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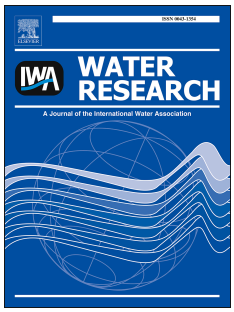

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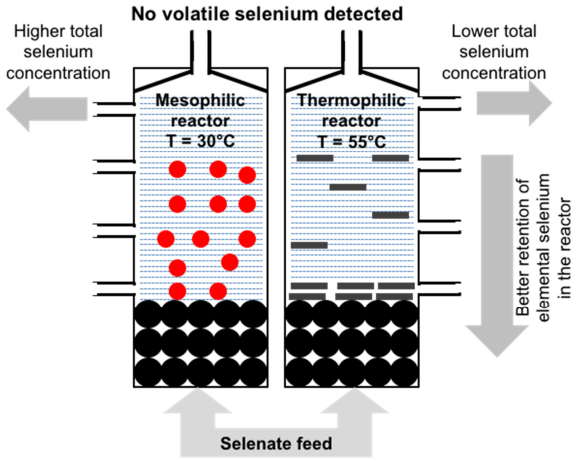
Graphical abstract



Effect of temperature on selenium removal from wastewater by UASB reactors

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Abstract

The effect of temperature on selenium (Se) removal by upflow anaerobic sludge blanket (UASB) reactors treating selenate and nitrate containing wastewater was investigated by comparing the performance of a thermophilic (55℃) versus a mesophilic (30℃) UASB reactor. When only selenate (50 µM) was fed to the UASB reactors (pH 7.3; hydraulic retention time 8h) with excess electron donor (lactate at 1.38 mM corresponding to an organic loading rate of 0.5 g COD L^{-1} d^{-1}), the thermophilic reactor achieved a higher total Se removal efficiency (94.4 ± 2.4%) than the mesophilic reactor (82.0 ± 3.8%). When 5000 µM nitrate was further added to the influent, total selenium removal was again better under thermophilic (70.1 ± 6.6%) when compared to mesophilic (43.6 ± 8.8%) conditions. The higher total effluent Se concentration in the mesophilic UASB reactor was due to the higher concentrations of biogenic elemental Se nanoparticles (BioSeNPs). The shape of the BioSeNPs observed in both UASB reactors was different: nanospheres and nanorods, in respectively, the mesophilic and thermophilic UASB reactors. Microbial community analysis showed the presence of selenate respirers as well as denitrifying microorganisms.

Keywords: selenate, thermophilic, nitrate, selenium nanoparticles, UASB

1. Introduction

There is only one order of magnitude difference between the nutritional requirement (30 − 85 µg Se d^{-1}) and toxicity level (400 µg Se d^{-1}) of selenium (Se) for humans (Lenz and Lens, 2009). Therefore, the United States Environmental Protection Agency (US EPA) has set a discharge limit at 0.63 µM (50 µg Se L^{-1}) in drinking water (US EPA, Drinking water contaminants). Both physical (nanofiltration, reverse osmosis) and chemical (ion exchange, ferrihydrite adsorption and zero valent iron reduction) methods have been tested for the removal of Se oxyanions from wastewaters (Lenz and Lens, 2009). However, their application in full−scale systems is limited due to rather low efficiencies or economic reasons. The microbial reduction of Se oxyanions to elemental Se in bioreactors is a promising alternative to treat Se rich wastewater (Chung et al., 2006; Lenz et al., 2008a). Biological reduction of Se oxyanions yields colloidal biogenic elemental Se nanoparticles (BioSeNPs) that remove the Se oxyanions from the wastewater, although a large fraction (25-68% of influent Se concentration) of these BioSeNPs remains present in the effluent of the bioreactors (Lenz et al., 2008b). The presence of these BioSeNPs in the effluent results in a higher total Se concentrations, and thus an additional post-treatment step is required to meet the discharge limits (Buchs et al., 2013; Staicu et al., 2015) thereby leading to higher operating costs.

One of the major reasons for the presence of BioSeNPs in the effluent is their extracellular bio-production (Jain et al., 2015b). Dissimilatory reduction of selenium oxyanions by selenate respiring microorganisms (e.g. *Sulfurospirillum barnesii*) leads to extracellular production of BioSeNPs, while the detoxificaton of selenium oxyanions leads to their intracellular production (Nancharaiah and Lens, 2015). The extracellular production of

BioSeNPs is known to be more pronounced when nitrate-grown microorganisms (*Sulfurospirillum barnesii*, *Bacillus selenitireducens* and *Selenihalanaerobacter shriftii)* are used to reduce selenite (Oremland et al., 2004). In many selenate containing wastewaters, nitrate is present as well (e.g. agricultural drainage wastewater in the San Luis Drain that disposes water in the Kesterson Reservoir in the San Joaquin Valley contains 48 mg L^{-1} nitrate and 325 µg L^{-1} selenium) (Tanji and Kielen, 2003) which might lead to a larger extracellular production of BioSeNPs resulting in higher total Se concentrations in the effluent when such wastewaters are treated by UASB reactors. However, so far no study has been carried out to confirm this hypothesis in continuous bioreactors.

The microbial community present in a mesophilic UASB reactor, when fed with selenate, evolved towards selenate respirers or dissimilatory reduction of selenate (Lenz et al., 2009; Stolz et al., 2006). Under thermophilic conditions, a different microbial community might evolve over time (Khemkhao et al., 2012), with different SeO_4^{2-}removal mechanism, and thus different location. Furthermore, an elevated temperature can change the allotrope (glass transition temperature of Se is 31℃), size (Lee et al., 2007; Tam et al., 2010) and shape of Se nanoparticles (unpublished results), which may allow them to be better retained in the UASB reactor. However, to the best of our knowledge, no study has so far reported the microbial reduction of selenate under thermophilic conditions.

In this study, the biological reduction of selenate under thermophilic (55℃) conditions was investigated in an upflow anaerobic sludge blanket (UASB) reactor inoculated with anaerobic granular sludge. Another UASB reactor, operating at identical conditions but at mesophilic (30℃) conditions, was used as a control. The effect of temperature on the total, dissolved Se and selenate removal efficiency was determined, together with the

BioSeNPs concentration in the effluent and microbial communities present in the UASB granules. The effect of nitrate at different influent concentrations (100, 500 and 5000 µM corresponding to NO_3^-/SeO_4^{2-} ratio = 2, 10 and 100, respectively) was also investigated.

2. Materials and methods

2.1 Source of biomass

The seed sludge used in this study, described in detail by Roest et al. (2005) was obtained from a full scale UASB reactor treating wastewater of four paper mills (Industriewater Eerbeek B.V., Eerbeek, The Netherlands). Both UASB reactors were inoculated with 200 g wet weight anaerobic granular sludge, as described by Lenz et al. (2008a).

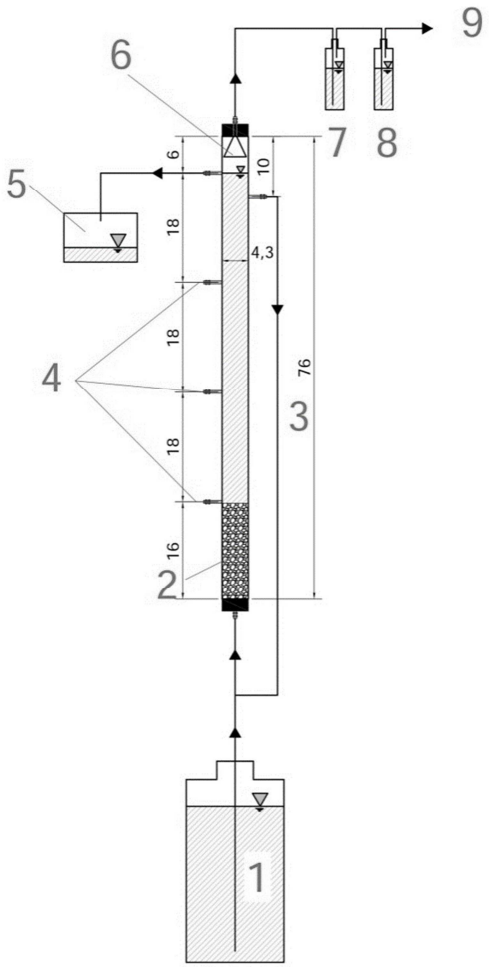
2.2 Composition of the synthetic wastewater

The composition of the synthetic wastewater was (in g L^{-1}): $Na_2HPO_4 \cdot 2H_2O$ (0.053), KH_2PO_4 (0.041), NH_4Cl (0.300), $CaCl_2 \cdot 2H_2O$ (0.010), $MgCl_2 \cdot 6H_2O$ (0.010) and $NaHCO_3$ (0.040). 0.1 mL of the acid as well as alkaline trace metal solutions was added to 1 L of the synthetic wastewater. The composition of acid and alkaline trace elements are described in Stams et al. (1992).

2.3 UASB setup

Two UASB reactors (1 L volume) were operated at a hydraulic retention time (HRT) of 8 h (Figure 1). The influent was fed from the bottom of the reactor and a recirculation ratio of 2 was maintained for mixing. The influent and recirculation flow were maintained constant at 2.2 and 4.4 mL min^{-1}, respectively, resulting in an upflow velocity of 0.3 m h^{-1}. The thermophilic UASB reactor was maintained at 55 (± 2)℃ using a water jacket, while the mesophilic UASB reactor was maintained at 30 (± 2)°C in a temperature controlled room.

123 Two gas traps were connected to the exit of each UASB reactor. The first gas trap (G1)
124 contained 200 mL of concentrated HNO_3 to trap alkylated Se compounds and the second
125 gas trap (G2) contained 100 mL of 6 M NaOH to trap H_2Se (Lenz et al., 2008a). The
126 sampling ports of both reactors were named S1, S2 and S3, from the bottom to the top
127 (Figure 1).



128

129 **Figure 1.** Schematic overview and dimensions (in cm) of the UASB reactors used in this
130 study. Influent tank (1), anaerobic sludge (2), recirculation system (3), sampling ports S1,
131 S2 and S3 from the bottom to the top (4), effluent tank (5), gas separator (6), HNO_3 trap
132 (7), NaOH trap (8) and gas outlet (9).

133

134 **2.4 UASB operating conditions**

Five different operational periods were investigated in both reactors. Sodium lactate ($C_3H_5NaO_3$), Sodium selenate (Na_2SeO_4) and potassium nitrate (KNO_3) as a source of carbon, selenate and nitrate, respectively, were added at different concentrations during the experiment. The pH was maintained at 7.0-7.5 by the use of phosphate buffer in the influent. During period I (1-25 days), sodium selenate (Na_2SeO_4) was added to the influent solution at a concentration of 10 µM (30 µM d^{-1}), as operated in the study carried out by Lenz et al. (2008a).

Table 1. Different operating periods of the UASB reactors used in this study

Period	Operating days	Lactate influent conc. (mM)	SeO_4^{2-} influent conc. (µM)	NO_3^- influent conc. (µM)
I	1 - 25	1.38	10	0
II[a]	26 - 43	1.38	50	0
III	44 - 60	1.38	50	100
IV	61 - 82	1.38	50	500
V	83 - 98	13.8	50	5000

Note: [a] The reactors were inoculated with fresh biomass during this period

At the start of period II (26-43 days), both UASB reactors were re-inoculated with fresh anaerobic granules to avoid the interference of trapped Se in the biomass from period I when determining the BioSeNPs concentration along the reactor length. During period II, both UASB reactors were fed with a selenate concentration of 50 µM (150 µM d^{-1}).

During period III (44-60 days), nitrate (100 µM, 300 µM d^{-1}) was added to the influent along with selenate (50 µM, 150 µM d^{-1}) at a NO_3^-/ SeO_4^{2-} ratio of 2. The influent

concentration of nitrate was increased 5 times (500 µM, 1500 µM d^{-1}) during period IV (61-82 days) which changed the NO_3^-/ SeO_4^{2-} ratio to 10. In the period V (83-98 days), the influent nitrate concentration was increased to 5000 µM (15000 µM d^{-1}; NO_3^-/ SeO_4^{2-} ratio = 100).

Both UASB reactors were operated by supplying the electron donor lactate in excess during the entire duration of the study to avoid the incomplete selenate and nitrate reduction due to the lack of electron donor. The reactors were fed with a synthetic wastewater containing 1.38 mM (corresponding to an organic loading rate of 0.5 g COD L^{-1} d^{-1}) of sodium lactate as the sole electron donor till the end of period IV. The influent lactate concentration was increased to 13.8 mM (corresponding to an organic loading rate of 5 g COD L^{-1} d^{-1}) during period V.

2.5 Sampling

50 mL of samples from the influent and effluent of both UASB reactors were collected daily. To investigate the BioSeNPs stratification in the UASB reactors, samples were collected every 2-3 days from different sampling ports during Period II (26-43 days). Samples from the gas traps were analyzed for Se at the end of every period. Samples for scanning electron microscopy−energy disperse X−ray analysis (SEM-EDXS) of the BioSeNPs was collected from the effluent at the end of study (end of period V).

2.6 DGGE analysis and sequencing

A denatured gradient gel electrophoresis (DGGE) analysis followed by sequencing of selected bands was carried out to describe the microbial populations in the reactors due to

their exposures to selenate, nitrate and two different operating temperatures. The DNA extraction, polymerase chain reaction and denatured gradient gel electrophoresis has been carried out as described in a previous study (Jain et al., 2016). The samples for DGGE analysis were taken on day 0 (inoculum), day 43 (end of period II) and day 98 (end of period V). Selected bands obtained in the DGGE analysis were excised from the gel. The individually excised gel bands were further cut into smaller pieces with a sterile blade, and kept overnight in 30 µL of Tris-EDTA (TE) buffer at 4℃. The overnight incubated samples at 4 ℃ were heated to 95 ℃ for 10 min to elute DNA into the buffer. Thereafter, 1 µL of each of the eluted samples was used as a template in the next PCR, which was carried out using the same primer but without the GC clamp. PCR amplification products were checked on ethidium bromide stained 1.5% agarose gel before their sequencing. All sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on the ABI Prism 377 DNA sequencer (Applied Biosystems, Waltham, Massachusetts). All sequence results were compared with known 16S rRNA sequences in the GenBank database using the basic local alignment search tool (BLASTn).

The obtained band patterns were subjected to digital analysis by using XR+ Image Lab 2.0 software (Bio-Rad, Hercules, California). Peak areas of band patterns in the DGGE fingerprints were used to indicate the intensities. The peaks that were detected were adjusted to remove the unresolved peaks because of background, and the peaks with an area < 1% of the maximum peak in a DGGE profile were excluded. The diversity analysis was carried out using Gel Compar II version 6.6 (Applied Biomath, Sint-Martens-Latem, Belgium). Dice coefficients, i.e. unweighted data on the basis of band presence or absence, were calculated to have a better understanding of the banding patterns. A cluster

analysis using the unweighted pair group method with arithmetic mean (UPGMA) on the basis of the Dice coefficient was performed.

2.7 Analytics and statistics

Samples of influent, effluent and from the sampling ports were analyzed for residual total Se concentration, using an atomic absorption spectroscopy − Graphite Furnace (AAS-GF) (ThermoElemental Solaar MQZe GF95) and a Se lamp at 196.0 nm. Samples taken from the HNO_3 and NaOH gas traps were diluted two times with 6 M NaOH (G1) and 14.4 M HNO_3 (G2), respectively, to adjust the pH before AAS-GF analysis. To separate the BioSeNPs from liquid phase, 15 mL of the effluent was centrifuged for 10 min at 37,000g (Hermle Z36 HK high speed centrifuge). The supernatant was analyzed by AAS-GF to obtain the dissolved Se concentrations in the effluent. The BioSeNPs concentration in the effluent was calculated by subtracting the dissolved Se concentration from the total Se concentration in the effluent. The samples were acidified prior to measurement by adding a few drops of concentrated HNO_3.

Selenate, nitrate and lactate in the influent and effluent were determined by Ion Chromatography (IC) (Dionex ICS 1000), equipped with an AS4A column with an eluent composition of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate. The eluent flow rate was maintained at 0.5 mL min^{-1}. The retention time of lactate, nitrate and selenate was 1.3, 3.9 and 11.3 min, respectively. SEM-EDXS analysis was carried out according to the protocol described in earlier study (Jain et al., 2015c).

The average percentage removal and standard deviation were obtained by taking the last five data points at the end of each period.

3. Results

3.1 Selenium removal in the absence of nitrate (Period I-II)

The performance of both reactors was not similar during the first few days of operation (0-9 days). The removal of selenium was almost instantaneous in the mesophilic reactor (Figure 2a), achieving a total and dissolved Se removal efficiency of > 85% after the first day of operation. The thermophilic reactor was able to achieve comparable efficiencies only after a ~ 15 days adaptation period (Figure 2a). However, at the end of period I, the removal efficiency of both the total and dissolved Se was nearly identical (∼ 90% of 10 µM added selenate) for both UASB reactors, irrespective of their operational temperature (Table S1 in SI). On day 25 of UASB reactor operation, the concentrations of total and dissolved Se in the effluent of both UASB reactors were lower than the US EPA limit of 0.63 µM Se for drinking waters (US EPA, Drinking water contaminants), but higher than the flue gas desulfurization wastewater discharge criterion of 0.063 µM (US EPA, 2015).

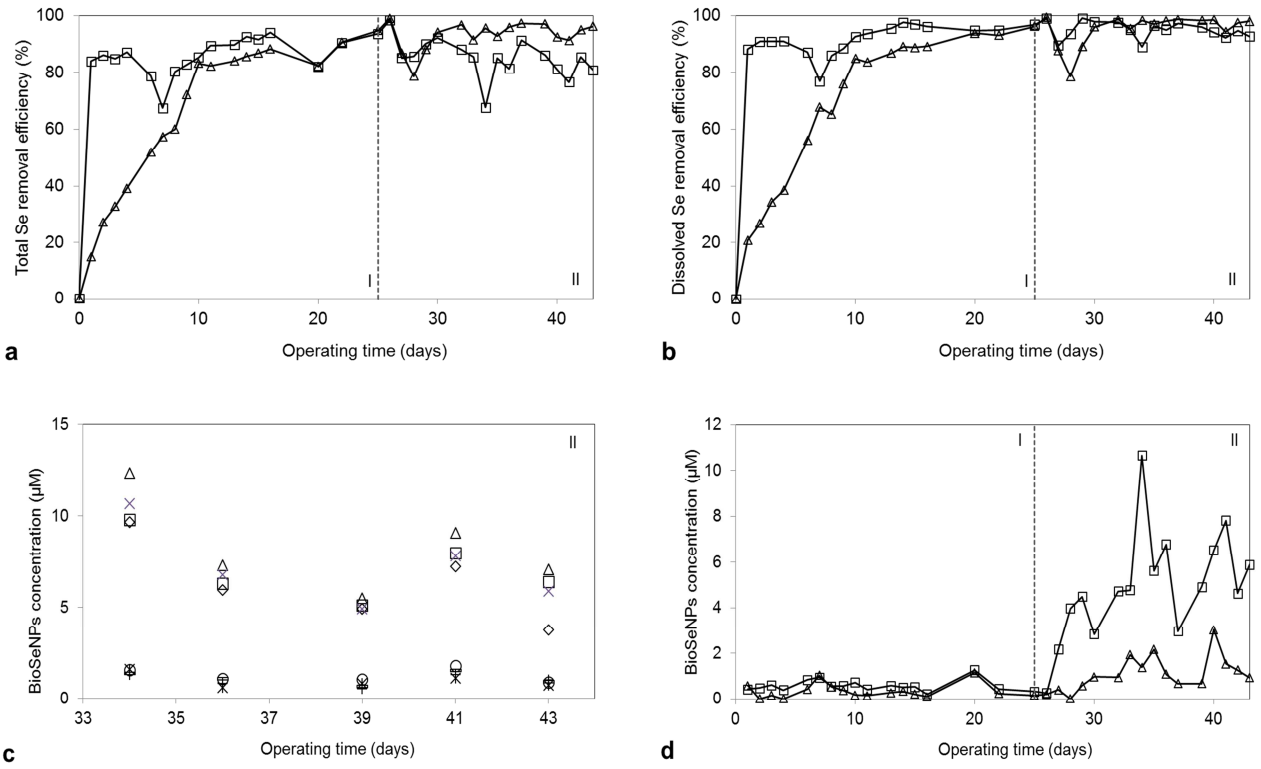


Figure 2. Comparison between the total Se (a) and dissolved Se (b) removal efficiency (%) in period I-II under mesophilic (30℃, □) and thermophilic (55℃, Δ) conditions. The concentration of BioSeNPs (c) in the samples recovered from sampling ports S1 (◇), S2 (□), S3 (Δ) and effluent (×) of the mesophilic reactor and S1 (*), S2 (○), S3 (+) and effluent (—) of thermophilic reactor and effluent BioSeNPs concentration (d) in the mesophilic (30℃, □) and thermophilic (55℃, Δ) UASB reactor during period I-II. The vertical dotted line represents the switch between the different operating periods.

In period II, higher selenate loading rates (5 times more than period I, 50 μM, 150 μM d^{-1}) did not affect the Se removal efficiency of the mesophilic reactor and high total Se removal efficiencies were obtained immediately (Figure 2a, Table S1 in SI). For the thermophilic reactor, the removal of the total Se started instantaneously, in contrast to period I when the influent selenate concentration was 5 times lower. In period II, the average removal efficiency of the total Se after achieving steady state was 94.4 (± 2.4)% and 82.0 (± 3.8)%, respectively, under thermophilic and mesophilic UASB conditions (Figure 2a, Table S1 in SI). The average dissolved Se removal efficiency was better for the thermophilic (97.3 ± 1.7%) compared to mesophilic (93.9 ± 1.5%) UASB reactor (Figure 2b, Table S1 in SI). The selenate removal efficiencies (> 99%) were similar for both the thermophilic and the mesophilic UASB reactor (Figure S1a in SI, Table S1 in SI). Lactate was completely consumed (> 99%) during this period of reactor operation (Figure S1b in SI).

3.2 BioSeNPs concentration in the absence of nitrate (Period II)

The BioSeNPs concentration measured in each sampling port (S1, S2 and S3) in the thermophilic UASB reactor during period II was lower than the corresponding values measured in the mesophilic UASB reactor (Figure 2c). The concentration of BioSeNPs

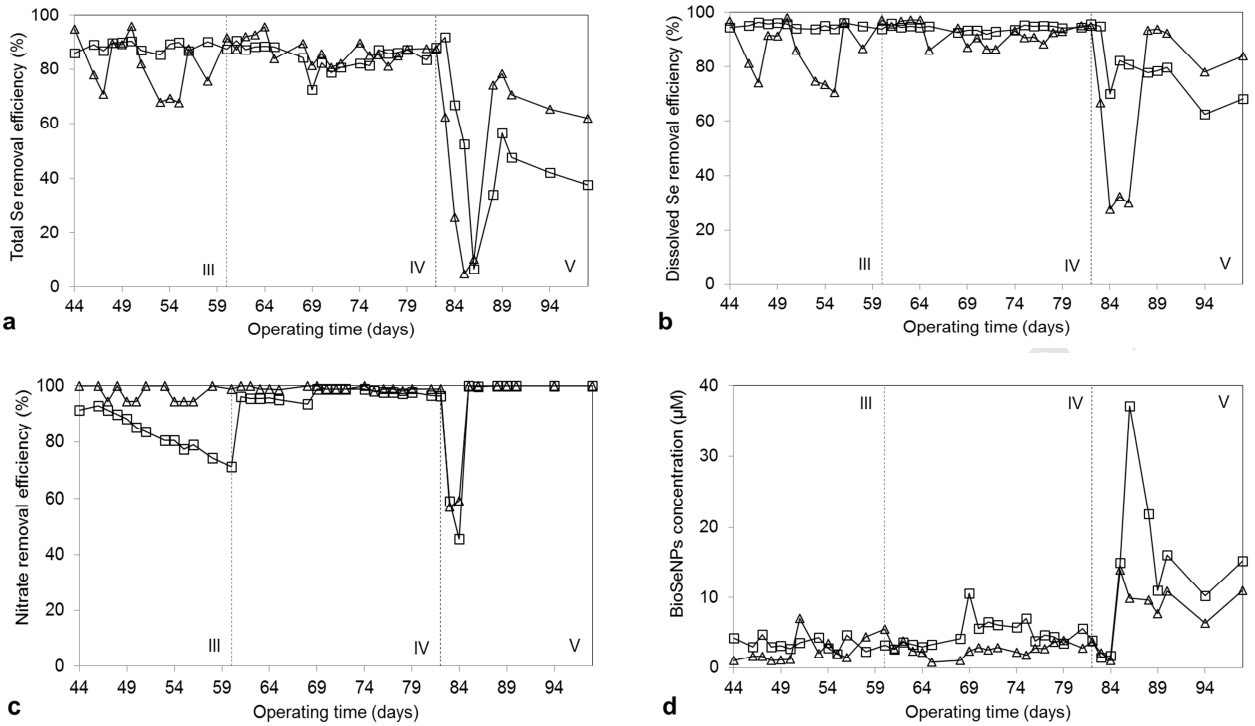
264 measured in the different ports of the thermophilic UASB reactor ranged between 0.6 and
265 2 µM along the reactor height. On the other hand, the concentrations of BioSeNPs along
266 the height of the mesophilic UASB reactor varied from 10 µM on day 34 to 5 µM on day 39
267 (Figure 2c).
268
269 Figure 2d showed that the BioSeNPs concentration was consistently lower in the
270 thermophilic UASB reactor effluent when compared to the mesophilic UASB reactor for
271 period II and slightly lower in period I. The analysis of UASB reactor effluents suggests
272 that the BioSeNPs fraction was 2.1% (56.2 µmol) and 9.2% (248.7 µmol), respectively, in
273 the thermophilic and mesophilic reactor of the total Se fed (2700.0 µmol) during the entire
274 period II (Table 2). During period I, the BioSeNPs fraction in the effluent of the thermophilic
275 (4.0%) UASB reactor was also lower than in the mesophilic reactor (6.0%) (Table 2).
276
277 **Table 2.** Total BioSeNPs present in the effluent of thermophilic and mesophilic UASB
278 reactors during different operational periods.

Period		**I**	**II[a]**	**III**	**IV**	**V[b]**
Operating days		**1 - 25**	**26 - 43**	**44 - 60**	**61 - 82**	**83 - 98**
Total Se fed (µmol)		750	2700	2550	3300	2400
Total BioSeNPs in	M[c]	44.8	248.7	172.3	316.4	710.1
effluent (µmol)	T[d]	29.8	56.2	142.0	164.7	402.4
% of BioSeNPs in the	M	6.0	9.2	6.7	9.6	29.6
effluent to total Se feed	T	4.0	2.1	5.6	5.0	16.8

279 Note: [a]The reactors were inoculated with fresh anaerobic granules during this period;
280 [b]Lactate concentration increased to 13.88 mM on day 85; [C]M refers to mesophilic and [d]T
281 refers to thermophilic

3.3 Selenium removal in the presence of nitrate (Period III-V)

During period III (44 − 60 days), the addition of 100 µM (300 µM d^{-1}) of nitrate (NO_3^-/ SeO_4^{2-} ratio = 2) in the influent had very little effect or even slightly improved the Se removal efficiency of the mesophilic reactor. Both the total (88.8 ± 1.5%) and dissolved (94.8 ± 0.9%) Se removal efficiencies in the effluent of the mesophilic UASB reactor were comparable to the concentrations measured prior to the addition of nitrate (Figures 3a, 3b and Table S1 in SI). The selenate removal efficiency in the mesophilic reactor during period III exceeded 99% (Figure S2a in SI). A decrease in the total (73.6 ± 8.5%) and dissolved Se (80.4 ± 10.9%) removal efficiency was observed in the thermophilic UASB reactor after the addition of 100 µM of nitrate (NO_3^-/ SeO_4^{2-} ratio = 2) (Figure 3a and 3b, Table S1 in SI). During the first 4 days of period III (days 44 - 47), the removal efficiency of both the total and dissolved Se in the thermophilic UASB reactor effluent decreased by ~10% after the addition of nitrate (Figures 3a and 3b). The selenate removal efficiency (85.1 ± 10.9%) in the effluent of the thermophilic UASB reactor followed the same trend of the dissolved Se (Table S1, Figure S2a in SI). The nitrate removal efficiency of the thermophilic UASB reactor (97 ± 3.1%) was better than that of the mesophilic reactor (76.5 ± 3.7%), suggesting that the effluent nitrate concentration was below the detection limit of ~ 16 µM (Figure 3c, Table S1 in SI). Lactate was completely consumed by both UASB reactors at 1.38 mM (4.14 mM d^{-1}) of feed concentration (Figure S2b in SI).



304 **Figure 3.** Removal efficiency (%) of total Se (a), dissolved Se (b) and nitrate (c) during the
305 nitrate fed periods III, IV and V (□ mesophilic, Δ thermophilic). Effluent BioSeNPs
306 concentration (d) in the mesophilic (30℃, □) and thermophilic (55℃, Δ) UASB reactor
307 during period III-V. The vertical dotted line represents the switch between the different
308 operating periods.

309

310 During period IV (61 - 82 days), when the nitrate concentration was increased to 500 μM
311 (1500 μM d^{-1}) and thus the NO_3^-/ SeO_4^{2-} ratio to 10, the removal efficiency of total Se
312 (86.1 ± 1.8%), dissolved Se (94.9 ± 0.6%) and selenate (> 99%) in the mesophilic UASB
313 reactor was similar to the efficiency observed in period III (Figures 3a, 3b, Table S1 and
314 Figure S2a in SI). The removal efficiencies of total Se (85.3 ± 2.6%), dissolved Se (92.1 ±
315 2.5%) and selenate (95.6 ± 6.1%) in the thermophilic UASB reactor in period IV improved
316 when compared to period III and became comparable to the ones achieved in the
317 mesophilic UASB reactor (Figures 3a, 3b, Table S1 and Figure S2a in SI). However, the

total Se and dissolved Se removal efficiency achieved in period IV for the thermophilic reactor were still less than those achieved during period II in the same reactor. The selenate removal efficiency was comparable in the thermophilic UASB reactor in periods II and IV (Table S1, Figures S1a and S2a in SI). The nitrate removal efficiency in the thermophilic and mesophilic UASB reactor were, respectively, 98.7 (± 0.3)% and 97.1 (± 0.6)%. This suggests that nitrate removal efficiencies were not affected in the thermophilic UASB reactor, while improved in the mesophilic UASB reactor when compared to period III (Figure 3c, Table S1 in SI). Lactate was completely consumed in both UASB reactors during this period (Figure S2b in SI).

In period V (82 - 97 days), the influent nitrate concentration was increased by 10 times, when compared to period IV, to achieve a NO_3^-/ SeO_4^{2-} ratio of 100. Both reactors had a decreased efficiency at the outset when compared to period IV. The total Se removal efficiency for the thermophilic UASB reactor (70.1 ± 6.6%), when compared to the mesophilic UASB reactor (43.6 ± 8.8%), was better (Figure 3a, Table S1 in SI). The dissolved Se removal efficiency for the thermophilic UASB reactor (88.3 ± 7.0%) was marginally better than the one observed in the mesophilic UASB reactor (73.7 ± 7.6%) (Figure 3b and Table S1 in SI). The selenate and nitrate removal efficiencies in both UASB reactors exceeded 98 and 99%, respectively (Figures S2a and 3c). The lactate removal efficiencies in period V was greater than 80% in both UASB reactors (Figure S2b). It is important to note that lactate was not in excess on the first two days of period V.

3.4 BioSeNPs concentration in the presence of nitrate (Period III-V)

The concentration of BioSeNPs in the effluent of both UASB reactors during period III was similar (Figure 3d, Table 2). However, during period IV and V, when the NO_3^-/ SeO_4^{2-}

ratio was 10 and 100, respectively, the BioSeNPs concentration in the thermophilic UASB reactor effluent was lower than in the mesophilic UASB reactor effluent (Table 2). It is important to note that when compared to period II, BioSeNPs fraction in effluent increased by more than 10% and 20% under, respectively, thermophilic and mesophilic operating conditions after the addition of nitrate in the influent at 5000 µM (NO_3^-/ SeO_4^{2-} ratio = 100).

3.5 Characteristics of the BioSeNPs in thermophilic and mesophilic UASB reactors

The color of BioSeNPs present in the effluent of the thermophilic and mesophilic UASB reactor was different from the day 1 onwards and remained different throughout the study (Figure 4a). Grey colored BioSeNPs were observed in the thermophilic UASB reactor, while they were red colored under mesophilic conditions. BioSeNPs present in the effluent of the thermophilic UASB reactor were nanorods (Figure 4b1, Figure S3 in SI), whereas those in the effluent of the mesophilic UASB reactor were spherical (Figure 4b2). The effluents of both the UASB reactors showed that microorganisms were of similar shape and were present in minor quantities (Figure 4b1 and 4b2). EDXS spectra confirmed that the nanoparticles were composed of Se (Figures 4c1 and 4c2, Points 2), while the washed out microorganisms in the effluent did not shown any Se peak (Figures 4c1 and 4c2, Points 1).

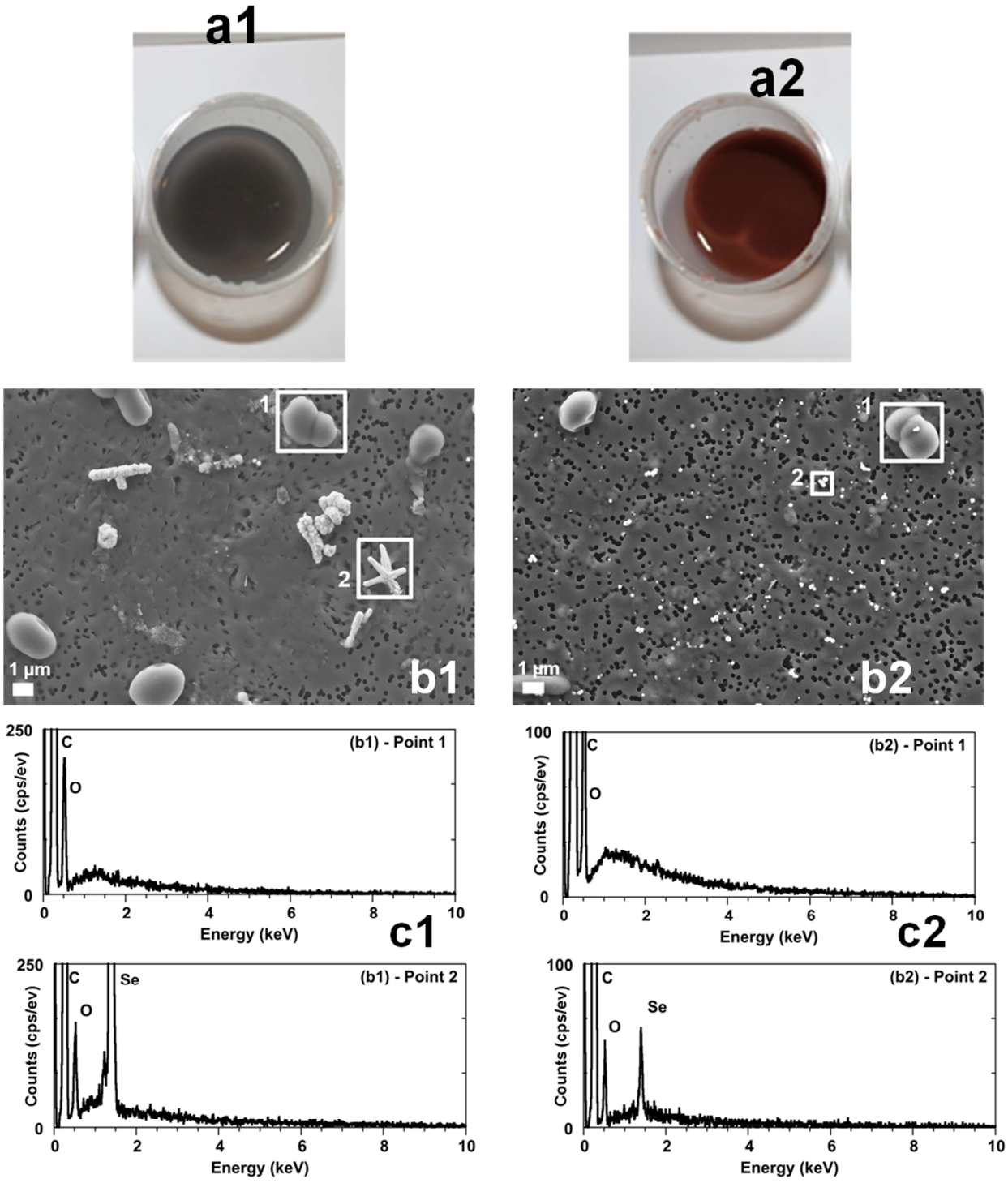
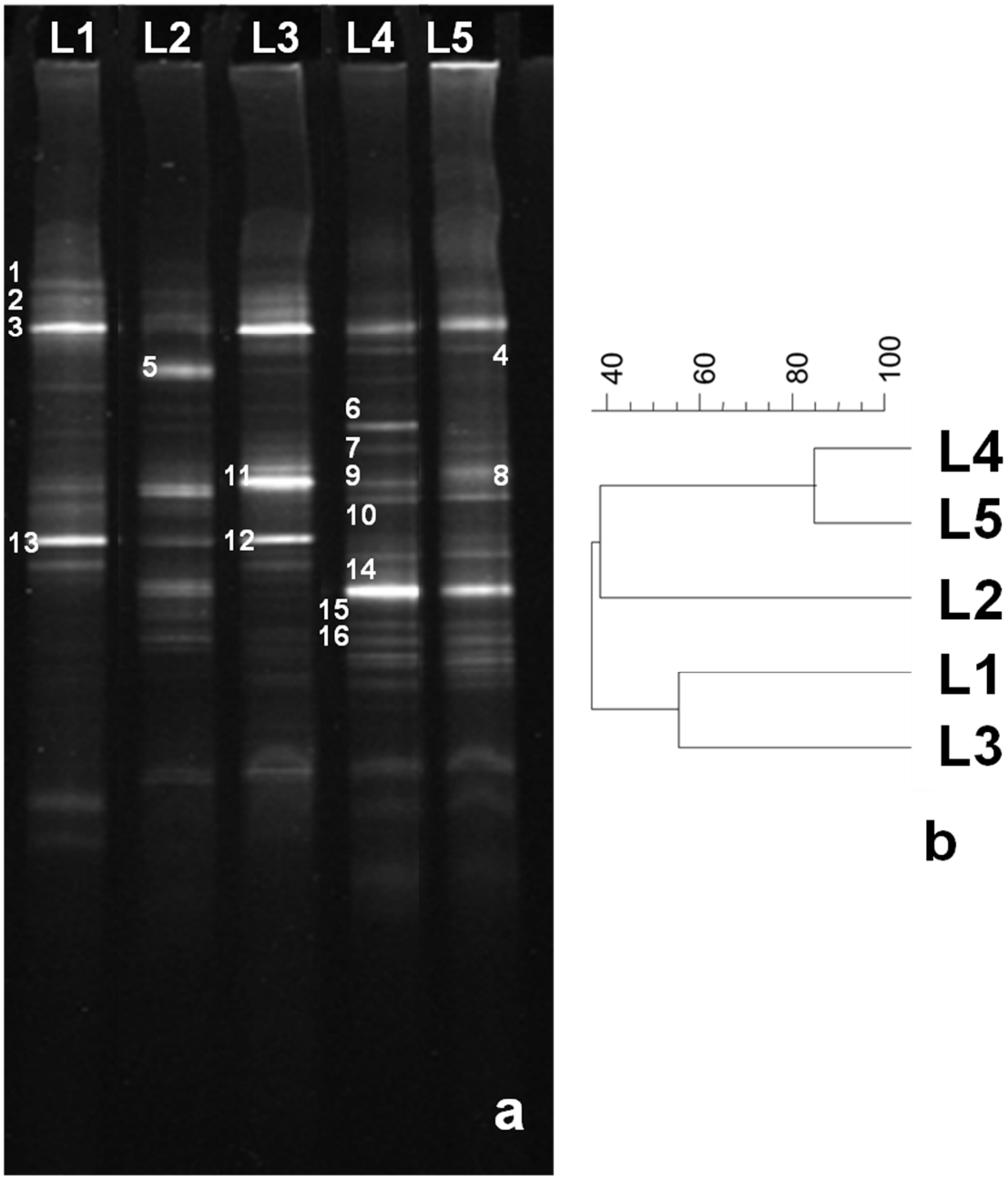


Figure 4. Color of elemental Se (a) produced, SEM images (b) and EDXS analyses (c) of effluents from thermophilic (1) and mesophilic (2) UASB reactors sampled on day 98 of reactor operation (Selenate = 50 µM, Nitrate = 5000 µM, lactate = 13.8 mM). Please note that the color of the effluent of both UASB reactors was different from day 1 of the reactor operation.

368 **3.6 Microbial community analysis of the UASB reactor granules**

369 The DGGE clearly showed the differences between the bands of the anaerobic granules at
370 different operating temperatures as well as at different points of reactor operation (Figure
371 5a). The diversity analysis carried out suggested that the microbial community in both
372 reactors was only 40% similar after the addition of selenate for 18 days (end of period II)
373 (Figure 5b). The similarity of the microbial community increased to 80% at the end of
374 period V in both reactors after addition of nitrate (Figure 5b).



375

376 **Figure 5.** DGGE (a) and diversity analysis (b) of bacteria in both UASB reactors at
377 different points of time during reactor operation: inoculated anaerobic granules (L1),
378 mesophilic anaerobic granules at the end of period II (L2) and V (L4) and thermophilic
379 anaerobic granules at the end of period II (L3) and V (L5).

380

381 Sequencing of the selected bands showed the presence of *Geovibrio ferrireducens* in the
382 inoculum, at the end of period II in the thermophilic UASB reactor and in both UASB
383 reactors at the end of period V (Figure 5a and Table 3). *Sulfurospirillum barnesii* and
384 *Pseudomonas stutzeri* were found present in, respectively, the mesophilic and
385 thermophilic UASB reactor, while *Desulfotomaculum nigrificans* was found present in the
386 inoculum and in both reactors at the end of period II (Table 3).

387

388 **Table 3.** Presence of different microorganisms in the UASB reactors: inoculated anaerobic
389 granules (L1), mesophilic anaerobic granules at the end of period II (L2) and V (L4) and
390 thermophilic anaerobic granules at the end of period II (L3) and V (L5).

Band no. in DGGE (Figure 5a)	Microorganisms	L1	L2	L3	L4	L5
1	*Marinifilum flexuosum*	+	–	–	–	–
2	*Alkalitalea saponilacus*	+	–	+	+	–
3	*Geovibrio ferrireducens*	+	–	+	+	+
4	*Clostridium thermobutyricum*	–	–	–	–	+
5	*Sulfurospirillum barnesii*	–	+	–	–	–
6	*Cesiribacter andamanensis*	–	–	–	+	–
7	*Desulfurispirillum indicum*	–	–	–	+	–
8, 9, 11	*Pseudomonas stutzeri*	–	–	+	+	+
10	*Paracoccus denitrificans*	+	–	–	+	+
12	*Desulfotomaculum nigrificans*	+	+	+	–	–
13	*Odoribacter laneus*	+	–	+	–	–
14	*Denitrovibrio acetiphilus*	–	+	–	+	+
15	*Geovibrio thiophilus*	–	+	–	+	+
16	*Paracoccus versutus*	–	+	+	+	+

391

Upon the addition of nitrate during period III-V in both reactors, *Paracoccus denitrificans,* present in the inoculum, but not detected in both UASB reactors at the end of period II, was found again present in both UASB reactors at the end of period V (Table 3). *Denitrovibrio acetiphilus*, initially present only in the mesophilic UASB reactor at the end of period II, was found in both UASB reactors at the end of period V. *Pseudomonas stutzeri*, which was found present only in the thermophilic UASB reactor at the end of period II, was found present in both reactors at the end of period V. *Desulfurispirillum indicum*, a selenate reducing bacterium, was detected in the mesophilic UASB reactor only at the end of period V.

4. Discussion

4.1 Biological removal of Se in UASB reactors

This study demonstrated that a better total Se removal efficiency can be achieved in an UASB reactor operating at thermophilic (55℃) as compared to mesophilic (30℃) conditions when the influent selenate concentration is 50 µM (Figure 2a). The higher total Se removal efficiency in the thermophilic UASB reactor was due to the lower BioSeNPs (elemental Se) concentrations compared to mesophilic UASB reactor in the effluent (Table 2, Figure 2d). The absence of Se in the gas traps of both UASB reactors suggests that the major selenate removal mechanism in both UASB reactors was selenate reduction to elemental selenium and its subsequent retention in the bioreactor, as confirmed by the different BioSeNPs concentration in the effluent of the UASB reactors (Figure 2d).

The lower concentration of BioSeNPs in the thermophilic UASB reactor effluent (Figure 2d, Table 2) when compared to the effluent of the mesophilic UASB reactor was probably due to the extracellular reduction of selenate to BioSeNPs under mesophilic conditions, as

417 indicated by the members of the microbial community (Figure 5, Table 3). *Sulfurospirillum*
418 *barnesii*, present in the mesophilic UASB reactor at the end of period II, produces
419 BioSeNPs extracellularly (Figure 5a, Table 3) (Oremland et al., 2004). *Pseudomonas*
420 *stutzeri*, present in the thermophilic reactor at the end of period II, reduces selenate
421 (Kuroda et al., 2011; Lortie et al., 1992); however, localization of the BioSeNPs formed in
422 this species have not yet been studied in detail (Figure 5a, Table 3).
423
424 The other possible reason for the lower BioSeNPs concentration in the effluent of the
425 thermophilic UASB reactor compared to the mesophilic UASB reactor can be the different
426 settling properties of the BioSeNPs produced under thermophilic conditions (nanorods)
427 and mesophilic conditions (nanospheres) (Figure 4b). BioSeNPs are known to interact with
428 cations, which were present in both UASB reactors, leading to a less negative ζ-potential
429 value resulting in a decrease in their colloidal stability and thus allowing them to settle
430 (Buchs et al., 2013; Jain et al., 2015a). The lowering of the ζ-potential depends on the
431 surface interactions of the cations with the BioSeNPs, which depend on the properties of
432 the BioSeNPs, including shape, size and surface charge. However, further research is
433 required to study the settling properties of differently shaped BioSeNPs in different
434 environmental and engineered settings.
435
436 It is interesting to note that at an influent concentration of 10 µM selenate, longer
437 adaptation times (9 days) were required for the thermophilic UASB reactor to achieve total
438 dissolved Se removal efficiencies comparable to those obtained instantaneous under
439 mesophilic conditions (Figures 2a, 2b). Surprisingly, this was not observed when the
440 reactors were restarted using a feed selenate concentration of 50 µM with fresh anaerobic
441 granules (Figures 3a, 3b). This can be attributed to the fact that the adaptation of

anaerobic granules to selenate fed to a continuous reactor has a positive dependence on the Se loading rate at non-toxic influent selenate concentrations (Takada et al., 2008), thus, making the adaptation by anaerobic granules faster.

4.2 Effect of nitrate on Se removal

Lai et al. (2014) demonstrated that the reduction of selenate is inhibited by the presence of nitrate at surface loading rates larger than 81.4 m moles m^{-2} d^{-1} (NO_3^-/ SeO_4^{2-} ratio = 56.2; 714 µM nitrate fed to the reactor). In this study, the selenate reduction efficiency was not affected by the presence of nitrate at a NO_3^-/ SeO_4^{2-} ratio of 100 in the mesophilic as well as thermophilic UASB reactor (Table S1, Figure S2a in SI). The higher selenate removal efficiency (> 99%) under thermophilic conditions can be due to presence of *Pseudomonas stutzeri* (Figure 5a and Table 3), known for the reduction of selenate (Kuroda et al., 2011; Lortie et al., 1992). The higher selenate removal efficiency under mesophilic conditions (> 98%) can be due to the presence of selenate-respiring microorganisms e.g. *Sulfurospirillum barnesii* and *Desulfurispirillum indicum* (Figure 5a and Table 3). Similar results were obtained in a *Sulfurospirillum barnesii* bioaugmented anaerobic UASB sludge when the nitrate and sulfate concentrations were 1500 and 200 times in molar excess compared to selenate (Lenz et al., 2009). It is important to note that unlike Lai et al. (2014), this study was carried out in presence of excess electron donor.

This study demonstrated that the presence of nitrate in the influent leads to excess release of BioSeNPs in the effluent of both the reactors (Figures 2d, 3d and Table 2). In the presence of nitrate as well, the thermophilic UASB reactor achieved ∼15% higher total Se removal efficiencies as compared to the mesophilic UASB reactor at a NO_3^-/ SeO_4^{2-} ratio of 5000 in period V (Figure 3a and Table S1 in SI). The excess presence of BioSeNPs in

the effluent of the mesophilic UASB reactor can be partially attributed to the extracellular production of BioSeNPs by selenate-respiring microorganisms such as *Desulfurispirillum indicum* (Rauschenbach et al., 2011), found present in the mesophilic UASB reactor at the end of period V (Tables 1 and 3).

The selenate-reducing microbial community is shaped by the presence of nitrate in a hydrogen-based membrane biofilm reactor (Lai et al., 2014). In this study as well, the addition of nitrate led to 80% similarity in the microbial community of both UASB reactors at the end of period V, despite they were only 40% similar prior to NO_3^- feeding at the end of period II (Figure 5b). The addition of nitrate also induced the growth of denitrifying bacteria such as *Denitrovibrio acetiphilus* and *Paracoccus denitrificans* (Table 3).

4.3 Se speciation

The presence of volatile Se fractions in the gas traps was negligible (< 5 µg of Se was trapped in the gas traps for the entire period) at 30℃, as observed in earlier studies with UASB (Lenz et al., 2008a, 2008b) and activated sludge (Jain et al., 2016) reactors. Interestingly, it was also negligible at 55℃ (< 5 µg of Se was trapped in the gas traps for the entire period), suggesting that the formation of alkylated compounds is not related to the temperature within the range investigated.

The selenide formation was also not observed during the long term reactor operation, as the concentration of Se in the second gas trap was negligible for both UASB reactors. This is plausible as microorganisms such as *Sulfurospirillum barnesii, Bacillus arsenicoselenatis, and Selenihalanaerobacter shriftii* are not known to readily produce selenide (Herbel et al., 2003). Nevertheless, it is also possible that some selenide was

formed in the reactor mixed liquor and then quickly oxidized to elemental Se or precipitate as metal selenide, and thus remained undetectable in both the liquid and gas phase.

4.4 Practical implications

This study showed the thermophilic UASB reactors can treat hot selenate and nitrate contaminated wastewater, e.g. flue gas desulfurization scrubbing waters that have an adiabatic temperature of about 55℃ (Akiho et al., 2010; Higgins et al., 2008), without the need to cool down the scrubbing water. Besides, a 10-15% higher total Se efficiency was achieved in the thermophilic UASB reactor when compared to the mesophilic UASB reactor. This difference may seem marginal in terms of percentage, however, a 10% difference corresponds to 395 µg L^{-1} or 1185 µg L^{-1} d^{-1}. Similarly, a 15% difference, as observed in period V, would translate to 593 µg L^{-1} or 1778 µg L^{-1} d^{-1}. This became even more significant when considering that the flue gas desulfurization wastewater discharge criterion is 5 µg L^{-1} (0.063 µM) (US EPA, 2015).

A simple cost-benefit analysis was carried out considering the base cost of 0.25€ per kg of Se of a biological selenium removal technology comprising of a series of anaerobic bed reactors operating at 15℃ coupled with sand filtra tion prior to discharge (MSE Technology Applications Inc., 2001). Assuming the cost of electricity to be 0.075 € per kWh and the thermal efficiency 95%, the cost for treating 1Kg of Se in the mesophilic and thermophilic UASB reactor, respectively, is 0.51 and 1.06€. Though the removal cost of Se treatment is higher for thermophilic conditions, the higher Se removal efficiency of the thermophilic UASB reactor would ensure lower post treatment cost to meet the 5 µg L^{-1} (0.063 µM) discharge criterion. Thus, a holistic view of the complete system composed of the bioreactor and secondary treatment step is required to fully identify the operating costs of

such systems. Future work needs to focus on understanding the limitations of a UASB reactor to internal hydrodynamics with emphasis on recovering the BioSeNPs. Low-cost electron donors, such as methane or ethanol, need to be tested to determine the maximum selenate and total Se removal rates while attempting to reduce the costs of the electron donor.

5. Conclusions

- Thermophilic UASB reactors achieved 10-15% higher total Se removal efficiency compared to the mesophilic UASB reactor
- Thermophilic conditions (55℃) led to the formatio n of different shaped BioSeNPs in the thermophilic (nanorods) and mesophilic (nanospheres) UASB reactors
- Extracellular BioSeNPs producing selenate-respiring microorganisms (e.g. *Sulfurospirillum barnesii*) in the mesophilic UASB reactor is one of the reasons for its lower efficiency
- Addition of nitrate triggers the higher concentration of BioSeNPs in the effluent of both the UASB reactors

Supporting information

The SI contains extra figures with experimental data as noted in the text.

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Author Contributions

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Highlights

UASB reactor at 55℃ achieves a 10-15% higher total Se removal efficiency than at 30℃

BioSeNPs produced at 55℃ are nanorods, whereas nan ospheres at 30℃

Adding NO_3^- to the feed decreases the Se removal efficiency