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Luis Carlos Reyes-Alvarado, Frédéric Habouzit, Eldon R. Rene, Gaelle Santa-Catalina, Renaud Escudié, et al.. Effect of ammonium, electron donor and sulphate transient feeding conditions on sulphidogenesis in sequencing batch bioreactors. *Bioresource Technology*, 2019, 276, pp.288-299. 10.1016/j.biortech.2018.12.087 . hal-02623546

**HAL Id: hal-02623546**

**<https://hal.inrae.fr/hal-02623546v1>**

Submitted on 26 May 2020

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PII: S0960-8524(18)31758-9  
DOI: <https://doi.org/10.1016/j.biortech.2018.12.087>  
Reference: BITE 20844

To appear in: *Bioresource Technology*

Received Date: 11 November 2018  
Revised Date: 22 December 2018  
Accepted Date: 24 December 2018

Please cite this article as: Reyes-Alvarado, L.C., Habouzit, F., Rene, E.R., Santa-Catalina, G., Escudie, R., Bernet, N., Lens, P.N.L., Effect of ammonium, electron donor and sulphate transient feeding conditions on sulphidogenesis in sequencing batch bioreactors, *Bioresource Technology* (2018), doi: <https://doi.org/10.1016/j.biortech.2018.12.087>

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Comment citer ce document :

Reyes-Alvarado, L. C., Habouzit, F., Rene, E. R. (Auteur de correspondance), Santa-Catalina, G., Escudie, R., Bernet, N., Lens, P. N. L. (2019). Effect of ammonium, electron donor and sulphate transient feeding conditions on sulphidogenesis in sequencing batch bioreactors. *Bioresource Technology*, 276. 288-299. . DOI : 10.1016/j.biortech.2018.12.087

**Effect of ammonium, electron donor and sulphate transient feeding conditions on sulphidogenesis in sequencing batch bioreactors**

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## Abstract

This work aimed to study the effect of transient feeding conditions on sulphidogenesis in 8 sequencing batch bioreactors (SBR). SBR L1 and H1, operated under steady-state conditions were used as the control reactors, while four SBR were tested under transient feeding conditions, using moderate (L2 and L3, feast and famine: 2.5 and 0 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>) and high (H2 and H3, feast and famine: 15 and 0 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>) loads. The sulphate removal efficiency (*RE*) was  $\geq 90\%$  in SBR L2, L3 and H1. The NH<sub>4</sub><sup>+</sup> famine conditions resulted in a higher sulphate *RE* ( $\geq 40\%$  H3) compared to feast conditions ( $\leq 20\%$  H2). Besides, the sulphidogenic first-order kinetic constant was 4 % larger and the use of electron donor was 16.6% more efficient under NH<sub>4</sub><sup>+</sup> famine conditions. Sulphidogenesis is robust to transient feeding conditions, but not when applying high loading rates (SBR H2 and H3).

**Keywords:** Sulphate reduction, sulphidogenesis, wastewater treatment, sequencing batch reactor, transient feeding conditions, feast-famine

## 1 Introduction

Sulphate rich wastewater is a serious environmental problem. Sulphate needs to be removed from industrial wastewater under controlled conditions. Adversely, if sulphate is reduced under uncontrolled conditions the corrosive sulphide can be produced (Reyes-Alvarado, 2018).

Sulphate rich industrial wastewaters possess characteristics such as low pH, high oxidative potential, can contain high concentrations of toxic metals and lack chemical oxygen demand (COD). Therefore, they can dramatically damage the flora and fauna of water reservoirs upon discharge without proper treatment (Mapanda et al., 2007).

Biotechnologically, sulphate reducing bacteria (SRB) reduce sulphate to sulphide by means of COD utilization at different stoichiometric relations (Liamleam and Annachhatre, 2007). Besides, SRB are capable to perform sulphate reduction using different electron donors,

e.g. hydrogen, ethanol, glucose, propionate, butyrate, among other complex mixtures like molasses (Liamleam and Annachhatre, 2007). However, most SRB lack the enzymes required to perform the hydrolysis of complex polymers and, therefore they use soluble organic monomers (Liamleam and Annachhatre, 2007). Most recently, slow release electron donors, such as carbohydrate based or lignocellulosic polymers, have been used for the removal of sulphate in batch (Reyes-Alvarado et al., 2017b; Reyes-Alvarado, 2018) as well as in continuous (Reyes-Alvarado et al., 2018a) bioreactors. Such slow release electron donors promise high sulphate *RE* in batch. For instance, potato, filter paper and scourer showed  $\geq 95$  % and cork 82 % sulphate *RE* (Reyes-Alvarado et al., 2017b, 2018a).

The removal of sulphate by SRB from sulphate rich wastewaters has been studied in different anaerobic reactors, i.e. batch reactor (Al-Zuhair et al., 2008), sequencing batch reactor (SBR) (Torner-Morales and Buitrón, 2010), up-flow anaerobic sludge blanket reactor (UASB) (Bertolino et al., 2012), extended granular sludge bed reactor (EGSB) (Dries et al., 1998), gas lift reactor (Sipma et al., 2007) and inverse fluidized bed bioreactors (IFBB) (Reyes-Alvarado et al., 2018b, 2017a). Among these studies, the effect of the COD:sulphate ratio and the HRT have been studied extensively for high sulphate removal efficiencies ( $RE > 75$  %). These different technologies offer diverse advantages in terms of biomass retention and, therefore, capacity of organic loading (Nicolella et al., 2000). SBR is a mature technology and tested for nutrient (C, N and P) removal from domestic wastewater (Cyzdik-Kwiatkowska et al., 2014), this bioreactor configuration is well known for high biomass retention. Hence, the nutrient removal is affected by the solid retention time (SRT) and this SRT also allows to control biomass aggregation such as flocs and granules in the SBR (Cyzdik-Kwiatkowska et al., 2014; Liao et al., 2006). Torner-Morales and Buitrón (2010) tested the SBR for sulphate removal and, using a cycle length of 6 h and a COD:sulphate ratio of 2 and 6, and reported a sulphate *RE* of 90 and 100%, respectively.

Bioprocesses including reactions mediated by bacteria produce new cells and structures (e.g. enzymes and exopolysaccharides), and use electron donors and acceptors as energy sources. During anaerobic reactor operation, approximately 5 to 15% of the total influent COD (100%) is used for biomass formation (de Lemos Chernicharo, 2007). Thus, SRB consume nutrients (e.g. C, N, S and P) during the sulphidogenesis and the process can be hampered by lack of nutrients or if the conditions in the bioreactors (operated as batch sequential or continuous mode) are not optimal. For instance, SRB need COD to produce sulphide during sulphidogenesis. COD starving, transient feeding or limiting conditions might thus dramatically, reduce the sulphate *RE* (Velasco et al., 2008). Only few studies have reported the effect of transient feeding conditions in sulphidogenic bioreactors (Janyasuthiwong et al., 2016; Papirio et al., 2013; Reyes-Alvarado et al., 2017a). For instance, Papirio et al. (2013) showed that sulphidogenic bioreactors completely fail when the influent pH is decreased from 7.0 to 3.0, but recover (sulphate *RE* > 95%) when the influent pH is increased to 5.0. Janyasuthiwong et al. (2016) reported that a sulphidogenic bioreactor decreases more than 35% of its performance when the reactor pH was decreased from 7.0 to 5.0, the sulphate *RE* dropped from 75% to less than 40%. Reyes-Alvarado et al. (2017a) showed that sulphidogenesis in a continuous bioreactor was not hampered after ten consecutive feast (the electron donor and acceptor were increased to 2133 mg.L<sup>-1</sup> of lactate and 1495 mg.L<sup>-1</sup> of sulphate) and famine (the electron donor and acceptor were removed from the influent) cycles of operation. During the feast feeding, the sulphate *RE* (67 ± 15%) was comparable with the previous operation period IV (71 ± 4%) of the same continuous bioreactor and also comparable with the performance of the control bioreactor (61 ± 15%) (Reyes-Alvarado et al., 2017a).

This study does not advise to operate bioreactors under transient feeding conditions as a strategy to reduce the start-up phase of sulphidogenesis. There is, however, lack of information about the performance of biological sulphate reduction processes during intermittent or transient

operation of bioreactors. Hence, this study aims to determine the robustness and resilience to changing conditions (COD,  $\text{SO}_4^{2-}$  and  $\text{NH}_4^+$ ) on the biological sulphate reduction in SBR. Hereby we report (i) the effect of the sulphate concentrations during the start-up of a sulphidogenic SBR using two different inoculum (a methanogenic anaerobic and a sulphidogenic sludge), (ii) the effect of the electron donor and acceptor at transient feeding conditions (feast to famine), (iii) the effect of the electron donor and acceptor transient feeding conditions at high concentrations and (iv) the effect of the presence and absence of nitrogen source ( $\text{NH}_4^+$ ) during the sulphidogenesis in SBR and batch bioreactors.

## 2 Material and methods

### 2.1 Synthetic wastewater

In this study, the synthetic wastewater had the following composition (in  $\text{mg}\cdot\text{L}^{-1}$ ):  $\text{NH}_4\text{Cl}$  (300),  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$  (120),  $\text{KH}_2\text{PO}_4$  (200),  $\text{KCl}$  (250),  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$  (15), yeast extract (20) and 0.5 mL of a mixture of micronutrients. The micronutrients solution contained (in  $\text{mg}\cdot\text{L}^{-1}$ ):  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$  (1500),  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$  (100), EDTA (500),  $\text{H}_3\text{BO}_3$  (62),  $\text{ZnCl}_2$  (70),  $\text{NaMoO}_4\cdot 2\text{H}_2\text{O}$  (36),  $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$  (40),  $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$  (24),  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$  (70),  $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$  (20) and  $\text{HCl}$  36 % (1 mL) (Villa-Gomez et al., 2012). Sodium lactate and sodium sulphate were used as the electron donor and acceptor, respectively. The source of nitrogen ( $300 \text{ mg } \text{NH}_4\text{Cl}\cdot\text{L}^{-1}$ ) was excluded from the preparation of the synthetic wastewater when it was required by the experiment. The pH of the influent synthetic wastewater was adjusted to 6.0 with  $\text{NaOH}$  (1 M) or  $\text{HCl}$  (1 M). All reagents used in this study were of analytical grade.

### 2.2 Sequencing batch reactor set up

Eight SBR were operated in this study, all made of glass with the same configuration but with different working volume: two of 6 L (SBR L (low) and H (high)) and six of 2 L (SBR L1-3 and

SBR H1-3). The SBR comprised of the following components: influent tank, two peristaltic pumps (Masterflex L/S), effluent tank, heating system (water bath), stirring system, sampling port and timer. The reactors operated at 3 cycles of 8 h per day. The schedule of each cycle was: 2 minutes to discharge, two minutes of feeding, 3 hours of agitation starting from time zero and 5 hours of settling from 3 to 8 h of the cycle. For the SBR of 6 L (SBR L (low) and H (high)), 1 L of supernatant was removed and the same volume was fed, resulting in an influent liquid flow ( $Q_{in}$ ) of  $3 \text{ L} \cdot \text{d}^{-1}$  or an HRT of 2 d. For the SBR of 2 L (SBR L1-3 and SBR H1-3), 0.33 L of supernatant was removed and the same volume was fed, resulting in an influent liquid flow ( $Q_{in}$ ) of  $1 \text{ L} \cdot \text{d}^{-1}$  or an HRT of 2 d. The stirring system consisted of an axle with two propellers, and the speed was fixed at 120 rpm. The temperature was controlled at  $30 \text{ }^{\circ}\text{C}$  with a water bath (Cole Parmer, Polystat 12112-00).

### 2.3 Batch reactor set up

Batch bioreactors were also used, operated and constructed as described elsewhere (Reyes-Alvarado et al., 2018a). These batch bioreactors consisted of serum bottles with a capacity of 0.12 L, these used butyl rubber stoppers and aluminium caps for sealing. Syringes with long needles were used for sampling the supernatant. This procedure was essential to maintain the desired anaerobic conditions. The batch bioreactors were incubated at  $30 \text{ }^{\circ}\text{C}$  and were agitated at 120 rpm on an orbital shaker (New Brunswick Scientific Innova 2100 platform shaker, Eppendorf, USA).

### 2.4 Source of biomass

A 6 L working volume SBR was inoculated with sulphate reducing biomass and used as a control reactor (L) (Figure 1A). SBR L was operated with a volatile suspended solid (VSS) concentration of  $8.9 (\pm 1.5) \text{ g VSS} \cdot \text{L}^{-1}$  and total suspended solid (TSS) concentration of  $14.1 (\pm 1.7) \text{ g TSS} \cdot \text{L}^{-1}$ .



At steady performance, the biomass was divided and used to inoculate the smaller (2 L volume) three SBR (Figure 1B) and batch bioreactors (Figure 1D).

A second 6 L working volume SBR named H (Figure 1A, of 6 L volume) was inoculated with anaerobic sludge from a methanogenic process treating vinasse wastewater, this contained  $36.5 (\pm 0.6) \text{ g VSS.L}^{-1}$  and  $75.6 (\pm 1) \text{ g TSS.L}^{-1}$ . SBR H was operated, at steady performance, with a concentration of  $15.9 (\pm 0.7) \text{ g VSS.L}^{-1}$  and  $25.8 (\pm 0.3) \text{ g TSS.L}^{-1}$ . Subsequently, the biomass was divided and used to inoculate another three SBR (Figure 1C, H1, H2 and H3 with 2 L working volume each) with 2 L of liquor volume containing  $15.9 \text{ g VSS.L}^{-1}$  of sulphate reducing biomass. Both SBR (L and H) were operated at a constant COD:sulphate ratio of 2.4 until steady biological sulphate *RE* was  $\geq 90\%$ .

The biomass of SBR L was divided in two parts (Figure 1): 5.2 L was used to inoculate the other three smaller SBR (L1, L2 and L3, Figure 1B) and 0.8 L was used for experiments in batch bioreactors (Figure 1D). The volume of 5.2 L was completed till 6 L with synthetic wastewater (lacking electron donor and acceptor), mixed-up and divided in portions of 2 L containing  $7.7 \text{ g VSS.L}^{-1}$  as sulphate reducing biomass for the new SBR operated at low sulphate concentrations (SBR L1, L2 and L3). The volume liquor of 0.8 L, from SBR L, was used to inoculate bench scale batch bioreactors (Figure 1D). Immediately after sampling, 0.040 L of the liquor was placed in the batch bioreactors, this contained  $8.9 (\pm 1.5) \text{ g VSS.L}^{-1}$  of sulphate reducing biomass. The batch bottles were capped and, after two hours of settling, 0.030 L of supernatant was removed carefully with a syringe. This was followed by the addition of new synthetic wastewater with the desired electron donor (lactate), acceptor (sulphate) and  $\text{NH}_4^+$  ( $\text{NH}_4\text{Cl}$ ) concentrations. This procedure was essential to maintain the initial biomass concentration in the batch bioreactors constant.

## 2.5 Experimental design

Two different biomass types were tested in two SBR, the control (L) and the experiment (H) bioreactor (Figure 1A and Table 1). SBR L with sulphate reducing biomass was fed with 0.417 g  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$  (140 mg S- $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ) sulphate and 1 g  $\text{COD} \cdot \text{L}^{-1}$  lactate. SRB H was fed with sulphate at 2.5 g  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$  (840 mg S- $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ) and 6 g  $\text{COD} \cdot \text{L}^{-1}$  of lactate. In both SBR, the influent COD:sulphate ratio was maintained at 2.4. In SRB H, the sulphate and lactate concentrations were 6 times higher than those used for SBR L. SBR L was operated at an HRT of 2 d along the experiment. SBR H was operated under the following schedule: phase 1) initially, 8 days at 2 d HRT, phase 2) followed by 6 days of batch conditions, the influent and effluent pumps were stopped at this time, and phase 3) the last 20 days operated at 2 d HRT. Both SBR L and SBR H were operated at constant  $\text{NH}_4^+$  concentrations (300 mg  $\text{NH}_4\text{Cl} \cdot \text{L}^{-1}$ ).

## 2.6 Transient, feast-famine, feeding conditions in SBR operation

Two SBR were used as control experiments, L1 and H1 (Figure 1B and C). SBR L1 was operated at low concentrations: 0.4 g  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$  (140 mg S- $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ) and 1 g  $\text{COD}_{\text{Lactate}} \cdot \text{L}^{-1}$ . SBR H1 was operated at high concentrations: 2.5 g  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$  (840 mg S- $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ) and 6 g  $\text{COD}_{\text{Lactate}} \cdot \text{L}^{-1}$ . Neither the COD or sulphate concentrations nor the HRT were modified during the 22 d of SBR operation.

SBR L2 and L3 were operated at transient feeding conditions. Both reactors followed the schedule described in Figure 1E. The first four days, both SBR were operated at steady feeding conditions at 0.4 g  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$  (140 mg S- $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ) and 1 g  $\text{COD}_{\text{Lactate}} \cdot \text{L}^{-1}$ , followed by a famine day. On a famine day, the influent pump was stopped and therefore any electron donor and acceptor were fed. The famine operation was followed by a feast day. During a feast day, 2.5 g  $\text{SO}_4^{2-} \cdot \text{L}^{-1}$  (840 mg S- $\text{SO}_4^{2-} \cdot \text{L}^{-1}$ ) and 6 g  $\text{COD}_{\text{Lactate}} \cdot \text{L}^{-1}$  were fed within the influent.

SBR H2 and H3 were also operated at transient feeding conditions (Figure 1E) but at higher concentrations (compared to L2 and L3). The first four days, both SBR were operated at steady feeding conditions ( $2.5 \text{ g SO}_4^{2-} \cdot \text{L}^{-1}$  or  $840 \text{ mg S-SO}_4^{2-} \cdot \text{L}^{-1}$  and  $6 \text{ g COD}_{\text{Lactate}} \cdot \text{L}^{-1}$ ) and further followed by a famine day. The famine operation (described above) was followed by a feast day at  $15 \text{ g SO}_4^{2-} \cdot \text{L}^{-1}$  ( $5000 \text{ mg S-SO}_4^{2-} \cdot \text{L}^{-1}$ ) and  $36 \text{ g COD}_{\text{Lactate}} \cdot \text{L}^{-1}$ .

SBR L1 was operated at constant  $\text{NH}_4^+$  concentrations ( $300 \text{ mg NH}_4\text{Cl} \cdot \text{L}^{-1}$ ) as well as SBR H1 ( $1800 \text{ mg NH}_4\text{Cl} \cdot \text{L}^{-1}$ ). For SBR L2 and H2, the  $\text{NH}_4^+$  source ( $\text{NH}_4\text{Cl}$ ) was increased also 6 times ( $1800 \text{ mg NH}_4\text{Cl} \cdot \text{L}^{-1}$ ) during steady and transient (feast) feeding operation. The  $\text{NH}_4^+$  source ( $\text{NH}_4\text{Cl}$ ) was only excluded ( $0 \text{ mg NH}_4\text{Cl} \cdot \text{L}^{-1}$ ) in SBR L3 and H3 influent feeding, from the start of their operation. Concerning the electron donor and acceptor, during the feast days of SBR operation, the COD:sulphate ratios was maintained at 2.4. The presence and absence, induced by the feast or famine operation, of COD, sulphate and  $\text{NH}_4^+$  in the influent was the sole disturbance applied to the different SBR, as specified above. The cycle length (8 h) and the feast (24 h) and famine (24 h) length periods were chosen based on background information from other SBR operations.

## 2.7 Sulphate reduction experiments in batch

The sulphate reducing activity of the biomass was investigated in batch under feast and famine conditions (Figure 1D). The feast and famine conditions were induced by altering the initial  $\text{NH}_4^+$  and sulphate concentrations. The lactate concentration ( $1,000 \text{ mg COD} \cdot \text{L}^{-1}$ ) was kept constant while the initial sulphate concentrations were 417, 666 and  $1491 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1}$ , in the respective batch bioreactors (Table 2). The initial  $\text{NH}_4^+$  concentration was excluded ( $0 \text{ mg NH}_4\text{Cl} \cdot \text{L}^{-1}$ , zero) for the experiments A, B, and C, while  $300 \text{ mg NH}_4\text{Cl} \cdot \text{L}^{-1}$  was the concentration for experiments A\*, B\* and C\*. Table 2 shows an overview of the 6 different initial feast and famine conditions

used to evaluate the sulphate reducing activity of the biomass. All batch experiments were performed in triplicate.

## 2.8 Evaluation of SBR performance

The activity in the reactor was evaluated in terms of the loading rates ( $LR$ ), removal rates ( $RR$ ), fraction ( $f$ ) of a component in the effluent and removal efficiencies ( $RE$ ). The robustness and resilience of the process were evaluated in terms of resistance and resistance index, according to the following equations (Eqs. 1-6):

$$LR = \frac{Q(A)}{V} \quad \text{Eq. 1}$$

$$RR = \frac{Q(A-B)}{V} \quad \text{Eq. 2}$$

$$f = \frac{(A-B)}{A} \quad \text{Eq. 3}$$

$$RE = \left( \frac{(A-B)}{A} \right) \times 100 \quad \text{Eq. 4}$$

$$\text{Resistance} = \frac{RE}{\Delta t} \quad \text{Eq. 5}$$

$$\text{Resistance index} = \frac{\text{Resistance of reactor experiment}}{\text{Resistance of reactor control}} \quad \text{Eq. 6}$$

The flow rate ( $Q$ ) of 3 L.d<sup>-1</sup> was equivalent to 3 cycles.d<sup>-1</sup> and was used for the SBR with 6 L working volume (SBR L and H). The SBR with 2 L working volume (L1, L2, L3, H1, H2 and H3) used a  $Q$  of 1 L.d<sup>-1</sup> (equivalent to 3 cycles.d<sup>-1</sup>). The operational reactor volume ( $V$ ) of the SBR was expressed in L. The initial or influent concentration ( $A$ ) and the final or effluent concentration ( $B$ ) of any compound fed to the biomass was used for calculation, e.g. compounds like the initial SO<sub>4</sub><sup>2-</sup>, sulphate sulphur (S-SO<sub>4</sub><sup>2-</sup>), sulphide (S-S<sup>2-</sup>), lactate or COD concentrations. The concentration of all influent and effluent carbon compounds, responsible for the COD, e.g. lactate and volatile fatty acids, were expressed in COD equivalents (with units of mg COD.L<sup>-1</sup>) and fractions (dimensionless). The time difference ( $\Delta t$ ) was defined between the time of starting a

new condition ( $t_{NC}$ ) and the time necessary to reach removal efficiencies  $\geq 80\%$  of any compound ( $t_{RE \geq 80\%}$ ). The batch volumetric ( $V_r$ ) and specific ( $S_r$ ) removal rates were calculated using the Eq. 7 and Eq. 8 at the time of evaluation ( $t_e$ ) corresponding to the steepest slope and using the initial VSS concentration ( $VSS_{t_0}$ ). For estimating the  $V_r$  of sulphate, the numerator of Eq. 7 was divided by the fraction of sulphur present in sulphate (0.3333).

The yield of sulphate removed on the COD (total COD used) was calculated using Eq. 9. In this equation, the fraction of sulphate ( $1-f_{S-SO_4^{2-}}$ ) consumed at  $t_e$  was multiplied by the initial sulphate concentration for the respective batch incubation and further divided by the COD concentration at the same  $t_e$ . In this study, the first order kinetic constant ( $k_1$ ) of sulphate removal was calculated from the slopes depicted by the plot of the  $V_r$  of sulphate against the initial sulphate concentration in the batch (Eq. 10). According to Schmidt et al. (1985), Eq. 10 is recommended for the kinetic evaluation of substrate consumption in non-supporting growth conditions. In the batch experiments, the cell growth was considered as zero or negligible due to the initial substrate (either lactate or sulphate) to biomass ratio. The standard deviation was calculated for all average values of data enclosed in a period of SBR operation ( $> 3$  data points) and, also, for every batch experiment in triplicate.

$$V_r = \frac{[1 - (f_A)_{t_e}] \times (B)}{t_e \times \left(\frac{1 d}{24 h}\right)} \quad \text{Eq. 7}$$

$$S_r = \frac{V_r}{VSS_{t_0}} \quad \text{Eq. 8}$$

$$Y_{SO_4^{2-}/TCOD} = \frac{(1 - f_{S-SO_4^{2-}})_{t_e} \times SO_4^{2-}}{(1 - f_{TCOD})_{t_e} \times TCOD_{t_0}} \quad \text{Eq. 9}$$

$$-\frac{dS}{dt} = k_1 S \quad \text{Eq. 10}$$

## 2.9 Analytical techniques

The pH was measured off-line using a sulphide resistant electrode (Prosense, Oosterhout, The Netherlands). Sulphate and ammonium were analyzed by ion chromatography (DIONEX 100)

using a conductivity detector (Mottet et al., 2014). The volatile fatty acids (VFA) concentrations (acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate) were measured using a gas chromatograph (GC-800 Fisons Instrument) equipped with a flame ionization detector (Mottet et al., 2014). Lactate was analyzed by high performance liquid chromatography (HPLC) using a Aminex HPX-87H [300 × 7.8 mm column (Biorad), 35 °C and 0.4 mL.min<sup>-1</sup> flow rate] with an isocratic elution solution of 0.005 M H<sub>2</sub>SO<sub>4</sub> and a refractometric detector (Waters R410) as reported in the literature (Quéméneur et al., 2012). The VSS, TSS and the sulphide or total dissolved sulphide (TDS, by the methylene blue method) were measured according to the procedures outlined in Standard Methods (APHA, 1999).

### 3 Results and discussion

#### 3.1 Sulphate reduction using enriched sulphate reducing biomass as inoculum at low sulphate concentrations

SBR L was operated during 34 days, when the S-SO<sub>4</sub><sup>2-</sup>-RR reached the value equal to the S-SO<sub>4</sub><sup>2-</sup>-LR, the sulphate RE was assumed to be 100% (Figure 2A-D). During the first 12 d of operation, a sulphate RE of 22 (± 15)% was observed. The time to reach > 80% of sulphate RE ( $t_{RE \geq 80\%}$ ) was after 12 d of SBR operation. After this time (12 d), SBR L was considered to be under steady performance (sulphate RE = 90 (± 9)%) until the end of the operation (Figure 2A). Sulphide concentrations were as low as 27 (± 21) and 60 (± 25) mg S<sup>2-</sup>.L<sup>-1</sup>, respectively, during and after the first 12 d of operation. The sulphide production rates ranged between 13 and 30 mg S<sup>2-</sup>.L<sup>-1</sup>.d<sup>-1</sup>, respectively (Figure 2A).

The lactate RR or consumption rates were equivalent to 100% RE along SBR operation (34 d). Likewise, the total COD (TCOD) RE was 99 (± 3)% during the 34 d of operation (Figure 2B). The presence of COD as VFA, alcohols or other analytes in the effluent was very punctual

but at very low concentrations (Figure 2C). For instance, iso-butyrate ( $f = 1$ ) was detected during the first two days of operation. Propionate ( $f = 1$ ) was detected as the sole VFA at day 8 of operation. Propionate ( $f = 0.39$ ) and acetate ( $f = 0.6$ ) were detected at day 20 of operation (Figure 2C).

Furthermore, the effluent pH was  $7.81 (\pm 0.14)$  during the non-steady performance, the first 12 d of SRB operation, and later it was maintained at  $7.75 (\pm 0.29)$  until the end of the steady performance (Figure 2D). The substrate to biomass ratio ( $\text{SO}_4^{2-}:\text{VSS}$ ) was 0.025 during the operation of SBR L. A summary of the performance of SBR L is shown in Table 1.

### 3.2 Sulphate reduction using methanogenic anaerobic sludge as inoculum at high sulphate concentrations

SBR H was operated in the three experimental phases during 34 days of semi-continuous operation (Figure 2E-H). At the beginning of the first phase, SBR H showed high  $\text{SO}_4^{2-}\text{-RR}$ , in the range of 1,165 - 1,192  $\text{mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$  (392-401  $\text{mg S-SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ ), corresponding to a sulphate  $RE > 90\%$ . This  $RE$  is supported by the initial  $\text{SO}_4^{2-}:\text{VSS}$  ratio (0.068) and, therefore, by the initial biomass concentration (36.5  $\text{g VSS} \cdot \text{L}^{-1}$ ). Nevertheless, the  $\text{S-SO}_4^{2-}\text{-RR}$  reached an average  $RE$  of  $62 (\pm 25)\%$  during phase I, suggesting the washout of bacteria with poor settling time ( $> 5$  h). An increase in the sulphide concentration ( $148 \pm 97 \text{ mg S}^{2-} \cdot \text{L}^{-1}$ ) was observed and the production rate was  $74 (\pm 48) \text{ mg S}^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$  during phase I (Figure 2E).

During phase II (from 8.3-14.3 d), the influent and effluent pumps were switched off and therefore SBR H was operated in batch mode. During this time, the sulphate  $RR$  ( $20 \pm 17 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$  or  $7 \pm 6 \text{ mg S-SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ ) and sulphide production rate ( $54 \pm 6 \text{ mg S}^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ ) decreased, but the sulphide concentration and the sulphate  $RE$  reached  $324 (\pm 34) \text{ mg S}^{2-} \cdot \text{L}^{-1}$  and  $95 (\pm 4)\%$ , respectively, in SBR H. After 14.3 d, the influent and effluent pumps were switched

on and the sulphate *RE* dropped to 65 ( $\pm 12$ )%, while a sulphide production rate of 142 ( $\pm 55$ ) mg  $S^{2-} \cdot L^{-1} d^{-1}$  was observed, corresponding to a sulphide concentration of 284 ( $\pm 111$ )% mg  $S^{2-} \cdot L^{-1}$ , at the beginning of phase III.

The performance from days 20 to 34 (phase III) reached a steady sulphate *RE* of 96 ( $\pm 10$ )%, corresponding to a sulphate *RR* of 1,193 ( $\pm 140$ ) mg  $SO_4^{2-} \cdot L^{-1} d^{-1}$  (or 401  $\pm 47$  mg  $S-SO_4^{2-} \cdot L^{-1} d^{-1}$ ). The sulphide production rate was 201 ( $\pm 46$ ) mg  $S^{2-} \cdot L^{-1} d^{-1}$  at the highest sulphide concentration observed (440  $\pm 41$  mg  $S^{2-} \cdot L^{-1}$ ) in SBR H (Figure 2E). The lactate *RR* or consumption rate was also equivalent to 100% *RE* in SBR H along the operation time (34 d). Only after changing the operation regime, from batch to semi-continuous, from phase II to phase III, the lactate in the effluent corresponded to fraction  $f = 0.6$ . This was the only time at which lactate was detected in the effluent. On the other hand, the TCOD *RR* showed different performances during the three phases, corresponding to a TCOD *RE* of 60 ( $\pm 21$ )% during phase I, 84 ( $\pm 23$ )% during phase II, 89 ( $\pm 4$ )% at the beginning of phase III and 90 ( $\pm 7$ )% during the steady performance of phase III (Figure 2F).

During the first 2 days of SBR H operation, acetate ( $f = 1$ ) was the main effluent COD component. But suddenly, after 2 and until 8 days of operation, propionate ( $f = 0.6 \pm 0.27$ ) was the dominant fraction followed by acetate ( $f = 0.4 \pm 0.27$ ) during phase I. During phase II, propionate ( $f = 1$ ) was the only VFA in the reactor after one day of batch operation. At the end of phase II, there was no VFA or any other analyte in the effluent. Lactate ( $f = 0.6$ ) and acetate ( $f = 0.39$ ) were detected in the effluent after one day of operation of phase III. On day 20, propionate ( $f = 0.6$ ) was observed as the main VFA followed by acetate ( $f = 0.4$ ). After 20 days of operation, SBR H reached steady sulphate *RE* (96  $\pm 10$ %) performance and acetate ( $f = 0.8 \pm 0.2$ ) was the major VFA followed by propionate ( $f = 0.2 \pm 0.2$ ) (Figure 2G).

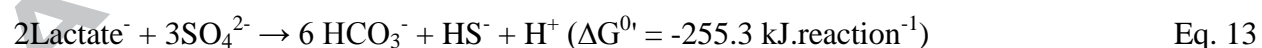
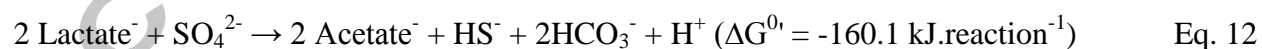


During the sulphate reduction process in SBR H, the observed effluent pH was  $> 7.0$  and  $< 8.0$  for 34 d (Figure 2H). The performance of SBR H is summarized in Table 1. During its steady performance, the VSS concentration was  $15.9 \text{ g VSS.L}^{-1}$  in SBR H and, therefore, the  $\text{SO}_4^{2-}:\text{VSS}$  ratio was equal to 0.15.

A fast sulphate removal by SBR L was expected due to the conditions tested: no COD limiting conditions, COD:sulphate ratio of 2.4 and sulphate:VSS ratio of 0.025. However, 12 days were required to reach steady performance and a sulphate  $RE = 90 (\pm 9)\%$ . Al-Zuhair et al. (2008) found that the specific growth rate of pure cultures of SRB is increased by high sulphate concentrations, as high as  $2.5 \text{ g SO}_4^{2-}.\text{L}^{-1}$ . There is evidence that SRB growth and activity is improved by increasing the sulphate concentrations also when anaerobic sludge is used as inoculum (O'Reilly and Colleran, 2006). Therefore, the sulphate reduction was higher in SBR H ( $62 \pm 25\%$ ) starting from the first phase of operation, when it was exposed to higher initial sulphate concentrations ( $2.5 \text{ g SO}_4^{2-}.\text{L}^{-1}$ ) (Figure 2E). In contrast, the control SBR L showed a poor sulphate  $RE$  ( $22 \pm 15\%$ ) using  $0.4 \text{ g SO}_4^{2-}.\text{L}^{-1}$  during the first 12 days of operation (Figure 2A). This difference can be supported by the high bacterial diversity present in the anaerobic sludge (Guo et al., 2014), such diversity is beneficial for the robustness and resilience of a biological system.

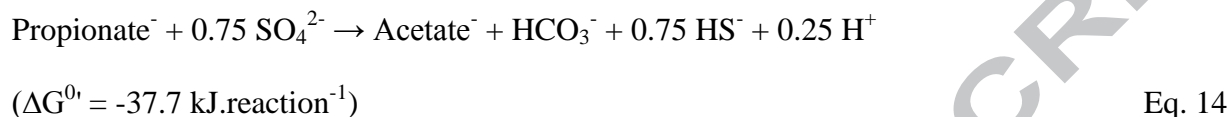
SBR H was inoculated with anaerobic sludge and compared to an enriched sulphate reducing biomass present in SBR L. In both SBR L and SBR H, the lactate was used efficiently ( $RE = 100\%$ ) from the beginning of the experiment till the end (Figure 2B and F). SBR H showed a low COD  $RE$  and  $RR$  ( $60 \pm 21\%$  and  $1,793 \pm 616 \text{ mg COD.L}^{-1}\text{d}^{-1}$ ) when compared to the control SBR L (COD  $RE = 99 \pm 3\%$  and a COD  $RR = 495 \pm 14 \text{ mg COD.L}^{-1}\text{d}^{-1}$ ). The propionate fraction became dominant in the effluent, compared to the acetate fraction, in the first phase and the beginning of the third phase of SRB H operation (Figure 2G). This propionate accumulation

was not observed during the performance of SBR L, propionate was detected as  $f = 1$  and  $f = 0.39$  at day 8 and 20 of operation, respectively (Figure 2C). This evolution in SRB H suggests that hydrolytic-fermentative bacteria can outcompete the SRB for the fermentation of lactate to propionate during its non-steady performance compared to SBR L which had a well established pathway of carbon utilization during sulphate reduction. Zhao et al. (2008) reported, for a sulphidogenic bioreactor, that lactate can be consumed by two different pathways: either directly used for sulphate reduction (e.g. *Desulfovibrio spp.*) or firstly fermented to propionate and ethanol e.g. *Clostridium spp.* and *Bacteroides spp.*) and secondly the fermented products can be used by SRB (e.g. *Desulfobulbus spp.* or *Desulfovibrio spp.*). For instance, the specific growth rates of hydrolytic-fermentative bacteria ( $\mu_{VFA\ mixture} \gg 1.2\ d^{-1}$ ) (Escudié et al., 2005) is larger compared to SRB (*Desulfovibrio desulfuricans*,  $\mu_{Lactate} \sim 0.052\ d^{-1}$ ) (Cooney et al., 1996) and methanogenic archaea ( $\mu_{Acetate} \sim 0.15-0.55\ d^{-1}$ ) (O'Flaherty et al., 1998). This supports the pathway of fermentation of lactate and further utilization of the fermented products as electron donors for sulphate reduction. Furthermore, the conversion of lactate to propionate (Eq. 11) has a lower standard free energy change of biological formation ( $\Delta G^{0'} = -54.9\ kJ.reaction^{-1}$ ) in contrast to sulphate reduction using the initial electron donor ( $COD_{Lactate}$ ) by means of Eq. 12 (-160.1  $kJ.reaction^{-1}$ ) and unlikely by Eq. 13 (-255.3  $kJ.reaction^{-1}$ ).

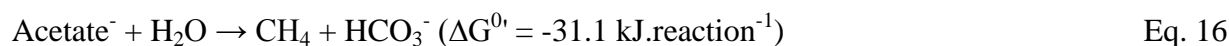
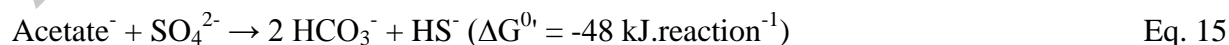


Eq. 12 describes sulphate reduction by SRB under no lactate limiting conditions and Eq. 13 suggests sulphate reduction by pure SRB species and avoid the competition for lactate by other microorganisms. However, Eq. 14 shows a  $\Delta G^{0'} = -37.7\ kJ.reaction^{-1}$  and suggests that sulphate reduction is also possible by means of propionate oxidation (Jr. Leslie Grady et al.,

2012). For instance, anaerobic sludge was reported to perform steady sulphate reduction at a  $RE = 99.5\%$  using a propionate:sulphate ratio of 1.31 (or a  $COD_{Propionate}$ :sulphate ratio of 2.0), at an HRT of 2 d and an initial sulphate concentration of  $1.97 \text{ g.L}^{-1}$  (Ghigliazza et al., 2000). The propionate:sulphate or  $COD_{Propionate}$ :sulphate ratios reported by Ghigliazza et al. (2000) are very much in agreement with the stoichiometry shown in Eq. (14):



High fractions of propionate in the effluent COD and low sulphate  $RE$  (e.g.  $62 \pm 25\%$  during phase I in SBR H) indicates a small population of propionate consuming SRB. On the other hand, low propionate fractions (as in SBR L) indicate a stable pathway of COD consumption during the sulphate reduction. *Desulfobulbus* like SRB are well known to utilize propionate as electron donor for sulphate reduction. Using conditions such as a COD:sulphate ratio of 2, *Desulfobulbus* like SRB accounted for 20 % of the bacterial community (Dar et al., 2008). Therefore, when bacteria consumed the propionate, the sulphate  $RE$  can be improved (i.e. to  $95 (\pm 4)\%$  in phase II) of SRB H and little or sporadic propionate formation was observed in the case of SRB L operation. During steady performance, the  $\delta$ -proteobacteria are the most abundant group rather than firmicutes during sulphate reduction using lactate as the electron donor (Zhao et al., 2010). Notwithstanding, the acetate fraction in the effluent is dominant during steady performance, despite acetotrophic sulphate reduction (Eq. 15):  $\Delta G^0$  of  $-48 \text{ kJ.reaction}^{-1}$  and acetotrophic methanogenesis (Eq. 16):  $\Delta G^0$  of  $-31.1 \text{ kJ.reaction}^{-1}$ .



Besides, SRB have a lower affinity ( $> K_S$ ) for acetate compared to acetotrophic methanogenic archaea ( $< K_S$ ) (Stams et al., 2005), suggesting that acetate is removed by means of

methanogenesis in both SBR L and H, but at different rates. In contrast, at low COD:sulphate ratios ( $< 1.3$ ), SRB can outcompete methanogenic archaea for acetotrophic sulphate reduction (Chou et al., 2008).

Changes on the operation mode of the bioreactors drive to improvements on the sulphate *RE*, e.g. changing from UASB to UASB with biomass recirculation operation (Boshoff et al., 2004). Increasing the HRT or SRT is beneficial for sulphate reduction, this change optimizes the contact time of bacteria (VSS) with the substrates. This suggest that slow growing SRB (in comparison to hydrolytic-fermentative bacteria) have the time to convert the propionate and overcome accumulation (e.g. in SBR H, Figure 2G) or a shock load during transient operations. In this study, the influent pumps were switched off (in period II from day 8.3 to 14.3) to increase the HRT and the SRT in order to overcome the accumulation of propionate during the start-up of sulphidogenesis in SBR H.

High sulphide concentrations influence the sulphate reducing metabolism and can help to outcompete methanogenic archaea (Icgen and Harrison, 2006). When the HRT was decreased (from 2 days to 6 days of batch operation) to start period II in SBR H, the accumulation of sulphide ( $324 \pm 34 \text{ mg S}^{2-} \cdot \text{L}^{-1}$ ) supported the growth of the SRB population (Figure 2E). Furthermore, Torner-Morales and Buitrón (2010) reported a decreasing sulphate *RE* by 1/5 and 1/2 during the operation of SBR with sulphide stripping, the previous sulphate *RE* was 100 and 90% for COD:sulphate ratios of 6 and 2, respectively. The SRB are more tolerant to high sulphide concentrations ( $< 1600 \text{ mg S}^{2-} \cdot \text{L}^{-1}$ ) than other bacteria and methanogenic archaea, the sulphide toxicity seems to be more specific or dependent for genus or groups (O'Flaherty et al., 1998). Continuous bioreactors, like the inverse fluidized bed bioreactors (IFBB), with biomass recirculation can operate at sulphide concentrations of  $1,200 \text{ mg S}^{2-} \cdot \text{L}^{-1}$  without affecting the

COD or sulphate *RE* (Celis-García et al., 2007). However, the toxicity due to high sulphide concentrations is higher when reactors are operated at long HRT (> 1 d) (Kaksonen et al., 2004).

A non-steady sulphate *RE* was observed during the beginning of the third period of operation in SBR H, for 6 d (from 14 to 20 d of operation). Decreasing the HRT could have hampered the sulphate reducing process in SBR H, the sulphate *RE* was 65 ( $\pm$  12)% and the COD *RE* was 89 ( $\pm$  4)% (Figure 2E). After 20 days of SBR H operation, a steady sulphate *RE* was achieved (96  $\pm$  10%), wherein acetate was the major by-product and propionate became a minor component in the effluent COD (~ 10% of the COD fed). The steady sulphate reducing conditions imply a well-established metabolism in the presence of sulphate, this performance was reached in SBR L and H after 12 and 20 d of operation, respectively. However, the lack of propionate consuming SRB can hamper the performance of bioreactors (O'Flaherty and Colleran, 1999; Lens et al., 1998; Qauibi et al., 1990). During operation of SBR H, this was a key factor to reach the steady sulphate reducing performance, a drawback that was not observed during the operation of SBR L.

### 3.3 Sulphate reduction under transient feeding conditions in the SBR

SBR L1 (Figure 3A1-A3) showed a sulphate *RE* of 79 ( $\pm$  17)% and COD and lactate *RE* of 100%. VFA were not detected in the effluent (Figure 3A2) and the average pH was 7.4 ( $\pm$  0.2) (Figure 3A3). If the  $S-SO_4^{2-}-RR$  reached a value equal to the  $S-SO_4^{2-}-LR$ , the sulphate *RE* was assumed to be 100%. On the other hand, SBR H1 (Figure 4A1-A3) achieved a sulphate *RE* of 94 ( $\pm$  8)%, lactate *RE* of 99 ( $\pm$  3)% and COD *RE* of 93 ( $\pm$  7)%. Lactate ( $f \leq 0.4$ ) was present in the effluent on the first 2 days of operation. However, during the operation time, acetate and propionate became the only VFA dominant in the effluent COD (Figure 4A2), 0.58 ( $\pm$  25) and

0.38 ( $\pm$  0.24) were the respective average fractions. The average effluent pH was 7.7 ( $\pm$  0.2) (Figure 4A3).

SBR L2 performed sulphate removal under transient feeding conditions (feast: 6 g COD<sub>Lactate</sub>.L<sup>-1</sup> and 2.5 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup> and famine: 0 g COD<sub>Lactate</sub>.L<sup>-1</sup> and 0 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>), 92 ( $\pm$  13)% and 90 ( $\pm$  11)%, respectively, during feast and famine conditions (Figure 3B1). Lactate was always consumed at 100% *RE* and the COD *RE* ranged between the feast (99 ( $\pm$  1)%) and famine (92 ( $\pm$  6)%) conditions (Figure 3B2). The average fractions of acetate (0.28  $\pm$  0.32) and propionate (0.31  $\pm$  0.34) were similar but the latter was in higher fractions after a feast day of operation. The pH was 7.7 ( $\pm$  0.1) during the feast and 7.5 ( $\pm$  0.1) during the famine conditions (Figure 3B3).

SBR H2 was fed with high concentrations of COD and sulphate (36 g COD<sub>Lactate</sub>.L<sup>-1</sup> and 15 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup> or 5 g S-SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>) during the feast periods. The sulphate *RE* was 55 ( $\pm$  19)% and 36 ( $\pm$  29)%, respectively, during the feast and famine conditions, also this parameter decreased stepwise from 88% to 14% from 7 to 22 d of operation. The S-SO<sub>4</sub><sup>2-</sup> *RR* (Figure 4B1) and COD *RR* decreased from, respectively, 2,224 mg S-SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>d<sup>-1</sup> (6670 mg SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>d<sup>-1</sup>) and 15,174 mg COD.L<sup>-1</sup>d<sup>-1</sup> to 451 mg S-SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>d<sup>-1</sup> (1,347 mg SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>d<sup>-1</sup>) and 441 mg COD.L<sup>-1</sup>d<sup>-1</sup> at the end of the experiments. Lactate was only consumed partially, 85 ( $\pm$  8)% *RE* during the feast and 86 ( $\pm$  14)% during the famine conditions. The average COD *RE* was 42 ( $\pm$  26)% during the feast and 35 ( $\pm$  35)% during the famine conditions. The average lactate fraction in the effluent was 0.2 ( $\pm$  0.2), but the rest of the effluent COD was composed by fractions as follows: acetate 0.43 ( $\pm$  0.29), propionate 0.30 ( $\pm$  0.23), butyrate 0.06 ( $\pm$  0.04) and iso-valerate 0.0022 ( $\pm$  0.0011). On the other hand, the lactate *RR* recovered after the famine days: at the end of the experiment (20 to 22 d of operation), it was totally oxidized to VFA (Figure 4B2) and propionate ( $f$  = 0.61) was the dominant VFA effluent fraction followed by acetate ( $f$  = 0.28) and butyrate ( $f$  = 0.1). The average

pH was 7.2 ( $\pm 0.3$ ) during the feast and 7.0 ( $\pm 0.3$ ) during the famine conditions, but the trend of the profile was decreasing until pH values  $< 7.0$  (Figure 4B3).

SBR L3 (Figure 3C1-C3) performed sulphate removal at transient feeding conditions (feast: 6 g COD<sub>Lactate</sub>·L<sup>-1</sup> and 2.5 g SO<sub>4</sub><sup>2-</sup>·L<sup>-1</sup> and famine: 0 g COD<sub>Lactate</sub>·L<sup>-1</sup> and 0 g SO<sub>4</sub><sup>2-</sup>·L<sup>-1</sup>) in the absence of influent NH<sub>4</sub><sup>+</sup>. The sulphate *RE* was 92 ( $\pm 11$ )% during the feast and 86 ( $\pm 11$ )% during the famine conditions (Figure 3C1), while the average lactate *RE* was 100% in the feast and (99  $\pm 4$ %) during the famine periods, only detected ( $f = 0.44$ ) on day 20. The COD *RE* was higher (96  $\pm 3$ %) during the feast in comparison to famine (85  $\pm 8$ %) conditions (Figure 3C2). Acetate (0.51  $\pm 0.12$ ) and propionate (0.46  $\pm 0.10$ ) were the VFA fractions in the effluent, but propionate was with the higher fraction ( $f = 0.65$ ) at the end of the operation. The pH was never below 7.0: it was 7.6 ( $\pm 0.1$ ) and 7.4 ( $\pm 0.1$ ), respectively, during the feast and famine conditions (Figure 3C3).

SBR H3 (Figure 4C1-C3) performed sulphate removal at transient feeding conditions, high concentrations of electron donor and acceptor (36 g COD<sub>Lactate</sub>·L<sup>-1</sup> and 15 g SO<sub>4</sub><sup>2-</sup>·L<sup>-1</sup> or 5 g S-SO<sub>4</sub><sup>2-</sup>·L<sup>-1</sup>) and in the absence of NH<sub>4</sub><sup>+</sup>. The sulphate *RE* was 57 ( $\pm 21$ )% at feast and 55 ( $\pm 28$ )% at famine conditions, this parameter did not decrease to lower than 30% and it recovered from  $\sim 30$  to  $\sim 43$ % during the last two famine operation days (Figure 4C1). Also, the lactate *RE* was very similar during the feast (93  $\pm 8$ %) and at famine (92  $\pm 10$ %) conditions and a fraction was left without consumption ( $f = 0.15$  to 0.47). But the COD was not totally consumed, the *RE* was 37 ( $\pm 26$ )% and 33 ( $\pm 34$ )% for the feast and famine conditions, respectively (Figure 4C2). For instance, the effluent COD showed an average fraction composition of acetate (0.45  $\pm 0.19$ ), propionate (0.36  $\pm 0.21$ ), butyrate (0.14  $\pm 0.002$ ), iso-butyrate (0.003  $\pm 0.001$ ) and iso-valerate (0.006  $\pm 0.002$ ). Nevertheless, propionate ( $f = 0.53$ ) was the highest fraction followed by acetate ( $f = 0.44$ ) in the effluent at the last 2 days of operation. During SBR H3 operation, the pH did not

reach values  $< 7.0$ ; the average pH was  $7.7 (\pm 0.3)$  and  $7.8 (\pm 0.3)$ , respectively, during the feast and famine conditions (Figure 4C3). In contrast, in SBR H2, the sulphate *RE* decreased until 14% (Figure 4B1) and the pH was also  $< 7.0$  (Figure 4B3) at the last 2 days of operation.

The sulphate reduction process was not affected by the transient feeding conditions in SBR L2 and L3 (Figure 3B1 and C1). The performance in these bioreactors (the sulphate *RE* was  $\geq 90\%$ , unlike the sulphate *RE* =  $86 \pm 11\%$  whereas famine conditions in L3) was comparable to that of the control bioreactor at low concentrations (L1 sulphate *RE* =  $79 (\pm 17)\%$ ) and also at higher concentrations (H1 sulphate *RE* =  $94 \pm 8\%$ ). There are sulphidogenic bioreactors operating at sulphide concentrations of  $1,215 \text{ mg.L}^{-1}$  without  $\text{H}_2\text{S}$  inhibition (Celis-García et al., 2007). However, the potential sulphide toxicity increases simultaneously to the increments of the HRT (Kaksonen et al., 2004). The maximum observed sulphide concentration was in the range of 818-919  $\text{mg.L}^{-1}$  for SBR H2 and H3.

The sulphate concentration of  $2.5 \text{ g SO}_4^{2-} \cdot \text{L}^{-1}$  showed the optimal specific growth rate ( $d^{-1}$ ) using SRB pure cultures, in contrast, lower or higher concentrations can affect the SRB and the biochemical sulphate reduction activity (Al-Zuhair et al., 2008). Thus, the biological sulphate reduction was affected using higher concentrations of electron donor and acceptor (SBR H2 and H3:  $36 \text{ g COD}_{\text{Lactate}} \cdot \text{L}^{-1}$  and  $15 \text{ g SO}_4^{2-} \cdot \text{L}^{-1}$ ). The sulphate *RE* was reduced to  $< 20\%$  in SBR H2 (Figure 4B1) and maintained slightly above 40% in SBR H3 (Figure 4C1) until the end of the operation time.

The average lactate *RE* was  $\geq 99\%$  in the control SBR (L1 and H1) and both SBR L2 and SBR L3 under feast and famine conditions. These SRB (L1, L2, L3, and H1) showed a COD *RE*  $\geq 90\%$ , but SBR L3 showed a COD *RE* of  $85 (\pm 8)\%$  during famine conditions. The average lactate *RE* (92-93%) was higher in SBR H3, regardless of the feast/famine conditions, when compared to SBR H2 (lactate *RE* = 85-86%). The COD *RE* dropped to very low values in SBR



H2 (~ 2%) as well as in SBR H3 (~ 6%) at the end of the reactor operation. The presence of propionate indicates that lactate was first consumed by hydrolytic-fermentative bacteria. Lactate can be degraded first by microorganisms other than SRB, such as the hydrolytic-fermentative or homoacetogenic bacteria (Dar et al., 2008; Wang et al., 2008). This evidenced that sulphate reduction was possible using lactate and also the propionate produced by hydrolytic-fermentative bacteria at the feast and famine conditions. However, sulphate reduction in the presence of acetate was less likely to occur. According to O'Reilly and Colleran (2006), at a COD:sulphate ratio > 2.1, acetate is used by archaea for methanogenesis. Janyasuthiwong et al. (2016) showed that the sulphate reduction is inhibited in batch bioreactors when the acetate concentration is increased from 100 to 500 mg.L<sup>-1</sup>. This suggests that acetate needs to be removed by means of other processes, *e.g.* methanogenesis, rather than sulphate reduction when sulphate is present in wastewater; if acetate is not removed then the sulphate reduction will be inhibited. The acetate concentrations were very low in SBR L2 and L3 (Figure 3B2 and C2), when the COD *RE* was ≥ 90% and 85 (± 8)% (only in SBR L3 at famine conditions) at 6 g COD<sub>Lactate</sub>.L<sup>-1</sup> in the influent. On the other hand, SBR H2 and SBR H3 showed inhibition of the sulphate reduction because of the presence of high acetate concentrations (Figure 4 B2 and C2), this can be seen at very low COD *RE* (SBR H2 ~ 2% and SBR H3 ~ 6%) and high average acetate fractions (SBR H2 *f* = 0.38 (± 0.18) and SBR H3 *f* = 0.47 (± 0.14)) at 36 g COD<sub>Lactate</sub>.L<sup>-1</sup> in the influent.

The influent pH is a factor affecting the sulphate reduction in bioreactors. Papirio et al. (2013) observed that the sulphate reduction process fails when the influent pH is decreased from 7.0 to 3.0, but this recovered to a sulphate *RE* of 97% when the pH was increased to an influent pH of 5.0. Janyasuthiwong et al. (2016) also observed that, when decreasing the influent pH from 7.0 to 5.0, the sulphate *RE* was reduced from ~ 75% to 40%. In this study, the influent pH was 6.0 in all the tested SBR, regardless whether steady or transient feeding conditions, while the

average effluent pH was  $> 7.0$ . Using lactate and propionate as electron donors, the biological sulphate reduction produces carbonate (Eq. 12-14), but SRB can also use other electron donors and produce carbonate (Liamleam and Annachhatre, 2007). The sulphidogenic process is responsible for the generation of the buffering capacity and achieving stable pH  $> 7.0$  while sulphate rich wastewater treatment (Reyes-Alvarado et al., 2017a).

### 3.4 Effect of $\text{NH}_4^+$ famine conditions on sulphate reduction in bioreactors performance

The experiments in batch using electron donor feast conditions (A and A\*, COD:sulphate ratio of 2.4) showed different slopes during the sulphate removal process, but in the experiments A and A\*, the sulphate removal fractions ( $f$ ) were, respectively, 0.96 and 0.98 within 8 h of operation (Table 2). The sulphate and lactate  $V_r$  were  $2,116 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$  and  $5,526 \text{ mg COD}_{\text{Lactate}} \cdot \text{L}^{-1} \text{d}^{-1}$  in the absence of  $\text{NH}_4^+$  (A) and  $1,834 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$  and  $4,140 \text{ mg COD}_{\text{Lactate}} \cdot \text{L}^{-1} \text{d}^{-1}$  in the presence of  $\text{NH}_4^+$  (A\*), respectively. Furthermore, the COD consumption  $V_r$  was lower in comparison to that of lactate (COD), these were  $3,448 \text{ mg COD} \cdot \text{L}^{-1} \text{d}^{-1}$  for A and  $2,240 \text{ mg COD} \cdot \text{L}^{-1} \text{d}^{-1}$  for A\*.

The sulphate reduction, under feast conditions at a COD:sulphate ratio of 1.5, showed larger  $V_r$  during the  $\text{NH}_4^+$  famine ( $4,065 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ ,  $6,697 \text{ mg COD}_{\text{Lactate}} \cdot \text{L}^{-1} \text{d}^{-1}$  and  $5,319 \text{ mg COD} \cdot \text{L}^{-1} \text{d}^{-1}$ , B) in comparison to the  $\text{NH}_4^+$  feast ( $3,488 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ ,  $4,920 \text{ mg COD}_{\text{Lactate}} \cdot \text{L}^{-1} \text{d}^{-1}$  and  $2,921 \text{ mg COD} \cdot \text{L}^{-1} \text{d}^{-1}$ , B\*) conditions in batch bioreactors. It is unlikely that the larger fraction of sulphate removed was at  $\text{NH}_4^+$  feast (0.91 in B\*) in comparison to the  $\text{NH}_4^+$  famine (0.78 in B) conditions within 24 h of operation (Table 2).

The sulphate reduction, using electron donor famine conditions (COD: $\text{SO}_4^{2-}$  ratio of 0.67), showed the largest  $V_r$  during the  $\text{NH}_4^+$  famine conditions ( $9,673 \text{ mg SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ ,  $7,033 \text{ mg COD}_{\text{Lactate}} \cdot \text{L}^{-1} \text{d}^{-1}$  and  $5,715 \text{ mg TCOD} \cdot \text{L}^{-1} \text{d}^{-1}$ , C) in comparison to the  $\text{NH}_4^+$  feast conditions

(9,019 mg  $\text{SO}_4^{2-} \cdot \text{L}^{-1} \text{d}^{-1}$ , 5,703 mg  $\text{COD}_{\text{Lactate}} \cdot \text{L}^{-1} \text{d}^{-1}$  and 3,553 mg  $\text{COD} \cdot \text{L}^{-1} \text{d}^{-1}$ ,  $\text{C}^*$ ) in batch reactors. The fractions of sulphate removed were very similar under the  $\text{NH}_4^+$  famine or feast conditions, 0.48 in C and 0.49 in  $\text{C}^*$ , respectively, within 24 h of operation (Table 2).

Figure 5A shows the  $V_r$  of sulphate removal increases but the fraction of sulphate removed decreases when the electron donor famine conditions increases. The  $k_l$  of sulphate removal was 4 % lower using  $\text{NH}_4^+$  feast ( $k_l=6.69 \text{ d}^{-1}$ ,  $r^2=1$ ), compared to  $\text{NH}_4^+$  famine ( $k_l=6.98 \text{ d}^{-1}$ ,  $r^2=0.9992$ ) conditions (Figure 5A). Under  $\text{NH}_4^+$  famine conditions, the  $S_r$  by SRB used less electron donor (mg  $\text{COD} \cdot \text{mg VSS}^{-1} \text{d}^{-1}$ ) for sulphate reduction (16.6 %) in comparison to the  $\text{NH}_4^+$  feast conditions (Figure 5B). The experiments under  $\text{NH}_4^+$  famine conditions are displaced to the left and top hand according to, respectively, the X-axis and the Y-axis in Figure 5B. This was also indicated on the slopes of the linear regression analysis using the  $S_r$  data and the sulphate removed on the COD consumed: 4.39 mg  $\text{TCOD} \cdot \text{mg VSS}^{-1} \text{d}^{-1}$  under  $\text{NH}_4^+$  feast ( $r^2 = 0.9895$ ) and 3.66 mg  $\text{TCOD} \cdot \text{mg VSS}^{-1} \text{d}^{-1}$  under  $\text{NH}_4^+$  famine ( $r^2 = 0.99$ ) conditions (Figure 5B).

In all the batch incubations, lactate and the COD were totally consumed, respectively, within 8 h and 24 h (Figure 5C and D). However, the lactate and COD  $S_r$  were affected by the sulphate concentration or increasing electron donor famine conditions (Figure 5C and D). Acetate was the major VFA produced after 4 h of incubation, but it was also completely consumed within 24 h. Propionate was produced ( $f < 0.1$ ) after 4 h and consumed within 8 h in all batch reactor incubations, except for experiment  $\text{A}^*$  where propionate was consumed only after 8 h.

Okabe et al. (1992) observed that *Desulfovibrio desulfuricans* decreased the electron donor uptake, the cell size was negatively affected and the carbon content of the cell decreased during sulphate reduction and  $\text{NH}_4^+$  famine conditions. During sulphidogenesis and sulphate feast conditions, *Archaeoglobus fulgidus* strain Z developed higher biomass concentrations compared to sulphate starving conditions, also, the carbon uptake for biomass formation was larger at

sulphate feast conditions (Habicht et al., 2005). Hence, the final sulphate removal was lower in SBR H2 ( $\leq 20\%$  sulphate *RE*,  $\text{NH}_4^+$  feast conditions) compared to SBR H3 ( $\geq 40\%$  sulphate *RE*,  $\text{NH}_4^+$  famine conditions). Under  $\text{NH}_4^+$  feast conditions, the  $k_l$  of sulphate removal was 4 % lower and the use of electron donor ( $\text{mg COD} \cdot \text{mg VSS}^{-1} \text{ d}^{-1}$ ) was 16.6% larger for sulphate reduction; therefore, higher sulphate was removed in SBR H3. These differences were not observed between SBR L2 and SBR L3, because of the lower influent concentrations of electron donor and acceptor tested for sulphate reduction. This study showed that (i) influent sulphate concentrations of  $2.5 \text{ g SO}_4^{2-} \cdot \text{L}^{-1}$  reduced the start-up time of sulphidogenesis in the SBR and therefore higher sulphate *RR* were observed, (ii) the sulphate reducing process is robust and resilience to transient feeding conditions even in low dynamic bioreactors as SBR and (iii)  $\text{NH}_4^+$  feast or famine conditions affect the kinetics of sulphate reduction in bioreactors. The COD:sulphate ratio is reported to be the most important parameter controlling the production of sulphide by means of dissimilatory sulphate reduction by SRB (Velasco et al., 2008). According to Torner-Morales and Buitrón (2010), a sulphate *RE*  $> 90\%$  is guaranteed at a COD:sulphate ratio of 2.4 using lactate as electron donor. The COD:sulphate ratio of 2.4 was constant along the experiments, thus, this ratio was never modified for avoiding disturbances different than those of the transient feeding conditions in the respective SBR.

#### 4 Conclusions

The sulphidogenic process is robust to transient feeding conditions. SBR L2 and SBR L3 reduced  $\geq 90\%$  of the supplied sulphate, similar to the control SBR H1 ( $94 \pm 8\%$ ). The sulphate reduction was affected by the  $\text{NH}_4^+$  feast (*RE* dropped to  $\leq 20\%$  in SBR H2) or famine (*RE*  $\geq 40\%$  in SBR H3) conditions. Furthermore, the  $k_l$  of sulphate removal under  $\text{NH}_4^+$  famine conditions, in the batch bioreactors, was 4 % larger and the use of electron donor was 16.6% more efficient in comparison to  $\text{NH}_4^+$  feast conditions.

## Supplementary information

E-supplementary data of this work can be found in the online version of the paper.

## 5 Acknowledgements

This research was funded by the European Union through the Erasmus Mundus Joint Doctorate Programme ETeCoS<sup>3</sup> (Environmental Technologies for Contaminated Solids, Soils and Sediments, grant agreement FPA no. 2010-0009). The authors thank the staff members from the laboratory of INRA (Narbonne, France) for analytical assistance.

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**List of figure captions**

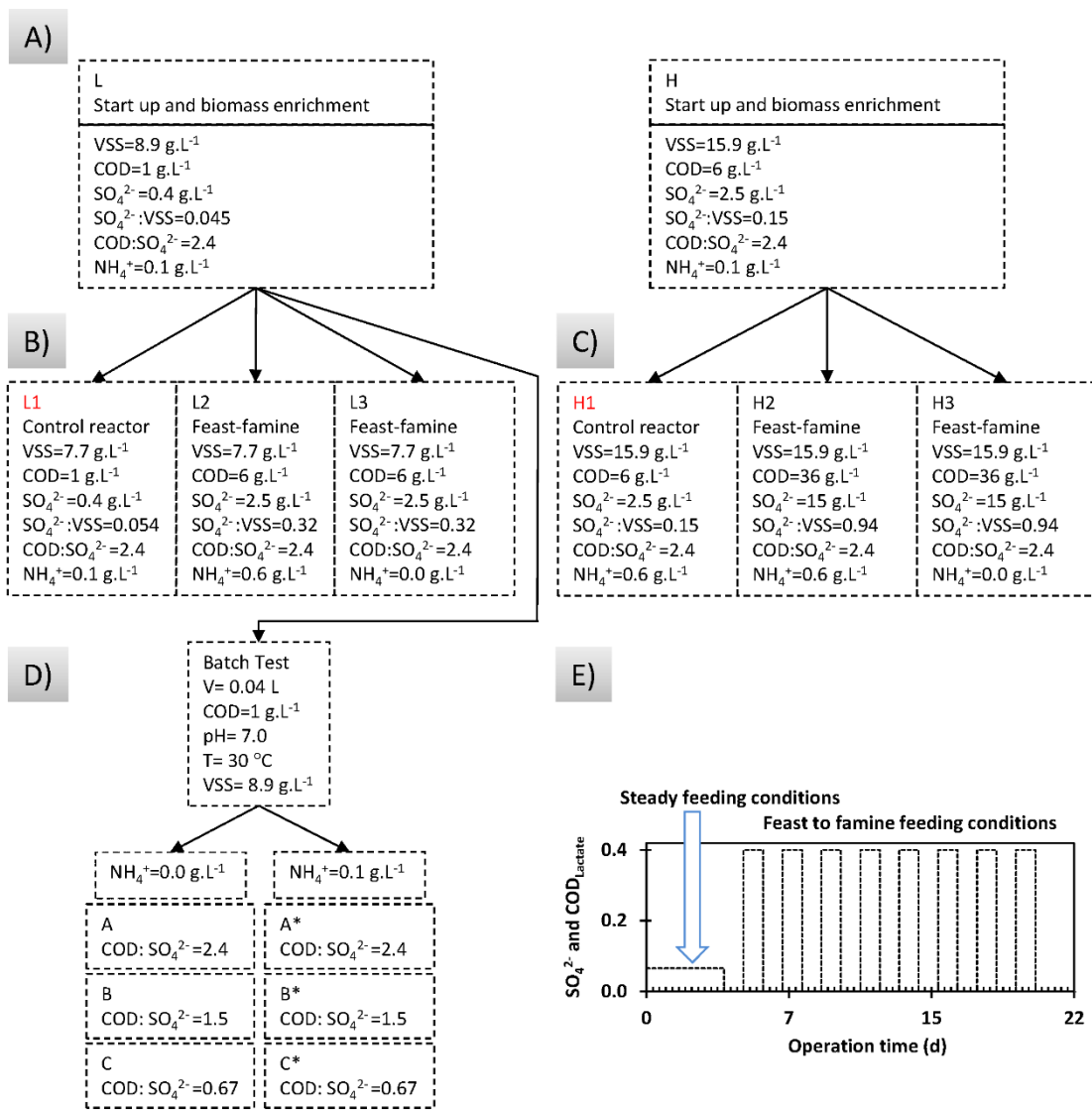
**Figure 1.** The pathway of using biomass during the experiments. A) SBR L (left) and SBR H (right), B) SBR operated under low concentration of electron donor and acceptor (R1L, R2L and R3L), C) SBR operated under high concentrations of electron donor and acceptor (R1H, R2H and R3H), D) Batch test and conditions for  $\text{NH}_4^+$  famine (A, B and C) and feast (A\*, B\* and C\*), and E) Schedule of feast and famine conditions in SBR. The parameter under steady and feast conditions are described inside the boxes. The famine conditions are not included in the boxes, but this refers to switching off the influent and effluent pumps

**Figure 2.** Performance of the SBR L (A-D) and SBR H (E-H). A) and E) Sulphur profiles:  $\text{S-SO}_4^{2-}$  loading rate (---),  $\text{S-SO}_4^{2-}$  removal rate ( $\blacklozenge$ ), production rate of  $\text{S-S}^{2-}$  ( $\blacklozenge$ ) and sulphate removal efficiency ( $\times$ ). B) and F) COD profiles: the COD loading rate (---), COD removal rate ( $\blacklozenge$ ), the lactate removal rate ( $\bullet$ ) and COD removal efficiency ( $\times$ ). C) and G) the fraction composition of the effluent COD: lactate ( $\Delta$ ), acetate ( $\circ$ ), propionate ( $+$ ) and iso-butirate ( $*$ ); D) and H) pH profiles: the  $\text{S-SO}_4^{2-}$  loading rate (---), influent pH at 6.0 (—) and effluent pH ( $\bullet$ ). The SBR L was operated at constant 8 h.cycle<sup>-1</sup> and the SBR H was operated in three periods (I: 8 h.cycle<sup>-1</sup> SBR; II: batch for 6 days; and III: 8 h.cycle<sup>-1</sup> SBR)

**Figure 3.** Profiles of the SBR under transient feeding conditions at low concentrations of electron donor and acceptor, SBR L1 (A1-3), SBR L2 (B1-3) and SBR L3 (C1-C3). A1), B1) and C1) Sulphur profiles:  $\text{S-SO}_4^{2-}$  loading rate (---),  $\text{S-SO}_4^{2-}$  removal rate ( $\blacklozenge$ ), production rate of  $\text{S-S}^{2-}$  ( $\blacklozenge$ ) and sulphate removal efficiency ( $\times$ ). A2), B2) and C2) COD profiles: the COD loading rate (---), COD removal rate ( $\blacklozenge$ ), the lactate removal rate ( $\bullet$ ) and COD fraction as VFA in the effluent. A3), B3) and C3) pH profiles:  $\text{S-SO}_4^{2-}$  loading rate (---), influent pH at 6.0 (—) and effluent pH ( $\bullet$ )

**Figure 4.** Profiles of the SBR under transient feeding conditions at high concentrations of electron donor and acceptor, SBR H1 (A1-3), SBR H2 (B1-3) and SBR H3 (C1-3). A1), B1) and C1) Sulphur profiles:  $\text{S-SO}_4^{2-}$  loading rate (---),  $\text{S-SO}_4^{2-}$  removal rate ( $\blacklozenge$ ), production rate of  $\text{S-S}^{2-}$  ( $\blacklozenge$ ) and sulphate removal efficiency ( $\times$ ). A2), B2) and C2) COD profiles: the COD loading rate (---), COD removal rate ( $\blacklozenge$ ), the lactate removal rate ( $\bullet$ ) and COD fraction as VFA in the effluent. A3), B3) and C3) pH profiles:  $\text{S-SO}_4^{2-}$  loading rate (---), influent pH at 6.0 (—) and effluent pH ( $\bullet$ )

**Figure 5.** The effect of electron donor and  $\text{NH}_4^+$  feast and famine conditions on sulphate reduction in batch bioreactors: A) Volumetric sulphate removal rate and the best fraction of sulphate removed against the initial sulphate concentration, B) Specific sulphate removal rate against the sulphate removed on the TCOD removed, C) Specific  $\text{COD}_{\text{Lactate}}$  removal rate and fraction of lactate removed (after 8 h of operation) as function of the initial sulphate concentration, and D) Specific TCOD removal rate and fraction of TCOD removed (at the end of the sulphate reduction process) as a function of the initial sulphate concentration. In the primary axis:  $\text{NH}_4^+$  feast ( $\blacklozenge$ ) and famine ( $\blacksquare$ ) conditions. In the secondary axis:  $\text{NH}_4^+$  feast ( $\diamond$ ) and famine ( $\square$ ) conditions



Figure

6.

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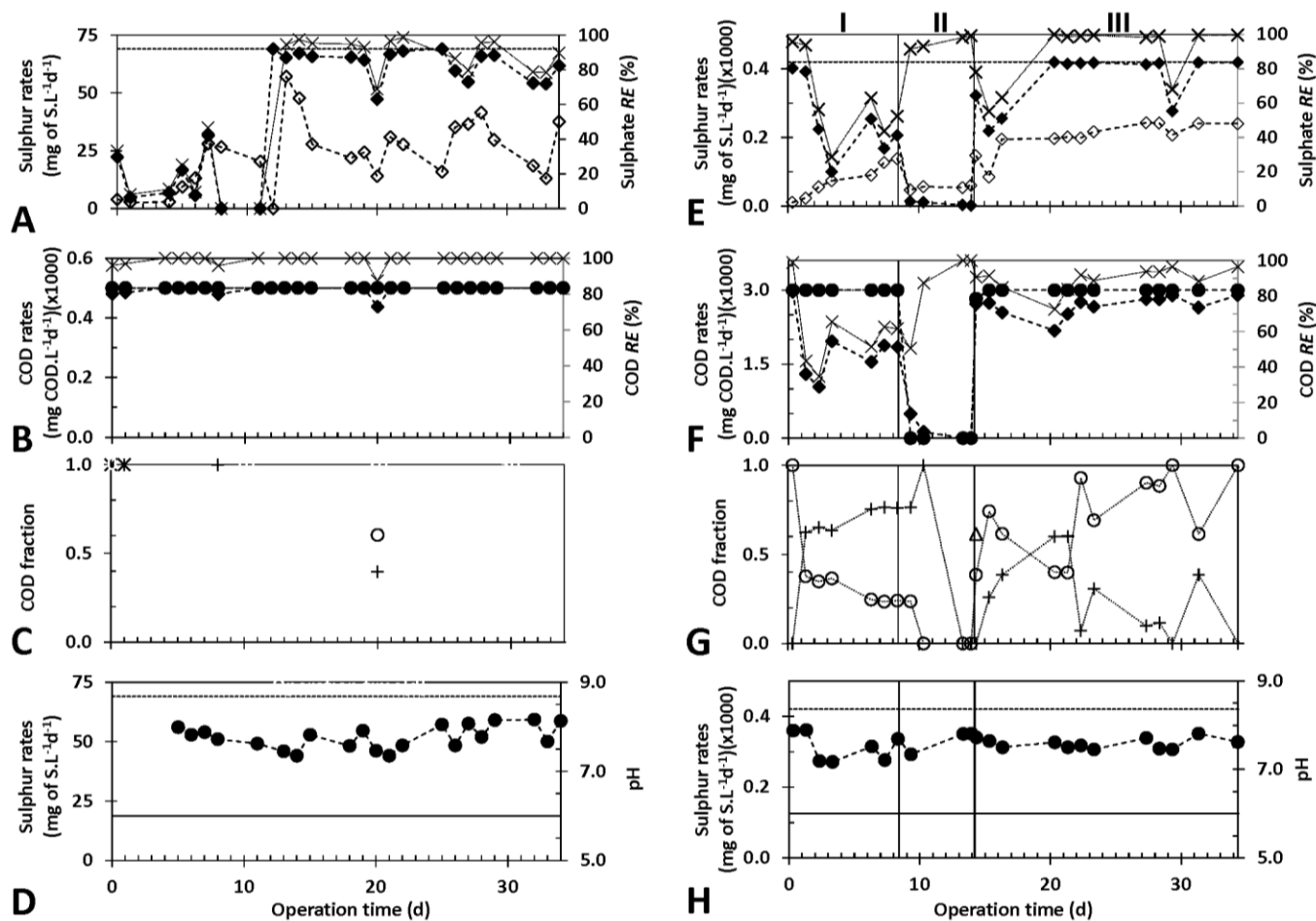


Figure 7.

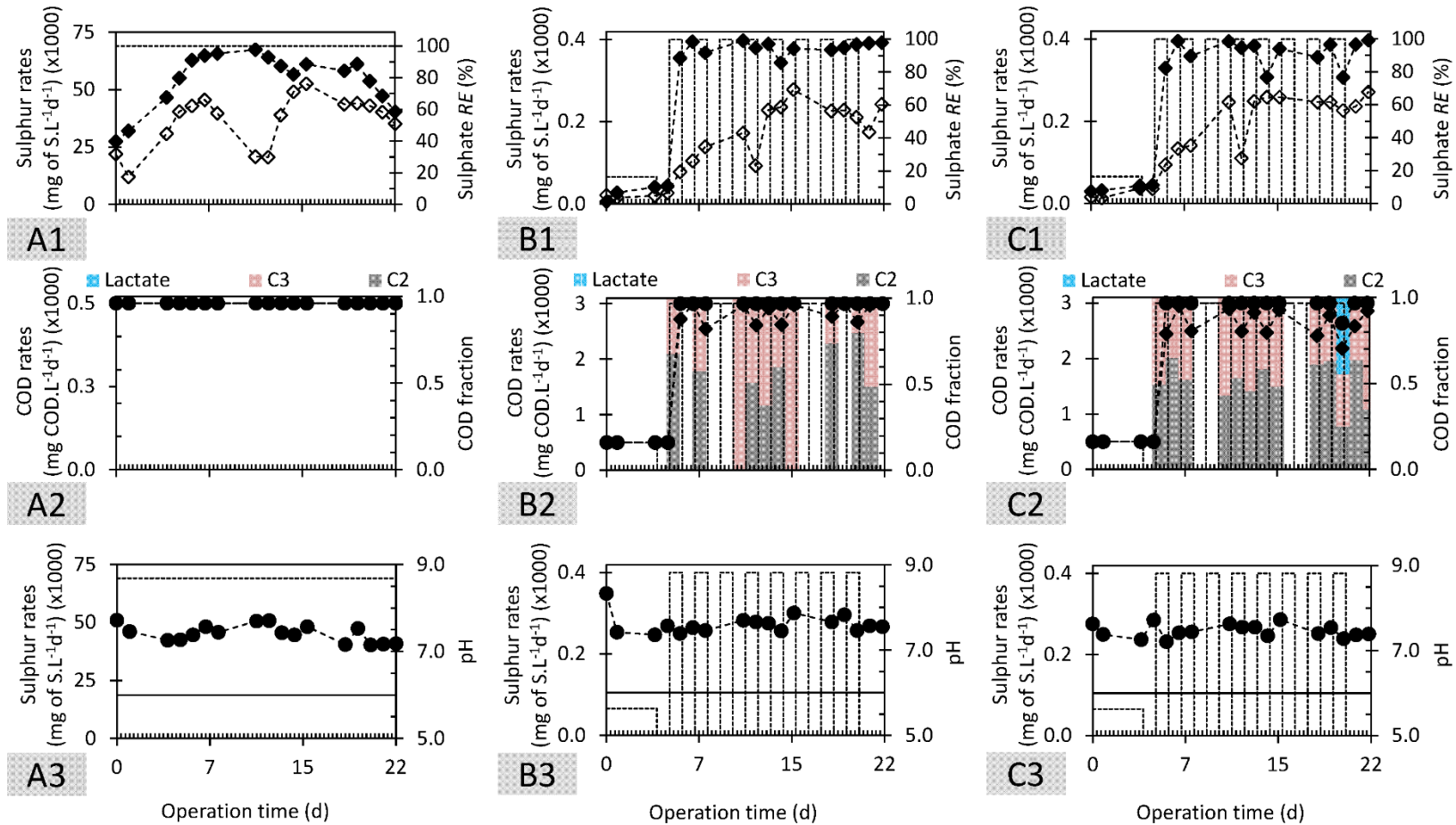


Figure 8.

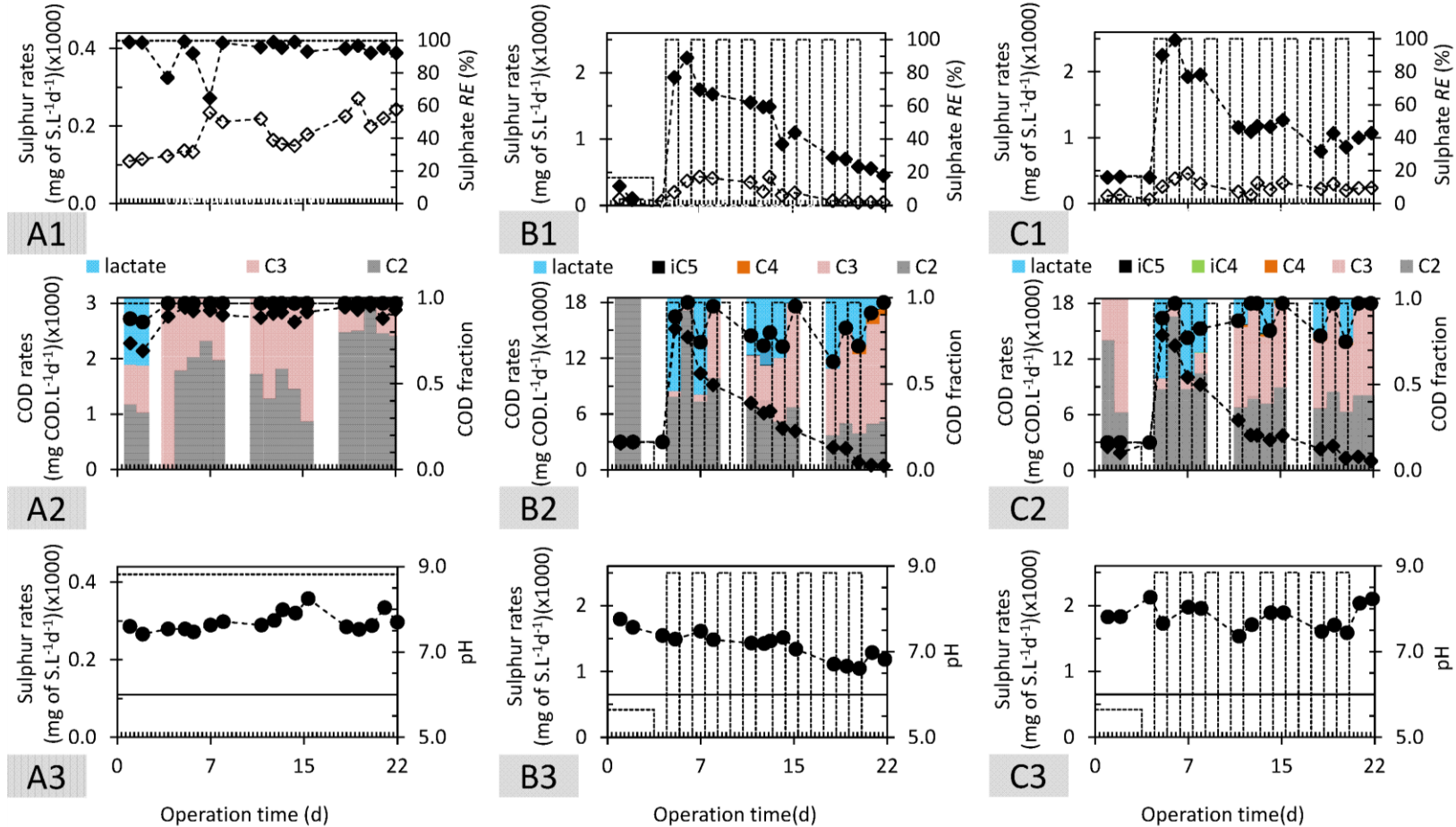


Figure 9.

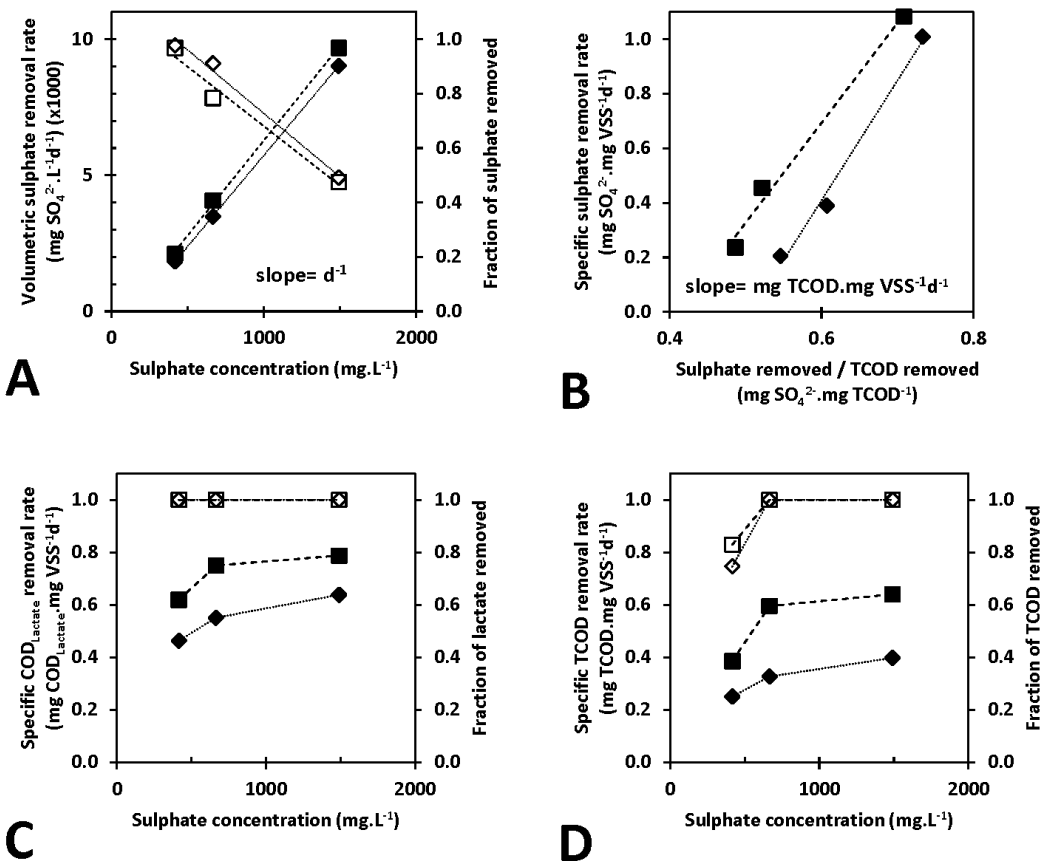


Figure 10.



Table 1. Operational conditions and performance of the control (L) and experimental (H) SBR

SBR L													
Phase	OT (d)	OM	Stage (d)	COD-LR	SO <sub>4</sub> <sup>2-</sup> -LR	S-SO <sub>4</sub> <sup>2-</sup> -LR	L-RR	L-RE (%)	TCOD-RR	TCOD-RE (%)	SO <sub>4</sub> <sup>2-</sup> -RR	SO <sub>4</sub> <sup>2-</sup> -RE (%)	S-SO <sub>4</sub> <sup>2-</sup> -RR
I	34	SBR	NSP (12)	500	209	69	500	100	495±14	99±3	45±33	22±15	15±11
			SP (22)								189±19	90±9	63±6
SBR H													
I	8	SBR	NSP (8)	3,000	1,250	420	3,000	100	1,793±616	60±21	737±335	62±25	249±112
II	6	Batch	(6)	0	0	0	0	100	155±234	84±23	20±17	95±4	7±6
III	20	SBR	NSP (6)	3,000	1,250	420	2,943±99	98±3	2,671±106	89±4	785±156	65±12	265±52
			SP (14)										

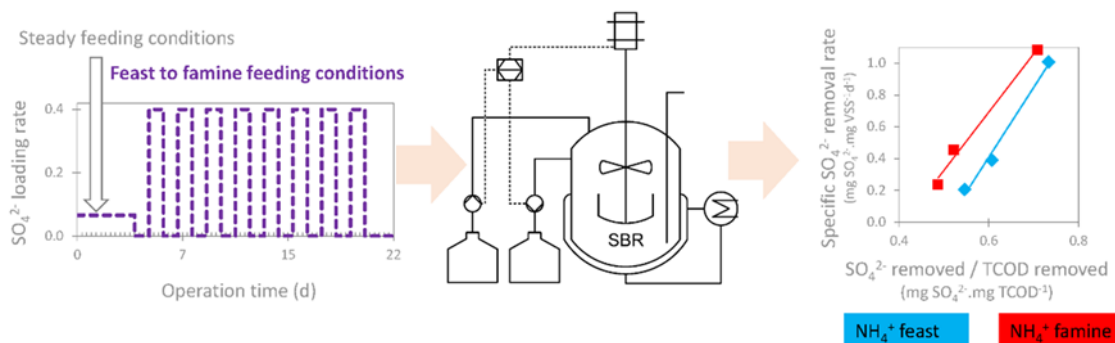
Note: Loading (LR), removal (RR) and production (PR) rates are shown with the units of mg.L<sup>-1</sup>.d<sup>-1</sup>. OT = Operation time, OM = Operation mode, NSP = non steady performance, SP = steady performance

Table 2. Feast to famine initial conditions and parameters used for kinetic evaluation of sulphate reduction in batch experiments

NH <sub>4</sub> <sup>+</sup> famine initial conditions (0 mg. L <sup>-1</sup> )							
Batch reactor	COD:SO <sub>4</sub> <sup>2-</sup> ratio	COD <sub>Lactate</sub> (mg COD.L <sup>-1</sup> )	Sulphate (mg SO <sub>4</sub> <sup>2-</sup> .L <sup>-1</sup> )	Fraction of SO <sub>4</sub> <sup>2-</sup> removed (time (h) of evaluation in brackets)	Fraction of TCOD removed (time (h) of evaluation in brackets)	Y <sub>SO<sub>4</sub><sup>2-</sup>/COD</sub> (mg SO <sub>4</sub> <sup>2-</sup> . mg TCOD <sup>-1</sup> )	Pe co of uti SC
Electron donor feast to electron donor famine	A	2.4	1,000	417	0.96 (8)	0.83 (8)	0.49
	B	1.5	1,000	666	0.78 (24)	1.0 (24)	0.52
	C	0.67	1,000	1,491	0.48 (24)	1.0 (24)	0.71
NH <sub>4</sub> <sup>+</sup> feast initial conditions (300 mg. L <sup>-1</sup> )							
Electron donor feast to electron donor famine	A*	2.4	1,000	417	0.98 (8)	0.75 (8)	0.55
	B*	1.5	1,000	666	0.91(24)	1.0 (24)	0.61
	C*	0.67	1,000	1,491	0.49 (24)	1.0 (24)	0.73

## Effect of ammonium, electron donor and sulphate transient feeding conditions on sulphidogenesis in sequencing batch bioreactors

### Graphical abstract



## Effect of ammonium, electron donor and sulphate transient feeding conditions on sulphidogenesis in sequencing batch bioreactors

### Highlights

- Sulphidogenesis was optimal at 2.5 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup> and a COD:sulphate ratio of 2.4
- Sulphidogenesis was robust to transient feeding conditions at 2.5 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup>
- Sulphate *RE* was ≥ 40% at 15 g SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup> influent sulphate concentration
- NH<sub>4</sub><sup>+</sup> feast-famine conditions affected the sulphate reduction process