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TECHNICAL ARTICLE

Biotic and Abiotic Sequestration of Selenium in Anoxic Coal Waste Rock

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Abstract

Mobile selenium oxyanions ($Se^{VI}O_4^{2-}$ and $Se^{IV}O_3^{2-}$) can be sequestered by biotic or abiotic reduction to non-mobile species or by adsorption to mineral surfaces. Microbial analyses and geochemical batch testing with samples collected from a coal waste rock dump in the Elk Valley, British Columbia, Canada were conducted to assess whether Se can be sequestered in anoxic, waste rock by these mechanisms. Bacteria that reduce Se(IV) and Se(VI) to Se(0) were isolated from the waste rock. Isolates that reduce Se(IV) to Se(0) were present in a water sample collected from an underlying rock drain. Three isolates were affiliated with *Pseudomonas* and *Arthrobacter*. One isolate was a putatively novel species. The production of Se(0) was confirmed by X-ray absorption near edge spectroscopy of a red precipitate isolated from a broth media containing rock-drain water. No adsorption or reduction of Se(VI) was observed in anoxic, abiotic (sterile) batch tests conducted with waste rock and a 1.0 mg/L Se(VI) solution, whereas Se(IV) was adsorbed by the waste rock and subsequently reduced to Se(0) in abiotic batch tests with a 0.7 mg/L Se(IV) solution. In non-sterile batch tests using waste rock and rock-drain water (0.39 mg/L Se(VI)), Se(VI) was biologically reduced to Se(IV), which was subsequently removed from solution by a combination of bioreduction, adsorption, and possibly abiotic reduction. This study suggests that, under anoxic conditions, Se sequestration in waste rock may occur via biotic reduction of Se(VI) to Se(IV) followed by adsorption of Se(IV) and abiotic and biotic reduction of Se(IV) to Se(0).

Keywords Abiotic batch tests · Selenate · Selenite · Adsorption · Reduction · Electron microprobe

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Introduction

Selenium (Se) is both an essential micronutrient for animal, plant, and human life and a toxic environmental contaminant at elevated concentrations (Lenz and Lens 2009). The toxic effects of Se, as well as its mobility and bioavailability in water (e.g. surface or ground water) are defined by its oxidation state. Selenium is typically present in surface water and shallow groundwater as the soluble, mobile oxyanions $Se^{VI}O_4^{2-}$ and $Se^{IV}O_3^{2-}$ (Dungan and Frankenberger 1999). The mobile nature of the Se oxyanions make aquatic environments, and the fish and wildlife associated with them, particularly susceptible to Se bioaccumulation and its concomitant toxic effects (Lemly 2004; Lenz and Lens 2009). Cases of Se contamination of fish and aquatic wildlife are often associated with irrigation drainage, landfill leachate, and wastes associated with the mining and processing of coal and metals (Lemly 2004).

Mobile species of Se can be removed from water by adsorption onto solids or precipitation when reduced

to insoluble species by abiotic or biotic processes. The Se(IV) oxyanion strongly adsorbs on minerals, such as clays (Bar-Yosef and Meek 1987), Fe(III)-oxyhydroxides (Duc et al. 2003; Rovira et al. 2008; Su and Suarez 2000), pyrite (Kang et al. 2011; Tachi et al. 1998), and siderite (Scheinost et al. 2008). The adsorption of the Se(VI) oxyanion occurs to a much lesser extent than the Se(IV) oxyanion, often via weak electrostatic bonds (Balistrieri and Chao 1987; Hayes et al. 1987). Once adsorbed, Se(IV) and Se(VI) can be reduced to Se(0) in abiotic reactions with Fe(II) or Fe(II)-bearing minerals (Charlet et al. 2007, 2012; Chen et al. 2009; Myneni et al. 1997). Removal of Se(IV) and Se(VI) oxyanions from solution by microorganisms via reduction to insoluble Se(0) and Se(-II), often referred to as bioreduction, occur by three primary mechanisms: (1) incorporation into amino acids or other essential compounds; (2) detoxification; and (3) respiration (Stolz and Oremland 1999). The most environmentally significant form of Se bioreduction is respiration, which occurs in the presence of electron acceptors, such as organic carbon and Fe(II), and the absence of more favourable electron acceptors, such as O_2 and NO_3^-, which can also be removed from solution by microorganisms (Herbel et al. 2000; Knotek-Smith et al. 2006; Maiers et al. 1988; Oremland et al. 1989; Stolz and Oremland 1999). Examples of this process have been observed in the Goddard Marsh and Fording River Oxbow in the Elk Valley, where Se is sequestered in the sediments as Se(0) and Se(-II), while the overlying water column maintains a Se(VI) concentration > 20 µg/L (Martin et al. 2011). Artificial bioreduction systems can also be created to treat Se-contaminated water (Lenz et al. 2008) or enhance abiotic immobilization systems (Knotek-Smith et al. 2006). Previous field studies have reported sequestration of mobilized Se by adsorption of Se(IV) onto Fe(III)-oxyhydroxides (Ziemkiewicz et al. 2011) and bioreduction of Se(VI) to insoluble Se species in a mine pit backfilled with water-saturated waste rock (Bianchin et al. 2013) and in unsaturated coarse coal reject piles (Kennedy et al. 2015). While these studies confirm that Se sequestration can occur within mine waste facilities, the sequestration processes (biotic and/or abiotic) and their possible underlying mechanisms were not investigated.

The Elk Valley, in southeastern British Columbia, Canada has been a major steelmaking coal mining region in Canada since the late 1960s (Goodarzi et al. 2009; Lussier et al. 2003). The Elk River, located in the Elk Valley (Supplemental Fig. S1), contains elevated concentrations of Se. For example, the concentration of Se at a monitoring station at the mouth of the Elk River (Fig. S1) has exceeded the British Columbia Ministry of Environment water quality guideline for aquatic life of 2 µg/L since the late 1990s (Beatty and Russo 2014; Swanson 2010). The dominant source of this Se load can be attributed to waste rock dumps at five coal mining operations in the Elk Valley (Fig. S1; McDonald and Strosher 1998; Wellen et al. 2015).

The primary sources of aqueous-phase Se in the Elk Valley waste rock are pyrite (FeS_2) and sphalerite (ZnS), which contain 21% of the solid-phase Se in the waste rock (Hendry et al. 2015). Oxidation of Se(-II)-containing pyrite and sphalerite by O_2 (Essilfie-Dughan et al. 2017), and possibly NO_3^- introduced to the waste rock during blasting (Mahmood et al. 2017), generates mobile Se(IV) and Se(VI) oxyanions that enter the Elk River (Day et al. 2012; Hendry et al. 2015). Excess carbonate minerals in the waste rock, including siderite ($FeCO_3$) and ankerite ($CaMg_{0.5}Fe_{0.5}(CO_3)_2$), maintain a circumneutral pH in the waste rock dumps (Biswas et al. 2017).

One possible management mechanism to control the discharge of Se-rich waters from the waste rock to receiving waters could be the establishment of anoxic zones. These anoxic zones could include mined-out pits filled with saturated waste rock or constructed water-saturated zones at the base of the oxic waste rock dumps through which the Se-rich drainage must migrate before discharging to receiving waters. The development and assessment of such Se management initiatives require an understanding of the potential Se sequestration mechanisms within the waste rock. As such, the objectives of the current study were to assess the potential for adsorption, abiotic reduction, and bioreduction of Se(IV) and Se(VI) in anoxic coal waste rock from the Elk Valley. These objectives were attained using sterile and non-sterile batch tests and culture-dependent microbial methods on samples of waste rock and associated rock drain water. The presence of bacteria capable of reducing Se(VI) and Se(IV) to Se(0) was confirmed using culture-dependent methods. Adsorption and abiotic reduction of Se(IV) and Se(VI) were quantified in anoxic, sterile batch tests. The minerals associated with sequestered Se were identified using aqueous and solid analytical techniques. In addition to assisting with the development and assessment of Se management initiatives at waste rock dumps in the Elk Valley, the findings of this study may be of value in controlling Se contamination from other sources, including irrigation drainage, landfill leachate, and waste associated with the mining and processing of metals. Furthermore, the methods described in this study are not specific to Se contamination or the mechanisms of Se sequestration. There are many environmental contaminants controlled by both abiotic and biotic controls that can be investigated using experiments designed to simultaneously detect biotic and abiotic reactions.

Materials and Methods

Site Description and Sample Characterization

Samples of waste rock and water from the waste rock drain outlet were collected from the West Line Creek (WLC) waste rock dump in the Elk Valley (Fig. S1). The dump was constructed at the Line Creek Mine between 1981 and 2012 (Mahmood et al. 2017; Villeneuve et al. 2017). As of 2011, it contained 210 M m^3 of waste rock, covered an area of about 2.7 km^2, and had average and maximum heights of 115 and 255 m, respectively (Villeneuve et al. 2017). Data presented by Mahmood et al. (2017) show that WLC has some "transient zones of O_2 depletion" but is mostly oxic, with O_2 concentrations greater than 10% by volume at most sampled intervals. Boulders segregate from the waste rock during end dumping and form a coarse permeable basal drain below the waste rock dump, referred to as a rock drain. The WLC rock drains convey O_2-rich water from upstream of the dump as well as water draining from the dump downstream to Line Creek, a tributary of the Elk River (Villeneuve et al. 2017). Between 2002 and 2012, the annual volume of water discharged from the rock drain was on the order of 1 M m^3. This annual water discharge was estimated to carry about 900 kg/a of dissolved Se out of the dump (Hendry et al. 2015). Currently the discharge water is being treated at the WLC active water treatment facility (Teck Resources Limited 2016).

A rock drain water sample was collected from the rock drain outflow in September 2014 as part of a routine sampling campaign. Key constituents of the drain water sample included (as mg/L): Ca, 302; Mg, 174; Fe, 0.09; Se, 0.39; SO_4^{2-}, 1032; NO_3^-, 21.1; and alkalinity (as $CaCO_3$), 256. The pH of the sample was 7.8. Although the dissolved O_2 content of the water sample was not measured at the time of sampling, the average O_2 content at the rock drain outflow was measured to be 73.3 ± 2.1% of saturation (mean ± standard deviation, n = 21; Sean Carey, McMaster University, personal communication) in June 2012. As such, the rock drain water sample was assumed to be oxic at the time of sampling and allowed to equilibrate under atmospheric conditions at laboratory temperature (22 ± 2 °C) prior to use in the experiments described below.

The solid waste rock sample tested in this study was collected in 2012 as part of a dump sampling program described by Hendry et al. (2015). It was collected from borehole LCO-WLC-12-02a at a depth of 53.7 m below the ground surface from a zone of oxic, unsaturated waste rock placed in the dump between 1990 and 1994 (Mahmood et al. 2017). The gravimetric water content of the sample was 4% (Barbour et al. 2016). Immediately after collection, the sample was vacuum sealed and stored at laboratory temperature prior to geochemical characterization by Hendry et al. (2015). The pyrite and sphalerite concentrations in the sample were determined to be 1265 and 415 mg/kg, respectively, using LECO analysis (Hendry et al. 2015). X-ray diffraction (XRD) analysis showed that siderite and ankerite made up 1 and 5% of the sample, respectively (Supplemental Table S1 in the Online Resource). The remainder of the sample was made up of quartz and clay minerals. As part of the current study, the sample was further characterized using electron microprobe wavelength dispersive spectroscopy analysis (WDS) to confirm the presence of ankerite, which is difficult to distinguish from dolomite using XRD. These measurements showed that the ankerite contained an average Ca:Mg:Fe:Mn cation ratio of 1.10:0.46:0.43:0.01 and the siderites an average Ca:Mg:Fe:Mn cation ratio of 0.03:0.09:0.87:0.01. The XRD and WDS analyses are presented in the Online Resource. Prior to testing and analyses of the solid sample, it was air dried and ground with an agate mortar and pestle. The ground sample had a specific surface area of 3.5 m^2/g as measured by the Brunauer–Emmett–Teller (BET) method.

In addition to batch testing of the waste rock sample, batch tests were also conducted on quartz to represent an inert solid. The quartz sample was prepared by grinding synthetic silica sand (99.7% SiO_2, U.S. Silica, Ottawa, Illinois, USA) with a tungsten carbide swing mill. The specific surface area of the ground quartz was measured to be 0.86 m^2/g using the BET method.

Overview of Methods and Analyses

Three sets of experiments were undertaken to investigate the biotic and abiotic controls on Se, as summarized in Table 1. Biotic and abiotic processes were assessed using standard culture-dependent microbial analyses and sacrificial, sterile batch testing, respectively. The combined effect of biotic and abiotic processes was investigated using non-sterile batch tests. Analyses conducted in each set of the experiments are presented in Table 1. Solution analyses included pH measurements, elemental quantification of Se, Fe, and Mn by inductively coupled plasma mass spectrometry (ICP-MS), NO_3^- quantification by ion chromatography (IC), and Se speciation quantification by high performance liquid chromatography (HPLC) coupled with ICP-MS. The methods' detection limit for Se, Fe, Mn, NO_3^-, Se(VI), and Se(IV) were 0.5, 2, 0.02, 50, 10, and 10 µg/L, respectively. Solid analyses included mineral characterization by XRD, semi-quantitative elemental analysis and collection of backscattered electron (BSE) images by electron microprobe energy dispersive X-ray spectroscopy (EDS), imaging by scanning electron microscopy (SEM), Se redox speciation by X-ray

Table 1 Summary of experiments conducted

Experiment	Technique	Conditions	Analyses
Microbial analyses			
Isolation and identification of Se-reducers	Culturing on agar media	22 ± 2 °C, oxic	16S rRNA gene sequencing
Confirmation of reduced Se	Culturing in broth media	22 ± 2 °C, oxic	XANES, EDS, SEM, XRD, ICP-MS
Sterile geochemical tests			
Control	Abiotic batch tests	33 ± 2 °C, N_2 glove box, sterile	ICP-MS, IC, plating
Se adsorption and reduction	Abiotic batch tests	33 ± 2 °C, N_2 glove box, sterile	ICP-MS, IC, HPLC, plating, XRD, EDS
Non-sterile combined biogeochemical tests			
Se(IV) and Se(VI) sequestration	Batch tests	33 ± 2 °C, N_2 glove box, not sterile	ICP-MS, IC, HPLC, plating, XRD, EDS

absorption near edge spectroscopy (XANES), and elemental quantification of Se by ICP-MS. Prior to ICP-MS analysis, solids were dissolved in hydrofluoric and nitric acids in a procedure modified from Jenner et al. (1990). Details of the XRD, EDS, and XANES analyses are provided in the Online Resource.

Se Bioreduction

The potential for Se bioreduction by the culturable microbial community in the waste rock and rock drain water was assessed using a culture-dependent method. Although not all the bacteria in the samples are culturable, culture-dependent methods make it possible to determine the capabilities of different microbial isolates. Sterile phosphate-buffered saline solution was used for sample suspension and serial dilutions used for culturing. The dilution range, which provided countable bacterial colonies, was pre-determined by plating the samples using eight tenfold serial dilutions. The fourth to eighth dilutions were selected to plate samples in duplicate on both Se(IV)- and Se(VI)-amended basal salt media (BSM). This medium was produced using a recipe modified from that described by Ike et al. (2000) and Maiers et al. (1988) and contained (in g/L): $(NH_4)_2SO_4$, 0.3; $CaCl_2{\cdot}2H_2O$, 0.2; $MgSO_4{\cdot}7H_2O$, 0.143; NaCl, 5.85; KH_2PO_4, 0.05; K_2HPO_4, 0.05; yeast extract, 0.5; 60% sodium lactate syrup, 1.9; agar, 15; and 1 mL trace mineral salt solution. The trace mineral salt solution contained (in g/L): H_3BO_3, 0.6; $Co(NO_3)_2$, 0.15; $CuSO_4$, 0.08; $MnCl_2{\cdot}4H_2O$, 0.99; and $Zn(O_2CCH_3)_2$, 0.35. After the medium was sterilized (single 20 min autoclave wet cycle, 121 °C, 120 kPa), 10 mL of filter-sterilized (0.02 µm, Whatman, Inc.) 100 mM Se(IV) or Se(VI) solution was added to the media to yield 1 mM Se. The Se(IV) and Se(VI) solutions were made with Na_2SeO_3 and Na_2SeO_4 salts (Sigma-Aldrich, St Louis, MO, USA), respectively. Cultured plates were sealed with plastic paraffin film and kept at room temperature for a minimum of 2 weeks prior to enumeration.

A common product of Se bioreduction is red elemental Se nanospheres (Stolz and Oremland 1999). The accumulation of the red nanospheres in Se-reducing colonies grown on solid media causes the colony or centre of the colony to appear red and allows for visual confirmation of Se bioreduction (Oremland et al. 2004). Four red colonies from Se(IV)-enriched BSM plates were subcultured on Se-free BSM plates to confirm the colonies were only red in the presence of Se. These bacterial colonies were subcultured on Se(IV)- and Se(VI)-enriched media to determine if they could reduce Se(IV) and Se(VI). Two bacterial colonies were isolated from each of the waste rock and rock drain water samples. These isolated bacterial colonies were subjected to DNA extraction using an EZ-10 spin column bacterial genomic DNA mini-preps kit (Bio Basic Canada, Markham, ON) following the manufacturer's recommended protocol. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using a 27F (5′-AGA GTTTGATCMTGGCTCAG) and 1492R (5′-GGWTACCTT GTTACGACTT) primer set, yielding an amplicon approximately 1400 base pairs long. The Econotaq plus master mix (Lucigen Corporation, Middleton, WI, USA) was used for a 50 µL reaction mixture and the final concentration of the primers was 1 µM. The thermal cycling profile was set according to the Econotaq recommended settings. The PCR amplicons were sequenced at Génome Québec (McGill University, Montreal, Canada). Identification and phylogenetic analyses were carried out according to Bondici et al. (2013). For taxonomic identification, the 16S rRNA gene sequences were compared to all the type strains within the Ribosomal Database Project (RDP) database (Cole et al. 2009). A phylogenetic tree was constructed by the neighbour-joining method using MEGA with 1000 bootstrap replicates (Tamura et al. 2011).

An enriched broth media was prepared to isolate red Se(0) particles for subsequent analysis. The media was created by dissolving the BSM ingredients, with the exception of the agar, in the drain water sample in two 500 mL high-density polyethylene bottles. The bottles were sealed

during incubation. To limit the potential toxicity impacts of the elevated Se(IV) concentrations, Na_2SeO_3 salt was added to the broth in two 2 mM increments and two 5 mM increments. After 28 days, a total of 15 mM of Se(IV) was added to each of the bottles. The media was then incubated an additional 14 days, transferred to 50 mL Falcon centrifuge tubes, and centrifuged at 4400 rpm for 20 min.

Abiotic and Non-sterile Batch Tests

All batch test solids, solutions, and bottles were sterilized to eliminate biotic processes from the batch tests. The ground waste rock and quartz samples were sterilized by exposing them to 30 kGy of ^{60}Co gamma radiation (Chemistry Dept., University of Saskatchewan) and autoclaving them in two 30 min dry (gravity, 121 °C, 120 kPa) cycles 24 h apart. Prior to autoclaving, the ground samples were split into 120 mg subsamples, transferred to glass crimp top batch test bottles, allowed to equilibrate with the atmosphere in a nitrogen (N_2) glove box (MBraun, < 100 ppmv O_2) for 2 days, and crimp-sealed with silicon septa. Dry autoclave cycles were used and the solids kept in sealed bottles during autoclaving to minimize oxidation of the minerals during the sterilization process. Concerns that the reduced minerals of interest in the solid waste rock sample may be oxidized during sterilization were addressed by performing the same sterilization procedures on pure-phase mineral samples of pyrite, sphalerite, and siderite. Characterization of these materials before and after sterilization confirmed that no measurable oxidation occurred. Details of the materials tested and analyses conducted are provided in the Online Resource. The autoclaved batch test bottles were reintroduced to the glove box prior to the addition of batch test solutions. The solutions were passed through a 0.02 µm syringe filter during addition to the batch test bottles. The bottles were then resealed with sterile septa. Multiple batch tests with identical solutions were conducted to allow separate bottles to be sacrificially sampled on each sampling day, thus eliminating the risk of contaminating the batch tests during sampling.

Batch tests were conducted in the N_2 glove box. The rock drain water and deionized water (Millipore Milli-Q 18.2 MΩ) used in the experiments were bubbled with N_2 for 1 h prior to being introduced into the glove box. Each abiotic batch test contained 120 mg of ground waste rock or quartz and 16 mL of one of five solutions: Se-free deionized water (control), deionized water with 1.0 mg/L Se(VI), deionized water with 0.7 mg/L Se(IV), deionized water with 86 mg/L Se(IV), or rock drain water. The Se(VI) and Se(IV) solutions were made using Na_2SeO_4 and Na_2SeO_3 salts, respectively. All solutions were adjusted to and buffered at pH 7 with 50 mM PIPES (1,4-Piperazinediethanesulfonic acid), sodium hydroxide pellets, and 1 M hydrochloric acid. The solid to solution ratio in these batch tests was 7.5 g/L, which is three orders of magnitude less than that of the coal waste rock dumps in the Elk Valley (Barbour et al. 2016). While batch tests with low solid to solution ratios do not properly reflect site conditions, they do provide a larger ratio of Se to solid surface while maintaining Se concentrations similar to those expected on site (Day et al. 2012). This results in reactions taking place over a longer period of time and a greater density of sequestered Se on solid surfaces. Similar solid to solution ratios have been used by Bruggeman et al. (2005), Kang et al. (2011), and Scheinost et al. (2008).

All sampling was conducted in the glove box. The days at which samples were collected and details of the sampling program are presented in Table 2. Immediately prior to sampling, each bottle was shaken, opened in the glove box, and, after most of the solid had settled out (generally after 30 min), the supernatant was decanted into a new 10 mL syringe and passed through a 0.2 µm filter. The pH of each sample was measured with an electrode (Thermo Scientific Orion ROSS ultra pH/ATC triode, accuracy: 0.03 pH units, precision: 0.01 pH units) after it was filtered but before it was split into subsamples for ICP-MS, IC, and HPLC analyses. Solution samples were removed from the glove box after

Table 2 Batch test sampling days

Batch test solution	Sample days
Control—no Se	0[a,b], 1[c], 4, 10[a], 22[c,d]
1.0 mg/L Se(VI)	0[a,b], 1[c], 4[a,b], 6, 14, 21[a,b], 30, 42, 60, 82, 100[b,c,d]
0.7 mg/L Se(IV)	0[a,b], 1[c], 3[b], 6, 10[a], 14, 20[b], 29[a], 41, 46, 60, 66, 81[b,c,d]
86 mg/L Se(IV)	0[a,b], 61[a,d]
Drain water (sterile)	0[a,b], 1[c], 4, 7, 14[a], 21[b], 30, 42[a], 47[e], 53, 60, 82, 100[b,c,d]
Drain water (non-sterile)	0[a,b], 1[c], 4[a,b], 7, 14, 21[a,b], 30, 42, 47[b], 53[b], 60[b], 67, 82, 100[b,c,d]

[a]Sampled in triplicate

[b]Sample analyzed by HPLC for Se speciation

[c]Sample plated to assess sterility/CFU enumeration

[d]Four samples combined for composite sample

[e]Sampled in duplicate

they were split into subsamples for analyses. Subsamples requiring preservation were immediately preserved after removal from the glove box while the remainder of the samples were kept sealed until analysis. On the final day (see Table 2), four bottles were combined into composite solution and solid samples. Selected solid samples were rinsed with degassed deionized water three times and air dried in the glove box. Dried solid samples remained in the glove box until analysis.

Plating was conducted on the abiotic batch tests sampled on days 1 and 81 or 100 to confirm their sterility. Approximately 1 mL of the solutions to be cultured was aliquoted into sterile 1.5 mL Eppendorf tubes prior to the solids settling. The samples, as well as two tenfold serial dilutions, were plated in duplicate on Se(IV)-amended BSM, Se(VI)-amended BSM, and Reasoner's 2A (R2A) media (Difco BD, Sparks, MD, USA). Plates were sealed with plastic paraffin film and incubated in the glove box for a minimum of 16 days prior to bacterial colonies counts. The absence of colonies on cultured plates was interpreted as confirmation of batch test sterility.

In addition to the sterile abiotic batch tests, one set of batch tests was conducted using non-sterile waste rock and rock drain water to simulate the potential effects of combined abiotic and biotic processes on Se removal. With the exception of sterilization and plating procedures, the non-sterile batch tests were conducted using the same preparation and sampling procedures as the abiotic batch tests. The ground samples were not irradiated or autoclaved and the rock drain water not filtered. The day 1 and 100 batch tests were plated using the same procedure as the sterile batch tests with the exception of increasing the number of plated dilutions from three to five to account for the higher number of bacteria present in the non-sterile batch tests. These non-sterile batch test results were compared to the abiotic test results to determine the impact of viable bacteria on the concentration of Se in solution.

Results and Discussion

Se Bioreduction

Plating on Se-amended media was conducted to determine whether viable bacteria capable of reducing Se(IV) and Se(VI) to Se(0) were present in the waste rock and drain water. Three days after cultivation, white bacterial colonies with red centres developed on the Se(IV)-amended plates cultured with the waste rock solid and the rock drain water. Bacterial colonies were also present on the Se(VI)-amended plates cultured with waste rock, but not on those cultured with the rock drain water. After 8 days, the colonies on Se(IV)-amended plates were mostly red while the colonies on the Se(VI)-amended plates were red in the centre and yellow on the edges. No colonies were observed on the Se(VI)-amended plates cultured with rock drain water, suggesting the absence of Se(VI)-reducing bacteria or a very small population of Se(VI)-reducing bacteria relative to the Se(IV)-reducing population.

Two colonies from each of the samples cultured on Se(IV)-amended media were isolated. Isolated colonies were sub-cultured on Se-free media, which resulted in the development of yellow colonies within 3 days for colonies originating from waste rock and 10 days for colonies originating from drain water. These results confirm that the colonies only appeared red when Se was available for reduction and that their growth was not dependent on the presence of Se. Isolates grown on Se-free media were sub-cultured on Se(IV)- and Se(VI)-amended media. Colonies grown on the Se(IV)-amended media were similar in size, shape, and colour to those originally selected for isolation. On Se(VI)-amended media, colonies originating from the waste rock sample were yellow with red centres after 8 days while colonies originating from drain water remained yellow, consistent with those that developed on the Se-free plates. The presence of viable Se(IV)- and Se(VI)-reducing bacteria in the waste rock sample and Se(IV)-reducing bacteria in the drain water sample suggest there is potential for Se(IV) and Se(VI) bioreduction to occur within the WLC waste rock dump, given the necessary nutrients and favourable geochemical conditions.

Sequence analyses of almost full-length 16S rRNA gene sequence showed that the isolates originating from the waste rock sample, WR1 and WR2, were closely related to species of *Erwinia* and *Pseudomonas*, respectively (Fig. 1). The isolates obtained from the drain water sample, DW1 and DW2, were closely related to species of *Arthrobacter*. Pairwise comparison of the 16S rRNA gene sequences (≈1400 base pairs) showed that the drain water isolates were 100% identical to each other. Percent identities of the three distinct isolates to the type strains present within the RDP database were as follows: WR1 97% to *Erwinia amylovora*, WR2 99% to *Pseudomonas brenneri*, and DW1 and DW2 99% to *Arthrobacter subterraneus* and *Arthrobacter tumbae*.

Several species of *Pseudomonas* originating from Se-contaminated environments can reduce Se(IV) or Se(VI) to Se(0) under a range of laboratory conditions (Macy et al. 1989; Oremland et al. 1989). *Pseudomonas stutzeri* can reduce Se(VI) to Se(IV) and Se(IV) to Se(0) similarly to the pseudomonad WR2 isolated and cultured in this study (Kuroda et al. 2011; Lortie et al. 1992). Also, *P. aeruginosa* (Macy et al. 1989), *P. fluorescens* (Garbisu et al. 1996; Steinberg et al. 1992), and *P. moraviensis* (Staicu et al. 2015) reduce Se(IV) to Se(0). Reduction of Se(VI) by some *Pseudomonas* species is inhibited by NO_3^-, which was present in the rock drain water sample. *Pseudomonas*

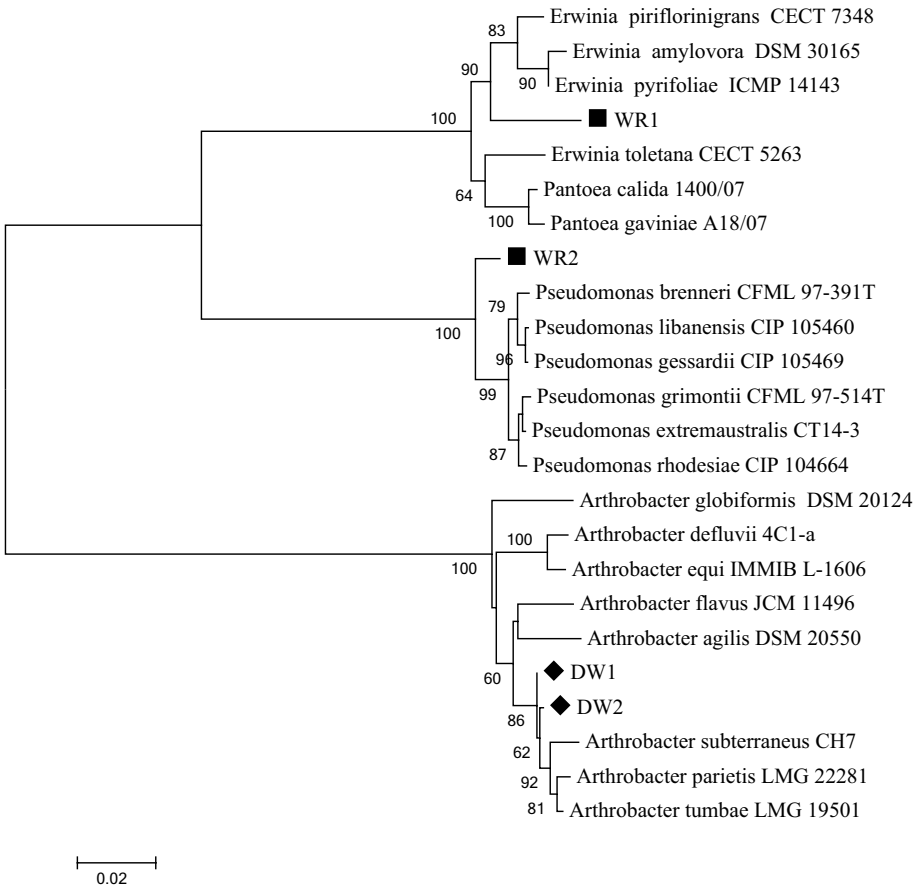

Fig. 1 Neighbour-joining phylogenetic tree of four bacterial isolate sequences and reference 16S ribosomal sequences. Bootstrap values greater than 60 are indicated at the nodes. Isolates were named according to the location of isolation: WR (waste rock), DW (drain water). Bar represents 0.02 substitutions per nucleotide position

fluorescens and some other pseudomonads can remove NO_3^- from solution by denitrification (Hunter and Manter 2009; Lortie et al. 1992; Steinberg et al. 1992).

In contrast to *Pseudomonas*, the ability of *Arthrobacter* to remediate Se-contaminated sites or to treat Se-contaminated waters has not been studied extensively. While several studies identified species of *Arthrobacter* in Se-reducing environments (Maiers et al. 1988; Yang et al. 2016), only two reported Se reduction by an *Arthrobacter* species. One *Arthrobacter* species isolated from a reservoir in California with low Se concentrations reduced Se(IV) to Se(0) but not Se(VI) (Burton et al. 1987). A second species isolated from a coal mine-affected marsh in the Elk Valley reduced both Se(IV) and Se(VI) to Se(0) (Yang et al. 2016). Although the latter species was retrieved from an environment similar to that of DW1 and DW2, it is unlikely to be the same species as DW1 and DW2, given the absence of Se(0) on the Se(VI)-amended media cultured with DW1 and DW2. Finally, we found no information on the Se tolerance or reduction capacity of *Erwinia*; the *Erwinia* genera consists of facultative anaerobic plant pathogens, such as *E. amylovora*, responsible for fire blight in fruit trees (Zhao 2014). Given this difference in capabilities and the marginal sequence homology (97% to known 16S rRNA sequences) for WR1 and *E. amylovora*, WR1 is a putatively novel species.

Broth media was prepared to allow the isolation of reduced Se precipitate from the media for analysis. The broth media appeared pink after 6 days and a red precipitate accumulated at the bottom of the bottles after 17 days. The addition of Se(IV) to the enriched broth resulted in the accumulation of additional red precipitate. The collected XANES spectrum, EDS spectrum, and SEM images corresponding to the red precipitate are presented in Fig. 2. Linear combination fitting analysis of the XANES spectrum (Supplemental Fig. S4 and Table S4) indicates that Se(IV), Se(0), and Se(-II) make up 70.2 ± 3.3, 13.5 ± 1.0, and 16.3 ± 1.1%, respectively of the Se present in the isolated red precipitate. The presence of Se(0) and Se(-II) shows that Se(IV) reduction did occur in the broth media. The Se(IV) content was attributed to the precipitation of $CaSeO_3$, which was supersaturated in the broth (Seby et al. 2001). The sub-micron particles visible in the SEM images (Fig. 2c, d) are of comparable size and shape to other Se(0) nanospheres (Hockin and Gadd 2006; Lenz et al. 2008; Oremland et al. 2004; Yang 2011). Furthermore, the largest peaks of the red precipitate XRD pattern (Supplemental Fig. S3) are consistent with trigonal elemental

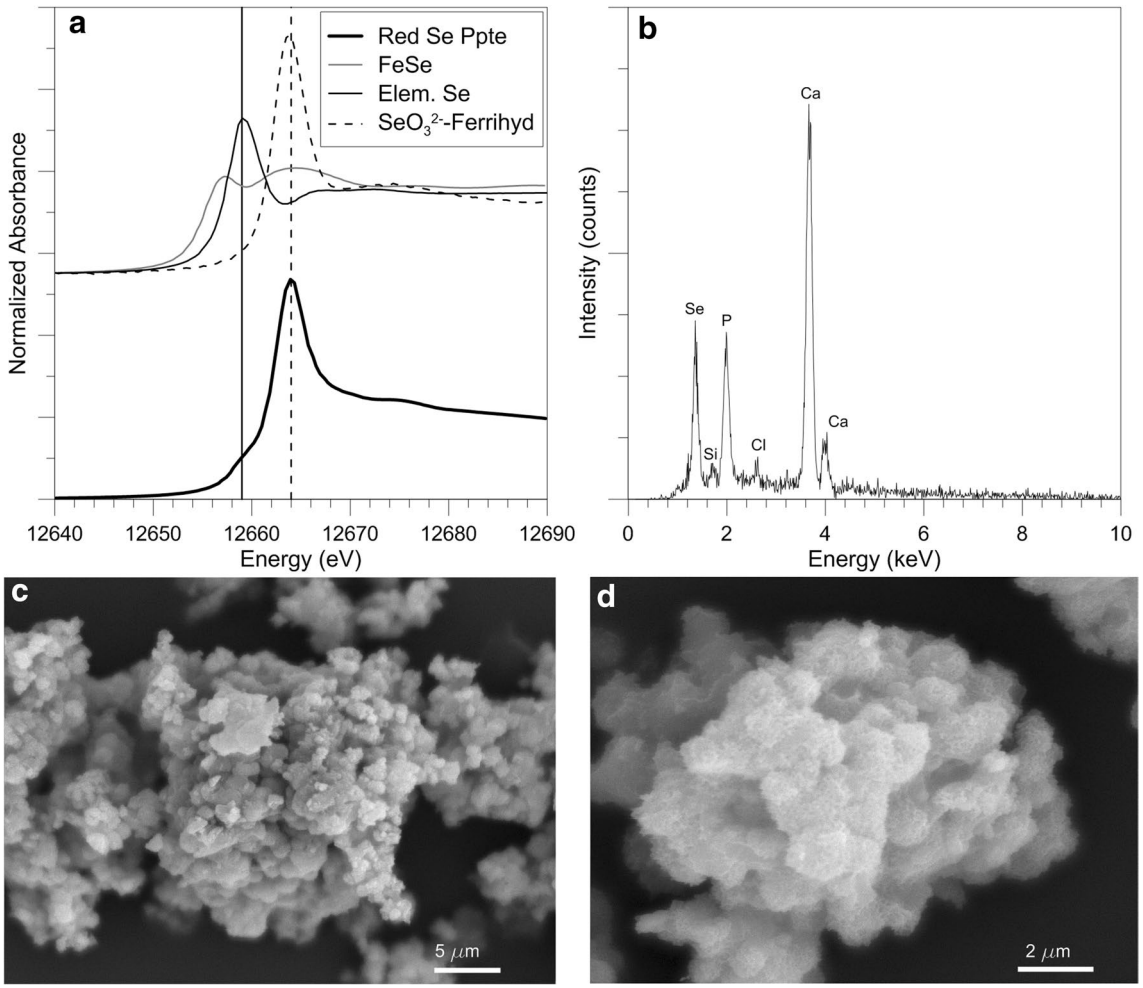

Fig. 2 XANES spectrum (**a**), EDS Spectrum (**b**), and SEM images (**c, d**) of a red precipitate isolated from the broth media

Se measured by Keller et al. (1977). The precipitation of Se(0) nanospheres in the broth confirms the red hue that developed at the centre of the bacterial colonies on Se-amended plate media was solid-phase Se(0) and that both Se(IV) and Se(VI) can be sequestered as solid Se(0) by bacteria present in the WLC waste rock dump.

In addition to being supersaturated with $CaSeO_3$, the broth was also supersaturated with calcite ($CaCO_3$), and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; Geochemist's Workbench Spec8, data not presented). The presence of these Ca and phosphate compounds is supported by the EDS spectrum (Fig. 2c) and the ICP-MS results, which determined that the digested precipitate was comprised of 20% Ca, 14.9% Se, and 6.8% P, by weight. There is no indication of these minerals or any other minerals in the XRD pattern (Supplemental Fig. S3). Conversely, XRD analysis of the Se(0)-containing bioreactor sludge by Lenz et al. (2008) suggests the presence of calcite and brushite ($CaHPO_4{\cdot}2H_2O$), but not Se(0). Although the form and importance of P and Ca in the precipitate is unclear, their co-precipitation with Se may act as a marker indicating precipitation of Se(0) when EDS is used to characterize sequestered Se on waste rock.

Abiotic and Non-sterile Batch Tests

The absence of colonies on the plates cultured from the control, 0.7 mg/L Se(IV), and 1.0 mg/L Se(VI) abiotic batch tests confirms that the batch tests were sterile. All but one of the plates cultured from the abiotic rock drain water batch tests were also free of colonies. Eleven colonies (the equivalent of 110 Se-reducing, colony forming units per millimetre; CFU/mL) with red centres grew on one of the Se(IV)-amended BSM plates cultured with the undiluted day 100 abiotic drain water batch test. The presence of Se(IV)-reducing colonies on the one plate has been attributed to contamination of the abiotic rock drain water batch tests during the addition of the filtered solution because the glassware and ground samples for all four sets of abiotic batch tests were sterilized together. Plating of the day 1 non-sterile batch test yielded 1.5×10^7 and 7.5×10^4 CFU/

mL on R2A and Se(IV)-amended plates, respectively. The R2A count decreased to 8.5×10^4 CFU/mL in the day 100 batch test and the Se(IV)-amended media count remained at 7.5×10^4 CFU/mL. These values are much higher than that of the contaminated abiotic batch test and, as such, changes in solution chemistry due to biological activity should be much more evident in the non-sterile batch test results. Furthermore, since not all abiotic drain water batch tests were expected to be contaminated, any biotic processes should yield inconsistent results when compared to the non-contaminated samples. The colonies that developed on the abiotic and non-sterile batch test-cultured plates were not investigated further nor was the cause of the absence of colonies on all Se(VI)-amended plates cultured in the glove box.

Abiotic batch tests conducted using inert quartz yielded no change in solution chemistry. In the quartz batch tests, the standard deviations of measured Se and NO_3^- concentrations were $<7\%$ of the mean when the batch test concentrations were more than two times the detection limit. The Fe and Mn concentrations were 0.069 ± 0.086 and 0.0078 ± 0.0029 mg/L, respectively. While variation in these values is greater than the sensitivity of ICP-MS, no patterns with time were observed and the mean values are much less than that of the waste rock batch tests (Figs. 3, 4, 5). These data confirm that changes in the solution chemistry of the waste rock batch tests can be attributed to reactions related to the waste rock.

The evolution of pH and the redox elements (i.e. Se, Fe, and Mn) measured in the Se-free (control), 1.0 mg/L Se(VI), and 0.7 mg/L Se(IV) batch tests on waste rock with time are presented in Figs. 3, 4, and 5, respectively.

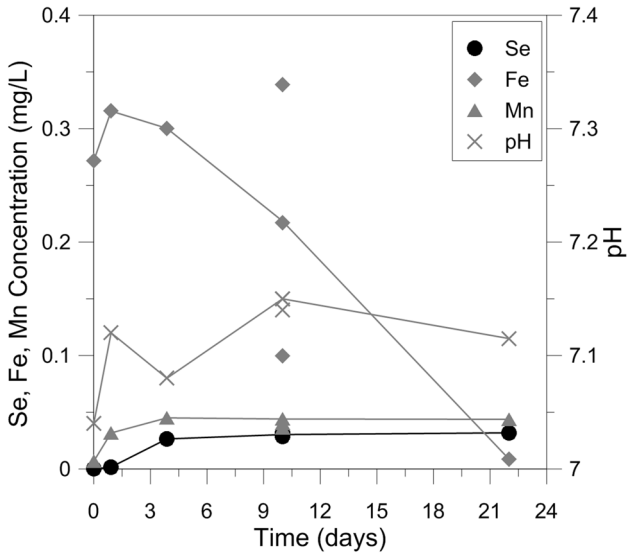

Fig. 3 Measured pH and Se, Fe, and Mn concentrations in sterile, Se-free (control) waste rock batch tests. Points represent single measurements. Lines pass through the average measurement of a given day

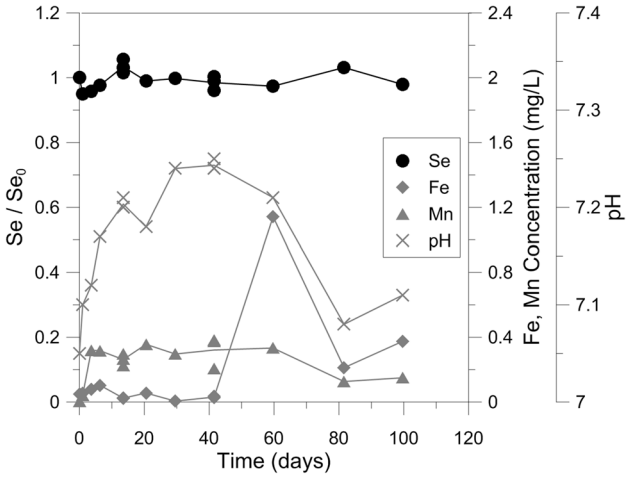

Fig. 4 Measured pH and Se, Fe, and Mn concentrations in sterile, 1.0 mg/L Se(VI) waste rock batch test solutions. Points represent single measurements. Lines pass through the average measurement of a given day

The solutions in each of these batch tests were prepared with deionized water and buffered at pH 7. The Se concentration in the control batch test solutions increased from 0.004 mg/L on day 0 to 0.030 mg/L on day 4. This increase was attributed to the dissolution of Se salts that precipitated in the waste rock pore space during air drying. No measurable change in Se concentration was measured in the 1.0 mg/L Se(VI) batch tests nor in the oxidation state of the aqueous Se. An increase of ≈ 0.03 mg/L relative to the initial 1.0 mg/L is less than the precision of the ICP-MS measurements. In the 0.7 mg/L Se(IV) batch tests, 40% of the Se(IV) was removed from solution on the first

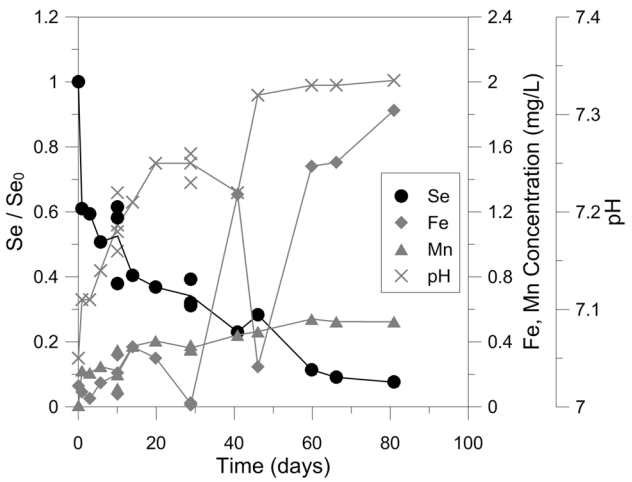

Fig. 5 Measured pH and Se, Fe, and Mn concentrations in sterile, 0.7 mg/L Se(IV) waste rock batch test solutions. Points represent single measurements. Lines pass through the average measurement of a given day

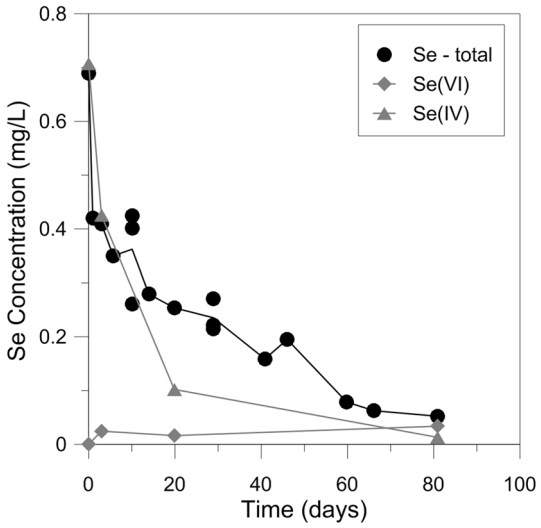

Fig. 6 Measured Se species concentrations in sterile, 0.7 mg/L Se(IV) waste rock batch test solutions. Points represent single measurements. Lines pass through the average measurement of a given day

day and the remainder by day 81 (Fig. 6). The concentration of Se(VI) increased initially and remained around 0.030 mg/L, following the same trend as Se in the control batch tests (Fig. 3). The initial uptake of Se(IV) by the waste rock is attributed to adsorption and the long term uptake is attributed to reduction from Se(IV) to insoluble Se(0). Siderite and pyrite remove Se(IV) from solution by these two mechanisms (Bruggeman et al. 2005; Kang et al. 2011; Scheinost et al. 2008).

In each of the waste rock batch tests, the increases in pH and concentrations of Fe and Mn in the first 2–4 days are attributed to dissolution of the carbonate minerals, siderite and ankerite. The solubility of Fe(III) is very low at neutral pH with the precipitation of goethite or other Fe(III)-oxyhydroxides (Appelo and Postma 2005). The cause of the decreasing Fe concentration with time in the control batch tests is not clear—it may have resulted from the oxidation of Fe(II) to Fe(III) by trace amounts of O_2 (Fig. 3). The increase in Fe concentration in the Se(VI) batch tests after day 42 is attributed to the reduction of insoluble Fe(III) to soluble Fe(II) and, thus, reflects a decreasing redox potential in the batch tests (Fig. 4). As such, the high concentrations of Fe in the Se(IV) batch tests relative to the control and Se(VI) batch tests suggest a lower redox potential and that Se(IV) was reduced to Se(0) (Fig. 5). After day 29, the increased Fe concentration was accompanied by an increase in pH and Mn concentration, confirming the source of Fe(II) and the associated low redox potential was the carbonate minerals in the waste rock. No explanation for the anomalous Fe concentration measurement in the 60 day 1.0 mg/L Se(VI) batch test (Fig. 4) was determined and, because of its anomalous nature, it will not be considered further.

The impact of sample heterogeneity and the precision of analyses on trends in pH and the concentrations of Se, Fe, and Mn are apparent in Figs. 3, 4, and 5, especially on days where batch tests were sampled in triplicate (see Table 2 for list of triplicate sampling days). In most cases where sampling was conducted in triplicate, variations in pH and elemental concentrations were not great enough to obfuscate overall concentration–time trends. One example of this is the day 10 triplicate Se and Mn measurements in the control batch tests (Fig. 3).

The Fe concentrations, however, varied greatly. The variation in Fe on day 10 was likely due to a combination of analytical error and differences in the rate of Fe(II) oxidation to Fe(III) in the three different batch tests. The effects of sample heterogeneity on reaction commencement time and rate are evident during rapid changes in solution chemistry, such as when Se(IV) was reduced to Se(0) and Fe(III) was reduced to Fe(II) (Fig. 5). In this case, the Fe concentration increased between days 29 and 41, decreased between days 41 and 46, and then increased again after day 46. The opposite trend was measured in the Se concentrations. These apparent fluctuations are attributed to differing reaction rates in the batch tests and not to fluctuations in any single batch test. While the use of identical, sacrificial batch tests can make it difficult to differentiate reactions from heterogeneity, it allows us to confirm that the batch test results are repeatable. For example, an equilibrium Se concentration of 0.032 ± 0.005 mg/L was measured in all six of the control batch tests sampled after day 3.

The sequestration of Se by the waste rock as Se(0) precipitate and Se(IV) sorbed to Fe minerals is supported by the EDS spectra (Fig. 7). In preparation for EDS, the waste rock was subjected to sterile, 61 day 86 mg/L Se(IV) batch tests, which resulted in the solid Se concentration increasing from 4.9 to 510 mg/kg. Given that over 99% of the Se on the analyzed waste rock was derived from the batch testing, all Se measured by EDS was assumed to be the result of Se sequestration rather than naturally-occurring Se in the waste rock minerals. In the BSE images corresponding to the EDS spectra (Fig. 7), relatively light elements (e.g., Si, Al, Mg, and Na) appear darker than the heavier elements (e.g. Se, Fe, and Ca). In Fig. 7a, two high-Se locations, C1 and C2, were present on a silicate (grey) mineral. At C1, Se was associated with Ca and P consistent with bioreduced Se(0) (Fig. 2a) indicating that Se(0) was present at C1. At C2, Se(IV) was sorbed to Fe minerals. The ratio of Fe to S at C2 suggests a combination of pyrite and siderite or an Fe(III)-oxyhydroxide. Given that Fe(III)-oxyhydroxides are a common secondary mineral associated with pyrite in the waste rock (Hendry et al. 2015), it is likely that one or more Fe(III)-oxyhydroxides were present at C2; however, we cannot confirm this statement because EDS analyses cannot identify the oxidation state of Fe nor can it detect

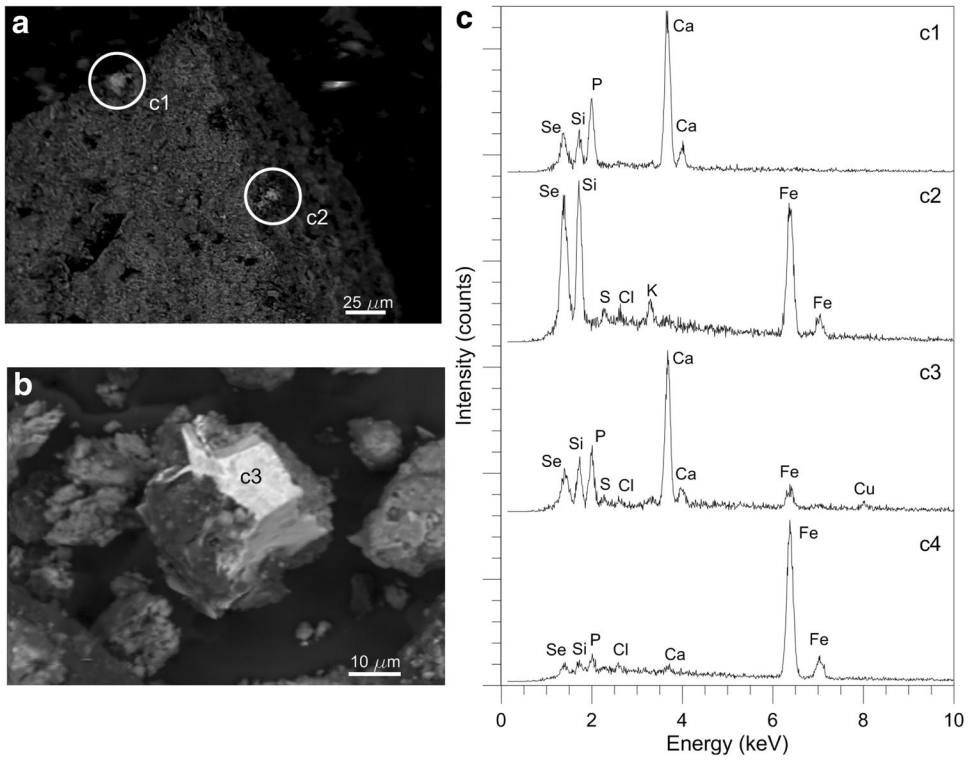

Fig. 7 BSE images (**a, b**) and EDS spectra (**c**) collected from waste rock reacted for 61 days in sterile 86 mg/L Se(IV) batch tests. The corresponding BSE image for EDS spectrum C4 is not presented

the presence of carbonate, hydroxides, or oxides. At C3 and C4, Se was associated with Ca and P, as well as the Fe-minerals chalcopyrite and siderite, respectively. This adds evidence to the hypothesis that reduction of Se(IV) to Se(0) by pyrite "involves an adsorption step" (Bruggeman et al. 2005). Adsorption and reduction of Se(IV) by chalcopyrite (C3) has been found to be very similar to pyrite (Naveau et al. 2007). Sequestration of Se at C4 is attributed to adsorption of Se(IV) and subsequent reduction to Se(0) by siderite, which was observed by Scheinost et al. (2008).

The initial Se(VI) concentration in the abiotic and non-sterile rock drain water batch test solutions was 0.39 mg/L. Consistent with the control and Se(VI) batch tests (Figs. 3, 4), the concentration of Se increased by ≈0.03 mg/L and there was no change in aqueous Se oxidation state (Fig. 8). In the non-sterile batch tests, all Se(VI) was reduced to Se(IV) between days 47 and 53 and 97% of the Se was removed from solution by day 67 (Fig. 9). The reduction of Se(VI) to Se(IV) is attributed to biotic processes given the absence of Se(VI) reduction in the abiotic batch tests. This is supported by the presence of Se(VI)-reducing bacteria in the waste rock sample isolated using culture-dependent methods. Confirmation of reduction of Se(VI) to Se(IV) in the non-sterile batch test solutions highlights the importance of abiotic sequestration of Se(IV) in waste rock dumps where aqueous Se is predominantly present as Se(VI). After the Se was reduced to Se(IV) in the non-sterile drain water batch tests, it was removed from solution much faster than in the

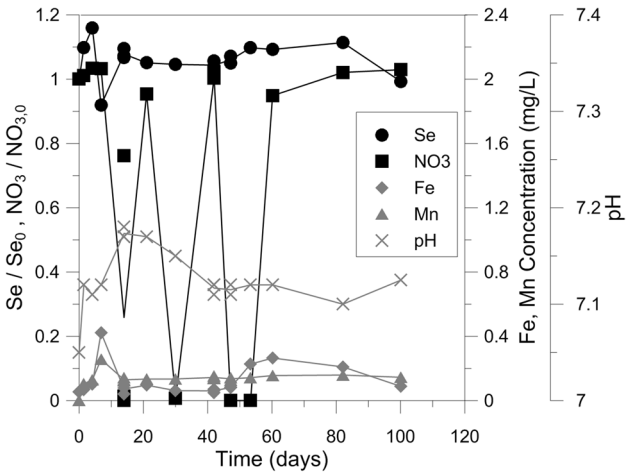

Fig. 8 Measured pH and Se, NO_3^-, Fe, and Mn concentrations in sterile drain water batch test solutions (0.39 mg/L Se(VI), 21.1 mg/L NO_3^-). Points represent single measurements. Lines pass through the average measurement of a given day

sterile 0.7 mg/L Se(IV) batch tests, suggesting that bioreduction of Se(IV) to Se(0) occurred concurrently with adsorption of the Se(IV) to the waste rock and abiotic reduction to Se(0).

A large shift in redox potential prior to Se removal was apparent in the non-sterile rock drain water batch tests (Fig. 9). The initial high NO_3^- concentration is characteristic of oxidizing conditions (Hockin and Gadd 2006;

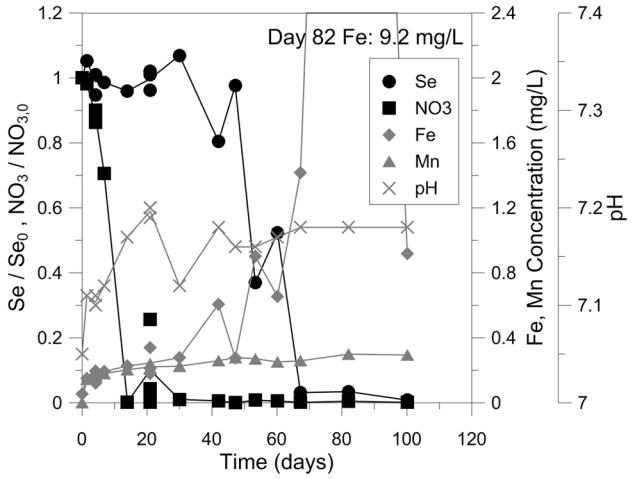

Fig. 9 Measured pH and Se, NO_3^-, Fe, and Mn concentrations in non-sterile drain water batch test solutions (0.39 mg/L Se(VI), 21.1 mg/L NO_3^-). Points represent single measurements. Lines pass through the average measurement of a given day

Masscheleyn et al. 1990) while the high Fe concentration after day 47 is characteristic of reducing conditions (Couture et al. 2015; Kang et al. 2011). The reduction of Se(VI) to Se(IV) only in the absence of NO_3^- is consistent with other studies that have shown that NO_3^- inhibits Se(VI) reduction and is preferentially reduced by several Se(VI)-reducing bacterial species (Oremland et al. 1989; Steinberg et al. 1992). Three of the Se-reducing isolates collected from waste rock and drain water samples are affiliated with NO_3^- reducers in the genera *Pseudomonas* (Steinberg et al. 1992) and *Arthrobacter* (Eschbach et al. 2003). Abiotic NO_3^- reduction by Fe(II) or organic carbon occurs very slowly or not at all unless in the presence of a catalyst (Parmentier et al. 2014; Picardal 2012); however, complete removal of NO_3^- from the drain water did occur in some abiotic batch tests (Fig. 8). Given the inconsistency of NO_3^- removal and the presence of bacterial contamination determined by plating, NO_3^- removal from the abiotic batch tests is attributed to biotic processes.

The delay in Se(VI) reduction in the non-sterile batch tests (47 days) relative to the Se(VI) reduction apparent on the waste rock-cultured Se(VI)-amended BSM plates (3 days) is attributed to the presence of NO_3^- in the drain water, as well as the absence of a carbon amendment, such as lactate. Given the presence of NO_3^-, the non-sterile batch tests should not be used for determining rates of microbial Se(VI) reduction. Further testing using columns that can allow a microbial community to reach equilibrium with anoxic, Se(VI)-laden, NO_3^--depleted water would allow reduction rates representative of anoxic coal waste rock to be determined. Columns packed with waste rock should have a solid to solution ratio representative of the WLC dump rather than the low solid to solution ratio used in this study's batch tests (7.5 g/L). While the non-sterile batch tests were not suitable for determining rates of Se(VI) bioreduction, their use in parallel with abiotic batch tests was very effective in demonstrating the mechanisms of Se sequestration that may take place in anoxic coal waste rock.

Conclusions

Laboratory experiments showed that biotic and abiotic sequestration of Se in coal waste rock from the WLC dump in the Elk Valley, Canada is possible under anoxic, water-saturated conditions. A bacterial isolate affiliated with the genus *Pseudomonas* and a putatively novel species that can reduce Se(IV) and Se(VI) to Se(0) were present in the solid waste rock sample. Further, isolates affiliated with the genera *Arthrobacter* that can reduce Se(IV) to Se(0) were present in water sampled from an associated rock drain. Under anoxic, abiotic conditions, 40% of the initial 0.7 mg/L Se(IV) was removed from solution in 1 day by adsorption to the solid waste rock sample and the remainder was removed by reduction to insoluble Se(0) by day 81. Adsorption of Se(IV) to pyrite, siderite, and/or Fe(III)-oxyhydroxide, and abiotic reduction of Se(IV) to Se(0) by siderite and chalcopyrite were confirmed with EDS. Conversely, under the same environmental conditions, Se(VI) was not removed from solution. Finally, non-sterile batch tests confirm that bacteria in the waste rock reduce Se(VI) to Se(IV) under anoxic conditions without the addition of nutrient amendments, which allowed subsequent bioreduction, adsorption, and abiotic reduction of Se(IV). Based on our findings, the sequestration of Se under anoxic conditions should be considered as a viable method to decrease the concentration of Se in water migrating through coal waste rock dumps in the Elk Valley before it discharges to the Elk River and its tributaries.

Although this study shows Se sequestration within the WLC dump is possible under anoxic, water-saturated conditions, additional research is warranted. This additional research could include characterizing the effects of greater solid to solution ratios on Se sequestration (i.e. the solid to solution ratios under in situ conditions are approximately three orders of magnitude greater than those tested) and the design and management requirements to develop and maintain anoxic zones within the dumps.

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