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Abstract

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

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

Introduction

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

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

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

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

Materials and methods

Water sampling and batch experiment setup

Water was collected in June 2015 from the source of Reigous creek at the abandoned Carnoulès mine (Southern France: 44°7'2.14"N; 4°0'6.00"E). Its physicochemical characteristics were as follows: pH 4.7, 2.69 mg O_2 l^{-1}, 14.9°C, 2.56 mS cm^{-1}, 590.6 mV, 511 mg Fe l^{-1} (100% Fe(II)), 53.8 mg As l^{-1} (14% As(V)).

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

Batch experiment monitoring

The experiment was conducted for eight days. Water samples (~3 ml) were collected from the bottles for chemical analysis at days 0, 2, 3, 4, 5, 6, 7 and 8. At day 3, 5 and 8, pH and dissolved oxygen were measured with a multiparameter analyser (Ultrameter™ Model 6P). Dissolved oxygen remained stable, with an average of 7.8 ± 0.5 mg O_2 l^{-1} in all batch (data not shown). pH decreased during incubation from 4.7 to 2.7 ± 0.2 and 3.6 ± 0.1 in the biotic and abiotic experiments respectively (data not shown), in relation with Fe(II) oxidation and subsequent Fe(III) hydrolysis (Nordstrom and Alpers 1999). After sampling, water was immediately filtered through disposable filters (cellulose acetate, pore size 0.22 µm) and the filtrate was analyzed for Fe(II), total Fe, As(III) and As(V) concentration according to routine procedures described in Fernandez-Rojo et al. (2017).

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

Chemical analyses

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

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

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

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

where $As(III)_d$ and $As(III)_p$ were the concentration of dissolved and particulate As(III) (in mg l^{-1}), respectively, at the beginning of experiment (t^0) or after 8 days of incubation (t^{final}).

Biomass quantification

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

DNA extraction and quantification

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

Quantification of *aioA* genes

The abundance *aioA* genes encoding the catalytic subunit of the As(III)-oxidase was determined by quantitative real-time PCR (qPCR). The reverse primers aoxBM2-1R and forward primer aoxBM4-1F were used to target a 110 bp fragment of the *aioA* gene (Quéméneur et al. 2010). For each sample, 2 ng of DNA were used in a 20 µl PCR reaction with 0.3 µM of each primer, 100 ng of T4GP32 (MP Biomedicals) and 1X IQ SYBR Green Supermix (BioRad). The program was run in a CFX Connect (BioRad) and consisted in an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 54 °C for 20 s, 72 °C for 10 s, and a data acquisition step at 80 °C for 10 s. At the end, a melting curve analysis was performed through measurement of the SYBR Green I signal intensities during a 0.5 °C temperature increment every 10 s from 65 °C to 95 °C.

Sequencing of 16S rRNA gene

Bacterial diversity was determined by Illumina high-throughput sequencing of bacterial 16S rRNA genes. V4-V5 region (about 450 bases) was amplified by PCR using primers PCR1_515F (Barret et al. 2015) and PCR1_928R (Wang and Qian 2009). For each sample, 10 ng of DNA were used in a 50 µl PCR reaction conducted under the following conditions: 94°C for 2 min, 30 cycles of 1 min at 94°C, 65°C for 40 s and 72°C for 30 sec, followed by 10 min at 72°C. The PCR products were checked by gel electrophoresis and quantified using a fluorometer (Qubit®, Invitrogen) and sent to GeT-PlaGe platform (Toulouse, France) for Illumina MiSeq analysis using a 2×300 bp protocol.

Bioinformatic analyses of 16S rRNA gene sequences

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

Statistical analysis

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

Results

Bacterial biomass

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

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

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

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

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

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

Iron and arsenic species in the particulate phase

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

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

Iron and arsenic oxidation

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

Diversity of bacterial communities

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

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

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

($1.4 \pm 0.4 \times 10^4$ genes copies per ng of DNA) and to the initial water ($4 \pm 2 \times 10^3$ genes copies per ng of DNA^{-1}). The highest number of *aioA* gene copies was obtained as a consequence of the incubation at 35°C.

Discussion

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

Bacterial diversity in AMD water and its evolution in batch experiments

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

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

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

Effect of nutrient supply on bacterial communities and on pollution attenuation

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

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

Effect of temperature on bacterial communities and on pollution attenuation

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

Environmental significance

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

Seasonal variations of As speciation were observed in Carnoulès AMD (Morin et al. 2003; Egal et al. 2010) with the preferential formation of As(III)-rich precipitates during the coldest season, mainly in the form of tooeleite. Conversely, during summer, As(V) dominates in the amorphous ferric arsenate form. These field observations are in agreement with our results that clearly revealed a positive effect of higher temperature on microbially mediated As(III) oxidation. As a consequence, in case of an *in situ* biological treatment, contrasted performance in term of efficiency of arsenic removal and sludge composition is expected depending on the climate or the season. The formation of stable As(V)-rich biogenic precipitates might be favored at temperatures higher than 20°C.

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

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

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Compliance with Ethical Standards

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

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

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

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

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

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

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

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