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Biological treatment of selenium-laden wastewater containing nitrate and sulfate in an upflow anaerobic sludge bed reactor at pH 5.0

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GRAPHICAL ABSTRACT

ABSTRACT

This study investigated the removal of selenate (SeO_4^{2-}), sulfate (SO_4^{2-}) and nitrate (NO_3^-) at different influent pH values ranging from 7.0 to 5.0 and 20 °C in an upflow anaerobic sludge blanket (UASB) reactor using lactate as an electron donor. At pH 5.0, the UASB reactor showed a 20–30% decrease in reactor performance compared to operation at pH 5.5 to 7.0, reaching removal efficiencies of 79%, 15%, 43% and 61% for NO_3^- , SO_4^{2-} , Se_{total} and Se_{diss} , respectively. However, the reactor stability was an issue upon lowering the pH to 5.0 and further experiments are recommended. The sludge formed during low pH operation had a fluffy, floe-like appearance with filamentous structure, possibly due to the low poly-saccharide (PS) to protein (PN) ratio (0.01 PS/PN) in the soluble extracellular polymeric substances (EPS) matrix of the biomass. Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) analysis of the sludge confirmed Se oxyanion reduction and deposition of Se^0 particles inside the biomass. Microbial community analysis using Illumina MiSeq sequencing revealed that the families of *Campylobacteraceae* and *Desulfomicrobiaceae* were the dominant phylotypes throughout the reactor operation at approximately 23% and 10% relative abundance, respectively. Furthermore, approximately 10% relative abundance of both *Geobacteraceae* and *Spirochaetaceae* was observed in the granular sludge during the pH 5.0 operation. Overall, this study demonstrated the feasibility of UASB operation at pH values ranging from 7.0 to 5.0 for removing Se and other oxyanions from wastewaters.

1. Introduction

Selenium (Se) is both an essential and toxic trace element for living cells, with only 5- to 10-fold difference between essential and toxic concentrations (Nanchaiaiah and Lens, 2015). Se accumulation and intoxication can cause mutations, reproductive failures and teratogenic effects, particularly with aquatic egg-laying organisms upon exposure to toxic Se levels (Lemly, 2014). As such, it is now widely recognized that removal of Se from contaminated waters is essential for the protection of animal, human and ecosystem health (Tan et al., 2016).

Compared to physical and chemical technologies, biological treatment has emerged in recent years as a promising Se removal technology due to the possibility of simultaneous treatment of various oxyanions as well as yielding elemental Se (Se^0) nanoparticles that can potentially be recovered and reused (Tan et al., 2016). Despite these recent advances, there are still many challenges in applying this bioprocess to real wastewaters. One such challenge is the low pH of some Se-laden wastewaters, e.g. acid mine drainage (AMD) wastewater. AMD wastewater is characterized by low pH values (varying between pH 2 to 5) and varying heavy metal concentrations depending on the mining site (Koschorreck and Tittel, 2007). Se speciation depends on pH, ionic strength and redox properties of a system (Erosa et al., 2009). Adjustment of the initial pH entails additional costs when the process is transferred into full-scale applications treating real wastewaters. Espinosa-Ortiz et al. (2015) reported selenite (SeO_3^{2-}) removal from pH 4.5 medium using *Phanerochaete chrysosporium* fungal pellets and achieved a total soluble selenium (SeO_3^{2-}) removal efficiency of 70%. However, the fungal pellets were incapable of reducing and removing selenate (SeO_4^{2-}).

Table 1 gives an overview of bioreactor studies related to the treatment of Se-laden wastewaters. It shows that there are very limited studies investigating the effect of pH on Se removal. The majority of the studies, as summarized in Table 1, adjusted the initial pH to a circum-neutral pH during laboratory testing or used a buffer medium before performing Se bioremoval to avoid large pH

fluctuations and associated impact on the biological system. Therefore, bioprocesses capable of Se removal from wastewaters with low pH without prior neutralization need to be developed.

This study investigated the effect of decreasing influent pH (from 7.0 to pH 5.0) on the performance of a continuously operated upflow anaerobic sludge blanket (UASB) reactor (referred to as low pH reactor) treating SeO_4^{2-} , SO_4^{2-} and NO_3^- using anaerobic granular sludge at room temperature. The aim of the study was to observe the lowest possible influent pH at which the UASB can be operated for the removal of the targeted oxyanions. The protein, polysaccharide, and Se content of the UASB granules were determined at various time points and compared to the inoculum and granular sludge from a control UASB reactor operated at pH 7.0. Low pH reactor biomass morphology was also analyzed at the end of the reactor run and compared to inoculum and control reactor. The microbial community composition of the inoculum and sludge sampled from the low pH reactor was analyzed and related to low pH UASB reactor performance and operational strategies.

2. Materials and methods

2.1. Source of biomass

Experiments were performed with anaerobic granular sludge taken from a full-scale UASB reactor treating paper-mill wastewater in Eerbeek (The Netherlands). Biomass was supplied at 10 g (wet weight) to 100 mL medium as described in Lenz et al. (2008). No additional biomass was added during the entire reactor run. Prior to each operational change (see below), a small portion of the biomass was withdrawn from the UASB reactor for analysis of microbial community, protein and polysaccharide content.

2.2. Synthetic wastewater

All experiments were carried out using synthetic mining wastewater containing (in g L^{-1}): NH_4Cl (0.30), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01), and NaHCO_3 (0.04). Acid and alkaline trace

Table 1
Comparison of studies on Se removal in different bioreactors in the presence of co-oxyanions.

Reactor type	Se_{inf}	Other Ions	Inoculum	Operational Conditions	Removal Efficiency	References
H ₂ -MBfR	0.55 mg SeO_3^{2-} -Se L^{-1}	5 mg NO_3^- -N L^{-1} 79 mg SO_4^{2-} L^{-1}	Biofilm from pilot plant treating groundwater	pH 7.0–9.0; H ₂ gas	90% Se; 100% NO_3^- ; 75% SO_4^{2-}	Chung et al. (2006)
MBfR	0.55 mg SeO_4^{2-} -Se L^{-1}	0–10 mg NO_3^- -N L^{-1}	Denitrifying anaerobic methane oxidation (DAMO) culture	pH 7.0; 30 °C; CH ₄ gas	94% SeO_4^{2-} (without NO_3^-); 60–75% SeO_4^{2-} ; 75% NO_3^-	Lai et al. (2016)
H ₂ -MBfR	0.55–6.6 mg SeO_4^{2-} -Se L^{-1}	40–90 mg SO_4^{2-} L^{-1}	Anoxic sludge from wastewater treatment plant	pH 7.0–9.0; HRT 3.3 h; 20 °C; H ₂ gas	>90% SeO_4^{2-} ; 25% SO_4^{2-}	Ontiveros-Valencia et al. (2016)
Upflow fungal pelleted reactor	0.79 mg SeO_3^{2-} -Se L^{-1}	No co-contaminants	<i>Phanerochaete chrysosporium</i>	pH 4.5; HRT 24 h; 30 °C; Glucose	70% SeO_3^{2-}	Espinosa-Ortiz et al. (2015)
UASB	0.79 mg SeO_4^{2-} -Se L^{-1}	<2500 mg SO_4^{2-} L^{-1}	Eerbeek anaerobic granular sludge	pH 7.0; HRT 6 h; 30 °C; lactate	99% Se (methanogenic condition); 97% (SO_4^{2-} -reducing condition); 20–90% SO_4^{2-}	Lenz et al. (2008)
UASB	0.79 mg SeO_4^{2-} -Se L^{-1}	930 mg NO_3^- -N L^{-1} 192 mg SO_4^{2-} L^{-1}	Eerbeek anaerobic granular sludge with immobilized <i>Sulfurospirillum barnesii</i>	pH 7.0; HRT 6 h; 30 °C; lactate	97% Se; 100% NO_3^- ; 90% SO_4^{2-} (after NO_3^- had been removed)	Lenz et al. (2009)
UASB	13 mg Se L^{-1}	1–10 g L^{-1} heavy metals 35.6 g L^{-1}	Anaerobic sludge from mesophilic UASB reactor treating beverage wastewater	pH < 1.0 adjusted to 6.0–9.0; HRT 24 h; 30 °C; ethanol	97% Se_{total}	Soda et al. (2011)
UASB	5–12 mg SeO_4^{2-} -Se L^{-1}	47 mg NO_3^- -N L^{-1} 1500 mg SO_4^{2-} L^{-1}	Eerbeek anaerobic granular sludge	pH 5.0–7.0; HRT 24 h; 20 °C; lactate	43% Se_{total} ; 61% Se_{diss} ; 85% SeO_3^{2-} ; 92% NO_3^- ; 15% SO_4^{2-}	This study

*H₂-MBfR – hydrogen-based membrane biofilm reactors; UASB – upflow anaerobic sludge bed.

metal solutions (0.1 mL each) were added to 1 L of synthetic wastewater (composition of trace metal solutions is described in [Stams et al., 1992](#)). No phosphate or pH buffer was included in the medium and the pH was adjusted to the desired pH using 0.5 M HCl. Analytical grade sodium lactate was used as the electron donor and potassium nitrate, potassium sulfate and sodium selenate were added as electron acceptors. Their concentrations depended on the operational period, as described below.

2.3. UASB reactor

2.3.1. Low pH bioreactor configuration and operating conditions

The low pH UASB reactor had a working volume of 2.0 L, an inner diameter of 5.6 cm and a length of 1 m ([Fig. S1](#)). It was operated at room temperature ($20 \pm 2^\circ\text{C}$) at an average hydraulic residence time (HRT) of 24 h. The influent flow rate was set at 1.4 mL min^{-1} and the upflow velocity at 1.5 m h^{-1} with a recirculation flow of 62 mL min^{-1} . The organic loading rate was $2\text{ g COD L}^{-1}\text{ d}^{-1}$ using lactate as the electron donor.

The low pH UASB reactor was provided with excess electron donor and exposed to three oxyanions, i.e. SeO_4^{2-} , NO_3^- and SO_4^{2-} , for the entire duration of the study. An average of $48 (\pm 5)\text{ mg NO}_3^- \text{N L}^{-1}$ and $2 (\pm 0.5)\text{ g SO}_4^{2-} \text{L}^{-1}$ were fed into the low pH reactor for the entire UASB reactor operation. The low pH UASB reactor was operated in three different operational periods (Phase I to III). The first phase of the low pH reactor operation was divided into 5 parts. Phase Ia used approximately 10 mg Se L^{-1} as SeO_4^{2-} , $42\text{ mg NO}_3^- \text{N L}^{-1}$ and $2\text{ g SO}_4^{2-} \text{L}^{-1}$ at pH 7.0 and was considered the start-up phase, lasting for 29 days. Subsequent phases were maintained long enough to reach a pseudo-steady-state. The influent pH was gradually decreased from pH 7.0 (phase Ib, day 30–61) to pH 6.0 (phase Ic, day 62–90), to pH 5.5 (phase Id, day 91–129) and finally to pH 5.0 (phase Ie, day 130–168). Each phase was operated for approximately 32 days before the pH was changed. After phase Ie, the incoming feed pH was maintained at pH 5.0 and the removal efficiency stability of the bioreactor was assessed by changing the SeO_4^{2-} concentration from $\sim 12\text{ mg Se L}^{-1}$ (phase I) to a range of $1\text{--}5\text{ mg Se L}^{-1}$ (phase IIa, day 169–202) and then back to $\sim 12\text{ mg Se L}^{-1}$ (phase IIb, day 203–231). Finally, the effect of SeO_3^{2-} addition at pH 5.0 was investigated during phase III (day 232–266), where the total Se concentration ($\sim 10\text{ mg Se L}^{-1}$) in the influent consisted of 50% SeO_3^{2-} and 50% SeO_4^{2-} . The average influent Se concentrations (as SeO_4^{2-}) during phase I was $12.0 (\pm 0.8)\text{ mg Se L}^{-1}\text{ d}^{-1}$, $3.0 (\pm 0.8)\text{ mg Se L}^{-1}\text{ d}^{-1}$ (phase IIa) and $12.0 (\pm 0.8)\text{ mg Se L}^{-1}\text{ d}^{-1}$ during phase IIb. In phase III, the concentration of total Se was split into 50% SeO_4^{2-} and 50% SeO_3^{2-} in mg Se L^{-1} at a concentration of $5.0 (\pm 1.0)\text{ mg Se L}^{-1}$ and $4.0 (\pm 0.5)\text{ mg Se L}^{-1}$, respectively. Pseudo-steady state was considered for each phase once there were seven consecutive data points observed within $\pm 10\%$ relative error to each other.

2.3.2. Control reactor set-up

A control reactor was operated alongside the low pH reactor with an influent stream pH of 7.0. The control reactor set-up was operated similarly to phases Ia and Ib of the low pH reactor with phase I of the control reactor (pH 7.0) which only lasted for 92 days. The sludge characteristics of the control reactor (i.e. polysaccharide/protein ratio and biomass morphology) were reported and compared with sludge characteristics of the low pH reactor.

2.3.3. Analytical methods

Liquid samples were collected and centrifuged at $37,000\text{ g}$ for 15 min to remove suspended cells and Se^0 particles ([Jain et al., 2015](#)) before the supernatant was used for analysis. Chemical oxygen demand (COD), total dissolved sulfide (TDS), and total solids

(TS) measurements were carried out using standard methods ([APHA, 2005](#)). Ion chromatography (IC, Dionex ICS 1000 AS4A column) was used to measure the concentration of NO_3^- and SO_4^{2-} at retention times of 3.9 and 7.5 min, respectively. Due to SO_4^{2-} peak interference with the SeO_4^{2-} peak, SeO_4^{2-} was not determined by IC. Instead, all influent and effluent Se concentrations were analyzed as Se using a graphite furnace atomic absorption spectrometer (GFAAS, ThermoElemental Solar MQZe GF95, Se lamp at 196.0 nm) and reported as total Se (Se_{total}) and dissolved Se (Se_{diss}). Se_{total} includes both dissolved Se and colloidal Se present in the collected liquid sample, while Se_{diss} includes only dissolved Se remaining in the effluent after centrifugation ($37,000\text{ g}$) and filtration ($0.45\text{ }\mu\text{m}$, acetate membrane, Sigma-Aldrich, USA). The SeO_3^{2-} concentration in the reactor was measured using a spectrophotometric method as described by [Li et al. \(2014\)](#).

2.3.4. Statistical analysis

Averages and standard deviations were calculated for the oxyanion removal efficiencies. Statistical differences in lactate, NO_3^- , SO_4^{2-} , Se_{total} and Se_{diss} removal efficiencies between the low pH reactor operation phases were evaluated using analysis of variance (ANOVA) with Tukey tests, where a $P\text{-value} \leq 0.05$ was considered statistically significant. Minitab 17 Statistical (Minitab Inc., US) and OriginPro 9.0 (OriginLab Corporation, US) software were used for statistical analysis and for presenting results in the form of graphs, respectively.

2.4. Anaerobic granular sludge characterization

2.4.1. Microbial community analysis

Granular sludge samples from the UASB reactor at the end of each period and from the inoculum were analyzed for microbial community composition. Prior to sampling, vigorous biomass mixing within the reactor was performed by briefly increasing the upflow velocity. Genomic DNA was extracted from the samples following the protocol of [Lueders et al. \(2004\)](#). Extracted DNA was quantified using a NanoDrop-1000 spectrophotometer and amplified by PCR using the primers Pro 341 forward and Pro 805 reverse targeting the V4 region of the 16S rRNA gene of bacteria and archaea ([Takahashi et al., 2014](#)). Amplicons were checked by agarose gel electrophoresis and sequenced using the Illumina MiSeq sequencing platform following the standard protocol. Sequences produced were analyzed using the standard operating procedure of the bioinformatics platform Mothur ([Schloss et al., 2009](#)).

2.4.2. Protein, polysaccharide and selenium content

On days 168 (end of phase Ie) and 266 (end of phase III), granular sludge was taken from low pH reactor for analysis of protein, polysaccharide and Se content. Liquid suspension with biomass (100 mL) was taken and the granular sludge was homogenized using a glass potter homogenizer. Protein and polysaccharide concentrations were determined after extracting soluble extracellular polymeric substances (EPS) following the protocol of [Geyik and Çeçen \(2014\)](#). Briefly, a known weight of sludge was homogenized using a potter homogenizer and centrifuged at $12,000\text{ g}$ for 10 min. The supernatant was considered the 1st soluble EPS fraction. The pellet was re-suspended in PBS and centrifuged again at $12,000\text{ g}$ for 10 min. This supernatant was taken as the 2nd soluble EPS fraction. The two EPS fractions were pooled, filtered using a $0.45\text{ }\mu\text{m}$ membrane and stored at -20°C until analysis.

Analyses of protein and polysaccharide content were carried out using the Pierce™ BSA protein assay kit (ThermoScientific) and phenol-sulfuric acid method ([Albalasmeh et al., 2013](#)), respectively. The total Se in the granular sludge was measured after acid

digestion with 65% HNO₃ (MARS 5 pKo Temp CEM Microwave) using GFAAS.

The morphology of granular sludge from the inoculum, control reactor and low pH reactor was determined using stereo-microscopy and scanning electron microscopy (SEM, Zeiss Supra™ 55VP) equipped with energy dispersive X-ray spectroscopy (EDX, Princeton Gamma-Tech). EDX was used for detection of Se and S on the surface of the granular sludge. For SEM-EDX analysis, the sludge samples were prepared by gentle rinsing with Milli-Q water (18 MΩ cm⁻¹) and leaving them to dry on a dish at ambient temperature. Dried samples were deposited on carbon tape and coated with iridium before imaging.

3. Results

3.1. Low pH reactor performance at decreasing pH

The low pH reactor performance in terms of COD, NO₃⁻, SO₄²⁻ and Se removal efficiencies is shown in Fig. 1, while the averaged effluent concentrations are reported in Table S1. Statistical groupings of the different phases during the low pH reactor operation were developed for comparison of the removal performances (Table S2). Phases that did not exhibit statistically significantly different removal efficiencies were grouped together. The pH of the feed was gradually lowered from 7.0 to 5.0 to avoid a sudden pH shock on the biomass. Although the influent pH was lowered from 7.0 to 5.0, the effluent pH values were always between pH 7 and 8, with an average pH of 7.4 (±0.3).

The low pH reactor demonstrated relatively stable COD, NO₃⁻ and Se removal for influent pH values between 7 and 5.5. The COD removal efficiency was, on average, 74 (±3)% from start-up until the end of phase Id. However, the COD removal efficiency diminished to 58 (±9)% when the influent pH was decreased to 5.0 in phase Ie (*P*-value ≤ 0.0001). The NO₃⁻ removal efficiency during phase Ia to Ic (influent pH 7.0 to 6.0) was on average 97 (±2)% and decreased slightly to 90 (±3)% during phases Id and Ie (influent pH 5.5 and 5.0). Overall, NO₃⁻ removal efficiencies were not statistically significantly different during phases Ia through Ie at an average of 94 (±5)% (0.30 ≤ *P*-value ≤ 0.52). SO₄²⁻ removal, on the other hand, increased from 14 (±9)% in phase Ia to an average of 42 (±8)% during phases Ib-Ic (influent pH 7.0 to 5.5, *P*-value ≤ 0.0001), but decreased again to 14 (±9)% in phase Ie (influent pH 5.0, *P*-value ≤ 0.0001). The sulfide concentrations in the effluent were initially at 70 (±9) mg TDS L⁻¹, increased to 359 (±137) mg TDS L⁻¹ during phases Ib-Ic and then exhibited a sudden decrease to 83 (±34) mg TDS L⁻¹.

Se_{total} removal followed a similar trend from phase Ib until halfway through phase Id with high removal efficiencies of 91 (±6)%, 91 (±5)%, 84 (±6)%, 78 (±14)% Se_{total}, for phases Ia-d, respectively. Se_{total} removal efficiencies were not statistically significantly different during phases Ia to Id (0.45 ≤ *P*-value ≤ 1.0) at an average of 88 (±7)% but decreased to 43 (±13)% for phase Ie (*P*-value ≤ 0.003). Se_{diss} removal efficiencies exhibited a similar trend to the Se_{total} removal efficiencies, showing good removal at 95 (±3)% from start-up to halfway into phase Id. During phase Id (day 113–121), the reactor continuous operation had to be stopped for approximately one week due to maintenance needs such as replacement of tubing. Mixing was still maintained by providing an internal recirculation (from the biomass sampling port to the effluent port, Fig. S1) to maintain biomass activity. Due to the internal circulation arrangement, only a small portion of the sludge was not properly mixed during maintenance. This could have caused the temporary decrease in reactor performance during the end of phase Id which appeared to have recovered within a few

days. During phase Ie (operated at pH 5.0), the removal efficiency of Se_{diss} decreased to 61 (±17)%. The activity test of the biomass taken during phase Ie (Fig. S2) indicated that the biomass still had the potential to be highly metabolically active.

Removal performance results for the control reactor over 92 days are shown in Fig. S3 and were found to not be statistically different from the low pH reactor (Table S2) for COD, NO₃⁻, SO₄²⁻ and Se removal during phases Ia to Id.

3.2. Low pH reactor performance with changing Se concentration

The COD removal efficiency decreased from 58 (±9)% from phase Ie to an average of 43 (±5)% during phase II and finally to 20 (±9)% when SeO₃²⁻ was added to the low pH reactor (phase III, *P*-value ≤ 0.0001). Similarly, the low pH reactor showed an overall 20% decrease in NO₃⁻ removal efficiency after phase Ie (*P*-value ≤ 0.01), while the SO₄²⁻ removal performance was fairly consistent at an average of 13 (±4)%, similar to phase Ie (0.52 ≤ *P*-value ≤ 1.0). Sulfide concentrations in the effluent consistently decreased during each phase, reaching a concentration of 8 (±6) mg TDS L⁻¹ at the end of the reactor run. Phase II and phase III attained a Se removal performance comparable to the one in phase Ie (0.13 ≤ *P*-value ≤ 0.95), calculating an average Se_{total} and Se_{diss} removal efficiency of 40 (±18)% and 60 (±14)%, respectively. A SeO₃²⁻ removal efficiency of 85 (±6)% was achieved during phase III.

3.3. Granular sludge characterization

During the beginning of phase Ie, floating biomass was frequently observed in the low pH reactor as depicted in Fig. S4a, while no floating biomass was observed in the control reactor (Fig. S4b). However, starting with phase IIb until the end of the low pH reactor run, floating biomass was rarely observed. The sludge from the control reactor retained its granular shape with red particles deposited on the surface (Fig. 2 b1). In contrast, the biomass from the low pH reactor became fluffier with yellowish/reddish particles deposited on the surface (Fig. 2 c1).

The total Se concentration in the low pH reactor biomass at the end of phase Ie (day 168) was lower than in the control reactor biomass (Table 2). However, the Se concentrations increased by the end of phase III (day 266), indicating that the biomass was still capable of accumulating additional Se. SEM images (Fig. 2 b2 and c2) and EDX spectra (Fig. 2 b3 and c3) indicated that the biomass from both reactors formed Se⁰ within the granules. Spherical as well as a few rod-shaped Se⁰ structures were observed within the low pH reactor biomass, similar to Se⁰ spheres observed in other studies at neutral pH (Jain et al., 2015; Oremland et al., 2004).

The soluble EPS protein content from the low pH reactor biomass at the end of phase Ie (Table 2) was significantly higher at 432 (±17) mg protein g TS⁻¹ (*P*-value ≤ 0.001) compared to the inoculum and control reactor at 153 (±2) and 80 (±4) mg protein g TS⁻¹, respectively. In contrast, the soluble EPS polysaccharide concentration of the low pH reactor biomass during phase Ie was the lowest at 6 (±2) mg polysaccharide g TS⁻¹ (*P*-value ≤ 0.009). At the end of phase III (day 266), the soluble EPS protein content of the low pH reactor biomass had decreased considerably (*P*-value ≤ 0.0001), while the polysaccharide concentration had increased (*P*-value ≤ 0.002) as shown in Table 2.

3.4. Microbial community analysis

The microbial composition of the inoculum and with each change in reactor operation are reported as prokaryotic relative abundance at the family-level (Fig. 3). The inoculum was

Fig. 1. Removal efficiencies (%) of the UASB reactor operated for 266 days: COD (black square), NO_3^- (green square), SO_4^{2-} (brown triangle), total Se (red diamond), dissolved Se (blue asterisk) and SeO_3^{2-} (yellow star). Effluent pH (orange line) was measured along with dissolved sulfide (blue & white inverted triangle). *Continuous operation was stopped to conduct maintenance (tubing and connector replacement) but internal recirculation for the reactor was maintained to minimize biomass activity loss. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dominated by archaea such as the *Methanosaetaceae* (19.1%) and *Methanobacteriaceae* (18.3%); the most abundant bacterial groups were *Anaerolineaceae* (11.4%) and *Syntrophobacteraceae* (8.7%). A major change in microbial community was observed between the inoculum and the low pH reactor biomass samples due to a change of the relative archaeal abundance from 39% to 5%. A shift in bacterial diversity was noticed within the low pH reactor biomass samples, but to a lesser extent than between the inoculum and low pH reactor samples.

The proteobacterial families *Campylobacteraceae* and *Desulfomicrobiaceae* were enriched in the reactor relative to the inoculum and changed from non-detectable to relative abundances of 34.4% and 20.2%, respectively. The *Synergistaceae* and *Desulfovibrionaceae* increased from 6.9% and 3.4% (phase Id) to an average of 13 (± 4)% and 8 (± 2)%, respectively, from phase Ie to III. *Spirochaetaceae* and *Geobacteraceae* started appearing during phase Ie (at pH 5.0) and their abundance increased until the end of phase III (Fig. 3).

4. Discussion

4.1. Selenium bioremoval at low pH

This study, to our knowledge, is the first to investigate Se removal performance in a laboratory-scale UASB reactor treating a wastewater close to a real acid mine drainage: influent pH values as low as pH 5, the presence of co-electron acceptors (i.e. NO_3^- and SO_4^{2-}) and ambient temperature (20 °C). The Se removal efficiencies obtained at an influent pH value of 5.0 showed a trend of approximately 20–30% decrease in reactor performance reaching 43 (± 13)% Se_{total} and 61 (± 17)% Se_{diss} removal efficiencies compared to operation at pH 5.5 to 7.0. However, it was noted that the reactor stability was an issue after phase Ie. Though there was an observed trend of 20–30% decrease in performance due to the instability of the reactor, further experiments are required to establish the long-term reactor performance at low pH operation for Se removal in the

Fig. 2. Morphology of granular sludge samples taken from (a) inoculum, (b) control-reactor and (c) low-pH-reactor after 266 days of continuous operation, assessed using a (1) stereomicroscope and (2) SEM with full frame and spot-based elemental analysis at 20 kV; (3) representative EDX spectra of selected region.

Table 2

Protein, polysaccharide and Se concentrations in the inoculum, control reactor and low pH reactor granular sludge. Soluble EPS was extracted from the granular sludge prior to quantification of protein and polysaccharides while Se content in the biomass was determined after combined acid-heat digestion.

Granular sludge	Inoculum	Control reactor (pH 7.0)	Low pH reactor (pH 5.0)	
Conditions	Untreated (day 0)	$\text{NO}_3^- + \text{SO}_4^{2-} + \text{SeO}_4^{2-}$ (day 92)	$\text{NO}_3^- + \text{SO}_4^{2-} + \text{SeO}_4^{2-}$ (day 168, phase Ie)	Addition of 50% SeO_3^{2-} (day 266, phase III)
Protein (mg g TS^{-1})	153.00 (± 2.00)	80.00 (± 4.00)	432.00 (± 17.00)	128 (± 6.00)
Polysaccharide (mg g TS^{-1})	31.00 (± 2.00)	17.00 (± 2.00)	6.00 (± 2.00)	52 (± 19.00)
Polysaccharide/Protein (PS/PN)	0.20 (± 0.06)	0.20 (± 0.06)	0.01 (± 0.01)	0.4 (± 0.20)
Total Se in biomass (mg Se g TS^{-1})	0.00	45 (± 6.00)	12 (± 1.00)	86 (± 12.00)

presence of co-electron acceptors.

It should be noted that other studies pre-conditioned the system for optimal performance at neutral pH and mesophilic conditions. Dessì et al. (2016) showed that the Se removal efficiency and Se retention within the biomass were greater at higher temperatures (55 °C), presumably due to the increase in crystallinity size, change in particle shape and decrease in zeta-potential of the biogenic Se^0 , allowing for better settleability and lowering the release of colloidal Se (Jain et al., 2017). Additionally, most of the previous studies did not consider the presence of co-electron acceptors, which can result in an added stress with regards to Se removal, e.g. competition or hindrance of bioreduction and toxicity. A study by Soda et al. (2011) used real Se wastewater taken from a Se refinery plant to evaluate biological Se reduction using a UASB reactor. The authors pre-conditioned the wastewater to increase the pH from

<1.0 to 6–9 and to decrease salinity from 7% to 1% before feeding the wastewater into the biological treatment system. With this pretreatment approach, Soda et al. (2011) achieved > 90% Se removal in the biological system regardless of the presence of various heavy metals (<5 mg L^{-1}).

Espinosa-Ortiz et al. (2015) demonstrated 70% SeO_3^{2-} removal using pure culture fungal pellets at pH 4.5 under aerobic conditions and immobilization of biogenic Se^0 within the fungal pellets. However, compared to SeO_4^{2-} , SeO_3^{2-} is highly reactive and readily reduced to Se^0 by a number of microorganisms (Nanchaiah and Lens, 2015). Both low pH studies by Espinosa-Ortiz et al. (2015) and Kenward et al. (2006) employed pure cultures and as such, contamination becomes a concern when scaling-up. Concerns about contamination are largely eliminated when using mixed culture-based wastewater treatment systems, such as granular

Fig. 3. Microbial community analysis of granular sludge taken from the UASB reactor at different sampling times shown as heat map at the family level in % relative abundance of each phase of reactor operation. Samples were correlated and clustered according to relatedness as shown in the dendrogram. Taxa with <5% relative abundance (only if relative abundance are <5% for all samples) were grouped together as “others” for all samples.

sludge as in this study. In any industrial operation, the stability of an anaerobic process is a key factor when scaling-up a system (Aslanzadeh et al., 2013). Long-term re-use and biosorption capacity of both fungal pellets and pure bacteria culture in a continuous bioreactor under low pH operation have yet to be established. In contrast to the previously mentioned studies, the UASB reactor used in this study was operated for a total of 176 days at pH 5.0 and managed to maintain a consistent Se removal efficiency (40% Se_{total} and 60% Se_{diss}). Furthermore, the bioreactor was able to adapt quickly to operational changes and no irreversible toxicity to the granular sludge occurred due to the presence of SeO₃²⁻ at the low

concentrations tested (4 ± 0.5 mg Se L⁻¹). In contrast, Jain et al. (2016) observed SeO₃²⁻ toxicity to the activated sludge under aerobic conditions leading to repeated reactor crashes and failure in continuous operation.

The specific Se removal mechanisms in the presence of both NO₃⁻ and SO₄²⁻ at low pH are largely unknown. Se oxyanion reduction to Se⁰ can be facilitated by dissimilatory metal reduction or unspecific detoxification pathways of SO₄²⁻ reducers or NO₃⁻ reducers (Nanchaiah and Lens, 2015). Unspecific detoxification pathways pertain to the use of non-Se specific reductases in other microorganisms, i.e. SO₄²⁻, NO₃⁻ or NO₂⁻ reductases for SeO₄²⁻

reduction (Lenz et al., 2009; Tucker et al., 1998). On the other hand, recent studies on the metal adsorption properties of EPS showed that under acidic conditions, bacteria-associated EPS might control anion and metal binding through reactive functional groups (d'Abzac et al., 2013). This was observed by Kenward et al. (2006), where *Shewanella putrefaciens* sorbed SeO_4^{2-} to its cell wall optimally at pH 3. Sorbed SeO_4^{2-} was then reduced to Se^0 from within the cell by an intracellular electron donor, presumably ubiquinone. Further studies are required related to the microbial sorption and role of EPS in SeO_4^{2-} reduction at different pH values in the presence of NO_3^- and SO_4^{2-} to fully decipher the Se removal mechanisms.

Though the influent pH was at 5.0, the system was always able to neutralize the incoming low pH to an effluent pH of >7.0 . This is most likely due to both the reactor re-circulation and the ongoing denitrification and sulfate reduction processes that generate alkalinity and, therefore, neutralize the pH during reactor operation (Koschorreck and Tittel, 2007; Nanchaiaiah et al., 2017; Shen et al., 2013). Alkalinity produced during denitrification and SO_4^{2-} reduction processes were estimated to be approximately 188 (± 30) mg $\text{CaCO}_3 \text{ L}^{-1}$ (Fig. S5).

NO_3^- removal was high and stable during phase I which lasted for 169 days. During this period, removal of NO_3^- was associated with nitrite concentrations $<1 \text{ mg NO}_2^- \text{ N L}^{-1}$ (Fig. S6). However, NO_3^- removal became unstable in phases II and III coinciding with higher nitrite concentrations in the effluent reaching up to 3 mg $\text{NO}_2^- \text{ N L}^{-1}$. Low pH values have been suggested to hinder or even inhibit denitrification because of nitrite accumulation (Nanchaiaiah et al., 2017; Shen et al., 2013). Nitrite at low pH can exist as nitrous acid and has been shown to inhibit denitrification activity at a concentration as low as 0.04 mg $\text{NO}_2^- \text{ N L}^{-1}$ (Baeseman et al., 2006). Shen et al. (2013) observed an approximately 28% decrease in denitrification rates when the initial pH was gradually lowered to 4.5 from 7.2. Similarly, Nanchaiaiah et al. (2017) observed that it took longer for complete NO_3^- removal to occur in a sequential batch reactor when the initial pH was lowered from 7.0 to 5.0 and 4.0. Therefore, unstable and decrease in NO_3^- removal efficiency (by 20%) can be due to the increase in nitrite levels from approximately 0.5 to 3 mg $\text{NO}_2^- \text{ N L}^{-1}$ caused by the low pH (5.0) of the influent.

The SO_4^{2-} removal and sulfide production performance of the low influent pH reactor were fairly low (Fig. 1), which could be due to the presence of NO_3^- (He and Yao, 2010) and/or the low temperature (20°C) operation (Costabile et al., 2011). At pH 5.0, the SO_4^{2-} reduction was even lower, presumably because SO_4^{2-} reducers present in the Eerbeek sludge are neutrophilic (Roest et al., 2005), though there are studies on acid tolerant and acidophilic SO_4^{2-} reducers (Sánchez-Andrea et al., 2014). In contrast to this study, Lopes et al. (2008) were able to achieve nearly complete SO_4^{2-} reduction at pH 4.0; however, the system was operated at thermophilic conditions, SO_4^{2-} was the sole electron acceptor and the organic loading rate was lower ($0.8\text{--}1.9 \text{ g COD L}^{-1} \text{ d}^{-1}$).

4.2. UASB reactor biomass characterization and formation

Unlike in other UASB reactor studies conducted at low pH (Gonzalez-Gil et al., 2012; Lopes et al., 2008), the granular shape of the low pH reactor biomass was not well maintained during low pH UASB reactor operation (Fig. 2). The biomass growing at pH 5.0 was fluffier with filamentous structures and sticky substances surrounding the biomass, the bulk of which is likely EPS. The gradual formation of light, fluffy flocs was one of the likely causes for the floating biomass (Fig. S4a) observed during phase Ie, potentially caused by gas (N_2 , H_2S or CH_4) entrapped within the biomass. Loss of minerals from the low pH biomass can also contribute to the change in granular structure, though both studies at low pH from Gonzalez-Gil et al. (2012) and Lopes et al. (2008) maintained a

granular structure despite mineral leaching (Fe, Zn, Cu and Al).

Another possible factor explaining the floating biomass from the low pH reactor is the polysaccharide (PS) to protein (PN) ratio (PS/PN) of the EPS, which can affect biomass properties. Shin et al. (2000) were able to demonstrate that higher PS/PN ratios correlate with a lower negative surface charge of the biomass or higher hydrophobicity of the cells. This correlates well with the results from this study with the PS/PN ratio of the biomass in the low pH reactor being 15 times lower ($P\text{-value} \leq 0.02$) than the PS/PN ratio of both the inoculum and the control reactor (Table 2, phase Ie). Furthermore, floating biomass was noticed less frequently during operation of the low pH reactor at pH 5.0, coinciding with an increase in PS/PN ratio reaching similar values to both the inoculum and the control reactor (phase III, $P\text{-value} = 1.0$). This could indicate either adaptation of the microbial community to the low pH conditions or a decrease in EPS production. Geyik and Çeçen (2014), for instance, observed that a lower PS/PN correlated with larger flocs of less compact biomass. These observations were attributed to the increase in hydrophobicity and cohesion between aggregates.

Another important aspect in microbial SeO_4^{2-} reduction is the immobilization of Se^0 within the biomass to prevent discharge into the environment. Red deposits observed in the low pH granular sludge (Fig. 3 c1) are strong indicators of elemental Se precipitation in the biomass. Further evidence of Se precipitation was obtained from the detection of Se, at $86 (\pm 12) \text{ mg Se g TS}^{-1}$ (Table 2), in the biomass and the SEM-EDX imaging analysis (Fig. 3) of the low pH granular sludge. The Se immobilization capacity for long-term operation of the UASB reactor at low pH has yet to be fully defined, despite a clear indication of Se^0 entrapment within the biomass. Although there were no changes in Se removal efficiency between days 138–266 (during pH 5.0 operation), the eventual release of Se^0 can be expected due to biomass cell wall rupture/damage (Gonzalez-Gil et al., 2016). Therefore, further studies on biogenic Se^0 toxicity to biomass and Se^0 retention by granular sludge in a given time frame should be conducted in order to establish the necessary frequency of biomass removal and the addition of fresh biomass.

4.3. Microbial ecology of anaerobic granular sludge at low pH

The microbial community composition changed noticeably from the inoculum (day 0), where the majority of the community consisted of archaeal methanogens to a bacterially-dominated diverse community mainly composed of *Deltaproteobacteria* and *Epsilonproteobacteria*. In contrast, no major changes in bacterial diversity were observed among the biomass samples taken during different phases of reactor operation.

Microorganisms capable of reducing SeO_4^{2-} are found in a wide range of phylotypes and appear to be largely related to both denitrifying and sulfate-reducing bacteria (Nanchaiaiah and Lens, 2015). Families of *Campylobacteraceae* and *Desulfomicrobiaceae* were dominant and consistently found in the Se removing granular sludge (Fig. 3). Additionally, analysis at the genus level (Fig. S7) indicated that the population shifted from predominantly known methanogenic to denitrifying and sulfidogenic (SO_4^{2-} reducing) community members. This was evident by the increase in relative abundance of *Sulfospirillum* gen. (belonging to the *Campylobacteraceae* family), followed by *Aminivibrio* gen. (belonging to the *Synergistaceae* family), *Desulfomicrobium* gen. and *Desulfovibrio* gen. (both belonging to the *Desulfomicrobiaceae* family). These genera are not only recognized as denitrifiers, e.g. *Sulfospirillum barnesii*, and SO_4^{2-} reducers, e.g. *Desulfovibrio desulfuricans*, but have also been reported to reduce SeO_4^{2-} along with NO_3^- and SO_4^{2-} (Lai et al., 2016; Lenz et al., 2009; Nanchaiaiah and Lens, 2015; Ontiveros-Valencia et al., 2016; Tomei et al., 1995; Truong et al., 2013). This

is most likely the reason for the high Se removal efficiencies noticed in phases Ia to Id before low pH 5.0 impacted the reactor performance. Moreover, the dominant phyla were *Proteobacteria* and *Synergistetes* which have many known denitrifying bacteria representatives that can explain the high NO_3^- removal efficiencies (>70%) attained throughout the low pH reactor operation. In addition, low SO_4^{2-} removal efficiencies (>30%) noticed during the entire reactor operation could be due to the lower abundance of SO_4^{2-} reducers in the granular sludge.

Interesting to note was the increase of *Spirochaetaceae* and *Geobacteraceae* when operating at pH 5.0. The various species of *Geobacteraceae* are well-known to be metal reducers, including SeO_3^{2-} (Nancharaiyah and Lens, 2015; Pearce et al., 2009), at circum-neutral pH. In this study, the enrichment of *Geobacteraceae* was only observed after the pH was decreased to approximately 5.0. Correspondingly, *Geobacteraceae*-related sequences have been identified abundantly in an acidic mine lake at pH 5.5–5.9 (Lu et al., 2013). Likewise, the family *Spirochaetaceae* was found to be one of the dominant phylotypes in an acidic (Sánchez-Andrea et al., 2014) as well as a NO_3^- and SO_4^{2-} rich (Liao et al., 2014) system. There are, however, thus far, no reports on Se oxyanion reduction by *Spirochaetaceae*.

Further studies regarding changes in microbial community structure at low pH are interesting to pursue, particularly during the start-up operation of the UASB reactor. In order to avoid a pH shock to the system, gradual lowering of the operational pH was employed in this study and therefore it took more than 100 days before the reactor was operated at pH 5.0. Using sludge bio-augmentation with *Geobacteraceae* and *Spirochaetaceae*, it might be possible to accelerate reactor start-up at pH 5.0 without major concerns for reactor failure due to high acidity.

4.4. Industrial implications

This study indicates the possibility of treating acidic Se-laden wastewater directly in a bioreactor without the additional cost of pre-treatment for wastewater neutralization. Assuming a wastewater treatment capacity of 15,000 m^3 per day and caustic soda cost of \$0.44 per kg, the cost of increasing the pH of a wastewater from 4 to 5, using 30% caustic solution, would be approximately \$35 per day, compared to \$39 per day when increasing the pH to 7. It might be possible to further lower the pH adjustment cost, depending on the load of acidic Se-laden wastewater, by increasing the HRT (e.g. by increasing the reactor volume or decreasing the influent flow rate).

There are many aspects that need further investigation in order to further improve the understanding of reactor operations at low pH values. Exploration of possible changes in reactor design or operation should be considered to evaluate the optimal removal performance of Se operated at low pH with exposure to the co-electron acceptors NO_3^- and SO_4^{2-} . Changes in reactor operation that can be explored to increase the Se removal performance of a bioreactor are: (a) reactor configuration, e.g. promoting a biofilm-dominated system as described in Ontiveros-Valencia et al. (2016); (b) increasing reactor temperature (Dessi et al., 2016); or (c) varying NO_3^- and SO_4^{2-} concentrations (Tan et al., 2018).

A techno-economical assessment of the treatment process should be conducted to assess whether Se removal at low pH values would be competitive with pH adjustment of acidic Se-wastewaters. Establishing the granular sludge Se retention capacity of a UASB reactor over time to prevent a sudden Se release potentially caused by biomass lysis (Gonzalez-Gil et al., 2016) would be an important aspect of this assessment. Another aspect to consider is the long-term Se toxicity to the biomass and microbial stress caused by low pH exposure. Working knowledge regarding

the biomass stress response would be useful in order to establish a proper biomass removal cycle and the frequency of fresh biomass addition.

5. Conclusions

UASB bioreactor performance for the removal of SeO_4^{2-} , SO_4^{2-} and NO_3^- using lactate as the electron donor at 20 °C and at pH values between 7.0 and 5.0 was investigated over 266 days. Upon operation of the UASB reactor at influent pH 5.0, the reactor performance showed a trend of 20–30% decrease, achieving a Se_{total} and Se_{diss} removal efficiency of 43 (± 13)% and 61 (± 17)%. A higher removal efficiency was achieved for NO_3^- (>70%), while SO_4^{2-} removal efficiencies were lower than 50% for all pH values investigated. Long-term reactor performance and stability could not be completely assessed at pH 5.0 operation and as such, longer-term studies would be required for future experiments. The biomass which developed at pH 5.0 consisted of large, fluffy and filamentous granules. Microbial community analysis showed a shift from a dominantly archaeal methanogenic population in the inoculum to a bacterially dominated community during reactor operation. The bacterial community in the reactor was mainly composed of known denitrifiers and SO_4^{2-} reducers, i.e. *Sulfospirillum* and *Desulfovibrio*. An enrichment of *Geobacteraceae* and *Spirochaetaceae* was observed once the reactor was operated at pH 5.0.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.chemosphere.2018.07.079>.

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