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Original Research

High rate production of concentrated sulfides from metal bearing wastewater in an expanded bed hydrogenotrophic sulfate reducing bioreactor

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ABSTRACT

Metallurgical wastewaters contain high concentrations of sulfate, up to 15 g L^{-1}. Sulfate-reducing bioreactors are employed to treat these wastewaters, reducing sulfates to sulfides which subsequently co-precipitate metals. Sulfate loading and reduction rates are typically restricted by the total H_2S concentration. Sulfide stripping, sulfide precipitation and dilution are the main strategies employed to minimize inhibition by H_2S, but can be adversely compromised by suboptimal sulfate reduction, clogging and additional energy costs. Here, metallurgical wastewater was treated for over 250 days using two hydrogenotrophic granular activated carbon expanded bed bioreactors without additional removal of sulfides. H_2S toxicity was minimized by operating at pH 8 ± 0.15, resulting in an average sulfate removal of 7.08 ± 0.08 g L^{-1}, sulfide concentrations of 2.1 ± 0.2 g L^{-1} and peaks up to 2.3 ± 0.2 g L^{-1}. A sulfate reduction rate of 20.6 ± 0.9 g L^{-1} d^{-1} was achieved, with maxima up to 27.2 g L^{-1} d^{-1}, which is among the highest reported considering a literature review of 39 studies. The rates reported here are 6–8 times higher than those reported for other reactors without active sulfide removal and the only reported for expanded bed sulfate-reducing bioreactors using H_2. By increasing the influent sulfate concentration and maintaining high sulfide concentrations, sulfate reducers were promoted while fermenters and methanogens were suppressed. Industrial wastewater containing 4.4 g L^{-1} sulfate, 0.036 g L^{-1} nitrate and various metals (As, Fe, Tl, Zn, Ni, Sb, Co and Cd) was successfully treated with all metal(loid)s, nitrates and sulfates removed below discharge limits.

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1. Introduction

Sulfate-rich wastewaters originate from various processes such as the food processing industry, tanneries, paper industry and metallurgy. In metallurgy, acid mine drainage, bioleachate, electrowinning- and refining bleeds, sulfuric acid leachates and off-gases from smelters and roasters all result in the production of acid, sulfate-rich wastewaters contaminated with metal(loid)s. Sulfate-reducing bioreactors have been used to treat these wastewaters, neutralizing the acid, reducing sulfates and removing/recovering metal(loid)s [1–4]. Three key parameters for the operation of sulfate-reducing bioreactors are (1) the amount of sulfate reduced, (2) the concentration of sulfides generated, and (3) the sulfate reduction rate [5]. High concentrations of sulfides and a high degree of sulfate removal allow efficient treatment of the wastewater without additional treatment steps. The produced concentrated sulfide stream can be used to remove or recover metal(loid)s, while the residual sulfides can be recovered as elemental sulfur or sulfuric acid [6,7]. High reduction rates permit the use of smaller reactors, lowering capital expenditures [8,9].

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Sulfate-reducing bioreactors are limited by autotoxic H_2S, inherently limiting the ability to reduce/produce high concentrations of sulfate/sulfides [10–14]. Several strategies can be employed to limit this toxic and inhibitory effect [15]. Removal of H_2S via metal sulfide precipitation has been shown to benefit sulfate reduction: In 39 peer-reviewed publications, 33 used some form of sulfide removal, reporting an average sulfate reduction rate of 10 ± 13 g SO_4^{2-} L^{-1} d^{-1}, while publications not employing sulfide abatement strategies reported an average reduction rate of 1 ± 1 g SO_4^{2-} L^{-1} d^{-1} (Fig. S1) [5,16–18]. However, sulfide removal may imply the addition of metals and the production of extra sludge. Additionally, the sulfate-reducing bacteria (SRB) can be inhibited by the metals, and the sludge can clog the system [19–21]. Furthermore, metals will precipitate non-selectively, complicating their recovery. A second option is the removal of sulfides through stripping of H_2S. However, efficient stripping of sulfides requires a pH of 7 or lower and relatively high concentrations of aqueous H_2S, both of which can increase the toxicity of sulfides towards SRB [10]. A third option is the dilution of wastewater prior to reduction. This approach requires higher flow rates, lower hydraulic retention times (HRT), mixing and polluted water [22]. Sulfide oxidation methods have also been employed, but this may compromise the anaerobic conditions of the bioreactor [23,24]. A final option to maximize sulfate reduction and sulfide production consists of maximizing total aqueous sulfides while minimizing aqueous H_2S. This can be accomplished by increasing pH from to 7 to 8, lowering the ratio of H_2S:HS^- from 1:1 to 1:9 [14,15,25–27].

In the absence of organic substrate, H_2 is a desirable electron donor for sulfate reduction [26,28,29]. Complex organic substrates such as molasses are not directly metabolically available, may inhibit microorganisms at high concentrations and may result in the presence of residual organic matter in the effluent [1,2,30]. H_2 has the advantage that it can be produced on-site via electrolysis [5]. However, using H_2 as the electron donor is associated with several challenges. The use of H_2 implies working in an ATEX certified environment and exposure to energy prices (gas in the case of methane reforming, electricity in the case of electrolysis). However, this has not proven a significant obstacle, as hydrogenotrophic sulfate reduction has been proven to be safe and economical at the full scale [5,29]. The low solubility of hydrogen gas makes H_2 transfer a limiting factor, however, and as a result, most H_2 based systems use gas-lift bioreactors (GLBs) [31–33]. However, GLBs have several drawbacks. To ensure satisfactory gas-liquid transfer rates, a sufficient reactor height is required, and H_2 needs to be recirculated, resulting in further costs due to the high back pressure and energy requirements for the ATEX pump [15]. Additionally, when no biological carrier is used, GLBs are sensitive to biomass washout, which is detrimental to the achievable sulfate reduction rate [1,2,34]. Few studies have investigated the use of other hydrogenotrophic sulfate-reducing bioreactors such as membrane bioreactors (MBRs), continuously stirred tank reactors (CSTRs) and packed bed bioreactors (PBRs), all of which employed some form of sulfide removal to maximize sulfate reduction [16,23,35,36]. CSTRs typically require high reactor volumes and are sensitive to biomass washouts, while PBRs and MBRs frequently clog and suffer from high back pressures [5,17].

In this study, we evaluated the operation of two expanded bed bioreactors (EBRs) with granular activated carbon (GAC) as biocarrier for treating sulfate-rich industrial wastewater. EBRs packed with GAC have the advantage of providing a large surface area for biofilms, while avoiding the height required for GLBs or fluidized bed reactors (FBRs), additional energy required for fluidization and biomass loss due to high shear forces [1,2]. Optimal reactor performance (maximum amount of sulfate reduced and sulfide production) was first evaluated by sequentially increasing the sulfate concentration in the influent. The sulfate reduction rate was maximized by sequentially increasing the flow rate in one reactor. To identify the dominant genera and assess whether sulfate levels and flow rates impacted the sulfate reducing population, the microbial community was monitored. As a proof of concept, industrial wastewater originating from urban smelter off-gases and electrowinning bleed was treated for 48 days. Here, the capacity of the hydrogenotrophic EBRs to precipitate metal(loid)s and reduce sulfate and nitrate was assessed. This is an important step as metal(loid)s and other components such as nitrates may affect the operation of the bioreactor due to their toxicity or the clogging of the EBRs. By treating industrial wastewater, the technology is validated as being able to treat complex industrial wastewater.

2. Materials and methods

2.1. Medium and reagent preparation

The synthetic medium was adapted from the protocol described in Van Houten et al. (1994) [37]. The sulfate concentration was increased during the experiment from 3 to 6 and 9 g L^{-1} by adding 4.438 g, 8.875 g and 13.313 g Na_2SO_4 per liter of medium. Trace elements and vitamins were prepared and added according to DSMZ 141, used for methanogenic media [38]. Prior to use, the medium was stored at 4 °C and sparged 30 min with N_2. Industrial wastewater was sampled from a metallurgical refining plant in November 2019 ("raw" wastewater). The raw wastewater was sparged with H_2S prior to application in the bioreactor. Phosphate was added to the industrial wastewater by diluting 0.21 mL of 15.2 mol L^{-1} H_3PO_4 in 10 L of wastewater. The composition of the industrial wastewater is listed in Table 1.

When synthetic wastewater was used, 1 M HCl was used to maintain a pH of 8. In the case of industrial wastewater (pH 1.2) 5 M NaOH was used to maintain a pH of 8 ± 0.15 and CO_2 at a rate of 2.3 mL min^{-1} was used as a carbon source. The electrolyte used for H_2 generation consisted of 0.25 mol L^{-1} Na_2SO_4 diluted in demineralized water. All reagents were supplied by Sigma-Aldrich and analytical grade. 2 L of ultrapure water used for dilution of samples was deaerated with N_2 for 20 min. Sulfide anti-oxidation buffer (SAOB) was prepared fresh as described in Vaiopoulou et al. [39].

2.2. Setup

Two up-flow expanded bed bioreactors were used for hydrogenotrophic sulfate reduction. The setups were constructed according to Fig. 1. The bioreactor consisted of a glass column of 50 mm diameter, 60 cm length, a three-phase separator and closed off with a rubber stopper at the top. The bioreactors were filled with 53 g dry GAC (Carbsorb 40, Calgon Carbon Corporation). A recirculation and upflow of 280 mL h^{-1} was applied, resulting in an expanded bed volume of 250 mL, which is considered as the active biological volume. This resulted in a net hydraulic volume of 0.75 L. Gas bags filled with N_2 were connected to 10 L influent bottles to maintain anaerobic conditions. Inoculum and GAC were collected from heterotrophic industrial scale bioreactors used for the treatment of metallurgical wastewater with a proven presence of sulfate reducers. Sampling ports were present in the bioreactor for sampling of the activated carbon, gas phase and effluent. Watson-Marlow 300 peristaltic pumps were used to pump the influent into the bioreactor and provide liquid circulation. Hydrogen gas was generated via electrolysis using external, current-controlled electrochemical cells [39,40]. The electrolyzers were constructed using 5×20 cm perspex frames, a stainless steel mesh as cathode, and a dimensionally stable iridium mixed metal oxide titanium electrode as an anode. Cation exchange membranes (Membrane

Table 1
Wastewater composition.

Compound	Raw concentration (mg L^{-1})	Stripped concentration (mg L^{-1})
As	2.31	2.17
Cd	0.06	0.01
Co	0.21	0.20
Cu	99.46	0.00
Fe	1353.21	1353.19
Ni	17.71	17.70
Sb	0.39	0.10
Se	1.91	1.90
Tl	0.21	0.20
Zn	0.63	0.62
SO_4^{2-}	4434.21	
NO_3^-	36.31	
Cl^-	3947.02	
Na^+	2509.11	
Ca^{2+}	20.15	
NH_4^+	61.07	
Mg^{2+}	8.61	
K^+	191.26	

Internation, Ultrex CMI-7000) were used in the electrolysis cells to separate cathode and anode. 0.25 mol L^{-1} Na_2SO_4 was used as electrolyte, which was pumped using Watson-Marlow 300 peristaltic pumps. Two 500 mL Scott bottles were used as buffer vessels and gas-liquid separators for the electrolyte. A direct current power supply (Velleman LABPS3005 0–30 V, 0–5 A, Belgium) was used to control the electrochemical cell under a constant current regime.

The produced oxygen gas was vented into the atmosphere and the hydrogen gas was injected into the bioreactor. The current of the electrolysis cells was maintained in order to have a stoichiometric excess of 200% H_2 provided to the reactors at all times. Given the relationship between the current, hydrogen gas production and sulfate reduction (see reaction (1)), the current of the electrolysis cell was controlled as in Table 2 and calculated using the Faraday's constant.

The volumetric flow of gas leaving the system could be measured using a displacement tube filled with 1 M HCl. Outgoing gas was vented outside to the atmosphere after passing a scrubber

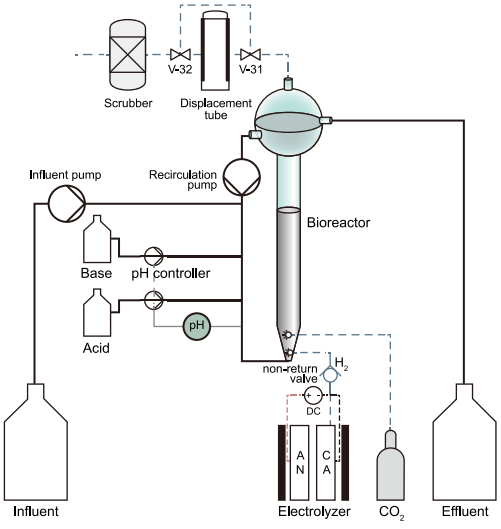

Fig. 1. Up-flow expanded bed bioreactors used during the experiment. Full black lines indicate liquid streams, while dotted blue lined indicate gas flows. An external electrochemical cell was used for hydrogen gas generation, while a pH controller was used to maintain a pH of 8 ± 0.15 via the addition of HCl or NaOH.

containing 32% NaOH (Carl Roth). pH was measured using a sulfide resistant probe (HA405-DXK-S8/120, Mettler-Toledo) connected to a Prominent Dulcometer® for pH control. pH probes were calibrated every week to ensure continued accurate measurement of pH. The temperature was registered every 3 days using an analogue graduated ethanol thermometer. The lab temperature was controlled, and the bioreactor temperature was on average 23 ± 1 °C.

2.3. Experimental procedure

In a first series of tests the concentration of reducible sulfate and the attainable concentration of sulfides were evaluated by increasing the influent concentration of sulfate from 3 to 6 and 9 g L^{-1}. After this series of experiments had been completed, the effect of flow rate and attainable sulfate reduction rate were assessed. This was done by increasing the flow rate of reactor 2 from 20 mL h^{-1} to 40 mL h^{-1} and 60 mL h^{-1}. To provide a proof-of-concept reactor 1 was used to treat industrial wastewater from a metallurgical refining plant (see Table 1).

2.4. Sampling and analyses

Liquid samples were first filtered 0.45 μm syringe filter (Chromafil PVDF, Macherey Nagel) and diluted with deaerated ultrapure water. Samples for anion and sulfide analysis were diluted with deaerated ultrapure water to dilute concentrations below the upper detection limit of the method (100 mg L^{-1} and 3 mg L^{-1}, respectively). Dissolved sulfide (DS) analysis was executed immediately via Nanocolor tests (ref. 985073, Macherey-Nagel) and subsequent spectrophotometric analysis (Filter photometer nanocolor 500 D, Macherey-Nagel). Samples for sulfate analysis were conserved using SAOB. Sulfate was analyzed using a Metrohm 930 Compact Ion Chromatography Flex system (Metrohm, Switzerland). Gas (H_2S, H_2, CH_4, CO_2, N_2) samples were sampled using a syringe and analyzed immediately on a CompactGC (Global Analyser Solutions, Breda, the Netherlands).

Samples of GAC for 16S rRNA gene amplicon sequencing were extracted from the reactor through the sampling ports. Samples were preserved at −20 °C until DNA extraction. DNA extraction was executed as previously reported in De Vrieze et al. [41,41]. In short, frozen samples were bead beaten with a Powerlyzer® 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., Carlsbad, CA, USA), which was followed by a phenol/chloroform extraction. DNA quality was assessed via a 1% (w/v) agarose gel and a polymerase chain reaction (PCR). Bacterial primers 27F (GAGTTTGATCMTGGCTCAG) and 1492R (GGYTACCTTGTTACBACTT) were used for this PCR. 1 μL of the DNA extract was added to the master mix, which consisted of 10 μM forward primer (27F) and reverse primer (1492R), 10x Taq buffer, 25 mM $MgCl_2$, 10 mM dNTP, 5 U μL^{-1} Taq polymerase, 20 mg mL^{-1} BSA and DNA/RNA free water. PCR started with a pre-denaturation, 7 min at 95 °C, followed by 29 cycles of 1 min at 95 °C for denaturation, 56 °C at 1 min for

Table 2
Summary of the applied current for each influent sulfate concentration, influent rate, and the corresponding voltage of the electrochemical cell. The current was controlled in such a manner that H_2 gas was supplied in stoichiometrical excess.

Influent (g SO_4^{2-} L^{-1})	Flow rate (mL h^{-1})	Applied current (mA)	Voltage (V)
3	20	268	2.93 ± 0.05
6	20	536	3.58 ± 0.09
9	20	804	4.0 ± 0.1
9	40	1608	4.6 ± 0.2
9	60	2412	5.0 ± 0.3

annealing and 2 min at 72 °C for extension, at the end a final extension of 10 min at 72 °C was performed. DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany) for sequencing on an Illumina Miseq platform with v3 chemistry with the primers 341F (5′-CCT ACG GGN GGC WGC AG -3′) and 785Rmod (5′-GAC TAC HVG GGT ATC TAA KCC-3′) [42].

Read assembly and clean-up was largely derived from the MiSeq SOP described by the Schloss lab [43,44]. In brief, Mothur v1.44.3 was used to assemble reads into contigs, perform alignment-based quality filtering (alignment to the mothur-reconstructed SILVA SEED alignment, v138), remove chimeras (vsearch v2.13.3), assign taxonomy using a naïve Bayesian classifier and SILVA NR v138 and cluster contigs into OTUs at 97% sequence similarity [45]. All sequences that were classified as *Eukaryota*, *Archaea*, *Chloroplasts* and *Mitochondria* were removed. Also, if sequences could not be classified at all (even at (super)Kingdom level), they were removed. For each OTU, representative sequences were picked as the most abundant sequence within that OTU. Further data trimming and analysis were performed in R (v4.1) using statistical packages like *Phyloseq* (v1.36.0), *vegan* (v2.5.6) and *deseq2* v1.26.0. Further library clean-up was performed based on the suggested analysis in McMurdie and Holmes [46,46]. To assess significant changes in beta-diversity or composition due to treatments and conditions, (PERM)ANOVA and ANOSIM analysis were performed, using a cut-off of (adjusted) p-value < 0.05.

3. Results and discussion

3.1. Maximizing sulfate removal and dissolved sulfide concentrations

In the first operational period, 3 g SO_4^{2-} L^{-1} was provided to both reactors (period 1) at a rate of 20 mL h^{-1} (period I). Sulfate reduction commenced quickly, resulting in residual sulfate concentrations of 0.05 ± 0.03 g SO_4^{2-} L^{-1} and dissolved sulfide concentrations of 0.44 ± 0.04 g DS L^{-1} (reactor 1). Near-complete removal down to 0.2 ± 0.1 g SO_4^{2-} L^{-1} resulted in 0.38 ± 0.01 g DS L^{-1} in reactor 2 after 16 days (Fig. 2). Influent sulfate concentrations were increased from ca. 3 to ca. 6 g SO_4^{2-} L^{-1} (period II), resulting in a temporary increase in effluent sulfate concentrations up to 1.3 ± 0.5 g SO_4^{2-} L^{-1} and 2.5 ± 0.8 g SO_4^{2-} L^{-1} in reactors 1 and 2 respectively, subsequently stabilizing at 0.1 ± 0.1 g SO_4^{2-} L^{-1} and 0.5 ± 0.5 g SO_4^{2-} L^{-1}. At an influent concentration of ca. 6 g SO_4^{2-} L^{-1} dissolved sulfide concentrations in the effluent plateaued at 1.0 ± 0.2 g DS L^{-1} and 0.9 ± 0.1 g DS L^{-1}. After increasing the influent sulfate concentration up to ca. 9 g L^{-1} (period III), the outgoing sulfate concentrations temporarily increased to 4 ± 1 g SO_4^{2-} L^{-1} and 3.7 ± 0.6 g L^{-1}. During this period, an accidental increase in O_2 concentrations due to malfunctioning of the electrochemical cells resulted in a near-complete inhibition of sulfate removal and drop in dissolved sulfide concentrations on days 172 (reactor 1) and 127 (reactor 2). After anaerobic conditions had been re-established, sulfate removal stabilized at levels of 3.3 ± 0.2 g SO_4^{2-} L^{-1} (reactor 1) and 2.3 ± 0.2 g SO_4^{2-} L^{-1} (reactor 2), resulting in a sulfate removal of 5.9 ± 0.4 g SO_4^{2-} L^{-1} and 7.08 ± 0.08 g SO_4^{2-} L^{-1}, respectively. Maximal DS concentrations of 2.3 and 2.1 g DS L^{-1} and average concentrations of 2.1 ± 0.2 g DS L^{-1} and 1.9 ± 0.2 g DS L^{-1} was measured for the two reactors.

A review of current research on sulfate-reducing bioreactors reveals that, to our knowledge, no previous studies have achieved higher dissolved sulfides concentrations than reported here. The highest dissolved sulfide concentrations have been published in Stucki et al., Weijma et al., Maillacheruvu et al. and Van Houten reporting 1.2 g DS L^{-1} (pH 8), 1.15 g DS L^{-1} (pH 7.5), 1 g DS L^{-1} (pH 7) and 0.9 g DS L^{-1} (pH 7), respectively [25,37,47,48]. The concentration of H_2S is cited as one of the key factors limiting the operation of sulfate-reducing bioreactors [10,11,14,49–52]. Lens and his colleagues reported short term concentrations up to 1.6 g DS L^{-1} followed by a drastic drop in performance and concentrations stabilizing at 0.15 g DS L^{-1} [36].

The key role of H_2S also emphasizes the effect of pH, as a higher concentration of sulfides can be tolerated due to a lower proportion of H_2S. While increasing the pH even further will decrease the fraction of H_2S, other inhibitive effects will take over when deviating from physiological conditions. Sulfate reduction will become less energetically favorable, while methanogenesis remains unaffected (see reactions (1) and (2)). The specific activity of sulfate reducers is high at pH between 7 and 8, while specific growth rates are maximized between pH 8 and 9. As a result, an overall pH of 8 is ideal for maximizing growth rates and specific activity [27].

H_2S has been found to inhibit heterotrophic SRB when acetate was provided as an electron donor [14]. Minimizing inhibition by H_2S is consequently one of the key strategies to maximize sulfate reduction. This can be achieved by either stripping, precipitation as metal sulfides, oxidation and/or dilution of the wastewater. However, as described previously, all these sulfide abatement strategies come with several adverse side effects. As an alternative to these sulfide abatement strategies, our results show that the use of a hydrogenotrophic expanded bed bioreactor operated at pH 8 ± 0.15 allows for the reduction of 7.08 ± 0.08 g SO_4^{2-} L^{-1} and production of a concentrated sulfide solution up to 2.1 ± 0.2 g DS L^{-1}, while not suffering from the mentioned drawbacks.

3.2. High sulfate reduction rates without H_2S removal

After increasing the influent concentrations of reactor 2 to 9 g SO_4^{2-} L^{-1}, the flow rate was increased from 20 mL h^{-1} to 40 mL h^{-1} and 60 mL h^{-1} (periods IV and V) to assess the effect of flow rate and maximum reduction rate (see Fig. 3). During period I, II and III, the sulfate reduction rate increased in each period, reaching a maximal reduction rate of 16.6 ± 0.4 g SO_4^{2-} L^{-1} d^{-1} on days 147–161. During period IV, the flow rate was increased to 40 mL h^{-1}. While the amount of sulfate reduced declined from 6.7 ± 0.2 g SO_4^{2-} L^{-1} to 4.7 ± 0.7 g SO_4^{2-} L^{-1}, the reduction rate increased to 20.6 ± 0.9 g SO_4^{2-} L^{-1} d^{-1} as the proportional increase in flow rate was higher than the decrease in the amount of sulfate reduced. However, when increasing the flow rate from 40 to 60 mL h^{-1} (period V) at first, an instantaneous increase in sulfate reduction rate was observed up to 27.2 g SO_4^{2-} L^{-1} d^{-1}. Afterwards, the amount of sulfate reduced decreased more than the proportional increase in flow rate, resulting in a net decrease in reduction rate to a level of 16 ± 2 g SO_4^{2-} L^{-1} d^{-1}. After day 244, the reactor performance became unstable, resulting in fluctuating reactor performance. The instability was attributed to the disturbance of the packing material at the bottom of the reactor. As a result, GAC carrier material was able to enter tubing used for recirculation, resulting in clogging and poor recirculation. As the correct operation of reactor 2 could no longer be guaranteed, it was decided to shut down the reactor on day 275.

High sulfate reduction rates are typically achieved in reactors that employ active sulfide/H_2S removal through stripping, metal sulfide precipitation or oxidation. Accordingly, the removal of H_2S can increase the achieved reduction rates [1,2,10,11]. In a limited literature review of 39 peer-reviewed publications, we established that 33 used some form of sulfide removal (Fig. S1). On average, a sulfate reduction rate of 10 ± 13 g SO_4^{2-} L^{-1} d^{-1} was reported with peaks up to 30 g SO_4^{2-} L^{-1} d^{-1} [53,54]. In contrast, the average achieved reduction rate reported in publications without active sulfide removal was 1 ± 1 g SO_4^{2-} L^{-1} d^{-1} with a reported maximum of 3.1 g SO_4^{2-} L^{-1} d^{-1}. It can also be observed that higher reduction

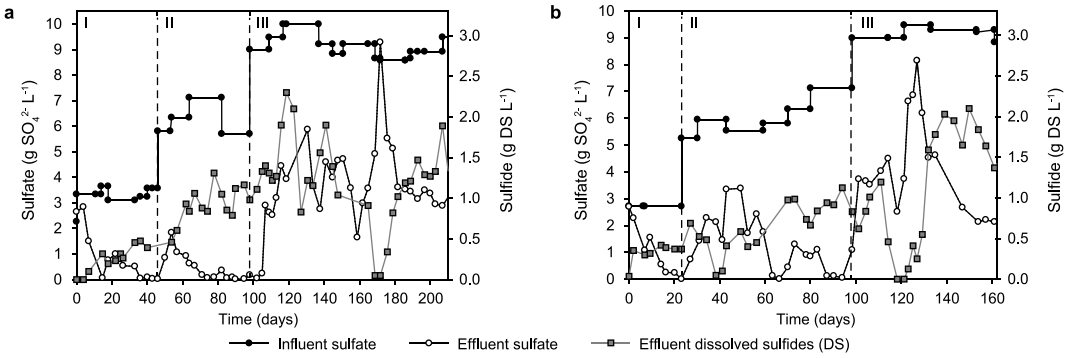

Fig. 2. Sulfate and dissolved sulfide concentrations in the influent and effluent of sulfate-reducing expanded bed bioreactors 1 (**a**) and 2 (**b**) with influent sulfate concentrations increasing from 3 g SO_4^{2-} L^{-1} (period I) to 6 g SO_4^{2-} L^{-1} (period II) and 9 g SO_4^{2-} L^{-1} (period III).

rates are usually achieved when a relatively high bioreactor pH is maintained, which can also be attributed to a low H_2S concentration (Fig. S1). This is in agreement with Visser et al., which has shown that based on the specific growth rates and activity of sulfate reducers that sulfate-reducing bioreactors are ideally operated at pH 8 (see the earlier section "Predominance of sulfate reducers and repression of fermenters and methanogens") [27]. A high sulfate reduction rate was achieved by keeping H_2S concentrations below 0.05 g H_2S L^{-1}. At the reported peak rate of 65 g SO_4^{2-} L^{-1} d^{-1}, the reactor performance became unstable due to accumulating H_2S, resulting in a reactor failure [47].

Overall, the average observed reduction rate of 20.6 ± 0.9 g SO_4^{2-} d^{-1} L^{-1} ranks as the 6th highest sulfate reduction rate reported among 39 publications, while the peak rate of 27.2 g SO_4^{2-} d^{-1} L^{-1} ranks as the 4th best. The rates reported here are 6–8 times higher than those reported for other reactors without active sulfide removal and the only in its kind reported for expanded bed sulfate-reducing bioreactors using H_2 as electron donor. Additionally, due to the alkalizing effect of the sulfate reduction (see (1)), a high sulfate reduction rate will lower the costs associated with maintaining a slightly higher pH of 8, resulting in a feedback loop: high reduction rates allow a higher pH, which allows for higher reduction rates. In any case, the results here show that this type of reactor is able to maintain high sulfide concentrations and achieve sulfate reduction rates equal to or exceeding those now applied in industrial, full scale settings for the treatment of concentrated, high flow rate metallurgical wastewater.

3.3. Predominance of sulfate reducers and repression of fermenters and methanogens

Microbial community analysis showed that during the corresponding periods of similar influent sulfate concentrations, the beta-diversity of the microbial communities did not differ significantly from each other (PERMANOVA: $p_r = 0.335$, ANOSIM: $p = 0.402$), indicating that the bioreactors could be considered as biological replicates and the microbial communities evolved in a similar manner over time and during the conditions applied in periods I-III (Fig. S2, Table S1). When taking a closer look at period III, it appears that the community composition of bioreactor 1 diverges from bioreactor 2 over time (Fig. 4). In bioreactor 1, *Desulfovibrio* remained one of the dominant SRB genera, while in bioreactor 2, *Desulfomicrobium* and *Sulfurospirillum* appear to have become more dominant. The microbial communities of the bioreactors in the 3 g SO_4^{2-} L^{-1} period differed significantly compared to the 6 and 9 g SO_4^{2-} L^{-1} periods (PERMANOVA: $p_r = 0.015$). Relatively, sulfate-reducing genera such as *Desulfovibrio, Desulfomicrobium* and others were more abundantly present (log fold changes up to 15) during periods with increased influent sulfate concentrations, indicating that SRB indeed became more dominant when sulfate concentrations were increased. At the same time, genera associated with fermenting species decreased in relative abundance, implying that fermenters were repressed while sulfate reducers were promoted (Fig. 4, Fig. S3). The high sulfide concentrations achieved in the experiment may result from SRB adapting to higher sulfide concentrations [25]. Sulfate reducers are often more tolerant to higher aqueous H_2S and sulfide concentrations [27,55]. As a result, the high sulfide concentrations generated by sulfate reducers may be self-selecting, i.e. creating an environment in which only sulfide tolerant sulfate reducers can thrive, while less sulfide tolerant organisms are outcompeted. It has been shown that microbial communities can be affected by sulfide removal, and in sulfide-rich conditions, less diverse communities are found [1,2,10,11,56]. However, when looking at the taxonomic alpha diversity, calculated using the Hill numbers, there is neither a clear negative nor positive effect of the dissolved sulfide concentrations on the observed diversity (Fig. S5). Increasing the flow rate did not also significantly affect the microbial community (PERMANOVA: $p_r = 0.069$, Fig. S4).

Heterotrophic organisms such as *Soehngenia* spp. (average relative abundance: 1.7–48%) and *Rikenellaceae* spp. (average relative abundance: 0.2–7%) indicate the presence of carbohydrates in the medium [57,58]. However, as the medium recipe lacked organic substrates, these carbohydrates are most likely the product of homoacetogenic bacteria such as *Acetobacterium* spp., which was present at average relative abundancies varying between 0 and 14.1% [56]. This was confirmed by subsequent experiments. Consequentially, SRB such as *Desulfovibrio* spp. and *Desulfomonas*

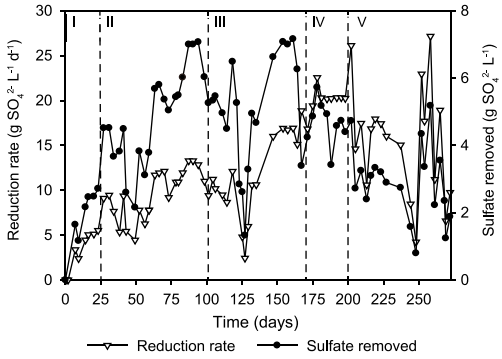

Fig. 3. Sulfate reduction rate and removal of expanded bed bioreactor 2 during period I (3 g SO_4^{2-} L^{-1}, 20 mL h^{-1}), period II (6 g SO_4^{2-} L^{-1}, 20 mL h^{-1}), period III (9 g SO_4^{2-} L^{-1}, 20 mL h^{-1}), period IV (9 g SO_4^{2-} L^{-1}, 40 mL h^{-1}) and period V (9 g SO_4^{2-} L^{-1}, 60 mL h^{-1}.

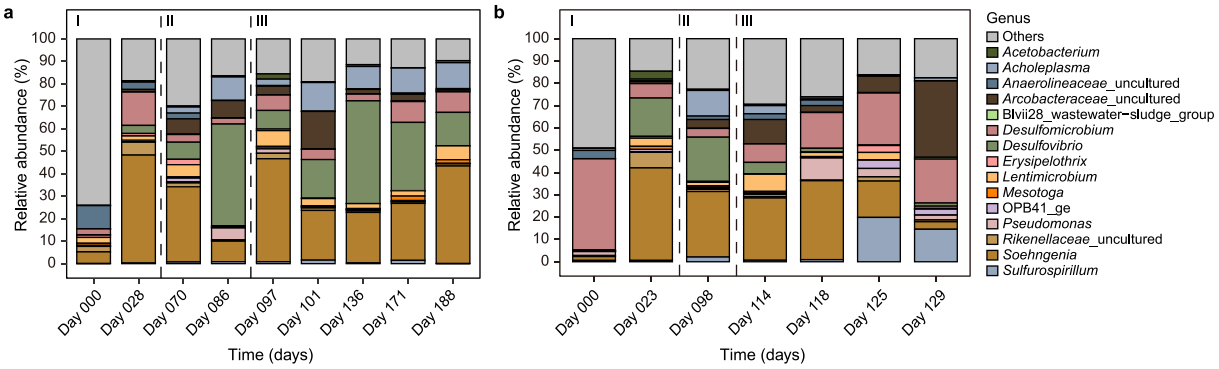

Fig. 4. Relative abundance of the top 15 most abundant genera in samples from bioreactor 1 (**a**) and reactor 2 (**b**) plotted over time with sulfate concentration increasing from 3 g SO_4^{2-} L^{-1} (period I) to 6 g SO_4^{2-} L^{-1} (period II) and 9 g SO_4^{2-} L^{-1} (period III).

spp. may rely on homoacetogens to produce intermediate organic acids as electron donors instead of directly utilizing H_2 and CO_2, as has been observed in other H_2/CO_2 fed syntrophic sulfate-reducing bioreactors [59,60]. Other genera such as *Pseudomonas* were also present at an average relative abundance of 0.01–10.07%. However, *Pseudomonas* are metabolically very diverse, and as a result, it is difficult to determine their functional role [61,62].

Operation at higher pH values has been known to promote methanogenesis, while sulfate reduction becomes energetically less favorable due to the depletion of H^+ (see reactions (1) and (2)) [30,59,60].

(1) $4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$ ($\Delta G^0 = -38.1$ kJ mol^{-1})
(2) $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G^0 = -32.7$ kJ mol^{-1})

In our experiments, methanogenesis was observed up to day 16 at a maximum volumetric CH_4 concentration of 1.16%. No methanogenesis was observed after increasing the influent sulfate concentrations up to 6 g SO_4^{2-} L^{-1}. At sulfide concentrations of ca. 1 g DS L^{-1} methanogenesis appeared to be inhibited, resulting in a selective process [1,2]. Additionally, only a few anaerobes are able to grow on H_2 (or acetate) and when H_2 is used as an electron donor, sulfate reducers should outcompete methanogens [30,63]. High sulfide concentrations also inhibit methanogenesis: at pH 8 ± 0.15, 50% of methanogenic growth and activity is inhibited by concentrations of 0.246 and 0.59 g DS L^{-1}, respectively [27]. Consequently, maintaining high sulfide concentrations may increase the predominance of sulfide-tolerant sulfate-reducing bacteria in the community.

Collectively, these results indicate that the microbial communities in the reactors were able to adapt to these sulfide-rich, high rate conditions, allowing for the successful operation of sulfate-reducing bioreactors at high sulfide concentrations. Additionally, while sulfate reduction is energetically less favorable at high pH, maintaining high sulfide concentrations allows sulfide-tolerant sulfate reducers to outcompete other competing microorganisms such as methanogens or fermenters.

3.4. Proof-of-concept: successful treatment of industrial wastewater

After maximizing the sulfate removal in reactor 1, the reactor was maintained to test the ability of the bioreactor to treat industrial wastewater. Industrial wastewater was sampled from a metallurgical refining plant and pretreated with H_2S to remove copper (see Table 1). On day 323, industrial wastewater was supplied to the reactor at a flow rate of 20 mL h^{-1} and CO_2 was supplied at 2.3 mL min^{-1}. Sulfate was removed from 4.4 g SO_4^{2-} L^{-1} to 0.073 ± 0.044 g SO_4^{2-} L^{-1}, resulting in the production of 0.384 ± 0.097 g DS L^{-1}.

74% of the sulfides were lost through precipitation with metals, as was evidenced by the formation of visible suspended solids and turbid appearance of the bioreactor liquid. Metal(loid)s were nearly completely precipitated, resulting in effluent concentrations of As (173 ± 178 μg L^{-1}), Fe (76 ± 70 μg L^{-1}), Tl (47 ± 8 μg L^{-1}), Zn (11 ± 8 μg L^{-1}), Ni (9 ± 7 μg L^{-1}), Sb (7 ± 10 μg L^{-1}), Co (1.1 ± 0.7 μg L^{-1}), and Cd (0.2 ± 0.6 μg L^{-1}), values below the most stringent discharge limits currently applied in the Flemish region, where this research was performed (Fig. 5). Only selenium was insufficiently removed to concentrations of 0.8 ± 0.5 mg L^{-1}, whereas selenium discharge limits are as low as 0.1 mg L^{-1}. Consequently, additional removal technologies may be required to lower selenium concentrations [64]. Nitrate is known to inhibit sulfate reducers at high concentrations or compete with sulfate as electron acceptor, but 36 mg NO_3^- L^{-1} was removed down to 7 ± 3 mg NO_3^- L^{-1} with concurrent sulfate reduction [65–67]. This is most likely due to the comparatively small concentration of nitrate and the surplus of electron donors. Despite the formation of suspended solids and the utilization of GAC as biocarrier, no clogging occurred, and metal sulfide precipitates did not adversely affect the operation of the bioreactor. Due to the upflow and expanded bed of the bioreactor, precipitates were able to pass through the bed without resulting in clogging and reactor failure. Metals precipitated in the bioreactor can therefore be recovered from the effluent of the bioreactor through the use of conventional solid liquid separation techniques such as decanters and settlers. During the treatment of industrial metallurgical wastewater 5 ± 1 mmol NaOH h^{-1} was consumed to maintain a stable pH of 8.

4. Conclusion

This research showed the first effective use of an upflow expanded bed hydrogenotrophic bioreactor for treating metallurgical wastewaters at high rates in the presence of high sulfide concentrations. The reactors operated under extreme sulfidic conditions reducing 7.08 ± 0.08 g SO_4^{2-} L^{-1} to 2.3 ± 0.2 g SO_4^{2-} L^{-1}, resulting in the production of 2.1 ± 0.2 g DS L^{-1}. Despite high sulfide concentrations and lack of active sulfide removal, reduction rates up to 20.6 ± 0.9 g SO_4^{2-} d^{-1} L^{-1} and 27.2 g SO_4^{2-} d^{-1} L^{-1} were achieved. By maintaining high sulfide concentrations and increasing influent sulfate concentrations, sulfate reducers became dominant while fermenters and methanogens appeared to be repressed. Furthermore, the reactor treated industrial metallurgical wastewater and removed almost all metalloids (excl. Se), sulfate and

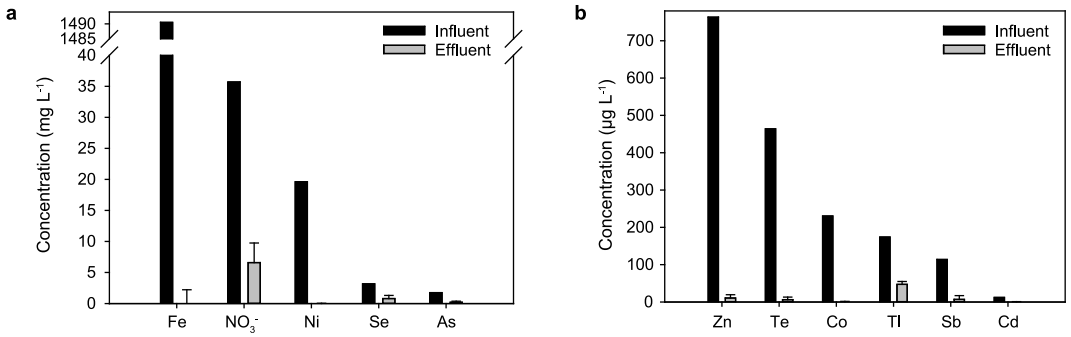

Fig. 5. The concentrations of Fe, NO_3^-, Ni, Se and As (in mg L^{-1}) (**a**) and of Zn, Te, Cp, Tl, Sb and Cd (in µg L^{-1}) (**b**) in the influent and effluent of the bioreactor when treating industrial metallurgical wastewater.

nitrate below the most stringent discharge limit currently applied in Flanders (Belgium).

By employing this new type of upflow expanded bed, hydrogenotrophic bioreactors without active sulfide removal, high rates can be achieved with minimal risk of clogging, issues with gas backpressure and biomass washout. Focusing on the core function of the bioreactor, i.e., maximal sulfate reduction, without additionally removing the produced sulfides allows the production of a concentrated sulfide stream, which can be subsequently treated or utilized under optimal conditions by either stripping, oxidation or mixing it with a metal(loid) bearing stream, separate from the bioreactor.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2022.100173.

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