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Bio-precipitation of arsenic and antimony in a sulfate-reducing bioreactor treating real acid mine drainage water

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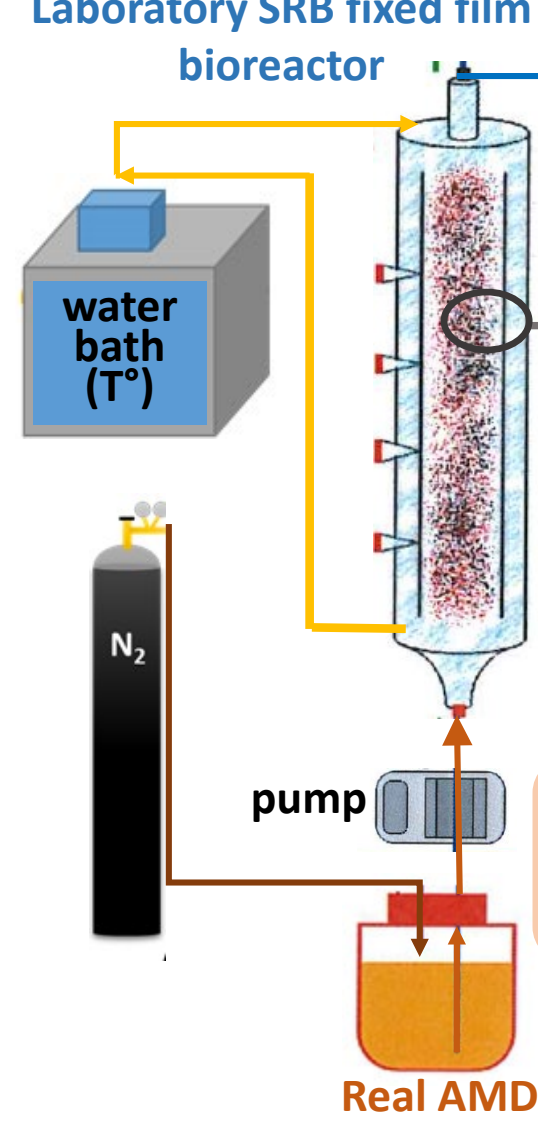
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**Bio-precipitation of antimony in a sulfate-reducing
bioreactor treating a real As-rich acid mine drainage water**

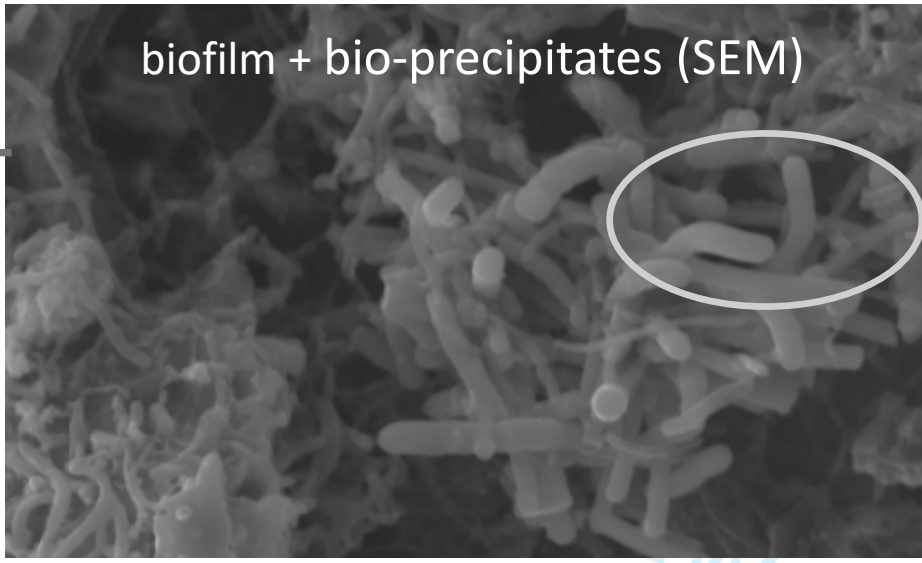
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Keywords:	antimony, arsenic, bioremediation, acid mine drainage, sulfate-reducing bacteria, Desulfosporosinus

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Laboratory SRB fixed film bioreactor



> 96 % As removed
> 97% Sb removed



16S rRNA gene metabarcoding
↓
Desulfosporosinus,
Cellulomonas,
Microbacter...

As 1mM
Sb from 0.01 mM to 1 mM

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3 **1 Bio-precipitation of antimony in a sulfate-reducing bioreactor treating a**
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6 **2 real As-rich acid mine drainage water**
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14 4 Elia Laroche^{1,2}, Catherine Jouliau¹, Cédric Duee¹, Corinne Casiot², Marina Héry², Fabienne
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36 **12 Abstract:**
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39 13 Arsenic (As) and antimony (Sb) can be disseminated from mining sites to aquatic ecosystems
40
41 14 by acid mine drainage (AMD). Here, the possibility to remove As and Sb concomitantly from
42
43 15 acidic waters by precipitation of sulphides induced by sulfate-reducing bacteria (SRB) was
44
45 16 investigated in a fixed-bed column bioreactor. The real AMD water used to feed the
46
47 17 bioreactor contained nearly 1 mM As and increasing Sb concentrations (0.008 ± 0.006 to 1.01
48
49 18 ± 0.07 mM) in order to reach a molar ratio $Sb/As = 1$. Results showed that the addition of Sb
50
51 19 did not affect the efficiency of As bio-precipitation. Sb was removed efficiently (up to 97.9%
52
53 20 removal) between the inlet and outlet of the bioreactor, together with As (up to 99.3%
54
55 21 removal) in all conditions. Sb was generally removed as it entered the bioreactor. Appreciable
56
57 22 sulfate reduction occurred in the bioreactor and could be linked to the stable presence of a
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3 23 major SRB OTU affiliated to the *Desulfosporinus* genus. The bacterial community included
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5 24 polymer degraders, fermenters, and acetate degraders. Results suggested that sulfate-reduction
6
7 25 could be a suitable bioremediation process for the simultaneous removal of Sb and As from
8
9 26 AMD.

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13 27 **Keywords:** antimony, arsenic, bioremediation, acid mine drainage, sulfate-reducing bacteria,
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15 28 *Desulfosporosinus*
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30 **Introduction**

31 Elevated concentrations of arsenic (As) and antimony (Sb) have been measured in diverse
32 natural environments due to anthropogenic emissions (Smedley and Kinniburgh, 2002; Herath
33 et al., 2017). These toxic metalloids are considered by the European Union and United States
34 Environmental Protection Agency as pollutants of priority interest (Council of the European
35 Communities, 1976; US EPA, 1982). Mining activities are a major source of As and Sb
36 dissemination in the environment (Smedley and Kinniburgh, 2002; He et al., 2019). Wastes
37 generated by mineral extraction can contain up to 10,000 ppm As and 1,000 ppm Sb
38 associated with sulfide minerals (Smedley and Kinniburgh, 2002; Wu et al., 2011; Cidu et al.,
39 2014). As result of inappropriate waste storage, oxidation of the sulfide minerals can generate
40 mine drainage characterised by high concentrations of these elements, which can enter aquatic
41 ecosystems (Manaka et al., 2007; Resongles et al., 2013; Paikaray, 2015; Sun et al., 2016).
42 However, solubilised As and Sb can be immobilised by co-precipitation with insoluble metal
43 (hydro)oxides (Cullen and Reimer, 1989; Filella et al., 2002). These processes have been
44 exploited in water treatment technology, and the combined removal of these two elements
45 following iron (Fe) addition was considered. However, variable efficiencies of As and Sb
46 adsorption onto iron compounds have been reported due to synergic or competitive behaviour

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2
3 47 according to the speciation of these elements (Lan et al., 2016; Qi and Pichler, 2017; Inam et
4
5 48 al., 2018; Wu et al., 2018) and pH (Inam et al., 2018). Moreover, the disadvantages of Fe-
6
7 49 based treatments are the need of a constant maintenance and elevated costs. The development
8
9 50 of passive treatment systems exploiting biological processes is a promising low-cost strategy
10
11 51 for the remediation of metals and metalloids present in acid mine drainages (Skousen et al.,
12
13 52 2000). Biological processes including direct bio-reduction or bio-oxidation can be exploited
14
15 53 in bioremediation solutions. Therefore, Kujala et al. (2022) studied the passive treatment of
16
17 54 mining-affected neutral water containing both Sb and As (around 2 and 0.5 μM respectively)
18
19 55 in peatlands, involving bio-oxidation and bio-reduction processes. Among the anaerobic
20
21 56 biological reactions, microbial sulfate-reduction has proven itself as an efficient way to
22
23 57 remove metals from mine waters at large scale (Mattes et al., 2011). In anaerobic bioreactors
24
25 58 or wetlands, the biological sulfide precipitation exclusively precipitates elements that are
26
27 59 already in the mine water. No external element (calcium or iron) is added, thus the final mass
28
29 60 of produced waste is lower than in competitor acid mine drainage technologies (Battaglia-
30
31 61 Brunet et al., 2021). Sulfate reduction has been shown to efficiently treat As and Sb
32
33 62 individually (Wang et al., 2013; Zhang et al., 2016; Alam and McPhedran, 2019; Xi et al.,
34
35 63 2020, Chen et al., 2020). Liu et al. (2018) also reported the concomitant removal of 5 mg/L
36
37 64 As and Sb from artificial waste water at neutral pH by bacterial sulfate reduction. In this batch
38
39 65 experiment, the removal of As depended on the presence of Fe(II) in the synthetic medium,
40
41 66 whereas Sb was removed even in absence of Fe. Although microbial sulfate-reduction has
42
43 67 been described for a long time as a bioprocess inhibited in acidic conditions, progress has
44
45 68 been made recently in the field of bio-precipitation of sulfides at low pH (Ñancucheo and
46
47 69 Johnson, 2012 and 2014). More specifically, bio-precipitation of arsenic sulfide at low pH
48
49 70 was reported (Battaglia-Brunet et al., 2012; Le Pape et al., 2017). From a chemical point of
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51 71 view, precipitation of As sulfide should be favored at low pH, while soluble thio-As
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3 72 complexes form at neutral and alkaline pH. The same geochemical behaviour was observed
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5 73 with Sb in geothermal systems (Planer-Friedrich and Scheinost, 2011). The pH is likely to
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7 74 affect the biogeochemical behaviour of Sb in sulfate-reducing bioreactors, as it was the case
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10 75 for As. Finally, the combined removal by bacterial sulfate-reduction of both As and Sb, when
11
12 76 they co-occur in an AMD has not yet been studied. In this context, the aim of the present
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14 77 study was to evaluate the efficiency of an upflow anaerobic fixed-bed column reactor to treat
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16 78 concomitantly As and Sb present in an AMD by biological sulfate-reduction in acidic
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18 79 conditions. The removal of these pollutants was investigated during ten months along the
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20 80 bioreactor in which Sb concentration was increased progressively. Physico-chemical
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22 81 parameters were monitored, together with mineral characterisation of the bioprecipitates. The
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24 82 spatial and temporal evolution of the bacterial community colonizing the reactor was
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26 83 investigated by 16S rRNA gene metabarcoding. Both results will document the understanding
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28 84 of the biogeochemical behaviour of As and Sb, in the context of sulfate reduction under acidic
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30 85 conditions.
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40 87 **Material and methods**

41 42 88 **Bioreactor design and operating conditions**

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45 89 The lab-scale experiment was performed in a fixed-bed reactor, consisting of a 360 mm glass
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47 90 column with an internal diameter of 36 mm (Fig. 1). A water jacket regulated the column
48
49 91 temperature at 25°C during the experiment and rubber-stopped sampling ports allowed liquid
50
51 92 sampling from the middle, bottom and upper levels. The column was sterilised by autoclaving
52
53 93 (120°C, 30 min) before filling with sterile materials composed of a mixture of pozzolana and
54
55 94 nutritive agar in order to enhance both bacterial attachment and growth, as previously
56
57 95 described (Battaglia-Brunet et al., 2021). The filling mixture was prepared with a total volume
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3 96 of 400 mL pozzolana, 400 mL agar at 25 g/L, 20 mL nutritive solution at pH 4.5 (20 g/L yeast
4
5 97 extract and 7.8 g/L KH_2PO_4) and 0.4 mL trace element solution (3 g/L EDTA; 1.1 g/L FeSO_4 ,
6
7 98 $7\text{H}_2\text{O}$; 65 mg/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 89 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 28 mg/L $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$; 18 mg/L
9
10 99 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 0.3 g/L H_3BO_3 ; 2 mg/L CuCl_2 ; 130 mg/L $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$).

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12
13 100 The filled column was de-oxygenated by flushing with sterile N_2 during three days, then
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15 101 inoculated with an acid tolerant microbial consortium containing sulfate-reducing bacteria
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17 102 (SRB) originating from the AMD of Carnoulès, France (Le Pape et al., 2017). Ten mL of this
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19 103 inoculum were centrifuged and the pellet stored at -80°C prior molecular analyses. A first step
20
21 104 promoting bacterial growth inside the column was performed in batch mode during 23 days.
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23 105 Then, the column was fed in continuous mode with AMD water sampled in January 2016 at
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25 106 the Carnoulès mine (Gard, France) and stored at 4°C under nitrogen until use. The main
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27 107 characteristics of this AMD are a pH close to 3, around 100 mg/L total dissolved As, 1000
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29 108 mg/L total dissolved Fe, 20 mg/L total dissolved Zn (Le Pape et al., 2017), and less than 1
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31 109 mg/L total dissolved Sb (Resongles et al., 2013). For the experiment, its pH was increased
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33 110 between pH 4.0 and 4.5 by adding NaOH. The water was filtered ($0.22 \mu\text{m}$) and
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35 111 complemented with 0.5 g/L glycerol as electron donor for the SRB. The feeding water was
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37 112 maintained in anaerobic condition under N_2 atmosphere.

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43 113 The variable parameter was the Sb concentration in the feed water: it was increased by adding
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45 114 Sb in the real AMD water, as potassium Sb(III) tartrate, in three steps (corresponding to phase
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47 115 1, 2 and 3) up to the molar equivalence between Sb and the As naturally present in the AMD.
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49 116 The experimental parameters of each phase, flow rate, pH, As and Sb concentration in the
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51 117 feed solution prepared with the real AMD, are summarised in Table 1. Analyses of
52
53 118 exploitable data started after the physico-chemical parameters (outlet pH, As, and Fe
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55 119 concentration in the outlet) had stabilised for each Sb concentration in the feed in order to
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57 120 compare the acquired results during each experimental condition.

121 **Monitoring**

122 Inlet and outlet waters were collected under anaerobic conditions and monitored over a 217
123 days period after the first 59 days dedicated to stabilisation of physico-chemical parameters.
124 The real flow-rate was calculated by weighing the cumulated outlet water collected in a bottle
125 (every day except Saturdays and Sundays). Inlet and outlet water pH were measured, and 10
126 mL were filtered at 0.45 μm , acidified with one drop of concentrated nitric acid and stored at
127 4°C until chemical analyses. The outlet water was sampled every day whereas the inlet water
128 sampling was done at each renewal of feed water.

129 Vertical profiles of physico-chemical and microbial parameters were determined at the end of
130 each phase from liquid samples taken along the column. Five mL of liquid samples were
131 collected in triplicate inside the column through the bottom, middle and upper septa with a
132 sterile syringe. These samples were mainly constituted of solution, with bacteria in
133 suspension, but could also contain precipitates (with or without attached bacteria). Triplicate
134 samples were also collected from inlet and outlet water bottles. Samples were filtered with a
135 sterile 0.22 μm cellulose acetate filter which was stored at -80°C for molecular analyses. The
136 filtrate was acidified and kept at 4°C for chemical analysis.

137 At the end of the experiment (day 217), the column was opened in a glovebox under N₂
138 atmosphere to collect bioprecipitates deposited on the solid filling materials. Triplicate
139 samples of solids were taken from the bottom, middle and upper parts of the column and
140 stored at -80°C until molecular analysis. Solid samples were also collected along the column
141 and dried under N₂ flux in anaerobic Hungate glass tubes for mineralogical characterisation.

142 **Chemical analyses**

143 Total As and Fe concentrations were monitored on outlet samples to check that stabilisation
144 was reached before changing the operating conditions, by Atomic Absorption

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3 145 Spectrophotometry (AAS, Varian SpectrAA 220 FS). After stabilisation, a more complete
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5 146 chemical characterisation was performed on selected water samples from outlet and inlet
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7 147 (three for each feed phase, except the Sb concentration from the inlet water in phase 1 in
8
9 148 duplicate, Table 1) and from the vertical profiles collected at the end of each phase. In these
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11 149 samples, total dissolved concentrations of As, Sb, Fe, S and other elements (Zn, Tl, Pb) were
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13 150 analysed using ICP-MS on the AETE-ISO platform (University of Montpellier).
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17 151 **Characterisation of the bioprecipitates**

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20 152 The bioprecipitates collected at the end of the experiment were characterised by scanning
21
22 153 electron microscopy (SEM). Prior to SEM, samples were submitted to plasma cleaner
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24 154 (Gambetti, Colibri, 300 s under Ar plasma) in order to remove organic phases. Samples were
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26 155 then covered with a 20 nm carbon coating (Cressington, Carbon Coater 208C) to make them
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28 156 conductive. The SEM images were acquired on a field emission gun scanning electron
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30 157 microscope MIRA 3 XMU (TESCAN, Brno, Czech Republic), under high vacuum conditions
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32 158 with a 15 kV beam. EDS spectra and element mappings were recorded using an EDAX
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34 159 Pegasus EDS system.
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39 160 **Molecular analyses**

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42 161 Genomic DNA was extracted from the pellet of the inoculum, frozen filters (5 mL aliquot)
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44 162 and bioprecipitates (between 0.5 and 0.8 g), using the FastDNA Spin Kit for soil according to
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46 163 the manufacturer's recommendations (MP Biomedicals, Illkirch, France), with the FastPrep®-
47
48 164 24 instrument at a speed of 5 ms⁻¹ for 30s. DNA extracts were quantified with the Quantifluor
49
50 165 dsDNA sample kit and the Quantus fluorometer, according to the manufacturer's instructions
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52 166 (Promega, Madison, WI, USA), and stored at -20°C.
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57 167 Bacterial diversity was determined by 16S rRNA gene metabarcoding. Libraries of amplicons
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59 168 and sequencing were performed by MetaHealth metagenomic-based services (CIRAD, PHIM,
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3 169 Eco&Sols, Montpellier, France). The 16S rRNA V3-V4 gene region was first amplified with
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5 170 primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-
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7 171 GACTACHVGGGTATCTAATCC-3'), in duplicate in a Mastercycler ep 384 (Eppendorf)
8
9 172 thermocycler with 20 μ L PCR reaction (0.5 g of DNA, 10 μ L of Phusion Flash High-Fidelity
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11 173 2X Master Mix (Thermo Fisher), 300 ng T4 gene 32 (MP Biomedicals), 0.25 μ M for forward
12
13 174 and reverse primers). The following conditions were applied: initial denaturation at 95°C for 2
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15 175 min, 25 cycles of 95°C for 30 s, 55°C for 40 s, 72°C for 30 s and a final elongation at 72°C
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17 176 for 10 min. A second PCR was run using primers 341F and 785R modified with Illumina
18
19 177 specific overhang sequences to implement dual barcodes. PCR reactions (15 μ L) contained:
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21 178 7.5 μ L Biolabs Taq 2X mastermix, 2 μ L of 10 μ M for forward and reverse primers, and 2 μ L
22
23 179 of PCR product. The following conditions were applied: initial denaturation at 95°C for 2
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25 180 min, 8 cycles of 95°C for 30 s, 55°C for 40 s, 72°C for 30 s and a final elongation at 72°C for
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27 181 10 min. Final amplicons concentrations were quantified on a D5000 ScreenTape bioanalyzer
28
29 182 (Agilent) and validated by qPCR on a LC480 real-time thermocycler (Roche), using qPCR
30
31 183 primers recommended in Illumina's qPCR protocol and Illumina's PhiX control library as
32
33 184 standard (Illumina). All amplicon products and control library (5% PhiX library) were finally
34
35 185 sequenced using paired-end Illumina MiSeq sequencing (2 \times 300 bp). The raw datasets are
36
37 186 available on the European Nucleotide Archive system under project accession number
38
39 187 PRJEB50048.

188 **Bioinformatic analyses**

189 Illumina reads were processed with the bioinformatics pipeline FROGS v 3.2 (Find Rapidly
190 OTUs with Galaxy Solution) (Escudié et al., 2018) available on the GenoToul Galaxy
191 platform (Afgan et al., 2018). Paired-end reads were firstly merged with a maximum of 10%
192 mismatch in the overlapped region with VSERACH. Only sequences with expected length
193 (between 340 and 450 nucleotides), no ambiguous bases (N) were clustered in two steps using

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3 194 the SWARM algorithm to obtain Operational Taxonomic Units (OTUs) with minimal
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5 195 distance ($d_g = 1$), then with an aggregation distance of 3 (Mahé et al., 2014). The removal of
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7 196 chimeric sequences was performed with VSEARCH with de novo UCHIME method (Edgar
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9 197 et al., 2011; Rognes et al., 2016), combined with a cross-sample validation. A filtering tool
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11 198 was used to select OTU according to their abundance (threshold = $5 \cdot 10^{-5}$, Bokulich et al.,
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13 199 2013), leading to a total of 1,595,159 high quality sequences. Subsampling to the lowest
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15 200 dataset (7,910 reads) was performed by random selection in order to efficiently compare the
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17 201 datasets and avoid biased community comparisons. Each OTU was affiliated against the
18
19 202 SILVA reference database (v138). Statistics tools included in FROGS pipeline were used to
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21 203 generate OTU table and to calculate the alpha and beta diversity (diversity indices and bray-
22
23 204 Curtis distances).

29 205 **Statistical analysis**

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32 206 Statistical analyses were performed with R version 3.4.3 (R Core Team, 2018) according to
33
34 207 Laroche et al. (2018). The “ade4” R package was used to perform a co-inertia analysis (CIA)
35
36 208 and two Principal Component Analyses (PCA) (Dray and Dufour, 2007). These analyses
37
38 209 highlighted the common structure between the physico-chemical parameters (pH and
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40 210 dissolved iron, sulfur, arsenic and antimony concentrations) and the dominant bacterial taxa in
41
42 211 the liquid samples at each position in the column. These dominant taxa represented more than
43
44 212 90% of the relative abundance of sequences in every sample during the monitoring period.
45
46 213 Before the CIA, the physico-chemical and biological variables were respectively standardised
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48 214 and Hellinger transformed (Legendre and Gallagher, 2001). The significance of the analysis
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50 215 was checked with a Monte-Carlo test (999 random permutations).
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218 **Results**

219 **Evolution of pH**

220 The pH was always increased between the inlet and the outlet water of the bioreactor (Fig.
221 2A). The average pH increased from 4.29 ± 0.03 to 5.10 ± 0.25 during the phase 1, from 4.03
222 ± 0.06 to 5.05 ± 0.19 during the phase 2 and from 4.35 ± 0.20 to 4.91 ± 0.29 during phase 3
223 (Fig. 2B). This pH increase was gradual in phase 1. In phases 2 and 3, there was a strong pH
224 increase (around 1 unit) between the inlet and the bottom of the column, followed by little pH
225 variation (< 0.3 unit) between the bottom and the upper section and finally a pH decreases
226 between the upper section and the outlet.

227 **Removal of As, Sb, Fe and S from the mine water**

228 The reactor removed 96 to 99% of dissolved As, during each of the three phases of
229 experiment (Fig. 3A). Arsenic removal occurred at different levels of the column bioreactor
230 depending on the experimental conditions. At the end of phase 1, 72% of As was removed
231 between the bottom and the middle of the column. In phase 2, As concentration decreased
232 gradually from 0.5 to 0.1 mM from the bottom to the top. In phase 3, As was mainly removed
233 between the feed point and the bottom of the reactor (99.8%).

234 During phase 1, Sb concentration in the feed solution was that of the natural AMD (less than
235 0.01 mM), thus it was too low to determine a precise removal rate. Sb removal during the two
236 next phases, in which Sb concentrations were adjusted to about 0.7 and 1 mM, averaged 97%
237 and 98% respectively. Most Sb was removed in the bottom zone of the reactor in each phase
238 (Fig. 3B and C). As for arsenic, its concentration decreased gradually from 0.018 to 0.004
239 mM along the column in phase 2. The dissolved Sb concentration in the outlet water was
240 always higher than in the upper zone of the bioreactor. This phenomenon, also observed for S
241 and Fe in phase 2, might be explained by oxidation reactions. Despite the constant flow of N_2

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3 242 flux in the outlet bottle, there may to have been some oxygen diffusion through the outlet
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5 243 pipe. Other toxic chemical substances (Pb, Tl and Zn) present in minor concentrations in the
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7 244 feed water were removed inside the bioreactor (Supplementary Table SM1).
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10 245 The average dissolved Fe and sulphur (S) concentrations in the inlet water were respectively
11
12 246 21 ± 2 and 38 ± 3 mM (Fig. 3 D and E). Maximum Fe removal reached 20, 35 and 14% in
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14 247 phase 1, 2 and 3, respectively (Fig. 3D). Sulfur removal reached 16, 17, and 11% in phases 1,
15
16 248 2 and 3 respectively. Inside the column, the S concentration decreased gradually from 33.8 to
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18 249 30.4 mM at the end of the phase 1 (Fig. 3E). For the others phases, it stabilised around $32.3 \pm$
19
20 250 0.05 mM (phase 2) and 30.0 ± 0.5 mM (phase 3) between bottom and upper section and
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22 251 increased slightly in the outlet water.
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27 252 **Observation of the bioprecipitates**

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30 253 SEM observation of the biofilm-colonised filling materials collected inside the bioreactor
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32 254 revealed the presence of mineral-encrusted rod-shaped bacteria of different sizes, around 5
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34 255 μm long and 0.5 to 2 μm wide (Fig. 4 A). The EDS spectra of the mineral phases associated
35
36 256 with this biofilm revealed major peaks of S and As. However, since the interaction volume of
37
38 257 the electron beam is ca. 1 μm^3 , it cannot be excluded that part of the EDS signal comes from
39
40 258 the surrounding phases. Other zones of the biofilm showed aggregates including rod cells-
41
42 259 shaped particles (Fig. 4C), enriched in Sb together with As and S as major peaks on the EDS
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44 260 spectrum (Fig. 4D). The elemental map of one bioprecipitate (Fig. SM2) showed differences
45
46 261 between the distribution of As and Sb in the As-Sb-S bearing phase. Moreover, the areas
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48 262 corresponding to high Fe concentrations were distinct to those of high As and Sb
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50 263 concentrations in this bioprecipitate.
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56 264 **Evolution of bacterial diversity**

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3 265 The 16S rRNA genes retrieved by metabarcoding from inoculum, liquid samples and
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5 266 bioprecipitates collected inside the bioreactor were classified into 78 OTUs. Overall, the
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7 267 richness (number of observed OTU) and the Shannon index (reflecting both richness and
8
9 268 evenness) indicated a low bacterial diversity in all the samples (Fig. SM3). The highest
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11 269 bacterial diversity was observed in liquids collected at the end of phase 1 in the bottom and in
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14 270 the upper of the column (30 ± 2 OTU observed).

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18 271 Ten taxa identified at the genus level had relative abundance of reads $\geq 2\%$ in at least one
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20 272 sample (Fig. 5). The inoculum, able to reduce sulfate at acidic pH and enriched from the
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22 273 Carnoulès AMD, was mainly composed of *Desulfosporosinus* (77.0%), *Microbacter* (11.6%)
23
24 274 and *Cellulomonas* (10.9%) These three genera were identified in all liquid samples collected
25
26 275 inside the column, whatever the level and the phase, with relative sequence abundances
27
28 276 varying from $1.9 \pm 0.4\%$ to $76.5 \pm 5.9\%$ (mean of triplicates) for *Desulfosporosinus*, $4.4 \pm$
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30 277 1.3% to $41.3 \pm 2.4\%$ for *Cellulomonas* and $16.0 \pm 4.6\%$ to $66.5 \pm 1.2\%$ for *Microbacter*.

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33 278 Other genera were detected in lower proportions inside the column: *Acidithiobacillus* ($0.7 \pm$
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35 279 0.2% to $16.9 \pm 2.0\%$), *Luteococcus* ($0.3 \pm 0.04\%$ to $9.3 \pm 1.7\%$), *Acidocella* (0 to $4.9 \pm 1.1\%$),
36
37 280 *Metallibacterium* (0 to $5.1 \pm 0.2\%$), *Sediminibacterium* (0 to $2.6 \pm 2.2\%$), *Streptococcus* (0 to
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39 281 $2.7 \pm 4.6\%$) and *Thiomonas* (0 to $3.1 \pm 0.3\%$). There was a clear evolution in both space and
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41 282 time of the taxonomic composition of bacterial communities inside the column.

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44 283 *Desulfosporosinus* proportions were lower during phases 1 and 2 in all levels ($1.9 \pm 0.4\%$ to
45
46 284 $35.7 \pm 8.9\%$), than in the inoculum. However, it became again dominant ($76.5 \pm 5.9\%$) during
47
48 285 phase 3 at the bottom of the column. *Cellulomonas* was the dominant genus in bottom and
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50 286 middle column samples in phase 1 (33.9 ± 0.7 and $41.3 \pm 2.4\%$), and co-dominant ($41.2 \pm$
51
52 287 5.8%) in the bottom level in phase 2. *Microbacter* was dominant in the upper level in phase 1
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54 288 and 2 ($53.3 \pm 3.9\%$ and $66.5 \pm 1.2\%$) and middle and upper levels in phase 3 ($36.6 \pm 9.1\%$
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56 289 and 53.9 ± 10.1). *Luteococcus* was always present but at low relative abundance (less than 9.3
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2
3 290 $\pm 1.7\%$) and particularly during the phases 1 (bottom and middle) and 2 (bottom). *Acidocella*
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5 291 was more abundant in the bottom level during phase 1 ($4.9 \pm 1.1\%$); its relative abundance
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7 292 was the lowest during phase 3.

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10 293 At the end of the experiment (phase 3), the main difference between communities from
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12 294 bioprecipitates and liquid samples was the relative abundance of the *Desulfosporosinus* genus.
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14 295 Its proportion was close for the bottom level and higher for the middle and top levels on the
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16 296 solids ($71.6 \pm 1.7\%$ bottom, $81.0 \pm 1.3\%$ middle and $47.6 \pm 5.6\%$ top) than in the liquid (76.5
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18 297 $\pm 1.7\%$ bottom, $30.0 \pm 15.6\%$ middle and $14.8 \pm 2.9\%$ top) samples. It was thus largely
19
20 298 dominant in the bioprecipitates collected from bottom and middle levels. *Microbacter*,
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22 299 *Cellulomonas* and *Luteococcus* were also identified in the bioprecipitates.

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26 300 A co-inertia analysis was performed to interpret the relationship between the physico-
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28 301 chemical parameters and the dominant bacterial taxa (Fig. 6). The Monte-Carlo test revealed a
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30 302 significant co-structure between the physico-chemical and biological variables (p-value =
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32 303 0.03). Ninety seven percent of the total variance in the two datasets were explained by the
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34 304 first two axes of the CIA. Samples were separated according their position in the column,
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36 305 particularly at the bottom, along the second axis of the analysis (Fig. 6A). The dotted arrows
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38 306 represent the difference between the ordination of the biological (origins of arrows) and
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40 307 physico-chemical parameters (arrowheads). The principal component analysis (PCA) of
41
42 308 physico-chemical parameters shows that samples at the bottom of the column were associated
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44 309 with lower pH values and higher dissolved As concentrations, at the opposite to those at the
45
46 310 middle and upper levels of the column (Fig. 6B). *Acidithiobacillus* were more abundant in the
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48 311 upper level of the column according the PCA of the dominant bacterial taxa (Fig. 6C).
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50 312 Iron and S were positively correlated with *Thiomonas* and *Metallibacterium*, but negatively
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52 313 with *Desulfosporosinus* (Fig. 6B and 6C). A positive correlation was observed between pH
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54 314 and *Microbacter*, whereas pH correlated negatively with *Cellulomonas* and

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3 315 *Luteococcus*, *Cellulomonas* and *Luteococcus* were also correlated positively with As, unlike
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5 316 *Microbacter*.

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10 11 12 318 **Discussion**

13 14 15 319 **Removal of As and Sb along the experiment**

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18 320 The upflow anaerobic fixed-bed bioreactor efficiently removed As, Sb and some metals (Pb,
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20 321 Tl and Zn) present in a real arsenic-rich AMD water amended with Sb. Antimony removal
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22 322 was efficient even when Sb concentration was increased: it averaged 97% for initial
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24 323 concentrations 0.7 mM (phase 2) and 1.01 mM (phase 3). The improvement of Sb sulfide
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26 324 precipitation by lowering pH was observed by Chen et al. (2020), however their experimental
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28 325 condition was less acidic (pH 6.5) than ours. Here, more than 96% of As was removed inside
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30 326 the column reactor even with the increase of Sb concentration in the feeding water. Similarly,
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32 327 Liu et al. (2018) reported the concomitant precipitation of As and Sb (up to 98.2% and 99.4%)
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34 328 from synthetic neutral pH solution containing 0.07 mM of As(V) and 0.04 mM Sb(V), by
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36 329 bacterial sulfate-reduction. Here, the increase in the fed Sb concentration did not affect As
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38 330 bio-precipitation. Increasing the total metalloids concentration in the feed might have allowed
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40 331 a more efficient and rapid consumption of dissolved sulfide, in particular near the acidic feed
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42 332 supply at the bottom of the reactor, thus decreasing the toxicity-related stress of bacteria
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44 333 induced by H₂S in acidic conditions.

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46
47 334 The SEM-EDS observations strongly suggest that As and Sb precipitated inside the reactor as
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49 335 solid sulfides. Li et al. (2022) already observed by SEM-EDS precipitates of amorphous
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51 336 precipitates identified as Sb₂S₃ in their batch culture of SRB grown in presence of high Sb
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53 337 concentrations. These As and Sb sulfide phases were detected in zones showing rod bacterial
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55 338 shapes, possibly because they precipitated around the cell that excreted the dissolved sulfide.

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3 339 Such a phenomenon was already observed in batch conditions with As sulfide precipitating as
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5 340 coatings around bacterial cells growing in batch (Le Pape et al., 2017) and continuous
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7 341 (Battaglia-brunet et al., 2021) conditions with AMD from Carnoulès site. Liu et al. (2018)
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9 342 suggested that As adsorption could occur on already formed sulfides. As a global way, the
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11 343 sorption/co-precipitation of As with metal sulfides was suggested by other studies (Jong and
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13 344 Parry, 2003; Sahinkaya et al., 2015). Among them, Fe sulfides were regularly cited to enhance
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15 345 the removal of As inside sulfate-reducing bioreactor (Luo et al., 2008; Altun et al., 2014; Liu
16
17 346 et al., 2018). In our column, the soluble Fe and S concentrations varied in a similar way,
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19 347 suggesting their concomitant precipitation. However, the elemental map and the low Fe
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21 348 signals in EDS spectra indicated a minor presence of Fe sulfide in Sb-As-bearing phases.
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23 349 Then, Fe does not appear to play a significant role in the removal of As or Sb in the present
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25 350 study. This difference of behaviour with previous studies can be explained by the stronger
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27 351 acidic condition of our experiment that favoured the precipitation of As and Sb as sulfide
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29 352 minerals (Le Pape et al., 2017; Han et al., 2018).

353 **Bacterial diversity and key functional groups**

354 The bacterial communities colonising the bioreactor resulted from inoculation with a
355 microbial consortium including sulfate-reducing bacteria, originating from the AMD of
356 Carnoulès. Most of the taxa identified in the inoculum and in the bioreactor were previously
357 observed in the Carnoulès ecosystem (Delavat et al., 2012 and 2013) and in anaerobic
358 bioreactors treating artificial or real mine effluents by sulfate reduction (Battaglia-Brunet et
359 al., 2012; Sánchez-Andrea et al., 2014). A single known SRB was detected inside the reactor,
360 affiliated to the genus *Desulfosporinus*, which is in agreement with previous finding in
361 similar systems (Battaglia-Brunet et al., 2012; 2021). This genus includes acid tolerant SRB
362 isolated from mining environment (Kimura et al., 2006; Church et al., 2007; Alazard et al.,
363 2010; Jameson et al., 2010; Sánchez-Andrea et al., 2015; Mardanov et al., 2016; Panova et al.,

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3 364 2021). It was the main bacterium composing the sulfate-reducing microbial consortium
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5 365 inoculated in the bioreactor, and was maintained inside the column, being often one of the
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7 366 main (bottom and/or middle levels in phases 1 and 2) and even the dominant (bottom level in
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9 367 phase 3) genus. Its relative abundance represented thus a considerable portion of the microbial
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11 368 community inside the reactor (up to $76.5 \pm 5.9\%$ in liquid and $81.0 \pm 1.3\%$ in bioprecipitates).
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13 369 Bacterial communities gathering SRB, fermenters and cellulolytic bacteria have already been
14
15 370 observed in bioreactors treating mine water (Morales et al., 2005; Pruden et al., 2007; Pereyra
16
17 371 et al., 2008 and 2010; Vasquez et al., 2016). In the bioreactor, interactions between the
18
19 372 different identified metabolic groups are expected. Fermentative bacteria able to degrade
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21 373 complex organic molecules accounted for a large proportion of the detected genera. Members
22
23 374 of the *Cellulomonas* genus are able to degrade cellulose and other complex polysaccharides
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25 375 (Lynd et al., 2002) and were already found associated to acidophilic SRB consortia (Dev et
26
27 376 al., 2021). Cellulose degraders (*Cellulomonas*) and fermenters (*Microbacter* and *Luteococcus*)
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29 377 may possibly be able to depolymerise and consume sugars from the agar phase, and generate
30
31 378 metabolites for SRB. The genus *Microbacter* has been first discovered in an acid rock
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33 379 drainage of the Rio Tinto and is described as an anaerobic propionigenic bacterium able to
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35 380 produce propionate, lactate and acetate from various sugars (Sánchez-Andrea et al., 2014).
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37 381 *Luteococcus*, a genus belonging to the Propionibacteraceae family, which members are known
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39 382 to produce mainly acetate, propionate and CO₂ from sugar fermentation (Stackebrandt et al.,
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41 383 2006), is consistently detected inside the column, although at lower proportion. More
42
43 384 precisely, the OTU sequence classified within the genus *Luteococcus* matches with that of the
44
45 385 acidophilic Propionibacteraceae strain H7p isolated from sediment of the Carnoulès AMD
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47 386 (Delavat et al., 2012). SRB might use metabolites produced by the complex polysaccharides
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49 387 degraders and the fermenters as carbon and energy sources. Association of these diverse
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51 388 metabolic groups inside an anaerobic process can promote synergic activities (Logan et al.,
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3 389 2005; Pereyra et al., 2010; Sánchez-Andrea et al., 2014; Vasquez et al., 2016).
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5 390 *Desulfosporosinus* species usually oxidise organic compounds incompletely, producing
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7 391 acetate (Alazard et al., 2010; Mayeux et al., 2013; Sánchez-Andrea et al., 2015; Vandieken et
8
9 392 al., 2017); however, a recent study has reported complete oxidation of propionate, lactate, and
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11 393 acetate to CO₂ by a *Desulfosporosinus* strain (Hausmann et al., 2019). On another hand, as the
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13 394 bioreactor was fed with glycerol, a well-known substrate for acidotolerant *Desulfosporosinus*
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15 395 strains but also a substrate for *Cellulomonas* and Propionibacteraceae, a competition for
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17 396 glycerol may also occur and limit the organic carbon availability for SRB. As some anaerobic
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19 397 bacteria can perform fermentation of tartrate (Schink et al., 1984), the supply of this organic
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21 398 acid (with Sb) as additional potential electron donor, i.e. 0.7 mM in phase 2 and up to 1 mM
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23 399 during phase 3, could also contribute to influence the evolution of bacterial community
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25 400 composition during the experiment. *Acidocella* is a heterotrophic acidophilic genus that also
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27 401 presents a metabolic interest. For instance, *Acidocella aromatica* uses a limited range of
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29 402 organic substrates, such as acetate (Jones et al., 2013; Ñancucheo et al., 2016). Acetate is a
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31 403 toxic compound at low pH, potentially produced by several bacteria in our system, including
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33 404 SRB. In 2006, Kimura et al. proposed a syntrophic interaction between *Acidocella* and
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35 405 *Desulfosporosinus*. *Acidocella* generated carbon dioxide and hydrogen from the acetate
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37 406 produced by *Desulfosporosinus*, hydrogen being used as electron donor by
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39 407 *Desulfosporosinus*. In our system, *Acidocella* was mainly present in phases 1 and 2, and
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41 408 might have been replaced by other acetate degraders in phase 3.
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52 410 **Relation between the bacterial communities, the physico-chemical parameters, and the**
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54 411 **sulfate reducing activity**
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3 412 There was a clear evolution in both space and time of the taxonomic composition of bacterial
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5 413 communities inside the column (Fig. 6). The co-inertia analysis showed a significant
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7 414 correlation between the evolution of biological and some physico-chemical parameters.
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10 415 As a fact, pH, S, As and Sb concentrations were among the most important factors explaining
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12 416 the percentages of the main bacterial genera (Fig. 6). However, these parameters are directly
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14 417 influenced by the sulfate-reducing reaction which consumes H^+ and SO_4^{2-} , and leads to the
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16 418 precipitation of As and Sb as sulfides. Sulfate reduction occurred inside the column, as
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18 419 evidenced by the decrease of dissolved S concentration and metal sulfide precipitation, and
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20 420 the gradual increase of pH in the bioreactor. In the liquid samples, the higher proportion of
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22 421 *Desulfosporosinus*-related OTU at the bottom of the column during the phase 1 and 3 is in
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24 422 agreement with the increase of pH between the inlet and the bottom of the column, but also
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26 423 with the As and Sb precipitation between the bottom and the middle of the column. The
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28 424 lowest precipitation efficiency was observed during phase 2 (Fig. 3), corresponding to the
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30 425 lowest feed pH and lowest proportion of *Desulfosporosinus*-related OTU at the bioreactor
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32 426 bottom (Fig. 6). The highest proportion of this sulfate reducing bacterium was found in the
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34 427 bio-precipitates suggesting that *Desulfosporosinus* was mainly present in the biofilm
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36 428 compartment. This may be linked to the development of micro-environments that protected
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38 429 the cells against stressing parameters such as acidity or oxygen diffusion from the outlet (Yin
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40 430 et al., 2019; Tran et al., 2021). The presence of *Acidithiobacillus*- and *Thiomonas*-related
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42 431 OTU in the upper zone of the bioreactor, that appeared red coloured (SM4), may be linked to
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44 432 oxygen diffusion from the top of the column. *Acidithiobacillus* and *Thiomonas* are S- and Fe-
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46 433 oxidising bacteria already detected in Carnoulès site (Delavat et al., 2012, 2013). The glycerol
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48 434 fed at the bottom of the bioreactor could explain higher proportions of *Cellulomonas*,
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50 435 *Luteococcus* and *Desulfosporosinus* (all genera consuming this substrate) in the bottom zone
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52 436 of the bioreactor (Fig. 6). The metabolism of organic substrates might have exerted influence
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3 437 on the dynamics of the bacterial community. Hessler et al. (2018) observed a stratification of
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5 438 microbial communities throughout a biological sulfate-reducing up-flow anaerobic packed
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7 439 bed reactor that was attributed to variations of lactate and the volatile fatty acids produced by
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9 440 microbial metabolisms. Further analyses would be required in order to determine the effect of
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11 441 organic molecules resulting from the metabolism of glycerol and tartrate and biodegradation
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13 442 of agar, on the diversity and structure of bacterial communities.
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17 443 Globally, this study demonstrated for the first time the possibility to remove concomitantly As
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19 444 and Sb as sulfides in a continuous column bioreactor fed with a real AMD. The reactor
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21 445 removed up to 98%-99% of dissolved As and Sb, with no competition between As and Sb for
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23 446 precipitation with sulfides when they co-occurred at concentrations up to 1 mM each in the
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25 447 AMD. *Desulfosporosinus*, the only known SRB genus detected in the bioreactor, was present
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27 448 as a major contributor to the bacterial community, which also included polymers degraders
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29 449 and fermenters. The biological reduction of the sulfate contained in the AMD provided
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31 450 enough dissolved sulfide to precipitate efficiently As and Sb. The co-occurrence of SRB,
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33 451 polymers degraders and fermenters can be an advantage for future *in situ* passive treatment of
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35 452 AMD, in the perspective to use low-cost complex organic substrates to sustain long-term
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37 453 bacterial activity in a bioreactor. Results are promising for the perspective of passive bio-
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39 454 treatments of AMD containing metalloids.
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7 3 **Figure 1:** Schematic representation of the upflow anaerobic fixed-bed bioreactor (A). Picture
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9 4 of the column with filling material and yellow biogenic precipitates (B).

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15 6 **Figure 2:** pH variations of inlet and outlet waters represented by white and black circles
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17 7 respectively (A). Histogram of pH vertical-profiles at the end of each phase in the column
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19 8 bioreactor (B); the white circles represent the average pH of inlet and outlet waters along each
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21 9 phase, and the error bars indicate standard deviations of the mean for three measurements for
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23 10 each feed condition.

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30 12 **Figure 3:** Vertical punctual profiles of dissolved arsenic (A), antimony (B, focus on low
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32 13 concentration C), iron (D) and sulphur (E) concentrations in the column bioreactor at the end
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34 14 of each phase (data missing for the Sb concentration in the inlet water in phase 1). Error bars
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36 15 indicate analytical error (2%). The white circles represent the average concentrations of inlet
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38 16 and outlet waters for each phase (three measures), and the error bars indicate standard
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40 17 deviations of the mean for three measurements for each feed condition.

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47 19 **Figure 4:** SEM observation of bottom bioreactor filling at the end of experiment in
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49 20 backscattering electron mode showing biofilm morphology in a first zone (A) and the
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51 21 corresponding EDS spectrum (B), and another zone showing Sb-enriched aggregate (C) and
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53 22 the corresponding EDS spectrum (D).

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Figure 5: Taxonomic composition of bacterial communities (at the genus level) in the inoculum, the liquid and the solid samples collected along the reactor at the end of each phases (analyses performed in triplicates except for the inoculum). “Others” represent the phylogenetic groups with a relative abundance < 1% calculated on the whole dataset.

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Figure 6: Co-inertia analysis of the physico-chemical parameters and the dominant bacterial taxa at each position in the column. (A) Plot represents the projection of the samples, grouped according their position inside the column, on the first two co-inertia axes. The dotted arrows represent the difference between the ordination of the biological (origins of arrows) and physico-chemical parameters (arrowheads); dotted arrows length are inversely proportional to the strength of the relationship between parameters. The upper-left barplot indicates the eigenvalues. (B) PCA of the physico-chemical parameters at each position in the column. Chemical parameters are represented by their chemical symbols as: Fe (dissolved iron concentration), S (dissolved sulfur concentration), As (dissolved arsenic concentration) and Sb (dissolved antimony concentration). (C) PCA of the dominant bacterial taxa at each position in the column.

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Figure 1

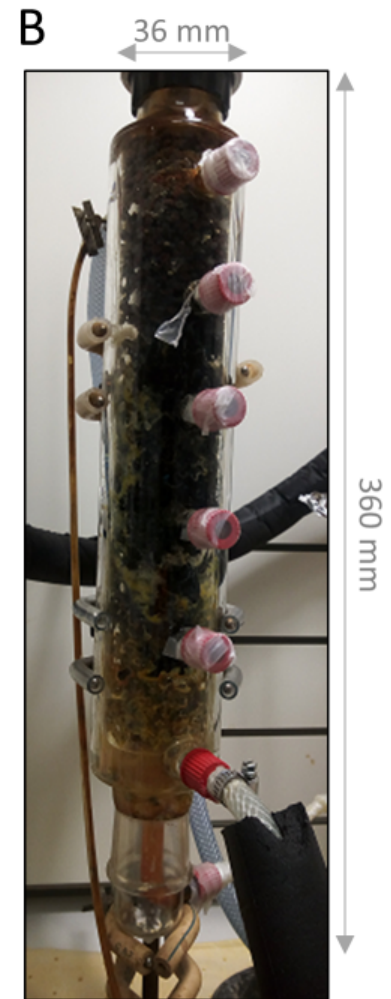
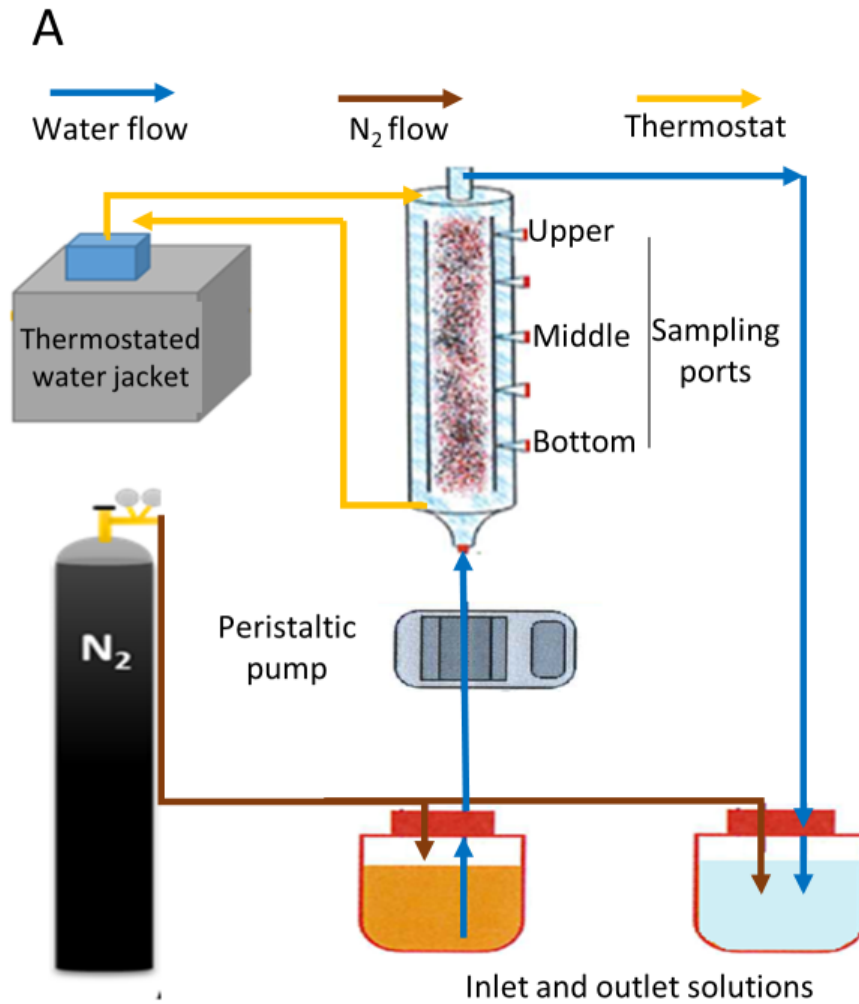


Figure 2

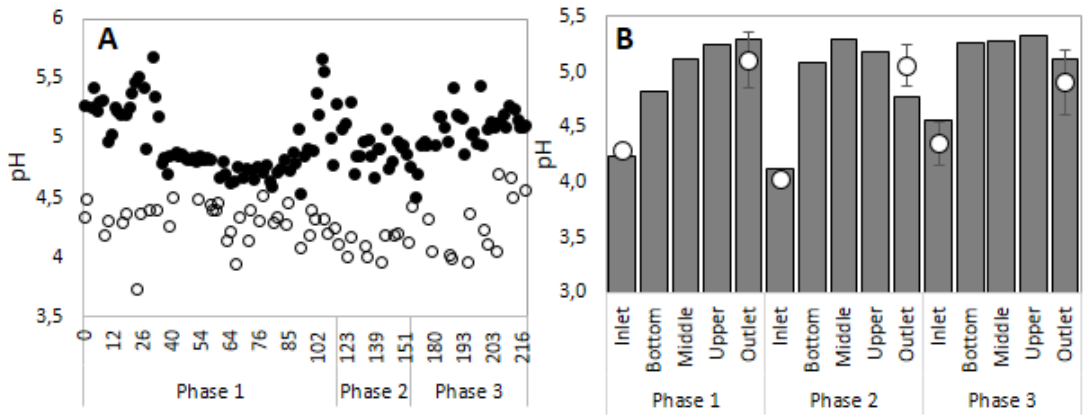


Figure 3

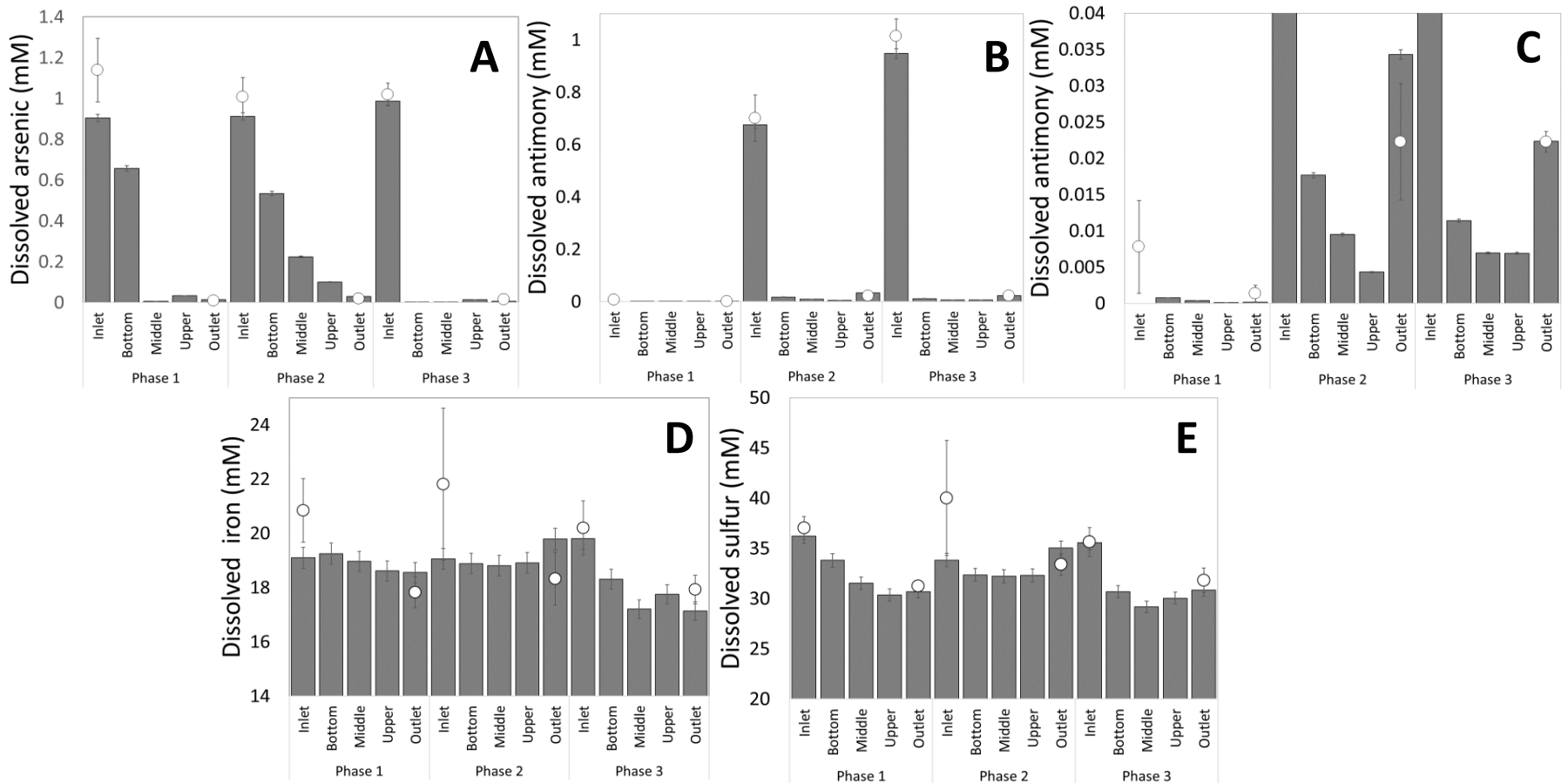


Figure 4

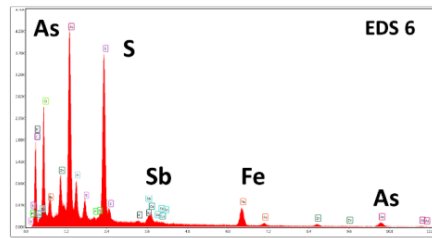
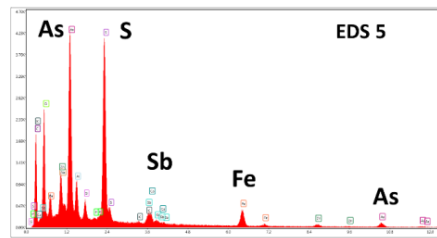
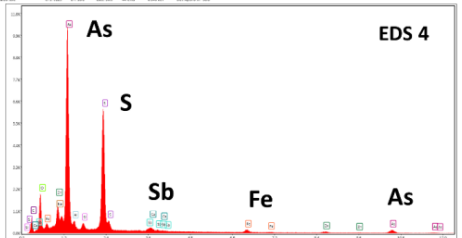
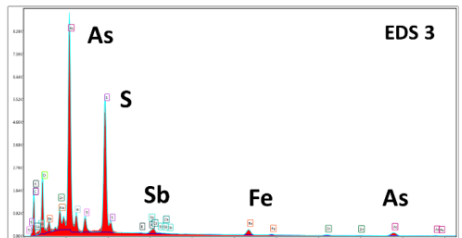
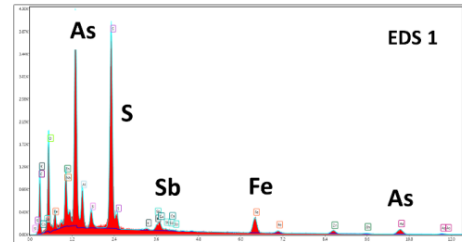
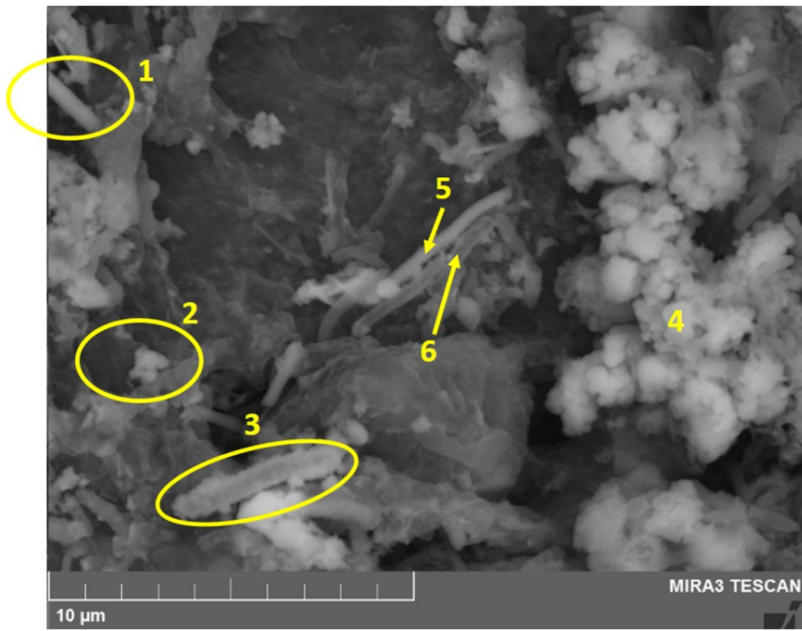
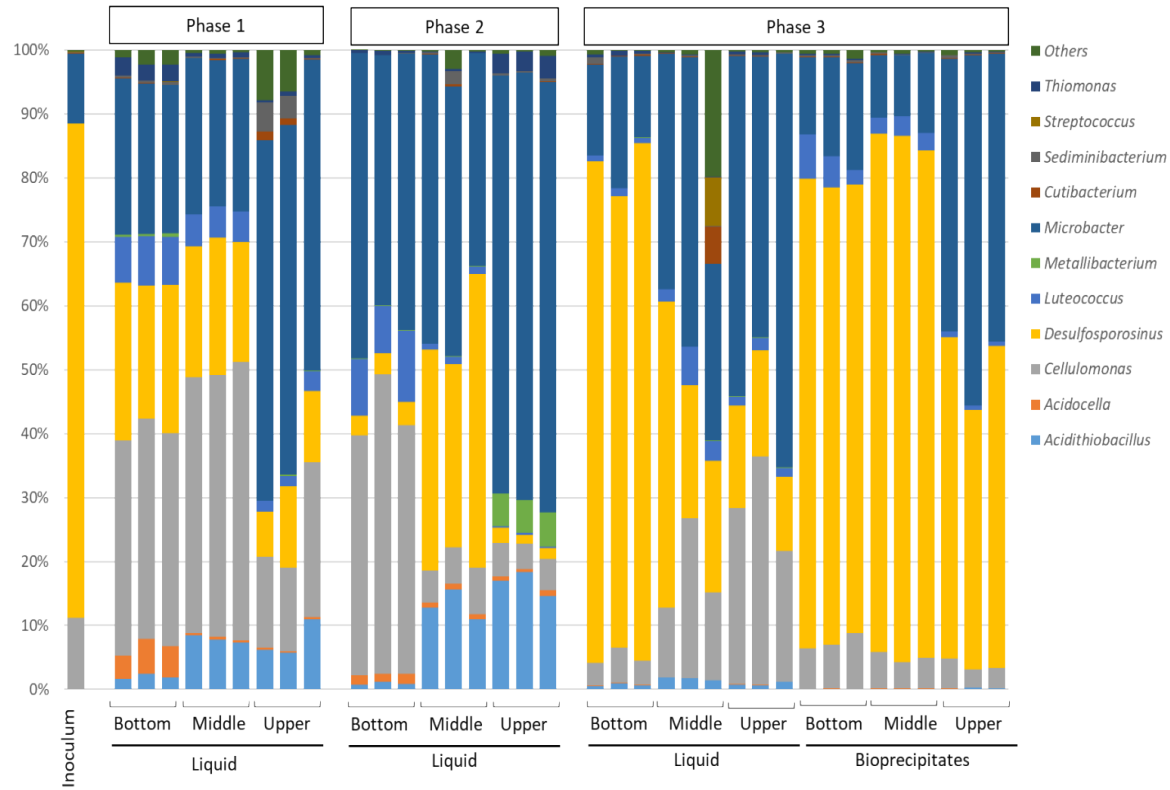


Figure 5



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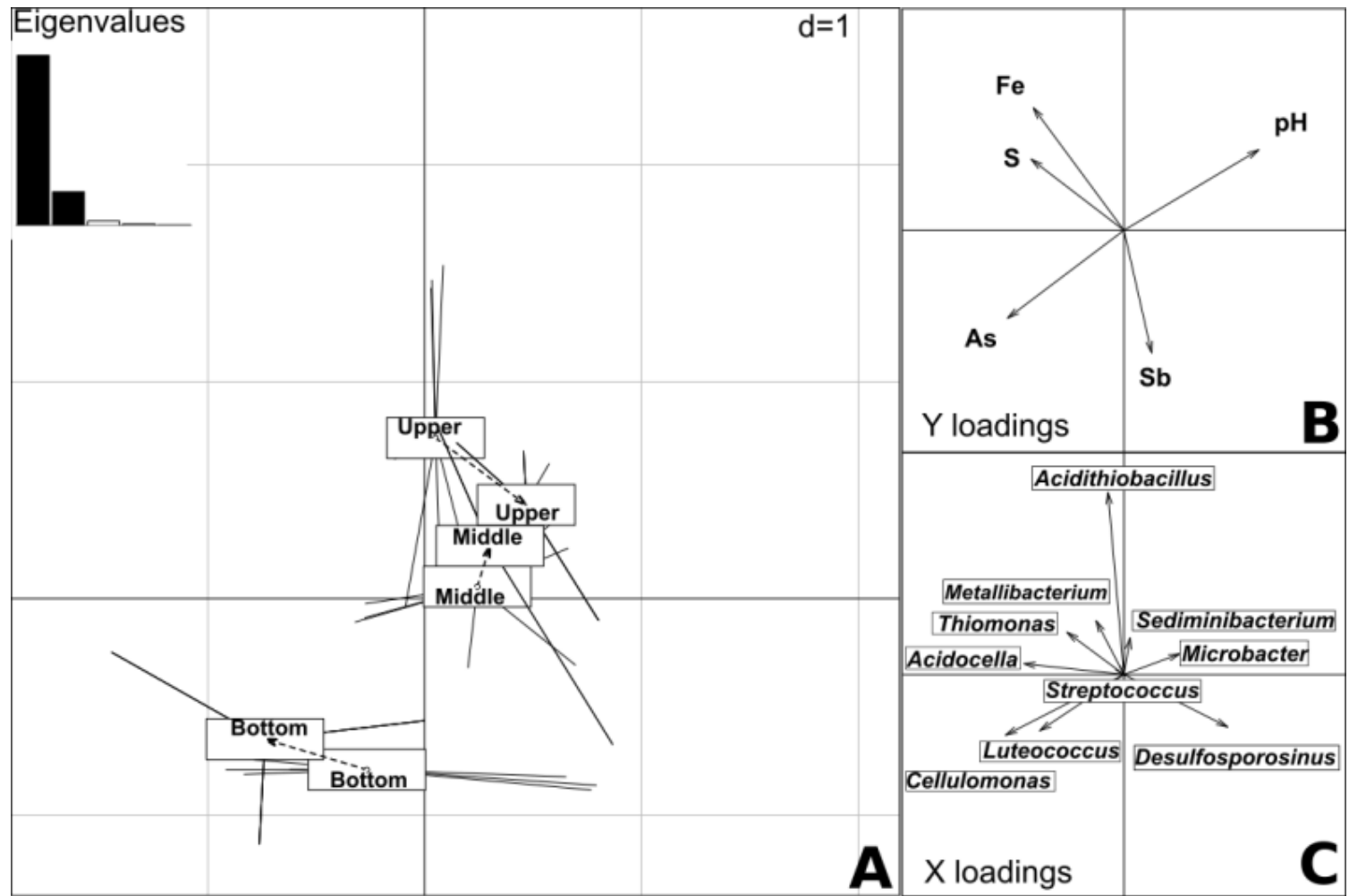


Table 1: Experimental conditions

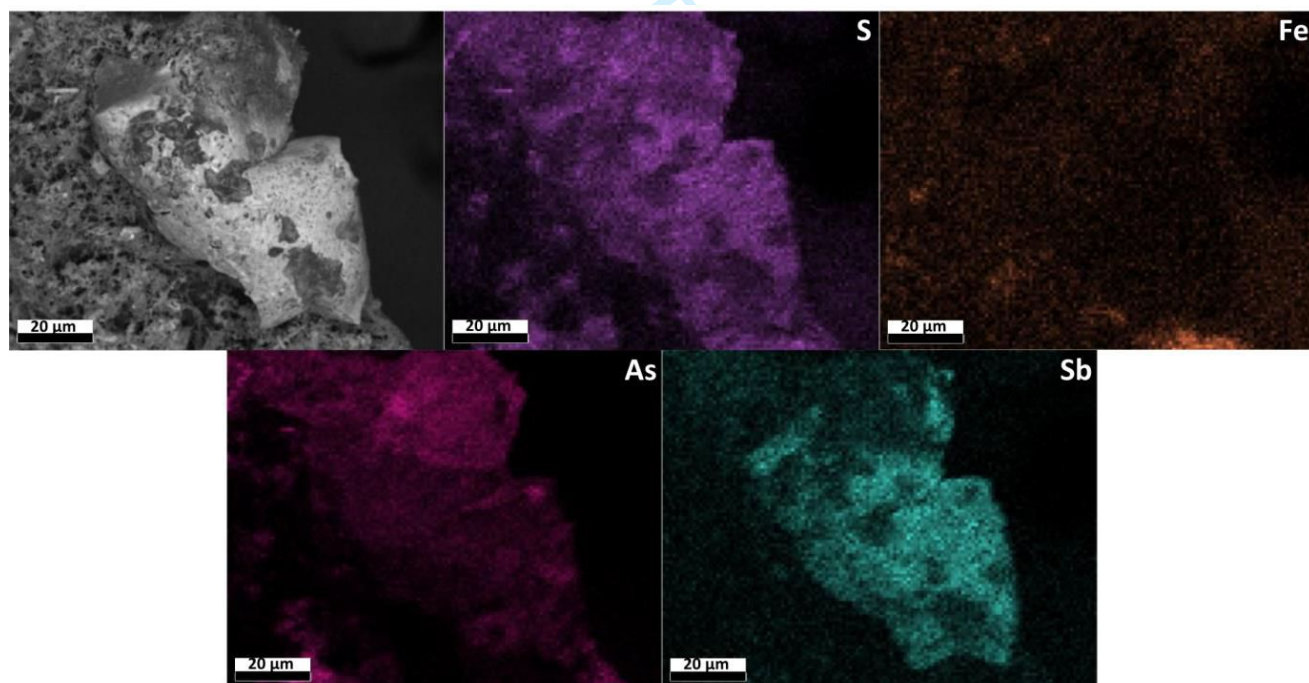
Phase	Duration		Hydraulic				
	of the phase (days)	Sampling day for ICP-MS analysis	Flow (ml/h)*	retention Time (h)*	Feed pH*	Feed [As] (mM)*	Feed [Sb] (mM)*
1	117	0, 82 and 117	1.5 ±	132 ± 10	4.29 ± 0.03	1.14 ± 0.16	0.008 ±
			0.1				0.006
2	35	123, 141 and 152	1.3 ±	163 ± 19	4.03 ± 0.06	1.01 ± 0.09	0.70 ± 0.09
			0.1				
3	65	152, 155, 204 and 217	1.4 ±	149 ± 15	4.35 ± 0.20	1.02 ±	1.01 ± 0.07
			0.1				

(*) average values

SM1. Average removal and concentrations of minor toxic elements from inlet and outlet waters during each phase.

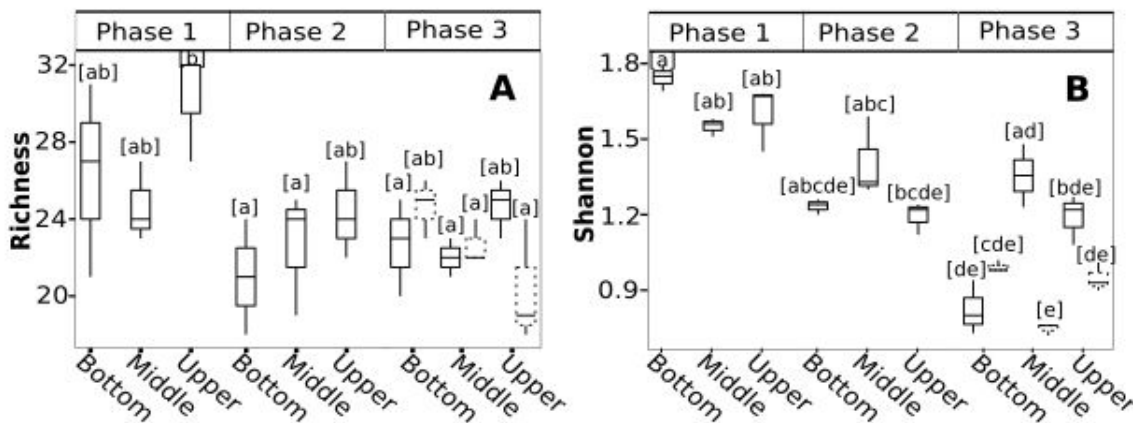
Phase		1	2	3	
Pb	Concentration (mM)	Inlet	1.0 ± 0.9	0.7 ± 0.4	1.2 ± 0.5
		Outlet	$2.10^{-3} \pm 2.10^{-3}$	$< 2.10^{-5}$	$4.10^{-3} \pm 5.10^{-3}$
	Removal (%)	99.9 ± 0.1	100	99.7 ± 0.4	
Tl	Concentration (mM)	Inlet	$1.5.10^{-3} \pm 1.0.10^{-3}$	$1.5.10^{-3} \pm 3.2.10^{-2}$	$1.5.10^{-3} \pm 6.2.10^{-5}$
		Outlet	$2.1.10^{-5} \pm 1.3.10^{-5}$	$1.6.10^{-5} \pm 1.1.10^{-5}$	$2.0.10^{-5} \pm 1.5.10^{-6}$
	Removal (%)	98.7 ± 0.8	99.0 ± 0.8	98.7 ± 0.1	
Zn	Concentration (mM)	Inlet	0.36 ± 0.02	0.38 ± 0.05	0.35 ± 0.05
		Outlet	$2.9.10^{-3} \pm 1.9.10^{-3}$	$9.8.10^{-3} \pm 5.6.10^{-3}$	$7.2.10^{-3} \pm 9.0.10^{-3}$
	Removal (%)	99.2 ± 0.5	97.4 ± 1.4	97.9 ± 2.7	

SM2. SEM-EDS map showing the distribution of selected elements on bioprecipitate collected at the end of the experiment (middle zone of the column bioreactor).



SM3.Bacterial Diversity indexes

Bacterial species richness in number of OTUs observed (A) and Shannon diversity index (B) in the liquid samples (solid lines) and solid samples (dashed lines) collected inside the reactor. The different letters represent significant differences between samples ($p < 0.05$).



SM4. Pictures of the column bioreactor at the end of experiment



Bottom of the column bioreactor
At the end of experiment



Top of the column bioreactor
At the end of experiment