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Chungman Moon  
*University of Windsor*

Rajesh Singh  
*University of Windsor*

Sathyanarayan S. Veeravalli  
*University of Windsor*

Saravanan R. Shanmugam  
*University of Windsor*

Subba Rao Chaganti  
*University of Windsor*

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Effect of COD:SO$_4^{2-}$ Ratio, HRT and Linoleic Acid Concentration on Mesophilic Sulfate Reduction: Reactor Performance and Microbial Population Dynamics

Chungman Moon $^{1,†}$, Rajesh Singh $^{1,‡}$, Sathyanarayan S. Veeravalli $^1$, Saravanan R. Shanmugam $^1$, Subba Rao Chaganti $^2$, Jerald A. Lalman $^{1,*}$ and Daniel D. Heath $^{2,3}$

1 Department of Civil and Environmental Engineering, University of Windsor, 401 Sunset Ave., Windsor, ON N9B 3P4, Canada; E-Mails: chungman.moon@gmail.com (C.M.); rajesh.singh@cug.ac.in (R.S.); sevilim@uwindsor.ca (S.S.V.); ramiah@uwindsor.ca (S.R.S.)

2 Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Ave., Windsor, ON N9B 3P4, Canada; E-Mails: chaganti@uwindsor.ca (S.R.C.); dheath@uwindsor.ca (D.D.H.)

3 Department of Biological Sciences, University of Windsor, 401 Sunset Ave., Windsor, ON N9B 3P4, Canada

† Current address: Korea Institute of Energy Research, 152 Gajeong-ro, Yuseong-gu, Daejeon 305343, Korea.

‡ Current address: Central University of Gujarat, School of Environment and Sustainable Development, Sector-30, Gandhinagar 382030, India.

* Author to whom correspondence should be addressed; E-Mail: lalman@uwindsor.ca; Tel.: +1-519-253-3000 (ext. 2519); Fax: +1-519-971-3686.

Abstract: Biological sulfate (SO$_4^{2-}$) reduction was examined in anaerobic sequential batch reactors (ASBRs) operated under different hydraulic retention times (HRTs) ranging from 12 to 36 h and COD (Chemical Oxygen Demand)/SO$_4^{2-}$ ratios of 2.4, 1.6 and 0.8. Competition between SO$_4^{2-}$ reducing bacteria (SRBs), methane producing archaea (MPAs) and homoacetogens (HACs) was examined in controls and cultures treated with linoleic acid (LA). The ASBR performance was influenced by the COD/SO$_4^{2-}$ ratio in control cultures with a SO$_4^{2-}$ reduction of 87% at a COD/SO$_4^{2-}$ ratio of 0.8. At a 12 h HRT, in both control and LA treated cultures, greater than 75% SO$_4^{2-}$ removal was observed.
under all the conditions examined. In control reactors operating at a 36 h HRT, high levels of MPAs belonging to *Methanobacteriales* and *Methanosarcinales* were detected; however, in comparison, under low COD/SO₄²⁻ ratio and with decreasing HRT conditions, a relative increase in SRBs belonging to *Desulfovibrio* and *Desulfatibacillum* was observed. Adding 0.5 g·L⁻¹ LA suppressed *Methanobacteriales*, while increasing the LA concentration to 1 g·L⁻¹ completely suppressed MPAs with a relative increase in SRBs. HACs belonging to Bacteroidetes were observed in the control and in cultures operated at 12 h HRT with a COD/SO₄²⁻ ratio of 1.6 and fed 0.5 g·L⁻¹ LA; however, with all other LA levels (0.5 and 1.0 g·L⁻¹) and HRTs (12, 24 and 36 h), HACs were not detected.

**Keywords:** sulfate reduction; sulfate reducing bacteria; methanogens; anaerobic sequencing batch reactor; COD/SO₄²⁻ ratio

1. Introduction

Sulfate (SO₄²⁻), an abundant anion in the environment, is discharged in effluents from various industrial sectors including edible oil processors, tannery operations, food processors and pulp and paper mills. Typically these effluents contain chemical oxygen demand (COD) and SO₄²⁻ concentrations ranging from 0.5 to 50 g·L⁻¹ and 0.3 to 7 g·L⁻¹, respectively [1,2]. In another sector such as mining, the management of sulfide ores is important as oxidation of ores exposed to precipitation results in the production of acid mine drainage which can cause severe environmental damage when discharged into receiving water bodies [1,3].

Many studies have employed up-flow anaerobic sludge blanket reactors (UASBRs) and continuous stirred tank reactors (CSTRs) to treat effluents containing high COD and SO₄²⁻ concentrations [4–6]. However, using anaerobic sequential batch reactors (ASBRs) is advantageous because the reactor configuration allows for treatment in a single tank without the need for a final clarifier. In addition, the important features of ASBRs include a lower cost reactor configuration combined with higher organic removal efficiency.

Advantages of employing mixed anaerobic cultures when compared to pure cultures include the ability of sourcing cultures from natural ecosystems and engineered bioreactors, ability to operate under non-sterile conditions, capability of adapting to fluctuating the operational conditions and the potential ability to utilize a variety of substrates. According to Colleran *et al.* [7] and Muyzer and Stams [8] using mixed anaerobic cultures is associated with a major challenge of enriching SO₄²⁻ reducing bacteria (SRBs) which compete with methane producing archaea (MPAs) and homoacetogens (HACs) for substrates such as H₂ and acetate. Evidence by Schonheit *et al.* [9] has shown that SRBs have a greater affinity for acetate when compared to MPAs. In comparison, studies by Isa *et al.* [10] using anaerobic filters continuously operating at a high organic loading concluded that 15% of the acetate added was utilized for SO₄²⁻ reduction while as much as 85% was converted into methane. Both the SRBs and MPAs are able to utilize acetate as the major source of organic carbon and energy source under anaerobic conditions [8]. A reaction sequence showing the microbial degradation of
organic matter in an anoxic environment in the presence and absence of SO$_4^{2-}$ is shown in Figure 1. Also shown are H$_2$ and acetate consumption reactions by SRBs, MPAs and HACs.

![Diagram of organic compound degradation](image)

**Figure 1.** Pathways of organic compound degradation under methanogenic and sulphidogenic conditions (Colleran *et al.* [7]). Notes: VFAs = volatile fatty acids; H$_2$S = hydrogen sulfide; CH$_4$ = methane; HPr = propionic acid; HLa = lactic acid; HBu = butyric acid; HAC = homoacetogenic bacteria; MPA = methane-producing archaea; SRB = sulfate-reducing bacteria; OHPA = obligate hydrogen-producing acetogen.

Bacterial abundance and activity are affected by factors such as the presence of terminal electron acceptors [11]. For example, in the presence of HCO$_3^-$, MPAs are dominant over SRBs. At high SO$_4^{2-}$ concentrations, SRBs belonging to *Desulfobacter* sp. and *Desulfobacca acetoxidans* are able to out-compete MPAs such as *Methanosaeta* sp. and *Methanosarcina* sp. for acetate [6,9]. Dar *et al.* [12] reported that in upflow anaerobic sludge blanket reactors (UASBRs) the detection of *Desulfovibrio-Desulfomicrobium* group indicated the presence of a large diversity of SRBs. In addition, they reported detecting members of other phylogenetic SRB groups, *Desulfotomaculum*, *Desulfobulbus*, and *Desulfococcus-Desulfobacca-Desulfosarcina*. In comparison to work reported by Raskin *et al.* [13], *Desulfobactarium* and *Desulfobacter* were not detected by Dar *et al.* [12]. This variation in the SRB populations is likely due to the different electron donors used by these researchers.

Factors affecting competition between the co-existence of SRBs, MPAs and HACs include pH, temperature, substrate and reactor type, COD to SO$_4^{2-}$ ratio (COD/SO$_4^{2-}$ ratio), hydraulic retention time (HRT) as well as physical structure of microbial cultures [5,14,15]. White and Gadd [5] claimed that interaction between the COD/SO$_4^{2-}$ ratio and HRT controlled SO$_4^{2-}$ reduction and COD/SO$_4^{2-}$ ratio 1.0 to 3.0 was preferred for SRBs. In comparison, Dar *et al.* [16] reported that at a low COD/SO$_4^{2-}$ ratio of 0.34, SRBs out-competed MPAs and to some extent HACs. These authors
reported that at a limiting \( \text{SO}_4^{2-} \) concentration (high COD/\( \text{SO}_4^{2-} \) ratio), the relative abundance of MPAs and HACs was approximately 45% of the total microbial consortia. These results indicate that further investigation on COD to \( \text{SO}_4^{2-} \) ratio is necessary to clearly understand the competition between MPAs, HACs and SRBs.

Competition between different microbial species can also be controlled by altering operational factors such as pH, temperature and adding methanogenic inhibitors. Sipma et al. [14] reported that both pH and temperature can impact MPAs more in comparison to HRT. Chaiprapat et al. [17] observed that for an ASBR operating at neutral pH, increasing \( \text{SO}_4^{2-} \) removal was observed in comparison to under low pH conditions. In the neutral pH range, SRBs and MPAs are active but suppressed under low pH conditions [18]. Inhibiting MPAs by utilizing chemical inhibitors could be of great significance in reducing methanogenesis by diverting electron fluxes to SRBs and subsequently improve \( \text{SO}_4^{2-} \) removal. Inhibiting methanogens with long chain fatty acids (LCFAs) have gained some attention over other inhibitory chemicals [19] because they are environmentally friendly, relatively abundant and biodegradable [20].

The presence of recalcitrant compounds, heavy metals, \( \text{SO}_4^{2-} \) and total dissolved solids can affect the anaerobic microbial dynamics and hence, the process efficiency. Sulfate-reducing anaerobic bioreactors have been treated as ‘black boxes’ without a thorough understanding of the microorganisms involved in \( \text{SO}_4^{2-} \) reduction [21]. The operation of these bioreactors is highly dependent on microbial activities and a better understanding of the role of microbial communities in these systems will assist in improving their design and performance [22]. Hence, further work is required to understand methanogenesis and \( \text{SO}_4^{2-} \) reduction from a more fundamental perspective. Hence, the objectives of this study were as follows: 1. To investigate the effect of \( \text{SO}_4^{2-} \) reduction at varying levels of COD/\( \text{SO}_4^{2-} \) ratio, HRT and LA (methanogenic inhibitor) concentration. 2. To investigate the microbial population dynamics at varying levels of these experimental variables. An integrated approach to characterize the microflora using terminal restriction fragment length polymorphism (T-RFLP) together with chemical analysis of the fermentation byproducts was used to analyze trends between the microbial community structure and changes in the operational parameters.

2. Materials and Methods

2.1. Inoculum Source

The anaerobic inoculum was procured from an UASBR located at a brewery wastewater treatment facility (Guelph, ON, Canada) (A) and at the municipal wastewater treatment plant (Chatham, ON, Canada) (B). Culture A and culture B were selected based on sources of MPAs and SRBs, respectively. The volatile suspended solid (VSS) of the culture A and B was 50 and 20 g VSS·L\(^{-1}\) respectively. The cultures (A and B) were diluted with basal medium to 25 and 12 g VSS·L\(^{-1}\) in 10 L reactors, respectively (designated as reactor A and B). The bioreactors were operated in accordance to procedures reported by Ray et al. [23]. The cultures in reactors A and B were maintained at 37 °C and at pH 7.0 ± 0.5 in sequencing batch mode with a 14 d (days) HRT and a feed concentration of 2000 mg glucose·L\(^{-1}\). In addition to glucose, reactor B was acclimated incrementally to increasing \( \text{SO}_4^{2-} \) levels from 250 to 2000 mg·L\(^{-1}\) over 2 months. During the acclimation period, the quantity of gas and VFAs
were monitored to establish quasi-steady state conditions. Inoculum for the experiments under consideration was combined from reactors A (80%) and B (20%) and diluted with basal medium to 8 g VSS·L\(^{-1}\). The basal medium composition used for dilution and feed was adapted from Wiegant and Lettinga [24]. All the chemicals (99% purity) for preparing the basal medium were procured from ACP Chemicals Inc. (Montreal, Quebec, Canada) and Sigma Aldrich, (Oakville, Ontario, Canada). Glucose (99% purity) and LA (99% purity) were procured from Spectrum Chemicals (Gardena, CA, USA) and TCI America (Portland, OR, USA).

2.2. Sulfate Reduction Studies

Two 7 L (total volume) reactors (New Brunswick Scientific, New Brunswick, NJ, USA) with a 5 L working volume were used to conduct the experiments. The reactors (R1 and R2) were operated as ASBRs at 37 ± 1 °C. Liquid samples were collected at the end of each cycle. Continuous mixing of the reactor contents during the reaction phase was conducted at 200 rpm using a stirring plate mixer. The pH (6.5 ± 0.1) was maintained using 1 M NaOH and 0.5 M HCl. The ASBRs (R1 and R2) were seeded with the inoculum from reactors A and B (8 g VSS·L\(^{-1}\)) and then purged with nitrogen (N\(_2\)) (99.99% purity, Praxair, Windsor, ON, Canada) for 5 min to maintain anaerobic conditions. A three factor three level Taguchi design was used for conducting the experimental run (Table 1) as described by Singh et al. [25]. The factors investigated in this study include LA concentration, COD/SO\(_4^{2-}\) ratio and HRT. The reactors (R1 and R2) were operated under the same conditions with a feed concentration of 2000 mg glucose·L\(^{-1}\) (2.134 g COD·L\(^{-1}\)) as a carbon source and SO\(_4^{2-}\) concentration varied according to the COD/SO\(_4^{2-}\) ratios shown in Table 1. The reactors were operated as follows: 40 min settling; 10 min decanting and 10 min fill. The reaction times maintained were 5, 11 and 17 h for HRT values of 12, 24 and 36 h, respectively. The volume decanted per cycle was constant at 2.5 L and the HRT was calculated using Equation (1):

\[
\text{HRT} = \frac{\text{(Working volume of the reactor)}}{\left(\text{Volume decanted per cycle} \times \text{No. of cycles per day}\right)}
\]

The reactors under each HRT were operated until they achieved quasi-steady state condition (constant SO\(_4^{2-}\) reduction with ±10% variation). Different LA levels (0, 0.5 and 1.0 g·L\(^{-1}\)) were fed to cultures according to experimental conditions shown in Table 1. During the inhibition studies, the cultures were incubated with LA for 24 h prior to initiating the experiment (adding SO\(_4^{2-}\) and glucose).

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**Table 1.** Experimental outline to elucidate the effect of operational parameters on sulfate removal in ASBRs.

| Experiment #s | LA Concentration (g·L\(^{-1}\)) | COD/SO\(_4^{2-}\) Ratio | HRT (h) | Initial SO\(_4^{2-}\) Concentration (g·L\(^{-1}\)) |
|---------------|-------------------------------|------------------------|--------|---------------------------------|
| 1             | 0                             | 0.8                    | 12     | 2.67                            |
| 2             | 0                             | 1.6                    | 36     | 1.34                            |
| 3             | 0                             | 2.4                    | 24     | 0.89                            |
| 4             | 0.5                           | 0.8                    | 24     | 2.67                            |
| 5             | 0.5                           | 1.6                    | 12     | 1.34                            |
| 6             | 0.5                           | 2.4                    | 36     | 0.89                            |
Table 1. Cont.

| Experiment # | LA Concentration (g·L\(^{-1}\)) | COD/SO\(_4^{2-}\) Ratio | HRT (h) | Initial SO\(_4^{2-}\) Concentration (g·L\(^{-1}\)) |
|--------------|----------------------------------|------------------------|--------|-----------------------------------------------|
| 7            | 1.0                              | 0.8                    | 36     | 2.67                                          |
| 8            | 1.6                              | 24                     | 1.34   |
| 9            | 0.8                              | 12                     | 0.89   |

Notes: The pH and influent substrate concentration were 6.5 ± 0.1 and 2 g·glucose·L\(^{-1}\), respectively; COD = Chemical oxygen demand; LA = linoleic acid; SO\(_4^{2-}\) = sulfate and ASBRs = anaerobic sequential batch reactors; The experiment design is based on the Taguchi model described by Singh et al. [25]; Cultures fed 0 g·L\(^{-1}\) LA is referred as control cultures; All the experiments were conducted in duplicate reactors designated as R1 and R2.

2.3. Analytical Methods

Biogas production was monitored using a tipping bucket gas meter [26] and the composition of biogas was quantified using a gas chromatograph [19]. 25 μL of each gas was injected and the detection limits for CH\(_4\) and H\(_2\) were 0.0032 kPa [0.5 mL/bottle (160 mL)] and H\(_2\)S was 0.0315 kPa [5 mL/bottle (160 mL)], respectively.

The liquid samples collected at the end of each cycle were analyzed for VFAs, dissolved sulfur compounds (SO\(_4^{2-}\) and sulfide) and alcohols. The sulfur compounds were analyzed according to methods described by Moon et al. [19] using an ion chromatograph (IC). The detection limits for the sulfide and SO\(_4^{2-}\) were 2.0 and 0.5 mg·L\(^{-1}\), respectively.

The IC and high performance liquid chromatograph (HPLC) methods used to analyze alcohol and VFAs in the effluent liquid samples were conducted using the methods described by Chowdhury et al. [27] and Moon et al. [19], respectively. The detection limit for alcohols (ethanol, \(i\)-propanol, \(n\)-propanol, \(n\)-butanol, and \(i\)-butanol) and VFAs (lactate, acetate, propionate, formate and butyrate) were 5 and 2 mg·L\(^{-1}\), respectively. The total suspended solids (TSS) and VSS were measured according to Standard Methods [28].

2.4. Microbial Methods

The microbial community diversity in the mixed culture subjected to different operational conditions was determined using nested polymerase chain reaction (PCR) of the 16S rRNA gene followed by terminal restriction fragment length polymorphism (T-RFLP) analysis. The cultures samples collected at the end of the experiment were used for microbial community analysis. Details for DNA isolation, PCR amplification and T-RFLP methods were previously reported by Chaganti et al. [29]. The data obtained from the T-RFLP analysis comprised the peaks reflecting the size of terminal restriction fragments (T-RFs) in base pairs (bp) together with the area of each peak measured in fluorescence units. For the T-RFs generated by the digestion of PCR-amplified 16S rRNA genes from culture samples in the current study, a phylogenetic assignment was performed using a modified database generated for T-RFs which were previously described by Chaganti et al. [29].

The relative abundances of the terminal restriction fragments (T-RFs) was used to detect phyla of samples observed under different operational conditions based on the taxonomy annotation using the
modified database generated from Microbial Community Analysis (MiCA) plus experimentally determined TRFs for microorganisms identified from the 16S rRNA gene clone library analysis.

3. Results and Discussion

3.1. Comparison of Sulfate Removal and the Degradation Byproducts at Different Operating Conditions

The SO$_4^{2-}$ removal based on an influent SO$_4^{2-}$ concentration range of 0.89 to 2.67 g·L$^{-1}$ varied from approximately 56% to 94% (Figure 2). The mean SO$_4^{2-}$ removal showed a small improvement when 0.5 g·L$^{-1}$ of LA was added; however, with a further increase in LA concentration to 1 g·L$^{-1}$, the SO$_4^{2-}$ removal increased from 69% to 83%. In comparison, according to Moon et al. [19], increasing the LA concentration from 0.5 to 1.5 g·L$^{-1}$ in batch reactors showed no significant effect on SO$_4^{2-}$ reduction. Moon et al. [19] observed that adding LA improved SO$_4^{2-}$ reduction by ≥15% in comparison to the control cultures operating at a similar COD/SO$_4^{2-}$ ratio and pH.

In the current study, varying the COD/SO$_4^{2-}$ ratio had less of an effect on the percent SO$_4^{2-}$ removal while decreasing the HRT from 36 to 12 h increased the SO$_4^{2-}$ removal efficiency from approximately 68% to 84% [25]. Studies using a lactate feed revealed that the HRT and the COD/SO$_4^{2-}$ significantly influenced the SO$_4^{2-}$ reduction with increasing SO$_4^{2-}$ removal detected with decreasing HRT (from 20 to 10 h) and with increasing the COD/SO$_4^{2-}$ ratio from 1 to 3 [5].

Dissolved sulfide (DS) was the major liquid degradation by-product from SO$_4^{2-}$ reduction. In control cultures, the DS concentration varied between 100 and 250 mg·L$^{-1}$ at elevated COD/SO$_4^{2-}$ ratios ranging from 1.6 to 2.4 (Figure 2a). In comparison, for cultures fed 0.5 g·L$^{-1}$ LA and operating at COD/SO$_4^{2-}$ ratios of 1.6 to 2.4, the sulfide concentrations ranged from 100 to 400 mg·L$^{-1}$ (Figure 2b). Increasing DS concentration ranging from 450 to 650 mg·L$^{-1}$ were detected in control cultures at a low COD/SO$_4^{2-}$ ratio of 0.8 while sulfide concentrations ranging from 250 to 400 mg·L$^{-1}$ were detected in cultures fed 0.5 g·L$^{-1}$ LA (Figure 2a,b). Alvarez et al. [30] reported a maximum sulfide concentration (300 to 500 mg·L$^{-1}$) could be obtained with maximum SO$_4^{2-}$ removal (50% to 62%) with a COD/SO$_4^{2-}$ ratio ranging from 0.7 to 1.5 for packed bed bench-scale biofilm reactors operating at a 100 h HRT, 20 °C and a pH at 7.5.

A maximum SO$_4^{2-}$ removal efficiency of 90% was observed at a 36 h HRT and with a COD/SO$_4^{2-}$ ratio of 0.8 in cultures fed 1 g·L$^{-1}$ LA. Under these conditions, the sulfide concentration observed was approximately 550 mg·L$^{-1}$ (Figure 2c). For a feed containing, 2500 mg·L$^{-1}$ of SO$_4^{2-}$, Chaiprapat et al. [17] reported an elevated sulfide levels reaching 929 mg·L$^{-1}$ in ASBRs fed a rubber skim wastewater and operating at a 10 d HRT with a pH at 7.0. Chaiprapat et al. [17] attributed the high sulfide concentrations at high COD/SO$_4^{2-}$ ratios to a high rate of sulfidogenesis. In the current study, high DS concentrations reaching 641 mg·L$^{-1}$ were observed under low COD/SO$_4^{2-}$ ratio conditions with a feed SO$_4^{2-}$ concentration of approximately 2670 mg·L$^{-1}$ and a 12 h HRT (Figure 2). Similarly, Dar et al. [16] reported sulfide levels of approximately 250 mg·L$^{-1}$ in reactors operating at a 50 h HRT, a high COD/SO$_4^{2-}$ ratio of 1.94 and cultures fed lactate containing wastewater.
Figure 2. Effect of HRT and COD/\(\text{SO}_4^{2-}\) ratio on \(\text{SO}_4^{2-}\) reduction and sulfide formation with cultures fed (a) \(0 \text{ g·L}^{-1}\) LA; (b) \(0.5 \text{ g·L}^{-1}\) LA; (c) \(1.0 \text{ g·L}^{-1}\) LA. Notes: 1. HRT = hydraulic retention time; Ratio = COD/\(\text{SO}_4^{2-}\) ratio; \(\text{SO}_4^{2-}\) = sulfate and DS = dissolved sulfide; 2. Initial \(\text{SO}_4^{2-}\) concentration with respect to COD/\(\text{SO}_4^{2-}\) ratios of 0.8, 1.6 and 2.4 are 2667, 1333 and 889 mg·L\(^{-1}\) of sulfate, respectively; 3. Reactor 1 (R1) and Reactor 2 (R2) were operated in sequential batch mode under the conditions in Table 1.

3.2. Effect of COD/\(\text{SO}_4^{2-}\) Ratio and HRT on Biogas Production Using LA Treated and Untreated Cultures

Increasing \(\text{CH}_4\) yield (mol·mol\(^{-1}\) glucose) with increasing COD/\(\text{SO}_4^{2-}\) ratios revealed that decreasing the \(\text{SO}_4^{2-}\) concentration showed increasing methanogenic activity. In the case of control cultures, the \(\text{CH}_4\) yield (mol·mol\(^{-1}\) glucose) increased from approximately 0.09 to 0.5 when the COD/\(\text{SO}_4^{2-}\) ratio increased from 0.8 to 2.4 (Figure 3a). Sarti and Zaiat [31] reported decreasing \(\text{CH}_4\) yields with decreasing COD/\(\text{SO}_4^{2-}\) ratio (3.5 to 2.5) for an ASBR operating with a 48 h HRT using butanol as a carbon source. In cultures fed \(0.5 \text{ g·L}^{-1}\) LA, increasing \(\text{CH}_4\) yield (from 0.16 to 0.95) was detected in the reactor designated as R1 and from 0.46 to 1.1 in R2 when the COD/\(\text{SO}_4^{2-}\) ratio was increased from 0.8 to 1.6 (Figure 3b). In contrast, for cultures receiving \(1 \text{ g·L}^{-1}\) LA, increasing \(\text{CH}_4\)
yield was initially observed with increasing COD/\text{SO}_4^{2-} \text{ ratio from 0.8 to 1.6; however, the CH}_4 \text{ yield decreased to approximately 0.07 mol·mol}^{-1} \text{ glucose, when the COD to SO}_4^{2-} \text{ ratio was increased to 2.4 (Figure 3c).}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Effect of HRT and COD/\text{SO}_4^{2-} \text{ ratio on CH}_4 \text{ and H}_2\text{S production with cultures fed (a) 0 g·L}^{-1} \text{ LA (b) 0.5 g·L}^{-1} \text{ LA (c) 1.0 g·L}^{-1} \text{ LA. Notes: 1. HRT = hydraulic retention time; Ratio = COD/\text{SO}_4^{2-} \text{ ratio; CH}_4 = \text{ methane; LA = linoleic acid and H}_2\text{S = hydrogen sulfide; 2. Hydrogen detected under the conditions examined is } \leq 0.03 \text{ mol·mol}^{-1} \text{ glucose; 3. Reactor 1 (R1) and Reactor 2 (R2) were operated under the same conditions shown in Table 1.}}}
\end{figure}

In both control and LA fed cultures, no clear trend was observed in CH\textsubscript{4} yield with varying HRT conditions. Isa \textit{et al.} [10] reported decreasing the HRT from 10 to 0.5 d decreased CH\textsubscript{4} production and increased H\textsubscript{2}S production coupled with high SO\textsubscript{4}\textsuperscript{2-} reduction. These authors also observed increasing competition between SRBs and MPAs at high substrate concentrations (\textit{i.e.}, high COD/\text{SO}_4^{2-} \text{ ratio) using high-rate anaerobic reactors operating with a feed containing a mixture of acetate and ethanol. At high substrate concentrations (5 g COD·L\textsuperscript{-1}), Isa \textit{et al.} [10] observed that the quantity of electron
equivalents diverted towards CH$_4$ production reached 89% and at 0.5 g COD·L$^{-1}$, the quantity diverted to CH$_4$ production was 66%.

The trend observed for the H$_2$S yield (mol·mol$^{-1}$ glucose) was similar to CH$_4$ yield. Increasing the COD/SO$_4^{2-}$ ratio resulted in an increase in the H$_2$S yield except for cultures fed 0.5 g·L$^{-1}$ LA. In control cultures and cultures fed 1 g·L$^{-1}$ LA and operating at 1.6 COD/SO$_4^{2-}$ ratio, the H$_2$S yield increased to approximately 0.30 and 1.1 mol·mol$^{-1}$ glucose, respectively (Figure 3a,c). This indicates that an increase in LA concentration results in more electron diversion to sulfide (H$_2$S) production coupled with SO$_4^{2-}$ reduction in comparison to the CH$_4$ production. Wei et al. [32] reported for a mesophilic fluidized bed reactor operating with SO$_4^{2-}$ concentrations ranging from 1.0 to 3.0 g·L$^{-1}$, the quantity of H$_2$S produced ranged from 150 to 370 mg·L$^{-1}$. In the current study, for an influent SO$_4^{2-}$ concentrations ranging from 0.8 to 2.6 g·L$^{-1}$, the corresponding H$_2$S concentration ranged from 50 to 225 mg·L$^{-1}$. In comparison, Wei et al. [32] reported elevated H$_2$S levels reaching 370 mg·L$^{-1}$ in jet-loop anaerobic fluidized bed reactors operating at a 6 h HRT, 3000 mg·L$^{-1}$ SO$_4^{2-}$ and a pH at 5.8.

3.3. Volatile Fatty Acid Production

Acetate was the major VFA observed under all the experimental conditions (Figure 4). In control cultures, the acetate concentration ranged from 190 to 900 mg·L$^{-1}$ within the range of HRT conditions (Figure 4a). In the control cultures maintained at high COD/SO$_4^{2-}$ ratios, propionate and formate were also observed with concentrations ranging from 80 to 300 mg·L$^{-1}$ and 150 to 450 mg·L$^{-1}$, respectively. Cultures fed 0.5 and 1.0 g·L$^{-1}$ LA had lower acetate levels ranging from 200 to 650 mg·L$^{-1}$.

In comparison to the control cultures, a possible reason for the low acetate levels could be due to the inhibition of methanogenesis and hence, acetate utilization by SRBs in LA treated cultures (Figure 4b). According to Visser et al. [33], in a heat treated culture to inhibit methanogenesis, acetate rapid consumption by SRBs resulted in increased SO$_4^{2-}$ reduction.

In studies conducted by Dar et al. [16], increasing the COD/SO$_4^{2-}$ ratio from 0.34 to 20.9 led to decreasing acetate production while increasing levels of propionate was observed. In the current study, except for control cultures, increasing COD/SO$_4^{2-}$ ratio caused the accumulation of acetate while no trend was observed for propionate. Propionate and formate levels ranging from 70 to 150 mg·L$^{-1}$ at COD/SO$_4^{2-}$ ratios $\leq$1.6 was observed in cultures fed 0.5 g·L$^{-1}$ LA while at a high COD/SO$_4^{2-}$ ratio of 2.4, the quantity of propionate and formate was negligible (Figure 4b). McCartney and Oleszkiewicz [34] reported that at high COD/SO$_4^{2-}$ ratios (non-SO$_4^{2-}$ reducing pathway), propionate and acetate were the major end products while at low COD/SO$_4^{2-}$ ratios (SO$_4^{2-}$ reducing pathway), acetate was the only end product. Supporting evidence in the current study showed low propionate and formate levels were observed at low COD/SO$_4^{2-}$ ratio (0.8) in comparison to ratios at 1.6 and 2.4 (Supplementary Figure S1).

In cultures fed 1 g·L$^{-1}$ LA and operating at a COD/SO$_4^{2-}$ ratio of 0.8, both acetate and butyrate were dominant while butyrate was not detected at a COD/SO$_4^{2-}$ ratio of 1.6 (Figure 4c). Lactate levels ranging between 200 to 350 mg·L$^{-1}$ as well as acetate (450 to 600 mg·L$^{-1}$) and butyrate (50 to 70 mg·L$^{-1}$) were observed in cultures operating at a COD/SO$_4^{2-}$ ratio of 2.4 and fed 1 g·L$^{-1}$ LA (Figure 4c). Increasing SO$_4^{2-}$ reduction at a COD/SO$_4^{2-}$ ratio of 2.4 for cultures fed 1 g·L$^{-1}$ LA is
likely due to preferred lactate consumption by SRBs when compared to propionate and butyrate (Figure 2c) [15,34].

Figure 4. Effect of HRT and COD/\(\text{SO}_4^{2-}\) ratio on volatile fatty acids production with cultures fed (a) 0 g·L\(^{-1}\) LA; (b) 0.5 g·L\(^{-1}\) LA; (c) 1.0 g·L\(^{-1}\) LA. Notes: 1. HRT = hydraulic retention time; LA = linoleic acid; Ratio = COD/\(\text{SO}_4^{2-}\) ratio; HAc = acetic acid; HLa = lactic acid; HPr = propionic acid; HFr = formic acid; HBu = butyric acid; 2. Only the major volatile fatty acids produced under each condition are shown; 3. Data shown in the plot represent averages from reactors R1 and R2.

3.4. Competition and Coexistence of Sulfate-Reducing Bacteria, Homoacetogens and Methanogens

Microbial samples for quantifying various microorganisms were collected after completing the experiments. However, the discussion in this section is based on data for samples #1, #2, #4, #5, #6 and #9 because microbial amplification of the samples from experiments #3, #7 and #8 did not yield any quantification data after conducting the analyses twice.

Variation in the microbial community structure is based on the reactor operational conditions. The four major phyla associated with anaerobic \(\text{SO}_4^{2-}\) reduction in the ASBRs belonged to *Firmicutes*, *Proteobacteria*, *Euryarchaeota* and *Bacteroidetes*. The relative abundance of these phyla in samples
obtained under different operating conditions is shown in Figure 5. In addition to the classified phylum, each sample containing T-RFs with a relative abundance of 22%–38% could not be assigned to known microbial phyla (shown as Uncultured in Figure 5).

Figure 5. Microbial communities consisting of bacteria and Archaea in cultures operated under sulfate rich and limiting conditions at phylum level. The x-axis label describing the culture conditions is as follows: 1. COD/\(\text{SO}_4^{2-}\) ratio–HRT (h)–LA concentration (g L\(^{-1}\)); 2. The phyla with abundance greater than 1% in all samples are shown; 3. Sequences that could not be assigned at phylum level were marked as ‘Uncultured’; 4. Refer Supplementary Table S1 for the organisms at genus level.

Species belonging to *Euryarchaeota* accounted for 32% and 5% in control cultures operating at 36 and 12 h HRT, respectively (Figure 5). The quantity of electron diverted to MPAs under these conditions corresponds to 4.8% and 1.4%, respectively (Table 2). The low CH\(_4\) yields at high HRTs is likely due to the inactivity of HACs. In cultures operating at a 36 h HRT with a COD/\(\text{SO}_4^{2-}\) ratio of 1.6, HACs which belonging to *Bacteroidetes* accounted for approximately 8% of the population (Figure 5). Note acetate was the major byproduct observed under this condition (Experiment #2, Supplementary Figure S1). Similarly, work by Dar et al. [16] indicated that at high COD/\(\text{SO}_4^{2-}\) ratios and at a 50 h HRT, HACs were able to out-compete MPAs and SRBs at low \(\text{SO}_4^{2-}\) concentrations.

The quantity of *Proteobacteria* and *Firmicutes* in control cultures in ABSRs operating at 12 h HRT was approximately 55% and 14%, respectively (Figure 5). Members of this group consisted of species belonging to *Desulfovibrio*, *Desulfatibacillum* and *Desulfomicrobium* (Supplementary Table S1). Several studies have reported that species belonging to this group are able reduce \(\text{SO}_4^{2-}\) under high \(\text{SO}_4^{2-}\) concentrations (low COD/\(\text{SO}_4^{2-}\) ratios) [7,30,35]. Low HRTs and high \(\text{SO}_4^{2-}\) levels are major factors leading to the suppression of MPAs and HACs. Under these conditions, a relative increase in SRBs activity was associated with an 87% \(\text{SO}_4^{2-}\) reduction (Experiment #1, Table 2; Figure 2a and Supplementary Table S1).

In cultures fed 0.5 g·L\(^{-1}\) LA, *Euryarchaeota* was in the range from 3% to 10% of the species detected (Figure 5). Cultures operating at 36 h HRT reached a 10% maximum of the species belonging to *Methanosarcinales* [36] (Supplementary Table S1). The percent electrons diverted to MPAs accounted for approximately 13% under this condition (Experiment #6, Table 2). Results from the current study
suggest that long HRTs and high COD/SO$_4^{2-}$ ratios are favorable to MPAs when compared to SRBs at a 0.5 g·L$^{-1}$ LA (Supplementary Figure S1 and Supplementary Table S1). Evidence by Isa et al. [10] suggest that at high COD/SO$_4^{2-}$ ratios and long HRTs more electrons are diverted towards MPAs in comparison to that of SRBs. In addition, species belonging to methylotrophic methanogens and *Methanosarcinales* are able to out-compete SRBs at high acetate levels [7,37]. In the current study, high acetate levels were observed in the cultures fed 0.5 g·L$^{-1}$ LA, *Methanococcoides methylutens* and *Methanosaeta concilii* belonging to methylotrophic methanogens and aceticlastic methanogens were detected (Supplementary Table S1) [36].

**Table 2.** Percent electron flow from substrate to SRBs and MPAs.

| Experiment # | LA Concentration (g·L$^{-1}$) | COD/SO$_4^{2-}$ Ratio | HRT (h) | COD Equivalent (mg·L$^{-1}$) SO$_4^{2-}$ Reduced | COD Equivalent (mg·L$^{-1}$) Methane Produced | Percent Electron Flow to SRBs | Percent Electron Flow to MPAs |
|--------------|--------------------------------|-----------------------|---------|-----------------------------------------------|-----------------------------------------------|-----------------------------|-----------------------------|
| 1            | 0                              | 0.8                   | 12      | 1281.4                                        | 28.7                                          | 60.0                        | 1.4                         |
| 2            | 1.6                            | 36                    |         | 543.6                                         | 101.7                                         | 25.4                        | 4.8                         |
| 3            | 2.4                            | 24                    |         | 351.8                                         | 194.7                                         | 16.4                        | 9.1                         |
| 4            | 2.4                            | 0.8                   | 24      | 971.3                                         | 91.9                                          | 45.4                        | 4.3                         |
| 5            | 0.8                            | 1.6                   | 12      | 663.8                                         | 265.4                                         | 31.0                        | 12.4                        |
| 6            | 1.6                            | 36                    |         | 394.8                                         | 288.8                                         | 18.5                        | 13.5                        |
| 7            | 0.8                            | 1.6                   | 36      | 1187.1                                        | 5.4                                           | 55.5                        | 0.3                         |
| 8            | 1.6                            | 2.4                   | 12      | 681.1                                         | 26.6                                          | 31.8                        | 1.2                         |
| 9            | 2.4                            | 24                    |         | 562.4                                         | 21.8                                          | 26.3                        | 1.0                         |

Notes: LA = linoleic acid; SO$_4^{2-}$ = sulfate; SRB = sulfate reducing bacteria; MPA = methane producing archaea and HRT = hydraulic retention time; COD equivalent and percent electron flow values are average of duplicate reactors; COD equivalent of SO$_4^{2-}$ reduced corresponds to the sulfide produced; 1 mol of sulfate reduced = 1 mol of H$_2$S produced = 2 mol of COD = 64 g of COD; 1 mol of CH$_4$ produced = 2 mol of COD = 64 g of COD; Electron flow to SRB = mol of SO$_4^{2-}$ reduced × 64 g COD·mol$^{-1}$ SO$_4^{2-}$ = A g COD; Electron flow to MPA = mol of CH$_4$ produced × 64 g COD·mol$^{-1}$ CH$_4$ = B g COD; Percent electron flow by SRB = [A/(COD influent)] × 100 and percent electron flow by MPA = [B/(COD influent)] × 100; Complete balance of COD is presented in supplementary Figure S1.

The amount of SO$_4^{2-}$ reduction (63% to 75%) observed in cultures fed 0.5 g·L$^{-1}$ LA could be due to the presence of SRBs belonging to *Proteobacteria* (29% to 45%) and *Firmicutes* (10% to 20%) (Figures 2b and 5). Under these conditions, *Deulfovibrio* sp. and *Desulfatibacillus* sp. were abundant (Supplementary Table S1). According to Sousa et al. [20] and Tan et al. [38], these microorganisms were detected in SO$_4^{2-}$ rich or SO$_4^{2-}$ limiting conditions and in the presence of electron donors such as fatty acids and alkanes. In cultures fed 0.5 g·L$^{-1}$ LA, *Dethiosulfovibrio* sp. and *Clostridium* sp., organisms belonging to the *Firmicutes* phylum, were detected (Supplementary Table S1). The presence of *Clostridium* sp. and species belonging to *Bacteroidetes* is consistent with the findings from other studies, where these species were observed under SO$_4^{2-}$ reducing conditions and capable of producing a mixture of VFAs from solid organic substrates and sugar beet molasses [39,40]. In the current study, acetate, propionate and formate which constituted approximately 30% to 40% of the
initial COD fed to the reactors were observed in cultures fed 0.5 g·L\(^{-1}\) LA (Experiments #4–#6, Supplementary Figure S1).

In cultures fed 1 g·L\(^{-1}\) LA and operating at a 12 h HRT, the relative abundance of *Proteobacteria* and *Firmicutes* was 58% and 6%, respectively (Figure 5). The low HRT (12 h) in combination with 1 g·L\(^{-1}\) LA resulted in the suppression of MPAs belonging to *Euryarchaeota*. The SO\(_4^{2-}\) removal obtained under this condition corresponded to 90% with only 1% of the electrons diverted to MPAs (Figure 2c and Table 2, Expt. #9). Studies by Moon *et al.* [19] using batch reactors suggest that high LA concentrations inhibited methane production in the presence of SO\(_4^{2-}\). Similarly, the suppression of MPAs was observed in SO\(_4^{2-}\) fed fluidized bed reactors operating at an HRT of approximately 16 h [41]. The results from this current study indicate that under low HRT conditions, SRBs were able to out-compete MPAs under SO\(_4^{2-}\) limiting conditions in the presence of 1 g·L\(^{-1}\) LA (Table 2 and Figure 5). Under SO\(_4^{2-}\) rich conditions, the SO\(_4^{2-}\) reduction efficiency was greater than 75% and the percent relative abundance of SRBs was approximately 50 (Figure 2 and Supplementary Table S1).

In work related to this study, Weijma *et al.* [42] suggest that sulfidogenic and acetogenic cultures favors high SO\(_4^{2-}\) reduction under low HRT conditions by outcompeting MPAs. In addition to the SRBs belonging to *Desulfovibrio* sp. *Desulfomicrobium* sp. and *Desulfatibacillum* sp., *Geobacter sulfurreducens* was detected in cultures fed 1 g·L\(^{-1}\) LA and operating at a 12 h HRT.

Phylogenetically, *Syntrophus aciditrophicus*, along with SO\(_4^{2-}\) reducers and Geobacter species, are members of the delta subdivision of *Proteobacteria*. Levels of *S. aciditrophicus* ranging from 3% to 7.5% were observed under all the conditions examined. Studies by Jackson *et al.* [43] suggest that *S. aciditrophicus* were able to degrade fatty acids in syntrophic association with SRBs under SO\(_4^{2-}\) rich and limiting conditions. This relationship could enhance the SO\(_4^{2-}\) reduction in the process as short chain fatty acids such as propionate, butyrate could be degraded to acetate which is a more preferred carbon substrate for SRBs in comparison to propionate and butyrate [15]. Studies by Sousa *et al.* [20] also suggest that these syntrophic bacteria existed along with SRBs in LCFA degrading communities in the presence of SO\(_4^{2-}\).

### 4. Conclusions

This study demonstrated that different levels of SO\(_4^{2-}\) reduction in ASBRs were observed under different operating conditions. A link between the microbial population dynamics and metabolic byproducts (from substrate and SO\(_4^{2-}\) reduction) to changes caused by HRT and COD/SO\(_4^{2-}\) ratio under control and LA treated conditions was established. Based on the factors under consideration in the study, the conclusions from this study are as follows:

1. Operating the ASBRs was suitable for SO\(_4^{2-}\) reduction with SO\(_4^{2-}\) removal efficiencies greater than 60%, under the conditions examined in this study.
2. ASBRs operating under low COD/SO\(_4^{2-}\) ratio (0.8) with low HRT (12 h) is preferred for SO\(_4^{2-}\) removal efficiencies greater than 75%.
3. When compared to the control cultures, in cultures fed 1 g·L\(^{-1}\) LA and at COD/SO\(_4^{2-}\) ratios 1.6 and 2.4, the SO\(_4^{2-}\) removal efficiencies improved by approximately 20% and 28%, respectively.
4. Dissolved sulfide and butyrate were associated with cultures fed with low COD/SO\(_4^{2-}\) ratios, while H\(_2\)S, acetate and propionate were linked with high COD/SO\(_4^{2-}\) ratios.
5. Methanogens belonging to *Methanobacteriales* and *Methanosarcinales* were abundant in control cultures operating at high HRTs while the addition of LA suppressed *Methanobacteriales* alone; however, with a reduction in HRT, washout of *Methanosarcinales* was observed.

6. In controls and cultures fed 0.5 g·L\(^{-1}\) LA, HACs belonging the *Bacteroidetes* phylum was abundant.

7. *Desulfovibrio* sp. and *Desulfatibacillum* sp., the major SRBs responsible for SO\(_4^{2-}\) reduction, were able to out-compete MPAs and HACs at low HRTs and low COD/SO\(_4^{2-}\) ratios.

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Author Contributions

The experimental work was conducted by Chungman Moon, Rajesh Singh, Sathyanarayanan S. Veeravalli and Saravanan R. Shanmugam. The manuscript was written by Sathyanarayanan S. Veeravalli and Jerald A. Lalman. Engineering data analysis was performed by Sathyanarayanan S. Veeravalli, Saravanan R. Shanmugam, Jerald A. Lalman. Microbial analysis was performed by Subba Rao Chaganti. Daniel D. Heath provided lab facility and funding for conducting the microbial analysis.

Conflicts of Interest

The authors declare no conflict of interest.

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