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In search of sulfate-reducing consortia able to degrade acetate under acidic conditions

Short Title: Searching for sulfate-reducing consortia consuming acetate at acidic conditions

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ABSTRACT

BACKGROUND: Sulfate-reducing microorganisms (SRM) can help to remediate acidic effluents containing metals. One drawback of sulfate reduction is that some SRM do not oxidize completely the substrate to CO_2 and acetic acid may remain as a byproduct, affecting the process efficiency. Acidic environments are a potential source of sulfate-reducers able to thrive acidic conditions. This work aimed to develop cultivable consortia of sulfate-reducing microorganisms able to consume acetate at acidic pH and analyze their community composition.

RESULTS: Starting from sediment enrichments from a natural acidic source, by successive transfers and combinations of electron donors and pH we obtained seven sulfate-reducing consortia. All the consortia consumed the acetate produced from the incomplete oxidation of the substrate (lactate or glycerol) and used 53-75% of the reducing equivalents for sulfate reduction. The sulfide production rate of the consortia was between 0.22-0.26 mmol/L·day in the range of pH 3 – 6, being slightly higher at acidic conditions (4 – 5). The microbial diversity of the consortia was dominated by 21 OTUs, including taxa of acetotrophic sulfate reducers (i.e., *Desulfotomaculum* and *Desulfatirhabdium*) and fermenting bacteria.

CONCLUSION: The consortia reported here have the potential to serve as inoculum for sulfate-reducing bioreactors and could help to overcome acetate accumulation at low pH.

Key words: acetate, acidic pH, acidophilic, consortia, community, sulfate reduction

INTRODUCTION

The biological sulfate reduction process is based on the oxidation of an electron donor, which can be an organic substrate or molecular hydrogen, coupled to the reduction of sulfate (terminal electron acceptor) to produce sulfide. Sulfate-reducing microorganisms (SRM) are responsible for sulfate reduction and are a group of prokaryotes, remarkably adaptable, that can be found in terrestrial and aquatic environments, mainly in sulfate-rich anoxic environments in very diverse natural environments such as saline, alkaline, acidic, or thermal habitats.[1, 2]

Recently, sulfate reduction at low pH raised interest for the treatment of metal-containing effluents, such as acid mine drainage (AMD),[3, 4] the biologically produced sulfide can react with heavy metals such as Fe^{+2}, Zn^{+2}, Cu^{+2}, or Cd^{+2} and precipitate them as insoluble metal sulfides.[5–8] Such effluents are somewhat acidic (pH < 5) due to the acidification of the waste generated from the exploitation of minerals, either by chemical or biological processes and generally contain low amounts of organic carbon (< 10 mg/L), these characteristics diminish the efficiency of the sulfate reduction process.[9]

The activity of SRM retrieved from environmental samples (i.e. sediments or streams) has been observed under extremely (pH 1-3) and moderately (pH 4-5) acidic conditions and many efforts have been made to enrich, cultivate, and eventually isolate SRM at those conditions.[10] The development of several types of reactors for the treatment of AMD became possible by using communities from this kind of acidic environments. For instance, Ňancucheo and Johnson[11] treated synthetic AMD successfully in a continuous reactor inoculated with an enrichment obtained from the stream of an abandoned copper mine, and bioaugmented with pure cultures of *Desulfosporosinus* M1 and *Desulfobacillus acidavidus.* The community developed on glass beads was the key to the successful operation of the reactor at pH as low as 2.1.

In another work, sulfate-reducing consortia and four isolates of SRM were eventually retrieved from the extremely acidic environment of Rio Tinto in Spain.[10] The isolates were cultivated at pH 5.5-4.0 using glycerol, methanol, and lactate as substrates, but glycerol and lactate were incompletely oxidized to acetate. Up to date, only a few isolates of the genera *Desulfovibrio*, *Desulfosporosinus*, *Desulfobacillus,* and *Desulfurella* have been identified as acid-tolerant or acidophilic; none of these isolates can oxidize acetate.[12]

Lactate and ethanol are the substrates typically used to promote the activity and growth of SRM at neutral pH.[13, 14] However, a challenging area in the field of sulfate reduction at acidic pH is that when incomplete oxidation of these substrates occurs, the efficiency of substrate oxidation *via* sulfate reduction is lower because acetate remains as a by-product.[15] The acidic pH adds another constraint to the use of acetate by SRM because at pH values lower than 4.76 (*i.e.* the pKa of acetic acid), undissociated acetic acid is the predominant form, and this non-ionized molecule will cross the cell membrane and inhibit cellular respiration.[12, 16] In contrast, glycerol has been used successfully as a substrate for the enrichment, cultivation, and even isolation of SRM, at acidic conditions (pH $\leq$ 4.0).[10, 17] Glycerol does not ionize at acidic pH, avoiding the harmful effects that ionizable substrates such as organic acids may cause, but acetate is still a common by-product of glycerol oxidation.[11]

Therefore, to efficiently apply sulfate-reduction for AMD treatment, it is critical to count with acetate consuming sulfate-reducing communities thriving at acidic pH. This work aimed to expand the scope of SRM at acidic pH by developing and characterizing sulfate-reducing consortia. Using the acclimation approach, we were able to obtain seven sulfate-reducing communities cultivated at low pH (3 or 4) that can consume acetic acid.

MATERIALS AND METHODS

Source of microorganisms

Enrichments previously cultured were used as inoculum to develop the acetotrophic sulfate-reducing consortia reported here; these enrichments originated from the sediments of the acidic leachates from an abandoned sulfur mine and were cultivated with different carbon sources (acetate, lactate, or glycerol) at different pH (3, 4, or 5) as reported elsewhere.[18] To start the cultures of the consortia, we screened 45 enrichments and selected a total of 38 to be used as inoculum, based on the sulfide production and acetate consumption capacity of each enrichment (Fig. S1). In this work, we aimed to obtain consortia free of sediment.

Culture medium and cultivation conditions

The following minimal anaerobic medium was used to develop the consortia (mM): 50 NH_4Cl, 30 NaCl, 40 $MgCl_2 \cdot 6H_2O$, 75 $CaCl_2 \cdot H_2O$, 1 mL/L trace element solution (50 mM HCl, 1 mM H_3BO_3, 0.5 mM $MnCl_2$, 7.5 mM $FeCl_2$, 0.5 mM $CoCl_2$, 0.1 mM $NiCl_2$ and 0.5 mM $ZnCl_2$), and 0.1 g/L of yeast extract, modified from Stams et al.[19] The medium was supplemented with 10 mM Na_2SO_4 as the electron acceptor and the stoichiometric amount of electron donor: 10 mM acetate, 6.6 mM lactate, or 5.71 mM glycerol. All cultures were developed in 120 mL serum bottles, containing 80 mL of minimal anaerobic medium supplemented with the corresponding substrate, sodium sulfate; anaerobic atmosphere (N_2/CO_2; 80:20%) and were incubated at 30°C in the dark without agitation.

Development of the consortia by successive transfers

To develop the consortia by successive transfers, we started from the 38 initial enrichments selected as inoculum. These enrichments were divided into two groups.

Group 1: those initial enrichments incubated at initial pH 4.0 and fed with lactate, acetate, or glycerol, six bottles each. The successive transfers of this group were inoculated with 20% of slurry from the enrichment or 20% of the previous transfer (see Figure S1). Group 2 consisted of 20 bottles in total, enriched at initial pH 3 with lactate or acetate (6 bottles each) or glycerol (8 bottles); for starting-up the successive transfers from this group, we assayed two ways of inoculation: 1) inoculation with 10% supernatant (liquid fraction after sedimentation) and 2) inoculation with 20% slurry (the mixture of liquid media and sediment after vigorous agitation). The development of the cultures was monitored periodically through the concentration of substrates (acetate, lactate, sulfate), the concentration of sulfide, and the pH until the sulfide concentration was constant and almost complete consumption of acetate was observed (around 30 days). At this point, the cultures that showed sulfide production and acetate consumption were transferred again to new media with the corresponding substrate and initial pH; in this way, another transfer was obtained. In total, five successive transfers were needed to obtain each one of the seven consortia presented here; all the consortia were devoid of the original sediment. Those cultures that did not produce sulfide and did not consume acetate were discarded (Figure S1). During the successive transfers, the pH of the cultures was not controlled.

Characterization of the final consortia

Each final consortium (fifth transfer) was characterized by sulfide production, sulfate consumption, acetate production, pH, and optical density (600 nm) in triplicate. The time profiles obtained in this assay were used to calculate the maximum rates of lactate and acetate consumption and sulfide production to verify the reproducibility of the activity of the consortia.

Favorable pH interval

The final consortia were cultivated (in duplicate) with their corresponding substrate but varying the initial pH of the culture medium with the addition of 1 N HCl or 1 N NaOH. For the consortia originally cultivated at pH 4.0, we screened the following initial pH values: 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0. For the consortia originally cultivated at pH 3.0, the initial pH was adjusted to 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0. The concentration of sulfide, pH, and optical density were determined every seven days; the sulfate reduction rate was indirectly obtained from the slope of the sulfide production curve (in mM) *vs.* time. Subsequently, the rates obtained were plotted at each pH value to obtain the interval of favorable pH of each consortium.

Chemical analyses

Dissolved sulfide was quantified by the Cord-Ruwisch method[20] with the corresponding calibration curve (0-20 mM, in triplicate; maximum error 5%) using $Na_2S \cdot 9H_2O$ as standard. Volatile fatty acids (lactate and acetate) and sulfate were determined by capillary electrophoresis with a diode array detector according to the method of Soga and Ross (1999)[21] from calibration curves (50-1000 mg/L), using high purity standards, after centrifugation (10000 *g*) and filtration (0.22 μm) of the samples. The pH was measured with a Thermo Scientific TM Orion TM VersaStar potentiometer. To quantify the increase of biomass, the optical density (600 nm) was determined from fresh samples of the cultures.

Molecular characterization

To characterize the diversity of each final consortium (fifth transfer), the DNA was extracted from each bottle of the triplicate assay (Characterization of the final consortia) using the SPIN FastDNA-T DNA Extraction Kit for Soil (MP Biomedicals,

Santa Ana, CA, United States) according to the manufacturer's instructions. Then, the DNA was pooled into one composite sample, amplified, and cloned. Amplification of the 16S rRNA gene was performed with primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) to obtain a 1465 bp fragment. The PCR mix (50 μL) contained: 5X PCR Green GoTaq® reaction buffer, 0.2 mM dNTPs, 0.1 μM of each forward and reverse primer, GoTaq® DNA Polymerase (1.25 u), and 1 μL of template DNA. The PCR program was: 97ºC for 5 min, followed by 30 cycles at 95 °C for 2 min, 52 °C for 40 sec, 72 °C for 1.3 min, and a final extension at 72 °C for 10 min. The PCR products with the expected size (1465 bp) were cleaned by DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, United States), and ligated (overnight) using the pGEM-T Easy vector (Promega) following the manufacturer's instructions. Ligation was plated on Luria-Bertani (LB) agar with ampicillin (100 mg/L), IPTG (0.00238 mg/L) and X-gal (0.0040 mg/L) as selection media. Positive white colonies were selected (48 per sample) and grown in LB medium for 18 h at 37 °C, the grown cultures were plated into GATC plates and sent for Sanger sequencing with SP6 primer (Eurofins GATC Biotech, Konstanz, Germany). The DNA sequences were checked using Chromas (version 2.32, Technelysium Pty. Ltd.), and contigs were constructed from the partial sequences using DNAbaser (version 2.71.0, Heracle Software, Lilienthal, Germany) resulting in sequences of 800-1200 bp of the 16S rRNA gene. To find the phylogenetic affiliation of the clones, the bacterial 16S rRNA sequences were checked for anomalies using Pintail online software[22] and compared to the blastn GenBank (NCBI). Sequences were also aligned with SINA (v1.2.11), of the SILVA ribosomal database project, to find the phylogenetic affiliation of the clones using SILVAngs (*version*: 1.9.4 / 1.3.9) for Sanger sequencing analysis and to construct rarefaction curves. The sequences are deposited in the NCBI nucleotide sequence

database GenBank under accession numbers MT022112-409. We used the R Studio program[23] to calculate the Euclidean distance matrix, and construct a dendrogram using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) building the tree by the upside down approach. The richness, Shannon-Wiener index, Simpson index of dominance, evenness, and principal component analysis (PCA) were calculated with R Studio program using the vegan community ecology R package (version 2.5.6).

RESULTS

Development and performance of the consortia-successive transfers

Acetate-consuming sulfate-reducing consortia were enriched from previous incubations of sediments at acidic conditions (pH < 4.0) by successive transfers. To be transferred again, the cultures should produce sulfide and consume acetate completely. Only the first transfers inoculated with 20% of slurry and lactate showed sulfide production and acetate consumption; the cultures inoculated with 10 or 20% of the supernatant produced less than 2 mM of sulfide and consumed less than 80% of the substrate. Therefore, the following successive transfers with lactate as substrate were inoculated with 20% (v/v) of slurry. Interestingly, Consortium 7, fed with glycerol, was the only one that was obtained using 10% (v/v) of the supernatant as inoculum in the first transfer; nonetheless, due to the long-time needed (68 days) to consume the acetate completely and produce sulfide, the successive transfers were also inoculated using 20% (v/v) of supernatant.

Following this methodology, from a total of 365 incubations, only seven consortia were obtained after five successive transfers (Fig. 1 and Figs. S2-3). These consortia were free of sediment, able to produce sulfide at acidic conditions (pH 3 or 4) and consume acetate using lactate (Consortium 1-6) or glycerol (Consortium 7) as

the substrates. It is worth noting that, at this stage of the experiment, each consortium was unique because there was only one culture of each consortium. Table 1 shows the combinations of pH, substrate, and type of inoculum that yielded the seven consortia coupling sulfate-reducing activity with complete oxidation of the substrates. Figure 1 shows the time profiles of acetate production/consumption and sulfide production during the five successive transfers of the seven consortia. In most of the transfers, acetate accumulated between days 5 and 30; later, the communities consumed acetate and continued producing sulfide. Most probably, acetate accumulated due to the incomplete oxidation of lactate or glycerol; according to the stoichiometry 1 mM of lactate can produce 0.5 mM of H_2S, 1 mM of acetate and 1mM of CO_2, and 1 mM of glycerol can produce 0.75 mM of H_2S, 1 mM of acetate and 1 mM of CO_2.[24]

The first two transfers of Consortia 1 to 6 still had remains of the sediment due to the strategy of using 20% of slurry as inoculum. Nevertheless, from the third transfer onward, all the cultures were planktonic and free of sediment, producing sulfide and consuming acetate in a more reproducible way. In the third transfer, sulfate-reduction and acetate consumption were slower compared with the previous transfers, possibly as a consequence of getting rid of the remaining sediment, all the seven consortia behave the same (Figs. 1 and S2). The pH profiles showed that no matter at which pH value each of the consortia started, the pH increased to values between 6.1 and 7.3 (Fig. S3). Interestingly, the consortia started to consume acetate when the pH reached a value close to 5.5, this trend occurred in all the transfers (Figs. 1 and S3). Attempts of developing consortia using acetate as the sole electron donor for sulfate-reduction, at initial pH 3 or 4, were unsuccessful due to the high concentration of undissociated acetic acid (9.8 mM and 8.4 mM at pH 3 and 4, respectively). The sulfate-reducing rates of the successive transfers varied widely

(Table S1) and did not show any clear tendency to increase; on the contrary, the sulfate-reducing rates decreased from transfer 1 to 3. Eventually, in the last two transfers (4 and 5), the sulfate-reducing activity increased in some cases.

Reproducibility of the acetate-dependent sulfate-reducing activity

In the fifth successive transfer, the cultures were devoid of sediment, and the sulfate-reducing activity remained. At this point, we considered that the consortia were cultivable and reproducible, as shown by the assays performed in triplicate (Fig. 2). From these results, it was possible to calculate the percentage of substrate used for sulfate reduction of each consortium based on the stoichiometry of sulfide production (Table 2). Consortia 2 and 7 used around 75% of the electron donor (lactate or glycerol) to perform sulfate reduction, the rest of the consortia used close to 50% of the substrate for sulfate reduction that was the target activity of the culturing approach. These results indicated that the consortia were not only composed of sulfate-reducers and the successive transfer technique was accurate and appropriate for the cultivation of sulfate-reducers.

We also calculated the acetate consumption, once acetate concentration reached a maximum and started to decrease, and sulfide production rates (Table 2). The rates of acetate consumption varied between 0.20 to 0.44 mmol/L·d. Consortium 1, fed with lactate, showed the highest acetate consumption rate; the rest of the consortia were also able to use acetate as substrate at lower acetate consumption rates. Regarding sulfide production rates, these were between 0.22 and 0.28 (mmol/L·d), and Consortium 7 showed the highest sulfide production rate.

Range of favorable pH

We attempted to determine the most favorable pH at which the sulfate-reducing activity occurred comparing the rates of sulfide-production at each pH (Fig. 3); the selection criterion was that the difference of the sulfide production rate obtained at the different initial pH values, was lower than 0.2.

The results showed that there was not one favorable pH value but a range at which each consortium carried out sulfate reduction optimally (Fig. 3 and Table 2). The consortia developed at initial pH 4 and fed with lactate (Consortium 1-3), performed better in the range of pH 4-6 than at pH 3.5 or pH 7. On the other hand, the consortia initially cultivated at pH 3.0 and fed with lactate performed better in a pH interval from 2.5 to 6.0 than at pH 7.0 (Consortia 4-6). Consortium 7, fed with glycerol, showed a clear preference for acidic pH (3.0 to 5.5) to perform sulfate reduction. The initial optical density increased from a value around 0.019 ± 0.001 to values between 0.23 and 0.34 in all consortia, which is in agreement with the optical density values obtained in the sulfate-reducing activity assays (Fig. 2), confirming that the microorganisms of the consortia are cultivable, showing growth and not just activity.

Microbial composition of the consortia

A total of 21 OTUs (genus level) were obtained per sample at 80-99% similarity (from 336 sequences) (Figure 4). At the phylum level, all the consortia were composed of members belonging to *Bacteroidetes* (20-70%), *Firmicutes* (6-58%), and *Proteobacteria* (2-17%). Other taxa were found exclusively in some consortia. For instance, only Consortium 2 and 7 contained sequences resembling *Caldiserica* (2-7%); and sequences related to *Sphaerochaeta* (2-71%) were only present in Consortium 1, 4, and 7. Interestingly, sequences related to the unclassified *Synergistetes JGI-0000079-D21* (2-11%) were present in all the consortia except in

Consortium 7. Uncultured bacteria were retrieved from almost all the consortia (2-9%) except from Consortium 3, 4, and 6, while unclassified bacteria (non-relative) amounted to 2-15%. According to the diversity indices (Table S2), consortia 3 and 7 showed the highest richness value (S=12) in comparison with the rest of the consortia, but the Shannon-Wiener index indicated that Consortium 7 was the most diverse (H=2.106) and the less diverse was Consortium 4 (H=1.145). Consortium 6 (dominated by *Lentimicrobium*) and Consortium 4 (dominated by *Sphaerochaeta*) showed the lowest Simpson' index values, whereas the rest of the consortia were equally dominated. The rarefaction curves of all the consortia are shown in Figure S4.

The consortia grouped in two different clusters (Fig. 4), Consortium 1 and 3 (lactate, initial pH 4) showed the most similar microbial structure, as well as Consortium 2 (lactate, initial pH 4) and 5 (lactate, initial pH 3), because they grouped in the same branch. Consortium 4 and 6 (lactate, initial pH 3) clustered together in another branch having a different microbial structure from the rest of the consortia. Consortium 7 (glycerol, initial pH 3) showed a more similar structure to the cluster formed by consortia 2 and 5. The most dominant members of the communities at the genus level (21 OTUs) were mainly fermentative bacteria and SRM. The PCA showed no clear relationship between the initial pH value (3 or 4) and the substrates (glycerol or lactate) with the composition of the microbial community in each of the seven consortia (Figure S5).

Using glycerol or lactate as electron donors, we retrieved sequences similar to *Desulfovibrio* (delta-Proteobacteria) representing 2-11% of the sequences in consortia 2, 4, 5, 6, and 7. Sequences similar (92-96%) to the genus *Desulfotomaculum* (Firmicutes) were obtained from consortia 1 and 3 representing 2% of the sequences; while *Desulfatirhabdium* (delta-Proteobacteria) was present in six consortia (91-93% similarity), with relative abundances between 2 and 13%. Sequences 94-96% similar

to *Desulfurella* (delta-Proteobacteria) were found in all the consortia except in Consortium 1, the relative abundance of sequences was between 2-22%.

DISCUSSION

Here, we report the enrichment and cultivation of seven sulfate-reducing microbial consortia able to consume acetate coupled to sulfate reduction at acidic pH. The microbial communities thriving in these enrichments carried out sulfate reduction, for over a year, in successive transfers using lactate or glycerol as the substrates. We pursued sulfate-reducing consortia free of sediment to avoid the "endogenous noise" that the sediment may cause in their characterization and further studies with them.

The percentage of substrate used to perform sulfate reduction confirmed the main function of the consortia (Table 2). Although the consortia came from the same source of inoculum (sediment) and despite using the same substrate in six of them (lactate, consortia 1-6), each consortium showed different consumption rates, denoting the presence of distinct active members in each community, in agreement with their composition (Fig. 4), diversity indexes (Table S2), and PCA (Fig. S5). This result may be due to the unpredictable processes shaping the communities, such as random dispersal and stochastic drift, as these forces have been identified to cause some systems to exhibit divergent communities when culturing microorganisms from a heterogeneous source, such as sediments or soils.[25, 26]

During the course of each transfer, all the consortia presented the same tendency to increase the pH gradually, from the corresponding initial pH 3 or 4 to values close to neutrality (Figure S3). This fact is related to the conversion of a strong acid such as sulfuric acid to a weak acid like hydrogen sulfide and the CO_2 produced from microbial metabolism that in turn contribute to the alkalinity of the system and increment of pH.[27, 28] Therefore, if sulfate reduction occurs, the drift of

the pH is unavoidable in batch assays and the initial conditions (pH and substrate) have a strong influence on the functional traits (consumption/production rates) of the communities developed under such conditions.[18]

We also observed that acetate accumulated and then consumed when the pH reached a value close to 5.0; at this pH, only 35% of acetic acid will remain undissociated, contributing to decreasing the potential toxicity of this organic acid (Figs. 1, 2, and Fig. S3). Possibly, when reaching pH 5, acetotrophic SRM could have coupled the oxidation of acetate with sulfate reduction (Fig. 2). In this study, the consortia were cultivated at initial pH 4 or 3 (Table 1), which in principle constrained the cultures fed with lactate; it is well known that organic acids (lactic and acetic, among others) are inhibitory at low pH because the undissociated form predominates and can cross the cell membrane lowering the intracellular pH.[29] The amount of the undissociated species depends on the dissociation constants; the pKa of lactic acid is 3.08 and for acetic acid is 4.76.[30] Therefore, in the experiments initiated at pH 4 or 3, the undissociated species of lactic acid amounted to 42% or 87%, respectively. In the case of undissociated acetic acid, the percentages were higher (84% at pH 4 and 98% at pH 3). Most probably, these high percentages of acetic acid prevented the cultures to succeed when we used acetate as the sole substrate. Sánchez-Andrea et al.[10] reported the inhibition of the acidophilic sulfate reducer *Desulfosporosinus acididurans* strain D with 5 mM lactic acid at pH 5, while nonionic substrates (glycerol, H_2, and methanol) allowed sulfate-reduction at pH values of 4.0. Given that glycerol is not toxic at acidic pH, because it does not ionize, this substrate has been used successfully to obtain sulfate-reducing consortia from natural environments; [10, 31, 32] nevertheless, the cultures obtained do not consume acetate.

We identified a range of pH at which each consortium performed sulfate reduction (Table 2 and Fig. 3). All the consortia showed the highest rates of sulfide

production in a range of pH predominantly acidic (i.e. between 3 and 6), indicating that the enrichment technique was appropriate to obtain cultures with reproducible activity in a wide range of pH values. According to the previous classification of acidophilic microorganisms,[32] all of the consortia obtained in the present work could be considered as moderately acidophilic because the communities exhibited sulfate-reducing activity at pH lower than 4. Overall, the performance of the seven consortia was very reproducible at acidic pH, which shows the robustness of the microbial communities; the consortia also consumed acetate, making them an asset for further application in the treatment of acidic effluents that contain metals. As expected, the structure of the consortia was not only composed of SRM and also included fermenters and chemoheterotrophs, in agreement with previous reports when enriching SRM from marine sediments or wastewater treatment reactors.[33, 34]

The majority of the consortia contained approximately 2-9% of the sequences related to thus far non-cultivable microorganisms. The sequences related to known species were between 80 to 99% similar to their closest relative, denoting the relevance and potential novelty of some of the microorganisms in the consortia. Most of the fermenters had the lowest percentage of similarity 80%, highlighting their novelty.

In all the consortia, at least one SRM was present in the community, and their global relative abundance was low (< 17%), concurrently with previous observations in sulfate-reducing communities enriched from peatlands where SRM were present in low abundances.[35] Regarding the SRM found in the consortia, members of *Desulfovibrio* can incompletely oxidize a wide variety of substrates including lactate, ethanol, and a few of them use glycerol.[24] They also can use hydrogen as electron donor, which was possibly produced by the fermenters present in the consortia. Microorganisms resembling *Desulfovibrio* could be responsible for the initial

consumption of lactate or glycerol in the consortia and left the residual acetate for other microorganisms able to consume it, such as *Desulfotomaculum* or *Desulfatirhabdium*. Some members of the genus *Desulfotomaculum* (Firmicutes) can degrade a great variety of simple organic compounds, including acetate, formate, ethanol, lactate, and glycerol.[36] The genus *Desulfotomaculum* includes spore-forming microorganisms that enable them to survive and grow in habitats that exhibit desiccation periods and low pH.[37] This characteristic may explain their presence in the consortia since the primary inoculum (sediment) was retrieved from a semi-arid zone. Microorganisms resembling *Desulfatirhabdium* could be the main contributors to the sulfate-reducing activity in most of the consortia because they are classified as complete oxidizers that can use a wide variety of long- and short-chain fatty acids, including acetate.[38] The draft genome of *Desulfatirhabdium,* reconstructed from a metagenome, includes heavy metal and acid resistance traits that could be important for AMD remediation.[39]

Fermentative bacteria are ubiquitous in sulfate-reducing communities, and bacteria of the genera *Lentimicrobium*, *Clostridium*, *Sphaerochaeta*, *Sedimentibacter*, *Ruminiclostridium, Sporotomaculum* and *Macellibacteroides*, may compose anaerobic microbial communities. All of them gain energy from the fermentation of complex organic matter and most probably played a key role in providing hydrogen and acetate to sulfate reducers.[40–42] For instance, *Clostridium* and *Desulfovibrio* coexisted in mixed sulfidogenic cultures and cooperated in the resistance of heavy metals like Cu, Zn, and Fe.[43]

Overall, the performance of the seven consortia showed that the successive transfer approach was appropriate to develop stable cultures of sulfate reducers from environmental samples (*i.e.* sediments) with lactate or glycerol as substrates at low pH (3 or 4). Despite that obtaining the consortia was time-consuming (245 days), after

five successive transfers, the cultures were devoid of the original sediment and allowed to corroborate the cultivability of the consortia and confirm that the sulfate-reducing activity remained. Our results showed that although the enrichments were cultivated at the same initial conditions, each one of the consortia turned out to be unique, as confirmed by the molecular analysis. These consortia, retrieved from the same source, represent an opportunity to use them as model communities that could help to understand the complexity of the natural community. Also, the value of the consortia is in their potential biotechnological application, given the reproducibility of the sulfate-reducing activity at acidic pH.

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Conflict of interest

The authors declare that they not have any conflict of interest.

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LEGENDS TO FIGURES

Figure 1. Kinetic profiles of sulfide (●) and acetate (■) of the seven consortia, Consortium 1 (C1) to Consortium 7 (C7), from successive transfer one (T1) to successive transfer five (T5). C1 - C3 substrate lactate, initial pH 4; C4 - C6 substrate lactate, initial pH 3; C7 substrate glycerol, initial pH 3.

Figure 2. Profiles of sulfide (●); sulfate (◆); lactate (▲) and acetate (■); optical density at 600 nm (O); and pH (▼) in the triplicate assays of the seven consortia (C1 - C7) after successive transfer 5.

Figure 3. Sulfide production rates obtained at different initial pH values for each cultivable consortium.

Figure 4. Dendrogram based on relative abundances of the 21 OTUs, at the genus level, obtained from the seven consortia (C1-C7).

Table 1. Initial pH, electron donor, and type of inoculum used to obtain the consortia.

Consortium	Initial pH	Electron donor	Inoculum
1	4	Lactate	20% of slurry
2	4		
3	4		
4	3		
5	3		
6	3		
7	3	Glycerol	10% of supernatant[a]

[a] just in transfer 1; transfers 2 to 5 where inoculated with 20% of supernatant

Table 2. Rates of sulfide production and acetate consumption, percentage of substrate used to perform sulfate-reducing activity, and interval of favorable pH of the seven cultivable consortia.

Consortium	Sulfide production rate (mmol/L day)	Acetate consumption rate (mmol/L day)	Percentage of substrate used to perform sulfate-reducing activity	Interval of favorable pH
1	0.22 ± 0.017	0.44 ± 0.076	53.9 ± 2.17	4.0-6.0
2	0.25 ± 0.008	0.20 ± 0.070	77.8 ± 8.75	5.0-6.0
3	0.25 ± 0.018	0.28 ± 0.053	60.6 ± 4.93	4.0-6.0
4	0.26 ± 0.019	0.39 ± 0.073	59.1 ± 4.54	3.0-6.0
5	0.26 ± 0.002	0.39 ± 0.123	58.3 ± 0.625	2.5-6.0
6	0.25 ± 0.011	0.34 ± 0.059	54.1 ± 2.09	3.0-5.5
7	0.28 ± 0.007	0.25 ± 0.018	[a]75.1 ± 3.74	3.0-5.5

[a]Theoretical value