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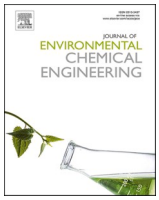

Biomass concentration enhances the stability of the biological desulfurization process through biochemical transformation of (poly) sulfides

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ABSTRACT

The biological desulfurization process utilizes sulfide-oxidizing bacteria (SOB) to transform toxic hydrogen sulfide (H_2S) gas into elemental sulfur that is recovered and reused in various applications. The recent addition of an anaerobic bioreactor enhanced sulfur selectivity by exposing SOB to alternating highly sulfidic conditions followed by micro-oxic conditions. These alternating conditions led to an interest in polysulfide anions (S_x^{2-}), which are formed as a result of the equilibrium between HS^- and elemental sulfur and enhanced with longer sulfidic retention times. Here, the effect of sulfide concentration was investigated to determine the influence on the uptake of (poly)sulfides in the sulfidic bioreactor and the influence in combination with the total biomass concentration for process stability. For the first time, internal sulfane was found to be stored in SOB from a continuous dual-reactor process. Interestingly, a maximum relative concentration of 0.1 mg-S mg-N^{-1} was found at the lowest sulfide concentration, indicating the conversion of sulfane into other species rather than storage. The S_x^{2-} chain length distribution changed the most with both an increased sulfide and biomass concentration, where the relative abundance of chain length 5 increased by ~10 % and chain length 7 decreased by ~5 %. Chain length 4 only increased when the sulfide concentration increased. Here, it is proposed that the nutrient dosing and sulfide concentration in the sulfidic bioreactor should be balanced. By balancing the biomass concentration and sulfide concentration in the sulfidic bioreactor, increased process stability can be achieved through the enhancement of (poly)sulfide uptake and prevention of chemical oxidation.

1. Introduction

Dihydrogen sulfide (H_2S) is a toxic component of various gas streams from anaerobic digesters (biogas), landfills, natural gas processing, and oil refineries [1]. The emission of gas streams containing H_2S is regulated through environmental guidelines, and therefore, various treatment technologies have emerged to mitigate H_2S emissions [2]. One such treatment technology is the biological desulfurization process under haloalkaline conditions, which was developed in the 1990s [3]. The biological desulfurization process utilizes sulfide-oxidizing bacteria (SOB) to convert H_2S into elemental sulfur (S^0), which can be further processed for use as a biosulfur product [4,5].

The biological desulfurization process is a multi-step process that begins with an absorber column, where haloalkaline (bi)carbonate solution containing both SOB and sulfur crystals comes into contact with H_2S containing gas. The H_2S gas gets absorbed into the (bi)carbonate medium, forming mainly bisulfide (HS^-) in solution (Table 1, Eq. 1). After the absorber column, the HS^- "rich" solution enters a micro-oxic bioreactor where SOB oxidize sulfide into S^0 utilizing oxygen as the end electron-acceptor. After oxidation, the sulfide-free or "lean" solution is then transported back to the top of the absorber column to be reused for absorption.

This process has been recently improved by the addition of a bioreactor between the absorber column and micro-oxic bioreactor, creating a dual-reactor process. In this additional bioreactor, hereafter referred to as the sulfidic bioreactor, an anaerobic environment is created after the

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Table 1
Major biological and chemical reactions in biological desulfurization processes.

Equation	Reaction	Reaction type
1	$H_2S_{(g)} \leftrightarrow H_2S_{(aq)} \leftrightarrow HS^- + H^+$	Chemical deprotonation
2	$HS^- + \frac{1}{2}O_2 \rightarrow \frac{1}{8}S_8 + OH^-$	Biological sulfide oxidation
3	$\frac{1}{8}S_8 + 1\frac{1}{2}O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$	Biological sulfur oxidation
4	$2HS^- + 2O_2 \rightarrow S_2O_3^{2-} + H_2O$	Chemical sulfide oxidation
5	$S_2O_3^{-2} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$	Biological thiosulfate oxidation
6	$HS^- + S_{x-1} \leftrightarrow S_x^{2-} + H^+$, $x \geq 2$	Chemical polysulfide formation
7	$S_x^{2-} + 1\frac{1}{2}O_2 \rightarrow S_2O_3^{2-} + (x-2)\ S^0$	Chemical polysulfide oxidation

Adapted from [6–8].

absorber column. These sulfidic conditions have been shown to increase the selectivity for sulfur and reduce the selectivity for the undesired byproducts thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}) [9]. Limiting the formation of both $S_2O_3^{2-}$ and SO_4^{2-} not only increases sulfur selectivity but also decreases the operational costs as their formation decreases the alkalinity of the process solution, and their buildup creates the need for a bleed stream (Table 1, Eq. 3 and 5). The reduction in the formation of $S_2O_3^{2-}$ has been hypothesized to mainly occur due to the partial removal of (poly)sulfides in the sulfidic environment by the SOB [10]. This partial removal limits the amount of (poly)sulfides that chemically oxidize in the micro-oxic bioreactor and additionally limits the production of SO_4^{2-} by decreasing biological $S_2O_3^{2-}$ oxidation [6,7] (Table 1, Eq. 4, Eq. 5, and Eq. 7).

The partial removal of (poly)sulfides was observed in previous experiments where the measured total sulfide concentration was lower than the theoretical sulfide concentration in the sulfidic bioreactor [10]. This difference led to an interest in the underlying mechanisms of the SOB to remove sulfide, mainly in the form of polysulfides (S_x^{2-}). In the biological desulfurization process, S_x^{2-} are initially formed when HS^- comes into contact with sulfur globules and/or crystals in the absorber column (Eq. 6). Here, HS^- cleaves the S-S bond in sulfur rings, and then, a chemical equilibrium is established in solution between S_8, HS^-, and S_x^{2-} [11–13]. S_x^{2-} are known to be present in the sulfidic zones of the process, i.e. absorber column and sulfidic bioreactor and are present in chain lengths ranging from 2 to 8 sulfur atoms, with 5 being the most prevalent [14].

Polysulfides are thought to play a key role in the biological desulfurization process as they are an intermediary sulfur species utilized in many biochemical processes [15–19]. For example, in *Beggiatoa* strains, deposited sulfur globules contained a mixture of S_8 rings and linear S_x^{2-} [20]. For SOB in the biological desulfurization process, two main enzyme systems, sulfide:quinone oxidoreductase (Sqr) variations and flavocytochrome c oxidoreductase (Fcc), have been shown to be active [6,21]. Research has illustrated that Sqr variations can both produce and utilize S_x^{2-}, while Fcc has been experimentally proven to also interact with S_x^{2-} [6,21–25]. The removal of (poly)sulfides utilizing SOB has been measured in batch systems and hypothesized to be converted and stored in long-chain organic S_x^{2-} (RSS_nH and RSS_nR) [26]. However, the internal storage capacity of the SOB is unknown especially in the sulfidic bioreactor where HS^-, S_8, and S_x^{2-} are constantly forming an equilibrium with each other.

Recently, S_x^{2-} have been studied in the biological desulfurization process, where the concentration and chain length has been shown to vary based on the H_2S loading rate, biomass concentration, and pH of the process solution [27,28]. Previous research found that increasing the H_2S loading rate and subsequently the total sulfide concentration in solution leads to an increase in total S_x^{2-} concentration [27]. The same study reported that a high biomass concentration (~90 mg-N L^{-1}) in solution led to a decrease in total S_x^{2-} concentration compared to a low biomass concentration (~24 mg-N L^{-1}), implying an increase in the uptake of (poly)sulfides by the bacteria. However, the biomass concentration in that study was grown utilizing higher H_2S loading rates and then utilized in experiments with lower H_2S loading rates, putting the biomass in a "famine" scenario and sulfide limited conditions, which are not seen in typical operation.

In (full-scale) biological desulfurization systems, the sulfidic bioreactor contains an excess of HS^- in the solution while the biomass concentration is controlled via the nutrient dosing rate. By increasing the nutrient dosing, and thereby biomass concentration, the uptake of S_x^{2-} in the sulfidic bioreactor can potentially be increased, in turn lowering the concentration of sulfide at the inlet of the micro-oxic bioreactor. This can decrease the chemical formation of $S_2O_3^{2-}$ and thus increase the selectivity of the process towards sulfur. However, previous studies have reported excess biomass concentrations can lead to an increase in SO_4^{2-} concentration, which also decreases the selectivity for sulfur [21,29]. Therefore, the uptake of S_x^{2-} in the sulfidic bioreactor has yet to be quantified and directly related to the biomass concentration and the total sulfide concentration in solution.

To understand if the uptake of (poly)sulfides by SOB can be stimulated, this study aims to quantify the stored reactive sulfur compounds under various sulfide concentrations and biomass concentrations. Additionally, the process performance in terms of sulfur selectivity and bulk S_x^{2-} concentrations were determined to see if a relationship exists. To the best of the authors' knowledge, this is the first time that the internal sulfane potential of the biomass has been quantified in continuous lab-scale experiments. Here, the ability of the biomass to contain sulfane or reduced sulfur compounds was determined and subsequently quantified to see the impact of biomass concentration in the biological desulfurization process. These insights could potentially provide new insights into the interaction of (poly)sulfides and microorganisms in natural, oxygen-limited conditions. Ultimately, understanding the influence of varying sulfide concentrations and the sulfide-biomass concentration relationship has the potential to make the process more robust by providing an operational strategy for better sulfur selectivity.

2. Materials and methods

2.1. Experimental set-up

The experimental set-up consisted of a dual-reactor system with a falling film gas absorber column (~70 mL), sulfidic bioreactor (2.2 L), and micro-oxic bioreactor (3.0 L) (Fig. 1). A feed gas mixture of nitrogen (N_2), carbon dioxide (CO_2), and pure hydrogen sulfide (H_2S) entered through the bottom of the absorber column. Oxygen (O_2) was added to the system via the recirculation of the headspace in the micro-oxic bioreactor. Each gas was controlled individually using mass flow controllers (type EL-FLOW, model F-201DV-AGD-33K/E, Bronkhorst, the Netherlands), which were specified based on the gas supply rate needed: N_2 ranged from 0 to 350 mL min^{-1}, CO_2 ranged from 0 to 40 mL min^{-1}, H_2S ranged from 0 to 30 mL min^{-1}, and O_2 ranged from 0 to 30 mL min^{-1}.

Both N_2 and H_2S were continuously supplied to the system, with N_2 supplied at a rate of 350 mL min^{-1} and H_2S supplied at a rate between 2 and 11.6 mL min^{-1}. CO_2 and O_2 were pulse-wise controlled using a multiparameter transmitter (Liquiline CM442–1102/0, Endress+Hauser, Germany) connected to two separate sensors both located in the micro-oxic bioreactor. CO_2 dosing in the absorber column was regulated by a pH sensor and supplied when the pH of the bioreactor was above the set-point (Orbisint CPS11D-7AA51; Endress+Hauser, Germany). O_2 dosing in the micro-oxic bioreactor was regulated by a redox sensor equipped with an internal Ag/AgCl reference electrode (Orbisint CPS12D-7PA51; Endress+Hauser, Germany) connected to a feedback controller (PID) to control the oxidation-reduction potential (ORP). A gas compressor (N-820 FT.18, KNF Laboport, NJ, USA) was used to recycle the headspace and add O_2 to the micro-oxic bioreactor. A stirrer was used to ensure mixing in the sulfidic bioreactor.

Solution from the micro-oxic bioreactor was recirculated over the absorber column using a gear pump (EW-75211–30, Cole-Parmer, USA)

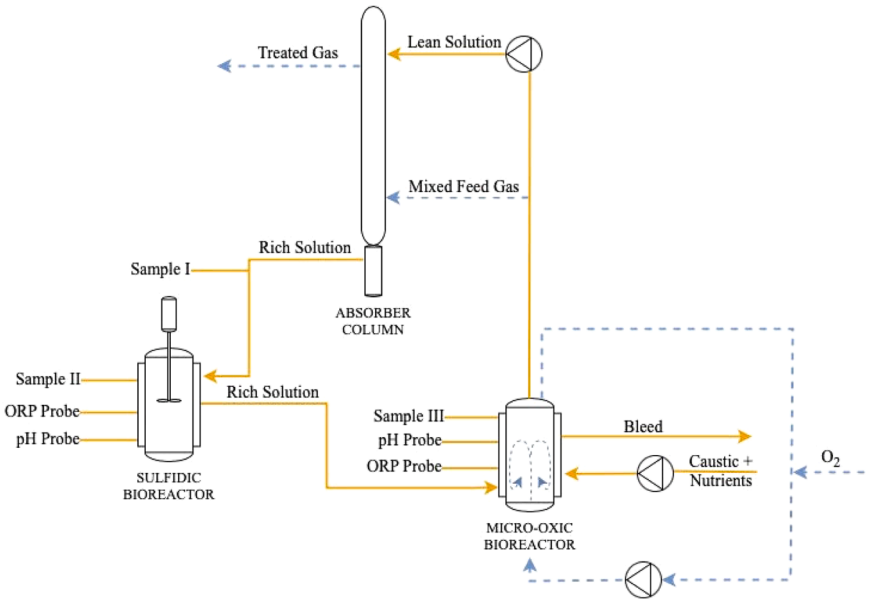

Fig. 1. Experimental set-up of dual-reactor system. Blue dashed lines indicate gas flows, and orange solid lines indicate liquid flows.

at a flow rate of 10 L h^{-1} throughout the entire operational run. In the absorber column, H_2S, N_2, and CO_2 came into countercurrent contact with the process solution, allowing absorption and transformation of H_2S into mainly HS^-. After the absorber column, the haloalkaline (bi) carbonate solution containing HS^- entered the sulfidic bioreactor via gravity flow. The HS^- rich solution subsequently entered the micro-oxic bioreactor via an overflow line. Make-up water consisting of a carbonate buffer ($NaHCO_3$ and Na_2CO_3), trace minerals [30], and urea was constantly fed to maintain the alkalinity of the system due to losses by the production of SO_4^{2-} and $S_2O_3^{2-}$ and to sustain the biomass concentration in the system. Excess process liquid left the system via a gravity overflow bleed line in the micro-oxic bioreactor, giving the system a total hydraulic retention time (HRT) of 14 days [21]. The absorber column and bioreactors were maintained at a temperature of 37.8 ± 0.4 °C utilizing water jackets attached to a thermostat bath (DC10, Thermo Haake, Germany). A photo of the actual set-up is provided in the supplemental information (SI 1).

Throughout the operational run, liquid samples were taken daily from the micro-oxic bioreactor, and gas samples were taken daily from the headspace of the micro-oxic bioreactor. Additional liquid samples were taken from the absorber column and sulfidic bioreactor for all (poly)sulfide analyses (Fig. 1). Liquid samples from the absorber column had a limited HRT due to the size of the lab-scale set-up, and therefore provided limited time for S_x^{2-} formation and are less representative of typical operational conditions. Thus, only liquid samples taken from the sulfidic bioreactor are presented in the results and discussion.

2.2. Experimental operation

2.2.1. Medium and inoculum composition

The haloalkaline (bi)carbonate medium with a starting pH of 8.5 and 1 M Na^+ was prepared according to Kiragosyan et al., (2019). The inoculum containing both microorganisms and sulfur was collected from previous experiments utilizing a biological desulfurization pilot system [27]. For start-up, 1 L of pilot effluent was mixed with 4 L of (bi)carbonate medium, 1 mL of concentrated trace element solution [30], and 5 mL of a urea stock solution (30 g-N L^{-1}).

2.2.2. Process conditions

During the entire operation of the system for 74 days, the pH was controlled at 8.5, while the ORP set-point was controlled at −395 mV. The ORP set-point is used as an approximate measurement for the O_2/H_2S supply ratio, where, ideally, an optimal amount of O_2 is supplied compared to the H_2S load to achieve a high sulfur selectivity. To test the conditions of different total sulfide concentrations to biomass concentration ratios, the H_2S loading rate was varied at two different biomass concentrations, with intended concentrations at 20 and 40 mg-N L^{-1} (Table 2). As the liquid circulation flow rate was constant at 10 L h^{-1} throughout the entire operational run, the sulfide concentration in the sulfidic bioreactor was directly proportional to the H_2S loading rate (g-S day^{-1}). Each H_2S loading rate was applied for a minimum of 24 hours. As precise biomass concentrations could not be achieved, the actual biomass concentrations are reported. Biomass concentrations were varied by changing the concentration of urea in the caustic and nutrient solution that was dosed to the system. Ratios between the theoretical sulfide concentration and biomass concentration provide a linear trend covering a range from 0.7 mg-S mg-N^{-1} to 2.7 mg-S mg-N^{-1}. A visualization of the experimental design of the ratios is provided in the supplemental information (SI 2).

2.3. Analyses

2.3.1. Analytical techniques

Each day, a liquid sample of ~10 mL was taken from the micro-oxic bioreactor to monitor process performance. This sample was split into two separate parts, where half was filtered using a 0.45 μm syringe filter (HPF Millex, Merck, the Netherlands), and the other half was left unfiltered. The filtered fraction of the sample was analyzed using ion chromatography (Metrohm 930 Compact IC flex, Switzerland) to measure SO_4^{2-} and $S_2O_3^{2-}$ using an anion column (Metrohm Metrosep A Supp 5, 150/4.0 mm, Switzerland) and pre-column (Metrohm Metrosep A Supp 4/5 Guard, Switzerland) and sodium (Na^+) and potassium (K^+) using a cation column (Metrohm Metrosep C4–150/4.0 mm, Switzerland). The unfiltered fraction of the sample was analyzed using high-temperature catalytic oxidation with a TOC-L CPH analyzer (Shimadzu, the Netherlands) to determine the total alkalinity by measuring the total inorganic carbon based on the total concentration of HCO_3^- and CO_3^{2-}. All liquid samples mentioned above were prepared and then stored at 4°C and analyzed within 3 days.

Due to the tendency of sulfur to adhere to the walls of the bioreactor, the biological sulfur selectivity was determined based on the mass balance of sulfur (Eq. 7) according to [9]. V_{in-H_2S} is the volumetric influent

Table 2
Process conditions in each experiment with varied H_2S loading rates and biomass concentrations.

		High biomass				Low biomass			
Experiment #		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
H_2S loading rate	*g-S day^{-1}*	7.2	12.4	16.3	23.9	4.3	7.4	12.4	10.7
Theoretical total sulfide concentration in sulfidic bioreactor	*mg-S L^{-1}*	30.1	51.5	67.8	99.6	18	30.9	51.5	44.6
Volumetric loading rate aerated reactor	*g-S L^{-1} day^{-1}*	2.4	4.1	5.4	8.0	1.4	2.5	4.1	3.6
Biomass concentration	*mg-N L^{-1}*	41	36	39	37	18	25	27	19
Ratio of S to biomass	*H_2S loading rate to biomass ratio (g-S day^{-1} per mg-N L^{-1})*	0.2	0.3	0.4	0.6	0.2	0.3	0.5	0.6
	Theoretical total sulfide to biomass ratio (mg-S mg-N^{-1})	0.7	1.4	1.7	2.7	1	1.2	1.9	2.4

of H_2S in mol-S day^{-1}, followed by the production rates for both SO_4^{2-} and $S_2O_3^{2-}$. These are based on the total volume of effluent V_{eff} (L), the average concentration of either SO_4^{2-} or $S_2O_3^{2-}$(mol-S L^{-1}), the total liquid volume V of the system (L), and the change in concentration of either SO_4^{2-} or $S_2O_3^{2-}$(mol-S L^{-1}) all over the time period Δt (days).

$$P_{S_8} = V_{in-H_2S} - \left[\frac{V_{eff}\left[SO_4^{2-}\right]_{avg.} + V\Delta[SO_4^{2-}]}{\Delta t}\right] - \left[\frac{V_{eff}\left[S_2O_3^{2-}\right]_{avg.} + V\Delta[S_2O_3^{2-}]}{\Delta t}\right] \quad (7)$$

Finally, the selectivity for SO_4^{2-} and $S_2O_3^{2-}$ were calculated by dividing the production rates of each component by V_{in-H_2S}. The system was assumed to be at pseudo "steady-state" after a start-up period of 2 weeks.

2.3.2. Biomass analyses

Total nitrogen was measured for both unfiltered and filtered samples based on the amount of organic nitrogen oxidized to nitrate by ammonium persulfate (LCK338, LCK238, LCK128, Hach Lange, USA). Unfiltered samples were used to determine the total nitrogen in the system, and filtered samples were used to determine the dissolved nitrogen in the bulk solution. The difference between these two measurements was the total nitrogen that could be attributed to the biomass in the process [6,7]. Each sample was diluted a minimum of 5 times to ensure no interference by the salt concentration of the haloalkaline (bi)carbonate medium.

To preserve biomass samples for next-generation sequencing (NGS), a 2 mL sample was taken each week and centrifuged at 15,000 g for 10 min. The supernatant was discarded, and the pellet was snap frozen using liquid nitrogen and kept at −70°C until extraction. A detailed extraction protocol as well as the NGS analysis is provided in the supplementary information (SI 3).

2.3.3. Polysulfide analyses

Extracellular S_x^{2-} located in the bulk solution (dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and higher dimethyl polysulfanes (Me_2S_4 to Me_2S_8)) were analyzed with ultra high-performance liquid chromatography (uHPLC) according to Roman et al., (2014). Samples were taken with glass syringes from the bottom of the absorber column and sulfidic bioreactor and quickly transferred to an anaerobic tent to prevent oxidation. Subsequently, samples were filtered using a 0.7 µm syringe glass fiber filter (AP40, 25 mm, Millipore, USA) enclosed in a metal casing (Microsyringe Filter Holder 25 mm, Merck). The samples were prepared by derivatizing the S_x^{2-} into more stable dimethyl polysulfanes (Me_2S_x) using methyl triflate (≥98 % pure, Sigma-Aldrich, the Netherlands) in methanol medium flushed with N_2. The volume of added methyl triflate was determined by the pH of the sample, which was measured prior to derivatization. After methylation of the sample, 20 µL of internal standard (dibenzo-a,h-anthracene, Supelco Analytical, USA) in benzene (Sigma-Aldrich, the Netherlands) was added. Finally, samples were centrifuged at 3300 g for 10 min to remove precipitates, and stored at 4°C and analyzed within 3 days. The uHPLC was equipped with a UV detector (Dionex UltiMate 3000RS, USA) and an extended column (Agilent, Zorbax Extended C-18 1.8 µm, 2.1 ×50 nm, USA). Samples were measured at a temperature of 20°C, a UV wavelength of 210 nm, a flow of 0.371 mL min^{-1}, and an injection volume of 1.25 µL. Detailed operation of the uHPLC can be found elsewhere [14]. Total S_x^{2-} concentration and chain length concentrations were determined based on the internal standard and response factors from Roman et al., (2014). The Milli-Q injection right before the start of the sampling sequence was used as a blank.

Intracellular S_x^{2-} were determined indirectly as "cell-bound" sulfane or reduced sulfur by using the methylene blue method [31]. Samples were taken using glass syringes from the absorber column and sulfidic bioreactor and immediately transferred to 2 mL Eppendorf tubes flushed with N_2. After sampling, the tubes were centrifuged (Eppendorf Microcentrifuge 5424, Eppendorf AG, Germany) in a two-step process. First, samples underwent an initial slow centrifuge (376 g for 2 min) to eliminate as much sulfur as possible. Afterward, 500 µL of the supernatant was transferred to another 2 mL Eppendorf tube flushed with N_2. Samples were centrifuged again (13,523 g for 5 min) to obtain a biomass pellet. The biomass pellet was then washed with 1 M $NaHCO_3$ buffer while under N_2. The 1 M $NaHCO_3$ buffer wash was then discarded, and the pellet was resuspended with 1 M $NaHCO_3$ buffer and vortex mixed with 10 % zinc acetate to stabilize the S^{2-} in the sample. Once stabilized, the methylene blue method was used to determine the concentration of S^{2-} in the sample at an optical density of 665 nm in a 1 cm× 1 cm macro cuvette (Shimadzu Spectrophotometer UV1800).

3. Results and discussion

Over the duration of the entire operational run, samples were taken at two distinct biomass concentrations (~20 and ~40 mg-N L^{-1}) and four different total sulfide concentrations (Table 2). For each biomass and total sulfide combination, the total S_x^{2-} concentration, sulfane storage within the SOB, and the chain length distribution in the bulk solution were analyzed. As the process solution was constantly recirculated through the different process sections (absorber, sulfidic, and micro-oxic bioreactor), the total theoretical sulfide concentration in the sulfidic bioreactor (mg-S L^{-1}) is proportional to the H_2S loading rate (g-S day^{-1}) of the system throughout the entire operational run. Therefore, all of the S_x^{2-} results are reported in terms of the total theoretical sulfide concentration. As the HRT of the absorber column in these experiments was short (0.5 min), limited effects were observed in the absorber column samples. Therefore, these results are provided in the supplemental information (SI 4).

3.1. Influence of total sulfide concentration on polysulfides and internal sulfane storage potential in the sulfidic bioreactor

3.1.1. Polysulfides based on total sulfides in the sulfidic bioreactor

To determine if the biomass concentration influenced the total S_x^{2-} concentration, the total S_x^{2-} concentration in the bulk solution was determined (Fig. 2A). During the experiments at lower biomass (~20 mg-N L^{-1}), the lowest theoretical sulfide concentration of 18 mg-S L^{-1}

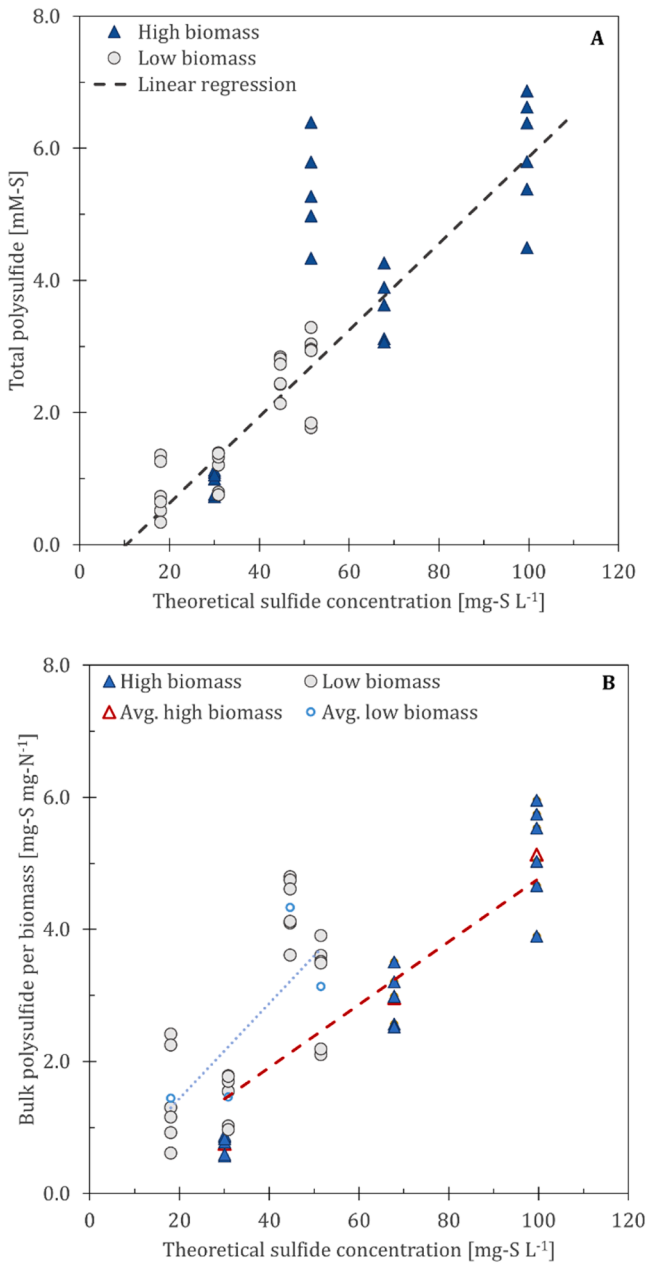

Fig. 2. Different theoretical total sulfide concentrations at both low biomass (~20 mg-N L^{-1}) and high biomass (~40 mg-N L^{-1}) concentrations, illustrated above: A) total S_x^{2-} concentration in the bulk solution and a linear regression analysis (dashed line) of all sampling points (N = 41) excluding the outliers for high biomass at a total sulfide concentration of 51.5 mg-S L^{-1}, with a slope of 0.066 mM-S/mg-S L^{-1} and a standard error of 0.003 mM-S/mg-S L^{-1} and B) total S_x^{2-} concentration per unit biomass in the bulk solution. Triplicate samples were taken with each sample analyzed in duplicate at each sulfide concentration. A linear regression analysis of the averages of these samples per biomass concentration are graphed as a dotted line with slope 0.08 ± 0.02 mg-S mg-N^{-1}/mg-S L^{-1} (low biomass average, N = 23) and a dashed line with slope 0.06 ± 0.004 mg-S mg-N^{-1}/mg-S L^{-1} (high biomass average, N = 17).

(Experiment 5) resulted in an average total S_x^{2-} concentration of 0.8 ± 0.4 mM-S. The highest theoretical sulfide concentration of 51.5 mg-S L^{-1} (Experiment 7) resulted in an average total S_x^{2-} concentration of 2.6 ± 0.6 mM-S. Comparatively, at the higher biomass concentration of ~40 mg-N L^{-1}, the lowest theoretical sulfide concentration of 30.1 mg-S L^{-1} (Experiment 1) resulted in an average total S_x^{2-} concentration of 1.0 ± 0.2 mM-S. The highest theoretical sulfide concentration of 99.6 mg-S L^{-1} (Experiment 4) resulted in an average total S_x^{2-} concentration of 5.9 ± 0.8 mM-S.

At high biomass concentration, three out of the four samples appeared to follow the same linear trend as the low biomass samples (Fig. 2A). However, the samples taken at a theoretical sulfide concentration of 51.5 mg-S L^{-1} had a total S_x^{2-} concentration twice as high as expected. This trend was not observed when the theoretical sulfide concentrations were lower (30.1 versus 30.9 mg-S L^{-1}) at the different biomass concentrations, where the average total S_x^{2-} concentration was 1.1 ± 0.3 mM-S versus 0.8 ± 0.2 mM-S for the low and high biomass concentrations respectively. Due to the linear appearance of the data, a linear regression analysis (N = 41, number of data points) was performed which resulted in a slope of 0.066 mM-S/mg-S L^{-1} and the standard error of 0.003 mM-S/mg-S L^{-1} excluding the apparent outliers for the high biomass at a total sulfide concentration of 51.5 mg-S L^{-1}.

Based on this observation and data from the absorber column (SI 4), it is most likely that the total S_x^{2-} concentration at a sulfide concentration of 51.5 mg-S L^{-1} and at high biomass are outliers in this case, potentially due to the fact it was the first experiment performed. Another potential explanation for the variability in this process is that the specific SOB that influence (poly)sulfides were not fully active yet or the sulfidic pressure was not high enough to influence the microbial community to give an advantage to the microorganisms that can survive in highly sulfidic conditions.

The increase observed for the average total S_x^{2-} concentration was expected due to chemical equilibrium as a higher concentration of total sulfide leads to a greater concentration of S_x^{2-} due to sulfur being present in excess in the system (Table 1, Eq. 6) [32]. Additionally, the autocatalytic nature of the S_x^{2-} assists with the increase in their concentration [33]. In these experiments, the difference in biomass concentration was not enough to influence the total S_x^{2-} concentration as observed in a previous study. This could be due to the fact that in this experiment, the biomass concentration was only ~2 times higher compared to an almost ~4 times increase in the biomass reported in the previous work [27].

The SOB present in the biological desulfurization process are believed to use (poly)sulfides as the main substrate due to the lipophilicity and partitioning coefficient of S_x^{2-} anions compared to HS^- [10, 34]. Therefore, the total S_x^{2-} concentration was also plotted in terms of the biomass (Fig. 2B). Here, a similar linear trend to the total S_x^{2-} concentration was observed. When separating the low biomass and high biomass samples, the average of the low biomass samples has a slightly steeper slope (~0.08 versus 0.06) than the average high biomass samples (excluding the outlier determined previously). These slopes lie in between those from a previous study conducted at pilot scale (~0.1 and 0.02) with the lower biomass concentration also producing the steeper slope [27]. This observation indicates that even though the total sulfide concentration has a direct influence on the total S_x^{2-} concentration in the sulfidic bioreactor, the biomass concentration can lower the amount of (poly)sulfides in solution per unit biomass. Therefore, the linear relationship between total sulfide concentration in the sulfidic bioreactor and the biomass concentration needs to be taken into account when designing the dual-reactor biological desulfurization process.

3.1.2. *Internal sulfane not influenced by total sulfides in the sulfidic bioreactor*

In addition to determining the bulk S_x^{2-} concentration in the process solution from the sulfidic bioreactor, biomass samples were analyzed for total internal sulfane and the amount of internal sulfane per biomass unit. This was used to determine if the uptake of (poly)sulfides through the storage of the reactive sulfide compounds could be stimulated. The total sulfane concentration in S_x^{2-} the bulk solution was determined based on the average chain length quantified, which can be found in the supplemental information as this is mainly determined through chemical equilibrium (SI 5).

The total internal sulfane concentrations showed no clear trend when the total sulfide concentration was changed. However, it was observed

that all samples remained below a total sulfane concentration of 3.0 mg-S L^{-1} (Fig. 3A). When looking at the low biomass samples, a decrease in the total internal sulfane concentration was observed with the average decreasing from 1.90 ± 0.27 mg-S mg-N^{-1} to 0.78 ± 0.18 mg-S mg-N^{-1}. For the high biomass samples, the average sulfane concentration started at 1.50 ± 0.18 mg-S mg-N^{-1} and ended with 1.78 ± 1.00 mg-S mg-N^{-1} at the highest total sulfide concentration. Although the average total sulfane increased with increasing total sulfide concentration for the high biomass samples, the deviation also increased with half of the samples resulting in ~1.0 mg-S L^{-1} and the other half closer to ~2.8 mg-S L^{-1}.

When analyzing the total internal sulfane concentration per unit of SOB (Fig. 3B), a slight difference was observed between the low and high biomass samples. Here, at all concentrations, with the exception of 18.0 mg-S L^{-1}, the average total sulfane per biomass remained below 0.1 mg-S mg-N^{-1}. This suggests that the SOB have a maximum sulfane uptake capacity per unit biomass. As this is the first time that the internal sulfane has been measured in the dual-reactor biological desulfurization process, previous results from separate studies that investigated the removal of (poly)sulfides from the bulk solution were used as a comparison [10,26]. Here, the internally measured reactive sulfur in this study was compared to the previously measured S_x^{2-} removals per unit of SOB. Previous research has shown the removal and uptake of (poly) sulfides to be 1.18 ± 0.51–4.11 ± 0.46 mg-S mg-N^{-1} in the sulfidic bioreactor in a continuous pilot system and up to 8 mg-S mg-N^{-1} in batch bottles [10,26]. Here, all specific sulfane measurements (Fig. 3B) were approximately below 0.1 mg-S mg-N^{-1}, more than 10x lower than previous measurements looking solely at the removal, indicating that the (poly)sulfides taken up by the SOB do not remain as reactive sulfane within the SOB.

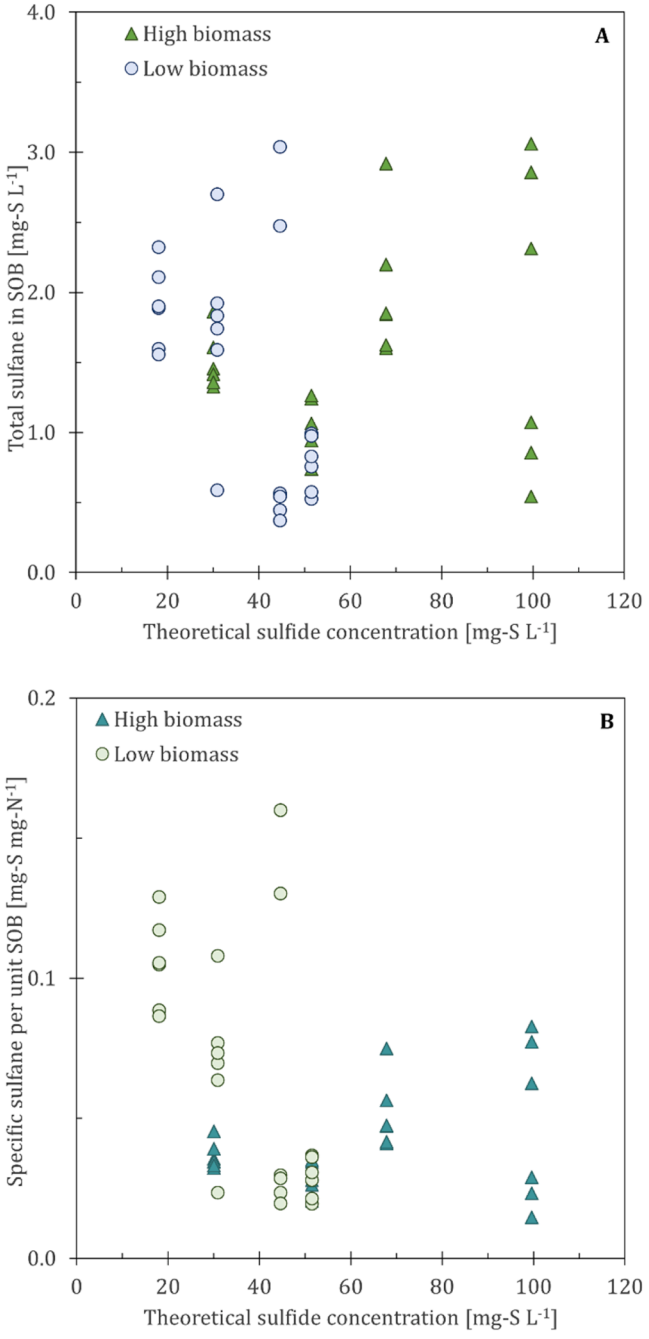

Fig. 3. At different theoretical total sulfide concentrations and at both low biomass (~20 mg-N L^{-1}) and high biomass (~40 mg-N L^{-1}) concentrations the A) internally stored total sulfane and B) internally stored total sulfane measured per unit biomass were determined. Triplicate samples were taken with each sample analyzed in duplicate, illustrated above at each sulfide concentration.

Instead of keeping sulfane stored in the periplasm, (poly)sulfide-sulfane compounds are assumed to undergo further (bio)chemical reactions into other compounds, such as persulfides (RSS_nH), glutathione (GSH), or even to elemental sulfur [23,26,35]. These reactions most likely occur due to the higher total sulfide concentration in the sulfidic bioreactor. The increased sulfide concentration leads to increased S_x^{2-} concentrations due to chemical equilibrium, and therefore, bioavailability. Increasing the bioavailability of S_x^{2-} to the SOB has been shown previously to suppress the cytochrome c oxidase (CcO) enzyme system typically present in the biomass in the biological desulfurization process [9,36]. The suppression of CcO/Fcc system potentially upregulates a different enzyme system, such as a Sqr or another system not reliant on the cytochrome C pool, to oxidize S_x^{2-} into different compounds and potentially influences the rate at which these (bio)chemical reactions occur [21]. Hence, the availability of (poly)sulfides may directly affect the rate of the enzymatic reactions, with an increased rate to limit the buildup of sulfane-containing ions within the periplasm.

Interestingly, the lowest sulfide concentration had the highest average specific sulfane concentration per unit biomass concentration at 0.11 ± 0.01 mg-S mg-N^{-1}, which was ~35 % higher than the next highest average. This was also observed in batch bottles in a previous study where the highest specific sulfide removal capacity in mg-S mg-N^{-1} was found at the lowest biomass concentration [26]. For the lowest sulfide concentration, the SOB could potentially be storing the reactive sulfane in a reduced form (i.e. RSS_nH) that is more readily oxidized, such as GSH [23]. Another hypothesis is that the enzyme systems within the SOB are producing more S_x^{2-} or reactive sulfur compounds that contain sulfane due to the lack of (poly)sulfides in solution. In the process, variations of the Sqr enzyme system are typically present with Sqr being known to produce S_x^{2-} as the main reaction product [15,37–39]. Since lower biomass concentrations have resulted in higher internal sulfane concentrations per unit SOB (Fig. 3B), more in-depth research into the role of sulfane and its dependency on Sqr in the biological desulfurization system is required.

3.2. Polysulfide chain length distributions change with both biomass and sulfide concentration

In addition to the total S_x^{2-} concentration, the individual S_x^{2-} chain lengths were determined. While chain lengths between 3 and 8 were detected in the sulfidic bioreactor samples, only chain lengths between 4 and 7 are depicted as these were always within the quantifiable range. Chain length 8 was detected only during the highest sulfide concentrations of 67.8 and 99.6 mg-S L^{-1} (Experiments 3 and 4, respectively). Chain length 3 was detected in all samples, however at relative concentrations of < 5 % in 85 % of samples compared to the other chain lengths. Therefore, all chain length data as well as the average chain lengths per sulfide concentration can be found in the supplemental

information (SI 6).

Four experiments with similar sulfide concentrations at two different biomass concentrations were selected to directly compare the influence of sulfide concentrations at different biomass concentrations on the chain length distribution (Fig. 4). First, an increase in the total sulfide concentration at low biomass concentration resulted in minor shifts (≤2 %) between chain lengths 5, 6, and 7. The most notable change between the two sulfide concentrations at low biomass was for S_4^{2-}, which increased from 16 ± 3 % to 20 ± 3 % in the sulfidic bioreactor. For the high biomass concentration, a similar trend was observed where an increase in total sulfide concentration resulted in another shift in the relative abundance of S_4^{2-} from 16 ± 2 % to 17 ± 2 %. This pattern was also seen for the absorber column (SI 4). An increase of ~10 % of S_5^{2-} was observed when comparing the low sulfide and low biomass concentration to the high sulfide and high biomass concentration. In comparison, a decline in S_7^{2-} was observed from 12 ± 2 % to 6 ± 1 % from low sulfide and low biomass concentration to high sulfide and high biomass concentration respectively. Chain length 6, S_6^{2-}, remained relatively constant throughout both total sulfide concentrations and changes in biomass concentration, fluctuating between 26 ± 1 % and 28 ± 2 %.

An increase in the sulfide concentration in the sulfidic bioreactor led to a change in distribution in both biomass concentrations, with the shift in chain length distribution most pronounced at both a high sulfide and high biomass concentration. When comparing the current results collected from the lab-scale system to the previous results reported utilizing a pilot-scale biological desulfurization system, variations in the S_x^{2-} chain length distribution were observed with an excess of biomass concentration (~90 mg-N L^{-1}) between H_2S loading rates of 27 g-S day^{-1} to 58 g-S day^{-1} [27]. At higher biomass concentrations, an increase in S_4^{2-} and a decrease in S_6^{2-} was observed in this pilot-system study. Here in this lab-scale experiment, a similar shift in S_4^{2-} was observed, but the decrease in S_6^{2-} was not observed. This suggests that the biomass concentration was not enough to see a similar distribution change in the experiments presented in this paper. Even though the H_2S loading rates were comparable between pilot-scale and lab-scale experiments, 2.5 and 5.0 g-S L^{-1} day^{-1} versus the 1.3 – 7.3 g-S L^{-1} day^{-1}, respectively (Table 2), this was not the main factor that contributed to the change in chain length distribution.

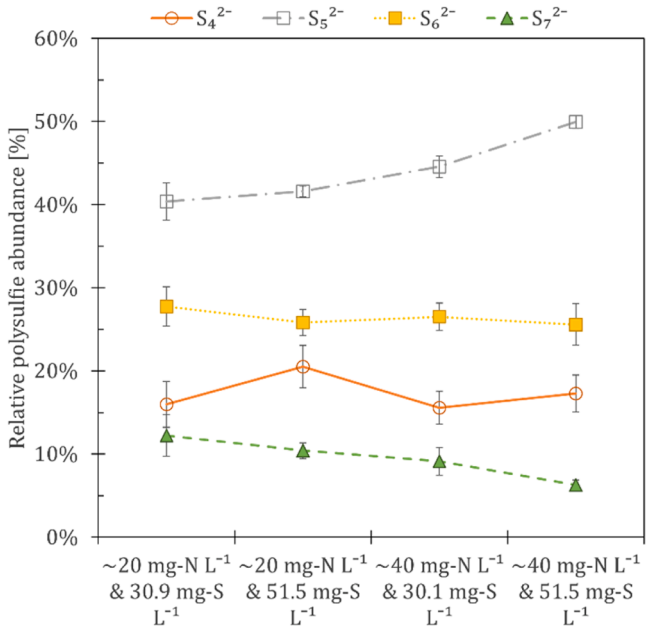

Fig. 4. Chain length variation in the sulfidic bioreactor in the bulk solution for four different sulfide-biomass concentration combinations: (from left to right) low sulfide and low biomass concentration, high sulfide and low biomass concentration, low sulfide and high biomass concentration, and finally high sulfide and high biomass concentration. Lines between data points were placed as a guide for the eye.

Higher biomass concentrations, up to some unknown level, could be beneficial to systems as these increased concentrations can change the chain length distribution of S_x^{2-}. It is currently unknown whether SOB can utilize certain chain lengths of S_x^{2-} more efficiently than others. However, the change in chain length distribution indicates that the bacteria are influencing the abiotic chemical reactions, as the distribution moves further away from the typical abiotic "normal" distribution. Most likely, an elevated concentration of S_x^{2-} anions increases the concentration of individual chain lengths, and thus, the selectivity of the system is steered towards sulfur. Based on the lipophilicity of the outer membrane, there are potentially certain chain lengths that both enter and exit the periplasm of the cell based on relative charge over the molecule and size [40,41]. This in turn influences the S_x^{2-} equilibrium if only certain chain lengths can pass through the membrane. If the SOB do have a preference for certain chain lengths of S_x^{2-} based on physical limitations, the increase in total S_x^{2-} concentration allows for an increase in these membrane interactions, and thus, biochemical reactions. When this occurs, the capacity of the system in terms of total sulfide is hypothesized to increase as more sulfide is transformed into S_x^{2-} and other reactive sulfur species, or ultimately sulfur. This transformation subsequently limits the chemical oxidation of sulfide into other byproducts later in the process. As the total sulfide concentration increased, the distribution of S_x^{2-} chain lengths became more similar to what would be predicted for an abiotic system. This shift suggests that chemical reactions are more dominant in the process than the biological reactions once a certain sulfide concentration is achieved. However, more research is needed to determine the exact point at which the total sulfide concentration dictates the S_x^{2-} distribution more than the biomass.

3.3. Operational results

During the 74 days of operation, data was continuously collected from the ORP and pH probes to monitor the process (SI 7). For the micro-oxic bioreactor, the average ORP was −365 ± 87 mV and an average pH of 8.6 ± 0.1. For the sulfidic bioreactor, the average ORP was −445 ± 21 mV, and an average pH of 8.3 ± 0.1. The high variability of the ORP in the micro-oxic bioreactor was attributed to fluctuations of the oxygen transfer to the liquid due to clogging of the air stone connected to the aeration system. The temperature was maintained at 37.8 ± 0.4 °C and 38.0 ± 0.4 for the micro-oxic and sulfidic bioreactors, respectively. Additionally, the conductivity, alkalinity, SO_4^{2-}, and $S_2O_3^{2-}$ were monitored throughout the entire operational run (Fig. 5A). Here, the average conductivity was 48.0 ± 3.5 mS cm^{-1} with a slight increase (~ ± 3 mS cm^{-1}) between day 36 and 60. This increase corresponded with the increase of $S_2O_3^{2-}$ in solution. The average alkalinity was 0.61 ± 0.07 M with two declines occurring on day 35 and 56. These two changes in alkalinity can be attributed to unstable, that is more variation, operation over the weekend where sulfide built up in the system due to issues with the recirculation of gas over the micro-oxic bioreactor.

The average concentration of SO_4^{2-} was 0.07 ± 0.02 M-S over the 74 days, increasing from 0.04 to 0.12 M-S over the entire operational run. In comparison, the average $S_2O_3^{2-}$ increased from 0.04 ± 0.02 M-S to 0.22 ± 0.07 M-S, ~5.5 times in the high biomass versus the low biomass experiments respectively. This increase in $S_2O_3^{2-}$ can be explained as a lack of biomass activity compared to the chemical oxidation of the (poly)sulfides [21]. Since the total sulfide concentration was varied throughout the entire operational run, the selectivity of the by-products provides more insight into the process performance and sulfur production (Fig. 5B). Each H_2S loading rate was applied for ~24 hours as the higher H_2S loading rates would produce too much elemental sulfur for the lab-scale system if applied over a longer time period. During the high biomass experiments (Experiments 1–4), the selectivity for sulfur was on average higher with a sulfur selectivity of 83 ± 16 %. Afterward, the selectivity for sulfur decreased to an average of 70 ± 39 %. Additionally,

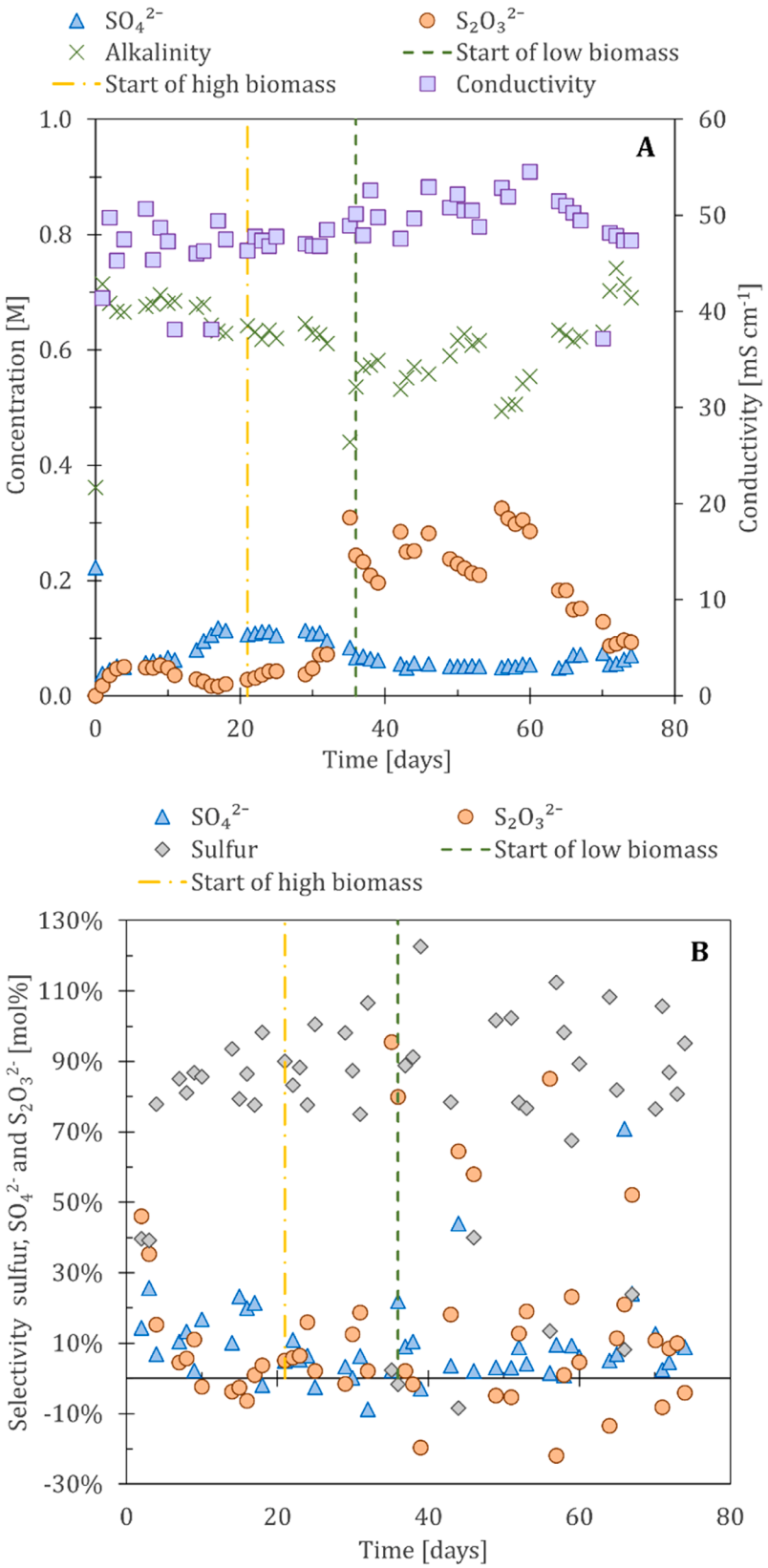

Fig. 5. Bioreactor data over the entire operational run: A) conductivity, alkalinity, SO_4^{2-}, and $S_2O_3^{2-}$ concentrations and B) selectivity of the system for sulfur, SO_4^{2-}, and $S_2O_3^{2-}$. The initial line (dash-dot) indicates when the start of the high biomass (~ 40 mg-N L^{-1}) experiments began with a theoretical total sulfide concentration of 51.5 mg-S L^{-1}. The second line (dashed) indicates was then low biomass (~20 mg-N L^{-1}) experiments began with a theoretical total sulfide concentration of 30.9 mg-S L^{-1}.

a linear regression analysis was performed on the sulfur selectivity values between the high (N = 10) and low (N = 25) biomass concentrations, where the slope was -0.03 ± 0.02 sulfur selectivity percent per day and -0.0041 ± 0.0064 sulfur selectivity percent per day respectively. For the low biomass experiments, the data shows that the system was more unstable, thus with more variations and a larger standard deviation of 37.6 %, when compared to the high biomass experiments with a standard deviation of 29.3 %. Over the entire period of operation, a few operational upsets were encountered, which caused sudden changes in conductivity and alkalinity, and also led to a high selectivity of (thio)sulfate, and therefore, a low selectivity for sulfur (Fig. 5B).

The change in theoretical total sulfide concentration and the change in biomass concentration over time were also plotted and followed a similar trend to each other (SI 8). As the total nitrogen (in the form of urea) dosed to the system was controlled for each set of experiments, this indicates the interconnectedness between the total sulfide concentration and biomass concentration in the lab-scale system. Here, the theoretical total sulfide concentration in the sulfidic bioreactor ranged from 30.1 mg-S L^{-1} to 99.6 mg-S L^{-1} at high biomass concentrations and from 18 mg-S L^{-1} to 44.6 mg-S L^{-1} at low biomass concentrations. Potentially, this illustrates an optimal range of sulfide concentration in the sulfidic bioreactor in relation to the biomass concentration. Based on this, it is hypothesized that a minimum amount of sulfide per biomass concentration is needed to ensure the formation of enough S_x^{2-} in solution. This could be achieved, to an extent, by modifying the operation of a system to achieve higher sulfide concentrations in the sulfidic bioreactor even at low H_2S loading rates.

3.4. Considerations for enhancement of the sulfidic bioreactor

When determining the application of increased nutrient dosing for full-scale biological desulfurization systems, multiple aspects must be taken into consideration. One of the challenges that exists is that in full-scale systems, the H_2S loading rate and sulfide concentration in the rich solution is driven by the sour gas stream, which in turn determines the design of the lean solution recirculation flow rate and absorber column. In contrast, the biomass concentration is controlled by the nutrient dosing rate. Here, the nutrient dosing rate is then set to maintain an adequate biomass concentration with, preferably, no excess nutrients present in the process solution. In this study, increasing the biomass concentration up to the experimental levels described was found to decrease the amount of (poly)sulfides that entered the micro-oxic bioreactor. This decrease in (poly)sulfide concentration most likely occurred due to the uptake, conversion, and storage of the (poly)sulfides into less easily oxidized forms such as RSS_nR. Due to the conversion and storage, a decrease in the chemical oxidation of S_x^{2-} into $S_2O_3^{2-}$ occurred, which in turn led to a decrease in the biological oxidation of $S_2O_3^{2-}$ into SO_4^{2-}. Thus, an increased biomass concentration in full-scale installations could be used to increase the absolute (poly)sulfide uptake. This, in turn, has the potential to benefit operations by decreasing the usage of caustic due to less byproduct formation and enhance the stability of the process. If an increase in nutrient dosing were to be implemented, the total sulfide concentration should be taken into consideration as to not increase the biomass concentration excessively.

However, increasingly higher biomass concentrations are not always advantageous. First of all, a higher biomass concentration requires more valuable nutrients and oxygen, both of which would increase operational costs potentially the usage of caustic [9,10,42]. Second of all, it was recently proposed that at higher biomass concentrations, the specific reduction degree of the bacteria decreases, which in turn increases the potential for SO_4^{2-} formation [29]. Therefore, at higher biomass concentrations, the ORP set-point of the micro-oxic bioreactor should be decreased to ensure the SOB are kept in a reduced state. This creates a smaller window of operation as the ORP set-point is also a measurement of the sulfide and oxygen concentration in solution, which could lead to operational challenges.

Potentially, a method that could be used to scale up the absolute uptake of (poly)sulfides without increasing the total biomass concentration is the implementation of a new process scheme. Here, a recirculation line from the micro-oxic bioreactor to the sulfidic bioreactor could be utilized to transport oxidized SOB back into reducing conditions in the sulfidic bioreactor. The SOB in this case scenario could be seen as "regenerated" (i.e. have already been exposed to micro-oxic conditions) where they (partially) converted the reduced compounds and are now able take up more (poly)sulfide. This could increase the uptake of (poly)sulfides without the need for increased nutrient dosing. However, more research is needed to evaluate whether this new process scheme could be used to increase the uptake of (poly)sulfides and subsequently the stability of the process.

In conclusion, further research is needed to determine how to best utilize the microbial community to obtain the highest sulfur selectivity. Based on this research, potentially the HRT of the sulfidic bioreactor enhances the uptake, and therefore, more research could be conducted looking into whether sulfidic pressure could also enhance the uptake. Additionally, since the biomass concentration appears to affect the rate at which the S_x^{2-} are forming versus their chemical conversion, more work should be done to look into the rates at which S_x^{2-} form under similar conditions in the biological desulfurization process.

4. Conclusions

In this experimental study, quantifying the increase of biomass concentration versus the decrease of S_x^{2-} concentration was explored for the first time in the continuous dual-reactor biological desulfurization process. SOB in the sulfidic bioreactor were found to contain reactive sulfane, and the "storage" was determined to have a relative limit of 0.1 mg-S mg-N^{-1} before the reactive sulfane is chemically transformed into other sulfur compounds. Increasing the total sulfide concentration in these experiments did not increase this storage of reactive sulfane. When determining whether a higher or lower biomass concentration was beneficial compared to the total sulfide concentration, a higher biomass concentration led to less variation in the process and, ultimately, a higher sulfur selectivity. The higher biomass concentration increased the total uptake of (poly)sulfide as it was found that the relative uptake of (poly)sulfide per unit biomass remained relatively constant over the various sulfide concentrations. Therefore, these results provide insight into the effect of sulfide concentration in the sulfidic bioreactor and show that the total sulfide concentration and biomass concentration must be kept in a balance with each other to take up and convert as much (poly)sulfide as possible. Based on the obtained results, enhancement of the biological desulfurization process could be achieved with higher biomass concentrations, with a potential optimum biomass concentration existing where the (poly)sulfide uptake is maximized, $S_2O_3^{2-}$ and SO_4^{2-} formation are limited, and operational costs are considered.

CRediT authorship contribution statement

Cees J.N. Buisman: Writing – review & editing, Project administration, Conceptualization. **Kestral A. K. Y. Johnston:** Writing – original draft, Visualization, Methodology, Formal analysis. **Annemerel R. Mol:** Writing – review & editing, Supervision, Project administration. **Karel J. Keesman:** Writing – review & editing, Supervision, Funding acquisition. **Celine B. Kugler:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Margo Elzinga:** Writing – review & editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2024.115121.

Data availability

Data will be made available on request.

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