Evaluating and modeling the biological sulfur production in the treatment of sulfide-laden streams containing ammonium

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ABSTRACT

Background

Biological treatment of effluents containing H_2S and ammonium are of great interest as both compounds can trigger serious environmental problems when disposed of. The aim of this study was to optimize the production of biosulfur from the partial oxidation of sulfide in sulfide- and ammonium-containing streams. Biological performance was evaluated under different aerating conditions and key kinetic parameters were adjusted based on an existing mathematical model adapted to this system.

Results

An optimal conversion of sulfide to S^0 of 86 % (w/w) was found at an oxidation-reduction potential (ORP) of -380 ± 10 mV and at an O_2/S^{2-} molar ratio of 0.44. Partial nitrification was observed at ORP higher than -200 mV and in excess of oxygen supply. Sulfide-oxidizing bacteria (SOB) outcompeted ammonium-oxidizing bacteria (AOB) in the competition for dissolved oxygen. In a modelling effort, the maximum specific growth rate for SOB, the sulfur shrinking kinetic constant, the maximum specific growth rate for AOB and the AOB oxygen half saturation constants were adjusted to 10.1 d^{-1}, 0.3 $mg^{2/3}$ VSS $mg^{-2/3}$ S, 1.75 d^{-1} and 1.5 mg L^{-1}, respectively, during model calibration.

Conclusions

Optimal S^0 production was found under limiting O_2 conditions in which AOB were not able to outcompete SOB. The mathematical model described satisfactorily the experimental profiles for ammonium, nitrite, sulfide and sulfate as a function of the aeration flowrate.

Keywords: sulfide; oxygen competition; biological sulfur production; partial nitrification; mathematical model

1. Introduction

Sulfate-rich streams generated in pulp and paper factories, food industry, tannery industry,[1] oil extraction and mining activities amongst others are ideal for sulfate reducing bacteria (SRB) growth under anaerobic conditions, which produces sour effluents with high sulfide content. H_2S is the most concerning S-compound because of its toxicity, low odor threshold and its potential as precursor of SO_x gases when combusted.[2] Also, sulfide-laden streams cause corrosion of pipelines[3] and plugging[4] when sulfide is completely or partially oxidized, respectively. Some sulfide-rich streams may also contain significant concentrations of ammonium. This is the case of those effluents generated in anaerobic reactors treating sulfate and nitrogen containing wastewater, such as tannery wastewater[5] and the two-step bioscrubbing process for the recovery of elemental sulfur (S^0) from combustion gases containing SO_x.[6] Valorization of sulfur from sulfur dioxide (SO_2) has important advantages as SO_2 is a contaminant with worldwide emissions for 2010 estimated to be 6×10^7 S-SO_2 tones,[7] that causes acid rains and formation of sulfate aerosols that affect solar radiation.[8] Additionally, SO_2 recovered as S^0 can be reused in sectors such as agriculture,[9] Li-S batteries[10] and S-based pigments production.[11,12] The reintegration of contaminants into the economy as valorized products is the main principle of a circular economy, an essential step for sustainability in which treatment of residues is aimed at diminishing environmental impacts and economical expenses.[13]

The biological production, recovery and valorization of elemental sulfur demand an optimization, not only of the biological process but also of the downstream stages, such as precipitation and purification, to enhance the overall process productivity. The biological sulfur production is mainly governed by operational parameters like dissolved oxygen (DO) and sulfide concentrations, which have major impacts on the oxidation-reduction potential (ORP). The latter has been reported as the key variable to control S^0 formation.[14] In a biotechnological process treating sulfide- and ammonium-containing effluents, operational parameters such as DO, stirring rate, hydraulic residence time (HRT), pH and temperature will affect the kinetics and, consequently, the proliferation of different microorganisms that

will compete for oxygen. Biological sulfide oxidation can be partial or complete (equations 1 and 2) depending on the O_2 and HS^- molar ratio. Similarly, ammonium oxidation will be governed by the O_2 and NH_4^+ molar ratio (equations 3 to 5). Biological ammonium oxidation implies the production of NH_2OH as an intermediate that, due to its high reactivity,[15] it is barely accumulated. Consequently, NH_2OH is not normally considered in the nitrification process.[16] However, under certain conditions NH_2OH is a precursor for the formation of N_2O (Eq. 6), which may be significant depending on process conditions. Finally, chemical precipitation of $(NH_4)_2SO_4$ (Eq. 7) can take place if ammonium and sulfate concentrations are high enough to reach its solubility product, often difficult for diluted effluents due to its high solubility (790 g $(NH_4)_2SO_4$ per liter of water).[17] Moreover, its production is enhanced at temperatures over 60ºC and acidic conditions (pH below 3).[18]

$$HS^- + 0.5O_2 \rightarrow S^0 + OH^- \quad (1)$$

$$S^0 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (2)$$

$$NH_4^+ + H^+ + O_2 + 2e^- \rightarrow NH_2OH + H_2O \quad (3)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (4)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (5)$$

$$NH_2OH + HNO_2^- \rightarrow N_2O + 2H_2O \quad (6)$$

$$2NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4 \quad (7)$$

Consumption and production rates can be described by kinetic equations together with mass balances already reported in the literature. Regarding ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), different experimental works and mathematical models have been proposed in the literature to describe AOB/NOB activity. Well-established models exist to describe the nitrification process,[19] and lots of previous works have modeled the nitrification process based on Monod-type models to estimate kinetic parameters.[20] In comparison, literature regarding mathematical modelling of SOB activity is more limited. Amongst others, Roosta *et al.*[21] described a fed-batch bioreactor using a mathematical model to understand the effect of sulfide load in the system. On the other hand, Mora *et al.*[22] reported a two-step sulfide-oxidation model that considered the strong effect of sulfide inhibition

using a Haldane-type equation coupled with a shrinking particle constant to describe the intracellular sulfur accumulation for a *Thiotrix spp.* SOB culture. However, to the authors knowledge, the competition among them in a bioreactor treating together sulfide- and ammonium-loaded wastewater has not been previously reported.

This work targets the production of biosulfur from the partial oxidation of sulfide in sulfide- and ammonium-containing streams. To this aim, this work evaluated the performance of a biological culture under different aeration conditions to promote biosulfur production and to study the microbial competition for the electron acceptor both from an experimental and a mathematical modelling approach.

2. Materials and Methods

2.1 Experimental setup and operating conditions

In this study the experimental assays lasted for 62 days and were performed in a 3L lab-scale biostat that was inoculated with a mixed culture obtained from a full-scale THIOPAQ™ process installed in a pulp and paper industry. This THIOPAQ™ process was fed with ammonium as nutrient and aims to treat high sulfide load wastewaters, being a niche for the growth of sulfide oxidizing and nitrifying microorganisms; that explains the high sulfide and ammonium oxidation activity from the beginning of the experiment (see section 3.1). The experimental setup is shown in Fig. 1. Mineral medium (MM) was fed at a flow rate of 110 ± 7 mL h^{-1} and a sodium sulfide stock solution of 5 g L^{-1} was prepared and fed at a flow rate of 15 mL h^{-1}, which resulted in an equivalent inlet total dissolved sulfide (TDS) concentration of 600 ± 50 mg S L^{-1}, a volumetric load of 24 ± 3 mg S L^{-1} h^{-1} and a hydraulic residence time (HRT) of 25h. The MM composition was as follows (in g L^{-1}): $NaHCO_3$ (3.89), NH_4Cl (1.1), KH_2PO_4 (0.13), K_2HPO_4 (0.17), $CaCl_2$ (0.02), $MgSO_4 \cdot 7H_2O$ (0.22) and trace element solution (1 ml L^{-1}). MM and sulfide were fed separately with peristaltic pumps to avoid sulfide salts precipitation. A CO_2 flow of 50 mL min^{-1} was mixed with an inlet air flow to supply the carbon source and the oxygen needed for the autotrophic mixed culture. CO_2 and air were supplied, measured and controlled with mass flow controllers (Bronckhorst, The Netherlands).

The experiment was divided in 7 periods that were defined by different air flow rates (Table 1) to study the biological competition of SOB and nitrifiers for O_2 and the S^0 production. Temperature, pH, DO and ORP in the biostat were monitored on-line. A home-made software developed in Visual Basic was used for data acquisition and control. The pH was controlled between 7.4-7.6 by an on/off controller adding NaOH (2M) and HCl (2M) solutions. As the case under study was the two-step bioscrubbing process for biosulfur recovery from SO_2, a thermostatic water bath was used to control the biostat temperature at 33ºC according to process conditions reported by Fernández *et al*.[6] It is worth mentioning that the optimal temperature for chemolitotrophic SOB is between 28 and 35ºC,[2] and AOB activity is favored at temperatures above 30ºC according to a SHARON process,[23] which means that SOB and AOB growth is enhanced at 33ºC. The stirring was kept at 230 rpm along the experiment. Since stirring also contributes to oxygen supply, a flat blade turbine type stirrer was used (IKA RW 20 digital). The biostat was cleaned periodically to avoid biofilm accumulation on its walls and stirrer and an activated carbon filter was used to avoid hydrogen sulfide release in air.

In addition, a set of abiotic experiments were performed to determine the total oxygen supplied in each of the seven periods using the dynamic gas in-gas out method.[24] The volumetric mass transfer coefficient was calculated from a mass balance model according to the standardized ASCE/EWRI 2-06 equation under dynamic conditions.[25] The mass transfer coefficient estimation is explained in detail in the Supporting Information (Section 1).

Table 1 shows the oxygen mass transfer coefficients (K_La), the oxygen-sulfide and oxygen-ammonium molar ratios of each period. The start-up of the biostat lasted 4 days keeping the oxygen supply in excess by setting an air flow rate of 0.8 L min^{-1} (data not shown).

2.2 Analytical methods

TDS was measured off-line by an ion-selective electrode (VWR International Eurolab, S.L.) connected to a bench-top meter (Symphony, VWR, USA). This electrode has a high sensitivity to the completely dissociated form of sulfide (S^{2-}), which made necessary to prepare the samples in an alkaline antioxidant buffer solution (SAOB) before measurement.[22]

Ammonium concentration in the MM and in the biostat was measured off-line with an ammonium analyzer (AMTAX sc, Hach Lange, Germany). The concentration of oxidized sulfur and nitrogen species (SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, NO_3^- and NO_2^-) were measured by ion chromatography (Dionex ICS-2000 HPLC system, Dionex, USA). Sulfite was not detected along the biostat operation and subsequently was not considered for the sulfur mass balance. The ion chromatography equipment has a suppressed conductivity detector and uses an IonPac AS18-HC column (4X250 mm – Dionex, USA). Prior to the analysis, samples were filtered with a 0.22 µm cellulose filter and bubbled with nitrogen to avoid changes in concentration due to chemical oxidation of H_2S.

DNA extraction of suspended biomass in the biostat was performed when the reactor was aerated with 0.35 L air min^{-1} (day 15 of operation) to analyze the microbial diversity grown in the biostat. The MoBio PowerBiofilmTM DNA extraction kit (MoBio Laboratories, USA) protocol was applied with two modifications in the extraction process as it was described by Reino *et al.*[26] The quality and quantity of the extracted DNA was measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Illumina sequencing of the DNA was performed using the primers Bakt-515F (5' GTG CCA GCM GCC GCG GTA A') and Bakt_909R (5' CCG TCA ATT YHT TTR AGT 3') with a minimum DNA concentration of 20 ng L^{-1} using the Illumina MiSeq platform (AllGenetics, A Coruña, Spain).

Elementary analysis was also performed during the last period when the elemental sulfur production was the highest and steady. The sample was centrifuged at 12000 rpm (Micro Centrifuge Model 16K, BioRAD, USA), lyophilized for 8 hours (Sentry 12525, The VirTis Company, New York) and stored at -25°C to avoid chemical oxidation of sulfur. Then, carbon, hydrogen, nitrogen and sulfur contained in the solid were analyzed by combusting the sample in an oxygen atmosphere at 1200°C and analyzing the produced gas by gas chromatography (Elemental Analyzer CHNS Thermo Scientific Flash 2000).

2.3 Mathematical model

The mathematical model is represented in Table SI3 following Peterson Matrix representation.[27] The set of kinetic equations used to describe SOB growth was partly

selected from the model proposed by Mora *et al*.[22] However, in the present study the intracellular S^0 accumulation term was not considered (see Table SI4) since the microbial analysis revealed the absence of intracellular S^0 accumulating SOB (see section 3.3). The set of kinetic and differential equations used in this study are shown in the Supporting Information (section 3).

The mathematical model was programmed in MATLAB R2013b. The set of differential equations was solved using the MATALB ODE15s function. A sensitivity analysis was performed to determine the most sensible kinetic parameters over an objective function (f_{obj}) defined as the norm of the difference between experimental and the mathematical model data for sulfide, sulfate, nitrite and DO (Eq. SI3).[22] This analysis was performed by changing by ±10% one at a time each kinetic parameter of the model and evaluating the objective function. The relative standard deviation (RSD) of the function was determined to identify the sensitivity. Afterwards, the model was calibrated using the fminsearch function in Matlab and using experimental data of the 6 first periods of operation of the reactor (Table 1). This fitting method minimizes the f_{obj} by adjusting the most sensible kinetic parameters, those determined from the sensitivity analysis, to fit model predictions to experimental data. Data from the last experimental period (after day 37) conducted under different experimental conditions than those along the calibration stage were used to validate the model.

Results obtained from the mathematical model fitting to the experimental data were statistically evaluated by using a two-tailed t-student distribution of independent samples. The evaluation consisted of the comparison between mean values of two data sets and the determination of a t-value and a p-value calculated using Eq. SI4 (Supporting Information) and standard t-tables, respectively.[28]

3. Results and discussion

3.1 Biological sulfur production and oxygen competition by sulfide oxidizing and nitrifying microorganisms

The biological sulfide and ammonium oxidation were analyzed along the operation of the biostat according to the operating periods shown in Table 1. Figure 2 shows the sulfur mass

balance along the experiment (Fig. 2A) and the concentration of the main sulfur (Fig. 2B) and nitrogen (Fig. 2C) species as an average concentration measured in each operating period (Table 1) once the steady-state was reached. The S^0 production per hour was determined by mass balance as the difference between the total sulfur species in the inlet and outlet (see section 2 of Supporting Information), a calculation that has been already reported in other works.[29,30]

Sulfur species mass flowrates in Fig. 2A corresponded to an inlet TDS of 89±3 % (w/w) plus a 4±3 % (w/w) of sulfate and 6±1 % (w/w) of thiosulfate of the total S-species in the inlet. From day 0 to 17 the air flowrate was stepwise reduced from 1 L min^{-1} to 0.35 L min^{-1} (Table 1). The oxidation of HS^- to SO_4^{2-} was 99 % as expected based on the O_2/TDS molar ratio, which was in excess with respect to the stoichiometric oxygen required to oxidize 1 mol of S^{2-} to S^0 (Eq. 1). Due to the low cleaning frequency of the biostat, that was initially performed every 10 days, the partial accumulation of S^0 over the walls and stirrer resulted in a higher net sulfur quantity in the outlet since the accumulated S^0 was further oxidized to sulfate. This explains the sulfur imbalance observed along the first 23 days of operation.

From day 19 to 24, the oxidation of sulfide to sulfate decreased to 62.3 % (w/w) at an air flow rate of 0.2 L min^{-1}. From day 24 to 37, the oxidation of sulfide to sulfate decreased down to 54.3 % (w/w) at an air flow rate of 0.05 L min^{-1} and an O_2/HS^- molar ratio of 1.5 (Table 1), which was lower than the stoichiometric oxygen required for a complete sulfide oxidation (Eq. 1 and 2). After day 37, the air flow was stopped, and oxygen was only supplied by stirring. The O_2/HS^- molar ratio supplied was 0.44, while the stoichiometric value for the partial oxidation to S^0 is 0.5 according to equation 1. As it can be seen in Figure 2A, the elemental sulfur production started to increase. On day 42, a sulfide load shock of 1200 mg $S\text{-}S^{2-}$ L^{-1} was applied and the process kept the partial oxidation of sulfide to S^0 over 75 % (w/w), reaching an 86 % (w/w) conversion to S^0 in the last days of this period. Other works have reported that O_2/HS^- molar ratios between 0.6 and 1 are needed to enhance S^0 production[31,32] while for ratios lower than 0.6, thiosulfate is the predominant product.[33] The lower molar ratio of 0.44 reported here imply a higher efficiency in terms of oxygen consumption at such a high sulfide conversion to S^0.

Analyzing the S-species based on the steady-state concentrations achieved during each period (Fig. 2B), the complete sulfide oxidation started to decrease progressively when the air flow rate was reduced from 0.35 to 0.2 L min^{-1} (Fig. 2B). The latter corresponded to an O_2/TDS molar ratio of 2.9, which was higher than 2, the stoichiometric molar ratio needed for a complete sulfide oxidation (Eq. 1 and 2), thus indicating that other aerobic species were competing with sulfide-oxidizing bacteria for O_2.

Regarding nitrogen species profiles in Figure 2C, AOB activity was observed until period IV, indicating that under those conditions, oxygen competition between AOB and SOB was taking place. Moreover, nitrate was not observed along the biostat operation (Fig. 2C). The lack of nitrate could be related with the kinetics of NOB. NOB and AOB have been reported to have a duplication time of 50h and 17h at 25ºC, respectively,[20] and the HRT of the biostat was set at 25h which was lower than the NOB duplication time. The accumulation of NOB in the biofilm was avoided by a periodical cleaning of the biostat wall; meanwhile AOB did not have a microbial growth limitation since the growth rate was higher than the dilution rate.

Moreover, a nitrogen mass balance was performed (Figure SI3). NO formation was not considered as its production relies on low pH conditions.[15] Since $(NH_4)_2SO_4$ formation is favored at pH below 3, temperature over 60ºC[18] and is highly soluble, its precipitation was not considered in the mass balance. Nitrogen uptake for biomass growth was estimated based on the biomass production estimated by the mathematical model (section 3.3) and the nitrogen content of the elemental composition of biomass, which is 6.1% (w/w) for sewage sludge.[34] Consequently, the nitrogen imbalance of 18 ± 9 mg N L^{-1} along the experimental periods could be due to a nitrous oxide (N_2O) formation as it has been reported in processes with nitrifying bacteria.[9,35,36]

Analyzing the conversion of ammonium to nitrite, all ammonium was oxidized when the air flow rate was 1 L min^{-1}. After that, ammonium oxidation decreased progressively along the following periods and nitrite was not further detected when air flow rate was 0.2 L min^{-1}. Under this condition, the DO/N-NH_4^+ and DO/TDS molar ratios were 3.2 and 2.9 (Table 1), respectively. These ratios are higher than the stoichiometric oxygen requirements for

ammonium and sulfide oxidation (Eq. 1 to 5) even if such oxygen consumption reduced the DO to below 1 mg L^{-1}, period V of Figure 3. Guisasola *et al.*[16] reported an oxygen half saturation constant for AOB of 0.74 mg L^{-1} while Mora *et al.*[22] reported a value of 0.15 mg L^{-1} for SOB. Consequently, the decrease in the AOB activity was attributed to the higher oxygen affinity by SOB, which means that under limiting oxygen environment, SOB outcompetes AOB.

Moreover, AOB are highly sensitive to low sulfide concentrations. The IC_{50} for AOB reported in literature ranges from 3 ± 0.3 mg TDS L^{-1},[37] to 0.7 mg TDS L^{-1}.[38] Since sulfide concentrations were around 1 mg L^{-1} in the reactor during the first 40 days, partial inhibition of AOB by free sulfide could have occurred in this stage. This inhibition effect was also studied by Sekine *et al.*[39] who implemented a sequential batch reactor with a HRT of 3 days and a solid retention time of 170 days to evaluate the biological oxidation of ammonium and sulfide. In that case, nitrification was not affected at inlet sulfide loads lower than 5.3 mg S L^{-1} h^{-1} while ammonium removal efficiency decreased from 100 to 50 % at inlet sulfide loads of 10.7 mg S L^{-1} h^{-1}. In the present work, a sulfide load of 24 mg S L^{-1} h^{-1} (see Section 2.1) and the major oxygen affinity by SOB could explain the rapid decrease of AOB activity. It is worth mentioning that Jiang *et al.*[40] reported ammonium removal efficiencies over 95 % for sulfide loads of 164 mg L h^{-1}; however, results were found in a biostat for simultaneous biological treatment of ammonium and sulfide with 17 days of acclimatization period. Further studies of acclimatization periods are warranted to couple ammonium treatment and biosulfur production.

To analyze the optimal conditions that enhance the production of nitrite and elemental sulfur, and considering that ORP and DO have been reported as key parameters to control the biological sulfide oxidation,[9] the ORP, DO and S^0 are depicted in Figure 3. It can be observed that in the last period (Fig. 3), when air flow rate was 0 L min^{-1} (Table 1), the ORP reached the most negative value while DO reached its lowest concentration. This occurred because the decrease in oxygen shifts the medium from an oxidative to a reductive environment. Based on the partial nitrification previously analyzed and results from Figure 3, no AOB activity was observed when ORP was less than -200 mV. Meanwhile, the optimal ORP for

sulfur production was found to be -380 ± 10 mV with a S^0 concentration of 594 mg L^{-1}, a sulfide to S^0 conversion of 86 % (w/w) and a TDS accumulation in the reactor of 30 mg L^{-1}, equivalent to 5 % (w/w) of the inlet sulfide. This conversion was obtained for a TDS/N-NH_4^+ molar ratio of 1.2 and an O_2/TDS of 0.44 (Table 1). Similar values have been reported in the literature for partial sulfide oxidation process under non O_2-competition with SOB/AOB by Krishnakumar *et al.,*[41] who implemented a reverse fluidized loop reactor for S^0 recovery; a 97 % (w/w) sulfide removal, 3 % (w/w) sulfide accumulation and 80 % (w/w) sulfur recovery under an ORP between -400 and -350 mV were reported. Similarly, Vannini *et al.*[42] used a membrane reactor for biological sulfur production and found an optimal ORP between -400 and -360 mV obtaining a sulfide conversion to S^0 of 79 % (w/w).

3.2 Microbial diversity characterization

Fig. 4 shows the results obtained from the microbial diversity analysis. The sample was taken from the suspended biomass on day 15 of operation, when air flow rate was 0.35 L min^{-1}. Halothiobacillaceae was the most abundant sulfide oxidizing family with a relative abundance of 45 % in the suspended biomass (Fig. 4); the high salt content in the mineral medium (see section 2.1) could also explain the large quantity of halotolerant microorganisms grown in the biostat. Betaproteobacteria class represented 25 % of the culture abundance (accounted in Fig. 4 in the unclassified group), which means that some other sulfide oxidizing microorganisms belonging to this class could be present in the culture medium such as *Thiobacillus* genera. Some other SOB species could have been also present in the biostat since 1 % and 9 % of the suspended culture were identified as Comamonadaceae and Xanthomonadaceae families (see Fig. 4), respectively. In fact, *Delftia*[43] and *Rhodanobacter* have been reported as SOB that belong to the abovementioned families, respectively.[44]

Regarding the nitrifying microbial diversity, 1.6 % of the suspended culture were AOB belonging to the Nitrosomonadaceae family (Fig. 4), which was probably highly active since the NH_4^+ oxidation in this period (0.35 L air min^{-1}) was 55 % (w/w) (Fig. 2C) for an ammonium load of 9.3 ± 0.3 mg N-NH_4^+ L^{-1} h^{-1}. NOB-type families were not identified in the suspended culture which is consistent with the fact that only partial nitrification

(nitritation) was observed. Additionally, as no autotrophic denitrifyiers such as *Thiobacillus denitrificans* were found and oxygen was in excess during the first 5 periods, it is unlikely that denitrification took place; . based on this, denitrification process was not considered for the mathematical modeling.

3.3 Modeling SOB and nitrifying activity

Mass balances, stoichiometric and kinetic equations were used to describe the performance of the biostat and to further assess the competition between SOB and nitrifying species for DO, which has not been reported previously to the authors knowledge. Existing models describing nitrifying (ASM models) and SOB activity[22] were used. However, significant changes were made to the model reported by Mora *et al*.[22] for sulfide oxidation. The latter considered that elemental sulfur was accumulated inside the cells during the partial oxidation of sulfide since the culture was mainly composed by *Thiotrix spp* (>95%). In our work, Illumina sequencing results did not show any SOB of the family Thiothrichaceae, which are the ones reported to accumulate intracellular S^0.[2] Therefore, model equations used to describe the kinetics of sulfide oxidation in the present work (see section 3, Supporting Information) were adapted accordingly. Thus, a Haldane-type kinetic equation was used to describe sulfide oxidation by SOB.

In order to understand the sulfide conversion to sulfate/sulfur and the competition with AOB, a sensitivity analysis was performed. The kinetic parameters $\mu_{max/SOB}$, k_{S^0}, $\mu_{max/AOB}$ and $k_{O_2/AOB}$ were the most sensitive parameters in this system (see Table SI7, Supporting Information). Consequently, these parameters were chosen to calibrate the model while all other parameters were kept as those reported in literature (see Table SI2, Supporting Information). Table 2 shows the parameters obtained after model calibration while Figure 5 shows model predictions of the whole experimental period, thus including the validation period after day 38. Remarkably, only 4 parameters out of 21 kinetic parameters were enough to satisfactorily model the experimental data for S and N species and DO in Figure 5, corroborating the high sensitivity of these parameters. Parameters calibrated were in the range of previously reported values; the latter are shown in Table 3. Munz *et al*.[45] reported a $\mu_{max/SOB}$ of 7.4 d^{-1} (25ºC) for a predominant culture of Halothiobacillaceae family in a

membrane reactor working with a solid retention time (SRT) of 5 days. Mora *et al.*[22] obtained a $\mu_{max/SOB}$ of 9.84 d^{-1} (25ºC) from respirometric experiments for a predominant culture of *Thriotrix* genus in a biostat with 2.8 days of SRT. Likewise, Mora *et al.*[22] reported values for k_{S^0} in the range of 0.833 and 0.03 (25ºC), similar to the results obtained from the model calibration performed in this work. In the case of $\mu_{max/AOB}$, Wu *et al.*[20] and Jubany *et al.*[46] reported values of 1.45 d^{-1} and 1.21 d^{-1} (25ºC), respectively; in this work, a higher value of 1.75 d^{-1} (Table 2) was achieved. Even though, the calibration resulted in a higher oxygen affinity constant for AOB ($k_{O_2/AOB}$ = 1.5 d^{-1}) compared to those reported in the literature; this could explain the AOB depletion under oxygen limitation. Pan *et al.*[47] obtained a $k_{O_2/AOB}$ of 0.6 mg L^{-1} (22ºC). Ge *et al.*[48] studied the partial nitrification in wastewater treatment plants and found that $k_{O_2/AOB}$ was between 0.22 and 0.56 mg L^{-1} (25ºC) for straight rods-type *Nitrosomonas*, while Guisasola *et al.*[16] found it to be 0.74 mg L^{-1} (25ºC). A value closer to the $k_{O_2/AOB}$ found in this work was reported by Regmi *et al.*[49] with a $k_{O_2/AOB}$ of 1.16 mg L^{-1} (25ºC) for a suspended culture.

In Figure 5A, model and experimental data profiles corresponding to the three main sulfur species – TDS, S^0 and sulfate – are depicted. It can be observed that sulfate profiles predicted by the mathematical model properly described the experimental profiles. Regarding sulfide profiles, the simulated profiles accurately describe the experimental profiles from the beginning until the end of the biostat operation. The S^0 profiles presented major deviations from the experimental data as S^0 was only determined by mass balance. Thus, the absence of a S^0 measurement and the successive solids accumulation and washing in the reactor due to biomass stuck over walls and stirrer lead to significant noisy S^0 concentrations along the experimental periods. Nitrogen species are represented in Figure 5B, the model shows a production of 7 mg L^{-1} of $N\text{-}NO_3^-$ during the first 5 days that was not observed experimentally; nevertheless, the mathematical model is in agreement with the experimental data for the rest of the operational periods. Regarding AOB activity, N_2O was not included in the mathematical model because N_2O was not measured and its production was estimated to be 7% (w/w) based on N imbalance. About DO, the experimental and the model data show similar behaviors with minor differences (Fig. 5C).

Although no biomass concentration as Volatile Suspended Solids (VSS) could be measured due to the presence of elemental sulfur, the active fractions of SOB, AOB and NOB concentrations along the experimental periods were calculated with the model (Fig. 5D). Because of biosulfur interference in the VSS analysis, the initial biomass concentration was estimated by performing a simulation of the start-up period (days 0 to 4) using all the kinetic parameters taken from the literature (Table SI2 in Supporting Information). This alternative was decided in order to avoid that the initial dynamics of the dissolved species were affected by an excessive offset of the simulated initial biomass concentration with respect to the real ones. According to the simulation, the SOB achieved the highest concentration during days 24 to 27 (129 mg L^{-1}) when complete oxidation occurred. When elemental sulfur accumulated after day 28, SOB decreased down to 113 mg VSS L^{-1} until day 41 because of the lower growth yield of SOB with sulfide, $Y_{X/S^{2-}}$, compared to elemental sulfur, Y_{X/S^0} (Table SI2 in Supporting Information). Afterward, the SOB concentration further decreased down to 65 mg VSS L^{-1} and after the sulfide load shock on day 42, SOB decreased to 45 mg VSS L^{-1} remaining steady until the end of the experimental period when the maximum elemental sulfur production was reached. In the case of AOB, during the first 4 days the model described small increase in the concentration to 24 mg VSS L^{-1} which is explained by the low ammonium load. A complete washout is observed after 30 days of operation. NOB did not show any growth as it was washed out at the HRT of this experiment.

The accuracy of the simulation was evaluated by a t-student test. The null hypothesis to run this test was that the experimental and model values were statistically similar, and the main results of the t-test are shown in Table SI8 of the Supporting Information. The t-values were in the range of the t-critical for sulfide, sulfate and nitrite which means that the null hypothesis cannot be rejected.

It is worth mentioning that more accurate calibration could have been performed if experimental VSS concentrations could have been determined. Moreover, for a deeper understanding of the biological sulfur formation, a routine method to measure not only S^0 but also other intermediate sulfur species like polysulfide are warranted to have a better

prediction of the S-species concentration along the process and to obtain more accurate results in the process modelling.

Beyond the limitations of the modelling approach, the statistical results showed good a matching among modelled and experimental data. Thus, this model can be further exploited in several ways, from helping in the design of future experiments[50] to its application to estimate the performance of biological systems where nitrogen and sulfide are involved.[51] Besides, this model can be applied to biostat-type systems to predict system behavior under different operational conditions such as sulfide/ammonium load ratios or hydraulic retention times. In addition, optimization of the oxygen supply as one of the most energy consuming operations in full-scale plants can be explored to, concomitantly, ensure the selectivity towards biosulfur formation. Similarly, the model could be used to predict the performance of AOB-enriched cultures as well as AOB cultures less sensitive to DO to explore the potential simultaneous, complete conversion of ammonium and sulfide to nitrite and biosulfur, respectively.

3.4 Assessment of the biosulfur production efficiency

Biosulfur can be used in different processes as row material. In the energy field, as a novel alternative of fossils fuels, Selvaraj *et al.*[10] was able to produce cathodes for Li-S batteries using sulfur produced biologically and able to recover 70% of it in these cathodes. In the agricultural sector, direct application of biosulfur as fertilizer has shown better efficiency than conventional sulfur.[9] Interestingly, since chemical sulfur is one of the main raw materials for S-based pigments such as methylene blue or ultramarine blue,[12] the use of biosulfur could also be evaluated as an alternative S-source for the synthesis of such pigments.

During the operational period with maximum sulfur production in the biostat (day 37 to the end of the experimental period) the solid phase composition was analyzed. Results showed that the dried solid contained a carbon, hydrogen, nitrogen and oxygen percentage (the main elements in biomass composition) of 25% (w/w) while elemental sulfur accounted for 75% (w/w). This was obtained at the end of the experimental period for a sulfide conversion to S^0 of 86% (w/w) at a sulfide loading rate of 25 mg S L^{-1} d^{-1}. It has been reported the use of

flocculants to enhance the sulfur precipitation. A 97.5% (w/w) efficiency in the sulfur recovery was found by Chen *et al.*[52] using polyaluminum chloride. Feng *et al.*[53] used an internal airlift loop reactor enriched with *halothiobacillus-type SOB* with flocculant-producer *Pseudomonas sp.* to enhance precipitation. A 96% (w/w) sulfur recovery efficiency was obtained. Despite flocculants can increase the sulfur recovery efficiency, they can affect sulfur purity and have a direct effect over the cost of the downstream process if biosulfur production as value-added product is targeted; Mora *et al.*[11] used a commercial flocculant (FL4820) for the effluent of a sulfur-producing biostat and obtained a 64.4% (w/w) and 74.3% (w/w) of solid recovered and sulfur purity, respectively. Moreover, Janssen *et al.*[54] studied the aggregation of microbial sulfur production and found that by increasing the sulfide load, sulfur aggregation was enhanced without the addition of flocculants. Furthermore, the work presented herein demonstrates that treatment of ammonium and sulfide containing wastewater should be subjected to an additional N removal process if both N and biosulfur recovery are sought since both cannot be achieved simultaneously in a CSTR.

4. Conclusions

Coupling of sulfide and ammonium biological oxidation in a biostat was studied to optimize biosulfur production at low HRT (25 h). The process showed that at a DO concentration of 0.07 mg L^{-1} and ORP of -380 ± 10 mV, 86% (w/) conversion of sulfide to S^0 with a purity of 75% working with DO/S-TDS molar ratios of 0.44 was reached. AOB were not able to outcompete SOB under these conditions for the oxygen consumption, which was confirmed through a microbial diversity analysis. The mathematical model proposed to simulate the process described satisfactorily the experimental profiles. Overall, results obtained in the present study confirmed that ammonium and sulfide rich effluents could be fully valorized to produce optimally elemental sulfur, but further studies are warranted to evaluate the effectiveness of acclimatization periods to couple biological ammonium oxidation with biosulfur recovery. Moreover, the biosulfur produced presented low settleability indicating that further research should be performed to enhance its aggregation towards its valorization as a value-added product.

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Table 1. Oxygen-sulfide (O_2/TDS) and oxygen-ammonium (O_2/N-NH_4^+) molar ratios and mass transfer coefficients (K_La) supplied for each of the period defined by the air flow rate evaluated during the biostat operation.

Period	I	II	III	IV	V	VI	VII
Air flow rate (L min^{-1})	1	0.7	0.5	0.35	0.2	0.05	0
Duration (days)	0-6	6-10	10-14	14-17	17-23	23-37	37-62
k_La (h^{-1})	22	18.6	14.6	12.2	9.1	5.5	1.8
O_2/TDS molar ratio	6.6	6	5.1	4.5	2.9	1.5	0.44
O_2/N-NH_4^+ molar ratio	7.3	7.2	4.8	4.2	3.2	2	0.5

Table 2. Values of the four parameters calibrated by the mathematical model.

Parameters	Units	Value
$\mu_{max/SOB}$	d^{-1}	10.1
k_{S^0}	mg $^{2/3}$ VSS mg $^{-2/3}$ S	0.3
$\mu_{max/AOB}$	d^{-1}	1.75
$k_{O_2/AOB}$	mg L^{-1}	1.5

Table 3. Kinetic parameters reported in the literature and determined at 25 ºC.

	$\mu_{max/SOB}$ $(d^{-1}$	k_{S^0}	$\mu_{max/AOB}(d^{-1})$	$k_{O_2/AOB}(mg\ L^{-1})$
Munz et al.[45]	7.4			
Mora et al.[22]	9.84	0.833 – 0.03		
Wu et al.[20]			1.45	
Jubant et al.[46]			1.21	
Pan et al.[47]				0.6*
Ge et al.[48]				0.22 – 0.56
Guisasola et al.[16]				0.74
Regmi et al.[49]				1.16

* This parameter was reported at 22ºC.