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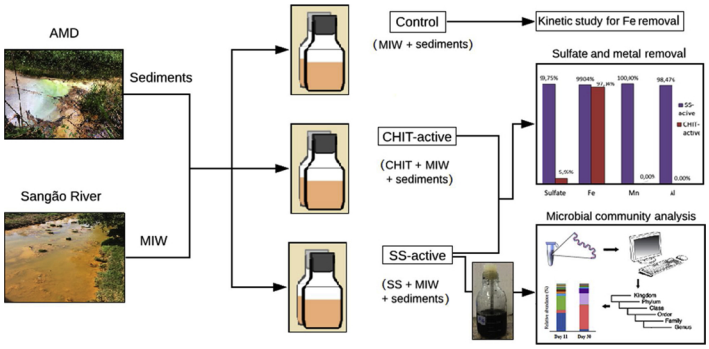
Chitin as a substrate for the biostimulation of sulfate-reducing bacteria in the treatment of mine-impacted water (MIW)

Caroline Rodrigues[a,*], Dámaris Núñez-Gómez[a], Daniele D. Silveira[a], Flávio R. Lapolli[a], María A. Lobo-Recio[a,b]

[a] Department of Environmental Engineering, Federal University of Santa Catarina (UFSC), 88040-900, Florianópolis, SC, Brazil
[b] Department of Energy and Sustainability, Federal University of Santa Catarina (UFSC), 88900-000, Araranguá, SC, Brazil

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to know the basis of sulfate-reducing bacteria (SRB) and chitin source relationship for the development of a biotreatment system for mine-impacted water (MIW). The MIW consists of river water impacted by coal acid mine drainage (AMD), an extremely acid effluent, rich in sulfate and dissolved metal ions, with a high pollutant potential. Chitin was used as metal ion sorbent and biostimulant of SRB, whose anaerobic dissimilatory metabolism reduces sulfate to sulfide. Microcosms were built in an oxygen-free atmosphere using chitin from two different sources: commercial chitin and shrimp shell waste, which contains calcium carbonate, an acidity removal agent, in addition to chitin. The results indicate that the shrimp shell performs best in removing sulfate (99.75%), iron (99.04%), aluminum (98.47%), and manganese (100%) ions. The iron ion sorption kinetics of the sediments were also studied; pseudo-second order behavior was observed. High-throughput sequencing analysis revealed the present bacterial community and its abundance in the microcosms after 11 and 30 treatment days: SRB were detected but were not the majority. Thus, this research aims to contribute to the sustainable treatment MIW through the employment of an abundant and low-cost biomaterial.

* Corresponding author.
E-mail addresses: caroline.rodrigues@posgrad.ufsc.br (C. Rodrigues), damaris_ng@hotmail.com (D. Núñez-Gómez), danid_bioeng@yahoo.com.br (D.D. Silveira), f.lapolli@ufsc.br (F.R. Lapolli), maria.lobo@ufsc.br (M.A. Lobo-Recio).

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1. Introduction

Acid Mine Drainage (AMD) from abandoned/inactive mines is a major environmental issue in countries with large-scale mining activities [1,2]. Therefore, the carboniferous basin in the south of the Santa Catarina (SC) State, Brazil, has been highly impacted by AMD [3]. This pollution has occurred over the last 30 years and fluvial water and sediments have been altered. Most rivers in this region are considered to be dead because of the high level of toxicity [4,5].

AMD forms via several chemical and biological processes in the presence of air and water, involving the oxidation of sulfides of geological origin (e.g., pyrite). An acid effluent is generated, which is characterized by a pH between 2 and 4 and high concentrations of sulfate (SO_4^{2-}) and dissolved metallic ions (e.g., Fe, Al, Mn, Zn, Cu, and Pb) depending on the geological strata of the mining area [6–11]. The AMD formation is a cycle of autocatalytic reactions, which is difficult to control, ceases only when pyrite is exhausted [11], and continuously contaminates surface and groundwater [2], known as mine-impacted water (MIW). Once MIW is generated, it is difficult to control the process and water treatment is expensive [11], can persist for centuries and reach extensive distances of watercourses [4].

An alternative substrate for MIW bioremediation method was tested, which is based on the ability of microorganisms to generate alkalinity and immobilize metals ions [8]. Sulfate-reducing bacteria (SRB) are a group of prokaryotes (from bacteria and archaea domains) that oxidize organic compounds under anaerobic conditions using sulfate as a final electron acceptor in the energy metabolism, perform dissimilatory sulfate reduction, and thus convert sulfate to hydrogen sulfide and release bicarbonate (Eq. (1)). The generic and simple organic compound CH_2O, available to SRB, is the electron donor and energetic source of the reaction [1,12–14].

$$SO_4^{2-} + 2\,CH_2O \rightarrow H_2S + 2\,HCO_3^- \quad (1)$$

Biological sulfate reduction can be performed by assimilatory and dissimilatory metabolisms. In the first, sulfate is incorporated to cellular growth, protein synthesis, etc. In the dissimilatory, sulfate is an electron acceptor, along with organic compounds (or H_2) as electron donors, being reduced to hydrogen sulfide and excreted in the medium [6]. This reduction process requires 8 electrons and is catalyzed by several enzymes (ATP sulfurylase, APS reductase and dissimilatory sulfite reductase), as illustrated in Fig. 1. Sulfate is activated through ATP (adenosine-triphosphate) and enzyme ATP sulfurylase, that catalyzes the sulfate binding in ATP, leading to the formation of APS (adenosine-phosphosulfate). This activation makes the standard reduction potential from sulfate to sulfite ($E^{\circ\prime} = -0.52$ V) be raised to near 0 V, enabling the reduction. The sulfate group in APS is reduced to sulfite (SO_3^{2-}), through enzyme APS reductase and releasing AMP (adenosine-monophosphate). Once sulfite is formed, the enzyme dissimilatory sulfite-reductase leads to the formation of sulfide [15,16].

Hydrogen sulfide may react with dissolved metals, preferably bivalents (M^{2+}), that are typically present in MIW and precipitate them in the form of sulfides (Eq. (2)), which is an important bioprocess for the removal of metals from AMD and MIW [1,13,17].

$$M^{2+}{}_{(aq)} + H_2S \rightarrow MS_{(s)} \downarrow + 2H^+ \quad (2)$$

These SRB are naturally present in anoxic sediments and places contaminated with AMD such as marine sediments and lakes [15,18]. The metabolism of SRB and environmental conditions in which they prosper are versatile [12]. Due to the potential for the combined removal of acidity, metals, and sulfate, biological sulfate-reduction appears to be a highly promising MIW treatment and metal recovery method [19,20].

In addition, it has been reported that sediments can retain over 90% of metals and metalloid water pollutants, which is important for metal cycling in ecosystems [21]. The particle size and sediment composition are related to the binding capacity of contaminants and the ability to retain metal ions via adsorption, chelation, and ion exchange mechanisms [22].

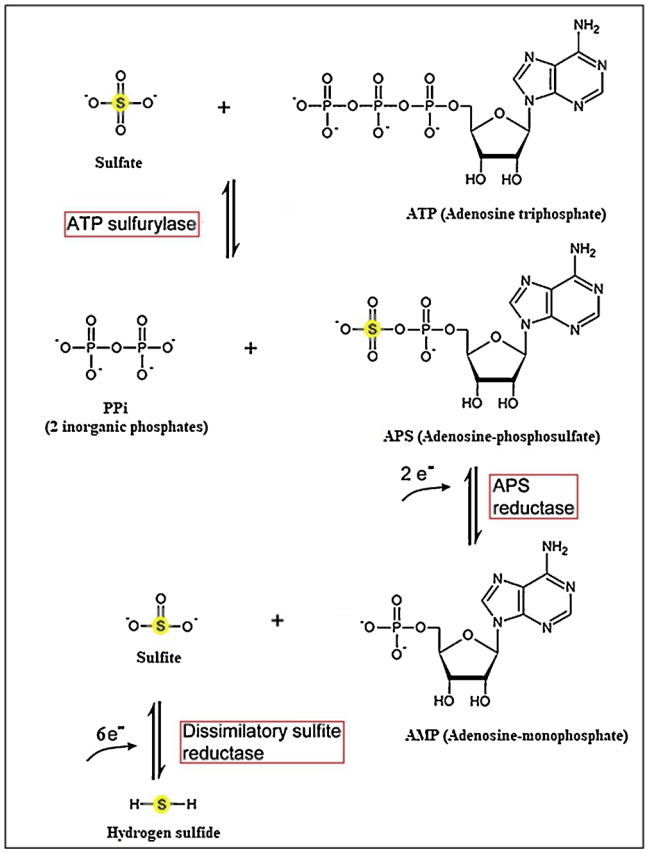

Fig. 1. Biochemical pathway of dissimilatory sulfate reduction.

Generally MIW contains very low concentrations of dissolved organic carbon and therefore an additional organic carbon source must be added as electron donor to adequately develop SRB [1,13,14]. Studies [9] concluded that the chitin chain may be used as an adequate substrate for the SRB culture because it has a suitable C/N ratio (6.86 on mass basis) and creates reducing conditions during its degradation, which promotes anaerobic processes. Previous studies [23] already showed that chitinous materials can be effectively used for AMD bioremediation.

Chitin is a biopolymer that is widely distributed in nature. Its main sources are the exoskeletons of many crustaceans and insects. Chemically, it is a high-weight molecular polymer comprising units of N-acetyl-2-amino-2-deoxy-D-glucose linked by β glycosidic bonds (1 → 4), forming a linear chain [5,7,24]. Chitin is also a metal-ion adsorbent; it can be used to remove metals from aqueous solution [5,7,25]. It has been successfully used in the MIW treatment [7] because of its acetamide and hydroxyl groups, which behave as Lewis bases, serve as coordination sites, and form stable complexes [26].

Shrimp shell (SS) is one of the sources of chitin. It is an abundant and low-cost residue, which is available in large quantities due to the processing of the fishing industry in the SC State [7]. It has the following composition: 17%–20% chitin, 33%–40% proteins, and 32%–38% mineral salts (mainly calcium carbonate) [27]. The latter guarantees the increase of the alkalinity in the medium, which is an advantage over other sources of chitin.

Thus, in this work, chitin was chosen as electron donor source for dissimilatory sulfate reduction. For comparative purposes, two different sources of chitin were tested: SS and commercial chitin (CHIT). Preliminary studies demonstrated that SS is a better AMD treatment agent than CHIT [5] because of its $CaCO_3$ content.

Table 1
Analytical methods.

Parameter	Methodology	Range
pH	pH meter Thermo Fisher Scientific	1–14
Fe	Ferrover[a]	0.02–3.00 mg L^{-1}
Mn	Periodate Oxidation[a]	0.0–20.0 mg L^{-1}
Al	Aluminon[a]	0.008–0.800 mg L^{-1}
SO_4^{2-}	Sulfaver[a]	2–70 mg L^{-1}
S^{2-}	Methylene Blue[a]	5–800 µg L^{-1}
CH_4	GEM 5000 gas analyzer Landtec	0–200 ppmv

[a] adapted from APHA [28].

This study aims to establish the basis for the development of a treatment system for coal MIW bioremediation based on the use of different sources of chitin as SRB biostimulant. The bacterial community and its abundance were also studied. Additionally, iron adsorption was observed in the sediment that was used as inoculum and the kinetics are described.

2. Materials and methods

2.1. Collection and characterization of mine-impacted water (MIW)

The MIW was obtained from the Sangão River in carboniferous basin of the southern SC State, Brazil. The samples were collected in non-sterile polypropylene bottles with no headspace [28], filtered using a 0.45 µm membrane under vacuum, and characterized before and after the treatments. Table 1 shows the methodology used for the analyses.

2.2. Chitin source

The SSs were acquired from fish markets, washed with tap water, dried for 72 h (for 48 h at 100 °C and 24 h at 50 °C), pulverized in a regular blender and sifted (to give greater homogeneity and adequate contact surface), and kept in glass desiccator prior to analysis, to avoid absorption of atmosphere moisture, as described by Núñez-Gómez et al. [5,7,29]. The CHIT consisted of chemically treated flake chitin for commercial purpose with 70% purity based on the manufacturer's information (*Polymar Ciência e Nutrição S/A*). Because the CHIT was already chemically treated, no additional steps were required; it was just pulverized in the blender and sifted.

2.3. MIW treatment: experimental setup for microcosms

To evaluate the potential of SRB for MIW bioremediation, experiments were carried out in batch microcosms under N_2 atmosphere to ensure anaerobiosis (dissolved oxygen ≤ 0.5 mg L^{-1}). The purpose was to biostimulate the development of SRB and thus remove the sulfate and dissolved metallic ions as insoluble sulfides [9].

The microcosms were prepared in 500 mL total capacity glass bottles, sealed with a silicone stopper and kept protected of light in a special room. Three sets of 20 bottles/microcosms were prepared (total of 60 microcosms). Two sets of active microcosms were prepared. Each flask contained 0.23 g of dry sediment (8.15 g of wet sediment), 260 mL of MIW and 10 g L^{-1} of source of chitin, CHIT or SS. The sets were labelled “CHIT-active” and “SS-active” respectively. The dry sediment weight was determined by drying the wet sediment in a stove at 105 °C during 24 h, to eliminate its humidity (this weight was used for kinetics calculations). The third set of bottles was analogously prepared but without the chitin source and was labeled “control group.” All bottles were purged with N_2, sealed, incubated in the dark at 20 °C, shaken manually once a day, and opened (sacrificed) after predetermined times (1, 2, 7, 9, 11, 16, 20, 24, 30 and 41 days). The temperature was constant during the tests and it was controlled with a wall thermometer, in a closed room with air conditioner, programmed for 20 °C continuously. The contents were filtered inside a fume hood in a N_2 atmosphere (to avoid oxidation of the compounds) and the filtrates were analyzed. Every experiment was performed in duplicate to minimize the experimental error and overcome any potential discrepancies of the biological system.

2.4. Adsorption kinetics of the sediments

Benthic sediments of an abandoned mine located in Urussanga, SC, were used as microbial inoculum source because this place is a promising source of SRB [14].

During the experimental process, parallel to the active essays, the control group flasks (MIW + sediments) were monitored, and a significant reduction of ion Fe was observed. The conditions were the same as described in item 2.3 (only without chitin source), since the purpose was to check if is there any other occurrence (biological or chemical) in the absence of source of carbon. This way, a kinetic study about the adsorption of iron by the sediment was carried out to determine the kinetic parameters and removal mechanism: the experimental data were analyzed using four kinetic models: pseudo-first order, pseudo-second order, intraparticle diffusion, and Elovich.

2.5. High-throughput sequencing

For the evaluation of the bacterial community involved in the sulfate reduction, two samples were taken throughout the operation of the SS-active essay, on days 11 and 30 (beginning and end of the log phase, the higher metabolism period), respectively [9]. The collected samples were stored at −20 °C prior to the analyses. High-throughput sequencing was used as molecular method to provide qualitative and quantitative results (relative abundance) for the bacterial community present in the samples. To determine the phylogenetic diversity of the bacterial communities of different treatments, the genus data were used.

All 16S rRNA data were analyzed using gene sequencing of the region V3–V4 on the extracted DNA [30,31]. Universal primers, such as 341 F 5′ CCTACGGGRSGCAGCAG-3′ [32] and 806R 5′ GGACTACHVGGGTWTCTAAT-3′ [33], were used because both have a great taxonomy coverage with respect to bacteria and archaea [31]. Finally, the libraries were sequenced using the MiSeq platform (MiSeq, Illumina Inc., USA) by Neoprospecta Microbiome Technologies, Inc. (Florianópolis, Brazil).

Chimeras were eliminated using the proprietary Neoprospecta filtering pipeline, which is based on the probability of Q-score errors. Operational Taxonomic Units (OTU's) with 97% similarity (0.03 phylogenetic distance) were selected and then subjected to taxonomic classification by comparing them with the 16S rRNA SILVA database [34]. Only representative sequences with hits of 99% identity in an alignment covering over 99% were considered. Subsequently, the data for relative abundance construction were processed using specialized bioinformatics software (Epiome®) and loaded onto a specific platform for the analysis and interpretation of the results [30].

3. Results and discussion

3.1. MIW characterization and microcosm tests

The characterization of the parameters of interest of the MIW is showed in Table 2 together with Brazilian and international guidelines to compare the MIW with the maximum allowable values based on legislations. None of the parameters analyzed agrees with the norms, indicating the poor quality of this river water, which is not adequate for irrigation and non-potable reuse and is off standards for effluent release.

Fig. 2 shows the sulfate and pH variations for CHIT- and SS-active essays over time and organizes them according to the maximum allowable value (MAV) for secondary non-potable water reuse

Table 2
MIW analytical data.

Parameter	Unit	Collection 1[a]	Collection 2[b]	CONAMA 357[c]	CONAMA 430[d]	FAO[e]
pH	–	2.61	3.14	6–9	5–9	–
SO_4^{2-}	mg L^{-1}	400	420	250	–	–
Fe	mg L^{-1}	32.4	35.8	5	15	5
Mn	mg L^{-1}	2.8	2.6	0.5	1.0	0.2
Al	mg L^{-1}	25.24	19.52	0.2	–	5

[a] Used for SS-active essay.
[b] Used for CHIT-active essay.
[c] Maximum allowable values for Class III water, adequate for non-potable reuse [35].
[d] Brazilian conditions and standards of effluent releases [36].
[e] Maximum recommended level in irrigation waters recommended by the United Nations Food and Agriculture Organization [37].

recommended by the Brazilian environmental legislation (CONAMA 357/2005 [35]). In the SS-active essay, sulfate removal and hydrogen sulfide formation (70 ± 34 μg L^{-1}) become apparent after 7–9 days of experiment. The characteristic smell and dark appearance of hydrogen sulfide (Fig. 3A, B and C) due to the formation of black precipitates, possibly metal sulfides, confirm the beginning of the sulfate-reducing activity [9,15]. Thus, an acclimation (lag) time of approximately 9 days can be inferred. After 30 days of treatment, the sulfate concentration was lower than the MAV for non-potable reuse water. The sulfate was almost completely removed after 41 days. The initial pH (2.61) strongly increased due to the increase of the alkalinity caused by $CaCO_3$. It reached a plateau near neutrality after one day (Fig. 2B) at an average value of 7.15 ± 0.50.

In contrast, neither the sulfate nor pH considerably changed in the CHIT-active essay (Fig. 2): the pH remained stagnant at a mean value of 4.29 ± 0.12, the sulfate maintained a mean of 388.10 ± 14.40 mg L^{-1}, and the visuals remained unchanged throughout the experiment (Fig. 3D, E and F). The sulfate removal reached only 10.71% after 41 days of experiment. The low pH of the CHIT-active essay may be related to the poor sulfate removal, possibly derived from inadequate physiological conditions during the development and establishment of SRB.

In this sense and based on previous studies [38–40], the vast majority of known species of SRB are neutrophilic, have a better growth at pH 6–8, and are highly sensitive to acidity [10]. Although acidophilic SRB were reported in the literature in recent years [6,14,17,39] and sulfate reduction was successfully carried out down to a pH of 2.5 [8] on CHIT-active essays, the bacterial development is inadequate for reaching a successful sulfate-reducing activity. Koschorreck [41] stated that there is no clear explanation for the absence of sulfate reduction under certain low-pH conditions because it is a widespread phenomenon. Different reports [1,18] on the isolation of acidotolerant SRB show that (i) organisms with a low metabolic energy yield, such as SRB, might be especially susceptible to a low pH and to the fact that metabolic products of anaerobic bacteria, such as hydrogen sulfide and organic acids, are potentially toxic at an acidic pH because neutral molecules (unionized) permeate easily to the cell membrane; (ii) low pH environments often contain elevated concentrations of dissolved metals, which might be toxic to bacteria (deactivating enzymes and denaturing proteins); (iii) there are cases in which sulfate reduction is stimulated by simple organic substrates but not by complex substrates, suggesting that other fermenting bacteria are inhibited by the low pH.

Based on data points from days 9 to 41 for the SS-active essay, the average rate of sulfate reduction (slope of the fitting curve) was calculated to be −13.172 mg $L^{-1}d^{-1}$ (Fig. 4). This rate is in agreement with the reported [9], ranging from −11.9 to −16.5 mg $L^{-1}d^{-1}$. If the sulfate decay is independent of the concentration, the kinetics are considered to be of zero order.

In addition to sulfate, Fe, Al, and Mn ions were successfully removed from the SS-active essay (Figs. 5 and 6). The Fe and Mn ion concentrations decreased to the MAV after 30 and 7 treatment days, respectively (Fig. 5A and B), reaching a 99.94% and 100% removal at the end of the experiment (41 days; Fig. 6). The Al ion removal has been fast and very efficient (98.47%) since the first day of treatment, although the concentration is slightly higher than the Brazilian MAV (0.2 mg L^{-1}, Fig. 5C and D) but adequate for irrigation based on the FAO (Food and Agriculture Organization of the United Nations) (Table 2). Aluminum is toxic for many life forms, even at relatively low concentrations, and its removal from acidic waste waters, such as MIW, is highly desirable [42].

In spite of the known metal adsorption capacity of chitin, the pH value close to neutrality of the SS-active essay (average of 7.15 ± 0.50, Fig. 2B) and black color of the obtained precipitates (Fig. 3C) suggest that metallic ion removal via precipitation of metallic hydroxides, carbonates, and mainly sulfides [43] is the principal mechanism.

Methane was not detected during the analysis of gases in the flasks that were opened on days 20 and 41 of the experiment, suggesting the absence of methanogenic bacteria in the microbial community.

In the CHIT-active essay, Mn and Al ions were not removed (Fig. 5B

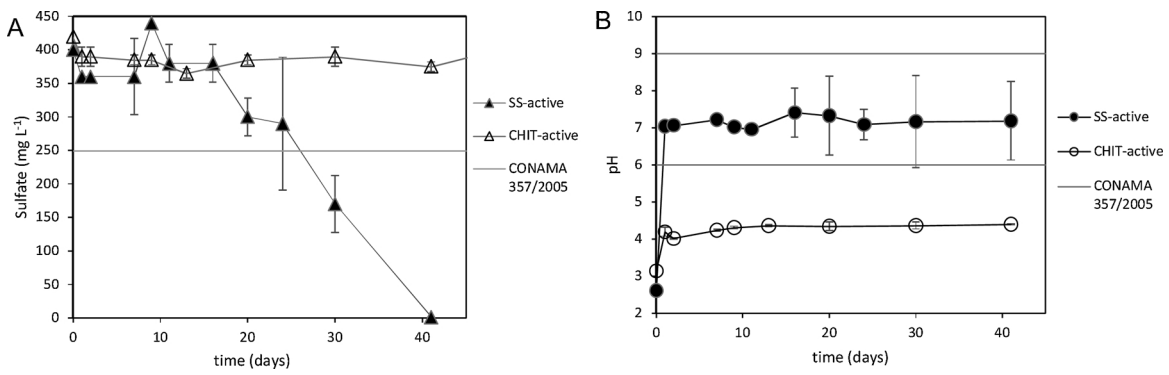

Fig. 2. (A) Sulfate concentration and (B) pH variations over time of the SS-active and CHIT-actives sets of microcosms. The data points represent duplicate average values and the error bars represent the standard deviation.

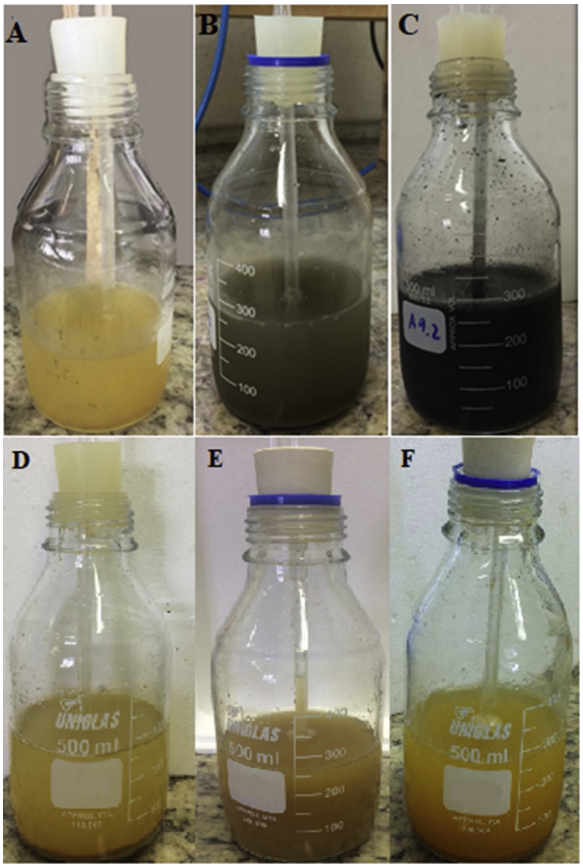

Fig. 3. Microcosm of the SS-active essay on (A) day 0, (B) day 7, and (C) day 30 and CHIT-active essay on (D) day 0, (E) day 7, and (F) day 30. This illustrates the evidences of black sulfides formation in the SS-active essay but not the same for the CHIT-active essay, in the same period.

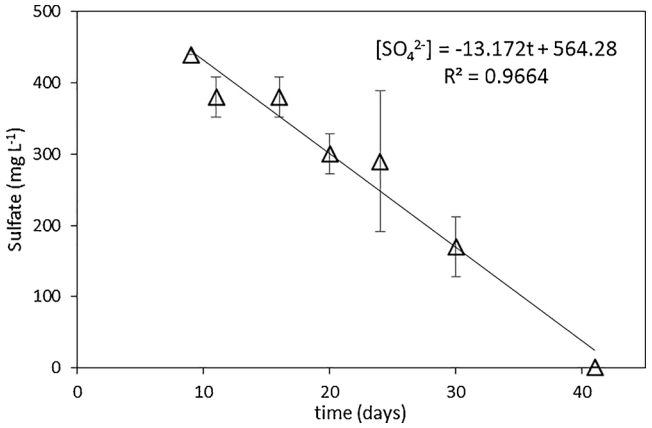

Fig. 4. Variation of sulfate concentration over time during log phase (higher metabolism) and its decay rate.

and C). Based on literature, the Mn ion is not easily removed at a pH < 6 [11] and the predominant form of Al in this pH range (4.29 ± 0.12) is the cation Al^{3+} [28], which is soluble. In contrast, 99.61% of Fe was removed and its concentration agrees with the MAV (Fig. 5A) after 12 days of treatment. However, an even higher Fe ion removal was observed in the control tests (water and sediments, pH = 3.82 ± 0.25; Fig. 7). After only 2 days, a 99% removal was obtained and the Fe concentration met environmental regulations. This indicates an adsorptive process of Fe ion in the sediment because a sulfate-reducing activity was not determined in the control tests.

The Fe ion adsorption on the sediment was investigated using adsorption kinetics, that is, the pseudo-first order, pseudo-second order, intraparticle diffusion, and Elovich models. The linearized equation and physical–chemical meaning for each model are provided in Table 3.

The pseudo-second order model provides the best fit of experimental data (R^2 = 0.9999). Consequently, it was considered that chemical adsorption is the rate-controlling step for uptake kinetics, which involves valence strength or electron exchange between sorbent (sediment) and sorbate (Fe^{2+}) because the sorption rate is proportional to the number of active sites [25,44]. Studies report that humic substances from the sediments (containing phenolic and carboxylic groups) may attract electrostatically cations until total saturation of its active sites [45]. Other authors also mention the presence of clay colloids [46]. The other models did not reach reliable fits or parameters. The high Fe removal in the CHIT-active essay was therefore attributed to the adsorption on the sediment and not to the process involving chitin and/or SRB.

This study shows that CHIT alone, under the studied experimental conditions, is not an adequate treatment agent neither as metal adsorbent nor as SRB biostimulant substrate. The pH below 5 of the experiment might have inhibited the SRB development and thus the sulfate removal. On the other hand, previous laboratory studies [5] showed that CHIT can remove metal ions from the same MIW in an acidic medium and air, where the removal strongly depends on the agitation. In the present experiment, the flasks were shaken once per day. The SRB acclimation and reducing environment prevented the Fe^{2+} oxidation, suggesting that Fe^{3+} is better adsorbed by CHIT or precipitated as hydroxide than Fe^{2+}. In comparison, the presence of $CaCO_3$ in SS buffered the pH value, allowing SRB development. This led to sulfate removal and strongly suggested that the metals are removed via precipitation as sulfides.

3.2. Microbial community analysis

Molecular analyses were used to reveal the bacterial community present in the SS-active essay because the evidence of sulfate-reducing activity was verified by the results mentioned above (sulfate removal and sulfide accumulation). The microbial communities of the SS-active essay were then analyzed by Illumina high-throughput sequencing. The media coverage contracted for the SS-active essay was 20,000 reads/sample, with an average paired-end fragment after quality analysis of 448 bp, on days 11 (log phase start) and 30 (log phase end).

Based on the genera found during the days of operation, the top 20 most abundant bacteria are shown in Fig. 8. The most abundant genus on day 11 was *Citrobacter,* belonging to Proteobacteria, followed by *Klebsiella*, *Clostridium*, *and Serratia*. On day 30, the most abundant genus was *Clostridium*, followed by *Enterobacter*, *Fonticella*, and *Citrobacter*. The other genera had low relative abundance values. Thus, a relative abundance of only 0.09% on day 11 and 1.05% on day 30 was found for *Desulfosporosinus*, a known SRB.

In relation to the most abundant genera found on day 11, *Citrobacter*, a facultative anaerobe Enterobacteriaceae family of bacteria that grows readily on ordinary media [47], has been reported to be able to reduce sulfate and produce H_2S [38]. Considering it does not belong to the traditional group of SRB, its sulfate-reduction ability has not been studied extensively [48,49]. Sulfide production is widespread among Enterobacteriaceae; however, *Citrobacter* is the only organism that can reduce sulfate [48]. *Citrobacter* sp. were isolated from a mining area in China and were discovered in cooperation with SBR on steel surfaces, forming a biofilm and producing H_2 [49]. Another species (*Citrobacter freundii*) with chitin-degrading activity and a chitinase gene was isolated from gastrointestinal microbiota of fish [50]. Chitinase is an enzyme that is able to break down the glycosydic bonds of chitin, which is the source of carbon used in this experiment. Zarasvand and Rai [48] stated that the isolation of *C. freundii* with sulfate-reducing capabilities shows that the diversity of SRB still has potential to be expanded. They

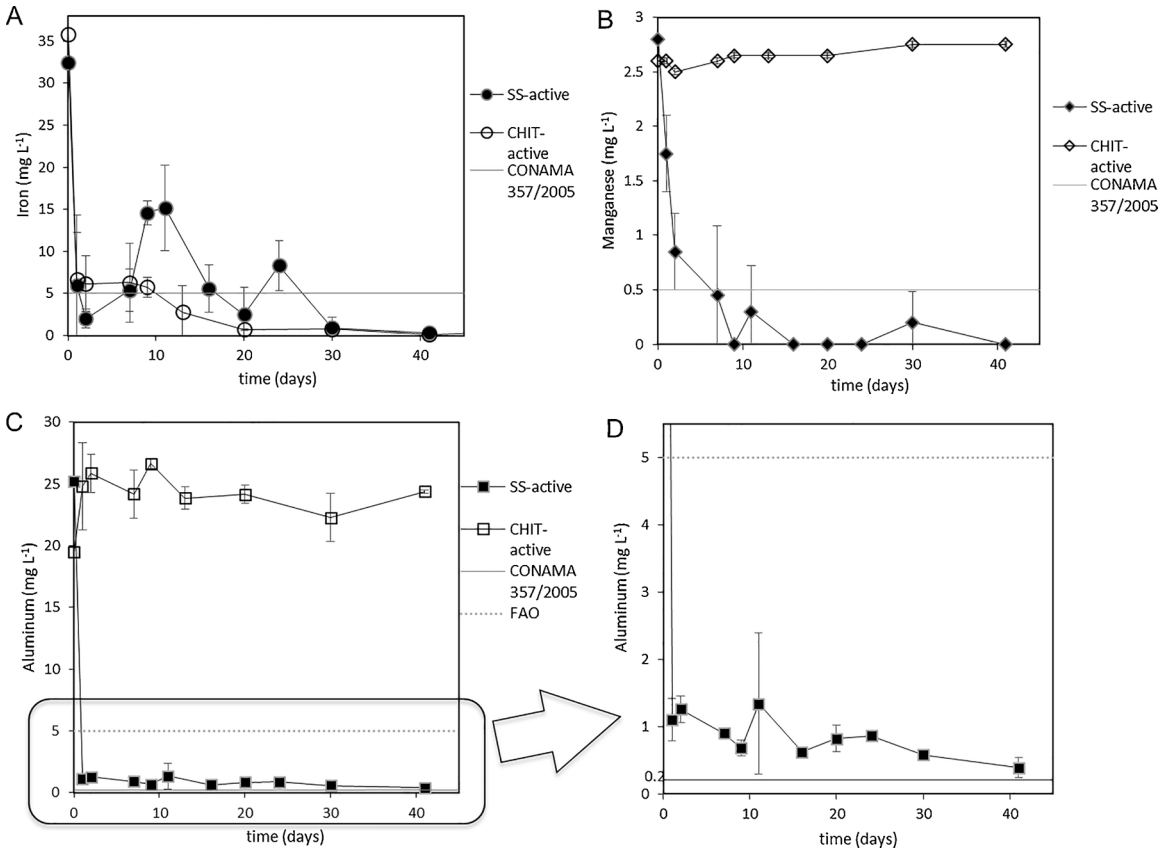

Fig. 5. Variations of (A) Iron, (B) Manganese, and (C and D) Aluminum ion concentration over time. (D) chart is an emphasis of Aluminum concentration. The data points represent duplicate average measurements and the error bars represent the standard deviation.

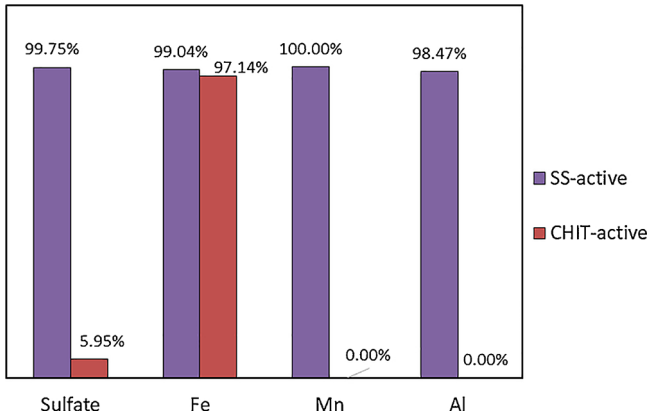

Fig. 6. Sulfate and metal removal (%) after 41 days.

considered strains of *Citrobacter* to be sulfate-reducing bacteria because of the sufficient amount of sulfides produced. The dissimilatory sulfite reductase (dsr) gene was detected in strain of *Citrobacter* sp. [48,51].

The most abundant genus on day 30 was *Clostridium,* which had greatly increased since day 11. These bacteria, belonging to Filo Firmicutes, are syntrophic anaerobic fermentative and can produce H_2, acetate, ethanol, and lactate; some are potential pathogens [52]. *Clostridium* can use hexose during fermentation [15], the monomer of chitin, and is a chitinase producer (*Clostridium paraputrificum* var. *ruminantium* isolated from the rumen of cows, rapidly degrades chitin and shrimp carapace) [53–55]. Based on literature, *Clostridium* occurs in the bacterial community related to AMD bioremediation [43] and in enrichments of acidic sediment of MIW [14]. Despite being a fermentative genus, it is also considered to be a SRB or able to carry out sulfate reduction [56–59]. Most SRB belong to 23 genera within Deltaproteobacteria, followed by the gram-positive SRB within Clostridia [12]. Pokorna and Zabranska [60] highlighted that there is no competition between SRB and rapidly growing fermentative bacteria. Based on similar experiments, it was reported that *Clostridium* species may provide electron donors that are utilized by *Desulfosporosinus* [61].

Traditional SRB found in this study was *Desulfosporosinus*. Together with *Sedimentibacter*, they belong to the phylum Firmicutes and clostridia class, respectively [62,63] and were found in very minute amounts on day 11 and in slightly larger amounts on day 30. Both were detected in a sulfidogenic fixed-bed bioreactor; genus *Desulfosporosinus* dominated [64].

Desulfosporosinus, a spore-forming curved rod, strictly anaerobic bacterium [62] and moderately acidophilic SRB, was isolated from MIW sediment with low pH and high dissolved metal ion concentrations [14,39,40,65,66] and from a MIW sediment with a pH range of 3.0–3.9, coexisting with *Clostridium* [56]. Its low relative abundance may be linked to the neutral pH of the system. Although SRB represent only a small portion of the bacterial population of AMD, it is in agreement with the literature (less than 0.5% of SRB) [67]. *Sedimentibacter* sp., whose name originated from sediments [63], was detected in sediments of a highly acidic river (Tinto, Spain; in addition to *Desulfosporosinus* and *Clostridium*) [68] and used to treat AMD with high metal concentrations [20].

Other detected genera were *Klebsiella*, *Enterobacter*, and *Serratia*. They are facultative anaerobes, gram-negative γ-Proteobacteria belonging to the family Enterobacteriaceae (enteric bacteria). They are also fermenters and have been associated with sewage from diverse industrial effluents [69], potential pathogens [15,70], and chitinase producers [53,54,71,72]. These genera can remove heavy metals from a variety of sources, for example, at petroleum-contaminated sites [73] used for biodesulfurization [74]. *Klebsiella* and *Clostridium* use hexose

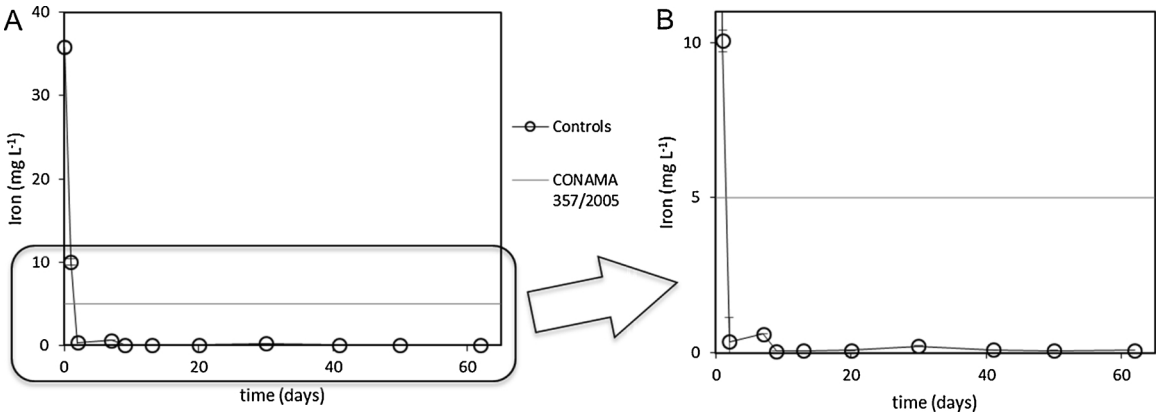

Fig. 7. (A) Iron concentrations in the control tests over time and (B) chart is an emphasis of the concentration.

Table 3
Kinetic parameters for Fe ion sorption on the sediment [25,29,44].

Model	Linearized equation	R^2	Parameter	Value	Unit
Pseudo-first order	$\ln(q_e - q_t) = k_1 . t + \ln q_e$	0.0290	k_1	−0.015	$time^{-1}$
			q_e	0.743	$m\ g^{-1}$
Pseudo-second order	$\frac{t}{q_t} = \frac{t}{q_e} + \frac{1}{k_2 . q_e^2}$	0.9999	k_2	1.918	$g\ mg^{-1} time^{-1}$
			q_e	36.101	$m\ g^{-1}$
			h_2	2,500	$mg\ g^{-1} time^{-1}$
Intraparticle diffusion	$q_t = k_{in} . \sqrt{t} + C$	0.5994	k_{in}	0.389	$mg\ g^{-1} time^{-0.5}$
			C	34.318	–
Elovich	$q_t = \frac{1}{\beta}\ln t + \frac{1}{\beta}\ln(\alpha\beta)$	0.8218	α	1.714×10^{22}	$mg\ g^{-1} time^{-1}$
			β	1.509	$g\ mg^{-1}$

q_t: amount of adsorbate in the adsorbent at time t.
q_e: amount of adsorbate in the adsorbent under equilibrium.
k_1: rate constant for pseudo-first order sorption.
k_2: rate constant for pseudo-second order sorption.
h_2: initial sorption rate ($k_2 . q_e^2$).
k_{in}: rate constant for intraparticle diffusion.
C: constant representing the resistance to mass transfer in the boundary layer.
α: initial sorption rate.
β: Elovich constant.

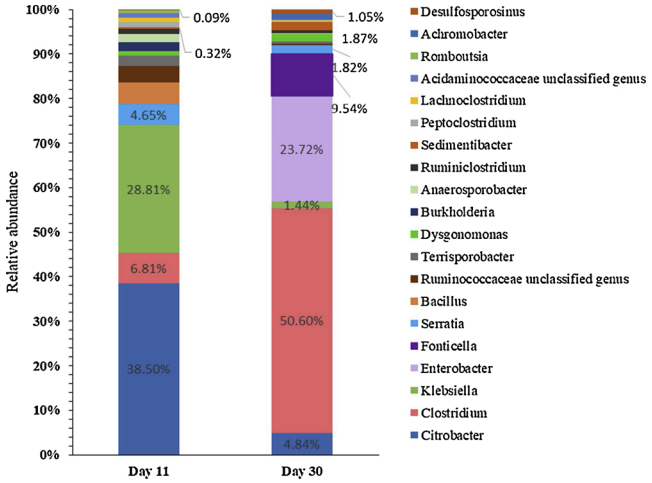

Fig. 8. Relative abundances at the genus level based on bacterial 16S rRNA gene classification of specimens with the highest number of sequences detected.

for fermentation [15] and are very abundant on day 11, although a decrease was observed on day 30. *Klebsiella* reduction may indeed have a positive effect on the treatment due to its pathogenic potential.

Based on the high-throughput 16S rRNA high-throughput sequencing analyses of this study, the *Clostridium* genus shows a greater abundance at the end of log phase. Novel high-throughput technologies might increase the success of identifying different novel SRB; although we have a huge amount of information about SRB, we only "scratched the surface" [12]. Nowadays, it is possible to detect microorganisms that may have not been identified before, which might indicate that a newly detected type of bacteria has always been a SRB with high activity potential. The results of this study also confirm the relationship between SRB and fermentative bacteria (or chitinase producers), suggesting a synergistic interaction between them. Some researchers [20,60,75] pointed out that SRB are unable to break down complex organic substances. As a result, the observed diverse bacterial community structure and functional role of the corresponding partners could be the reason for the effectiveness of this bioremediation process [43].

Methanogenic species were not detected, which agrees with the absence of methane in the analyses. Although methanogenic bacteria must coexist with SRB under anaerobic conditions based on literature, sulfate-reduction is kinetically and thermodynamically more favorable than methanogenesis in the presence of adequate sulfate concentrations and for simple substrates [6,76]. Moreover, sulfides are well-known inhibitors of methanogenesis [77] but the experiment time was too short for this group of methanogenic bacteria to develop. Methanogenic bacteria could negatively influence SRB because they compete for the same electron donor [6,12,41].

4. Conclusions

- Shrimp shell, a low-cost residue, proves to be adequate chitin source for MIW treatment with respect to the removal of sulfate, Fe, Al, and Mn ions: SS is an adequate SRB biostimulation substrate and electron donor under the studied conditions, achieves a very high sulfate removal via sulfide formation, and allows the removal of metal ions via precipitation of metallic sulfides. The carbonates in the SS cause a pH increase to neutrality, which is adequate for microbiological development. In addition, SS is an abundant and low-cost waste material, which is available in large quantities due to the processing of the fishing industry in the SC State. The MIW treatment methodology adds value to a residue (SS) whose uncontrolled disposal usually generates environmental problems, providing to the process sustainability.
- In the conditions tested, contrarily to the established in the literature for chitin, the CHIT essay was inadequate for the complete development and establishment of SRB and, consequently, to achieve adequate MIW treatment, demonstrating the importance of the pH for the SRB development and removal of sulfate and metal ions.
- The Fe^{2+} is adsorbed by the sediment; the process follows pseudo-second order kinetics.
- Although SRB was detected (*Desulfosporosinus*), it was not the most abundant bacteria. *Citrobacter* and fermentative *Clostridium* were most abundant in the beginning and at the end of the log phase, respectively. Both species are endorsed in the literature: *Citrobacter* reduces sulfates and there is evidence of *Clostridium* being a SRB. Thus, both of the bacteria seem to be key players in the anaerobic degradation of sulfate. There might be a possible synergic relation between SRB and fermentative bacteria. Furthermore, the detected bacteria, which produce chitinases (*Citrobacter*, *Clostridium, Serratia, Klebsiella*, and *Enterobacter*), form the majority, indicating the co-operation in degrading chitin and thus synergistic interactions between SRB and fermentative (or chitin degrader) bacteria. Therefore, revealing diverse microbial communities will provide new insights in the relationship between the genera found in this work.

In order to verify the occurrence of sulfate-reducing activity, other strategies varying conditions, such as no sediment addition, previous removal of metals in solution and check other factors that diverge from the established for chitin (not) being suitable substrate for SRB are being carried out.

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