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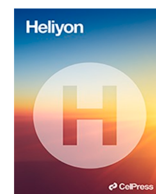

Research article

Developing a versatile tool for studying kinetics of Selenate-Se removal from aqueous solution using a chemostat bioreactor

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ABSTRACT

Understanding the impact of various parameters on the kinetics of dissolved selenium (Se) removal in bioreactors can be a challenging task, primarily due to the mass transfer limitations inherent in bioreactors employing attached growth configurations. This study successfully established a proof-of-concept for the efficient removal of Se from aqueous solutions using a chemostat bioreactor that relies solely on suspended growth. The research investigated the effect of selenate-Se feed concentrations under two distinct Se concentration conditions. One experiment was conducted at a considerably elevated concentration of 25 mg/L to impose stress on the system and evaluate its response. Another experiment replicated an environmentally relevant concentration of 1 mg/L, mirroring the typical Se concentrations in mine water. The bioreactor, featuring a working volume of 0.35 L, was operated as an anaerobic, fully mixed chemostat with hydraulic retention times (HRTs) ranging from 5 to 0.25 days. The outcomes revealed the chemostat's capacity to remove up to 25 mg/L of dissolved Se from water for all HRTs exceeding 1 day, under otherwise optimal conditions encompassing temperature, pH, and salinity. The research's significance lies in the development of a versatile tool designed to examine Se removal kinetics within a system devoid of mass transfer limitations. Furthermore, this study verified the ability of the bacterial consortium, obtained from a mine-influenced environment and enriched in the laboratory, to grow and sustain Se removal activities within a chemostat operating with HRTs as short as 1 day.

1. Introduction

Selenium (Se) exists naturally in the Earth's crust at concentrations between 0.05 and 0.5 mg Se kg^{-1} [1]. Anthropogenic activities that mobilize Se compounds into the environment include metal and coal mining. This may result in excessive concentrations of Se in receiving aquatic environments with total dissolved Se concentrations up to 12 mg-Se/L measured in some locations [2]. Regulated concentrations of total dissolved Se for discharge of contaminated industrial wastewater into receiving aquatic environments varies by region. The present selenium water quality guideline for the protection of freshwater and marine aquatic life set by the Canadian Council of Ministers of Environment is 1 μg/L, the British Columbia Ministry of Environment guideline is 2 μg/L, and the USA Environmental Protection Agency and Australia/New Zealand guidelines are 5 μg/L [3]. The reason that these guidelines are at the microgram level is because Se bioaccumulates up the food chain in the receiving aquatic environment, which results in toxic effects on wildlife such as deformity and reproductive failure in fish (Ohlendorf et al., 2011). Consequently, dissolved forms of Se, selenate

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(SeO_4^{2-}, Se [VI]) and selenite (SeO_3^{2-}, Se [IV]), must be removed from mine-influenced water (MIW) to meet the regulated concentrations.

Biological treatment of selenium from industrial wastewater is currently identified as the preferred alternative to chemical and physical technologies due to the lower reagent requirements, more compact sludge, and possibility of treating high volumes of wastewater [4–6]. In biological treatment of Se, microorganisms gain energy from oxidation-reduction reactions between an electron donor such as acetate (CH_3COOH) and an electron acceptor such as selenate or selenite in a controlled environment within the bioreactor [5]. Over the last few decades, active bioreactors in which nutrients and energy are added for better performance, have gained attention for Se bioremediation. The bioreactor is the core of the active biological treatment systems which is designed to facilitate the microbial growth and biological treatment of the target contaminant [7].

The performance of active bioreactors in dissolved selenium (Se) removal depends on the bioreactor configuration. Active bioreactors are configured as suspended-growth, attached-growth or hybrid (contains both attached growth and suspended growth) systems. The attached-growth bioreactors have been widely used as the well-established technology for metal-laden water treatment processes in the industry. In attached-growth systems the microorganisms form a biofilm on the surface of a solid media known as biomatrix [8] by forming extracellular polymeric substances (EPS). The formation of biofilm reduces the risk of the biomass being washed out from the system.

The removal of dissolved Se was examined in several studies in batch or continuous reactors including packed bed or fluidized bed reactors. Two commercially available attached-growth bioreactor configurations specifically developed for removal of selenate from Se-laden industrial wastewater include fluidized bed reactors (FBR), such as the one produced by the company Envirogen, and packed-bed reactors, such as the ABMet produced by Suez [6]. Envirogen bioreactor has been implemented in pilot and full scale for removal of up to 0.55 ppm Se down to 4.7 μg/L (99 % removal efficiency) [9,10]. The ABMet technology has also been evaluated for its capability for selenate and nitrate removal from MIW and have shown to be effective in removal of up to 1.95 ppm Se from industrial wastewater to below 2 μg/L in pilot and full scale [11,12]; MSE Technology Applications Inc., 2001). Depending on the reactor configuration and influent water chemistry (concentration of contaminants to be removed and water matrix), the operational hydraulic retention time (HRT) of industrial bioreactors can be in the range of hours to days.

In all these attached-growth systems, mass transfer limitations exist. To the best of authors' knowledge only one other bench-scale study investigated the removal of dissolved Se in a continuous reactor with only suspended biomass in the reactor [13]. There has been no full-scale proof of concept for Se treatment plants running solely as suspended-growth system [7]; due to the risk of washout and difficulty of maintaining anaerobic conditions at full-scale. Chemostats are typically used for kinetic studies, which must be performed in the absence of mass transfer limitations. In this work, the objective was to provide a proof-of-concept for the removal of total dissolved Se in a chemostat bioreactor containing only suspended biomass as the first step toward developing a laboratory tool for studying kinetics of dissolved Se removal in bioreactors. The chemostat bioreactor was used to assess the effect of HRT on extent and rate of Se removal.

It could be challenging to achieve the stringent regulated limits for total dissolved Se concentration using bioreactors relying solely on attached growth. Since the total dissolved Se concentration in mine-influenced water (MIW) is widely variable due to seasonal changes in the range of 0.002–12 mg/L measured in some receiving environments [2], operators of industrial bioreactors need to adjust the concentration of carbon source, that is provided as electron donor for bacterial growth and energy production, proportional to the influent Se concentrations as electron acceptor. In this work, it was hypothesized that the dissolved Se removal in a bioreactor, operating anaerobically at constant optimal temperature and pH where carbon source is supplied in excess, and in the absence of inhibitory compounds, is influenced by the concentration of selenium as the electron acceptor. The removal of dissolved Se was determined as a function of total dissolved Se concentration using Se in the form of selenate and excess stoichiometric amounts of carbon source acetate.

These variabilities in electron acceptor and electron donor type and concentration can change the Se transformation mechanisms and consequently the microbial community composition inside the bioreactor [14]. The microorganisms used in this study were enriched from sub-aqueous sediments collected from seepage ponds receiving MIW high in dissolved Se, as opposed to a pure culture. Environmentally sourced bacteria were chosen since mixed culture consortia are more robust and resistant to environmental changes compared to pure cultures [15].

This study represents a proof-of-concept for the efficient removal of selenium at concentrations up to 25 mg/L from synthetic wastewater, employing a chemostat bioreactor that exclusively relies on suspended growth system. The significance of this research lies in the development of a versatile tool designed for examination of Se removal kinetics in a system without mass transfer limitations.

This chemostat reactor configuration can be utilized for a broad spectrum of kinetic investigations. This includes, but is not limited to, the effect of diverse parameters such as Se concentration, carbon source concentration, or operational conditions (e.g., temperature, pH) on the Se removal process. By conducting such comprehensive kinetic studies using this innovative approach, there is great potential to assist mining companies in the optimization of their Se removal bioreactors. Ultimately, this advancement contributes to more effective and environmentally sustainable practices within the mining industry.

2. Materials and methods

2.1. Preparation of enrichments

The location and properties of the sediments used as the source for microorganisms to inoculate into the chemostat are described in Ref. [16]. Sediments from Mine 2 Site A were chosen since the study by Ref. [16] demonstrated that they contained selenate reducing bacteria. These collected sediments were stored in the laboratory in sealed containers with no headspace at room temperature (20–25 °C). Selenate reducing bacteria of sediments were enriched in modified [17] growth medium. The basal salts medium (BSM) contained (g/L final concentration): NaCl, 1.2; KCl, 0.3; KH_2PO_4, 0.2; NH_4Cl, 0.3; $MgCl_2.6H_2O$, 0.85; Na_2SO_4, 0.3; $CaCl_2.2H_2O$, 0.15, and was sterilized by autoclaving at 120 °C for 20 min. The SL-10 trace element 1000 X stock solution contained (g/L): $FeCl_2.4H_2O$, 1.5; $MnCl_2.4H_2O$, 0.1; $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, 0.036; 0.19; $ZnCl_2$, 0.07; $NiCl_2.6H_2O$, 0.024; H_3BO_3, 0.006; $CuCl_2.2H_2O$, 0.002; and HCl (25 %) 0.01 mL. This was sterilized using filtration (0.22 μm) and 1 mL added per 1 L growth medium. The enrichment growth medium contained 25 mg-Se/L added as sodium selenate (Na_2SeO_4, 60 mg/L). Acetate (234 mg/L) in the form of sodium acetate ($NaCH_3COO$, 325 mg/L) was added as the carbon source and electron donor in 10 times in excess of the stoichiometric ratio (10X stoich.) based on stoichiometric equations given in the following section assuming selenate is reduced to elemental Se in a two-step reaction (Equations (1) and (2)). The pH of the growth medium was in the range of 6.5–7.0 and was adjusted to 7.0, if needed, using 1 N NaOH. Prior to inoculation, N_2 gas was sparged through the growth medium to eliminate dissolved oxygen.

One hundred mL of homogenized sediment was added to 220 mL growth medium in sterile 320 mL glass bottles, which were sealed with butyl rubber stoppers and incubated statically in the dark at 30 °C (Hotpack model 5525, Waterloo, Ontario). Periodically, every 5–7 days, 220 mL of culture liquid in the glass bottles was replaced with fresh growth medium. The earliest appearance of an orange colour was used as an indication of selenate reduction to determine when to replenish the growth medium. This was continued for a total period of 30 days.

2.2. Stoichiometric Calculations

Due to lack of available data on cell biomass yield over carbon source acetate for selenium reducing bacteria, values reported for denitrifying bacteria were used instead due to the versatility of nitrate and nitrite reductases for selenate and selenite reduction, respectively [18]. However, it's important to note that this approach comes with inherent limitations due to the different and unknown metabolic pathways and growth characteristics of these bacteria. It was assumed that the fraction of electrons from the carbon source acetate used for cell synthesis (f_s) was $f_s = 0.4$ with the rest going to energy production ($f_e = 0.6$). This f_s value is based on the upper limit derived from experimental data in Ref. [19]. Using the method described in Chapter 2 of [20]; the overall stoichiometric equations for transformation of selenate to selenite, and selenite to elemental Se, respectively, were derived as follows:

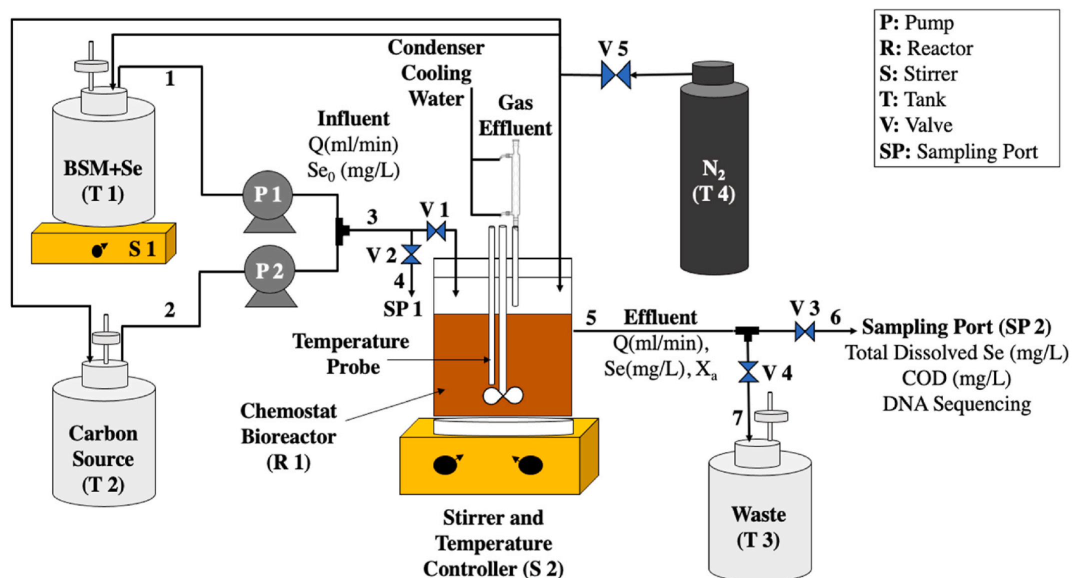

Fig. 1. Schematic diagram of the continuous experimental set-up. T-1 and T-2 are glass bottles containing the feed solutions, P-1 and P-2 are pumps, V-1 to V-5 are valves, S-1 is a stirrer, S-2 is a stirrer and temperature-controlled heating plate, R-1 is the chemostat bioreactor, T-3 received the effluent from the chemostat bioreactor, and SP-1 and SP-2 are sampling ports from which liquid and mixed liquor suspended solids, respectively, were removed for chemical and biological analyses.

$$0.3SeO_4^{2-} + 0.125C_2H_3O_2^- + 0.02NH_4^+ \rightarrow 0.3SeO_3^{2-} + 0.02C_5H_7O_2N + 0.045CO_2 + 0.105HCO_3^- + 0.105H_2O \quad (1)$$

$$0.15SeO_3^{2-} + 0.125C_2H_3O_2^- + 0.02NH_4^+ + 0.3H^+ \rightarrow 0.15Se^0 + 0.02C_5H_7O_2N + 0.045CO_2 + 0.105HCO_3^- + 0.255H_2O \quad (2)$$

By combining Eqns. (1) and (2) it was estimated that 1.25 moles of acetate are needed for reduction of 1 mol of selenate to elemental Se. A limitation of this study is that the provided stoichiometric equations are presented solely as a basis to calculate an estimated amount of carbon source to be added to the reactor. To ensure carbon source was not limiting, excess amount of carbon source was provided for both experiments. Although following the two-step reaction for selenate to selenite and further to elemental Se is the common pathway for the reduction of Se, they do not represent all possible reactions that could occur within the reactor.

2.3. Chemostat set-up and experiments

Continuous flow experiments were conducted using a CBR 90-3 Anaerobic CDC chemostat reactor with working volume during continuous operation of 350 mL (BioSurface Technologies Corporation (BST) Bozeman, Montana, USA) (Fig. 1). Feed to the chemostat was separated into two solutions. T-1 contained the inorganic components, which included BSM, 1 mL/L SL-10 trace solution and sodium selenate, the latter at concentrations to achieve either 25 mg-Se/L or 1 mg-Se/L in the combined feed to the chemostat, depending on the experiment. The carbon source sodium acetate was in a separate container (T-2) as a concentrated solution to prevent contamination. Concentrations of the components in each tank were adjusted taking into consideration dilution when the two flows mixed before entering the chemostat. The pH was adjusted to 7 in both feed bottles using 1 N NaOH. The chemostat bioreactor vessel, T-1 and T-2 were maintained anaerobic by continuous sparging with 99 % pure N_2 from T-4. The chemostat bioreactor was maintained at constant temperature (23 °C) with continuous mixing (220 RPM).

Two separate experiments were performed with different feed concentrations of selenate and acetate, respectively. In the first experiment, feed concentrations were 25 mg selenate-Se/L and 76.0 mg acetate-COD/L (estimated as 3 times the stoichiometric requirement using Eqns. (1) and (2)). In the second experiment, these were 1 mg selenate-Se/L and 10.1 mg acetate-COD/L (estimated as 10 times the stoichiometric requirement from Eqns. (1) and (2)). The rationale behind selecting two distinct concentrations for experimentation was to facilitate an investigation at a considerably elevated concentration (25 mg/L) compared to typical concentrations of Se in mine water to impose stress to the system and assess its response. Another experiment was conducted in an environmentally relevant concentration (1 mg/L), mirroring the typical Se concentration in mine water. This methodological approach was adopted to ensure a comprehensive examination of Se concentration impact, encompassing conditions reflective of real-world issue alongside an extreme scenario designed to evaluate the system's tolerance and potential adverse reactions.

Other than varying the Se and acetate concentrations, the same BSM and SL-10 components and concentrations as were used in the enrichment growth medium were provided in the feed to the bioreactor. At the beginning of each experiment, 225 mL of enrichment culture was inoculated into 450 mL of BSM medium including 1 mL/L SL-10 with a final concentration of 25 mg selenate-Se/L and 324 mg $NaCH_3COO$/L in the chemostat after mixing. The chemostat was operated in batch mode until the removal of dissolved Se was verified to be taking place based on the appearance of orange colour in the culture broth or decreasing concentrations of dissolved Se. Running the experiments in batch mode before proceeding with continuous mode serves several purposes. It enables the understanding of fundamental system behavior, assesses microbial activity and growth for maintaining a stable microbial population in continuous mode, validates the bioreactor setup and experimental protocols to address any issues proactively, and mitigates risks to the continuous phase by addressing challenges during batch experiments.

At the beginning of continuous flow, the initial hydraulic retention (HRT) time was 5 days for the first experiment and 3 days for the second experiment. The chemostat was run at constant HRT for a duration of time until the effluent dissolved Se concentration remained steady. Based on a previous experiment run for a long period of time at constant HRT, steady-state effluent dissolved Se concentration variations were less than 3 % of the influent Se-selenate concentration. Thus, this criterion was used to determine when to adjust to another HRT, which was done by increasing the flow rates of feeds to the chemostat. The HRT was gradually reduced in a stepwise manner until washout was observed for each experiment, occurring at different times throughout the course of each experiment. During each HRT, samples for dissolved selenium (Se) and chemical oxygen demand (COD) analyses were periodically collected from the effluent stream (SP-02) in both experiments. Furthermore, a modification was implemented in the chemostat setup, adding an influent sample port (SP-01) to enable the precise monitoring of dissolved Se and COD concentrations in the influent. Occasionally, on the day that the HRT was changed, two samples were taken for dissolved Se analysis, one before the HRT change and one 4 h after the change. Samples for biomass collection, and subsequent DNA extraction and 16S rRNA sequencing for microbial population analysis were taken from the effluent (SP-2). Dissolved oxygen (DO) concentration, pH, and temperature were monitored in the effluent taken from SP-2 at each time point.

2.4. Analytical methods

Samples for dissolved Se concentration analysis and soluble COD measurements were filtered (0.22 μm MF-Millipore Membrane Filter) immediately after collection to remove suspended solids, and the filtrates preserved with analytical grade nitric acid and sulfuric acid, respectively (2 mL/L), and stored at 4 °C until the day of analysis. Dissolved Se concentrations were measured in triplicate using inductively coupled plasma optical emission spectroscopy (ICP-OES) using an Agilent 5110 ICP-OES with a Multimode Sample Introduction System. The method detection limit for Se was 0.1 μg/L. ICP-EOS does not distinguish different Se compounds (such as selenate, selenite, and organo-selenium compounds). Thus, the amount of dissolved Se in all forms combined is reported here.

Selenium speciation analysis in the chemostat was not done because the sampling for Se speciation to conduct a mass balance is extremely challenging due to the availability of sample size, the presence of organic Se species, and the formation of volatile Se compounds. Chemical oxygen demand (COD) was measured as a proxy for the carbon source concentration using the HACH Reactor Digestion method 8000. The COD as opposed to direct measurement of acetate provides a comprehensive assessment of the total organic carbon content within the sample containing various organic compounds that may contribute to carbon availability, thereby offering a more holistic view of the carbon source concentration. This technique also simplifies the analytical process by employing a standardized and widely accepted method, making it more time and cost-efficient. Since the test was done on filtered samples, it gives the soluble chemical oxygen demand (SCOD).

While employing COD as a proxy for carbon source concentration offers various advantages, there are instances where measuring acetate directly could have provided specific benefits. The first notable advantage lies in the precision and specificity achieved through direct acetate measurement. This approach proves valuable when the research focus centers solely on acetate and demands an accurate determination of its concentration. Direct measurement ensures a targeted approach, free from the influence of other organic compounds that might coexist in a sample, in contrast to the broader assessment offered by COD. For studies requiring an in-depth comprehension of acetate dynamics, such as its consumption or transformation within a system, direct measurement can unveil insights that might be obscured when relying on a surrogate like COD.

In the context of the current study and others with similar requirements, considering direct acetate measurement is recommended. This is particularly relevant if specialized equipment, costs, and time constraints permit. Such an approach would contribute to a more nuanced understanding of acetate's role in the system, catering specifically to the study's objectives.

2.5. DNA extraction and sequencing

Genomic DNA was extracted from filter papers used for filtering effluent (SP2) samples using the DNeasy PowerSoil Pro Kit (QIAGEN) following the manufacturer's instructions. DNA concentrations were measured using an Invitrogen™ Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific). Purity of the extracted DNA was measured using a NanoDrop ND-2000 Ultraviolet–visible (UV–Vis) spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 nm for nucleic acid. To ensure the purity of extracted DNA, absorbance was also measured at 230 nm for organic contamination and at 280 nm for protein contamination. For pure DNA samples, the ratios of the absorbance should be A260/A280 = 1.8–2.0 and A260/A230 ≥ 2.0 [21].

If DNA did not pass QC, it was purified using DNeasy PowerClean Pro Cleanup kit (QIAGEN). The 16S rRNA variable regions V4 and V5 were amplified using primers 515F–Y and 926R [22,23] and sequenced on an Illumina MiSeq in the Biofactorial Core Facility in the Life Sciences Centre of the University of British Columbia. The raw sequence data were converted into relative abundance tables of bacterial taxonomic composition using bioinformatic software Qiime2 [24]. The Dada2 package was used to correct Illumina-sequenced amplicon errors due to its high sensitivity [25,26]. Qiime2 clustered filtered reads into amplicon sequence variants

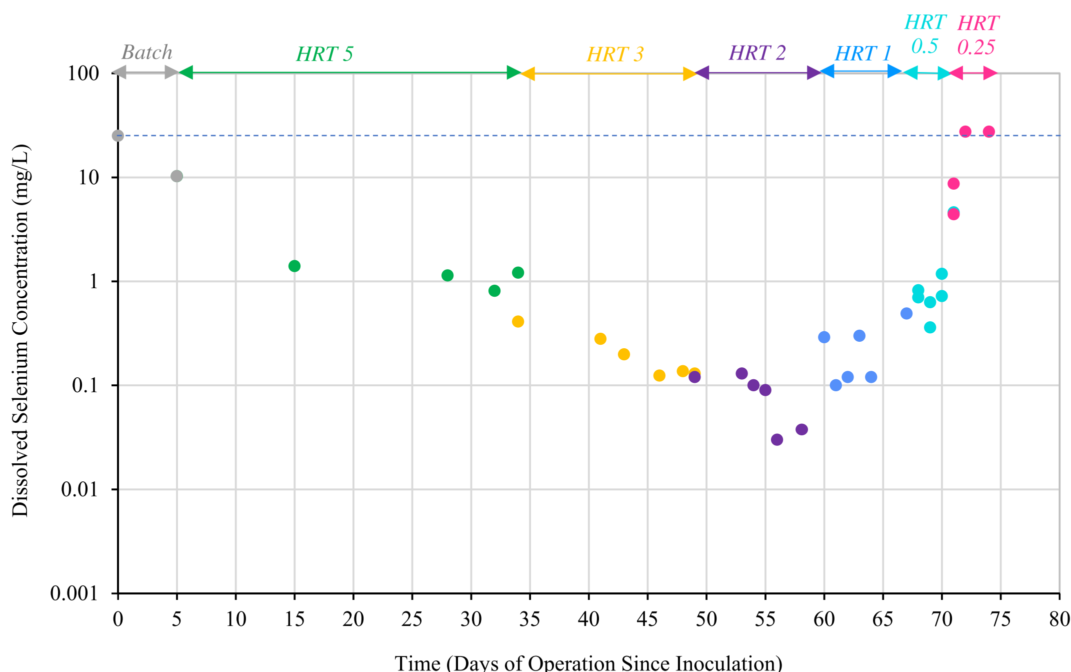

Fig. 2. Expected influent (dashed line) and measured effluent (circles) dissolved Se concentration over time in the chemostat with a feed concentration of 25 mg/L selenate-Se and 3 times stoichiometric amounts of carbon source acetate. Effluent concentrations are coloured according to HRT.

(ASVs) with a 97 % homology. Taxonomic classification was assigned for dominant sequences (more than 1 % of the population) by aligning the representative sequences to the Silva database (version 138.1) [27].

3. Results and discussion

3.1. Se removal

Dissolved Se concentrations in the chemostat effluent measured over time are plotted in Figs. 2 and 3 for Experiments 1 and 2, respectively. A logarithmic scale is used for dissolved Se concentration to observe more clearly the lowest effluent concentrations achieved, which is of interest given the strict regulatory requirements for the discharge of Se into aquatic environments. In Experiment 1 during the initial batch period, the dissolved Se concentration decreased from 25 to 10 mg Se/L within 5 days indicating an active selenate reducing microbial community. Upon the earliest appearance of orange colour on Day 5, the reactor was switched to continuous mode. Following the initiation of continuous flow, the chemostat was operated for 29 days at an HRT of 5 days. During this time, the effluent dissolved Se concentration achieved was 1.14 ± 0.21 mg Se/L. Subsequently, the HRT was reduced to 3 days for 15 days, during which the effluent dissolved Se concentration decreased over time reaching a steady-state concentration of 0.12 mg Se/L. The lowest effluent dissolved Se concentration achieved was 30 μg/L when the HRT was 2 days, which represents 99.9 % Se removal from the dissolved phase. When the HRT was shortened to 1 day and then 0.5 days, the effluent dissolved Se concentrations increased. Finally, when the HRT was further reduced to 0.25 days, the dissolved Se concentration increased to 27.5 mg/L, which is close to the concentration in the influent, meaning that dissolved Se was no longer being removed in the chemostat. This indicates that a minimum HRT was reached at which washout occurred. Washout happens when the concentration of microorganisms inside the bioreactor decreases to zero since the dilution rate (D), which equals the volumetric flow rate divided by the working volume of the bioreactor, and the inverse of the HRT, is greater than the cell specific growth rate (μ). Thus, for the conditions of this experiment, D becomes greater than μ for the selenate reducing bacteria in the chemostat somewhere between 2 and 4 day^{-1}.

In Experiment 2, the chemostat was switched from batch to continuous mode after 1 day due to the appearance of orange-coloured precipitates assumed to be elemental Se. The analytical data confirmed that Se was removed during the 1-day batch period from 0.72 to 0.61 mg Se/L. As opposed to Experiment 1, for this experiment a sampling port was added to the influent stream so that feed concentrations could be analyzed to confirm that they were as expected (Fig. 3). Measurements indicated that the feed dissolved Se concentration was 0.98 ± 0.10 mg Se/L for the entire duration of the experiment. As was observed in Experiment 1, the effluent dissolved Se concentrations decreased over time as the HRT was decreased from 3 to 2.5 and then to 2 days. The lowest dissolved Se concentration observed in the chemostat effluent was 1 μg/L (99.9 % Se removal) when the chemostat was running at HRTs of 2 days and 1.5 days. After the HRT was reduced to 1 day, the effluent dissolved Se concentration increased to 0.1 mg/L. Finally, when the HRT was further shortened to 0.5 days, the effluent dissolved Se concentration reached the same concentration as that in the feed signaling that washout had occurred. Thus, under the conditions for Experiment 2, the dilution rate, D, exceeded the cell specific growth rate somewhere between 1 and 2 day^{-1}.

Plotting steady-state effluent dissolved Se concentrations, obtained by averaging the final two or three data points in each HRT run,

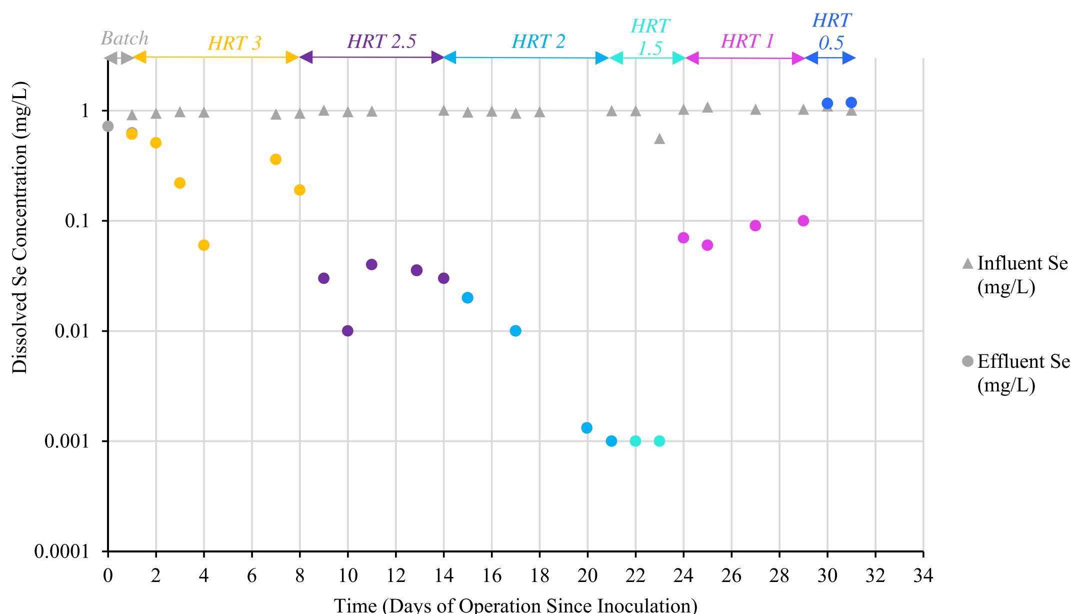

Fig. 3. Influent (triangles) and effluent (circles) dissolved Se concentration over time in chemostat with 1 mg/L feed selenate-Se and 10 times stoichiometric acetate. Effluent concentrations are coloured according to HRT.

as a function of HRT (Fig. 4) highlights similarities and differences between the two experiments. In both experiments, a similar pattern was observed where the steady-state effluent dissolved Se concentrations decreased as the HRT was reduced from 5 or 3, respectively, reaching a minimum at an HRT of 2 days. The second experiment, using 1 mg selenate-Se/L in the feed appears to achieve lower effluent concentrations that are close to regulatory requirements for some locations (1-2 μg-Se/L BCWQG) [3]. It was possible to operate the chemostat at an HRT of 0.5 days when the feed selenate-Se concentration was 25 mg/L, whereas washout occurred at this HRT when the lower feed concentration was used. Washout is expected to occur at lower HRTs for higher feed concentrations of substrate in a chemostat with microbial growth following Monod kinetics (Fig. 3.3, page 174 in Ref. [20]). However, in Experiment 1, the chemostat was run at an HRT of 0.5 days for only three days during which the effluent dissolved Se concentration was continuously increasing and steady-state may not have been achieved, thus there is some uncertainty as to if the chemostat could operate for longer than 3 days at this HRT.

By changing the HRTs from 5 to 2 days (for Experiment 1) and from 3 to 2 days (for Experiment 2), despite the fact that the HRTs were reduced, improving Se removal extent was observed. This trend was unexpected since longer retention times should lead to higher conversions. This could be potentially explained by the fact that longer HRTs (5 days for Experiment 1 and 3 days for Experiment 2) are leading to lower amounts of active biomass in the chemostat due to biomass decay, which might contribute to lower Se removal during long HRTs [20]. It is also possible that dead biomass releases elemental Se back into the solution while still in the chemostat.

One of the study's limitations was the inability to perform a comprehensive statistical analysis, primarily due to the limited number of data points available at each concentration. Attempting statistical analysis with such a small sample size heightens the risk of overfitting and may lead to inaccurate estimates or interpretations.

3.2. Carbon source consumption

The carbon source consumption was monitored for Experiment 1 by measuring SCOD versus time (Fig. 5). The concentration of SCOD in the chemostat at the beginning of the batch phase was expected to be around 253 mg/L based on the mass of sodium acetate added to the growth medium. However, the measured SCOD concentration was over 600 mg/L. The additional SCOD was likely associated with the inoculum. The inoculum for this experiment was prepared using fresh sediments removed from the mine site location two weeks prior to beginning the enrichment process. Organic compounds in the sediments might have persisted in the enrichment culture even though the enrichment period was 30 days. With the commencement of continuous flow, the effluent SCOD concentration remained much greater than the expected feed concentration of 76 mg/L due to this additional SCOD from the inoculum. The SCOD effluent concentration declined continuously until Day 53, thereafter remaining steady at 77 ± 6 mg/L until Day 68. It is unknown what chemical compounds contributed to the excess SCOD nor to what degree they were consumed by the microorganisms in the bioreactor. It is possible that they were slowly washed out from the bioreactor without reacting. From Day 68 until the end of the experiment, the effluent SCOD concentrations dropped to levels below the expected influent concentration and decreased steadily to 6 mg/L. The effluent SCOD concentrations did not trend upwards towards the end of the experiment similar to what was observed for effluent dissolved Se concentrations. This might have been due to the activity of other heterotrophic organisms that continued growing in the system whose growth was not coupled to selenate or selenite reduction, such as fermentative organisms, for example.

To test if the carbon source supply was sufficient, in addition to the SCOD of the effluent, the amount of SCOD in the influent was also monitored for Experiment 2. In Experiment 2, the influent SCOD concentration was expected to be 10.9 mg/L based on the amount of acetate provided and the flow rate of feed to the chemostat. Measured influent SCOD concentrations (Fig. 6) verified this (10.9 ±

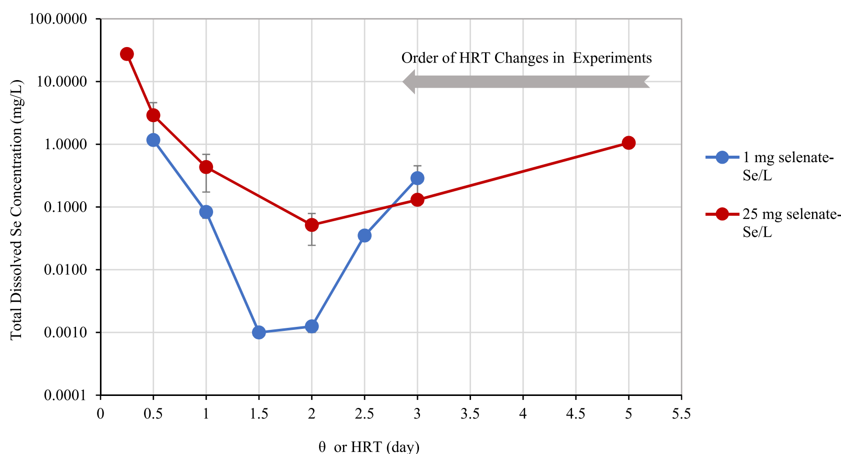

Fig. 4. The average steady-state dissolved Se concentrations at each HRT are plotted versus HRT with 25 mg/L Se-selenate and 3-times stoichiometric C-source (red circle) and 1 mg/L Se-selenate and 10-times stoichiometric C-source (blue circle) in the feed. The error bars show the standard deviation for the averaged dissolved Se concentrations at each HRT. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

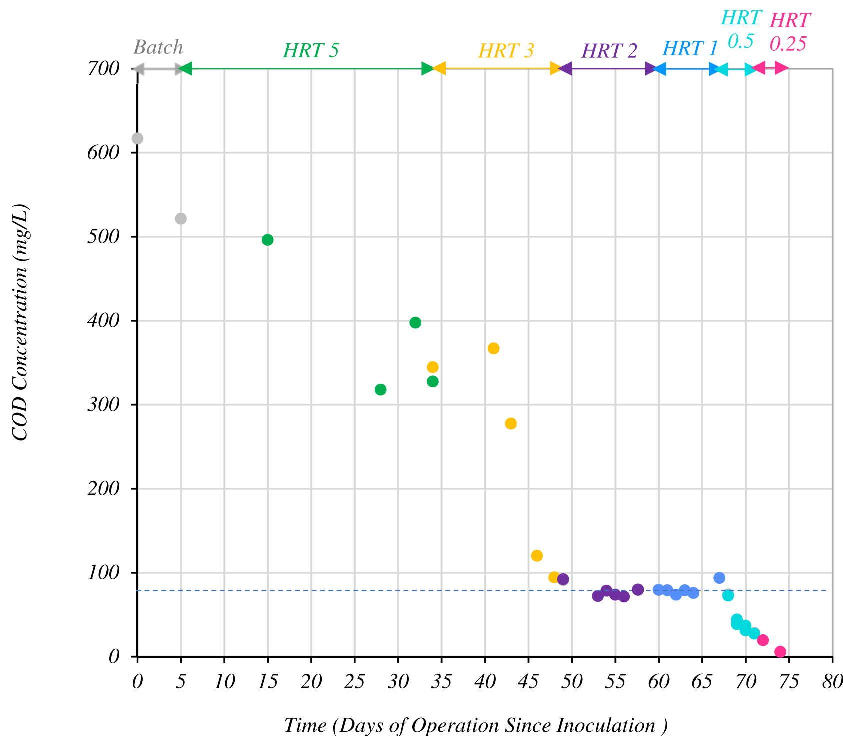

Fig. 5. Expected influent (dashed line) and measured effluent (circles) chemical oxygen demand (SCOD) versus time for an influent concentration of 25 mg Se-selenate/L and 3-times stoichiometric amounts of acetate in the feed. A selection of SCOD samples was analyzed in replicates and a standard deviation of less than 2 % was measured.

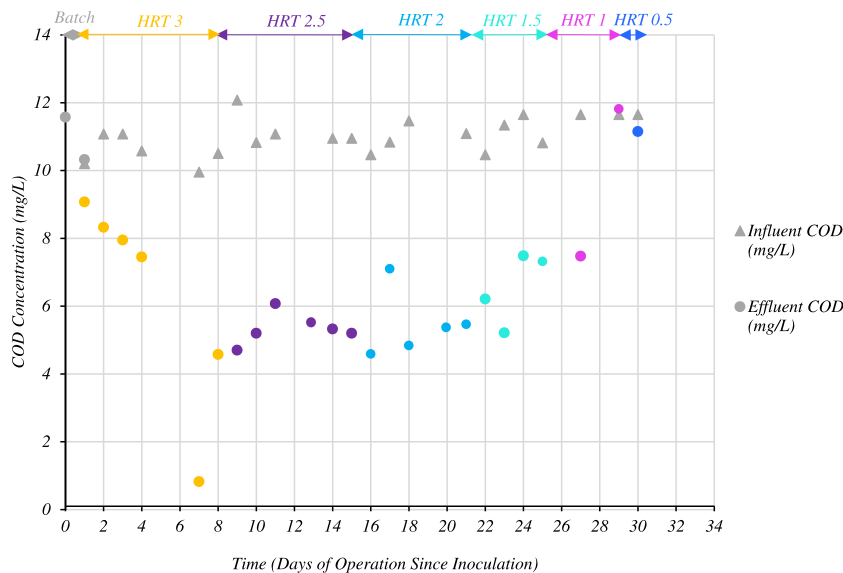

Fig. 6. Influent (triangles) and effluent (circles) SCOD concentrations over time in the chemostat with feed concentrations of 1 mg/L Se-selenate and 10-times stoichiometric amounts of acetate. Effluent concentrations are coloured according to HRT. A selection of SCOD samples were analyzed in replicates and a standard deviation of less than 2 % was measured.

0.5 mg/L) and acetate was confirmed to be the sole carbon source entering the chemostat. During the first HRT run tested during continuous flow (3 days), the effluent concentration of SCOD decreased consistently from 9.0 to 4.6 mg/L over 7 days. On Day 7, the concentration of SCOD decreased due to a clog in the SCOD influent tubing downstream of the influent sample port which was resolved. The effluent SCOD concentration was less variable during the periods when the HRT was 2.5 and 2 days, with an average of

5.39 ± 0.7 mg/L. When the HRT was changed to 1.5 days, the SCOD concentration started to trend upwards and by the time the HRT was reduced to 1 day, SCOD concentration increased to 11.8 mg/L meaning that SCOD not being consumed in the chemostat. This trend was consistent with that of dissolved Se in the effluent. The observation that SCOD was not being consumed confirms that washout of microorganisms occurred at the end of the HRT of 1 day run and that this was likely the cause of no removal of dissolved Se at that time.

Data from Experiment 2 were used to estimate the yield of carbon source consumed per amount of dissolved Se removed, $Y_{C/_{Se}}$, by dividing the moles of acetate used, calculated from Equation 3

$$(\text{mg COD}_{in} - \text{mg COD}_{out}) \frac{\text{mmole COD}}{32 \text{ mg COD}} \frac{\text{mmole acetate}}{2 \text{ mmole COD}} \tag{3}$$

by the moles of dissolved Se removed, calculated from Equation 4

$$(\text{mg Se}_{in} - \text{mg Se}_{out}) \frac{\text{mmole Se}}{78.9 \text{ mg Se}} \tag{4}$$

for each data point. The yield coefficients were averaged for each HRT (Fig. 7) and compared to the theoretical yield coefficient of 1.25 mol acetate/mole Se (dashed line in Fig. 7). The yield was most variable during an HRT of 1.5 days when the highest removal extents of dissolved Se were achieved. Calculated average yields $Y_{C/_{Se}}$ ranged from 4.8 to 8.5 mol COD/mole Se, which were 4–7 times higher than the theoretical yield. This suggests that more than ~74–85 % of acetate is consumed by microorganisms whose growth is not coupled with reduction of Se compounds, or the assumed f_S used to derive the stoichiometric equations is not valid for this microbial community.

3.3. Microbial population diversity

In the first experiment, an average of 23,173 high quality 16S rRNA sequences per sample were obtained from samples removed during HRTs of 3, 2, 1, 0.5 and 0.25 days. In the second experiment, an average of 13,715 high quality reads per sample were obtained from samples removed during the batch period and HRTs of 3, 2.5, 2 and 1.5 days. These were used to assess the diversity of the chemostat microbial population over time (Fig. 8 A and B). Overall, the average number of observed features in the samples from Experiment 1 was 115 (± 23) with the only observable trend being a much higher number of features on Day 67 when the HRT was 1 day. Overall, the number of observed features in samples from Experiment 2 were fewer, decreasing from 104 features during the batch phase to an average of 71 (±12) over the following 24 days indicating a trend towards a more constrained population.

Faith phylogenetic diversity which assesses the diversity with respect to the genetic relatedness of the population, reached a maximum of 15.7 on Day 67 and reduced to an average of 10 (± 1.5) for Experiment 1. For Experiment 2, the Faith index was

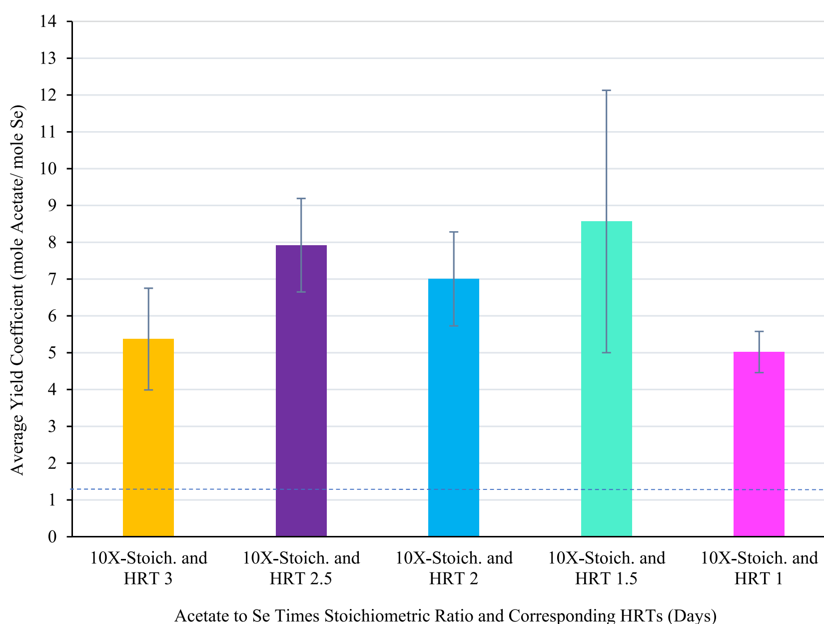

Fig. 7. Average yield coefficient for moles of acetate used per mole of Se removed at each HRT in presence of 10-times stoichiometric carbon source. The dashed line represents the theoretical yield coefficient based on balanced stoichiometric reactions. The error bars show the standard deviation for the averaged yields at each HRT.

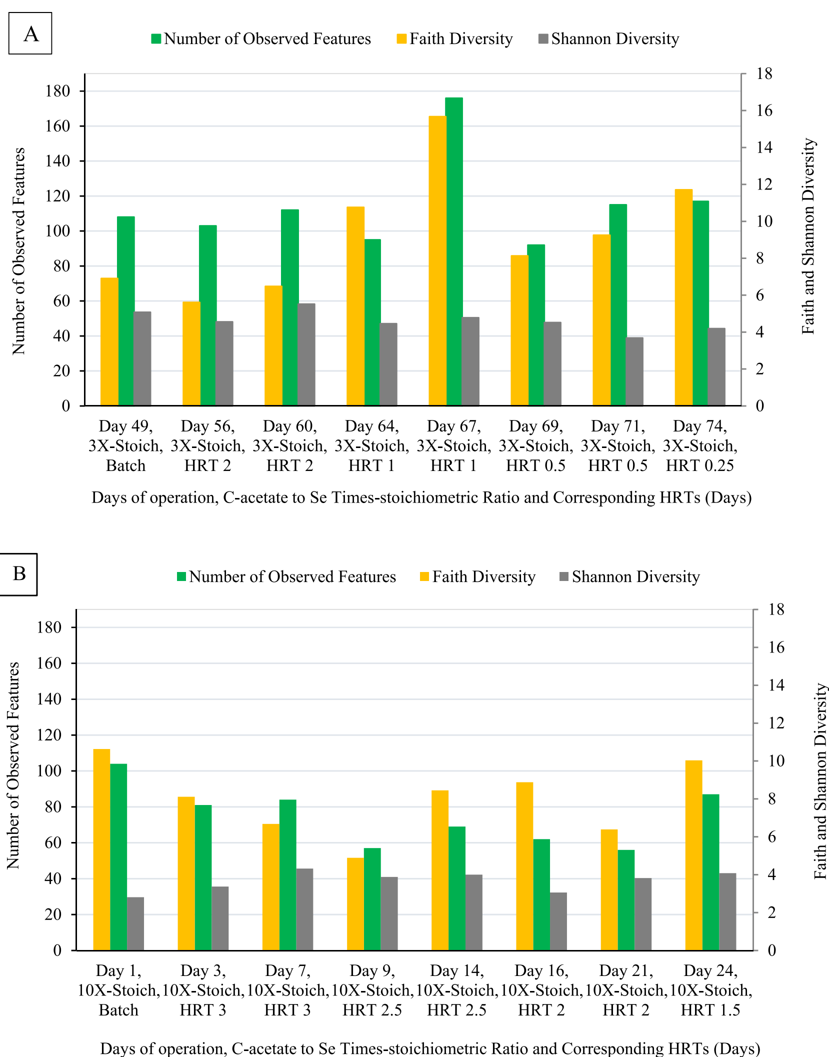

Fig. 8. Diversity indices versus time A) for Experiment 1 and B) for Experiment 2.

maximum on Day 1 at 10.6, then decreased to 4.9 on Day 9 and increased to a final value of 10 on Day 24 which was similar to Day 1. The Shannon index which is an indication of species richness and evenness in the community, was increasing and decreasing over the course of the experiments ranging between 2.8 and 5.5.

3.4. Microbial population composition

In Experiment 1, the first sample for DNA analysis was collected on Day 49. Between Days 49–60 in Fig. 9A when the HRT was 3 and 2 days, members of the *Moraxellaceae*, *Rhodocyclaceae, Comamonadaceae,* and *Clostridiaceae* families were dominant. Some *Moraxellaceae* were classified in the genus *Acinetobacter*. Members of the *Acinetobacter* genus are commonly present in soil, water, and sewage environments [28–30]. Some species such as *Acinetobacter* sp. VS3 are known for their ability to grow in selenate and selenite-rich environments [28]. After Day 64 and towards the end of the experiment, when the HRT was less than 1 day, *Dechloromonas* genus as well as some unclassified genera from the *Rhodocyclaceae* family were dominant. Multiple studies have reported the role of *Dechloromonas* species in the bioremediation of selenium oxyanions in the presence of acetate as electron donor [31,32]. The selenate reduction activity of these microorganisms is probably due to the similarity of its perchlorate reductase enzyme (pcrABCD) which is one nucleotide different from a selenate reductase (SerABC) [33]. In addition, metagenome-assembled genomes obtained from whole genome sequencing of samples collected from similar mine-impacted sediments in a previous study classified within *Rhodocyclaceae* family contained sequences for putative selenate reductases that were homologous with the NarG enzyme [34]. The NarG enzyme preferentially reduces nitrate, but it also has an affinity for selenate when nitrate is absent [14].

The *Comamonadaceae* and *Clostridiaceae* families were only dominant intermittently on (and potentially before) Day 49 and were not among the dominant families for the rest of the experiment. Family *Comamonadaceae* is in the beta-proteobacteria class and

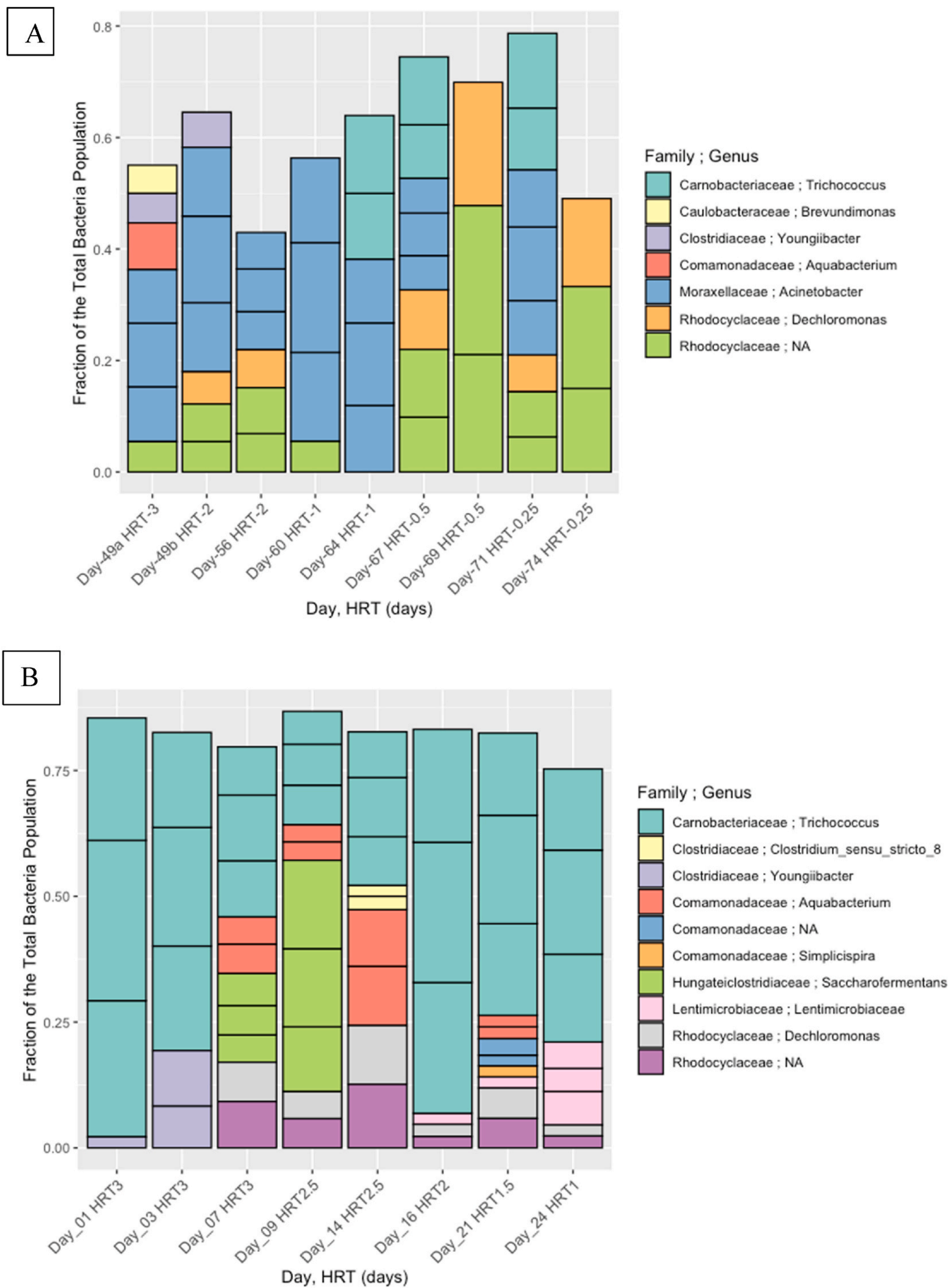

Fig. 9. Dominant ASVs (>2 % of the total bacterial population) families in the chemostat over time A) for Experiment 1 and B) for Experiment 2.

metagenome-assembled genomes for members of this family contained sequences for putative selenate reductases [34]. Some clades within the *Clostridiaceae* family are selenium dependent microorganisms that require selenium for the synthesis of selenoproteins that are needed for oxidoreductase pathways [35]. *Clostridium* sp. BXM from this family is capable of selenate and selenite reduction [36]. Some examples of selenium-dependent enzymes (selenoenzymes) from *Clostridiaceae* are glycine reductase protein A and B, proline reductase, purine hydroxylase, and glutaredoxin [40,41]. Dominance by organisms within this same clade (*Clostridium* sensu stricto 13) was observed also by Ref. [16] in their study in which the same source of sediment inoculum was used and was demonstrated to

remove dissolved Se-selenate in the presence of nitrate in batch cultures [16].

At the beginning of Experiment 2, the only dominant family was *Carnobacteriaceae* (Fig. 9B). This family is within the order *Lactobacillales* and the *Firmicutes* phylum that are commonly found in Se-contaminated mine sites [37,38]. After two days, the *Clostridiaceae* family became abundant in the community that was also dominant in Experiment 1. Between days 7 and 14, *Rhodocyclaceae* and *Comamonadaceae* families were abundant in the chemostat which were also abundant in Experiment 1.

This study was one of the few studies that were conducted in a continuous bioreactor by solely relying on the suspended growth of microorganisms in the bioreactor. In this study, the effect of influent Se concentration was investigated on its removal extent using a consortium of organisms that was collected from a mine-influenced environment and enriched in the laboratory instead of a pure culture. The use of a bacterial consortium that contains a variety of bacteria that are capable of selenate and/or selenite reduction is advantageous to pure cultures due to resistance of the microbial community to changes in the influent water chemistry. To the best of authors' knowledge, one kinetic study was conducted in a chemostat using a pure culture of *Bacillus* Sp. SF- 1 to study the kinetics of selenate and selenite removal [13]. The use of chemostat bioreactor is especially recommended for kinetic studies because it makes it possible to study the intrinsic kinetics of total dissolved Se removal when it can be assumed that there is no mass transfer limitation.

While there are advantages to using a bacterial consortium, it does introduce certain limitations, particularly in the context of microbial community analysis. Exploring microbial composition in diverse environmental samples presents significant challenges. These samples often contain an immense variety of microbial species, some of which may be present in low abundance or have specific growth requirements, making detection and characterization difficult. DNA extraction and sequencing of such samples generates massive datasets which require complicated bioinformatics analysis. Sample variability and the presence of unknown microorganisms, further contribute to the complexity. Despite ongoing technical advancements, the exploration of microbial diversity in environmental samples remains a scientific challenge. To address these challenges, DNA studies in this research were restricted to samples obtained from the chemostat, as they were anticipated to have a relatively limited microbial diversity. Taxonomic characterization of the enriched microbial consortia from the same mine-influenced site is reported in Ref. [16].

4. Conclusions

This study investigated the removal of total dissolved Se with 25 and 1 mg/L feed Se-selenate in a chemostat. The two experiments were conducted with different Se, carbon source stoichiometric ratios, and inoculums (same source but grown in different batch bottles). The following observations were made.

1. Proof-of-concept was provided for the use of a chemostat bioreactor as a laboratory tool with suspended biomass and well-mixed conditions in order to study intrinsic biological removal of Se-selenate in the absence of mass transfer limitations.
2. In two experiments with feed Se-selenate concentrations of 25 and 1 mg-Se/L, Se was removed to 30 μg/L and 1 μg/L, respectively. The removal percentage was 99.8 % at 25 mg/L and 99.9 % at 1 mg/L Se-selenate. This is a significant contribution because the total dissolved Se concentration in the feed of industrial bioreactors is highly variable.
3. The yield of carbon source consumed per amount of dissolved Se removed, $Y_{C/Se}$, was investigated for Experiment 2. The experimental yield was 4–7 times higher than the theoretical stoichiometric yield for all HRTs. This is a significant contribution for operation of industrial bioreactors as it provides information on the amount of carbon source that is required to achieve and maintain the removal of Se in bioreactors. Limiting amounts of carbon source can be inhibitory for microbial activity and too much carbon source can change the environment toward extremely negative oxidation-reduction potentials that result in permanently shifting microbial community toward sulfate reducing bacteria.
4. The bacteria that were collected from a mine-influenced environment and enriched in the laboratory were capable of growing and maintaining Se removal activities in a chemostat with HRTs as short as 1 day.
5. The study of microbial community compositions revealed that the composition of the community changes as a function of operating conditions (HRT and carbon source concentration) and over time. Some of the families such as *Rhodocyclaceae* and *Comamonadaceae* families were abundant in both experiments; otherwise, the families of microorganisms were different in the two experiments. A fraction of the bacteria was unclassified in the genus level for both experiments since the inoculum was started from an environmental source that possibly contained bacteria that had not been sequenced before.
6. The dominant microorganisms changed cyclically which is possibly due to the interactions between different families in the community; meaning that different populations can have facilitative or inhibitory effects on the growth of other populations.

[39].

CRediT authorship contribution statement

Elnaz Mohammadi: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Susan A. Baldwin:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no financial interests/personal relationships which may be considered as potential competing interests.

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