Journal of Environmental Chemical Engineering 14 (2026) 121731

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Enhancing sulfate-rich wastewater treatment through polyvinyl alcohol -immobilized sulfidogenic granules: Optimization of reactor configuration and bioaugmentation strategies

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ARTICLE INFO

Keywords:
Sulfate-containing wastewater
Sulfate reduction
PVA immobilization
Bioaugmentation
Glycerol oxidation
Anaerobic reactors

ABSTRACT

Sulfate-rich wastewater can pose significant environmental challenges due to its potential to acidify water bodies and disrupt ecosystems. This study evaluates the efficacy of polyvinyl alcohol (PVA)-immobilized sulfidogenic granules for sulfate removal and H_2S production in two continuous-flow reactor configurations, column reactor (CR) and continuous stirred tank reactor (CSTR). Using pure glycerol as electron donor, the CR demonstrated superior performance, removing > 95 % sulfate at initial loading rates ≤ 1.75 Kg S-SO_4^{2-}·m^{-3} ·d^{-1} , with minimal biomass washout (1.94 g volatile suspended solids (VSS)•L^{-1}in CR vs. 7.8 g VSS•L^{-1} in CSTR), setting a C/S ratio of 6.25 Kg O_2•Kg^{-1} SO_{42}^{-}. Parameter optimization minimized volatile fatty acids accumulation, with acetate constituting 92 % of total volatile fatty acids. After long-term operation (101 days), Desulfovibrio were enriched to 21.3 % of the total community whereas methanogens were almost fully removed (<0.03 %). After bioaugmentation with immobilized granules overgrown with sulfate-reducing and acetate-oxidizing bacteria (SRB-AO) acetate efflux was reduced by 54 %, highlighting the potential to control byproduct formation. The PVA granules achieved a sulfate removal rate of 0.19 Kg S-SO_4^{2-}·m^{-3} ·d^{-1} ·g VSS^{-1} ·L, which was similar to that of conventional suspended-growth sulfate-reducing systems (e.g., CSTR or flocculent UASB reactors) with fivefold higher biomass concentrations, while reducing startup time to 10 days. Total sulfide rate (including H_2S and HS^{-}) obtained at these conditions was 0.93 Kg•m^{-3}•d^{-1}. This work pioneers the integration of artificial granulation and granule bioaugmentation for scalable, highly-efficient sulfate removal, offering treatment options for industrial wastewater.

1. Introduction

Sulfate-rich wastewater streams are common, for example in industrial processes that use sulfuric acid or sulfite, such as the production of oil, fertilizer, paper, and pharmaceuticals as well as in metallurgy and seawater desalination [1–4]. In addition to these processes, the treatment of flue gases containing SO_2 also contributes to the generation of sulfate-rich waste streams [5–7]. These effluents are composed of a mixture of oxidized sulfur species (sulfite, thiosulfate, and sulfate) [8] that react to sulfate in air [9].

Sulfate-rich wastewater poses a significant threat to water-related ecosystems, as its improper discharge pollutes public water systems and endangers life. High sulfate concentrations not only acidify surface and groundwater but also degrade soil structure, ultimately reducing crop yields [8]. To mitigate these risks, many countries now enforce 'zero emission' policies for sulfate-rich wastewater [10], given that drinking water guidelines recommend sulfate levels not exceeding 300 mg SO_4^{-2} •L^{-1} [3].

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https://doi.org/10.1016/j.jece.2026.121731
Received 20 November 2025; Received in revised form 4 February 2026; Accepted 9 February 2026
Available online 10 February 2026
2213-3437/© 2026 Published by Elsevier Ltd.

Sulfate-rich wastewater has been traditionally treated using physicochemical methods such as chemical precipitation, ion exchange, and membrane filtration [11], which can be costly due to high operational costs [4,12]. Biological methods, especially anaerobic sulfate reduction with sulfate-reducing bacteria (SRB) together with the subsequent removal of sulfide, are becoming more sustainable alternatives [13]. Emerging technologies include coupling biological sulfate reduction with partial sulfide oxidation for elemental sulfur recovery [1,14]. These newer biological methods offer advantages like lower energy consumption, less sludge production, and the potential for resource recovery [8].

Sulfidogenesis has been studied in various reactor configurations and conditions [8]. Continuous Stirred Tank Reactors (CSTR) offer advantages such as reduced product accumulation, short start-up time as granulation is not needed, and high biomass yield from low solid retention time (SRT) [15], though biomass loss and low working volume remain to be challenging. Up-flow Anaerobic Sludge Blanket (UASB) reactors are widely applied [16]. These reactors rely on granular sludge containing a diverse microbial community that enhances the mechanical stability of the granules and the catalyzed functions [17,18]. Advanced reactor design enables high SRT and low hydraulic retention time (HRT), achieving high biomass concentration in small volumes [2]. However, UASB performance depends on sludge settling, which may decline under long-term operation due to poor granulation or sludge dispersion after pH and temperature changes [19]. The necessary time to start-up the reactor can be long, as it often requires weeks to fully develop granules; for instance, Wang et al. [20] observed large particles forming within 65 days, while Mora et al. [6] reported complete granulation within 50 days depending on organic loading rate (OLR) and biogas production.

Additionally, sulfide accumulation in the reactor can inhibit anaerobic bacteria and cause a system shutdown [2]. A failure of the sulfidogenic process can also be related to excessive growth of filamentous bacteria, gas entrapment due to substrate overload, presence of oil or grease in large amounts in the influent, or lack of mixing [15,16]. These problems can cause incomplete oxidation of the carbon source, leading to the accumulation of volatile fatty acids (VFAs), mainly acetic acid [17]. VFA accumulation can cause a decrease in pH inhibiting the activity of SRB [7]. Different strategies have been proposed to prevent these scenarios, including the thorough control of the COD/SO_4^{2-} ratio [18], the development of reactors with configurations that promote the complete oxidation of VFAs [7], the bioaugmentation with VFA-oxidizing bacterial species [19], or the development of immobilization methodologies, especially artificial granulation [20].

Artificial granulation is based on using polymeric matrices to encapsulate bacteria or enzymes, preserving their activity [24,25]. Several polymers have been studied to simplify biotechnological processes and reduce biomass stress [26,27]. Reported benefits include high biomass retention and concentration, stability and resistance, and protection of the bacteria against inhibitors [28,29]. Additional advantages are shorter start-up time, easier solid separation, and the possibility to extract biomass and preserve it at 3 ∘C for later use [2].

Hydrogel-forming materials are particularly attractive for wastewater treatment due to their non-toxicity, biocompatibility, and permeability [30]. Among them, PVA has received attention for its low cost and safety [28,29]. PVA-immobilized SRB have been applied to enhance heavy metal removal and it was shown that the increased activity was due to increased resistance against metal toxicity [30]. Di et al. [33] showed that PVA-immobilized SRB removed 94.1 % of sulfate and 84.4 % of manganese and ferrous ions from acid-mine drainage. However, PVA granules have been tested mainly in batch assays, and its performance in continuous-flow reactors are poorly studied. PVA has also been used to enrich slow-growing bacteria in Anammox stirred reactors, ensuring long-term stability [34].

Immobilization also provides important advantages in bioaugmentation [35], which involves introducing pure, mixed, or engineered cultures into activated sludge [36]. This approach is useful when influent compounds are toxic or when microbial diversity is inhibited [37], though adaptation, competition, and abiotic factors such as pH or temperature may limit performance [38]. Immobilization can protect cells from predation, reduces competition, and increases tolerance to adverse conditions [39]. Applications include simultaneous selenate and nitrate removal [40], denitrification [41], and degradation of polycyclic aromatic hydrocarbons [42]. Mohan et al. [43] studied SRB entrapped in alginate, improving COD removal from 35 % to 78 % and sulfate reduction from 27 % to 80 % in a lab-scale reactor. In this sense, bioaugmentation using an enriched culture of SRB was already assessed batch reactors in previous studies. However, bioaugmentation using a continuous flow reactor using artificial granules is a new alternative for improving sulfidogenic process.

Even though there are reports including advantages of immobilization method for improving performance of bacterial cultures, there are no evidence of using artificial granules using PVA to increase operational performance of a sulfidogenic process. This work aimed to improve sulfate removal rates in microbial reactors by immobilizing microbial populations using PVA, as well as overcoming disadvantages as long startup time, VFA accumulation and inhibition. Bioaugmentation was employed to overcome the remaining limitations of sulfate reduction processes that restrict their efficiency and applicability, such as acetate accumulation. In the first stage of this work, biomass immobilized on PVA matrices were prepared and inoculated into two different reactor configurations: Colum reactor (CR) and CSRT. Subsequently, sulfate removal efficiency (RE) and COD intake was monitored during sulfate reduction with glycerol as the electron donor. In the second phase of the work, operation conditions were optimized in CR to determine the maximum sulfate loading rate with sulfate RE above 90 %, while minimizing COD content and VFA concentrations in the effluent. Finally, an acetate-oxidizing sulfate-reducing enrichment was immobilized on a PVA matrix and amended to a sulfate-reducing reactor to evaluate the option to reduce the amount of acetate production. With the aim of support results of operational assays, characterization of microbial population was performed.

2. Methods

2.1. Analytical methods

Sulfate concentrations were determined using ion chromatography with conductivity detection, employing an ICS-1000 system (Dionex, United States) equipped with an Ultimate 3000 Autosampler and an IonPac AS18 column (ThermoScientific, United States). Glycerol concentrations in the media were determined as chemical oxygen demand (COD) following standard methods [21]. VFAs (formic acid, acetic acid, butyric acid, propionic acid and isobutyric acid) concentrations were quantified via high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 instrument (Dionex, USA) with an ICSep ICE-CPREGEL 87H3 column (Concise Separations, United States) and a UV-Vis detector set at 210 nm wavelength. To express all VFAs on a common basis, their measured concentrations were converted to chemical oxygen demand (COD) equivalents (mg $O_2 \cdot L^{-1}$) by applying the respective stoichiometric oxidation factor for each acid. H_2S was quantified in the effluent with an electrochemical needle-type sensor (SULF-NP, Unisense, Denmark). Biogas was not measured since minimal abundance of methanogenic archea was found. Sulfide data was not presented since variability of sulfide concentration are produced by pH fluctuations, these results are expressed as Total sulfur concentration. Protein was quantified with the Bradford method [22] and correlated with volatile suspended solids (VSS) measurements [21] to determine biomass concentration in media.

2.2. Inoculum immobilization

The inoculum was obtained from an anaerobic digestor at the Manresa and Sant Joan de Vilatorrada wastewater treatment plant (WWTP). Firstly, sulfate-reducing activity was enhanced through acclimation in sealed flasks using an anoxic mineral medium, which contained 3.5 g•L^{-1} K_2HPO_4, 0.5 g•L^{-1} NH_4Cl, 80 mg•L^{-1} SO_4^{-2} and glycerol (Molecular grade, Sigma Aldrich, Unite States) as the sole carbon source, to a COD of 500 mg COD•L^{-1}, according to Mora et al. protocol [31]. Glycerol stock solutions were prepared to achieve the target COD concentrations based on the theoretical COD-to-glycerol mass equivalence factor of 1.22 mg O_2 per mg glycerol, which was experimentally validated prior to reactor operation. Additionally, tap water and distilled water were mixed in a ratio 1:4 to supply trace elements. The pH of the mineral medium was adjusted to 7.5 and C/S ratio was set at 6.25 mg COD·mg^{-1} SO_4^{2-}.

PVA-biomass granules were prepared using high molecular weight polyvinyl alcohol (PVA) (Sigma Aldrich, United States) with a hydrolysis degree of 98–99 % and with a monomer molecular weight of 44.05 g·mol^{-1}. First, a 13.6 % w/w PVA solution was prepared and then blended in a 1:1 ratio with concentrated sludge (3.78 g VSS/L). Subsequently, the mixture was frozen at −15 ℃ for 24 h and finally cut into 5 mm side cubic-shaped granules. Immobilization protocol was obtained from [23] which includes fully physical an biological characterization of granules.

2.3. CR and CSTR experimental setup and operation

2.3.1. Evaluation of performance of CR and CSTR

To evaluate the performance of biomass in reducing sulfate to sulfide using glycerol as carbon source under different reactor configuration, as well as to test the stability of PVA granules during operation, a CR and CSTR were set up, both with a volume of 500 mL volume (Fig. 1a). CSTR stirring was set as 200 rpm. The reactors were inoculated with 100 mL of PVA-biomass granules (Fig. 1d) and fed with supplemented medium described in Section 2.2. The sulfate loading rate was increased from 0.2 to 4 Kg•$m^{-3}d^{-1}$, maintaining a C/S ratio of 6.25 Kg O_2•Kg^{-1} SO_4^{2-} in all the assay to avoid influence of this parameter. The reactors were operated with an HRT ranging from 6.4 to 3.4 h (Table 1). The reactors were operated for 101 days. pH and concentrations of sulfate, COD, H_2S, VSS and VFAs were measured in both the influent and the effluent. Error bars are not presented since samples were taken as one per day, every 2–3 days.

2.3.2. Optimization of operational conditions

The optimization of sulfidogenic activity was assessed to determine

Table 1
Operational conditions of CR and CSTR.

Period	Days	HRT (h)	Inlet sulfate concentration (mg S-SO_4^{2-}·L^{-1})	SLR (Kg S-SO_4^{2-}·L^{-1}·d^{-1})
I	1–13	6.4	26.6 ± 3.4	0.10
II	14–20	6.4	38.2 ± 2.7	0.14
III	21–26	6.4	48.7 ± 1.0	0.18
IV	27–38	6.4	71.6 ± 2.7	0.27
V	39–56	6.4	104.3 ± 1.3	0.39
VI	57–62	5	150.5 ± 3.8	0.72
VII	63–76	3.4	193.3 ± 4	1.41
VIII	77–82	3.4	178.3 ± 3.3	1.36
IX	83–89	3.4	138.1 ± 2.1	0.98
X	90–101	3.4	84.3 ± 3.0	0.59

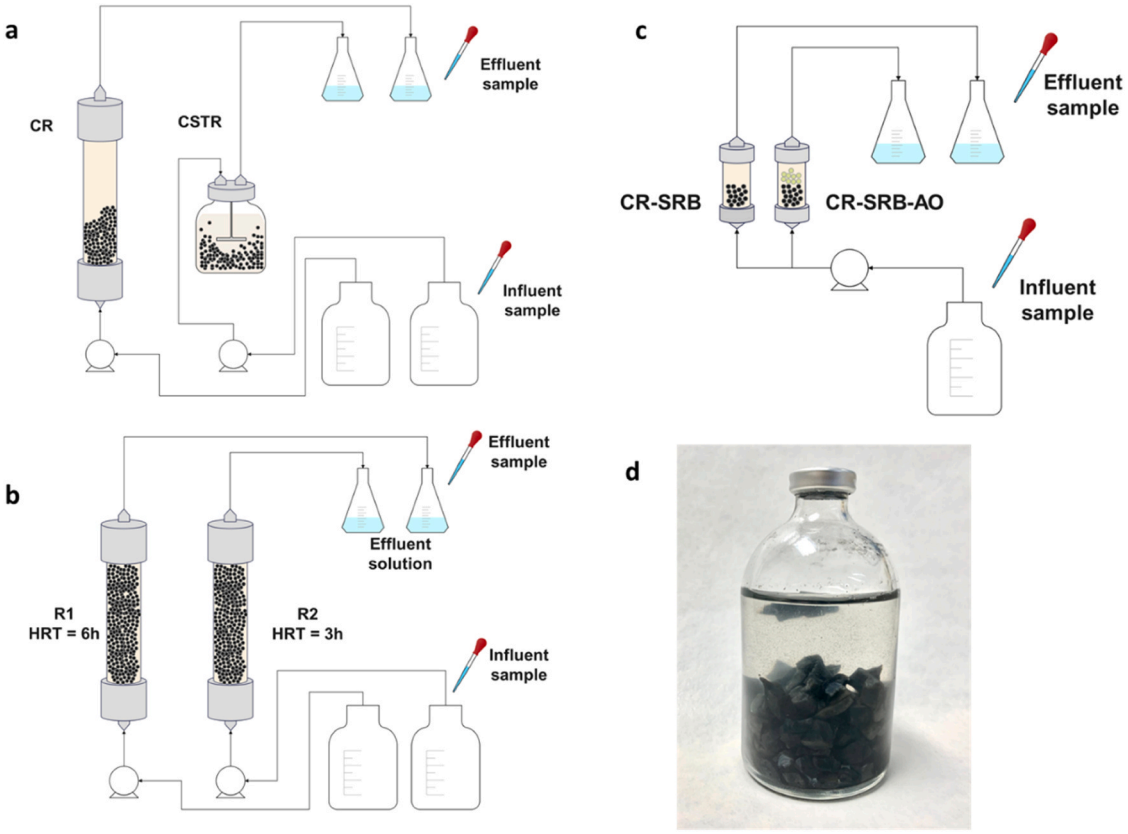


Fig. 1. Experimental setups used for the evaluation of artificial granules performance: (a) Diagram of the experimental system for evaluating the efficacy of sulfate reduction using a CR and a CSTR. (b) Diagram of the experimental system used for optimizing sulfate reduction in a CR. (c) Diagram of the experimental setup used for evaluating artificial immobilization as a bioaugmentation strategy. (d) Image of the PVA-SRB granules prepared with a 1:1 ratio of PVA.

the maximum sulfate loading rate that achieves more than 90 % of sulfate reduction while minimizing COD and VFAs concentration in the effluent. In this sense, C/S ratio and HTR were modified from Section 2.3.1 and set according previous studies on CR operation using natural granulation [6,34]. Specifically, two CR, designated R1 and R2, each with a volume of 500 mL, were inoculated with 300 mL of PVA-biomass granules and fed with the mineral medium described in Section 2.2 (Fig. 1c). Reactor R1 was operated with an HRT of 6 h and R2 with an HRT of 3 h. The sulfate loading rate (SLR) was increased from 35.2 to 130.3 mg $S\text{-}SO_4^{-2}\cdot L^{-1}\cdot h^{-1}$, with the C/S ratio varying from 4 to 7 mg $O_2 \bullet mg^{-1}$ SO_4^{2-} at each step (Table 2). The two reactors were operated for 70 days, during which pH, sulfate, COD, H_2S, and VFAs concentrations were measured both in the influent and in the effluent.

2.4. Bioaugmentation assay

A bioaugmentation assay was performed to decrease VFAs accumulation during CR operation, specifically acetate and propionate. For this purpose, an enriched culture of sulfate-reducing acetate-oxidizing bacteria (SRB-AO) from the same anaerobic digestor at the Manresa and Sant Joan de Vilatorrada WWTP was cultured at the Helmholtz Centre for Environmental Research-UFZ, Leipzig (Germany). The medium for SRB-AO cultivation was prepared following literature [24]. To enrich the sulfate-reducing sludge, 5 % of the sludge was inoculated into a fresh anoxic medium with a final volume of 30 mL. Sulfate concentration was monitored over time using ion chromatography. Once sulfate removal exceeded 50 %, 5 % v/v of the culture was transferred into fresh medium. This transfer was repeated twice, and scaling up was performed in the last transfer by inoculating 200 mL medium, again with a 5 % inoculum. After three transfers in 200 mL cultures, the culture was scaled up to a final volume of 500 mL.

Subsequently, pellets of the mixed SRB-AO cultures were used for immobilization onto PVA using the method described in Section 2.2. Bioaugmentation assays were carried out in 50 mL CRs inoculated with immobilized SRB (20 mL of overgrown granules) and the enriched culture of SRB-AO (Fig. 1c). Bioaugmentation strategy was evaluated by monitoring sulfate, COD and VFAs concentrations in the influent and effluent.

Table 2
Operational conditions of R1 and R2 used for optimization assay.

Operational conditions	R1 (HRT=6 h)	R2 (HRT=3 h)
Period I corresponding to C/S = 6.89 ± 0.06 mg $O_2 \bullet mg^{-1}$ SO_4^{2-}		
SLR (mg $S\text{-}SO_4^{-2}\bullet L^{-1}h$)	35.2	35.2
Inlet sulfate concentration (mg $S\text{-}SO_4^{-2}\bullet L^{-1}$)	194.3 ± 4.9	101.8 ± 9.4
Inlet COD concentration (mg $O_2 \bullet L^{-1}$)	1346.1 ± 10.4	696.1 ± 15.6
Period II corresponding to C/S = 4.02 ± 0.12 mg $O_2 \bullet mg^{-1}$ SO_4^{2-}		
SLR (mg $S\text{-}SO_4^{-2}\bullet L^{-1}h$)	35.2	35.2
Inlet sulfate concentration (mg $S\text{-}SO_4^{-2}\bullet L^{-1}$)	189.0 ± 13.4	100.1 ± 5.7
Inlet COD concentration ((mg $O_2 \bullet L^{-1}$)	736.6 ± 11.3	414.7 ± 10.2
Period III corresponding to C/S = 7.05 ± 0.05 mg $O_2 \bullet mg^{-1}$ SO_4^{2-}		
SLR (mg $S\text{-}SO_4^{-2}\bullet L^{-1}h$)	70.0	70.0
Inlet sulfate concentration (mg $S\text{-}SO_4^{-2}\bullet L^{-1}$)	415.0 ± 5.5	194.3 ± 4.9
Inlet COD concentration ((mg $O_2 \bullet L^{-1}$)	2943 ± 22.3	1359.9 ± 9.3
Period IV corresponding to C/S = 3.89 ± 0.06 mg $O_2 \bullet mg^{-1}$ SO_4^{2-}		
SLR (mg $S\text{-}SO_4^{-2}\bullet L^{-1}h$)	70.0	70.0
Inlet sulfate concentration (mg $S\text{-}SO_4^{-2}\bullet L^{-1}$)	413.3 ± 6.7	189.0 ± 13.4
Inlet COD concentration	1628 ± 27.3	723.4 ± 30.4
Period V corresponding to C/S = 7.02 ± 0.07 mg $O_2 \bullet mg^{-1}$ SO_4^{2-}		
SLR (mg $S\text{-}SO_4^{-2}\bullet L^{-1}h$)	130.3	130.3
Inlet sulfate concentration (mg $S\text{-}SO_4^{-2}\bullet L^{-1}$)	747.5 ± 9.4	419.0 ± 5.5
Inlet COD concentration	5195.4 ± 14.8	2967.2 ± 31.7
Period VI corresponding to C/S = 3.97 ± 0.09 mg $O_2 \bullet mg^{-1}$ SO_4^{2-}		
SLR (mg $S\text{-}SO_4^{-2}\bullet L^{-1}h$)	130.3	130.3
Inlet sulfate concentration (mg $S\text{-}SO_4^{-2}\bullet L^{-1}$)	741.3 ± 8.8	413.3 ± 6.7
Inlet COD concentration	2877.1 ± 23.6	1674 ± 19.5

2.5. Characterization of microbial communities

Microbial communities from all reactors were characterized by sequencing the 16S rRNA gene. First, the immobilized sludge attached to polyvinyl alcohol (PVA) carriers was disrupted using an ultrasonic probe (Sonoplus HD 400, Bandelin Electronic, Germany) until the biomass was completely suspended in the supernatant. Genomic DNA was then extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at −70 °C until further analysis. Next, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) using custom-designed fusion primers [25]. The resulting PCR amplicons were quantified with a 2100 Bioanalyzer (Agilent Technologies, United States) using the High Sensitivity DNA kit (Agilent Technologies, United States). Sequencing was subsequently performed on a Genestudio S5 platform (Life Technologies, United States) across three sequencing runs, employing the Ion 520 and Ion 530 Kit-Chef (Life Technologies, United States) for each experimental group. Raw sequencing reads were first subjected to quality assessment and filtering using the fastp program [25], applying a minimum read length of 50 bp and a minimum mean trimming quality score of 20 calculated over a 10 bp sliding window. Paired-end reads (R1 and R2) were then merged using the FLASH software [26] with default parameters. Finally, downstream bioinformatic analyses, including taxonomic assignment, statistical analysis, and data visualization, were carried out using the QIIME 2 platform (University of Colorado Boulder, United States).

3. Results and discussion

3.1. Study of reactor configurations

3.1.1. Long-term operation study

To analyze the sulfate reducing capacity and stability of PVA granules using glycerol as the sole carbon source, two reactors, a CR and a CSTR, were set up and operated over an extended period of time under identical sulfate loading rates, C/S ratios and HRTs. During the operation the loading rates of sulfate and glycerol were stepwise increased and the performance of the reactors was assessed by evaluating sulfate RE and glycerol oxidation, the latter measured as COD removal. A sulfate RE of 95 % was set as the target value to be achieved in the reactors.

Sulfate removal above 95 % was achieved in both reactors for increasing SLRs up to 0.72 Kg $S\text{-}SO_4^{2-}\bullet m^{-3}\bullet d^{-1}$ (Fig. 2) corresponding to periods I to VI (Table 1). Subsequently, in period VII, RE in CR and CSTR decreased to 38 % and 41 %, respectively, when SLR was increased to 1.36 Kg $S\text{-}SO_4^{2-}\bullet m^{-3}\bullet d^{-1}$. However, CR recovered a sulfate RE of 97 % after 10 days of operation in contrast with CSTR, which exhibited a maximum RE of 62 % for SLRs exceeding 1.0 Kg $S\text{-}SO_4^{2-}\bullet m^{-3}\bullet d^{-1}$ (during periods VII to IX). The difference in the behavior of the two reactors can be explained by the turbulence generated by agitation in the CSTR, which enhances the mass transfer rate through the granules, exposing the biomass to inhibitors such as H_2S and VFAs [27]. Inhibitors are present in high concentration while increasing inlet sulfate load, which can be detrimental to the process performance [28].

Conversely, COD was not completely removed in any of the reactors. COD RE ranged between 60 % and 80 % in the CR during the 100 days of operation, regardless of the sulfate loading rate. Similarly, the COD RE in the CSTR ranged between 90 % and 58 % throughout the entire operation.

This lower glycerol RE compared to sulfate RE was likely caused by the chosen high C/S ratio. This C/S ratio was chosen to guarantee the presence of carbon source to allow full sulfate conversion. This condition may lead to the accumulation of by-products from the partial oxidation of glycerol, such as volatile fatty acids (VFAs), which are widely reported as inhibitors that can trigger process failure [29]. To this aim, it was necessary to evaluate this phenomenon from VFAs quantification in both reactors during their operation.

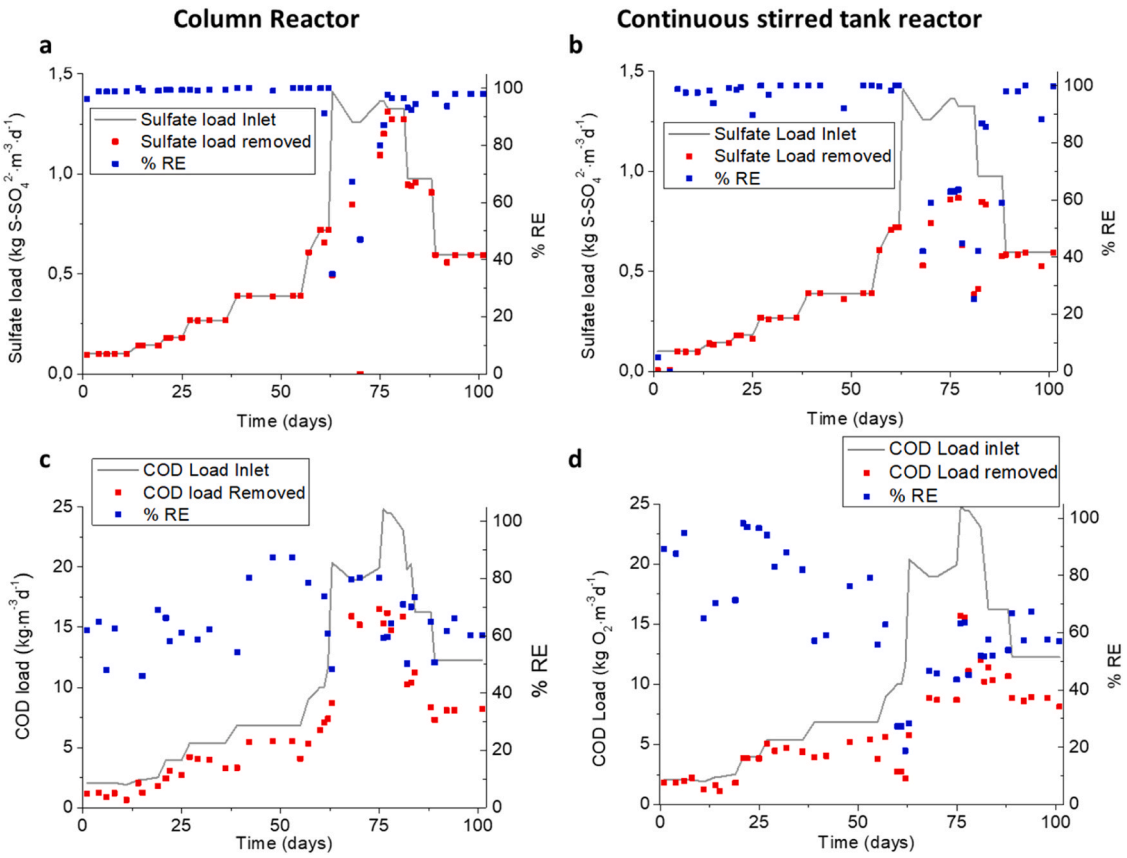


Fig. 2. Sulfate and COD removal performance in a,c) CR and b,d) CSTR.

3.1.2. *VFAs analysis*

In the next step we therefore investigated the accumulation of VFAs from C1 to C4 in both reactors (Fig. 3).

In general, the total concentration of VFAs at the outlet of both reactors increased when the glycerol loading rate increase (Fig. 3). However, accumulation effect can be observed since between 50 and 100 days, total VFA concentration increases but glycerol inlet concentration is constant. The accumulation of VFAs occurred in the form of low molecular weight VFAs such as formic acid, acetic acid, and propionic acid. This accumulation demonstrates the inhibition of microorganisms in the reactors that could utilize these compounds as electron donors for sulfate reduction. Additionally, it was observed that for the maximum COD loading rate (23.8 Kg $O_2 \bullet m^{-3} \bullet d^{-1}$), the concentration of VFAs at the outlet of the CR (1.62 mg O_2/L) was almost twice as high as in the CSTR (0.85 mg O_2/L). This indicates that operating in a better mixing system can help prevent the accumulation of by-products such as VFAs in the reactor media, which can be entrapped in PVA granules, as well as promoting stripping effect of VFAs due to an increase of mass transference between solid and gas phases [30].

3.1.3. *Study of granules degradation and wash out*

The stability of the PVA granules was analyzed by quantifying

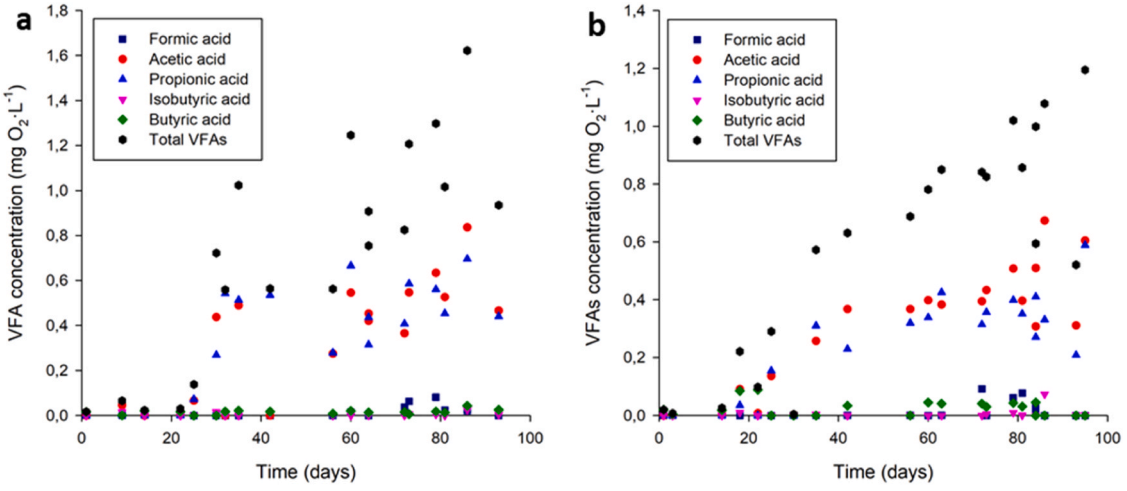


Fig. 3. Accumulation of VFAs over time in the two reactors. A) CR; b) CSRT. At day 50 the glycerol inlet concentration increase of VFAs accumulation could be observed due to an increase of COD load.

biomass in the reactor effluent (Fig. 4). These VSS values in the reactor effluent can be associated with the biomass released as a result of the partial degradation of the PVA granules. This data provided insights into the retention and washout of biomass, which are critical factors for maintaining reactor performance and stability.

The concentration of biomass, in the form of VSS, was measured at the outlet of CR and CSTR (Fig. 4). Samples were taken every 2–3 days and, with the objective of comparing the amount of biomass washed out in both reactors, cumulative VSS was calculated. The cumulated amount of biomass washed out in the CSTR over a time period of 100 days was 0.8 g VSS•L^{-1}, which was 3.3 times higher than the biomass washed out in the CR. The CSTR was inoculated with a total amount of 1.94 g VSS•L^{-1}. Therefore, these results indicate that during the entire operation according to VSS inoculated in the reactor, 4.0 % of the biomass was washed out from the CSTR. Conversely, in the CR, only 1.2 % of the biomass was washed out during the same period.

The larger biomass loss observed in the CSTR can be explained by the continuous mixing and higher shear forces which can lead to the disintegration of aggregates and subsequent release of biomass. This demonstrates that the PVA matrix can be a stable support for biomass retention but mechanical stirring can weaken its integrity [31], even though stirring speed was set to the minimum suitable. The degradation of PVA granules could be decreased by improving the mechanical strength of the used PVA, *e.g.*, by increasing PVA concentration in the polymer matrix, however, this can also be detrimental for mass transfer [32]. Moreover, CR has the advantage of allowing the increase of working volume. Regarding these results, the CR was selected as the superior configuration for the operation of PVA granules in the sulfate reduction process due to its higher efficiency and stability over an extended operational period, with less biomass loss and better sulfate RE under high loading conditions.

3.1.4. Microbial communities

Finally, the effect of long-term operation on the composition of microbial communities was evaluated using 16*S* rRNA gene sequencing. Overall, strong shifts in the microbial community were observed in both reactors after 100 days of incubation compared to the microbial community in the inoculum (Fig. 5). In contrast, the differences between the two reactors after long-term operation was less pronounced. Probably the largest shift was the loss of *Methanobrevibacter* populations in both reactors but also *Acetobacterium* populations were lost. In contrast, *Desulfovibrio* populations strongly increased in both reactors. These shifts can clearly be affiliated with the continuous supply of sulfate that supported growth of SRB as previously observed [33,34]. The presence of sulfate also suppressed methanogenesis and homoacetogenesis which both rely on the presence of H_2. Such washing out of methanogenic

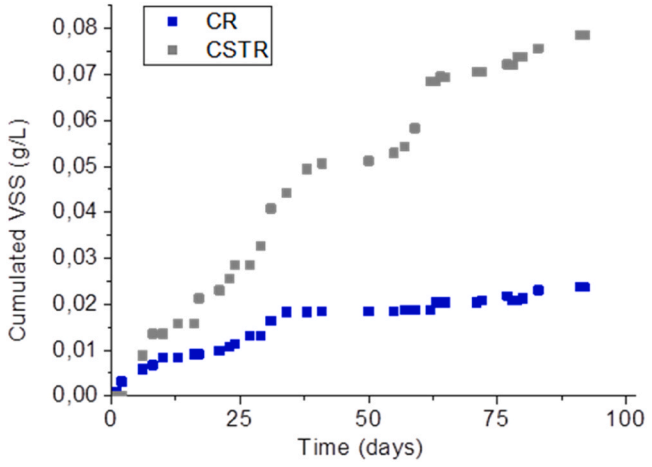


Fig. 4. Cumulated VSS in the outlet of CR and CSTR.

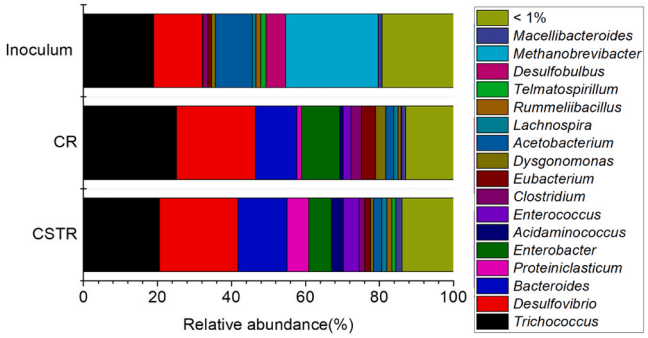


Fig. 5. Characterization of microbial communities in the inoculum and after 100 days of incubation in CR and CSTR.

Archaea has been described in other sulfidogenic reactors [35,36]. However, it was described that some *Methanobrevibacter* strains can use organic acids [37], but high sulfate concentrations and a slightly alkaline pH (7.5) has previously reported to inhibit methanogenic archaea [38,39]. In addition to this effect, it should be considered that immobilization methods can promote the selection and enrichment, as observed in [40,41], using PVA and alginate granules for the enrichment of anammox communities. The relative abundance of the genera *Bacteroides* and *Enterobacter* increased strongly. Both these genera have been described to be involved in VFAs production, mainly acetate [42,43], and are commonly observed in sulfidogenic reactors. The specific role of *Enterobacter* in glycerol and butyrate fermentation has been described [44,45]. Similarly, the role of *Bacteriodes* in VFAs production at pH values around 7.2 has been highlighted [46]; this pH was similar to the working pH used in the present work. Bacteria of the genus *Trichococcus* had constantly a share of the total microbial populations in all analyses (inoculum 19.0 %, CR 25.2 %, CSTR 20.6 %) indicating a persistent important role in the community. *Trichococcus* plays an important role as it can metabolize glycerol to generate VFAs and alcohols, which could be used as carbon source by SRB for sulfide production [47].

3.2. Improvement of operational conditions in column reactors

3.2.1. Sulfate reduction and COD intake at different loads

In the second phase of the study, the operation of the heterotrophic sulfate reduction process was optimized using glycerol as the sole carbon source in a column reactor. Therefore, two additional CR were set up (R1 and R2), operated at two different HRT (6 h for R1, 3 h for R2), and sulfate reduction was assessed. During the operation, the loading rate was gradually increased to determine the maximum sulfate loading rate that resulted in > 90 % sulfate reduction, lowest COD concentration in the effluent and still avoiding VFA accumulation. Simultaneously, two different C/S ratios (4 and 7 mg COD·mg^{-1} SO_4^{2-}) were tested.

Very small differences in the sulfate removal were observed between the two reactors (Fig. 6a). During reactors operation, sulfate RE above 90 % were obtained for sulfate load below 1.75 Kg S-SO_4^{2-}•m^{-3}•d^{-1}, regardless of the HRT. However, the RE decreased to values between 49.7 % and 64.6 % for a loading rate of 3 Kg S-SO_4^{2-}•m^{-3}•d^{-1}. The HRT had a minor influence on the sulfate RE. Only at high sulfate loading rates (above 2.7 Kg S-SO_4^{2-}•m^{-3}•d^{-1}) differences were observed when working with a C/S ratio of 4 and 7 mg COD·mg^{-1} SO_4^{2-}. At a C/S ratio of 7 mg COD·mg^{-1} SO_4^{2-}, R1 and R2 achieved sulfate removal rates of 1.88 and 1.99 Kg S-SO_4^{2-}•m^{-3}•d^{-1}, respectively, whereas these values were at 1.45 and 1.51 Kg S-SO_4^{2-}•m^{-3}•d^{-1} at a C/S ratio of 4 mg COD·mg^{-1} SO_4^{2-}.

These results suggest that working at high C/S ratios improves the process performance. Similarly, Mora et al. [33] obtained best sulfate removal at a C/S ratio > 7 mg O_2•mg^{-1} SO_4^{2-} in a reactor fed with glycerol. However, a high C/S ratio can lead to poor COD removal and VFAs accumulation [48]. In our study, complete sulfate removal was achieved for low sulfate loading rates (0.78 Kg S-SO_4^{2-}•m^{-3}•d^{-1}) in both

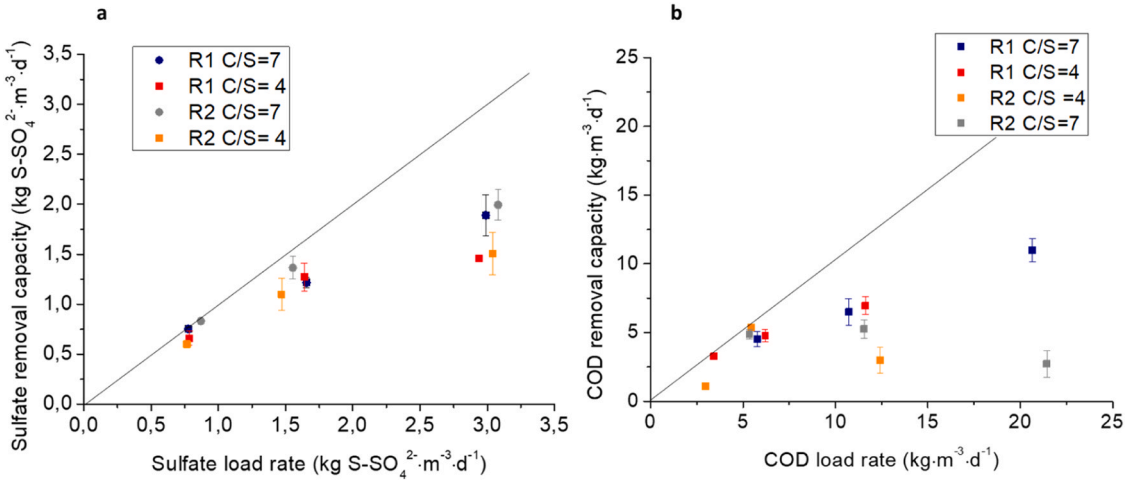


Fig. 6. Sulfate and COD removal in R1 (HTR = 6 h) and R2 (HTR = 3 h). The diagonal line represents 100 % removal. a) Sulfate removal capacity b) COD removal capacity.

reactors, which was a lower sulfate loading rate than applied in other studies investigating sulfate reduction with glycerol as electron donor. Nevertheless, biomass concentrations in R1 and R2 were 5 times lower than biomass concentration in reactors inoculated with natural granules [62]. In this sense, Fernandez-Palacios et al. (2019) [36] obtained a sulfate RE of 96 % at a sulfate loading rate of 4.3 Kg S-SO_4^{2-}•m^{-3}•d^{-1}, in a system operated with an overall biomass concentration of 28.0 g VSS•L^{-1}. In the present study, granules inoculated into R1 and R2 resulted in an initial biomass concentration of 4.12 g VSS•L^{-1} in the reactors. Bertolino et al. (2014) [49] achieved an almost complete sulfate removal in a sulfidogenic UASB reactor at a loading rate of 4.2 Kg S-SO_4^{2-}•m^{-3}•d^{-1} in a system with a biomass concentration of 25.5 g VSS•L^{-1}. Finally, a specific sulfate reduction rate of 0.15 (Kg S-SO_4^{2-}•m^{-3}•d^{-1} /g VSS•L^{-1}) was found in these studies, compared to 0.19 obtained in the present work. Therefore, these results demonstrate that the sulfate removal rate of the CR system presented in this work is equivalent to or slightly superior to the rates in previous studies. Although biomass concentration in PVA granules was low, this study improved biomass retention in a column up-flow reactor without compromising sulfate removal activity. Moreover, immobilization of biomass in PVA granules increased stability and allowed a start-up period of 10 days.

In regard to glycerol removal, results indicate that the only scenarios leading to almost complete consumption were obtained for low glycerol loading rates, below 7 Kg O_2•m^{-3}•d^{-1}, regardless of the C/S ratio and the HRT used (Fig. 6b). It was not possible to achieve COD RE above 60 % for higher loading rates. Specifically, COD RE values between 59.9 % and 60.5 % were obtained for R1, and between 24.0 % and 45.5 % for R2. These results demonstrate that the reduction of HRT decreases the glycerol removal rate of the system. Besides, these results agree with data reported in a previous study [50], indicating that low HTR increases COD in the effluent since it was not possible to achieve complete oxidation of the amended carbon source. In addition, low COD removal could generate an accumulation of by-products from glycerol fermentation, mainly VFAs that could cause inhibition of sulfidogenic activity.

For this reason, in order to assess the accumulation of process-inhibiting compounds, the concentration of VFAs was monitored at the outlet of both reactors.

3.2.2. *VFAs degradation and sulfide production*

VFAs concentration was measured during R1 and R2 operation (Table 3). According to the results presented in Fig. 3, only acetic and propionic acids were included in the monitoring of VFAs, as a result of the accumulation of byproducts from glycerol partial oxidation. Samples were taken for each period and VFAs production rate were calculated.

To assess the accumulation of process-inhibiting compounds, the concentration of VFAs was monitored at the outlet of both reactors. The accumulation of the different VFAs measured in the effluent of both reactors, presented in Table 3, is explained by the incomplete oxidation of the carbon source used. Thus, the metabolic pathways of glycerol under anaerobic conditions present two main routes in sulfate-reducing environments [34]. On the one hand, fermentation to 1,3-propanediol, which produces butyrate, acetate, and H_2; and on the other hand, the dihydroxyacetone pathway, which leads to pyruvate, subsequently convertible to acetate and propionate. Furthermore, it was observed that acetic acid accumulated to a greater extent than propionic acid, accounting for more than 92 wt% of the total VFA accumulation, due to the thermodynamically favored production of acetate.

Therefore, acetate is a common end-product of fermentative processes as well as glycerol-fed sulfate-reducing pathways, while only a limited number of sulfate-reducing bacteria (SRB) are able to directly

Table 3
Results of VFAs monitoring in R1 (HRT=6 h) and R2 (HRT=3 h) effluent, under different operational scenarios.

Sulfate Load[a]	Acetic acid[b]	Propionic acid[b]	Glycerol[b]	Total[b]	Sulfate Load[a]	Acetic acid[b]	Propionic acid[b]	Glycerol[b]	Total[b]
R1 C /S = 7					**R2 C/S= 7**				
0.78	1.48	0.62	1.10	3.19	0.87	0.81	0	2.36	3.17
1.66	2.26	1.67	2.79	6.42	1.56	2.82	0.83	6.15	9.81
2.99	2.43	1.69	3.16	7.36	3.02	4.06	1.09	7.30	12.5
R1C/S = 4					**R2C/S= 4**				
0.78	0.247	0	0.86	1.12	0.766	0.57	0	0.47	0.58
1.64	0.83	0.22	1.09	2.24	1.47	1.47	0	4.29	5.76
2.94	2.08	1.31	1.75	5.14	3.04	2.95	0.22	5.91	9.08

[a] Results are expressed as Kg S-$SO_4$2-•m-3•d-1.
[b] Results are expressed as Kg•m-3•d-1. For each VFA, Kg (VFA)•m-3•d-1

utilize acetate. In contrast, propionate is an intermediate that can be converted to acetate in both fermentative and sulfate-reducing pathways [34]. Increasing byproducts accumulation was observed in both reactors when the COD loading rate was increased to maintain C/S ratio under higher SLRs.

Additionally, the results demonstrate an influence of the HRT on the extend of full glycerol oxidation and, consequently, on the accumulation of VFAs (Table 3). Specifically, the effluent glycerol concentration was significantly higher in the reactor operated at an HRT of 3 h (median=4.80 Kg•m^{-3}•d^{-1}) than in the reactor operated at an HRT of 6 h (median=1.43 Kg•m^{-3}•d^{-1}), as determined by one-tailed Mann-Whitney *U* test (U=4.0, p = 0.012). Beyond the differences in glycerol efflux rate, the HRT also impacted VFA accumulation. Notably, VFA accumulation was 34.6–61.1 % higher across all operational scenarios when the HRT was reduced from 6 h to 3 h.

In both reactors, the concentration of sulfide in the effluent was dependent on the sulfate loading rate. Highest sulfide production was observed at a C/S ratio of 7 mg O_2•mg^{-1} SO_4^{2-}, which aligns with the findings reported in Section 3.2.2 (Table 4). However, across all operational scenarios, sulfide production was 11.7–54.3 % lower than the sulfate removal rate. This sulfur species imbalance can be explained by the gas-liquid equilibrium of sulfide species. Assuming equilibrium between aqueous and gaseous phases, the fraction of total dissolved sulfide present as volatile H_2S (aq) was estimated from the measured pH range (7.0–8.0) and the pKa of H_2S (≈7.0 at 35 °C). Across this pH interval, the calculated H_2S(aq) fraction varied from approximately 50 % at pH 7.0 to about 9 % at pH 8.5 [51,52]. These findings are consistent with experimental observations, where bubble formation on the surface of PVA granules was detected, indicating gaseous H_2S desorption into the gas phase [53].

3.2.3. Microbial communities

The impact of the HRT on microbial community composition was assessed through 16*S* rRNA gene sequencing in reactors R1 and R2 at the end of the operational period. Overall, the results revealed only modest differences between the two reactors (Fig. 7), which were inoculated using PVA granules obtained from Section 3.2.1. SRB were present in both reactors, affiliated to the genus Desulfovibrio (15.0 % in R1 vs 17.5 % in R2). Also, both reactors contained high proportions of Enterobacter (19.4 % in R1 vs 21.4 % in R2), a genus previously described as a thiosulfate reducer in an anaerobic system [54]. The slightly higher abundances of *Desulfovibrio* and *Enterobacter* in reactor R2 correlate with the enhanced sulfate RE in R2compared to R1 (Section 3.2.1). Results also show that R1contained higher relative abundance of Acinetobacter and γ-proteobacteria than R2, correlating with a higher VFA degradation efficiency in R1. Both genera have been associated with VFA oxidation in the past ([55−57], respectively).

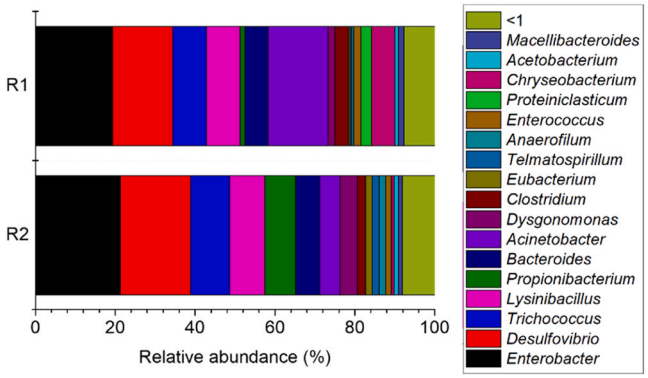


Fig. 7. Characterization of microbial communities in column reactors R1 and R2.

3.3. Bioaugmentation assay

While complete oxidation of glycerol by SRB is a desirable pathway, it was not observed in our system. As suggested by [34], glycerol is not directly oxidized by sulfate-reducing bacteria (SRB). Instead, it is first fermented by fermentative bacteria present in our system into intermediates such as 1,3-propanediol, ethanol, formate, propionate, and acetate. Subsequently, incomplete-oxidizing SRB (mainly *Desulfobulbus* and *Desulfovibrio*) utilize these intermediates, except acetate, as electron donors to reduce sulfate (SO_4^{2-}) to sulfide (H_2S). This incomplete oxidation mechanism explains why acetate tends to accumulate in sulfidogenic systems fed with glycerol.

For this reason, a bioaugmentation assay was performed to increase VFAs metabolization, especially acetate and propionate, during CR operation. Therefore, a mixed culture of SRB-AO was cultured and immobilized as PVA granules. Such granules were then inoculated into a 50 mL column reactor (CR-SRB-AO). A control reactor with PVA-SRB granules without acetate-oxidizing bacteria (CR-SRB) was set up and operated under identical operating conditions: HTR of 6 h, C/S ratio of 4 mg O_2•mg^{-1} SO_4^{2-} and a sulfate loading rate of 125 mg S-SO_4^{2-}•L^{-1}•h^{-1}. RE of sulfate, COD and VFAs were monitored throughout 30 days of operation.

3.3.1. Monitoring of VFAs during reactors operation

VFAs concentration during operation of CR-SRB and CR-SRB-AO, in order to evaluate VFAs degradation using bioaugmentation, was monitored (Fig. 8.a). The results show that the amendment with SRB-AO led to lower acetate concentrations compared to the CR-SRB reactor, amounting to a final acetate concentration of 45.3 ± 15.2 mg•L^{-1} (54 % of the acetate in the CR-SRB). No significant difference in propionate concentration was observed between the two reactors, as determined by an independent samples *t*-test (R1: 2.02 ± 1.78 mg·L^{-1}; R2: 3.28 ± 2.00 mg·L^{-1}; t = –1.16, p = 0.274). These results, demonstrate that

Table 4
Average sulfide production in R1 (HRT = 6 h) and R2 (HRT = 3 h) effluent, under different operational scenarios.

S-Sulfate Load[a]	S-Sulfate removed[a]	Total S-sulfur produced[b]	Total sulfur produced/ Sulfate removed[c]	S-Sulfate Load[a]	S-Sulfate removed[a]	Total S-sulfur produced[b]	Total sulfur produced/ Sulfate removed[c]
R1C /S = 7				**R2C/S = 7**			
0.78	0.75	0.67	0.89	0.87	0.83	0.50	0.60
1.66	1.22	0.82	0.67	1.56	1.36	0.86	0.63
2.99	1.89	0.99	0.52	3.02	1.99	0.91	0.45
R1C/S = 4				**R2 C/S = 4**			
0.78	0.66	0.58	0.88	0.77	0.60	0.27	0.45
1.64	1.27	0.89	0.70	1.47	1.09	0.61	0.55
2.94	1.46	0.93	0.64	3.04	1.51	0.69	0.46

[a] Results are expressed as Kg S-$SO_4$2-•m-3•d-1.
[b] Results are expressed as Kg•m-3•d-1.
[c] Results are expressed as Kg S-SO_4^{2-}•m^{-3}•d^{-1}/ Kg•m^{-3}•d^{-1}

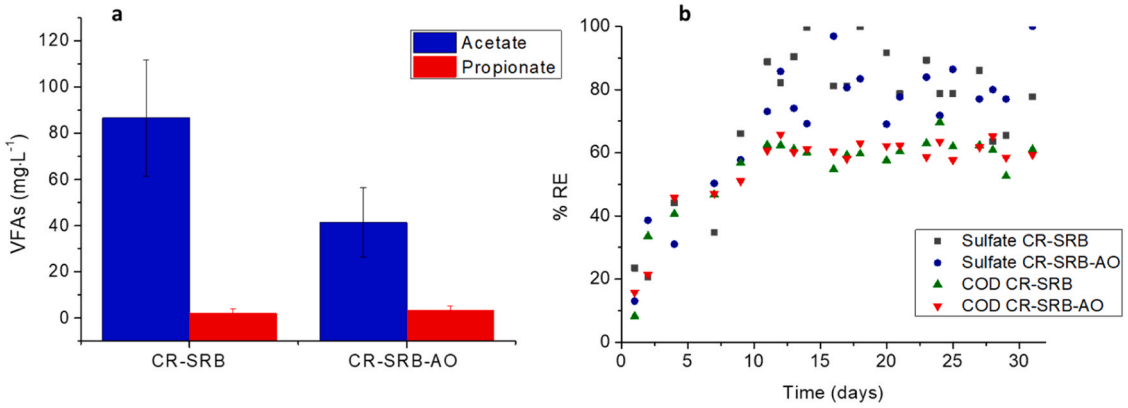


Fig. 8. a) Acetate and propionate concentrations in reactors inoculated with either original sludge ("CR-SRB") or with sludge bioaugmented with sulfate-reducing and acetate-oxidizing bacteria ("CR-SRB-AO").b) Removal of sulfate and COD during time during the operation of CR-SRB and CR-SRB-AO.

propionate oxidizing bacteria was not enriched. Also sulfate removal (83.6 % ± 10.5 % in CR-SRB vs 82.8 % ± 9.6 % in CR-SRB-AO) and COD removal (61.2 % ± 4.5 % in CR-SRB vs. 60.6 % ± 2.1 % in CR-SRB-AO) during the operation time at steady state were similar in the two reactors (Fig. 8.b). Since COD from added glycerol was not completely removed, the COD value cannot be used to differentiate VFAs consumption. As was described in results of Section 3.2.2, glycerol was present in the effluent of the CR using an HTR of 6 h and a C/S ratio of 4 mg O_2•mg^{-1} SO_4^{2-}. A decrease of acetate concentration in the effluent could have a crucial effect in a long-term operation of a CR-systems as the solution for achieving a full oxidation of glycerol.

Bioaugmentation has been described as a viable strategy to enhance the performance of anaerobic reactors, promoting the production of target compounds while reducing inhibitory effects [58–60]. However, challenges such as microbial survival, adaptation to reactor conditions, and competition with indigenous microorganisms have frequently been reported [61]. To date, the application of bioaugmentation with immobilized microorganisms in sulfidogenic reactors has been limited but one study employed a sulfidogenic sequencing batch biofilm reactor (SBBR) and inoculated it with an alginate-immobilized sulfidogenic consortium [28]. The results demonstrated that immobilization protected the added consortium from predation, ensuring stable reactor performance throughout the operational period. To the best of our knowledge, we here present the first investigation of bioaugmentation using artificially synthesized granules in a continuous-flow sulfidogenic completely mixed reactor (CR). The scalability of this process represents a promising alternative for future studies, supported by evidence from bioaugmentation assays conducted beyond laboratory-scale reactors using immobilized polymer granules [62]. Besides, there is potential to increase the acetate reduction rate by combining enriched microbial communities with the targeted addition of specific strains during the bioaugmentation process.

3.3.2. *Microbial communities*

To understand bioaugmentation effect using an enriched mixed culture of SRB-AO, as well as confirming that the reduction of VFAs in effluent was caused by SRB-AO introduction, a 16*S* rRNA gene sequencing assay was performed at the end of the operation period using samples of both reactor CR-systems (Fig. 9). The analysis of the results confirmed the presence of sulfidogenic bacteria in both samples. The genus *Desulfovibrio* was detected in the sludge and SRB-AO samples, representing 9.3 % and 7.4 % of the microbial community, respectively. However, the SRB-AO inoculum also contained VFA-oxidizing microorganisms such as *Pseudomonas*, *Comamonas* and *Flavobacterium* genus, which facilitated the complete metabolization of acetate into CO_2. Notably, the *Pseudomonas* genus exhibited a higher relative abundance in the SRB-AO granules. This genus has been previously identified as

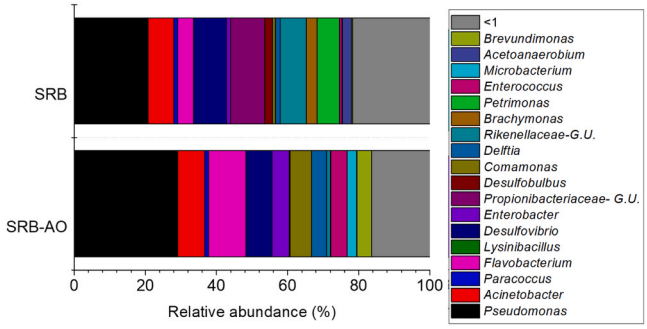


Fig. 9. Characterization of microbial communities in CR-SRB and CR-SRB-AO.

sulfate-reducing bacteria capable of remaining active at pH 9 [63]. Additionally, certain γ-proteobacteria, including *Pseudomonas*, have been reported as acetate oxidizing bacteria, suggesting their potential role in acetate degradation [57]. Furthermore, the *Flavobacterium* genus was detected at a high relative abundance in CR-SRB-AO. This genus comprises denitrifying bacteria capable of utilizing organic substrates for metabolic reactions [64]. Similarly, *Comamonas*—another genus with documented denitrifying activity—can use acetic acid as an electron donor [57]. The increased relative abundance of these genera can be attributed to the specific culture conditions to which the inoculum was exposed. Under long-term operation, these microbial groups are expected to persist in the reactor due to the sustained availability of acetate and propionate, as well as the prevailing sulfidogenic conditions.

Results of this lab-scale study demonstrated that bioaugmentation using an enriched mixed culture immobilized in artificial PVA granules can be a suitable method for decreasing byproducts such as acetate to avoid long-term system failure.

4. Conclusions

This study demonstrates that PVA-immobilized granules successfully achieved the objective of establishing a robust, high-rate sulfidogenic process in a continuous reactor. The system enabled > 95 % sulfate removal at loading rates exceeding 1.75 Kg S-SO_4^{2-}·m^{-3} ·d^{-1} , with superior biomass retention and mechanical stability compared to a CSTR. Optimal performance was found at an HRT of 6 h and a C/S ratio of 4 mg O_2·mg^{-1} SO_4^{2-}. Bioaugmentation with an SRB-AO consortium reduced inhibitory acetate accumulation by 54 %, confirming that targeted microbial enrichment can steer the process toward complete oxidation. The enriched community was dominated by *Desulfovibrio* (21.25 %), while methanogens were suppressed (<0.03 %). Future work should focus on optimizing granule cross-linking for long-term

durability and integrating real-time microbial monitoring for adaptive bioaugmentation in industrial applications.

CRediT authorship contribution statement

Rebeca Castro: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lorenz Adrian:** Writing – review & editing, Conceptualization. **Gemma Gabriel:** Writing – review & editing, Validation, Supervision. **David Gabriel:** Writing – review & editing, Project administration, Funding acquisition. **Xavier Gamisans:** Writing – review & editing, Project administration, Funding acquisition. **Xavier Guimerà:** Writing – original draft, Validation, Supervision, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors acknowledge the Spanish Government for the financial support provided to perform this research, through the project PID2021–126253OB-C22 funded by MCIN/AEI /10.13039/501100011033/ FEDER, UE.

Data availability

Data will be made available on request.

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