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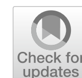

Acid mine drainage bioremediation using bacteria enriched from the confluence zone between its flow and treated sewage

A. Nobahar[1,2] · E. T. Fitas[1,2] · M. C. Costa[1,2] · J. D. Carlier[1]

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Abstract

Sediments from the confluence zone between an acid mine drainage stream and a water stream receiving the effluent from a municipal wastewater treatment plant were inoculated in a Postgate B medium base having methanol, glycerol, or ethanol as carbon source/electron donor, over a pH range between 2.00 and 6.00, aiming to obtain native cultures enriched in acidophilic/acid-tolerant sulfate-reducing bacteria. The most effective sulfate reduction observed in acidic conditions was in the enrichment cultures with methanol. Thus, the microbial consortium enriched in these conditions was further used in acid mine drainage bioremediation experiments at an initial pH of 4.50 and using different doses of nutrients medium base and methanol as carbon source/electron donor. The most promising results, with more than 99% removal of metals, were obtained in the mixture of 20% Postgate B medium base plus 80% acid mine drainage (v/v), which corresponds to 0.115% basal salts and 0.02% yeast extract (w/v). Metataxonomic analysis based on 16S rRNA gene sequences showed the presence of *Desulfosporosinus* spp. in all enrichment cultures, with the highest relative abundance in cultures at pH 4.00 with methanol. Furthermore, metataxonomic analysis in the acid mine drainage remediation tests revealed the presence of *Desulfosporosinus* spp. in all tested conditions. In addition, the study also revealed *Clostridium* members in all tests. Plus, their relative abundances were related to the dose of nutrient medium base and in balance with the abundances of *Desulfosporosinus* spp.

Keywords Acidophiles · Biotreatment · Metal attenuation · Sulfate reduction · Sulfidogenic bioreactor

Introduction

Mining activities are among the main anthropogenic source of sulfate emission to the environment, by exposing metal sulfide minerals to the environment causing their oxidation (Brahmacharimayuma et al. 2019). As a result, acid mine drainage (AMD) waters, with high acidity and rich in sulfate and different metals are introduced to the environment with several negative impacts to the aquatic and land organisms (Ighalo et al. 2022).

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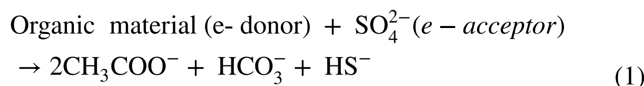
J. D. Carlier
jcarlier@ualg.pt

[1] Centre of Marine Sciences (CCMAR/CIMAR LA), University of Algarve, Gambelas Campus, Building 7, 8005-139 Faro, Portugal

[2] Faculty of Sciences and Technology, University of Algarve, Gambelas Campus, Building 8, 8005-139 Faro, Portugal

AMD treatment by sulfate reduction has been widely implemented (Muyzer and Stams 2008; Zhang et al. 2016b). Indeed, the sulfate reduction bioprocess by sulfate reducing bacteria (SRB) is a widely studied method applied to reduce the concentration of sulfate and different metals from AMD (Sánchez-Andrea et al. 2014a; Sun et al. 2020), and there are several review articles on this issue (eg. Ayangbenro et al. 2018). SRB is a diverse group of strict anaerobic bacteria and archaea with the ability of dissimilatory sulfate reduction into sulfide by oxidation of various substrates. Through the sulfate reduction bio-process, different organic materials or H_2 act as electron donors, while sulfate is electron acceptor, which results in sulfide production and energy conservation and proliferation of the SRB (Eq. 1) (Cabrera et al. 2006). As presented in Eq. 1, SRB consume H^+ and produce bicarbonate that rises the substrate pH (Sánchez-Andrea et al. 2014a; Zhang et al. 2016a)



$$\text{Organic material (e- donor)} + SO_4^{2-}(e-acceptor) \rightarrow 2CH_3COO^- + HCO_3^- + HS^- \quad (1)$$

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The produced sulfide reacts with heavy metal ions including Cu, Zn, Fe, Ni, Pb, Cd etc. and forms very insoluble metal sulfides (Eq. 2), that can be then recovered and used in different industrial operations (Foucher et al. 2001). Through this reaction, HS^- is hydrolyzed into H^+ and S^{2-} and will lower the pace of pH enhancement by microbial metabolism (Sánchez-Andrea et al. 2014a).

$$HS^- + Me^{2+} \rightarrow MeS \downarrow + H^+ \tag{2}$$

A major number of the studies on SRB showed that neutral pH between 6 and 8 is required for an optimal growth (Sen and Johnson 1999; Johnson and Sánchez-Andrea 2019). For these SRB, low pH is unfavorable since more energy is expended to pump protons across the cytoplasmic membrane that results in the availability of less energy for their growth (Meier et al. 2012; Sánchez-Andrea et al. 2014a). However, different studies have shown the existence of acidophilic or acid tolerant SRB (aSRB) in the extremely acidic environments (Koschorreck et al. 2003; Sánchez-Andrea et al. 2011; Ňancucheo et al. 2016; Serrano and Leiva 2017) such as highly acidic mine sediments (Alazard et al. 2010; Sánchez-Andrea et al. 2015; Ňancucheo et al. 2016; Dev et al. 2021), pyritic heap bioleaching residues (Phyo et al. 2020), acidic hot spring sediments (Willis et al. 2019) etc.. Most known aSRB belong to genera *Desulfosporosinus* (Jameson et al. 2010; Sánchez-Andrea et al. 2013, 2015; Phyo et al. 2020; Dev et al. 2021), *Clostridiaceae*, *Alicyclobacillaceae* (Phyo et al. 2020), *Thermodesulfobium*, (Sánchez-Andrea et al. 2015), *Peptococcaceae*, (Santos and Johnson 2017), *Desulfitobacterium* (Sánchez-Andrea et al. 2013) and *Desulfotomaculum*, (Dev et al. 2021). Estimations through thermodynamics analyzes reveal a higher Gibbs free energy of SO_4^{2-} reduction by SRB in low pH values (Meier et al. 2012; Sánchez-Andrea et al. 2022). Thus, when this additional energy compensates the energy required to export the protons across the cytoplasmic membrane, microbial growth occurs (Meier et al. 2012). Moreover, there are other mechanisms suggested for favoring aSRB growth at low pH, such as ATP synthesis by utilization of the proton motive force, proton elimination by vesicles, etc. (Sánchez-Andrea et al. 2014a; Ňancucheo et al. 2017; Brahmacharimayuma et al. 2019; Qian et al. 2019).

It has been shown that using aSRB in bioreactors allows efficient sulfate reduction at pH 4.00, and even at pH 3.5 but at a lower rate (Sheoran et al. 2010). On the other hand, it is for long known that a prior neutralization step in AMD bioremediation using SRB requires additional operational costs and/or reagents (van Houten et al. 1994). So, there may be advantages in bioremediation systems in which AMD is previously adjusted to pH values close to 4 instead of values close to neutral. Moreover, the addition of aSRB may turn the bioreactors more resilient to acidification caused by failures in the prior neutralization step or that may occur due to high contents of organic acids resulting from fermentation when sugar-rich substrates are used as carbon sources/electron donors (as for example described by Carlier et al. 2019, 2020b).

It is therefore evident the interest in obtaining and maintaining new microbial consortia enriched in aSRB as well as in studying their prokaryotic communities to better understand (and control) the possible relationships of symbiosis, competition, etc. with other taxonomic groups; or even in trying to obtain and maintain isolated aSRB strains. The search for aSRB adapted to the conditions of places where AMD is not yet treated could be even more interesting, in that it could contribute to the implementation of more resilient systems designed and built from scratch based on the specificities of the local characteristics. For example, Dev et al. (2021) reported that the failure of SRB-mediated treatment of AMD in cold regions due to inhibition of bacteria by acidic pH and low temperature can be overcome by enriching psychrophilic and acidophilic microbial consortia from local metal-rich sediments.

São Domingos mine is one of the most famous inactive metalworking mines in Portugal and the downstream area is significantly affected by AMD. A large amount of mining waste is exposed, producing AMD with pH values close to 2 and high concentrations of sulfate and metals (mainly aluminium, iron, zinc, and copper) (Álvarez-Valero et al. 2008; Alvarenga et al. 2012). The impact of AMD extends to multiple water bodies located within several kilometers. These include the São Domingos stream, which runs in proximity to the mine pit lake and courses through the valley within the mining area, as well as the scattered dam lakes in this region, until reaching the Chansa River reservoir (Pereira et al. 2004). Indeed, the Portuguese public company (Empresa de Desenvolvimento Mineiro—EDM) responsible for the environmental rehabilitation of the mine has plans to implement a process, as passive as possible, to treat the flow of AMD leaving the mine area (personal communication in a meeting with EDM representatives, February 2024).

The present study, carried out from 2020 to 2022 in Portugal, aimed to obtain a native aSRB enriched consortia from the sediments of a confluence zone between the AMD stream at São Domingos mine and a stream receiving a municipal wastewater treatment plant effluent, and test a selected consortium for the bioremediation of local AMD. Moreover, the changes on the prokaryotic communities in the enrichment cultures and in the bioremediation tests were studied aiming to better understand the main taxa with a putative role in the bioremediation and the adaptative evolution of the prokaryotic community to different conditions. Thus, the work reported contributes to support the possible

implementation of an in-site biological treatment process at Mina de São Domingos and contribute to the general knowledge on this subject.

Materials and methods

Enrichments of aSRB consortia

Inoculum collection and preparation

The sediments used as inoculum were sampled in March of 2020 at Mina de São Domingos, Portugal, an inactive mining area located in the Iberian Pyrite Belt (Fig. 1). The samples were collected from 5 sites at ~ 30 cm depth in the wet zone of the bank in the confluence between a water stream affected with AMD and a water stream receiving the effluent from the sewage wastewater treatment plant.

Samples were immediately transferred to the laboratory and measurements of pH in the sediments were performed using a 1:10 soil-to-water ratio (w/v) suspension as described in Thomas (1996) (Table 1). Afterwards samples were stored at 4 °C.

Table 1 pH of sediment and water in the sampled sites

Site	pH	
	Sediment	Water
1	3.13	2.90
2	4.04	4.86
3	5.55	3.19
4	3.96	5.01
5	3.66	2.96

Enrichment cultures

The enrichments were made in batch cultures using the micro and macronutrients base of Postgate B growth medium, which is a good and easy-to-prepare medium for SRB (Postgate 1984), but instead of lactate different carbon sources/electron donors were used (glycerol, methanol, and ethanol) and the pH was adjusted to different pH values (2.00, 3.00, 4.00, 5.00 and 6.00).

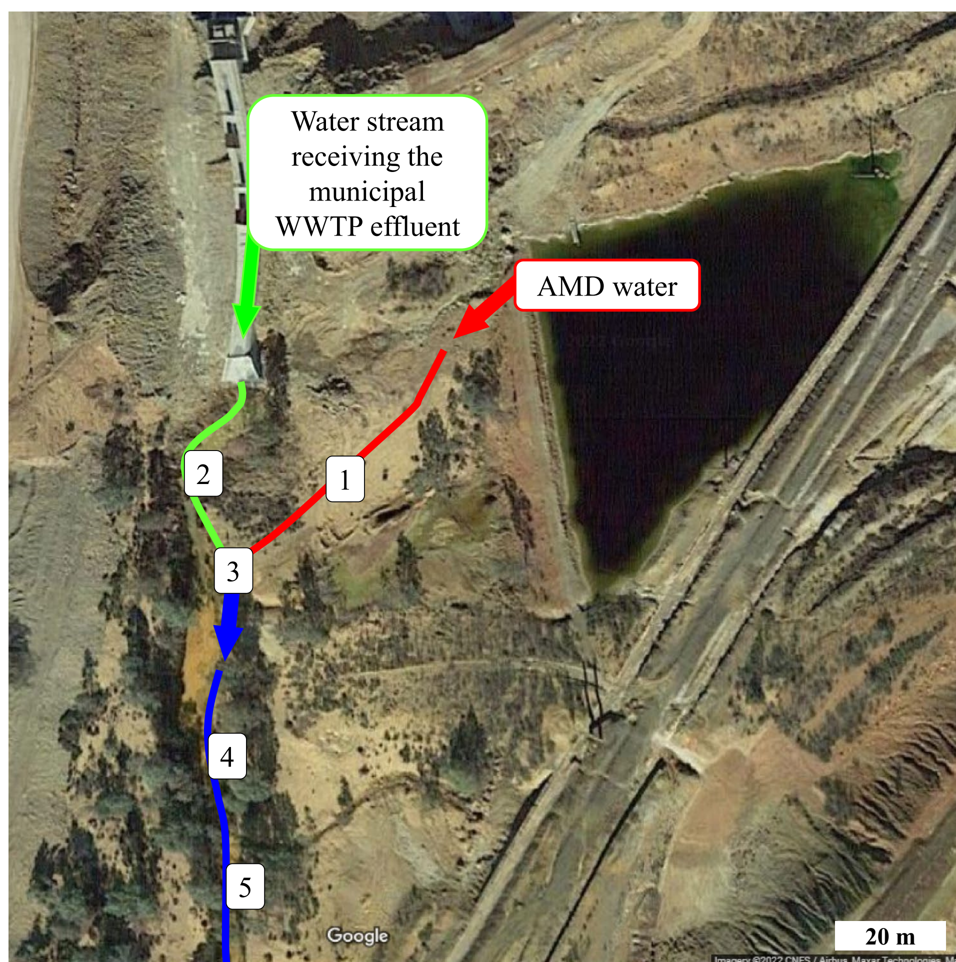


Fig. 1 Sampling area in the confluence zone between acid mine drainage and the stream receiving the sewage wastewater treatment plant effluent at Mina de São Domingos (adapted from google maps: 37°39′31.3″N 7°30′21.8″W)

At low pH lactate is present mainly as non-dissociated lactic acid and even micromolar concentrations of this compound are sufficient to inhibit the growth of SRB in acidic media (Ňancucheo et al. 2016). On the contrary, the tested organic substrates have been successfully used to enrich for acid-tolerant strains in previous works (e.g. Sen and Johnson 1999; Koschorreck 2008; Sánchez-Andrea et al. 2013).

To be used as inoculum, a mixture of sediments was prepared by adding 2 g of each of the five collected samples to 160 mL of medium without carbon source under magnetic stirring.

The Postgate B medium base used for enrichments was prepared by mixing the following reagents per liter of tap water: KH_2PO_4 (1 g), NH_4HCl (1 g), $FeSO_4•7H_2O$ (0.5 g), $MgSO_4•7H_2O$ (2 g), $CaSO_4•2H_2O$ (1.25 g), yeast extract (1 g), ascorbic acid (0.1 g) and thioglycolic acid (75 µL). Then, 30 mM of different carbon sources/electron donners were added to each enrichment culture as following: 1680 µL of 87% glycerol, 1140 µL of absolute ethanol and 786 µL of 99.9% methanol. Finally, the medium pH was adjusted to tested values using sulfuric acid or sodium hydroxide.

The assays were carried out in 100-mL glass bottles with 95 mL medium and 5 mL inoculum added to each flask. The cultures were covered with 7.5 mL of liquid paraffin to prevent oxygen diffusion. Finally, the bottles were sealed with butyl rubber stoppers and aluminum crimp seals and incubated at room temperature (25 ± 3 °C). Pipetting to inoculate was done with cut pipette tips to avoid clogging.

Simultaneously, as a positive control, a neutrophilic SRB consortium (Carlier et al. 2019, 2020b) was inoculated in original Postgate B medium (the base components and lactate as carbon source/electron donor) at pH 6.00.

To monitor the evolution of cultures, 2-mL samples were collected from the initial media and along the experiments, through rubber stoppers using syringes with needles. Sulfate and sulfide concentrations in the liquid phase were monitored as indicators of SRB activity. Redox potential (Eh) was monitored due to its importance as a limiting factor for SRB growth. The pH, as the variable parameter of the work, was monitored to evaluate its stability and its relations with SRB activity.

AMD bioremediation with aSRB enriched consortium

AMD characterization and preparation

The AMD used in this work was collected also in March of 2020, from the lagoon nearby the sediment sampling zone (Fig. 1). The sample was transferred to the laboratory and characterized before and after the pH adjustment for subsequent use in bioremediation tests (Table 2). Its pH was adjusted to 4.50 using sodium hydroxide and it was left to settle the formed metal hydroxides. This was repeated until pH was stable at 4.50 and finally the liquid fraction was decanted and used.

Table 2 Concentration of the main metals and pH of the AMD collected at the São Domingos mine before and after pH adjustment for the bioremediation tests

Metal	Before pH adjustment	After pH adjustment	
Cu	28 ± 1	25.74 ± 0.06	mg/L
Zn	45.4 ± 0.3	43.6 ± 0.4	
Fe	175 ± 13	0.24 ± 0.02	
Al	279 ± 12	12.3 ± 0.1	
Mn	19 ± 2	19.9 ± 0.2	
pH	2.60	4.50	

AMD bioremediation with aSRB

The aSRB enriched consortium obtained in cultures starting at pH 4.00 with methanol as carbon source/electron donor was tested for AMD bioremediation. A 10% (v/v) inoculum of this aSRB enriched culture was added to different ratios of Postgate B base of micro and macro nutrients (hereafter, referred as PB-m) to AMD at pH 4.5 and with 30 mM methanol. Then, sulfate reduction (SR) activity and metals removal were evaluated along the incubation. The PB-m to AMD (v/v) ratios tested were: 100% PB-m (no AMD) as positive control, 80% PB-m (20% AMD), 60% PB-m (40% AMD), 40% PB-m (60% AMD), 20% PB-m (80% AMD), No PB-m (100% AMD).

The assays were carried out in 200-mL glass bottles with 180 mL medium and 20 mL inoculum added to each flask. The cultures were covered with 10 mL of liquid paraffin to prevent oxygen diffusion. Finally, the bottles were sealed with butyl rubber stoppers and aluminum crimp seals and incubated at room temperature (25 ± 3 °C) for 34 days. 2-mL samples from the initial media and along the experiment (at intervals of 6 or 7 days) were collected through the rubber stoppers using syringes with needles, and used to monitor pH, redox potential, and the concentrations of sulfate, sulfide, Cu, Zn, Fe, Al and Mn.

Study of prokaryotic populations in enrichment cultures and AMD bioremediation tests To evaluate the transformations in the prokaryotic communities during the enrichment processes with the different carbon sources/electron donors and during the AMD bioremediation experiments, metataxonomic studies were carried by massive sequencing 16S rRNA genes through a so called Next-Generation Sequencing technology.

DNA extraction

The extraction and purification of DNA was performed using the DNeasy® PowerSoil® Pro Kit (QUIAGEN). For the sediments mixture used as the enrichments' inoculum, 250 mg was used as initial material. For the enriched cultures in liquid medium, 5 mL of each sample was centrifuged at 2500×*g* for 10 min at room temperature to collect cells and then the supernatant was discarded until the volume (pellet and remaining liquid) was 250 µL. Immediately after collection of these samples, they were used for DNA extraction following the manufacturer procedures. DNA concentration and quality was evaluated using a NanoDrop spectrophotometer (NanoDrop3300, Thermo Fisher Scientific).

Next-generation sequencing

Library preparation and sequencing workflow for full-length sequences of 16S rRNA gene amplicons were carried out at the Integrated Microbiome Resource (https://imr.bio/index.html). PCR amplifications were performed in duplicate using separate DNA template dilutions with the high-fidelity Phusion + polymerase and "fusion primers" (PacBio adaptors + barcodes + specific regions) in a single round of PCR. The specific regions of primers for the full-length target were the 27F (Paliy et al. 2009) = AGRGTTYGATYMTGGCTCAG and the 1492R = RGYTACCTTGTTACGACT (Lane 1991). The amplicons of these work, together with others to make a total of 96 (including four negative PCR controls and a positive control), were visualized by electrophoretic analysis and then cleaned up and normalized in one step using the high-throughput Charm Biotech Just-a-Plate 96-well Normalization Kit. Then, all the samples were pooled in one library which was quantified fluorometrically using the Invitrogen Qubit double-stranded DNA high-sensitivity (dsDNA HS) method, and finally the library was run on a PacBio Sequel II using a new chip.

Bioinformatics analyses

The analysis of sequences was performed through a pipeline available at the Microbiome Helper repository (Comeau et al. 2017). First, the standard operating procedure (SOP) PacBio CCS Amplicon SOP v1 (qiime2) (https://github.com/LangilleLab/microbiome_helper/wiki/PacBio-CCS-Amplicon-SOP-v1-(qiime2)) was used to prepare the reads and get *amplicon sequence variants* (ASVs, an alternative to operational taxonomic units—OTUs). Then the Amplicon SOP v2 (qiime2 2020.8) (https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2020.8)) was used for taxonomic classifications and diversity analysis, as summarized in the following main steps:

- Resolve orientation problems—list all sequences in the same orientation;
- Trim primers with cutadapt—to remove the primers from the reads and FILTER the reads by removing those that do not begin and end with the correct primer sequences and those that are out the size range 1300–1800 nt;
- Import FASTQs as QIIME 2 artifact—to import the trimmed reads into the QIIME 2 "artifact" file format (with the extension QZA);
- Summarize trimmed FASTQs—to get a report of the number of reads per sample and quality distribution across the reads;
- Denoising and pooling the reads into ASVs—by running the DADA2 workflow to: (i) denoise the reads by removing those with low quality (number of "expected errors" > 2) and trimming all to the same length (from where the quartiles of the quality score distribution per position drops below the value 25), (ii) infer ASV's from the denoised reads,, and (iii) remove chimeras by excluding variants that result from combinations of two "parent" reads;
- Assign taxonomy to ASVs—to classify the ASVs using the Naive-Bayes approach implemented in the scikit learn Python library (https://scikit-learn.org/stable/modules/naive_bayes.html) with the SILVA full-length 16S/18S classifier database;
- Filtering resultant table—to filter out highly rare ASVs (frequency < 0.05% of the mean sample depth) and contaminant (mitochondrial and chloroplast 16S sequences), as well as to exclude any ASV that is unclassified at the phylum level;
- Generate rarefaction curves—to calculate two alpha-diversity metrics (Shannon index and Faith's phylogenetic diversity index) for all samples using growing sequencing depths and plot the respective rarefaction curves, to determine if sufficient sequencing was performed;
- Calculating diversity metrics and generating ordination plots—to calculate the beta diversity metrics Bray–Curtis Dissimilarity and Weighted UniFrac distance and plot respective principal coordinates analysis (PCoA) graphs to visualize relations between samples;
- Generate stacked barchart of taxa relative abundances—to plot taxonomic abundances across samples (each sample individually).

Isolation identification and cultivation of acidophilic SRB strains Isolation

The isolation of aSRB strains was made in petri dishes with ~ 20 mL solid media and in 15 mL centrifuge tubes with ~ 14 mL solid medium, with carbon sources/electron donors and pH values selected according to the most promising results in enrichment cultures.

Five plates and five tubes were prepared for each selected condition. The plates were inoculated by spreading onto the solid medium 150 µL of successive dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) prepared from the enrichment cultures. The tubes, before medium solidification (kept at 60 °C in a water bath), were inoculated with 850 µL of the same successive dilutions and homogenized by tube inversion five times. Successive dilutions were made in sterile Ringer's solution, with the pH adjusted to the selected conditions.

The solid medium for strain isolation was prepared as follows: before adding the carbon source, bacteriological agar (15 g/L) was added, kept under magnetic stirring for 5 min, autoclaved and left to cool down to ~ 60 °C under magnetic stirring. Then, inside a sterilized laminar flow chamber (after 20 min UV exposition), the carbon source was added and the medium was divided into the petri dishes and left to solidify (before inoculation) or divided into centrifuge tubes and kept at 60 °C in water bath (until inoculation).

The solid medium in the petri dishes, after the inoculum had dried, was covered with another portion of solid medium and the plates were incubated at room temperature (25 ± 3 °C) in a closed five-liter glass bottle with 3 candles burning inside aiming to create anaerobic conditions. In the case of isolations in 15 mL centrifuge tubes, the inoculated solid medium was covered with 1.5 mL of liquid paraffin (previously sterilized by autoclave), the tube tightly closed with the screw cap, and the incubation also carried out at room temperature (25 ± 3 °C).

Isolations on solid media were monitored by direct observation of the plates and tubes looking for black colonies, a typical feature of SRB colonies in this type of media due to the formation of FeS precipitates (Postgate 1984).

Identification of isolates

The taxonomic classification of strains forming black colonies was performed using sequences from the 16S rRNA gene for prokaryotes and sequences from the 18S rRNA gene and from the ITS1-5.8S-ITS2 region for eukaryotes.

A part of each black colony was picked with a sterile spatula and used for DNA extraction using the DNeasy PowerSoil Pro Kit (QUIAGEN). The concentration and quality of extracted DNA was evaluated using a spectrophotometer (NanoDrop3300, Thermo Fisher Scientific). For the PCR amplifications the following primer pairs were used: 16S rRNA gene—the universal primers 8F (also known as fD1) (5′- AGA GTT TGATCC TGG CTC AG -3′) (Weisburg et al. 1991) and 1492R (5′-GGT TAC CTT GTTACG ACT T-3′) (Lane 1991); 18S rRNA gene—the universal primers 18S-F (5′- ACC TGG TTG ATC CTG CCA GT -3′) and 18S-R (5′- TCA GCC TTG CGA CCA TAC -3′) (Sogin 1990); ITS1-5.8S-ITS2 region—ITS1F primer (5' CTT GGT CAT TTA GAG GAA GTA A -3′) (Gardes and Bruns 1993) and the ITS4 primer (5'- TCC TCC GCT TAT TGA TAT GC -3′) (White et al. 1990). The reaction mixture was in 0.2 mL PCR tubes as follows: 2 µL of DNA sample (5–50 ng/µL), 5 µL of 10×DreamTaq buffer (ThermoFisher Scientific), 0.25 µL of DreamTaq DNA Polymerase (5 U/µL) (ThermoFisher Scientific), 1 µL dNTP solution (10 mM each), 1 µL forward primer solution (10 µM), 1 µL reverse primer solution (10 µM) and 39.75 µL sterile Milli-Q water. The PCR amplifications were performed in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, Foster City, EUA), with an initial denaturation step of 95 °C for 10 min, 35 cycles of 95 °C for 30 s, annealing temperature (57 °C for 16S rRNA and 50 °C for 18S rRNA and ITS1-4) for 30 s, 72 °C for 90 s and a final step of 7 min at 72 °C.

Then, before DNA sequencing, the sizes of PCR products were confirmed by electrophoresis on 1% (w/v) agarose gels in 1×buffer TAE (AMRESCO, Solon, EUA), stained by addition of 50 µL/L of GreenSafe Premium (NZYTech, Lisbon Portugal) in the gel.

Afterwards, the amplified products were sequenced with the respective primers by the Sanger method through a capillary electrophoresis sequencing system (Genetic Analyzer, Model 3130xl, Applied Biosystems, Foster City, EUA) at the Centre of Marine Sciences (CCMAR), university of Algarve, Portugal.

Finally, taxonomic classifications based on the 16S rRNA gene, the 18S rRNA gene and the ITS1-4 sequences were obtained by BLAST alignment in the NCBI databases "16S ribosomal RNA sequences (Bacteria and Arcaea)", "18S ribosomal RNA sequences (SSU) from fungi" and "Internal transcribed spacer region (ITS) from fungi", respectively.

Physical–chemical analysis A pH/E Meter GLP 21 (CRISON) was used to measure redox potential with a Pt electrode coupled with a reference saturated calomel electrode (CRISON, 52 61) and pH with a glass pH electrode (VWR, SJ 223). Redox measurements were converted to Eh values using a conversion factor of 241 mV for the Pt electrode. A UV–Visible spectrometer DR2800 (Hach-Lange) was used to determine the sulfate and sulfide concentrations using the sulfaVer4 (Method 8051, Hach-Lange) and the methylene blue (Method 8131, Hach-Lange) procedures, respectively at 450 and 665 nm. Samples collected for metals analysis

were initially centrifuged at 2500*g* and then were acidified with concentrated nitric acid (to 5% (v/v)). Microwave plasma atomic emission spectrometry with a 4200 MP-AES (Agilent) equipment was used to measure the concentrations of Cu, Zn, Fe, Al and Mn. Calibration curves were built using standards prepared from the following stock solutions in 0.5 M HNO_3: $Cu(NO_3)_2$, $Zn(NO_3)_2$, $Fe(NO_3)_3$ (Merck Certipur, Germany); $Mn(NO_3)_2$ and $Al(NO_3)_3$ (Panreac AA, Spain).

Data interpretation In both sets of experiments (enrichment cultures and AMD bioremediation tests), the parameters were monitored periodically over time to evaluate if data exhibited clear and easily discernible trends or patterns. For a first assessment and discovery of very evident trends in this type of data, statistical analysis may not be necessary, and visual inspection might be sufficient to draw meaningful suggestions and hypotheses.

Relying on this assumption, the experiments were performed without replicates (studies on the physical and chemical parameters in the enrichment cultures and in the AMD bioremediation tests) or with just two replicates (study on the prokaryotic communities in the AMD bioremediation tests).

Results and discussion

Sulfate reduction in the enrichments of aSRB consortia

The total conversion of sulfate to sulfide in the positive control (Figure S. 1) shows that the incubation strategy used in the enrichment cultures was appropriate. Aerobic microbes in inoculated microbial communities consume the oxygen initially present in the medium, creating optimal anoxic conditions for the growth of strictly anaerobic bacteria such as the SRB.

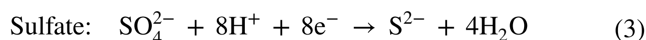
In what concerns the enrichments, the cultures starting at pH ≥ 4 (4.00, 5.00 and 6.00) with the three different tested carbon sources/electron donors (glycerol, methanol and ethanol) revealed physical–chemical changes indicative of SR activity: sulfate decrease, sulfide production, drop of redox potential to negative values and an increase of pH. On the contrary in the cultures starting with pH < 4 (2.00 and pH 3.00) theses parameters did not indicate SR activity.

Highest SR activity rates were observed in the samples enriched in the presence of methanol (Fig. 2). At pH 5.00 and 6.00, more than 99.9% of sulfate reduction was observed after 21 days of inoculation, and the most intense SR activity was from day 14 to 21 with a sharp decrease in sulfate concentration (reduction of almost 99% of the sulfate in this time range). In the cultures starting at pH 4.00, the highest intensity of SR was observed one week later, starting at day 21 and achieving complete SR at day 35. The final concentration of sulfide achieved was ~ 200 mg/L, ~ 100 mg/L and ~ 25 mg/L, in the cultures starting at pH 6.00, pH 5.00, and pH 4.00, respectively. A possible explanation for the lower sulfide concentrations in the most acidic conditions could be the reaction of the generated sulfide with metal ions present in the sediments that were leached to the solution, originating insoluble metal sulfides. The lower the pH, the more metals could have been solubilized from the sediments.

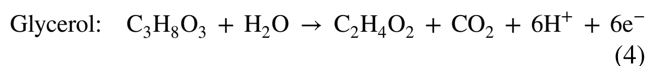
Regarding the enrichment cultures with glycerol as carbon source/electron donor, SR activity observed at different pH values had similar trends as in cultures enriched with methanol, but at slightly lower rates (Figure S. 2). Nearly 90% of the initial sulfate was consumed at pH 6.00, 5.00 and 4.00 after 42 days of inoculation.

The least sulfate reduction activity was observed in the enrichment cultures whit ethanol as carbon source/electron donor (Figure S. 3). Curiously, in the cultures starting at pH 4.00 more SR activity was observed than in cultures starting at pH 5.00 and 6.00. Even so, at pH 4.00 just moderate SR activity (~ 65% decrease of initial sulfate) was observed after 35 days.

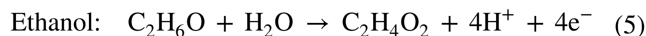
The same amount of each compound was used as a carbon and energy source (30 mM). Thus, the differences observed in SR efficiency may have been caused by the different stoichiometries for the oxidation of those compounds and the co-occurring reduction of sulfate. When 1 mol of glycerol or ethanol is partially oxidized to 1 mol of acetate, a concomitant reduction of 0.75 mol sulfate is expected in the case of glycerol, while 0.5 mol sulfate reduction is expected for ethanol. In the case of methanol, when 1 mol is completely oxidized to carbon dioxide, 0.75 mol of sulfate is expected to be reduced (Eqs. 3, 4, 5, and 6, adapted from Sánchez-Andrea et al. 2013). According to this, when the 30 mM of each compound is oxidized by SRB the expected amounts of reduced sulfate are 15 mM (1441 mg/L) in the case of ethanol and 22.5 mM (2161 mg/L) for glycerol and methanol.



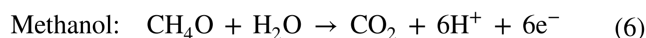
$$\text{Sulfate:} \quad SO_4^{2-} + 8H^+ + 8e^- \rightarrow S^{2-} + 4H_2O \tag{3}$$



$$\text{Glycerol:} \quad C_3H_8O_3 + H_2O \rightarrow C_2H_4O_2 + CO_2 + 6H^+ + 6e^- \tag{4}$$



$$\text{Ethanol:} \quad C_2H_6O + H_2O \rightarrow C_2H_4O_2 + 4H^+ + 4e^- \tag{5}$$



$$\text{Methanol:} \quad CH_4O + H_2O \rightarrow CO_2 + 6H^+ + 6e^- \tag{6}$$

In any case, in general, these results confirm the presence of aSRB in the sediments collected at the São Domingos

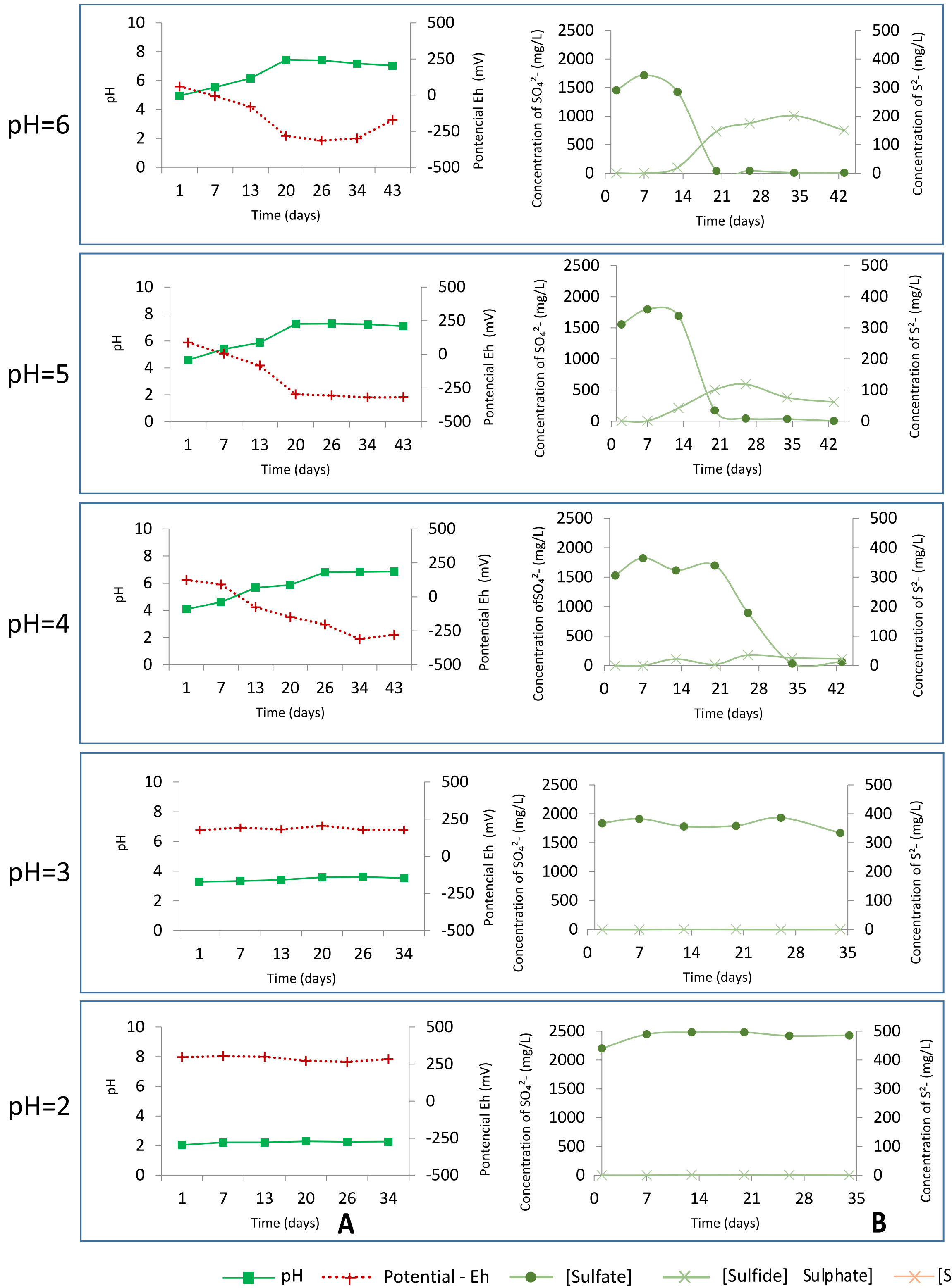


Fig. 2 pH, redox potential, sulfate and sulfide in the enrichment cultures in Postgate B media base with methanol as carbon source at different initial pH and room temperature (25 ± 3 °C)

mine, opening the possibility of installing at that location an AMD bioremediation process resilient to relatively low pH values using autochthonous microbial communities. Moreover, the results confirm previous works reporting alcohols and glycerol as non-toxic compounds to be used as carbon source/electron donors in sulfidogenic bioreactors performing at low pH. Indeed, sugars, alcohols, glycerol and hydrogen have been demonstrated as suitable electron donors in such conditions (Koschorreck 2008; Bijmans et al. 2010; Ňancucheo and Johnson 2012).

AMD bioremediation with aSRB enriched consortium

Batch experiments were carried out to evaluate AMD bioremediation using the consortium obtained through enrichment starting at pH 4.00 in a Postgate B medium base with methanol as carbon source/electron donor. For these experiments the pH of AMD was initially increased from 2.60 to 4.50, causing a previous precipitation of some metals mainly as hydroxides. The pH adjustment highly decreased Fe and Al concentrations in the AMD (Fe and Al concentrations dropped from ~ 173 to ~ 0.24 mg/L and from ~ 279 to 12 mg/L, respectively), while the concentrations of the other main metals including Cu, Zn and Mn did not undergo drastic changes (Cu, Zn and Mn were still ~ 25.74, ~ 43.6 and ~ 19.9 mg/L, respectively) (Table 2). On the other hand, since different ratios of Postgate B medium base (PB-m) to AMD were tested, the initial concentrations of the studied metals were not the same in all these tests. As higher as it was the fraction of PB-m, the greater dilution effect of Cu, Zn, Mn and Al metals from AMD occurred, while, on the contrary, it was observed that the PB-m fraction added Fe to the mixtures.

Apart from these previous phenomena, as shown in Fig. 3, metals removal in the test with minimum ratio of PB-m to AMD (20% PB-m plus 80% AMD), which contained 0.02% (w/v) yeast extract and 0.115% (w/v) of basal salts (originating from PB-m) was efficient as in the tests with higher PB-m contents (40%, 60% or 80% PB-m plus 60%, 40% or 20% AMD, respectively). When the inoculum was added to the test containing 20% PB-m plus 80% AMD, ~ 20 mg/L Cu and ~ 10 mg/L Al were immediately removed while the concentration of Zn dropped from ~ 30 to 7.64 mg/L. Then, during the first week after inoculation Zn decayed to bellow detection. On the other hand, Fe and Mn concentrations were almost constant in the solution (between ~ 20 and ~ 25 mg/L), despite the raise of sulfide concentration, until pH reached to 6.71 at day 20 and Fe was totally removed from solution, and until pH was 6.75 at day 28 and Mn was no more detected (Fig. 3, Fig. S. 4). Thus, 20% PB-m plus 80% AMD ratio was found as the most promising conditions for metals removal from the solutions due to less costly nutrient supplement requirement. In a study by Santos and Johnson (2017),

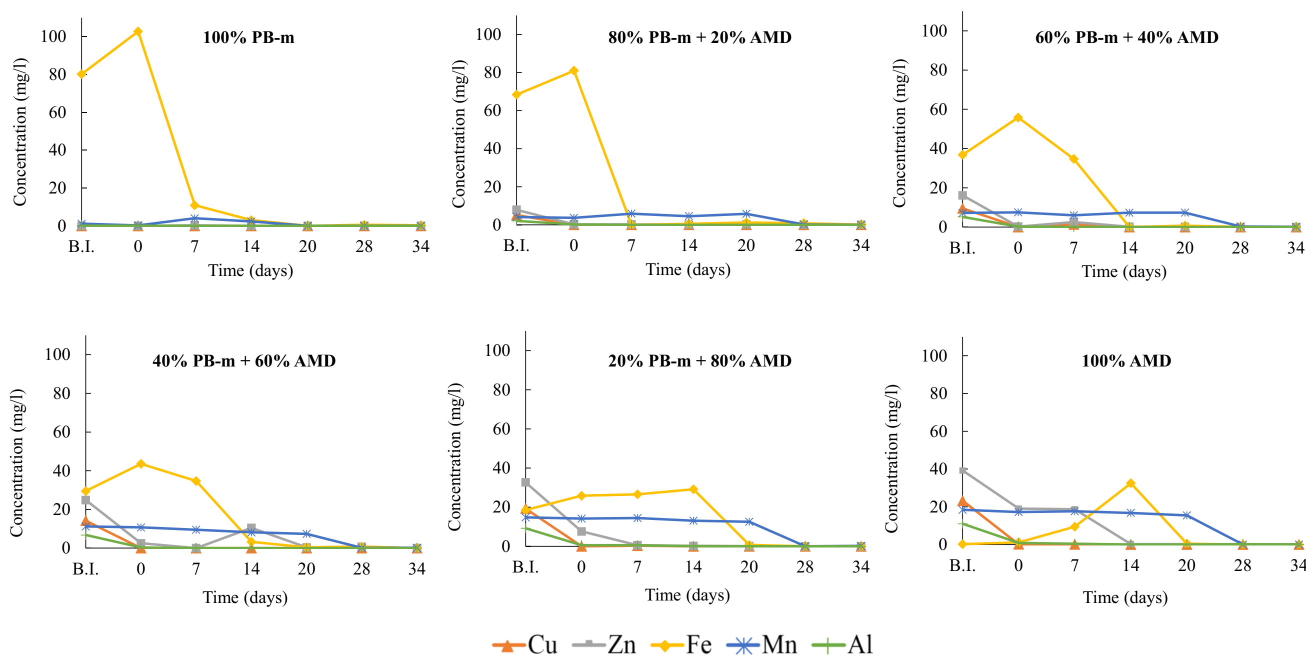


Fig. 3 AMD main metals (Cu, Zn, Fe, Al and Mn) in the treatment tests with different ratios of PB-m to AMD (v/v) inoculated with the selected aSRB consortium, at initial pH 4.50, with methanol as carbon source, and incubated at room temperature (25 ± 3 °C). B.I. in the X axis means before inoculation

an aSRB consortium enriched from acidic metal mine sites was employed for metal removal from a synthetic AMD supplemented with 0.01% (w/v) yeast extract and 0.12% basal salts in a laboratory-scale sulfidogenic bioreactor in a continuous flow system at pH values between 4.00 and 5.00. Their results showed the adaptability of the aSRB consortium in successful sulfate reduction and removal of more than 99% of all the transition metals including Zn, Co and Ni (except Mn). In another example, Ňancucheo and Johnson (2012) also had 0.01% (w/v) yeast extract and 0.12% (w/v) basal salts when used an aSRB consortium for the selective precipitation of transition metals including Cu and Zn from a synthetic AMD containing also Fe and Al, at different pH values.

Looking in detail to each monitored metal, the addition of a sulfide rich aSRB culture inoculum to the tested mixtures caused a rapid precipitation of Cu and its concentration dropped to almost zero in the beginning of experiments (Fig. S. 4). This is expected because of the low solubility product constant of copper sulfide ($Ksp = 1.3 \times 10^{-36}$) (Zhang et al. 2016b). In fact, the solubility of copper sulfide at pH between 1.00 and 5.00 is below 0.0001 mg/L (Lewis 2010), thus copper sulfide particles are formed even in highly acidic conditions.

On the other hand, the total Al content of solutions in all tested conditions also dropped to almost zero after inoculation. Al removal from the solution is important due to the toxicity effect of this metal on SRB growth (Falagán et al. 2017). Al does not form sulfide minerals and can precipitate by forming hydroxides in still relatively acidic conditions (pH ~ 5.00) (Pickering 1986; Falagán et al. 2017). The observed quick Al removal occurred because at the time the aSRB enriched culture was used as inoculum it had pH ~ 7 (despite having started at pH 4). Therefore, the addition of inoculum caused a slight increase of the initial pH to values between 4.73 and 4.91 in the tests.

Regarding zinc, under the tested conditions it can react with sulfide and precipitate, as the solubility product constant of zinc sulfide ($Ksp = 1.6 \times 10^{-24}$) is considerably low and it is the second most insoluble sulfide product (after copper sulfide) among the other metals present in the tested AMD (Zhang and Wang 2016). According to Lewis (2010), at pH between 4.00 and 6.00 the theoretical solubility of zinc sulfide is in the range of 0.1–0.001 mg/L. Therefore, Zn concentration immediately dropped after inoculation with the sulfide rich aSRB culture in all tested conditions. In the tests with less initial metals from AMD (60% and 80% PB-m plus 40% and 20% AMD, respectively) the concentration dropped immediately to almost zero. Nevertheless, in the samples with higher initial metal content, 20% PB-m plus 80% AMD and 100% AMD, zinc removal to almost zero content was achieved only after one and two weeks, respectively. The sharp initial drop of Cu and slight removal of Zn in these conditions by their reaction with sulfide caused a fast sulfide depletion; thus, just after the SR activity started to generate new sulfide, the rest of the Zn precipitated (Fig. S. 4).

In the case of iron, after adding the sulfide rich inoculum its content in the tested media increased and then started to decay as SR occurred and consequently the pH raised and iron hydroxides formed. The explanation for this initial increase can be the solubilization of iron sulfide (FeS) particles present in the inoculum (which was at pH ~ 7.00) when entered the in the acidic environment of the tested conditions (pH 4.50). Indeed FeS solubility rises with the decrease in pH and has low solubility in higher pH values (~ 0.1 mg/L at pH 6.00 and > 100 mg/L at pH 2.00) (Lewis 2010).

Mn was the last monitored metal removed from the solution. On one side MnS has much larger solubility ($Ksp = 2.5 \times 10^{-13} - 2.5 \times 10^{-10}$). On another side, Mn^{2+} requires pH > 8 to be oxidized to Mn^{4+} and to precipitate as carbonates or hydroxides (Hallberg and Johnson 2005). Yoo et al. (2004a; b) studied Mn removal from AMD by SRB and reported that pH, presence of citrate, co-existence of Fe and Zn and concentration of sulfide are the most important factors in Mn removal as MnS. They report successful precipitation of MnS in the absence of Fe and Zn with excess amount of sulfide at pH 5–7. In this work, as depicted in Fig. S. 4, Mn was removed from all samples when pH reached more than 5 and most of the Fe and Zn were removed from the solution beside the presence of excessive amounts of sulfide, which is in agreement with the reports of Yoo et al. (2004a; b).

In what concerns sulfate, this study showed that adding 20% (v/v) PB-m to AMD provided enough nutrients to promote SR activity as in the positive control with 100% PB-m, which occurred mainly during the second and third weeks (Table 3, Fig. S. 4). Interestingly, in the test with 100% AMD there was also some SR activity. However, it occurred mainly just after the third week. Curiously, SR in these tests was in general less efficient than in the

Table 3 Sulfate reduction in the AMD bioremediation tests with different ratios of PB-m to AMD (v/v) inoculated with the selected aSRB enriched consortium, at initial pH 4.50, with methanol as carbon source and incubated for 34 days at room temperature (25°C ± 3)

Culture medium	Sulfate reduction (%)
100% PB-m (no AMD)	46
80% PB-m (20% AMD)	45
60% PB-m (40% AMD)	36
40% PB-m (60% AMD)	35
20% PB-m (80% AMD)	30
no PB-m (100% AMD)	14

previous enrichment culture with 100% PB-m and same carbon source/electron donor (methanol), where all sulfate was consumed. Probably some components in the inoculated sediments played an important role in maintaining conditions favoring the activity of this SRB community in the enrichment cultures. Nevertheless, the obtained SR levels were enough to achieve efficient metals removals, as reported in other similar works. For example, Dev et al. (2021), using a bacterial consortium enriched from Arctic mine sediments in AMD remediation at pH 4.50 with glycerol as carbon source/electron donor achieved 34% SR and ~ 99% removal of metal content (mainly Fe and Zn) after 22 days.

Study of prokaryotic populations in enrichment cultures

Sequencing data validation and diversity analysis

After filtering and denoising the reads, the non-chimeric (total = 15,477) were clustered into 525 ASVs (alternative to OTUs), which were used for taxonomic classifications. Then, in the ASVs filtering/decontamination steps, one ASV was classified as chloroplast DNA and excluded, leaving a final number of 524 ASVs and 15,462 reads (Table S. 1). Neither highly rare ASVs, nor mitochondrial ASVs nor ASVs unclassified to the phylum level were discovered.

The rarefaction curves for both the Faith's phylogenetic diversity and the Shannon alfa-diversity indexes showed on all samples a growing trend reaching a plateau as the sequencing depth raise (Fig. S. 5) which indicates that the number of sequencing reads were enough to have the taxonomic diversity fully represented in all samples; even the sample with the lowest number of reads: sample E (Table S. 1). This gives confidence to the studies about the prokaryotic communities in the enrichment cultures. The Faith's phylogenetic diversity (faith_pd), which accounts only with the richness (number of taxa) within a sample, revealed a much higher value for the inoculated sediments (faith_pd ≈ 20), then for the enriched cultures with methanol or ethanol or glycerol (faith_pd ≈ 5–10). On the other hand, the Shannon index (sh), which considers both the richness and the evenness (whether the abundances of different taxa are more or less similar), showed more similar values among samples: the culture with methanol having the highest diversity (sh ≈ 6.5), followed by the enrichment culture with ethanol (sh ≈ 5.7), then the sediments inoculum (sh ≈ 5.3) and finally the culture with glycerol (sh ≈ 4.5).

In addition, for a better comparison of samples regarding their prokaryotic communities, two beta-diversity metrics were studied: the Bray–Curtis Dissimilarity, which is only a measure of shared ASVs between samples and the Weighted UniFrac distance, which accounts with the number of shared ASVs, their abundancies and with their phylogenetic relations (how closely related are the ASVs). The Bray–Curtis Dissimilarity showed that the prokaryotic communities on the enriched cultures with different carbon sources/electron donors (ethanol, glycerol, and methanol) were closer to each other than to the initial community on the inoculated sediments (Table S. 2). The Weighted UniFrac distance revealed that the ethanol-enriched culture went through a greater change in the prokaryotic community structure than the cultures with methanol and glycerol (Table S. 3).

Main prokaryotic communities

The structure of the prokaryotic communities highly changed in the enrichment cultures inoculated at pH = 4.00 with each of the three tested carbon sources/electron donors (Fig. 4). The most dominant genera (> 5% abundance) in the sediments were *Sideroxydans* (28.1%), *Sulfuriferula* (17.2%), *Acidithiobacillus* (11.9%) and *Granulicella* (7.4%), which is in accordance with previous reports in other sediments affected by low pH AMD (González-Toril et al. 2010; García-Moyano et al. 2015; Zhang et al. 2019; Carlier et al. 2020a).

In the enrichment cultures with methanol, the most abundant genera (with abundancies over 5%) where *Enterobacter* (12.1%), *Clostridium_sensu_stricto*_12 (8.8%), *Microbacter* (8.8%) and *Sedimentibacter* 8.1%. In the enrichment cultures with ethanol, the most dominant genera (> 5%) were *Clostridium_sensu_stricto*_10 (22.4%), *Clostridium_sensu_stricto*_12 (21.6%), *Desulfosporosinus* (12.2%), *Clostridium_sensu_stricto*_9 (7.4%), *Bacillus* (7.2%) and *Anaerocolumna* (5.8%). In the enrichment cultures with glycerol, *Microbacter* (18.5%), *Desulfosporosinus* (11.5%) and *Clostridium_sensu_stricto*_12 (6%) were the most dominant genera (> 5%).

Bacteria from the *Desulfosporosinus* genus are strictly anaerobic SRB and are known to have a key role in sulfate reduction in acidic mine wastes (Mardanov et al. 2016). As mentioned before, the bacteria from this genus were present in high relative abundances in the three enrichment cultures (35.4%, 12.2% and 11.5% in the enrichments with methanol, ethanol and glycerol, respectively). SRB from *Desulfosporosinus* genus are frequently reported to be present in reactors operating at low pH and they are normally originated from acidic sediments due to their high capacity to thrive at low pH conditions (Labrenz and Banfield 2004; Church et al. 2007; Senko et al. 2009; Lee et al. 2009; Moreau et al. 2010; Hallberg 2010; Sánchez-Andrea et al. 2011, 2012). Remarkably, in certain sites with oxidized mining waste materials the

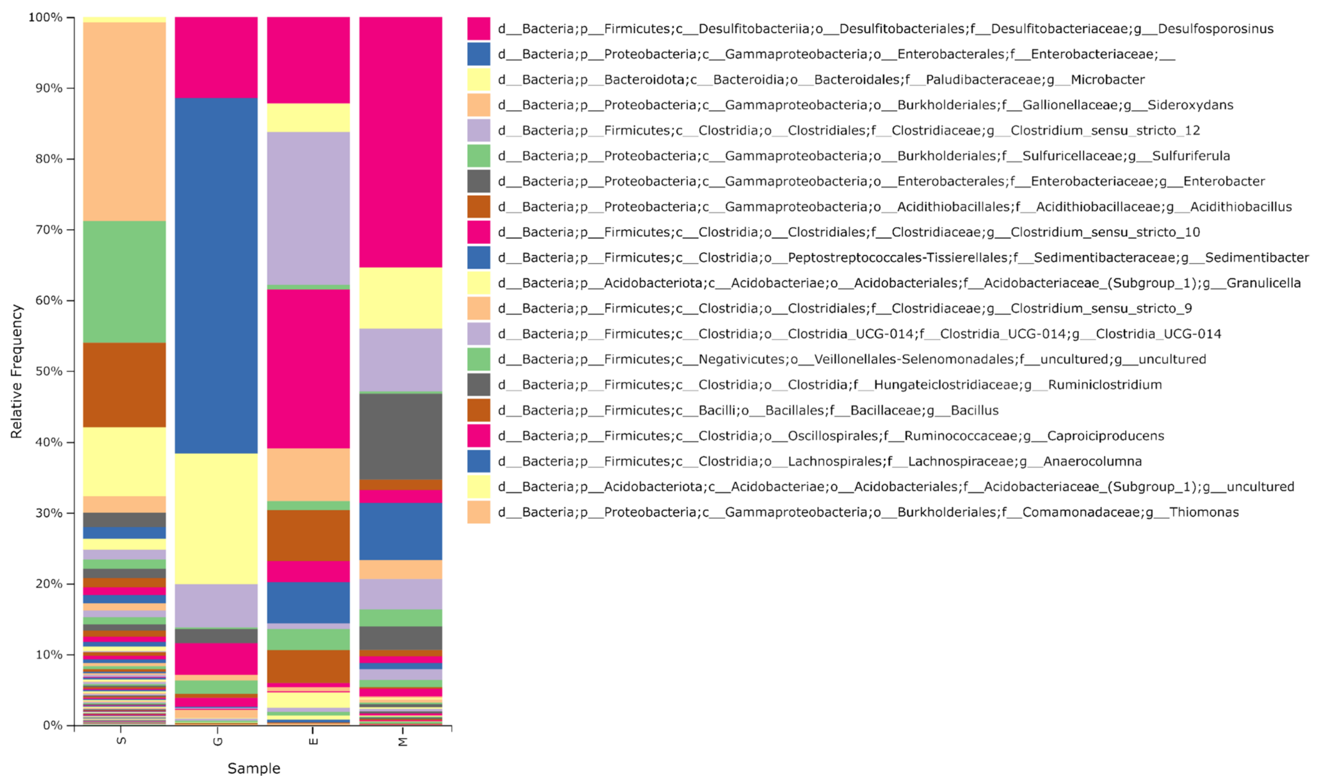


Fig. 4 Relative percentages (≥0.1%) of prokaryotic phylotypes at the genus level for the inoculated sediments (S) and for the enriched cultures starting at pH 4.00 with ethanol (E), glycerol (G) and methanol (M) as carbon sources/electron donors. The twenty most abundant are listed

only phylotype identified with SR capability was from *Desulfosporosinus* genus (Mardanov et al. 2016). The absence of sequences related to *Desulfosporosinus* genus in the sediments might be due to their presence as spores that hampered the DNA extraction (Labrenz and Banfield 2004), or to their very rare occurrence. Indeed, it is speculated that bacteria from *Desulfosporosinus* genus in sulfide tailings form a 'rare biosphere', since their rare abundancy but active components are masked by the predominant bacterial populations (Mardanov et al. 2016). By now, some studies have enriched and isolated acidophilic SRB of *Desulfosporosinus* genus from different acidic environments such as *D. acididurans* from sediments of Rio Tinto (Sánchez-Andrea et al. 2015) and *D. acidiphilus* isolated from acid mining effluent decantation pond sediments of the mining site of Chessy-Les-Mines (Alazard et al. 2010).

The analysis of 16S gene sequences in the enrichment cultures to the species level revealed the presence of *D. acididurans* in cultures grown with ethanol (3.8% relative abundance) and grown with glycerol (4.2%), as well as *D. acidiphilus* in cultures grown with ethanol (1.8%), with glycerol (0.3%) or with methanol (0.05%). It is reported that the pH range for *D. acididurans* growth is from 3.80 to 7.00, with an optimum pH of 5.50, and the ability of using different electron donors including glycerol, methanol, and ethanol, resulting in CO_2 and acetate generation (Sánchez-Andrea et al. 2015). In the case of *D. acidiphilus*, it is known to have a pH range for growth from 3.60 to 5.50 with an optimum pH of 5.20, and SR activity in the presence of glycerol, pyruvate, lactate, H_2, fructose and glucose as electron donors (Alazard et al. 2010). In the description of this strain, it is mentioned that it is not able to use ethanol and methanol as electron donors, which might be the reason for the very low abundancy of this group of bacteria in the cultures enriched with these compounds as carbon sources. They might have been growing in these cultures by using other electron donors available possible present in lower concentrations (e.g. released electron donors to the culture through the metabolic activity of other bacteria and/or from dead bacterial cells) (Alazard et al. 2010). Four other bacterial strains in *Desulfosporosinus* genus, which were not classified to the species level, were present in significantly high abundancies in the three enrichment cultures. They could be acidophilic SRB, which could reduce sulfate in acidic cultures and/or can be neutrophilic SRB that have grown after the pH rose as result of the activity of other aSRB like *D. acididurans* and *D. acidiphilus*.

Clostridium genera members were also present in the three enrichment cultures with high abundances. Members of this genera are identified as sulfate reducing (Zhang and Wang 2016; Zhang et al. 2016b; Hwang and Jho 2018) and acid generating/H_2-producing bacteria (Sánchez-Andrea et al. 2014a; Vilela et al. 2019, 2021), with adaptation potential to high dissolved organic carbon and turbidity (Zhao et al. 2017). Moreover, bacteria from *Microbacter* genera were observed in the enrichment samples with ethanol and methanol with high abundances. This group of anaerobic propionigenic bacterium usually co-exist with SRB either in natural or in engineered sulfidogenic environments (Lindsay et al. 2011; Sánchez-Andrea et al. 2013, 2014b). Although this does not demonstrate a direct contribution to SR activity, it seems to indicate some important role in the communities where it occurs. At least it is certain that the presence of a wide range of microorganisms in the cultures' communities increases their adaptability to different environmental conditions (Plugge et al. 2011; Ňancucheo and Johnson 2012; Ňancucheo et al. 2017), thus conferring an important plasticity in biotechnology processes.

Table 4 Alpha-diversity indexes using maximum sequencing depth of samples from the AMD treatment tests with different ratios of PB-m to AMD (v/v) at initial pH 4.50 and with methanol as carbon source, inoculated with the selected aSRB consortium and incubated at 25 ± 3 °C

	Day 0 (enriched inoculum)	Day 14	Day 34
Faith's phylogenetic diversity			
100% PB-m	7.0 ± 0.6	5.5 ± 0.5	5.83 ± 0.05
80% PB-m (20% AMD)		7.0 ± 0.3	5 ± 1
60% PB-m (40% AMD)		6.8 ± 0.5	4.7 ± 0.7
40% PB-m (60% AMD)		6.1 ± 0.6	5.4 ± 0.5
20% PB-m (80% AMD)		7.0 ± 0.3	5.8 ± 0.6
No PB-m (100% AMD)		4.4 ± 0.1	6.5 ± 0.4
Shannon index			
100% PB-m	6.2 ± 0.3	6.0 ± 0.2	5.70 ± 0.03
80% PB-m (20% AMD)		5.6 ± 0.8	5.1 ± 0.5
60% PB-m (40% AMD)		5.2 ± 0.7	5.0 ± 0.4
40% PB-m (60% AMD)		5.8 ± 0.7	5.6 ± 0.6
20% PB-m (80% AMD)		6.47 ± 0.01	5.3 ± 0.3
No PB-m (100% AMD)		5.2 ± 0.2	5.75 ± 0.09

Mean and mean deviations of duplicates are reported

Study of prokaryotic populations in AMD bioremediation tests

Sequencing data validation and diversity analysis

After filtering and denoising the sequenced reads, a total of 171,575 non-chimeric (Table S. 4) were clustered into 675 ASVs which were used for taxonomic classifications. Then, during the filtering/decontamination steps, one ASV classified as chloroplast DNA, another as mitochondrial DNA as well as 71 ASVs highly rare (< 0.05%) or unclassified to the phylum level, were excluded leaving a final number of 171,288 reads distributed in 602 ASVs.

The rarefaction curves for both the Faith's phylogenetic diversity and the Shannon alfa-diversity indexes showed in all samples a growing trend reaching a plateau as the sequencing depth raised (Fig. S. 6), indicating that the number of sequencing reads was sufficient to have the taxonomic diversity fully represented in all cases. In the test with 100% AMD (without PB-m supplement), both these alfa-diversity indexes revealed a sharp decrease in the prokaryotic community richness from the inoculum to the culture after 14 days of incubation followed by a slight recover of richness after 34 days of incubation, while in all the other tests the trend was a gradual decay of richness along all the incubation (Table 4).

For a better comparison of prokaryotic communities in the AMD treatment tests the beta-diversity metrics Bray–Curtis Dissimilarity and Weighted UniFrac distance were calculated (Tables S. 5, S. 6, S. 7 and S. 8) and then used to build Principal Coordinates Analysis (PCoA) graphs to facilitate visualization of relations between such high number of samples (Fig. 5). As a general remark, both these metrics revealed samples grouped in pairs, with both replicates from each sample close to each other, confirming a good representation of prokaryotic populations in both replicates. More in detail, the plot based on the Bray–Curtis Dissimilarity (Fig. 5A), which accounts just with shared ASVs (richness), displays the enriched inoculum (stars) and the tests just with AMD (squares) apart from each-others and separated from a major cluster of samples corresponding to tests with AMD plus supplements of PB-m as well as the controls without AMD (100% PB-m). Within this major cluster, the samples from tests with 80% AMD and just 20% PB-m (v/v) (rings) are on the side closer to the samples with 100% AMD while the controls of PB-m without AMD (diamonds) are on the other side. This reveals that the more similar the initial tested medium was, the more common ASVs the respective incubated cultures had. Moreover, the Weighted UniFrac distance (Fig. 5B), which accounts with ASVs' taxa as well as their common ancestors and abundances (evenness), also revealed the samples from the enriched inoculum (stars) and from the tests just with AMD (squares) apart from each-others and separated from a major cluster of samples from tests with AMD plus supplements of PB-m and the controls just with PB-m. However, in this case the samples in the major

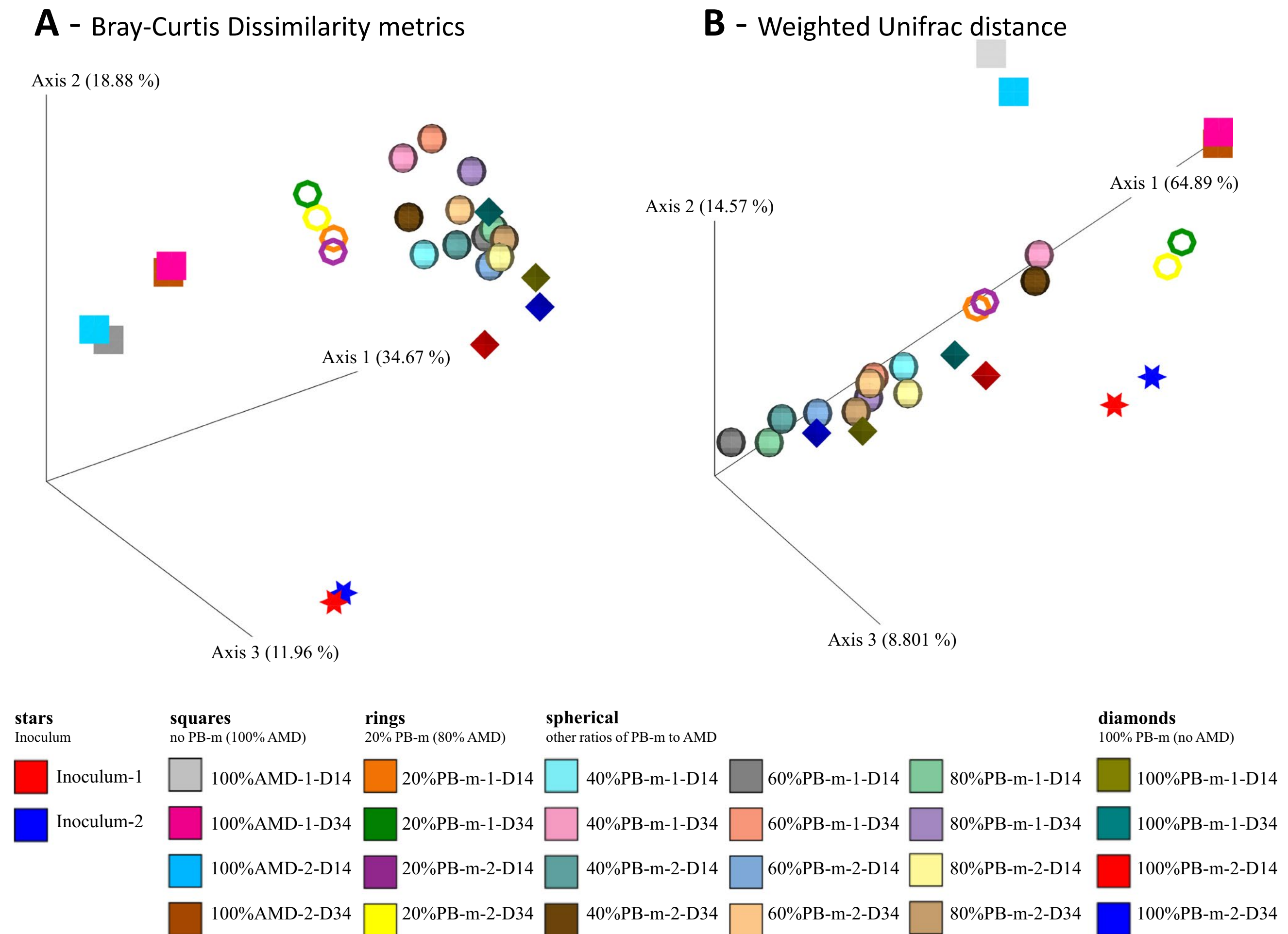


Fig. 5 Principal coordinates analysis (PCoA) plots based on two beta-diversity metrics (**A** and **B**) studied in the AMD treatment tests with different ratios of PB-m to AMD (v/v) inoculated with the selected aSRB consortium, at initial pH 4.50, with methanol as carbon source, and incubated at 25 ± 3 °C. Tests were sampled in duplicates (1 and 2) at days 14 and 34 (D14 and D34)

cluster were distributed without clear evidence of relation to the percentage of PB-m supplement, except for the test with 80% AMD (v/v) at day 34 PB-m (yellow and green rings) which remained closer to the samples with 100% AMD also at day 34 (pink and brown squares). Thus, the addition of any PB-m supplement to AMD caused an important impact on the evolution of prokaryotic communities after 14 days and that continued until day 34. On the other hand, the different amounts of PB-m tested did not cause drastic differences in the evolution of communities, except for the population in the test with the lowest amount of PB-m (20%) (v/v) which after 34 days differed from the other populations in the tests with different amounts of PB-m.

All together these diversity studies reveal an important plasticity of the consortium; that is, its capacity of gradual adaptation on the balances between taxonomic groups in relation to the gradual change in the composition of the ADM/PB-m supplement mixture.

Main prokaryotic communities

The evolution of most abundant prokaryotes in the tested conditions (different ratios of PB-m to AMD (v/v) is depicted in Fig. 6. The two most abundant families observed in each sample were always *Desulfitobacteriaceae* and *Clostridiaceae*, with the sum of their abundancies between 43 and 86%. The balance between these two populations was highly affected by the PB-m to AMD ratio: at day 14, the tests with 100% AMD or 20% PB-m plus 80% AMD (v/v) had higher relative abundancies of bacteria from *Desulfitobacteriaceae* family, while the tests with less AMD and more PB-m contents had higher relative abundancies of bacteria related to *Clostridiaceae* family. At day 34, this trend was even more evident and the *Desulfitobacteriaceae*

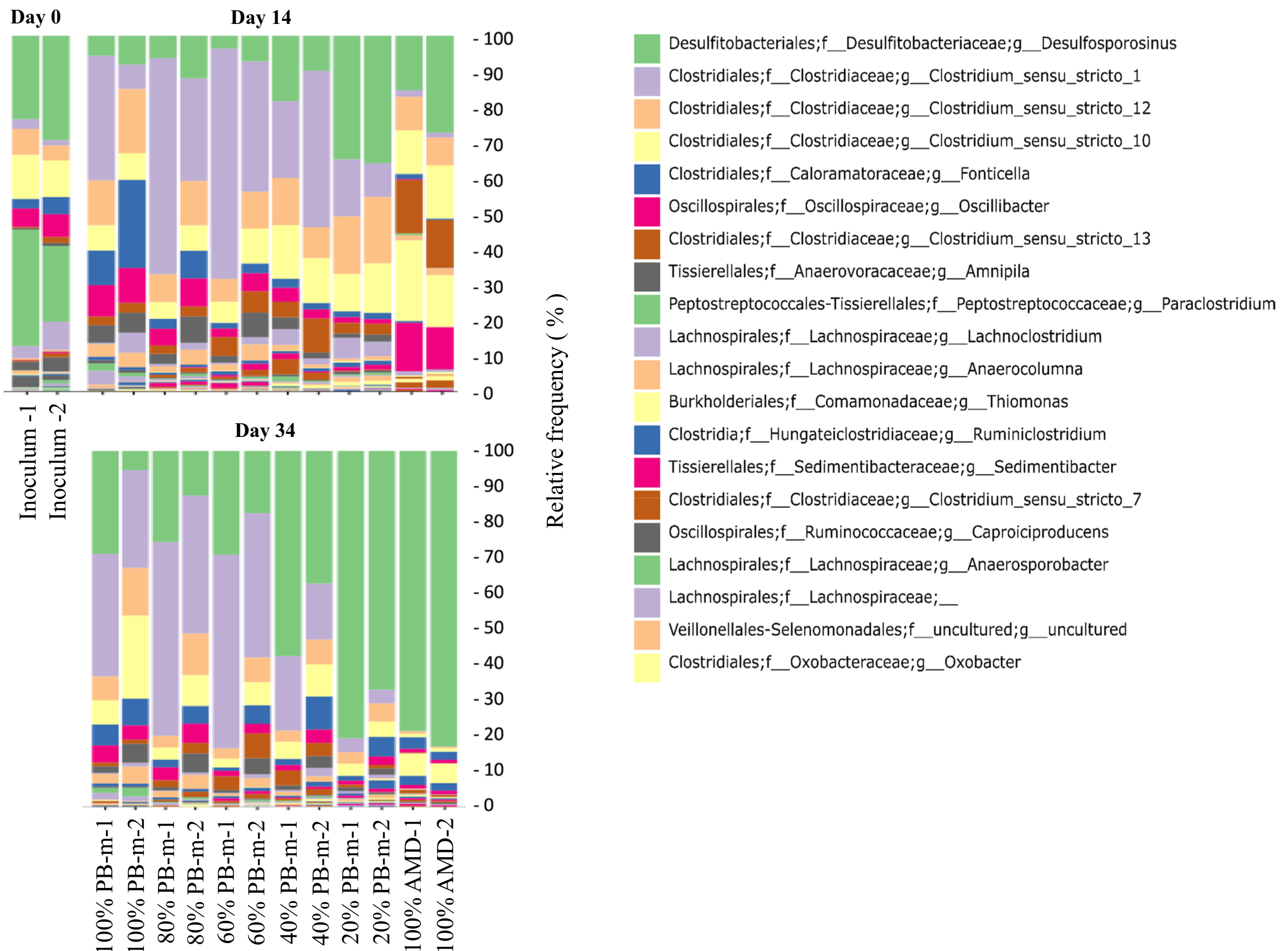


Fig. 6 Relative percentages (≥0.1%) of prokaryotic phylotypes at the genus level for the AMD treatment tests with different v/v ratios of PB-m to AMD inoculated with the selected aSRB consortium, at initial pH 4.50, with methanol as carbon source, and incubated at 25 ± 3 °C. Tests sampled in duplicates (1 and 2). The twenty most abundant are listed

family became dominant also in the test with 40% PB-m plus 60% AMD. The increasing relative abundancy of bacteria from *Clostridiaceae* by the raise of PB-m supplement (and less AMD content) can hypothetically be explained by the toxicity of AMD for certain taxa in this family and/or by their advantageous growth with higher nutrient contents through larger PB-m supplements.

Different works have reported the presence of *Clostridium* members together with *Desulfitobacteriaceae* members in sulfidogenic AMD bioremediation reactors (Hong et al. 2007; Martins et al. 2011; Lefticariu et al. 2015; Vasquez et al. 2018; Rodrigues et al. 2019). In fact, for example Aoyagi et al. (2017) remarked the importance of *Clostridium* members in SRB based bioreactors due to their capacities in breaking down complex organic substances into small molecules that can be used by *Desulfitobacteriaceae* members. Furthermore, sulfate reduction activity has been also attributed to *Clostridium* members in AMD treatment operations (Sallam and Steinbuchel 2009; Bao et al. 2012, 2017; Zhao et al. 2016; Rodrigues et al. 2020; Wu et al. 2022). For instance, in the investigation by Rodrigues et al. (2020), sediments from mine impacted waters from Sangão River (Brazil) were directly used as inoculum in AMD remediation studies with chitin as the only organic supplement and their meta-taxonomic studies of prokaryotes revealed very low abundances of *Desulfitobacteriaceae* members (< 0.28%) and relative high abundances of *Clostridium* members (27.04–12.75%), thus suggesting the *Clostridium* members were putatively the main sulfate reducers in their tested conditions. All this supports the idea that the *Clostridium* members present in the AMD treatment tests might have been acting as SRB and/or contributing to maintain suitable conditions for SRB from the *Desulfitobacteriaceae* family.

Looking to the genus level, bacteria from *Desulfosporosinus* genus consisted of about 26% of prokaryotes in the inoculum and then, after incubation in the AMD treatment experiments their relative abundances were increasingly larger from the tests with the highest to the lowest PB-m supplements: from 12.5% *Desulfosporosinus* spp. in the test with 80% PB-m plus 20% AMD to 83% *Desulfosporosinus* spp. in the test with 100% AMD (Fig. 6). These bacteria are known by their versatile behavior in extreme conditions, mainly as low pH and heavy metals toxicity, as well as their resistance to oxic conditions (Sánchez-Andrea et al. 2014a), and they are present in diverse SRB reactors treating AMD either in laboratory or in real scale experiments (Habe et al. 2020). They have been reported in studies on AMD bioremediation with a broad range of relative abundancies: from quite low (< 1%) fractions (Pester et al. 2010; Rezadehbashi and Baldwin 2018; Rodrigues et al. 2019) to high (26–60%) fractions (Sato et al. 2019; Valdez-Nuñez et al. 2022). Moreover, *Desulfosporosinus* spp. have demonstrated to contribute in high rates to SR activity, even when present in abundances as low as 0.006% (Pester et al. 2010). Therefore, their contribution to SR and consequent role in metals removal from AMD must be important. Indeed, other works point to this evidence. For instance, in the study by Dev et al. (2021) on AMD bioremediation at pH 4.5 with glycerol as electron donor, 34% of SR and ~ 99% metals removal was observed with a progressive domination of *Desulfosporosinus* spp. in the community during the incubation. The microbial metabolic process suggested by the authors was the initial fermentation of glycerol to acetic acid mainly by *Desulfosporosinus* when in low pH, followed by acetic acid oxidation to H_2 and CO_2 mainly by *Desulfitobacterium* when the pH raised to neutral values, and finally H_2 consumption by *Desulfosporosinus* (Dev et al. 2021).

In the present work there was probably also production of acetate, since it is known that the use of methanol in sulfidogenic anaerobic bioreactors results in acetate generation (Cao et al. 2012). Then, probably the produced acetate was consumed by other bacteria, including the highly abundant *Clostridium* spp.. In fact, it has already been shown that the oxidation of acetate can be undertaken by several bacteria present in this type of communities, including *Clostridium* spp. (Du et al. 2020).

Isolation identification and cultivation of acidophilic SRB strains

The attempt to isolate aSRB strains was done at two pH values (4.00 and 4.50) with all the three tested carbon sources (glycerol, methanol, and ethanol). Eleven black colonies become visible after about two weeks; just one in the petri dishes and ten in the tubes: one colony in a tube with solid medium at pH 4.50 and ethanol as carbon source and nine in tubes with solid medium at pH 4.00 and methanol as carbon source. The DNA from the colony isolated from petri dishes did not amplify the 16S rRNA gene but amplified the 18S rRNA gene and the ITS1-5.8S-ITS2 region, and both these sequences allowed to classify this colony as a fungus of the *Rachicladosporium* genus. On the contrary, the colony isolated from tubes with solid medium at pH 4.50 and having ethanol as carbon source just amplified the 16S rRNA gene sequence, which was classified as being from bacteria of *Desulfosporosinus* genus. Interestingly, all the nine colonies isolated from medium at pH 4.00 with methanol as carbon source amplified the 16S rRNA gene as well as both the 18S rRNA gene and the ITS1-5.8S-ITS2 region. The 16S amplicon sequences were classified as belonging to *Desulfosporosinus* genus, while the 18S and the ITS1-5.8S-ITS2 sequences were classified as being from a fungus of the *Trichoderma* genus. It may be possible that the presence of this fungus had a role in the fact that under these conditions more SRB colonies appeared (for example, by consuming oxygen from the medium). These results confirm the presence of acidophilic sulfate-reducing bacteria in the sampled area and suggest that the incubation of petri dishes in a jar with burning candles was not as efficient in creating anaerobic conditions as the strategy of solid medium incubation in closed tubes.

Conclusion

At the inactive copper mine at Mina de São Domingos, in Portugal, it is possible to obtain from sediments of the confluence zone between the AMD and the water stream receiving the municipal WWTP effluent microbial consortia with sulfate reduction activity at initial pH 4.00 when using methanol, glycerol or ethanol as carbon sources/electron donors.

Such consortia have among the most dominant prokaryotes *Desulfosporosinus* spp. and *Clostridium* spp. and can be used for the bioremediation of local AMD with pH adjusted to 4.50, having high adaptative plasticity to contents of AMD pollutants and/or available nutrients.

The isolation of aSRB colonies in closed centrifuge tubes with acidic solid medium was efficient and confirmed the growth of *Desulfosporosinus* spp. at low pH 4.00 and 4.50.

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Availability of data and materials Data is available in the manuscript and in the supplementary material.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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