	PS	WP
19.12.19	B	13.9	4.82	53.64	501	18	19	2.3	5.4	NA	NA
06.02.20	C	8.3	4.11	49.5	448.9	18	19	3.0	3.5	10.7	NA
12.03.20	D	14.1	3.87	70.7	665.2	9	10	2.9	3.0	9.1	10.0
18.06.20	E	22.3	3.79	88.1	732.3	18	19	NA	NA	6.1	5.0
27.07.20	F	20.2	4.12	93.1	820.7	9	10	4.0	4.6	0.2	3.1
15.09.20	G	20.5	4.44	91.0	768.6	18	19	4.6	4.1	NA	NA

*Total As; **Dissolved Oxygen measured inside the bioreactors

Supplementary Information

Table SI 1. Chemical characterization of inlet and outlet waters for the sampling dates where microbiological analyses were performed

Date (dd.mm.yy)	Period	Flow rate (L·h ⁻¹)		HRT (h)		pH inlet	Temp. (°C)	As T inlet* mg·L ⁻¹	Fe (II) inlet mg·L ⁻¹	As(III) inlet %	KOFe (min ⁻¹)		Fe rate prec. (mol·L ⁻¹ s ⁻¹)		As removal ratio (mol·L ⁻¹ s ⁻¹)		As(V) outlet %		TOC** outlet (mg·L ⁻¹)	
		PS	WP	PS	WP						PS	WP	PS	WP	PS	WP	PS	WP		
19.12.19	B	15	17.5	19.3	4.82	13.9	53.64	501	73		2.50	3.30	6.20	5.80	3.60	2.10			2.32	5.44
06.02.20	C	15	17.5	19.3	4.11	8.3	49.5	448.9	71		2.20	2.90	4.90	5.40	3.80	2.26			3.03	3.54
12.03.20	D	30	8.8	9.7	3.87	14.1	70.7	665.2	71		2.90	4.60	1.30	1.30	9.80	6.70			2.89	3.01
18.06.20	E	15	17.5	19.3	3.79	22.3	88.1	732.3	80		2.75	3.00	1.20	1.25	3.30	2.10			NA	NA
27.07.20	F	30	8.8	9.7	4.12	20.2	93.1	820.7	78		3.54	1.50	3.20	1.04	8.60	6.90			4.02	4.61
15.09.20	G	15	17.5	19.3	4.44	20.5	91.0	768.6	82		1.20	2.30	5.80	7.10	3.90	3.30			4.56	4.13

*Total As ; **Total Organic Carbon

Table SI 2. Performance indicators for the sampling dates where microbiological analyses were performed

Date (d.m.y)	Period	Total As (wt%)		Total Fe (wt%)		As/Fe (mol mol ⁻¹)*		%As V (wt%)	
		PS	WP	PS	WP	PS	WP	PS	WP
19.12.19	B	10.7	4.9	37	22	0.39	0.30	81	88
06.02.20	C	NA	NA	NA	NA	NA	NA	NA	NA
12.03.20	D	9.1	5.7	36	28	0.34	0.27	69	53
18.06.20	E	8.6	8.3	36	32	0.32	0.35	77	78
27.07.20	F	8.3	5.0	31	25	0.36	0.26	83	83
15.09.20	G	12.2	8.4	33	25	0.49	0.45	92	94

*Molar ratio

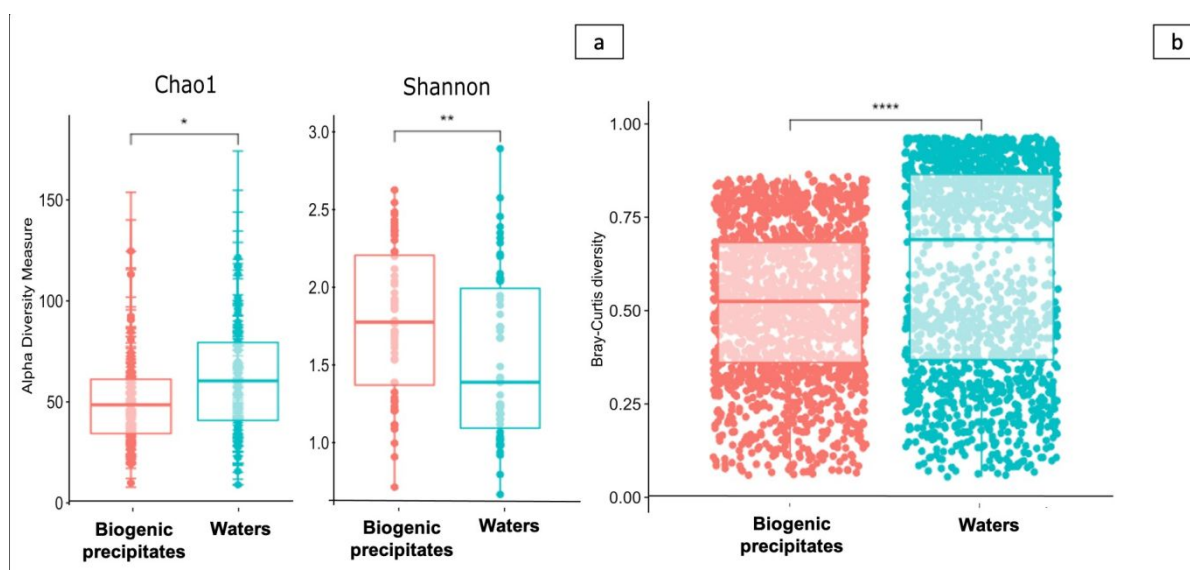


Figure SI 1. Indices of alpha (a) and beta (b) diversity obtained from the different types of samples (biogenic precipitates and water). ("****" = p-value < 0.0001, "****" p-value < 0.001, "***" = p-value < 0.01, "**" = p-value < 0.05). These analyses include total and active.

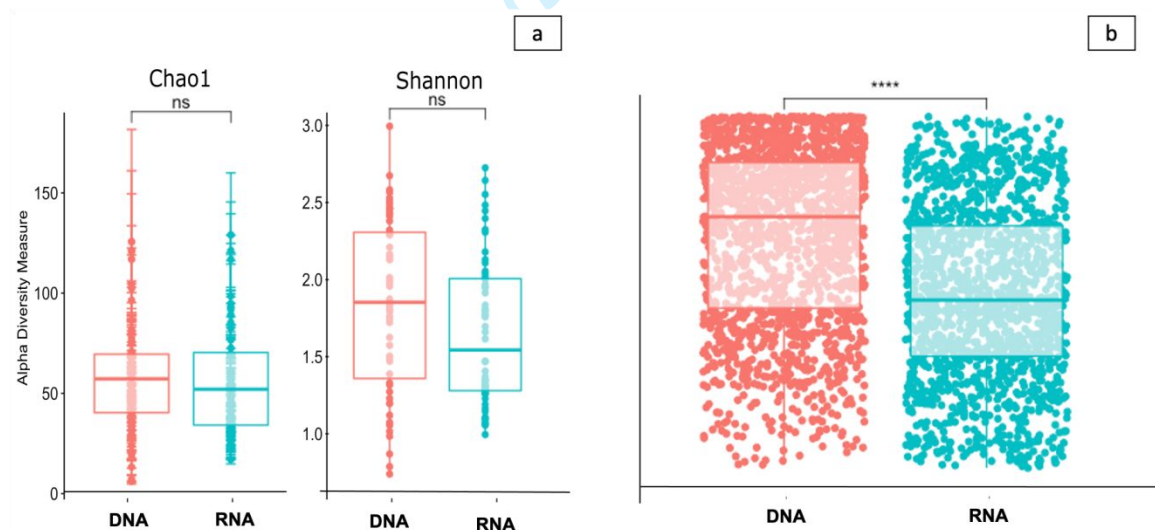


Figure SI 2. Indices of alpha (a) and beta (b) diversity obtained from the DNA and RNA extracts. ("****" = p-value < 0.0001, "ns" = p-value > 0.05).

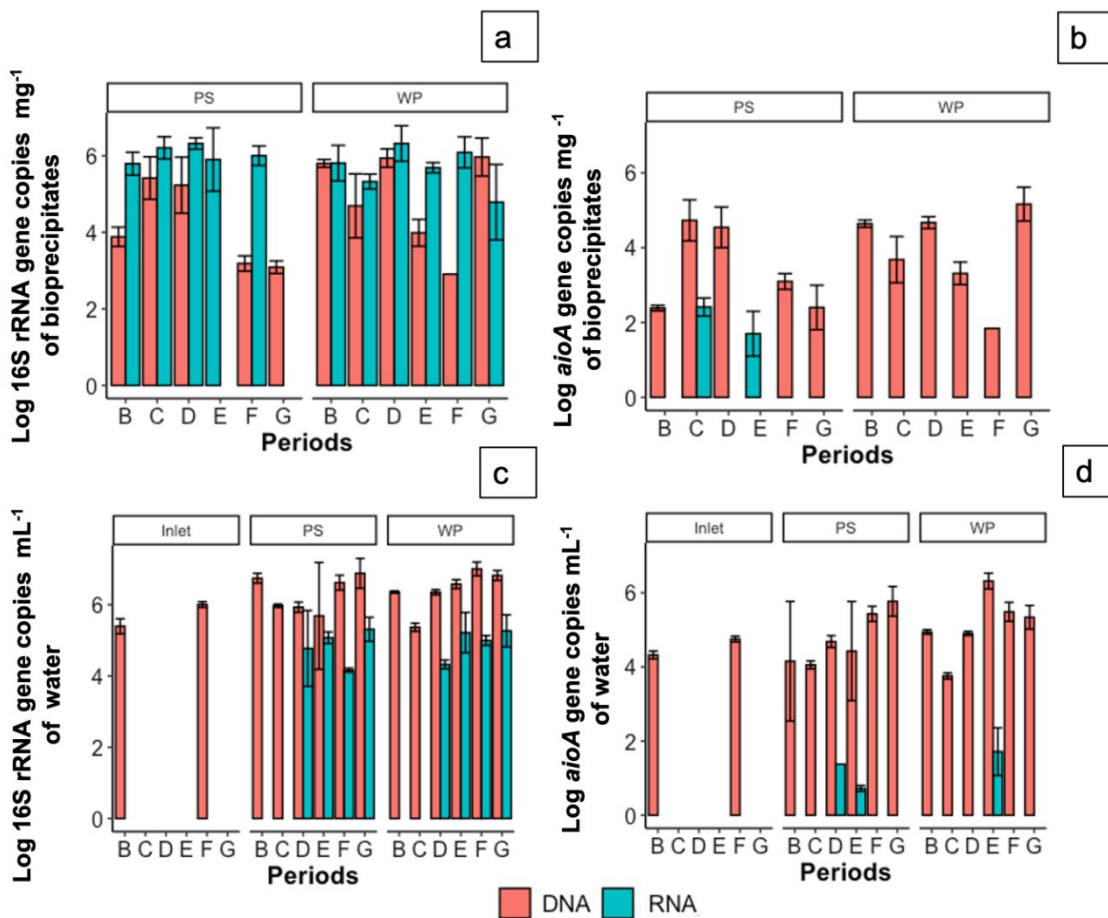


Figure SI 3. Number of copies of 16S rRNA gene (a) and *aioA* gene (b) in biogenic precipitates. Number of copies of 16S rRNA gene (c) and *aioA* gene (d) in water.