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POTENTIAL OF AUTOCHTHONOUS SULFATE-REDUCING MICROBIAL COMMUNITIES FOR TREATING ACID MINE DRAINAGE IN A BENCH-SCALE SULFIDOGENIC REACTOR

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Abstract - Biological acid mine drainage treatment depends significantly on inoculum origin, pH, COD/sulfate ratio, and carbon source. In this study, the performance and microbial diversity of anaerobic batch reactors used for sulfate reduction was evaluated. A medium COD/sulfate ratio of 1.14 ± 0.10 was used, and the evaluation was performed in two steps: Phase 1, based on the inoculum source (autochthonous, AUT, and non-autochthonous, N-AUT); and Phase 2, based on the carbon source (lactate, ethanol, and formate) and low pH. In Phase 1, the sulfate removal using both AUT and N-AUT biomasses were similar, 53% and 59%, respectively. In Phase 2, ethanol and lactate as electron donors yielded similar sulfate removal efficiencies of 42% and 44%, respectively, at neutral pH. When the initial pH was reduced from 4 to 3, sulfate removal using formate remained nearly constant at 34%, whereas it reduced from 43% to 30% with lactate, and dropped significantly from 18% to 7% with ethanol. Denaturing gradient gel electrophoresis analyses for sulfate-reducing bacteria revealed their presence in all samples. Microbial activity and sulfate removal obtained for AUT cultures indicated that they possess the potential for use in local acid mine drainage decontamination processes.
Keywords: Autochthonous microorganisms; Sulfate removal; Anaerobic batch reactor; Sulfate-reducing bacteria.

INTRODUCTION

The mining and metallurgical exploitation of sulfide minerals can lead to the formation of acid mine drainage (AMD) (Kaksonen et al., 2006). Acid mine drainage is produced by the oxidation of metal sulfides. This is possible when metal sulfides are exposed to air, water and catalyzed by autochthonous microorganisms existing in AMD (Sicupira et al., 2015). Besides its low organic carbon content, the resulting AMD has low pH, high sulfate and metal concentrations, causing serious environmental problems (Sicupira et al., 2015).

Traditional chemical processes for AMD treatment have several disadvantages, such as the high cost of chemical reagents, inefficient sulfate removal, and large amounts of sludge generation (Kaksonen et al., 2006). Thus, biological anaerobic treatment is an important substitute for conventional AMD treatment due to its high efficiency, cost-effectiveness, and environmental safety.

The bioremediation of AMD using sulfate-reducing bacteria (SRB) is a viable alternative. SRB can use numerous types of chemicals as electron donors such as lactate (Equation 1, 2, 3), ethanol (Equation 4), and formate (Equation 5). In addition, SRB reduce sulfate

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(SO_4^{2-}) using electron donors, producing hydrogen sulfide (H_2S) and bicarbonate (HCO_3^-), as shown in Equations 1, 4, and 5 for a COD/SO_4^{2-} ratio of 0.67, and Equations 2 and 3 for COD/ SO_4^{2-} ratios greater than 0.67 (Dar et al., 2008). The produced bicarbonate can increase the pH to neutral or alkaline values (Sahinkaya et al., 2011). Moreover, the produced sulfide enables metal removal by forming stable metal sulfide precipitates (Bekmezcia et al., 2011).

$$2C_3H_5O_3^- + 3SO_4^{2-} + 2H^+ \rightarrow 3H_2S + 6HCO_3^- \quad (\Delta G = -128.5\ kJ/mol) \quad (1)$$

$$4C_3H_5O_3^- + 3SO_4^{2-} \rightarrow 4C_2H_3O_2^{-1} + 4HCO_3^- + 3HS^{-1} + H^+ \quad (\Delta G = -37.7\ kJ/mol) \quad (2)$$

$$2C_3H_5O_3^- + SO_4^{2-} \rightarrow 2C_2H_3O_2^{-1} + 2HCO_3^- + HS^{-1} + H^+ \quad (\Delta G = -80.8\ kJ/mol) \quad (3)$$

$$CH_3CH_2OH + 1.5SO_4^{2-} + H^+ \rightarrow 2HCO_3^- + 1.5H_2S + H_2O \quad (\Delta G = -80.2\ kJ/mol) \quad (4)$$

$$4HCOO^- + SO_4^{2-} + 2H^+ \rightarrow 4HCO_3^- + H_2S \quad (\Delta G = -58.4\ kJ/mol) \quad (5)$$

Despite the extreme conditions of acid mine drainage, SRB communities have already been found in acid mine drainage sediment samples, as reported by Muyzer and Stams (2008). However, stable enrichment using cultures provided from acidic environments is scarce, and only a few acidophilic or acid-tolerant SRB have been identified (Sánchez-Andrea et al., 2013). Thus, the study of SRB based on their enrichment is important in enabling the application of autochthonous and extremophile SRB as biomass in sulfidogenic reactors. As the carbon source is a limiting nutrient in AMD, the choice of a suitable low-cost carbon source that allows high sulfate reduction efficiency is important (Rodriguez and Zaiat, 2011). Although lactate is the preferred carbon source of SRB for sulfate reduction, it is not cost-effective. It is, therefore, necessary to use different electron donors during the enrichment process, such as ethanol, which is a cheaper substrate (Bertolino et al., 2014).

The capacity of SRB to survive in diverse environments makes possible the use of autochthonous SRB for the bioremediation of sulfate-rich wastewater, such as acid mine drainage, as they can adapt to different environmental conditions. This also suggests the importance of studying and identifying extremophile SRB obtained from AMD sediments, as well as exploring their metabolic activities and their complexity (Sánchez-Andrea et al., 2012).

Non-autochthonous communities have been considered adequate for the treatment of acidic water (Altun et al., 2014; Singh et al., 2011; Zhang and Wang, 2016). Promising results have been reported for the use of autochthonous microorganisms as inocula for acid mine drainage treatment in anaerobic reactors (Sánchez-Andrea et al., 2013; Martins et al., 2009). However, few studies have compared the potential of autochthonous communities with that of non-autochthonous cultures for bioremediation processes (Luptakova and Kusnierova, 2005). Table 1 below contains some examples of sulfate removal efficiencies for autochthonous and non-autochthonous inocula, consisting of anaerobic sludge:

The uranium mine site located in Poços de Caldas, Minas Gerais, Brasil is composed of low-pH waters (mean pH 3), high concentrations of sulfate (15.6 mmol), ferric iron, and other heavy metals (Rodriguez et al., 2012). The physical-chemical treatment used to treat this wastewater is costly and generates a lot of sludge contaminated with uranium, thus failing to solve the environmental problem in a satisfactory way. As such, exploring the metabolic activities of

Table 1. Examples of sulfate removal efficiencies for non-autochthonous and autochthonous biomass.

	Non-autochthonous Biomass				Autochthonous Biomass	
Author	Altun et al. (2014)	Zhao et al. (2011)	Martins et al. (2011)	Singh et al. (2011)	Sánchez-Andrea et al. (2013)	Martins et al. (2009)
Sulfate removal efficiency (%)	80%	56%-80%	72%	82%	45%	33%-65%
Electron donor	Ethanol	Tannery wastewater	Ethanol	Lactate	Lactate	Ethanol or Lactate
pH	3.5-5	6-8	2.8	8.03	4.5	~1
COD/sulfate ratio	0.78	1-5	~0.6-1.2	Not indicated	Not indicated	0.8
Operational mode	Continuous fed fixed-bed bioreactor	Batch models	Continuous up-flow anaerobic packed bed systems	Static batch anaerobic sorption systems	Batch models	Batch models

the microbial communities present at this mine site (Sánchez-Andrea et al. 2012), especially SRB, is a very important method to analyze the biotechnological potential of the microorganisms living in that adverse environment.

The purpose of this study was to compare the use of autochthonous cultures of the sediment of an uranium mine located in Poços de Caldas, Minas Gerais, Brazil and non-autochthonous biomass in bioremediation of AMD in anaerobic batch reactors using Postgate C as the enrichment medium. The performance based on sulfate removal and the microbial community of the autochthonous culture was analyzed, comparing it with non-autochthonous culture, as a way to identify similar efficiencies and specific extremophile microorganisms. Afterwards, the sulfate removal efficiencies of the enriched cultures on a modified Postgate C medium were studied to better understand the preferential and more readily degraded carbon sources for growth in neutral pH (here considering lactate, ethanol, and formate). A complementary study of autochthonous SRB enrichment in acidic pH was also conducted, evaluating the capacity for sulfate removal, and sulfide and carbonate production, including a denaturing gradient gel electrophoresis (DGGE) profile to show their presence, even in acidic pH, and to study the bacterial community. The effect of the COD/sulfate ratio on sulfate removal efficiencies was also studied. Thus, the enrichment conditions based on carbon source, pH, and COD/sulfate ratio were modified to identify the potential of autochthonous SRB for application to the decontamination of their original uranium mine site.

MATERIALS AND METHODS

Phase 1: SRB enrichment in the Postgate C medium

Inoculum

Two different biomass samples were used as inoculum: one from a stable sulfidogenic batch reactor using lactate as a carbon source (original inoculum from a slaughterhouse wastewater treatment plant; hereafter: N-AUT culture) and the other from an enrichment culture of the sediment of a uranium mine located in Poços de Caldas, Minas Gerais, Brazil, containing acid mine drainage (hereafter: AUT culture). Duplicates of the N-AUT culture were prepared by suspending 10 mL of the inoculum from a sulfidogenic reactor operating for 88 days with lactate at a COD/SO_4^{2-} ratio of 1.0 and treating synthetic acidic wastewater with a sulfate removal efficiency of ~73% in 25-mL distilled water. Duplicates of the AUT culture consisted of uranium acid mine drainage samples enriched in a Postgate C medium. In total, 184 ± 20 mg/L of volatile solids from the N-AUT culture or 180 ± 20 mg/L of volatile solids from the AUT culture were added as inoculum to one of two 500-mL anaerobic batch reactors containing 250 mL of a Postgate C medium (Reactor N-AUT and Reactor AUT, respectively).

Enrichment Medium

SRB were enriched using a Postgate C medium composed of 3.0 g/L sodium lactate (carbon source and electron donor), 0.3 g/L sodium citrate, 0.1 g/L yeast extract, 0.5 g/L KH_2PO_4, 1.0 g/L NH_4Cl, 4.5 g/L Na_2SO_4, 0.04 g/L $CaCl_2 \cdot 2H_2O$, 0.06 g/L $MgSO_4 \cdot 7H_2O$, 0.004 g/L $FeSO_4 \cdot 7H_2O$, 0.001 g/L Resazurin, and 0.5 g/L Na_2S. The COD/sulfate ratio used was 1.20 ± 0.16 and 1.25 ± 0.18, for Reactors AUT and Reactor N-AUT, respectively.

Experimental Protocol

Both reactors were sterilized, maintained under a constant flow of nitrogen prior to inoculation, and subsequently, kept aseptic during the experiment. The systems were incubated at 30 ºC with agitation at 100 rpm for 41 consecutive days without medium replacement. A control reactor (Control), which was sterilized and maintained under a constant flow of nitrogen prior to inoculation, was kept aseptic after inoculation, containing no inoculum and 250 mL of a Postgate C medium, and was operated under the same conditions as used in experimental Phase I. The nitrogen flow was ceased in all reactors after inoculation.

Phase 2: SRB enrichment in the modified Postgate C medium

Inoculum

As inoculum, 180 ± 20 mg/L of total volatile solids from the AUT culture were added to 500-mL anaerobic batch reactors containing 250 mL of a modified Postgate C medium. AUT culture consisted of uranium acid mine drainage samples enriched in a Postgate C medium.

Enrichment Medium

The AUT culture was enriched using a Postgate C medium, which was modified by altering the carbon source from lactate (Reactors LAC) to ethanol (Reactors ETA) or formate (Reactors FOR; maintaining 26.8-mM concentration) and by reducing the enrichment medium initial pH, previously to inoculation (Table 2).

Experimental Protocol

The reactors were duplicated, except for reactor FOR 3 (initial pH 3) for which they were triplicated. The reactors were sterilized and maintained under a constant flow of nitrogen before use, and were kept aseptic during the experiment. The systems were incubated at 30 ºC, with agitation at 100 rpm

Table 2. Carbon sources and initial pH used on studied reactors.

Carbon Source	Initial pH	COD/sulfate ratio	Reactor Denomination
Lactate	-	-	Control LAC
	7.34 ± 0.10	1.08 ± 0.09	LAC 7
	3.96 ± 0.10	1.15 ± 0.10	LAC 4
	3.03 ± 0.10	1.27 ± 0.12	LAC 3
Ethanol	-		Control ETA
	7.41 ± 0.10	1.27 ± 0.10	ETA 7
	3.81 ± 0.10	0.95 ± 0.09	ETA 4
	2.70 ± 0.10	1.06 ± 0.09	ETA 3
Formate	-	-	Control FOR
	7.05 ± 0.10	1.20 ± 0.11	FOR 7
	4.02 ± 0.10	1.09 ± 0.10	FOR 4
	2.89 ± 0.10	1.02 ± 0.08	FOR 3

and without medium replacement. The experiments were conducted for approximately 35 days at neutral pH and for 24 days at an acidic pH. The enrichment period was determined based on the reduction of the cultivation medium, indicated by the redox potential. The enrichments were considered finished when the culture medium terminated sulfide production and the medium was no longer transparent, indicating that the redox potential had become greater than -200 mV, a suitable condition for SRB growth (Jing and Kjellerup, 2017). Thus, at the end of the experiments, when the medium turned pink, caused by the indicator Resazurin, the presence of a semi-reduced medium could be inferred, no longer capable of stabilizing the sulfate removal process. The duplicate reactors fed with lactate (Control LAC), ethanol (Control ETA), or formate (Control FOR), containing no inoculum and 250 mL of a modified Postgate C medium, were operated under the same conditions.

A summary of Phase 1 and Phase 2 experimental procedures are found in the flowchart below:

Physical and chemical analysis

The samples were centrifuged using an MCD-2000 HEMATOCRIT micro-type centrifuge at 8000 rpm for 10 min prior to COD and sulfate measurements. The COD (5220 D), pH (4500-H^+ B), total volatile solids (2540 Solids), sulfate (4500-SO_4^{2-} E), and dissolved sulfides (4500-S^{2-} D) were monitored based on American Public Health Association standards (APHA, 2012). The COD, sulfate, and sulfide concentrations were determined every 2-3 days, whereas the pH was measured only at the beginning and end of enrichment. SRB were enumerated using the most probable number (MPN) method, as described in APHA (2012).

Errors in analytical precision were estimated using standard errors and R software version 3.4.4 (R Core Team, 2018). Uncertainties were calculated using the protocols of Miller and Miller (2010) and Thompson et al. (2002). The t-student distribution for a 95% confidence interval was used for each analysis.

Microbial community analysis

For Phase 1, samples from both the AUT and N-AUT reactors were taken at different times during the one-month enrichment process to study the domain bacteria and SRB diversity via polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) analysis. In total, five samples were collected from each reactor. The biomass samples

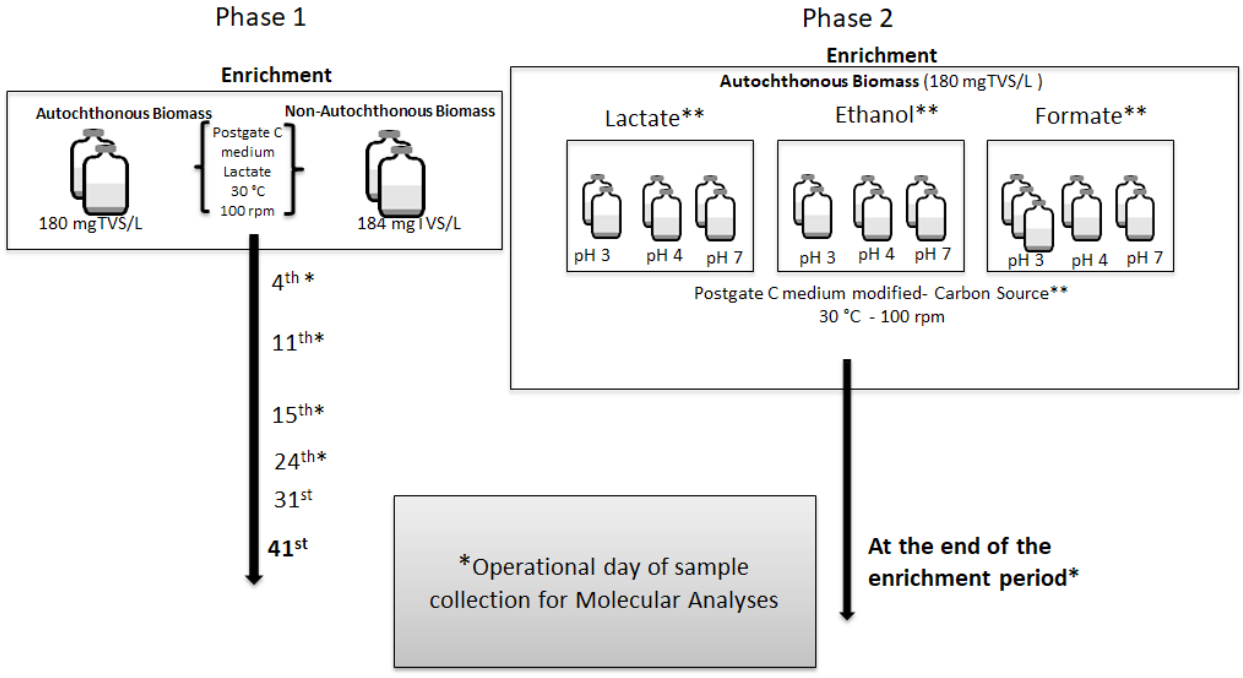

Figure 1. Summary of experimental phases (Phase 1 and Phase 2).

from the AUT reactor consisted of homogeneous cell suspensions in a Postgate C medium. The samples of the N-AUT reactor consisted of enriched cultures of N-AUT sludge in a Postgate C medium. The AUT 1 and N-AUT 1 samples were collected on operational day 4 from reactors AUT and N-AUT, respectively; the AUT 2 and N-AUT 2 samples were collected on operational day 11; the AUT 3 and N-AUT 3 samples were obtained on operational day 15; the AUT 4 and N-AUT 4 samples were taken on operational day 24; and the AUT 5 and N-AUT 5 samples were collected on operational day 31.

For Phase 2, biomass samples (cell suspensions) from reactors LAC 4, LAC 3, ETA 7, ETA 4, ETA 3, FOR 7, FOR 4, and FOR 3 were taken at the end of the enrichment period and named based on the reactor denomination. Molecular analyses were not performed for the LAC 7 reactor because it had been cultivated under the same conditions as the AUT reactor samples that were already analyzed in Phase 1. The collected samples were maintained at -20 ºC prior to deoxyribonucleic acid (DNA) extraction.

All molecular analyses were performed under aseptic conditions using aseptic techniques.

DNA extraction

The total DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega®), according to the manufacturer's instructions. The DNA extraction was confirmed by electrophoresis (DIGEL®) on 1.5% agarose gels at 120 V for 30 min (Geets et al., 2006; Lee et al., 2012).

PCR and DGGE analyses

All DNA-extracted samples were analyzed using PCR and DGGE. For PCR analysis, a 50-µL mixture consisting of 10-mM Tris-HCl buffer, 1.5-mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of Taq DNA polymerase (Promega), 0.2 µmol of each primer, and 50-100 ng of DNA was used. The dissimilatory sulfite reductase subunit B gene (dsrB) was amplified via PCR using the forward and reverse primers DSRp2060F (5'-CAACATCGTYCAYACCCAGGG-3') and DSR4R (5'-GTGTAGCAGTTACCGCA-3'), respectively (Lopez et al., 2014). The bacterial 16S rRNA gene was amplified using the forward and reverse primers 968F (5'-AAC GCG AAG AAC CTT AC) and 1401R (5' CGG TGT GTA CAA GGC CCG GGA ACG), respectively (Heuer et al., 1997). The 5' ends of the forward primers were GC-clamped (5' CGC CCG CGC GCGGCG GGC GGG GCG GGG GCA CGG GGG G) as suggested by Geets et al. (2006). Amplification was performed using a MaxyGene Gradient Thermal Cycler (Axygen) according to the following protocol: initial denaturation step at 94 ºC for 4 min; 35 cycles of denaturation at 94 ºC for 1 min, primer annealing at 55 ºC for 1 min and extension at 72 ºC for 1 min; and final extension step at 72 ºC for 10 min (Geets et al., 2006). Electrophoresis using a DIGEL® system with 1.5% agarose gels (Geets et al., 2006) at 120 V for 30 min was used to confirm the PCR amplification (Lee et al., 2012).

For DGGE analyses, a Loccus Biotecnologia system was used at 85 V and 60 °C for 17 h in a 7.5% polyacrylamide gel with a 40%-60% urea-formamide denaturing gradient (Ceccherini et al., 2009; Shi et al., 2012). BioNumerics® software (Applied Maths) employing the Dice coefficient and unweighted pair-group method with arithmetic mean (UPGMA) was used to analyze the DGGE profiles (Geets et al., 2006).

DsrB gene sequencing for SRB analysis

The bands from the dsrB DGGE were excised and reamplified using the same primers used for the initial DGGE, excluding the GC clamp, as suggested by Geets et al. (2006). A Wizard® SV Gel and PCR Clean-Up System (Promega) was employed to purify the reamplified products. An ABI 3730 DNA analyzer (Perkin-Elmer) was used to sequence the reamplified bands. The sequenced bands were registered in the GenBank database with the following accession numbers: KX351203, KX351204, KX351205, KX351206 and KX351207. Sequence alignment within the GenBank database was performed using a BLASTN program (Geets et al., 2006; Meyer and Kuever, 2007). An unrooted phylogenetic tree comparing the sequenced bands with some of their closest relatives retrieved from the GenBank database was constructed using the neighbor-joining method and MEGA 5.0 software.

RESULTS AND DISCUSSION

Phase 1: Performance of the AUT and N-AUT reactors

The extent of sulfate reduction by the AUT and N-AUT reactors was similar (summarized in Table 3 and Figure 2). A maximum sulfate removal of 53% and 59% was obtained in the AUT and N-AUT reactors, respectively, for a medium COD/sulfate ratio of 1.22 ± 0.24. Oyekola et al. (2010) also observed

Table 3. Physical-chemical parameters for Phase 1 cultivation.

Parameter*	Reactor AUT	Reactor N-AUT
Initial pH	6.55 ± 0.10	6.35 ± 0.10
Final pH	7.86 ± 0.10	7.91 ± 0.10
COD/SO_4^{2-}	1.20 ± 0.16	1.25 ± 0.18
Maximum COD removal (%)	27.3 ± 3.4	88.2 ± 3.7
Maximum SO_4^{2-} removal (%)	53 ± 15	59 ± 17
S^{2-} production (mmol)	19.0 ± 0.8	20.8 ± 0.8

*The parameters were calculated using the arithmetic average of the duplicate reactors.

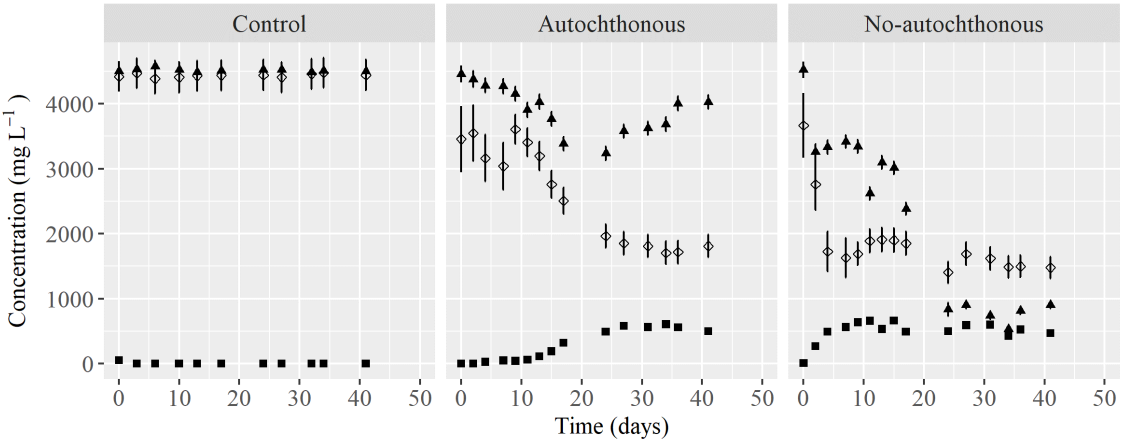

Figure 2. Performance of the control, autochthonous, and non-autochthonous reactors during the experiments (COD (▲), SO_4^{2-} (◇) and sulfide (■) concentrations). The error bars correspond to 95% confidence interval.

similar sulfate removal efficiencies, varying from 42% to 58% for a COD/SO4^{2-} ratio of 1.2. The poor sulfate removal observed in AUT and N-AUT reactors, lower than 70%, was probably due the limiting COD/sulfate ratio used in the sulfidogenic process. Dar et al. (2008) observed that when a COD/sulfate ratio of 2.0 was used, nearly all sulfate was converted to sulfide, which did not occur for a COD/sulfate ratio of 0.34, where almost all the sulfate remained in the effluent. The interesting point of view of this study is that SRB were the dominant group when using the lower stoichiometry COD/sulfate ratio (relative abundance ~ 75%), while the other condition presented a diverse microbial group in which SRB were not the most dominant (relative abundance ~ 25%). Lu et al. (2016) also reported that the decrease in the COD/SO_4^{2-} ratio from 5 to 1 caused the reduction in sulfate removal from 82% to 21%.

The COD removal of the AUT and N-AUT reactors was very different, 27% and 88%, respectively. Based on the stoichiometric relation of 0.67, a sulfate removal of 19.2 ± 5.6 mmol and lactate consumption of 12.8 ± 1.1 mmol are expected for the AUT reactor if lactate was completely oxidized, as shown in Equation 1. The lactate removal observed in the present experiment was 12.7 ± 0.6 mmol, which is similar to the expected value. The biomass of the AUT reactor was adapted and isolated from the acid mine drainage sediment of an uranium mine; the SRB richness of this inoculum was lower (Figure 3) than that of the N-AUT reactor because the AUT reactor was probably dominated by a sulfidogenic-specialized genus capable of complete oxidation.

The lactate consumption in the N-AUT reactor expected for a sulfate removal of 22.1 ± 5.9 mmol based on the stoichiometry for complete oxidation is 14.7 ± 1.3 mmol. A lactate removal of 41.6 ± 0.5 mmol was obtained, corresponding to the stoichiometry relation of Equation 3 (1 mol of SO_4^{2-} can consume 2 mol of lactate), an incomplete route. As the N-AUT inoculum includes a consortium of microorganisms, the incomplete consumption of lactate may have promoted the establishment of different microorganism species and syntrophic relationships of SRB with other microorganisms such as methanogenic archaea and acetogenic bacteria (Muyzer and Stams, 2008). In addition, it seemed that in reactor N-AUT a two-step reaction occurred. First, lactate was incompletely oxidized to acetate with sulfate and an excess of lactate, according to Equation 3. Then, the produced acetate could be assimilated by methanogens, as shown in Equation 6 below:

$$C_2H_3O_2^{-1} + H_2O \rightarrow CH_4 + HCO_3^- \quad (6)$$

AUT and N-AUT reactors showed similar sulfide production values. However, the AUT reactor presented two stages of sulfide production (Figure 2). The first stage occurred during the first 10 days in which no sulfide accumulation was observed. The second stage was 14 days long (days 11-25), corresponding to high sulfide production. Vainshtein et al. (2003) also noted two stages of sulfate reduction in a bacterial consortium isolated from a soil sample of an aerobic/anaerobic gradient ecosystem: a slow adaptation stage, with no accumulation of hydrogen sulfide, and a rapid sulfate removal stage, with sulfide accumulation and the generation of sulfur intermediates, such as thiosulfate and sulfite.

An adaptation time of ~10 days was necessary for the AUT culture (Figure 2), also observed by Martins et al. (2009) when studying metal-resistant SRB for acid mine drainage treatment in batch reactors under anaerobic conditions. The autochthonous SRB were more affected by the initial COD/SO4^{2-} ratio, as the sulfate loading rate was lower, 0.45 mmol.d^{-1} compared to 0.57 mmol.d^{-1}, for the N-AUT culture. According to Hwang and Jho (2018), a lag time is

necessary for the bacterial community to exhibit its sulfate reducing capacity. In addition, the lower sulfate loading rates found in autochthonous bacterial communities can also be attributed to the composition of their bacterial community. As we can see in Figure 3, non-autochthonous communities exhibited a diverse SRB group compared to the autochthonous reactor. In addition, during the AUT biomass lag phase, the sulfide and sulfate concentrations were mostly stable. This was not the case for the N-AUT reactor, as during the same period high sulfate removal and sulfide production were observed. Thus, because the N-AUT biomass was pre-acclimated in a stable sulfidogenic reactor at lower pH values and, perhaps, exposed to high sulfate content, it conferred a faster capacity to remove sulfate with no need for adaptation to the new culture medium. This was also observed by Rodriguez and Zaiat (2011), who reported no need of a lag phase when an anaerobic sludge was used as inoculum for common substrates of sulfate reduction, such as lactate. However, it is important to emphasize that after this acclimation period required by the AUT culture, both cultures had similar sulfate removal and sulfide production values (Table 3). Thus, it is important to conduct a complementary study of organic substrates more readily degraded by autochthonous bacteria than lactate (like ethanol and formate), as a way to reduce the lag phase found in Phase 1.

Comparative evaluation of the microbial community

Similarity dendrograms and DGGE profiles for the domain Bacteria and the SRB group are shown in Figure 3. The N-AUT and AUT reactors had distinct community structures, with a similarity of only 42% for the domain Bacteria and 12% for the SRB group. The diversities of the domain Bacteria and SRB were mostly stable during the enrichment. Both reactors showed a high microbial diversity for the domain Bacteria during the cultivation period (Figure 3a). For the SRB group, the AUT reactor showed one predominant band compared with the high diversity of the N-AUT reactor (Figure 3b).

It was not possible to amplify the SRB dsrB gene during the first two weeks of enrichment in the AUT reactor (samples AUT 1 and AUT 2; Figure 3b). This period corresponded to the adaptation time and low sulfide production (Figure 2). The low SRB numbers during this cultivation period were probably not sufficient to allow PCR amplification and DGGE analyses of that group. This did not occur during the Bacterial 16S rRNA gene PCR and DGGE analyses (Figure 3a).

The phylogenetic tree (Figure 4) and the phylogenetic affiliations of the sequenced dsrB DGGE bands (Table 4) indicate that Band 1 of the AUT reactor shows more similarity to the *Desulfotomaculum* genus, which belongs to the class Clostridia, whereas the other bands isolated from the N-AUT reactor were more similar to the *Desulfovibrio* genus, which belongs to the class Deltaproteobacteria. Species of the *Desulfovibrio* genus were the fastest growing SRB, using low molecular weight organic acids as substrates such as lactic acid, acetic acid, and ethanol (Cabrera et al., 2005). When lactate is

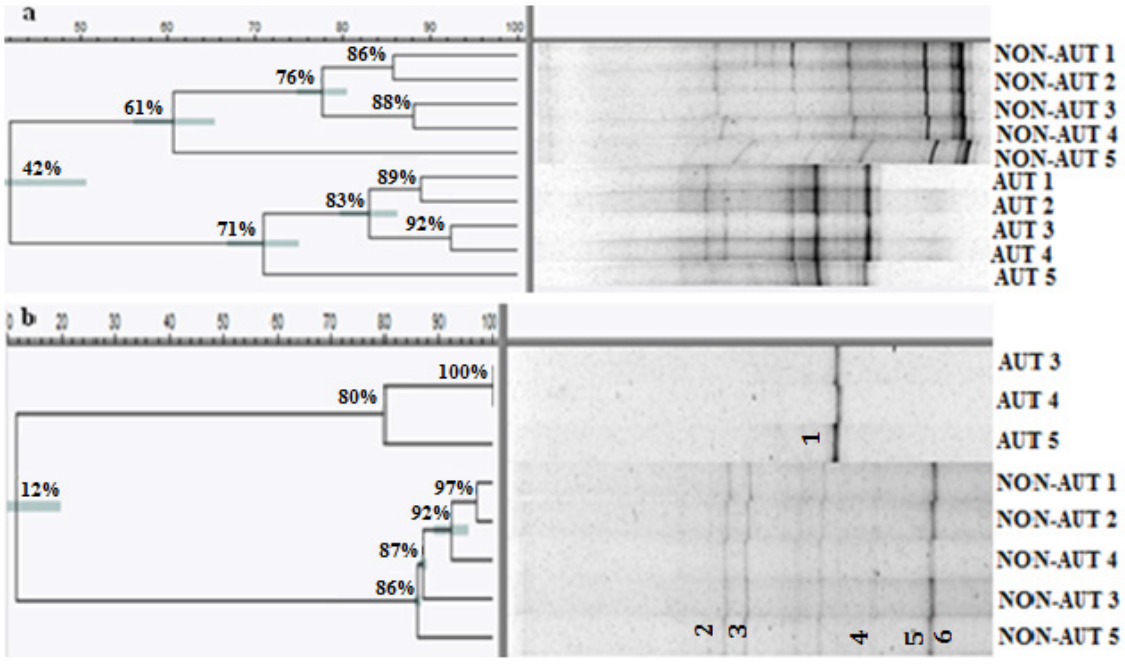

Figure 3. Similarity dendrograms (L) obtained using the BioNumerics program version 7.6 (Applied Maths, Belgium) and the Dice coefficient, UPGMA method and the DGGE gels (R) for the Bacterial 16S rRNA (a) and dsrB genes (b). The numbers in the gel images (b) represent sequenced bands.

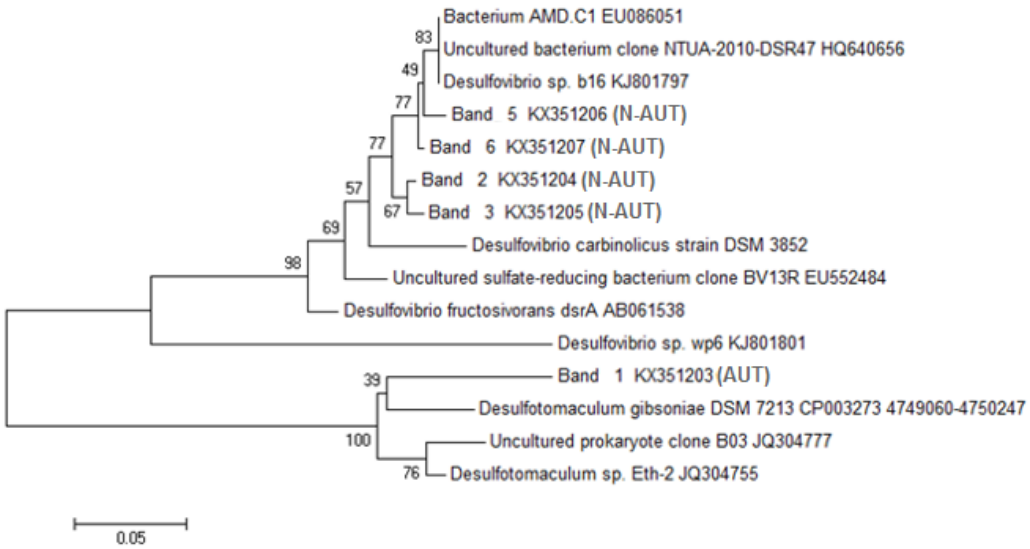

Figure 4. Phylogenetic tree constructed by comparing the sequenced bands from the dsrB gene DGGE profile with some of the closest relatives retrieved from the GenBank database. The tree was created using the neighbor-joining method and MEGA 5.0 software. The bootstrap values are indicated at the nodes. The scale bar represents two nucleotide substitutions per 100 nucleotides.

Table 4. Phylogenetic affiliations of bands sequenced from the dsrB gene DGGE profile.

Band (GenBank Accession Number[a])	Closest Sequence (GenBank Accession Number)	Similarity (Query Cover)[b]	Closest cultured strain (GenBank Accession Number)	Similarity (Query Cover)[b]	Phylogenetic group
1 (KX351203)	*Desulfotomaculum gibsoniae* DSM 7213 (CP003273)	91% (100%)	*Desulfotomaculum gibsoniae* DSM 7213 (CP003273)	91% (100%)	Clostridia
2 (KX351204)	Bacterium AMD.C1 (EU086051)	97% (100%)	*Desulfovibrio fructosivorans* JJ DSM 3600 (AF418187)	97% (95%)	Deltaproteobacteria
3 (KX351205)	Bacterium AMD.C1 (EU086051)	97% (100%)	*Desulfovibrio magneticus* RS-1 (AP010904)	94% (100%)	Deltaproteobacteria
5 (KX351206)	Bacterium AMD.C1 (EU086051)	98% (100%)	*Desulfovibrio carbinolicus* strain DSM 3852	94% (100%)	Deltaproteobacteria
6 (KX351207)	Bacterium AMD.C1 (EU086051)	99% (100%)	*Desulfovibrio magneticus* RS-1 DNA (AP010904)	94% (99%)	Deltaproteobacteria

[a]Accession number of the sequenced bands in this study in the GenBank database; [b]Defined using a BLASTN search tool.

used as an electron donor for sulfate reduction, these SRB incompletely oxidize it to acetate (Zhang and Wang, 2016). *Desulfotomaculum* genus, otherwise, is known for their capacity to grow on a large variety of substrates, including organic compounds, long-chain fatty acids, and several aromatic compounds. The oxidation of the substrates (to CO_2) is usually complete; however, acetate and other fatty acids can accumulate when high substrate concentrations are observed (Kuever et al., 1999).

Species of the *Desulfotomaculum* genus are also capable of sporulation (Kuever et al. 1999), which explains the presence of only one predominant band in the AUT reactor DGGE gel of the SRB group, which is probably a specialized SRB group. The spore formation capacity enables the survival and adaptation of these microorganisms under unfavorable conditions, such as extreme acidic conditions present in acid mine drainage. This, along with the above-mentioned metabolic adaptability, could also explain the significant differences in the DGGE profiles of the AUT and N-AUT reactors. This also could be the reason why the sulfate removal efficiency and sulfide production at neutral conditions were similar in both reactors, showing that this autochthonous population obtained at extreme conditions can be as effective with respect to the geochemical performance as non-autochthonous cultures. Moreover, despite the low diversity of the SRB group in the AUT reactor, the relatively high diversity found in the domain Bacteria could also have contributed to the overall performance of the reactor, with bacterial groups providing useful intermediary metabolic products for SRB in a cooperative relationship, contributing to COD removal rates. The diversity in the bacterial community and the functional role of the corresponding partners

may explain the effectiveness of acid mine drainage bioremediation (Martins et al. 2011).

The study of an acid mine drainage bioremediation system based on sulfate reduction during high acid treatment by Lu et al. (2011) showed that *Desulfotomaculum* species can tolerate extremely low-pH (1 and 2) conditions and heavy metal presence, such as copper (1.5 mg/L) and zinc (1.5 mg/L), while maintaining efficient sulfate removal. In the present work, an SRB group affiliated with *Desulfotomaculum* in the AUT reactor demonstrated sulfate removal similar to that of the SRB group in the N-AUT reactor (Figure 2), despite the lower diversity. This performance result and the acid tolerance of *Desulfotomaculum* organisms indicate that employing this SRB group can lead to significant environmental advances in local acid mine drainage bioremediation.

Phase 2: Performance and microbial communities of the LAC, ETA, and FOR reactors

Sulfate removal was observed in all reactors of Phase 2 (Table 5). The SRB presence in all reactors under neutral and acidic conditions was confirmed via the DGGE profile of the SRB group (Figure 5a). The efficiencies of sulfate removal for LAC 7, ETA 7, and FOR 7 reactors were 44%, 42%, and 34% respectively, values lower than expected based on other studies (Cao et al., 2012; Vilela et al., 2014; Bertolino et al., 2012). The COD/SO_4^{2-} ratio used in the experiments, lower than 1.27, might have affected the sulfate removal efficiencies and the SRB activity. According to Vilela et al. (2014), when a COD/sulfate ratio of 1 was used for ethanol as electron donor, the efficiency of sulfate removal was reduced from 80% (at a COD/sulfate ratio of 1.9) to 60%. In addition, a sulfate removal efficiency of 74% was found when a COD/sulfate ratio of 3 was used for formate as an electron donor. According to Bertolino et al. (2012), a COD/sulfate ratio of 2.0 is optimum for bacterial growth, resulting in sulfate removal rates of 98%. In addition, Yuan et al. (2015) also conclude that the sulfate removal efficiency is improved with an increase in the COD/sulfate ratio from 0.67 to 2.0. As discussed for Phase 1, a limiting COD/sulfate ratio of 1.08 ± 0.09 used for the LAC reactor might be responsible to the reduction in sulfate removal efficiency.

The pH was not controlled in the beginning of the experiments as, after inoculation, the value can be altered depending on the carbon source used, making this variable difficult to control. Besides that, all studied reactors were operated with neutral pH (7-7.4), in a range of optimal pH for most known SRB (Rampinelli et al., 2008). Thus, this variable might not have affected the sulfate removal efficiencies and SRB activity. The sulfate removal rates were 0.58 $mmol.d^{-1}$ for the LAC reactor, 0.70 $mmol.d^{-1}$ for the ETA reactor, and much higher for the FOR reactor, a value of 1.83 $mmol.d^{-1}$. According to Cao et al. (2012), the choice of electron donors has substantial impact on the rate of sulfate reduction. They also found that formate as electron donor promoted the fastest reduction of sulfate.

According to Table 5, similar sulfate removals were observed in the LAC 7, ETA 7, and FOR 7 reactors, as considering the calculated errors no significant differences were found. Sahinkaya and Yucesoy (2010) also found that substituting lactate with ethanol as a substrate in sulfidogenic reactors did not affect the sulfate removal efficiencies or system performance. Wolicka et al. (2015) obtained lower sulfate removals than those observed in this study (~25% and 30% for lactate and ethanol, respectively, as carbon sources) when SRB communities enriched on a modified Postgate C medium were used as inoculum in the

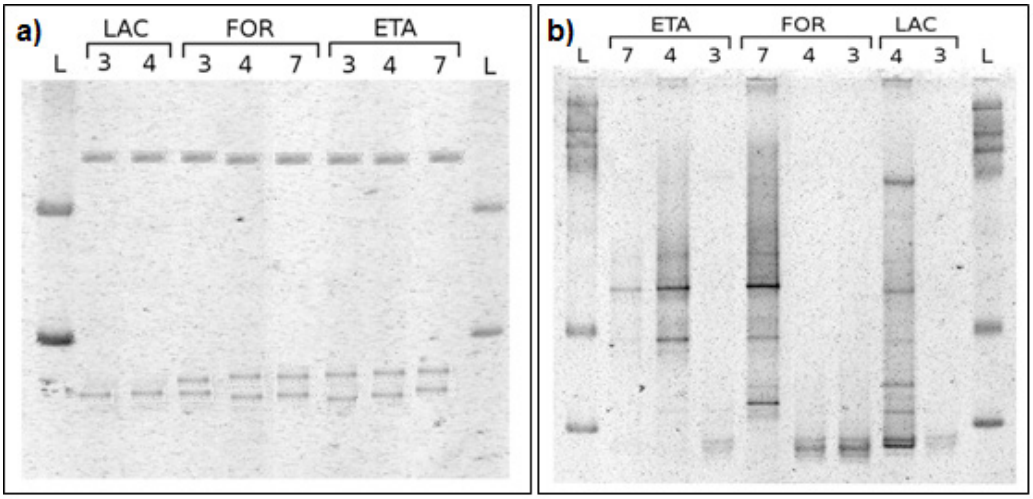

Figure 5. (a) Sulfate-reducing bacteria community profiles of the LAC, FOR, and ETA reactors under indicated initial pH conditions were compared using DGGE analysis. (b) Bacterial community profiles for the ETA, FOR, and LAC reactors under indicated initial pH conditions were compared using DGGE analysis.

Table 5. Physical-chemical parameters for Phase 2 cultivation under neutral and acidic pH conditions.

Parameter[a]	Reactor LAC 7	Reactor LAC 3	Reactor LAC 4	Reactor ETA 7	Reactor ETA 3	Reactor ETA 4	Reactor FOR 7	Reactor FOR 3	Reactor FOR 4
Initial pH[b]	7.41 ± 0.10	3.60 ± 0.10	5.12 ± 0.10	7.41 ± 0.10	5.97 ± 0.10	6.18 ± 0.10	7.05 ± 0.10	2.59 ± 0.10	4.15 ± 0.10
Final pH	8.04 ± 0.10	3.68 ± 0.10	4.74 ± 0.10	7.70 ± 0.10	5.45 ± 0.10	6.75 ± 0.10	7.15 ± 0.10	2.78 ± 0.10	4.24 ± 0.10
COD/SO_4^{2-}	1.08 ± 0.09	1.15 ± 0.10	1.27 ± 0.12	1.27 ± 0.10	0.95 ± 0.09	1.06 ± 0.09	1.20 ± 0.11	1.09 ± 0.10	1.02 ± 0.08
Maximum COD removal (%)	25 ± 3	11 ± 3	22 ± 3	25 ± 2	5.5 ± 3	9.6 ± 3	42 ± 2	22 ± 3	30 ± 3
Maximum organic matter removal (mmol)	11.9 ± 1.4	4.9 ± 1.3	12.6 ± 1.8	14.2 ± 2.6	2.7 ± 1.3	4.9 ± 1.6	156.2 ± 8.3	66.2 ± 9.4	101.1 ± 10.0
Stoichiometric organic matter value (mmol)[c]	13.1 ± 3.1	7.9 ± 2.6	13.9 ± 3.0	15.7 ± 3.7	4.9 ± 3.4	3.6 ± 2.9	73.1 ± 4.1	63.1 ± 16.9	84.4 ± 25.6
Maximum SO_4^{2-} removal (%)	44 ± 11	30 ± 11	43 ± 9	42 ± 10	18 ± 13	7 ± 6	34 ± 12	34 ± 11	39 ± 10
Maximum SO_4^{2-} removal (mmol)	19.6 ± 4.6	12.0 ± 4.0	20.8 ± 4.5	23.6 ± 2.6	7.3 ± 5.2	5.4 ± 4.4	18.3 ± 6.1	15.8 ± 4.2	21.0 ± 5.3
S^{2-} production (mmol)	16.0 ± 0,4	0.19 ± 0.07	0.44 ± 0.06	6.5 ± 0.4	0.22 ± 0.06	1.16 ± 0.09	2,5± 0.3	0.69 ± 0,09	0.31 ± 0.06
TVS (mg/l)	1742 ± 35	2.68 ± 0.35	1.86 ± 0.31	370 ± 36	2.19 ± 0.33	0.81 ± 0.29	3738 ± 43	3.26 ± 0.37	2.01 ± 0.31
MPN/mL	4.0×10^3	Not calculated	Not calculated	6.8×10^3	Not calculated	Not calculated	1.2×10^3	Not calculated	Not calculated

[a]The parameters were calculated using the arithmetic average of the duplicate reactors or the triplicate reactors (only for Reactor FOR 3). [b] pH measured after inoculation of the reactors. [c]The stoichiometric organic matter values were calculated using Reactions 1, 4, and 5 for the LAC, ETA, and FOR reactors, respectively; they indicate the organic matter removal that was expected based on the stoichiometry of these reactions.

bioreactors. Martins et al. (2009) reported a sulfate removal similar to this study using either lactate or ethanol as electron donors when studying acid mine drainage decontamination by metal-resistant SRB.

The experimental formate removal in the FOR 7 reactor was superior to the predicted organic matter removal based on the stoichiometry of Equation 5, probably because of formate consumption via pathways other than sulfate reduction such as methanogenesis. Although a $COD/SO4^{2-}$ ratio of 1 yields methanogens that are uncompetitive and completely displaced by SRB, some studies have suggested the coexistence of methanogens and SRB not only in low sulfate environments, but also in the presence of nonlimiting sulfate concentrations (Hu et al., 2015, Raskin et al., 1996). However, as the formate activity coefficient varies according to its molar concentration from 0.7 to 0.9 (Bonner, 1988), a $COD/SO4^{2-}$ ratio lower than expected may have been viable, causing lower electron availability for sulfate reduction.

The highest total concentration of volatile solids was observed in the FOR 7 reactor, indicating that the growth of a diverse microbial group is favored (Figure 5). In contrast, the ETA 7 reactor showed reduced microbial diversity when compared with the LAC 7 and FOR 7 reactors (Figure 5), which is probably due to a very specific SRB group capable of completely oxidizing ethanol (Rodriguez and Zaiat, 2011). The MPN quantification showed an SRB population of the same order of magnitude in the ETA, LAC, and FOR reactors, which was correlated with similar sulfate removal efficiencies obtained in the three reactors. However, the data obtained do not show a clear explanation for the decreased sulfide concentration in the liquid in ETA and FOR reactors, as the sulfide concentrations were below the expected stoichiometric values, suggesting the formation of compounds of intermediate oxidation state such as sulfite and/or thiosulfate, also seen in the Vilela et al. (2014) study.

The substitution of lactate with ethanol or formate as a carbon source in the Postgate C medium reduced the lag phase from 13 days to 3 and 2 days, respectively (Figure 6). Tsukamoto et al. (2004) and Zhou et al. (2015) also reported a smaller lag phase when ethanol was used as substrate for sulfate reduction. Lag phase reduction is important because it permits the faster growth of SRB, thus reducing the enrichment costs. This also proves that ethanol utilization during enrichment is more cost-effective than the usage of lactate. Ethanol was shown to be a better carbon source for enrichment at neutral conditions because the sulfate removal efficiency was maintained, with the occurrence of significant sulfide production, important for additional metal removal from acid mine drainage via sulfide precipitation during bioremediation. In addition, when a complete route is used to oxidize ethanol, alkalinization can be generated, allowing pH elevation in AMD (Oyekola et al., 2010).

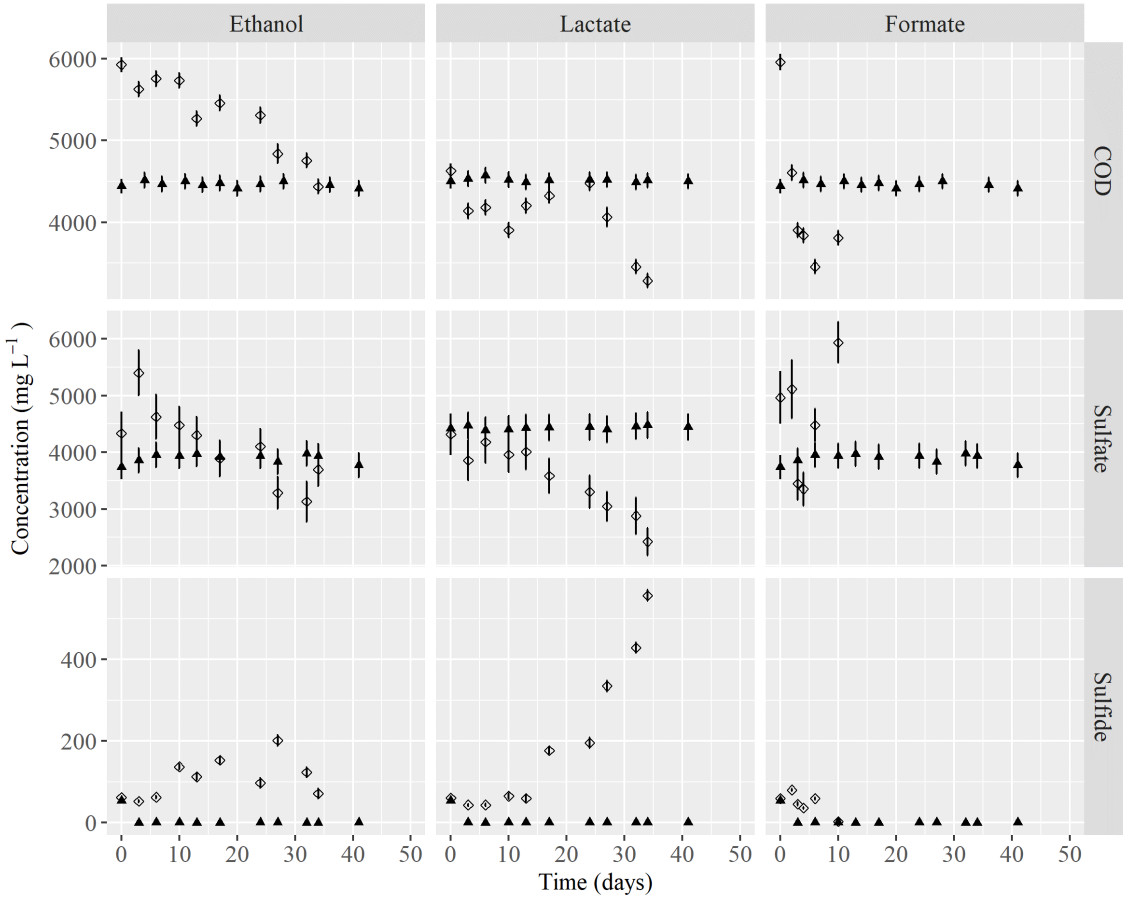

Figure 6. Performance of the LAC, ETA, and FOR reactors during the experiment in pH 7 (Control (▲) and pH 7 (◇)). The error bars correspond to 95% confidence interval.

The small cultivation period of the FOR 7 reactor is due to the oxidation of the enrichment medium after the 10-day experimental period. This can probably be explained by H_2 production after the initial sulfate consumption, as observed by Martins et al. (2016). It is important to emphasize that, despite this inferior enrichment period, sulfate removal in the FOR 7 reactor (~18 mM) was close to that of the LAC 7 and ETA 7 reactors (20 mM and 24 mM, respectively). However, sulfide production drastically decreased (Table 5), making the use of formate for biomass enrichment less viable. This also confirms that ethanol is the better carbon source for SRB cultivation under neutral pH conditions, but adjustments in the COD/SO_4^{2-} ratio, using values from 1.9 to 3, need to be made to optimize the sulfide production (Vilela et al., 2014).

Besides, a large amount of sulfate and organic matter continued to be available in the bioreactors, and the final pH is not inhibitory as the sulfate reduction stopped in all reactors. This might have occurred due to the inhibitory effect of residual sulfate, which affects the operating pH and redox potential (Oyekola et al., 2010). The high residual sulfate concentration observed in LAC, ETA, and FOR reactors most likely increased the redox potential at the end of the experiment, not allowing the continual growth of SRB. In addition, the high sulfide concentration produced by the sulfate reduction process may have become toxic for ETA and LAC reactor species, as suggested by Kaksonen et al. (2004), causing the sulfate removal process to fail. According to Oyekola et al. (2010), lactate is reported to decrease sulfide toxicity, which could explain how the LAC reactor could support higher sulfide concentrations than expected (16 mmol), compared to the ETA reactor (6.5 mmol). Also, sulfide inhibition is species specific; in other words, the level of inhibition experienced by each system also depends on the dominant bacterial groups under different operating conditions. For the FOR reactor, organic matter continued to be consumed even when sulfate reduction ceased, showing that another group of microorganisms has become more competitive than SRB, leading other routes to prevail over the sulfidogenic one.

In acidic experiments, it was not possible to control the initial pH, because it was significantly altered when sulfide and the inoculum was supplemented. Thus, it was not possible to compare the pH effect on each carbon source directly, especially because formate experiments were the only batch performed

under acidic conditions. In addition, the low COD/SO_4^{2-} ratio used in all experiments may have affected the activity of SRB due to limited electron availability for sulfidogenesis, as also suggested by Vilela et al. (2014). As a result, sulfate removal efficiencies may not have been affected only by pH in the reactors studied.

The sulfate removal efficiency was not affected by the pH reduction in the FOR 3 and FOR 4 reactors under acidic conditions (34% and 39%, respectively; Table 5; Figure 7). This shows that formate is a possible carbon source for the enrichment process under acidic conditions. As Rodriguez and Zaiat (2011) showed, the sulfate concentration of an acid mine drainage site located in Poços de Caldas, Brazil (the same location as the sediment collected in the current study), was ~16 mmol. The acidic FOR reactors were capable of removing sulfate around this order. Because formate contains only one carbon atom, it might be more readily oxidized by SRB than other substrates, such as lactate and ethanol (Rodriguez and Zaiat, 2011), which may have contributed to the maintenance of the sulfate removal efficiencies in low pH conditions. As low sulfide production was observed in these reactors, further analyses are necessary to prove that sulfate removal was effective even at low pH. The formate feed reactor showed efficiencies consistent with other reported studies using a medium COD/sulfate ratio of 1.05 ±0.13.

ETA experiments on acid tests were also performed close to neutrality (pH around 6.0), due to the high elevation of the initial pH when sulfide and the inoculum were added to the medium. However, a small pH reduction in the ETA 3 and 4 reactors (from 7.4 to 6.0) caused the sulfate removal process to fail, based on the calculated errors, and very low sulfide production was observed. As such, it was not possible to prove that the sulfate reduction process occurred. In addition, the low COD/SO_4^{2-} ratio used (1.00 ± 0.12), may have also affected the process efficiency, as according to Vilela et al. (2014), a COD/SO_4^{2-} ratio of approximately 1.9 is ideal for incomplete ethanol oxidizers to promote an efficient sulfidogenic process. Najib et al. (2017) also reported the effect of the COD/sulfate ratio and pH reduction from neutral 7 to 6 on an ethanol feed reactor. When COD/sulfate ~ 2 was used, efficiencies of 96% were obtained, likewise reduced to 39% for a COD/sulfate ratio of 0.5. The same occurred with the pH reduction from 7 to 6, causing the efficiency to decline to 36% in limiting substrate

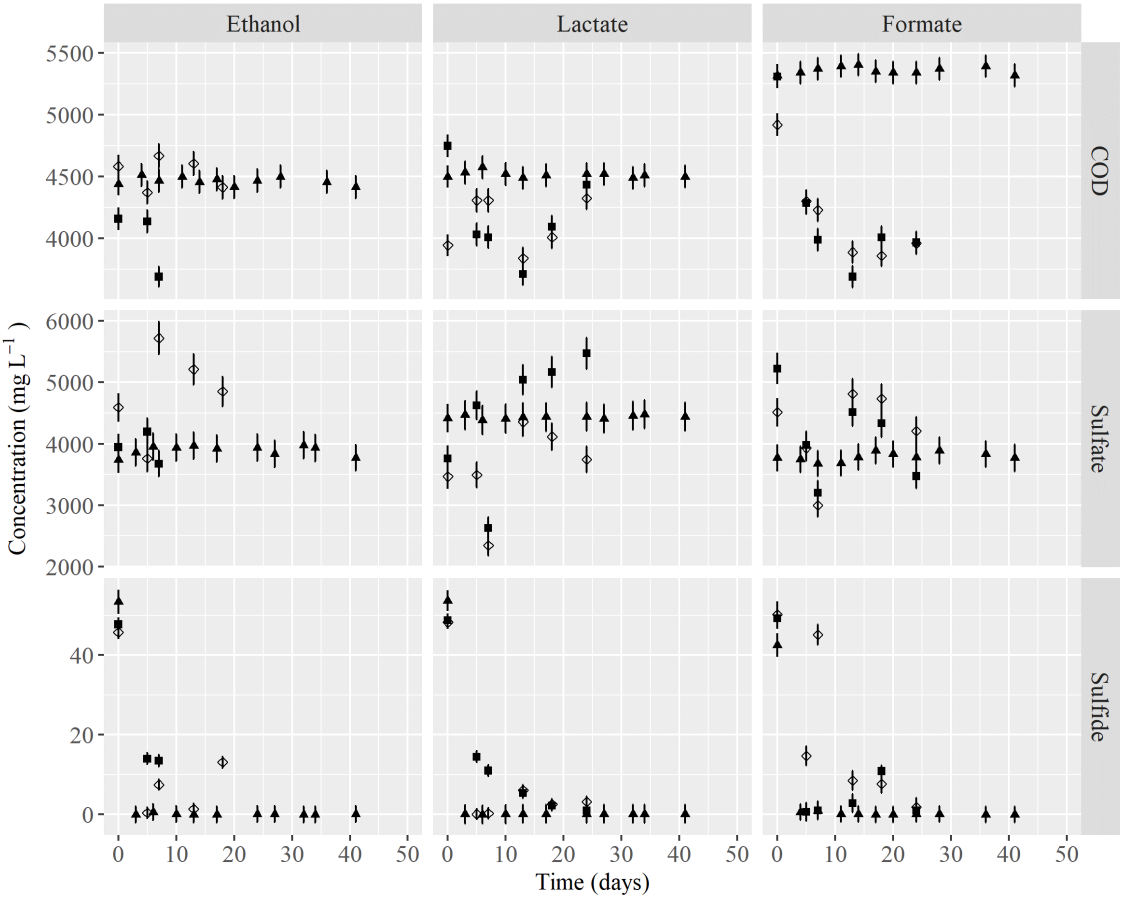

Figure 7. Performance of the LAC, ETA, and FOR reactors during the acidic pH experiment (Control (▲), pH 3 (◇) e pH 4 (■)). The error bars correspond to 95% confidence interval.

conditions. Thus, the limiting electron availability might have not favored the use of ethanol for sulfate reduction by SRB, causing fermentative bacteria and other microorganisms to compete with SRB for ethanol, as reported by Sun et al. (2016). As it was not possible to maintain the initial pH around 3 and 4 for ETA reactors, a complementary study is necessary to analyze the potential use of this carbon source as substrate for autochthonous SRB in low pH.

The sulfate removal efficiency with lactate as a carbon source was affected in reactor LAC 3 (reduced to 30%) and maintained in reactor LAC 4 (43%). Because the sulfate removal efficiency was reduced due to pH modification, lactate is not an ideal carbon source for enrichment at inoculation pH values lower than 3.6. For inoculation pH ~ 5, however, the observed sulfate removal (~21 mmol) was significant based on the AMD sulfate concentration (~16 mmol). Sulfate removal values at pH 5 (41%) and pH 7 (44%), similar to that in this study, were demonstrated by Verma et al. (2015). At pH 5, Jong and Parry (2006) also obtained sulfate reduction values similar to that in this experiment (43%). However, at pH 3, they determined values (6%) lower than the ones found in this study. Jong and Parry (2006) also observed that SRB activity decreased proportionally with increasing undissociated lactate concentration, as undissociated organic acids are able to diffuse across the cell membrane at low pH and prevent the bacterial cell from maintaining a membrane potential and proton motive force, causing an inhibitory effect on SRB activity. Therefore, it is also important to remember that very low sulfide production was observed on LAC reactors, indicating that more information is necessary to prove that the sulfate removal process was stabilized.

Based on the stoichiometry, reactors ETA 3, LAC 3, and LAC 4 had lower experimental organic matter removal than expected, likely because of acetate accumulation due to incomplete oxidation of organic matter by SRB, as reported by Bertolino et al. (2012). This acetate accumulation may also be caused by the pH reduction in reactors ETA 3 and LAC 4, as acetate can also be found in acetic acid formed in these reactors (Sánchez-Andrea et al., 2014). Sánchez-Andrea et al. (2013), in a similar study using acidophilic sulfate-reducing bacteria from Tinto River sediments, also reported acetate accumulation in all the enrichments performed and identified that the enrichments for sulfate-reducing microorganisms were dominated by the *Desulfotomaculum* species (Sánchez-Andrea et al. 2012).

The optimum pH value for the growth of most known SRB ranges between 5 and 9 (Postgate, 1984), which also explains the reduction in the sulfate removal efficiency in the acidic LAC and ETA reactors. A low pH reflects more energy investment in pumping protons across the cell membrane, thereby increasing the energy expenditure. However, the existence of acidotolerant and acidophilic SRB, such as the *Desulfotomaculum* species found in this study, identified in the AUT community in Phase 1 (Figure 4), and resistant to pH reduction in Phase 2 (Figure 5), provides the possibility of treating acidic waters directly, without previous effluent neutralization, thereby reducing the cost. Even though a decrease in bacterial biodiversity was noted in acidic conditions compared to neutral pH (Figure 5), the reactor performance in sulfate removal may not be adversely affected, as also demonstrated by Zhao et al. (2016), who reported efficiencies higher than 90% in acidic pH of 2.5, and COD/sulfate ratio ~ 2. However, Zhao et al. (2016) used a non-autochthonous biomass and compartmentalized bioreactor that can limit, to a great extent, the exposure of the biomass to adverse environmental conditions, such as low-pH shock. This can help explain the lower efficiencies that were obtained, considering the limiting COD/sulfate ratio used in this study. It is important to consider that SRB, even when present uniformly in all studied bioreactors in neutral and acidic pH, may have their activity reduced with pH modification. With this in consideration, the sulfate reduction process can be efficient without pH control, as long as this process generates alkalinity, allowing the pH to increase (Sánchez-Andrea et al. 2014). The activity of SRB might have been affected in the studied bioreactors because the pH did not increase. Church et al. (2007) also observed no pH elevation during the enrichment of extremophile SRB, and it probably occurred because the sulfidogenic process was not favored at low pH, so perhaps other routes were used by SRB that do not generate carbonate.

Low sulfide production was observed in all reactors cultivated under acidic conditions (Table 5 and Figure 7), likely because this condition does not favor sulfidogenic species. However, control reactors are present in Figure 7, suggesting that sulfide drops to zero after medium reduction and, thus, a small amount of sulfidogenic activity was observed in all reactors. Alternative pathways for sulfate removal and COD consumption may have been used by SRB, resulting in different reaction products, such as elemental sulfur (visually observed based on the formation of a white precipitate, thiosulfate and sulfite, as reported in Lee et al. (2014). Low sulfide production was also observed by Church et al. (2007), with a value of 0.015 mmol found during enrichment of sediments from an acid mine using a modified Postgate C medium and a pH around 4. They noted the presence of elemental sulfur in the low-pH experiments, instead of sulfide, due to the higher stability field for elemental sulfur in low-pH microcosms. They also reported that sulfur might not be a direct metabolic product in low pH. The

toxicity of H_2S, the predominant form of sulfide at pH values lower than 7, could be an impetus for different pathway utilization by SRB (Bijmans et al., 2008). Also, according to Koschorreck (2008), it is a common adaptation strategy of anaerobic bacteria to change their metabolic route at a low pH in order to avoid the build-up of toxic concentrations of organic acid. However, further analyses are necessary to prove that alternative pathways were used by SBR and to prove the effectiveness of the sulfate reduction process.

Different bacterial communities were observed in the studied reactors (Figure 5b), showing that the carbon source and initial pH considerably affect the microbial population, even when the same biomass is used as inoculum, as reported by Zhou et al. (2015). According to Sánchez-Andrea et al. (2014), the pH can affect the activity of different microorganisms, whereas the substrate can affect the complexity of these communities, thus having an important effect on the microbial community composition of a reactor. Uniformity of SRB diversity was observed in the SRB groups of all reactors, probably due to the low diversity of the AUT inoculum used, as shown in Figure 3. Besides, it was not possible to prove the establishment of an effective sulfate removal process. Figure 5a shows that autochthonous populations selected under different enrichment conditions were present in the bioreactors. Thus, even with the difficulty of using an autochthonous group to treat AMD in acidic conditions, AUT biomass was resistant to acidic pH, revealing a possible potential for autochthonous SRB but also indicating that more investigation is necessary to prove their effectiveness in bioremediation of AMD.

The acidic resistance of the AUT culture can probably be explained by the capability of these microorganisms to face the proton motive force across the cell membrane that can drive energy-dependent processes to promote pH homeostasis, as mentioned in Florentino (2017). Many strategies can be used by acidophilic and acidotolerant SRB to maintain physiological pH despite external acidic conditions, such as the utilization of specific transporters and enzymes for proton export (e.g., proton ATPases, antiporters, and symporters), adoption of particular permeability properties, incremental changes in the buffer capacity to sequester or release protons based on pH shifts, and enhancement of positive surface charges. These microorganisms generally have an impermeable cell membrane or low membrane fluidity that restricts the proton influx to the cytoplasm (Florentino, 2017).

Another important strategy used by the *Desulfotomaculum* species is spore formation, which enables them to survive under unfavorable environmental conditions. As cited by O'Sullivan et al. (2015), bacterial endospores can withstand multiple environmental stress conditions, such as extremes in pH and, thus, are remarkable survival systems that are able to persist over geological timescales. Although the AUT culture can survive under extreme pH conditions found in acid mine drainage, this condition prohibits the growth of the SRB population (Martins et al., 2011), which indicates the importance of the enrichment process in the establishment of an autochthonous sulfidogenic population capable of acid mine drainage treatment, even under acidic conditions. Giloteaux et al. (2013) also reported that SRB can survive in microenvironments associated with particles that provide protection from surrounding acidity and oxygen, enabling the tolerance of SRB to acidic conditions. According to Jing and Kjellerup (2017), microniches with elevated pH can form around SRB. Microniches, as defined in microbial ecology, are small areas with microbial actions that differ from those of the surrounding bulk soil or sediment habitat.

Sulfidogenic species were not favored under low pH conditions; low sulfide production was observed in all acidic experiments. However, these species were favored under neutral conditions; high sulfide production was observed when ethanol and lactate were the carbon sources. Because ethanol is a cost-effective carbon source and favors sulfidogenic species, the best alternative for enrichment of autochthonous SRB is the use of ethanol.

After the inoculation of this community in the sulfidogenic bioreactor used for acid mine drainage treatment, pH reduction using ethanol is possible, as reported by Bai et al. (2013) and Martins et al. (2011). Because the autochthonous SRB were resistant to acidic conditions and the DGGE analyses proved their presence in all reactors, gradual pH reduction will probably not reduce the sulfate removal efficiencies.

CONCLUSIONS

This study showed that the sulfate removal and sulfide production of the selected AUT population were similar to that of the N-AUT population after an acclimation period, showing that the AUT culture can have an interesting potential use for local acid mine drainage treatment. However, it was noted that the N-AUT community was capable of using lactate more quickly, avoiding an extended lag phase, also due to the identified species of *Desulfovibrio* genus present in this bioreactor, known for their capacity to grow faster when using lactate than other species. The N-AUT and AUT cultures had distinct community structures, both in the domain Bacteria and SRB groups. Low diversity was observed for the SRB group in the AUT reactor compared with that of the N-AUT reactor, probably due to the spore-forming capacity of the *Desulfotomaculum* species that allows their survival in

unfavorable conditions such as AMD and also causes the need of a lag phase for medium adaptation.

It is important to emphasize that the COD/sulfate ratio used, with a medium value of 1.14 ± 0.10, is a limiting condition for the sulfate reduction process, also contributing to efficiencies of sulfate removal lower than 70%. Therefore, based on similar sulfate removal efficiencies, effective sulfide production, and lag phase reduction, it was possible to infer that ethanol is a viable and low-cost alternative substitute for lactate as an enrichment carbon source under neutral pH conditions. Formate showed similar sulfate removal efficiency; however, it is not an effective electron donor for sulfate reduction under neutral conditions and adjustments of the COD/sulfate ratio are necessary to optimize sulfide production when this carbon source is used.

Sulfate removal efficiencies for the electron donors formate (inoculation pH of 3 and 4) and lactate (inoculation pH of 5) were similar in low pH to those obtained in neutral pH conditions used for enrichment. At low pH, a combination of organic acids accumulation and lower carbonate production probably hidered pH elevation, which may have inhibited the sulfidogenic route outcompetition for the electron donor oxidation. In addition, complementary data are necessary to corroborate the hypothesis that other intermediate routes were used by SRB, producing thiosulfate, sulfur, or sulfite. Carbon source modification and pH reduction to acidic levels significantly changed the bacterial diversity. Similar diversity was observed for the SRB group, confirming the presence and resistance of this community under acidic conditions.

According to the obtained results, the autochthonous SRB cultures presented potential for AMD bioremediation. However, this initial study is important due to the complexity of autochthonous microorganisms and represents a fundamental study of the enrichment process of extremophiles, raising the possibility of stabilizing an enrichment medium for their growth and application to AMD treatment.

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NOMENCLATURE

AMD	Acid Mine Drainage
AUT	Autochthonous
COD	Chemical Oxygen Demand ($mg \cdot L^{-1}$)
Control	Control Reactor
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ETA	Ethanol feed reactor
FOR	Formate feed reactor
LAC	Lactate feed reactor
MPN	Most Probable Number
N-AUT	Non-autochthonous
PCR	Polymerase chain reaction
S^{2-}	Sulfide ($mg \cdot L^{-1}$)
SO_4^{2-}	Sulfate ($mg \cdot L^{-1}$)
SRB	Sulfate-reducing bacteria
TVS	Total Volatile Solids ($mg \cdot L^{-1}$)

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