J Ind Microbiol Biotechnol (2006) 33:897–913
DOI 10.1007/s10295-006-0149-5

ORIGINAL PAPER

Microbial studies of a selenium-contaminated mine site and potential for on-site remediation

Heather M. Knotek-Smith · Don L. Crawford · Gregory Möller · Rachel A. Henson

Received: 1 November 2004 / Accepted: 17 May 2006 / Published online: 28 June 2006

Abstract Surface water Selenium (Se) concentrations are above regulatory standards at several active and inactive phosphate mine sites in the US Western Phosphate Resource Area. The focus of the present study was to examine the impacts of the microbial communities on the oxidation state of Se in overburden waste from the Smoky Canyon phosphate mine in Idaho, USA. Microbial populations were found that reduce soluble selenate (SeO_4^{2-}) to insoluble elemental Se. Microcosm experiments were conducted for molecular genetic analysis of this microbial community by rRNA gene profiling. An acetone pretreatment step was developed to remove interfering pre-petroleum hydrocarbons from the samples prior to extraction. PCR was used to amplify 16S and 18S rRNA genes present in the microbial community DNA. The amplified products were subjected to denaturing gradient gel electrophoresis (DGGE). Isolates and excised DGGE bands were amplified and sequenced for identification to determine the relative importance of culturable isolates to the total microbial population. Analysis of samples from different sites at the mine showed how Se contamination and previous remediation treatments changed the microbial populations across the site. Members of the family Enterobacteriaceae were dominant among the selenate reducing isolates from the site containing high Se levels. In particular, *Serratia fonticola* was isolated repeatedly from contaminated Smoky Canyon Mine site samples. Packed column studies were performed with seleniferous waste rock fractions from Smoky Canyon Mine. Column amendments consisted of combinations of iron, compost, and whey. Eh, pH, and extractable Se measurements were taken. Tests with infiltrated water showed columns containing an organic amendment combined with iron metal were the most resistant to Se leaching. Iron-based compounds from the corroding metal are thought to strongly bind the Se reduced by microbial activity, thereby stabilizing the Se in an insoluble form. We conclude that long-term stabilization of selenium at contaminated mine sites may require reductive microbial processes combined with abiotic immobilization by iron, either natural or engineered, to stabilize the Se and retard re-oxidation and release. Iron-selenide or iron-selenite compounds are more stable and resistant to leaching, especially when removed from active weathering.

Keywords Selenium · Microbial · Iron · Molecular · Remediation

H. M. Knotek-Smith
Department of Agricultural and Biological Systems Engineering, University of Idaho, Moscow, ID 83844-2060, USA

D. L. Crawford (✉)
Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, ID 83844-3052, USA
e-mail: donc@uidaho.edu

G. Möller
Department of Food Science and Toxicology, University of Idaho, Moscow, ID 83844-1052, USA

R. A. Henson
Environmental Science Program, University of Idaho, Moscow, ID 83844-3006, USA

Introduction

Selenium (Se) is an essential trace element for humans and animals but is toxic at high levels of exposure [6]. Se

has the lowest toxic-to-essential dietary exposure ratio (10) of any essential element [43]. The United States Recommended Daily Allowance (USRDA) for Se is 55 μg for the average adult or 0.8 μg/kg/day. The reference dose for chronic oral exposure (RfD) is defined as the amount of daily exposure to the human population, including sensitive subgroups, that is likely to be without an appreciable risk of deleterious effects during a lifetime [14, 15]. The RfD for Se is 5 μg/kg/day or 340 μg for a 68 kg adult. In the US, the maximum contaminant level drinking water standard for Se is 50 μg/L, and the U.S. Environmental Protection Agency (USEPA) national ambient water quality chronic criterion for aquatic life protection is 5 μg/L although there is a current proposal to monitor environmental Se using concentrations present in fish tissue.

In 1996, horses grazing on private land adjacent to the Caribou National Forest in southeastern Idaho were diagnosed with selenosis. Water for the horses and pasture irrigation had come from Maybe Creek, which contained Se concentrations in excess of regulatory water standards [65]. A phosphate mine overburden dump in Maybe Canyon was the source of the Se contamination. An assessment completed in the fall of 1997 showed that several of the region's active and inactive mine sites contained surface water Se concentrations that exceeded cold-water aquatic biota criteria for the element. Se was coming from waste rock shale and mudstones associated with the phosphate ore beds. This sedimentary mudstone rock had been used selectively for land reclamation due to its fertility.

The Smoky Canyon Mine (Fig. 1) is an open-pit operation in the Caribou National Forest in Caribou County, Idaho, USA approximately 6 miles due west of Afton, Wyoming. The mine is in a region called the US Western Phosphate Resource Area (WPRA). During open-pit phosphate mining operations, the topsoil is first stripped away and stockpiled for later reclamation of mined areas. After removing the topsoil, layers of ore and waste rock are removed from the pit using explosives and heavy equipment. The ore is transported via haul trucks to the processing facility while the waste material is separated and disposed of in a waste impoundment. Samples of waste rock types from the WPRA contain an average of 125 mg/kg selenium with a maximum of 1,500 mg/kg [28, 59]. As this waste rock shale is crushed, disturbed, and relocated, it becomes exposed to air and moisture, which subject them to natural oxidative weathering. This weathering promotes leaching of selenium by the rain and snowmelt that infiltrates these impoundments. The leachate often discharges near the bottom of the impoundment via a "seep" and enters nearby streams in this lotic

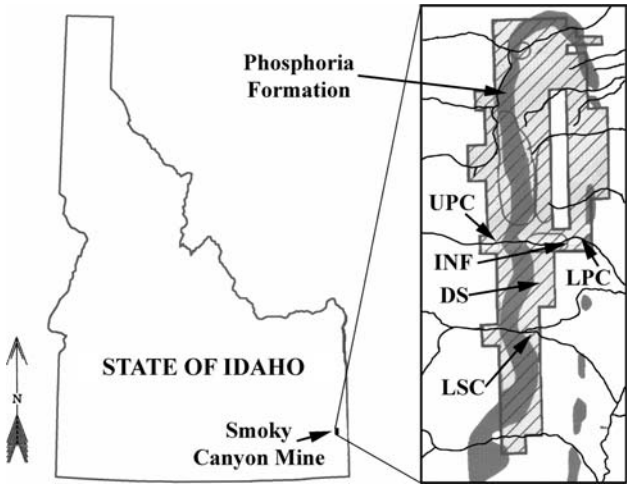

Fig. 1 State of Idaho map with Smoky Canyon Mine site. Local Phosphoria formation is shown in *dark gray* and sample locations are indicated by: *UPC* Upper Pole Creek, *LPC* Lower Pole Creek, *INF* Infiltration Trench, *DS* Deep Seep, and *LSC* Lower Sage Creek

environment. Removing or immobilizing selenium from these highly contaminated waste rock seeps is a priority for mining companies to prevent migration of Se from the mine site.

WPRA Se source materials have been shown to include a variety of geochemical types including iron-selenium compounds such as dzharkenite and Se-substituted pyrite, zero-valent Se, adsorbed and complexed selenium oxyanions, in addition to the expected organo-selenium compounds [53]. Selenium is chemically analogous to sulfur, as they are both Group VI elements. Like sulfur, Se resides in four oxidation states. The least mobile reduced forms of Se are selenide, Se(-II), and elemental Se, Se(0); the most mobile oxidized forms of Se are selenate, Se(VI), and selenite, Se(IV). Se in the Se(-II) oxidation state may exist as metal selenide or organoselenium compound, while Se(0) can reside in differing allotropic forms [2, 20]. In environmental samples, any or all of the four Se oxidation states may be present, each with its unique mechanism of retention [37]. The mobility, bioavailability, and toxicity of Se are controlled by this chemical speciation, therefore it is desirable to determine the concentration of individual Se species in a sample rather than total Se content. Lipton et al. [31] reported that soluble selenate and phosphate-extractable selenite composed less than 10% of the total Se in two soils from their study area. They found 30–40% of the total Se in these particular soils was associated as selenide with organic matter. Fio et al. [17] demonstrated that selenate is not adsorbed to soil, whereas selenite is rapidly adsorbed. Adsorbed selenite is resistant to leaching but

represents a potential long-term source of Se to groundwater. The concentration and speciation of Se in soil depends on the dynamics of pH, oxidation-reduction potential (ORP), solubility, complexing ability of soluble and solid ligands, and reaction kinetics [18, 20, 56].

Soil microbial populations can change any or all of the soil system dynamics, as well as the oxidation state of Se, thereby making it difficult to characterize a system based solely on abiotic laboratory experimentation. Microbial studies, used in parallel with traditional chemical characterization, kinetic analysis, and soil science can assist in understanding the environmental variables controlling Se transformations. This information can then be used to develop a stabilization strategy that includes microbe-mediated reactions.

Elemental Se and selenides, including most organically bound selenium, are less soluble and are therefore less mobile in the environment. They are the predominant species at low pH, especially in waterlogged soil, rich in organic matter [18]. This finding would seem to make the goals of bioremediation quite simple: find organisms that lower the pH and the ORP; however, soil microbial community diversity and weathering processes combine to challenge natural and engineered stabilization strategies.

Microbial organisms are known that reduce soluble selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) to insoluble elemental Se(0) [34] and to Se(-II) in organic compounds such as dimethylselenide and selenoamino acids [32, 60, 69]. Respiratory reduction of selenate to elemental Se is a mechanism whereby bacteria in sediments remove Se from overlying waters [48]. For many organisms the reduction of selenate and selenite is a toxicity resistance mechanism, though some will actually use the selenate as an electron acceptor for energy production [49]. The numbers of selenate-reducing organisms are higher in Se-contaminated environments, but they can also be found in environments that have not experienced Se stress [23].

Elemental Se produced by reduction is thought to be stable in the environment; however, there has been documentation of organisms that oxidize reduced forms of Se including elemental Se [35], selenite [20], and copper selenide [62]. These oxidations occur at relatively slow rates when compared to the reduction reactions [61].

The goal of this research was to characterize the microbial populations present in the Se-impacted environment at the Smoky Canyon Mine. Specific objectives were identification of cultivable indigenous Se reducers, evaluation of various amendments for enhancing Se reduction rates in soil, and improving overall resistance to leaching and environmental mobilization.

Materials and methods

Sample sites

The Smoky Canyon Mine site and the associated sample sites are shown in Fig. 1. The sample sites consist of the control sites, Lower Sage Creek (LSC) and Upper Pole Creek (UPC). LSC, located in the southern portion of the mine site, sits downstream of a culvert and fish ladder that were installed to allow the stream to flow through the site without coming into contact with the seleniferous waste impoundments or phosphoria formation. UPC, a variable-flow mountain steam located on the west side of the site, is upstream of any Se contamination. The Lower Pole Creek (LPC) site is located downstream of UPC after the creek has flowed beneath a canyon-fill, waste impoundment in a designed French drain that allowed flow beneath the mining waste rock impoundment. An infiltration trench (INF) was installed in the waste rock pile about 75 m before the French drain outflow of Pole Creek at the toe of the pile. This INF was a field test for large scale amendment and included a 1.5 m deep trench, 60 m across the almost 2 km-long and 150 m-high Pole Canyon waste rock dump. During this experiment iron metal powder (100 μm) and cheese whey (5% solids, pH 5, 230,000 L total) were added periodically for a one-month period to the excavated trench located above the Pole Creek outlet. During operation the infiltration trench lowered the amount of Se in the Creek from approximately 900 μg/L as selenate to 80 μg/L as selenite, however an increase back to original levels occurred within 60 d after amendment discontinuation (unpublished data). A sample from the trench was taken from the surface where past soil microbial growth was evident though the area had been partially dried. D-seep (DS) is a waste impoundment whose seep flows into nearby Sage Canyon; it was the location for an on-site subsurface bioreactor trial. The bioreactor consisted of a subsurface passive bioreactor/wetland made from a two-chamber, 16,800 L, polyethylene septic tank filled with cylindrical microbial support bio-rings that were 10 cm in diameter and 10 cm in length. Amendments of iron metal powder, whey, and compost were added to the first chamber via surface manholes. The bioreactor reduced an influent ranging from 250–1,000 μg/L, down to 10 μg/L at flow rate of 4.2 L/min over a period of several months (unpublished data). Samples were also taken from ponds and seeps above and below the bioreactor, as well as within the reactor, to identify the organisms present and to evaluate changes in microbial populations due to the bioreactor operation. In addition to

the site samples, two areas outside of Smoky Canyon Mine were sampled. These mining-impacted areas were the Wooley Valley wetland and Maybe Canyon stream [36]. At Wooley Valley, a wetland has formed at the base of an overburden dump. It reduces the influent Se concentration of 520 to 5 μg/L over a distance of 250 m [57]. Geochemical analysis at this site shows that the Se is associated with the ferrihydrite. Samples from this site were taken at the head of a flowing seep. The Maybe Canyon stream samples were taken from the streambed near a runoff seep. Se-contaminated seepage from an overburden dump, with levels around 1,800 μg/L, runs directly into this stream, which was the source of contamination for the previously described livestock selenosis events.

Microcosm experiments

Site soil samples (50 cc) were incubated at 12°C for 8 months in 500 mL beakers with 250 mL overlaying simulated site water to increase the microbial population numbers for molecular genetic analysis. The formulation used for the overlaying water is given in Table 1. Components were added to 1 L deionized water and autoclaved before addition to microcosms.

Enrichment and isolation of selenium-reducing organisms

Enrichments were made using anaerobic and aerobic liquid media. Anaerobic media were prepared using a modified Hungate technique [27]. Media included tripticase soy broth (TSB), Luria broth (LB), and ATCC 1957 Geobacter medium (1957) [5]. An iron deficiency variable in organism enrichments was introduced by adding 10 μM bipyridine to the TSB and LB media (TSB-P and LB-P, respectively) [10]. Liquid enrichments were incubated at 12 and 37°C. Anaerobic enrichments were transferred using syringes and diluted in degassed 1 g/L sodium pyrophosphate solution. Enrichments were incubated for 1 week at 37°C and 1 month at 12°C. After incubation, 2 mL of the enrichments were sampled and centrifuged. The cell pellet was retained and stored at −15°C for further analysis. Enrichments that developed the characteristic red color of Se(0) were used to inoculate solid media, which were TSB, TSB-P, LB, LB-P, and 1957 media with added agar. The dilution spread plate method using 1 g/L sodium pyrophosphate was used to isolate individual microbial cultures [66].

Soil and sediment samples from the site were also inoculated directly onto solid media. Prior to plate inoculation, bacterial cells were separated from soil particulates by placing 1 g of soil in 4 mL of 1 g/L sodium pyrophosphate, vortexing, allowing to incubate for 1 h, vortexing again, allowing to settle for 10 min, and inoculating spread plates with 0.1 mL of supernatant. Solid media used were as described above in addition to quarter-strength TSA with 6 g/L of 60% sodium lactate solution (TSAL). Anaerobic isolations were completed in an anaerobic glove box with degassed sodium pyrophosphate solution. Plates were incubated at 12 or 37°C. Anaerobic plates were incubated in anaerobic jars with hydrogen and carbon dioxide generator envelopes and palladium catalyst (BD Biosciences, Franklin Lakes, NJ). Plates were incubated for 1 week at 37°C and 1 month at 12°C or until colonies appeared. To verify that the red coloration of the colonies indeed resulted from reduced Se, they were re-streaked on solid media without Se.

DNA extractions

Colonies and enrichments. Cells were lysed using a modified Triton-X extraction method as described by Oho et al. [47]. Cell pellets from enrichments and single colonies picked from isolation media were boiled in 100 μL 1% Triton-X-100 for 10 min. Samples were

Table 1 Microcosm overlay water components designed to model environmental conditions

	Dseep above reactor	Dseep reactor flume	Dseep below reactor	Dseep catch pond	Lower pole creek	Maybe creek	Inlet to wooly wetland
NaF (mg/L)	1.35	1.65	1.44	1.59	2.02	1.68	0.30
$CaCl_2 \cdot 2H_2O$ (mg/L)	56.0	54.0	56.0	122	5.91	13.9	8.80
KOH (mg/L)	8.03	3.71	3.92	7.50	0.35	1.68	7.32
$MgSO_4 \cdot 7H_2O$ (mg/L)	465	495	505	703	416	653	812
$NaSeO_4$ (mg/L)	1.38	1.22	0.69	0.42	1.99	4.53	0.49
$NaHCO_3$ (mg/L)	0.00	3940	0.00	1068	384	753	478
$CaCO_3$ (mg/L)	836	962	861	941	545	590	893
$NaNO_3$ (mg/L)	9.05	11.2	16.5	10.0	9.05	12.3	39.8
Na_2SO_4 (mg/L)	26.4	24.6	23.3	78.2	7.58	19.7	25.5
H_2SO_4 (mg/L)	0.29	0.36	0.28	0.48	0.12	0.19	0.30

then cooled in ice for 10 min and centrifuged at 15,000 rpm for 2 min. Then 1.5 μL of supernatant was used for polymerase chain reaction (PCR) amplification of 16S rRNA. Triton-X extracts were stored at −15°C for any further analyses required.

Columns and microcosms. The Se waste rock soils used in these studies had a high level of kerogenic hydrocarbons, rendering standard DNA kit extraction techniques ineffective. A pretreatment step was [26] developed to remove the pre-petroleum hydrocarbon based on the method of Schwab et al. [55]. Tests were done to evaluate the effectiveness and to optimize the technique [26]. Acetone was selected over other common petroleum extraction solvents such as NaOH and chloroform [54] because of its high volatility and solubility in water. The complete removal of the solvent was important because the effect of the presence of the other solvents in the sample on DNA extraction efficiency was unknown. The solubility of solvent in water was important because the microcosm samples were not dried prior to treatment. Samples that had been previously frozen could not be successfully DNA extracted, most likely because freezing lysed the cells, leaving the DNA exposed. This DNA was then damaged by the acetone. Acetone extraction was followed by DNA extraction.

In the final process, 2 g or 2 cc of sample was placed in a 15 mL disposable centrifuge vial with 10 mL of acetone. Samples were mixed by rocking them by hand for 10 min. The vials were then centrifuged at 6,000 rpm for 6 min. Acetone was decanted and sample was allowed to air dry over night. DNA was extracted from samples using a soil DNA extraction kit (Qbiogene, Carlsbad, CA) followed by an additional clean-up step using a PCR clean-up kit (MoBio Labs, Solana Beach, CA).

Polymerase chain reaction (PCR)

PCR was used to amplify 16S rRNA genes of extracted DNA samples utilizing universal bacterial primers and fungi-specific primers. The prokaryotic PCR mixture contained 5 μL of 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl [pH 9.0], 1% Triton X-100), 2.5 μL of 25 mM $MgCl_2$, 2 μL of 10 mM (each) deoxynucleoside triphosphate, 25 pmol of each primer, 1.5 μL extracted rRNA, 2 U Taq DNA polymerase (Gibco BRL), and up to 50 μL 18-MΩ-cm water. The nucleotide sequences of the primers used for PCR were 5′-GCT GCC TCC CGT AGG AGT-3′ (*Escherichia coli* 16S rRNA positions 338 to 355 [3] (338F)) and 5′-CCG TCA ATT CMT TTR AGT TT-3′ [*Escherichia coli* 16S rRNA positions 907 to 926 [29] (907R)]. A GC-rich 40-mer clamp was attached to primer 907R for denaturing gradient gel electrophoresis (DGGE) analysis with a nucleotide sequence of 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′ (907R-GC) [41]. The fungi PCR mixture contained (50 μL) contained each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 2.5 U of Taq DNA polymerase (Gibco BRL), and the PCR buffer supplied with the enzyme [11]. The nucleotide sequences of the fungi primers used for PCR were 5′-CTG GTT GAT CCT GCC AG-3′ (*Saccharomyces cerevisiae* positions 4 to 20 (Euk1A)) and 5′-ACG AGA CTT GCC CTC C-3′ (*S. cerevisiae* positions 502 to 516 (Euk516R)) [3]. A GC clamp sequence was attached to primer Euk516R with a nucleotide sequence of 5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3′ [11]. The PCR program for both primer sets included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 92°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1.5 min. During the last cycle of the program, the length of the extension step was increased to 7 min. Denaturation temperatures were reduced from standard temperatures to lessen the rate of Taq degradation. Some samples required secondary amplification to get sufficient product for DGGE analysis. Reamplification was done as described above with 5 μL amplification product as template. It was found that soil extract PCR mixtures required 0.1 mg/mL BSA for successful amplification.

Denaturing gradient gel electrophoresis (DGGE)

Amplified samples were subjected to DGGE (DGGE-2001 system, CBS Scientific Company, Del Mar, CA) with samples of amplified 16S rRNA from selenate-reducing isolates to evaluate possible isolation and amplification biases and to determine the relative importance of culturable isolates to the total microbial population [9, 30]. Electrophoresis was performed with 0.75 mm thick, 6% polyacrylamide gels (acrylamide to bisacrylamide ratio, 37.5:1) containing 40–80% denaturant for prokaryote amplification product and 30–50% for fungi amplification product, where 100% concentration is defined as 7 M urea and 40% deionized formamide. Gels were submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) at 60°C. Twenty-μL samples of PCR product were applied to individual lanes in the gel. The electrophoresis conditions were 60°C and 85 V for 18.5 h.

DGGE band sampling

DGGE bands were excised, resuspended in 10 µL of 18 MΩ-cm water and stored at 4°C overnight [52]. The supernatant was then used for PCR reamplification. Some samples required secondary amplification in order to obtain sufficient product for sequencing. Reamplification procedures were as previously described with 5 µL amplification product as template. The reamplified band sample was run on DGGE to verify purity, and the reamplified PCR product was used for 16S rRNA gene sequencing.

16S rRNA sequencing

Amplified 16S rRNA genes from isolates and band samples were cleaned using a PCR clean-up kit (MoBio Labs, Solana Beach, CA). The sequencing reaction mixture consisted of 5 µL cleaned PCR product, 1 µL 12.5 mM 907R primer, and 4 µL BigDye Terminator v3.0 Cycle Sequencing Ready Reaction (Applied BioSystems, Foster City, CA). The thermocycler sequencing program consisted of an initial denaturation step at 9°C for 5 min, followed by 25 cycles of denaturation at 9°C for 30 s, annealing at 50°C for 30 s, and extension at 55°C for 4 min. Sequenced samples were cleaned using gel filtration cartridges (Edge BioSystems, Gaithersburg, MD) and desiccated in a DNA speed-vac (Savant, Farmingdale, NY). Samples were sequenced by the Laboratory for Biotechnology and Bioanalysis (Washington State University, Pullman, WA) using a 3100 Genetic Analysis System (Applied BioSystems, Foster City, CA). The sequences obtained were compared with public DNA database sequences by using the Basic Local Alignment Search Tool (BLAST®) [44]. All identifications scored at least a 98% match on the database.

Packed columns

Packed column studies were run with mixed seleniferous footwall mud and center waste shale (seleniferous waste) from the Smoky Canyon Mine. The seleniferous waste was dried, sieved using a No. 8, <2,380 µm sieve, and thoroughly homogenized prior to use. Characteristics of the resulting mixture are listed in Table 2 as analyzed by the Analytical Sciences Laboratory (University of Idaho, Moscow, ID).

Columns consisted of glass tubes 50 cm in height and 5 cm in diameter. They were constructed with six ports along the length of the column 7 cm apart. Eight test columns that contained the following amendment combinations were examined: no amendment control (C), compost (Cp), whey (W), compost and whey (CW), iron (I), iron and compost (IC), iron and whey (IW), and iron, compost, and whey (ICW). The columns were filled with 1,080 cc of seleniferous waste and amendments of 300 mL whey (Table 3), 100 g cast iron aggregate 8/50 mesh (Peerless Metal, Detroit, MI), and/or 300 cc compost (Table 4). Column components were placed in a 2 L beaker with 200 or 500 mL 18 MΩ-cm water in columns with or without whey respectively and mixed prior to column packing. Columns were packed by dropping 100 cc of mixed sample into the columns until all sample was added. A 10 cc sample of each column mixture was taken and analyzed for time-zero Se, pH, and Eh. Columns were incubated at 12°C for 1 month.

Table 2 Seleniferous waste characterization

Analysis	Value
pH	7
Phosphorus	26 µg/g
Potassium	58 µg/g
NO_3^-	15 µg/g
NH_4^-	1.9 µg/g
Organic matter	5%
SO_4^- S	75 µg/g
Boron	Below detection
Carbon	12%
Nitrogen	0.64%
Sulfur	2.9%
Selenium	30 µg/g
Cation exchange capacity	23 $cmol^+/kg$
Pore volume ratio	0.28

Packed column pH and Eh measurements

Eh and pH measurements were taken with micro-ORP (Ag/AgCl reference) and pH probes through the column ports (Microelectrodes Inc., Bedford, NH) using a portable pH/mV meter (Thermo Orion, Beverly, MA). All pH measurements were taken in triplicate and ORP measurements were taken in pentuplicate. ORP values were corrected to yield Eh.

Table 3 Composition of amendment whey

Analysis	Mass per 100 g
Acetic acid	74 mg
Citric acid	108 mg
Malic acid	ND
Pyroglutamic acid	10 mg
Succinic acid	161 mg
Lactic acid	3800 mg
Lactose	285 mg
Fat	0.16%
Protein	2.41%

Table 4 Composition of compost amendment

Analysis	Value
pH	5.8
Phosphorus	140 μg/g
Potassium	4700 μg/g
NO_3^-	Below detection
NH_4^-	6.9 μg/g
Organic matter	32%
Sulfate	50 μg/g
Boron	3.7 μg/g

Packed column sampling

Column sampling consisted of sliding the soil cores out of the columns and sampling the soil along the length of the column for DNA extraction, sequential extraction of Se, and for flow-through column experiments. Column samples were allowed to air dry and then crushed with a mortar and pestle prior to analysis.

Sequential extractions

A modified version of the sequential extraction procedure of Martens and Suarez [37] was used to determine species of soluble Se, species of Se adsorbed to the soil, Se associated with organics, and zero-valent Se. Sequential extraction was accomplished in four steps followed by a three-part speciation procedure. Two grams of sample were added to a disposable 15 mL centrifuge tube and mixed with 2 g washed sea sand (Fisher Scientific, Pittsburgh, PA). All sequential extraction samples were run in triplicate. Two blanks were run, each consisting of 4 g of washed sea sand. The first step of the procedure was a water extraction in which 10 mL of 18 MΩ cm water was added to the prepared sample, which was capped and shaken at 130 oscillations/min for 2 h at room temperature. After shaking, the tube was centrifuged at 3,000 rpm for 5 min and the supernatant decanted. The second step was a phosphate extraction in which 10 mL of 0.1 M (pH 7.0) K_2HPO_4-KH_2PO_4 was added to the pellet, which was then capped and shaken for 2 h at room temperature. The sample was again centrifuged and the supernatant decanted. The third step was a persulfate oxidation in which 10 mL of 0.1 M $K_2S_2O_8$ was added to the pellet, which was then capped and shaken for 2 h at 90°C and then centrifuged and decanted. The final extraction step was a nitric acid oxidation that consisted of addition of 1 mL 17 M nitric acid to the pellet, which was capped and heated to 90°C for 30 min. The samples were then removed from the heat, and cooled on ice; then 9 mL of 18 MΩ-cm water was added. The mixture was heated at 90°C for an additional 1.5 h then centrifuged and decanted.

Three 1 mL aliquots were removed from the water and phosphate extraction samples. The first was treated with 9 mL 50% v/v HCl and analyzed for selenite. The second was treated with 9 mL 50% v/v HCl followed by heating at 90°C for 30 min. Analysis of this second treatment measured the total selenite and selenate in the sample and thus selenate by difference. The third was treated with 1 mL of 0.1 M $K_2S_2O_8$ and heated at 90°C for 30 min followed by dilution with 9 mL 50% v/v HCl and additional heating at 90°C for 30 min. Se speciation was done on one set of the water and phosphate extraction samples to verify that there were no interferences in the analysis of total Se due to organics [15]. No interference was indicated because the final step in speciation resulted in the highest concentration of the three speciation steps. All later samples were analyzed using the final step in the speciation procedure ($K_2S_2O_8$ and HCl) as an analysis of total soluble Se.

Flow-through column experiment

Two grams of column sample was added to a disposable 15 mL centrifuge tube and mixed with 2 g of washed sea sand. This sample was placed in a 10 mL syringe plugged with cotton. The syringe was lowered into a 50 mL disposable vial for sample collection. Five pore volumes (10 mL) of 18 MΩ-cm water were added and collected every other day for 18 d. Columns were stored at 12°C during tests. All flow-through columns were run in triplicate. Two blank columns were run and consisted of 4 g of washed sea sand. Samples were collected, filtered by 0.45 μm syringe filter, and analyzed for total Se.

Selenium analysis

Sequential extraction and flow-through column samples were analyzed for Se content by flow-injection hydride generation atomic adsorption spectrophotometry [4, 70] using a Millennium Excalibur Atomic Fluorescence System (AFS) (PS Analytical, Orrington, Kent, UK) and the standard procedure for analysis of Se in water [50]. Additional total digested Se analysis by the University of Idaho Analytical Sciences Laboratory used the method of Tracy and Möller [63].

Results and discussion

Enrichment and isolation of selenium-reducing organisms

The purpose of the enrichment and isolation work was to qualitatively determine what Se-reducing organisms

were present at the site and how Se contamination impacted microbial populations in affected areas. We found 27 organisms that were capable of selenate reduction through enrichment followed by isolation on agar and formation of distinctly red monoclinic Se(0) (Table 5). Many of these organisms have been identified previously [39] [58]; however, 16 of the organisms we observed are newly identified as Se reducers.

These newly documented selenate-reducing organisms include *Aeromonas salmonicida* (*Aeromonas hydrophilia* was previously identified by Knight and Blakemore [25]). We also found *Bacteroides forsythus*, Carnobacteriaceae family member *Trichococcus pasteurii*, and Comamonadaceae family members *Aquaspirillum delicatum* and *Rhodoferax fermentans*. In the Enterobacteriaceae family, we documented *Acetobacterium malicum*, *Enterbacter amnigenus*, *Klebsiella pheumoniae*, *Morganella morganii*, *Rahnella sp*., *Serratia fonticola*, and *Yersinia intermedia* (many other Enterobacteriaceae family organisms have been identified as selenate-reducing [21, 22, 64]). We also identified *Dendrosporobacter quercicolus* (another Peptococcaceae family member *Desulfitobacterium* was previously reported by Stolz [58]) as a Se reducer). *Pseudomonas putida* was observed as a Se reducer in this work along with *Pseudomonas stutzeri* and *fluorescens*, two organisms that were previously identified by Lortie [33] and Ike [23] respectively. Rhizobiaceae family member *Agrobacterium tumefaciens*, Rhodocyclaceae family members *Propionivibrio limicola* and *Rhodocyclus tenuis*, and Sanguibacteraceae family member *Sanguibacter sp.* showed Se reduction capability. Our observations demonstrate that these organisms can reduce selenate under laboratory conditions; however, their ability to carry out this transformation in environmental or enrichment conditions remains in question.

The family Enterobacteriaceae was found in significant numbers in selenate-reducing isolates from the site in areas that had been exposed to high Se levels. In particular, the isolate *Serratia fonticola* was isolated and identified repeatedly in all three Smoky Canyon Mine site samples. The bioreactor microbial consortium was much more diverse, this is most likely due to whey and compost amendments from off-site sources.

We observed a plume of red forming in the solid media beneath many of the enterobacter colonies, an observation not previously reported. It appears that a compound excreted by the microbes reduced the selenate. It has been reported that cell-free extracts of *Pichia guillermondii* and *Micrococcus sp*. can reduce selenite but not selenate to zero-valent selenium [8].

Table 5 Selenate-reducing organisms identified by isolation on solid media

Sample site	Organism
D-Seep	Aeromonadaceae *Aeromonas salmonicida* (TSAL)
	Bacteriodaceae *Bacteroidales sp*. (TSAL)
	Enterobacteriaceae *Escherichia coli* (TSB)
	Enterobacteriaceae *Klebsiella pneumoniae* (TSAL)
	Enterobacteriaceae *Morganella morganii* (TSAL)
	Enterobacteriaceae *Serratia fonticola* (TSAL)
	Enterobacteriaceae *Yersinia intermedia* (TSAL)
	Oxalobacteraceae *Oxalobacter formigenes* (TSAL)
D-Seep reactor	Bacteriodaceae *Bacteroides forsythus* (TSAL)
	Carnobacteriaceae *Trichococcus pasteurii* (1957)
	Comamonadaceae *Rhodoferax fermentans* (1957)
	Comamonadaceae *Aquaspirillum delicatum* (1957)
	Enterobacteriaceae *Enterobacter amnigenus* (TSAL)
	Enterobacteriaceae *Rahnella sp* (TSB)
	Enterobacteriaceae *Serratia fonticola* (TSAL, 1957, LB)
	Eubacteriaceae *Acetobacterium malicum* (1957)
	Peptococcaceae *Dendrosporobacter (Clostridium) quercicolus* (TSAL)
	Pseudomonadaceae *Pseudomonas putida* (TSAL)
	Rhizobium *Agrobacterium tumefaciens* (1957)
	Rhodocyclaceae *Propionivibrio limicola* (1957)
	Rhodocyclus *Rhodocyclus tenuis* (1957)
	Sanguibacteraceae *Sanguibacter sp*. (TSAL)
Infiltration trench	Enterobacteriaceae *Escherichia coli* (TSB)
	Enterobacteriaceae *Morganella morganii* (1957)
	Enterobacteriaceae *Serratia fonticola* (TSAL)
	Pseudomonadaceae *Pseudomonas putida* (TSAL)
Wooley valley wetland	Comamonadaceae *Comamonas testosteroni* (1957)

1957 Geobacter media with agar, *LB* Luria Broth with agar, *TSAL* 1/4TSA with lactose, *TSB* TSB with agar

What we observed may be a sequential reduction in which the selenate is reduced to selenite via intracellular mechanisms and then excreted into the media, where it is then reduced by an extracellular compound similar to those found by Bautista and Alexander [8]. Alternatively, the factor may be an undocumented reducing compound. Lyophylized filtered samples of the liquid culture supernatant were incapable of selenate reduction, but this reducing compound may be oxygen sensitive. More characterization work is required before any conclusive statements can be made concerning this phenomenon.

An interesting finding was that no selenate reducers were found in non-contaminated (UPC) or control (LSC) areas. Also many selenate resistant organisms were present that we did not identify. Two resistant non-selenate reducing organisms from the UPC site were an Aeromonadaceae *Aeromonas salmonicida* and an Enterobacteriaceae *Yersinia intermedia*, both of which were identified as selenate reducers from the DS samples.

The characteristic red color of zero-valent Se was used as the only indicator of macroscopic selenate reduction and precipitation in these isolations. Selenium K-edge energies based on maximum of first derivative of XANES spectra for this microbially produced monoclinic red Se(0), 12,568.0 eV, were found to be the same as orthorhombic Se(0) [53]. The concentration of Se was not measured in the enrichment media. This may have caused us to miss enrichments that were reducing selenate to a lesser extent, or enrichments that formed reduced Se species other than the red Se(0). The goal of identifying highly active organisms in the consortia is well accomplished by this qualitative selection technique.

Denaturing gradient gel electrophoresis (DGGE)

The DGGE analysis of the enrichments is shown in Fig. 2 and the organisms identified by band amplification are shown in Table 6. In Fig. 2, enrichment samples are separated by media type. This was done to verify that there was no selection dominating the microbial populations due to media type. From these gels it is clear that the media were not selecting for specific organisms as evidenced by a scattered pattern of bands on the gels except in those lanes that were from the same sample site. Thus, the organisms enriched from the samples around the site can be compared in this figure. In general, the samples from UPC did not have as many Se-resistant organisms present as compared to the locations that had Se impact. This indicates that the mechanisms utilized by the organisms for

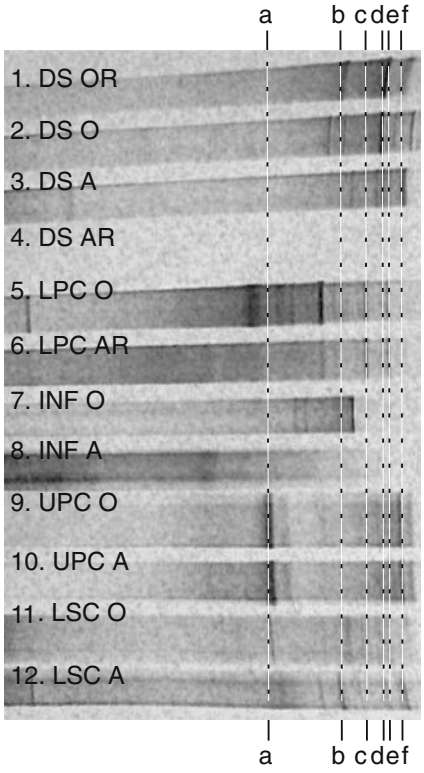

Fig. 2 Enrichment DGGE analysis by media type. Shown: trypticase soy broth with bipyridyl. Not shown: Luria Broth, Luria Broth with Bipyradil, and Trypticase Soy Broth. [*DS* D-Seep, *LPC* Lower Pole Creek, *INF* Infiltration Trench, *UPC* Upper Pole Creek, *LSC* Lower Sage Creek; *O* Oxic, *A* Anoxic, *R* selenate reduction; *a Aeromonas hydrophila*, *b Serratia fonticola*, *c Citrobacter werkmanii*, *d Bacillus subtilis*, *e Klebsiella ornithinolytica*, *f Aeromonas salmonicida*]

Se resistance probably require energy and organisms selected for this trait do not persist at sites with background levels of Se. Enrichments of LSC showed a diverse microbial population resistant to Se possible due to relatively lower concentrations of Se present in this stream relative to other sample sites. The INF samples did not amplify in any of the anaerobic cultures, a result that may be due to the over-abundance of aerobes in the highly amended trench that was completely dry at the time of sampling. None of the enrichments from LSC or UPC reduced selenate to red Se(0).

The same enrichment samples shown in Fig. 2 are reorganized in Fig. 3 based on sample site. This figure shows a consistent band patterning for all media types. The enrichments that turned red due to selenate reduction to zero-valent Se were difficult to amplify due to Se precipitate in the samples. Only a few of the enrichments that contained the red color were successfully amplified, and in those samples amplification was weak. The red enrichments that did successfully amplify, Figure 3 lanes 7 and 9, can be used for

Table 6 Organisms identified by band amplification

Sample site	Organism
Upper pole creek	Aeromonadaceae *Aeromonas hydrophila*
	Bacillaceae *Exiguobacterium acetylicum*
	Bacillaceae *Exiguobacterium antarcticum*
	Bacillaceae *Exiguobacterium undae*
Infiltration trench	Enterobacteriaceae *Raoultella (Klebsiella) ornithinolytica*
Lower pole creek	Enterobacteriaceae *Citrobacter werkmanii*
	Enterobacteriaceae *Raoultella (Klebsiella) ornithinolytica*
D-Seep	Aeromonadaceae *Aeromonas salmonicida*
	Bacillaceae *Bacillus subtilis*
	Enterobacteriaceae *Serratia fonticola*
	Enterobacteriaceae *Escherichia coli* CFT073
	Paenibacillaceae *Brevibacillus sp.*
Lower sage creek	Aeromonadaceae *Aeromonas hydrophila*
	Aeromonadaceae *Aeromonas salmonicida*
	Bacillaceae *Exiguobacterium undae*
	Bacillaceae *Bacillus weihenstephanensis*
	Enterobacteriaceae *Raoultella (Klebsiella) ornithinolytica*
	Enterobacteriaceae *Serratia fonticola*

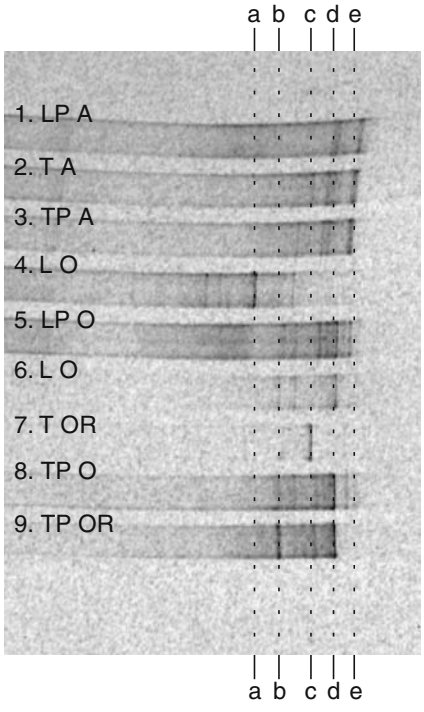

Fig. 3 Enrichment DGGE analysis by site location. Shown: *DS* D-Seep. Not shown: Lower Pole Creek, Infiltration Trench, Upper Pole Creek, and Lower Sage Creek. [*O* Oxic, *A* Anoxic, *R* selenate reduction, *L* Luria broth, *T* TSB, *P* bipyridyl; *a Brevibacillus sp*., *b Serratia fonticola*, *c Escherichia coli*, *d Bacillus subtilis*, *e Aeromonas salmonicida*]

comparison with the samples that did not turn red. This approach yields Se-reducing organisms that were active in precipitation of red Se. The Se-reducing organisms identified using these methods were the Enterobacteriaceae: *Escherichia coli a*nd *Serratia fonticola*. This finding is consistent with the high number of selenate reducers from the family *Enterobacteriaceae* found by isolation. Thus, results based on culturable isolates correlate well with those based on the DGGE characterization of non-culturable organisms. The results also show that organisms capable of selenate reduction under specialized isolated conditions many times do not carry out that function in enrichment or consortia conditions. We have not summarily shown that these organisms do not reduce selenate under environmental conditions.

Microcosm and packed column population studies

The purpose of the microcosm studies was to increase microbial numbers so that sufficient DNA was available for extraction and amplification. Analysis of the microcosms was used to compare microbial communities within the mine site, in other mine sites in the region, and in the laboratory column experiments. DNA was successfully extracted from all samples; however, amplification of the samples was not as successful. Despite the confirmation that DNA was present in the extracts, amplification was inhibited most likely due to the continued presence of humics or another interfering compound.

The samples that did amplify were the CW column at port 4 (CW4), Wooley Valley Wetland (WVW), and DS samples from above the bioreactor (ABR), the bioreactor outlet flume (BRF), and below the bioreactor (BBR). The DGGE analysis of these samples is shown in Fig. 4. The eukaryotic primer set amplified the DNA extracts very well as compared to the weak amplification of the prokaryotic primers that produced only faint bands in the WVW, DSABR, and DSBBR samples. A comparison of the CW4 sample to the other samples indicated that the predominant organisms in the CW4 sample were different from the microcosm samples. The WVW sample produced some bands that were similar to the DS samples on the eukaryotic gel but also some that were different. The DS samples changed from above the bioreactor (lane 3), to the bioreactor flume (lane 4), and ending with the bioreactor outlet stream (lane 5). This was expected, as iron, compost, and whey were added to the reactor. This should obviously shift the populations downstream of the reactor. It is interesting that the CW4 sample produced such different bands when compared to the DS samples. This indicates the effects of processing the samples in the laboratory, such as drying, mixing, and addition of amendments. The whey amendment added

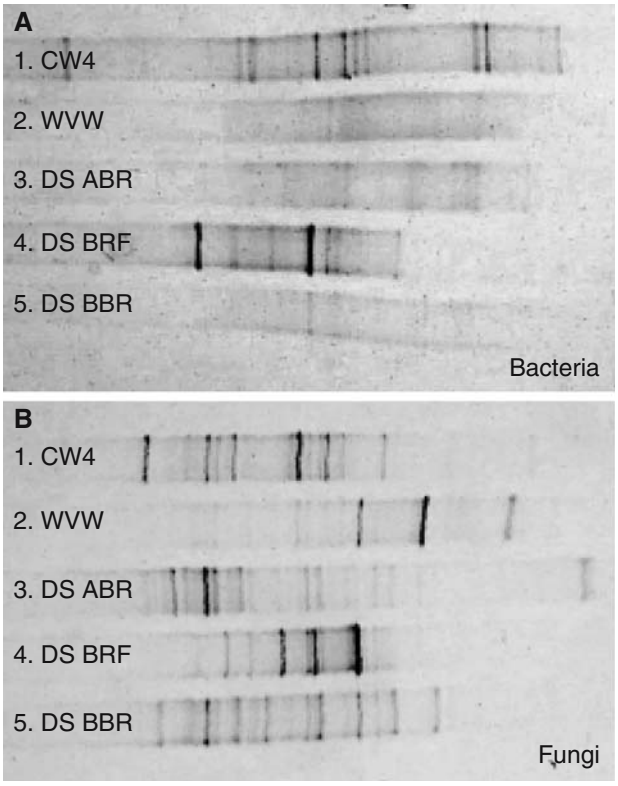

Fig. 4 DGGE analysis of Microcosm and Column DNA extractions **a** Bacteria amplified by 338F/907R-GC primer set, **b** Fungi amplified by Euk1A/Euk516R-GC primer set. [*CW4* Compost Whey Column port 4, *WVW* Wooley Valley Wetland, *DS* D-Seep, *ABR* Above Bioreactor, *BRF* Bioreactor Outlet Flume, *BBR* Below Bioreactor]

to the columns was the same as that added to the reactor except that the whey had been stored at 4°C for 1 month prior to use. Different compost products were added to the field reactor and the laboratory columns. It is expected that the primary source of community difference is the processing of the seleniferous waste, a process that involved drying, crushing, and mixing. These laboratory manipulations could have significant consequences as the possible anaerobic microsites in the soil would be exposed to air with the significant possibility of inoculation by soil grinding equipment. However, some of the organisms shown in the eukaryotic gel lanes 1 and 5 are similar. This similarity may be indicative of the ubiquitous nature of microbes and the selectivity imposed by carbon source. None of the organisms was successfully identified.

Packed column measurements

Time-zero measurements of the seleniferous waste with amendments are shown in Table 7. The pH profiles of the samples were: CW $\approx$ ICW $<$ W $<$ IW $<$ Cp $<$ IC $<$ C $<$ I, indicating that the whey amendment was the most significant factor affecting pH at time zero. The presence of iron increased the pH but not sufficiently to counteract the whey influence. The Eh profiles of the time-zero samples were: IC $<<$ ICW $\approx$ IW $<$ I $<$ W $\approx$ CW $<$ C $\approx$ Cp, indicating that as expected, the iron metal amendment reduced the Eh of the pore water. The soluble Se concentrations in the time-zero samples were: I = IC $<$ CW $<$ Cp = W = IW $<$ ICW $<$ C. This indicates that the iron compounds and reductive chemistry resulting from the spontaneous reaction of iron metal and water was reacting with the soil Se abiotically. Seleniferous waste was dried to ensure consistent components in each of the columns; however, drying of the sediments can significantly increase the amount of extractable Se [1]. The range of soluble selenium, 1.6 to 1.8 mg/L, is comparable to the higher levels found at DS during field monitoring.

The column pH and Eh measurements after 1 month of incubation at 12°C are shown in Fig. 5. The pH profile series for the columns was: W $\approx$ CW $<<$ Cp $<$ C $<$ IW $<$ ICW $<$ IC $<$ I. During the first week of incubation, the ICW and the CW columns generated large amounts of gas that displaced the seleniferous waste in the column, indicating the presence of gas-forming fermentative organisms. The IW and IC columns also produced gas but to a lesser extent. Fermentative organisms are indicated by the presence of acid. The circum-neutral pH profiles of the columns would seem to contradict the assumption that fermentative organisms were active, however iron chemistry may help explain the discrepancy. The presence of significant biologically generated acidity in the system would corrode the iron granules creating abundant iron salts and residual iron compounds. This may in turn autocatalytically decrease pH in the system through water hydrolysis and formation of iron hydroxide compounds

Table 7 Packed column time-zero measurements

	C	Cp	W	CW	I	IC	IW	ICW
pH	7.3 ± 0.12	6.8 ± 0.16	6.5 ± 0.04	6.3 ± 0.03	7.4 ± 0.14	7.1 ± 0.11	6.6 ± 0.04	6.3 ± 0.10
Eh (mV)	418 ± 17	429 ± 40	334 ± 4	345 ± 24	309 ± 24	69 ± 32	262 ± 42	243 ± 14
Se (μg/L)	1,830	1,610	1,610	1,560	1,170	1,170	1,610	1,670

Eh and pH measurements were taken in soil water and Se measurements from filtered supernatant

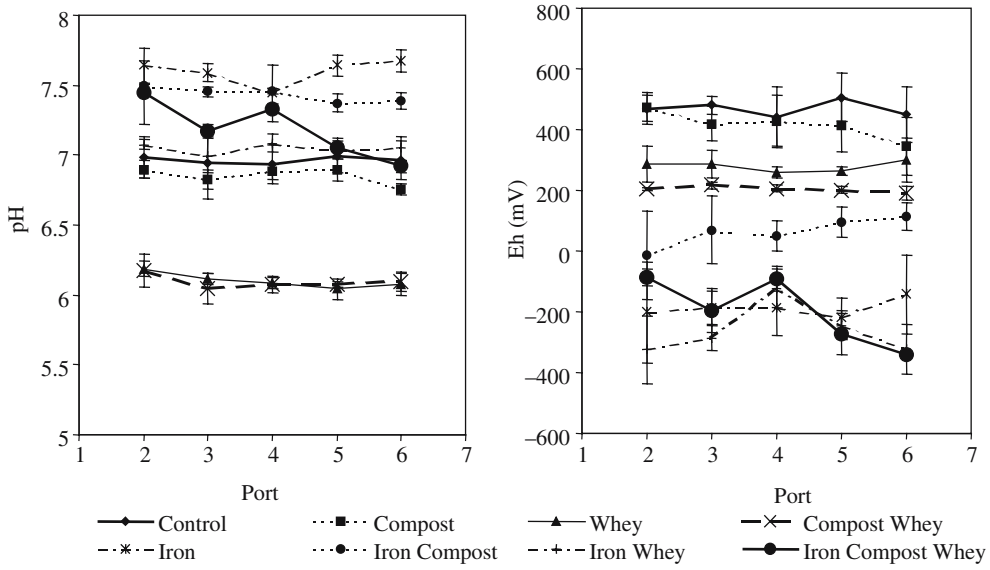

Fig. 5 Column profiles of pH and Eh (mV) after 1 month incubation at 12°C

such as ferrihydrite; natural buffering by the soil matrix in the column could diminish this chemical process significantly. The waste rock soils from the WPRA contain dolomitic limestone.

The Eh profile series of the columns was: IW ≈ I ≈ ICW < IC < CW < W < Cp ≈ C. The lowest ORP was present in the iron metal modified IW, I, and ICW columns. The W, IC, and Cp columns demonstrated Eh decreases relative to the control but to a lesser extent than IW, I, and ICW. The maximum decrease in Eh from the control column exceeded 750 mV for the iron-whey column. The measurements along the length of the columns were all within one standard deviation of one another.

Sequential extractions

Data from the sequential extractions is shown in Fig. 6. The profiles of the water extractions, which remove primarily selenate were: ICW ≈ IC ≈ IW < I < Cp < CW < C < W. The profiles of the phosphate extractions, which remove primarily selenite, were: ICW ≈ IW ≈ IC < CW < I < Cp < C. Selenite concentrations in the W column did not appear to correlate well, as it had the highest concentration near the top of the column and one of the lowest of all test columns for the middle and bottom sample. The profiles of the persulfate extractions, which remove primarily selenide, were: ICW ≈ I ≈ IC < CW < IW < Cp < C < W. The profiles of the nitric acid extractions, which remove primarily zero-valent Se, were: ICW ≈ I ≈ IW < IC < W < C < CW < Cp.

When comparing extractable soil Se to a total soil Se analysis using a vigorous, nitric-sulfuric-perchloric acid digestion in the method of Tracy and Möller [63], Se was not completely recovered. This is expected because sequential extraction determines a model solution extractable Se from the soil matrix and the digestion procedure quantifies all Se but the most recalcitrant geochemical inclusions in the soil matrix such as silicates. The ICW column was the lowest in extracted Se of all the column samples. Work done to evaluate extraction efficiency showed that this technique had 55% recoveries in seleniferous waste samples and 77% recoveries in spiked seleniferous waste samples compared to digested samples [26]. Thus, this procedure should only be used as a comparative technique for extractable Se rather than for total Se quantification by sum of the extracted masses. Our work did not directly evaluate the effects of the presence of iron, which is the variable with the most influence in this protocol. Jackson and Miller [24] found that phosphate buffer five times the concentration used in this protocol was required to recover 70% of selenate and selenite from iron-containing solutions. Iron metal amendments to soil will undoubtedly limit application of sequential extraction models of Se via formation of insoluble iron phosphates (vivianite) in phosphate extractions or direct acid consumption by oxidizable iron metal in nitric acid extractions. However, we anticipate that the relative stability indicated by lack of reactivity by model extractants is precisely the desired quality sought in an environmental stabilization technique.

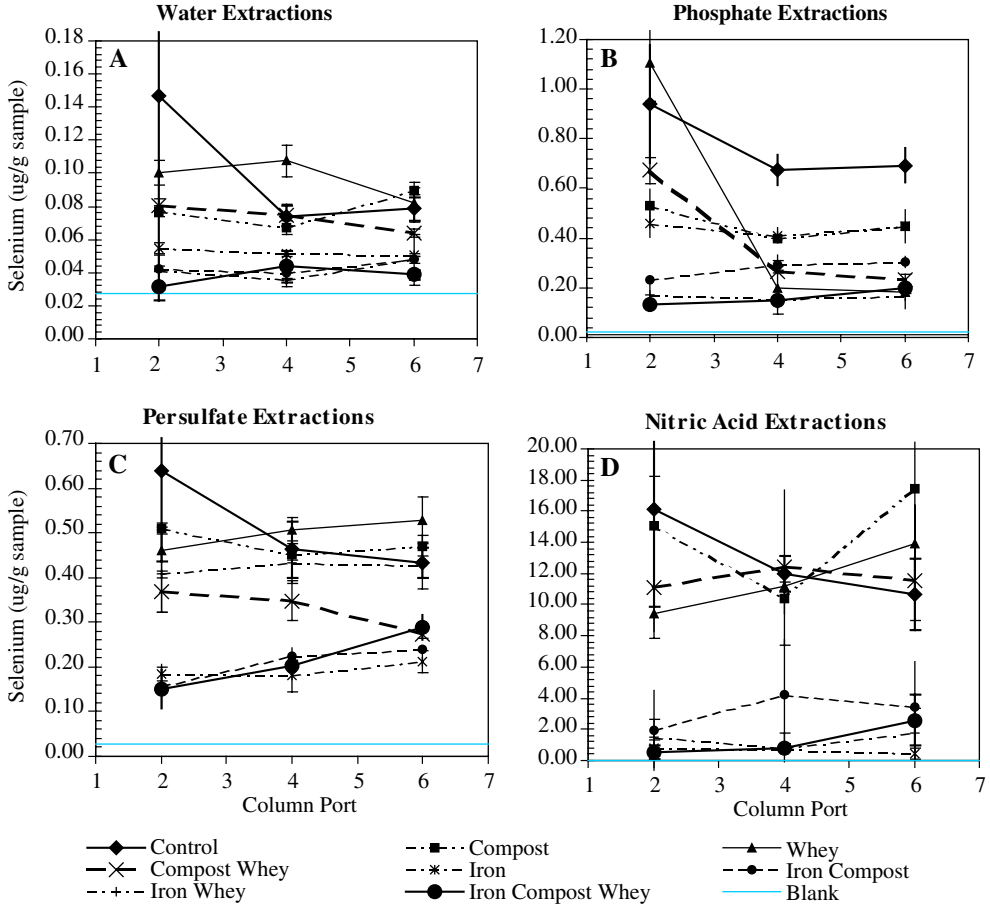

Fig. 6 Sequential Se extractions of seleniferous waste rock column samples. Graphs show the amount of Se extracted in μg/g of sample as a function of column port with 1 corresponding to the top port and 6 to the bottom port

The evaluation of the columns that did not have the iron amendment indicates that the highest level of mobilized selenate occurred in the W column, which actually increased the amount of selenate over the C column, probably due to the acidity (pH = 5) of the whey amendment solution. The highest level of selenite was detected in the C column followed by the Cp and CW column, while the lowest concentration of selenite was present in the W column. The highest level of selenide was present in the W column followed by the C, Cp, and CW columns. Many of the selenide concentrations, except the CW, fell within one standard deviation of one another indicating that levels of selenide were fairly consistent among the columns without the iron amendment. The levels of zero-valent Se were all within one standard deviation for the columns without the iron amendments. Interestingly, the amount of zero-valent Se decreased from the control for all but the Cp column.

The most significant information from this column experiment is in the comparison of the CW column to the C column. As stated earlier the compost whey column had significant growth. Based on the phosphate, persulfate, and nitric acid extractions, it appears that the Se was in fact reduced to or maintained as Se(0). It is confusing, however, that the control had a similar amount removed in the nitric extraction step; this is perhaps due to reaction of the native soil components, organic and inorganic, in this system. We hypothesize that the Se in the other columns was reduced to selenite and/or selenide, which then bound to the iron salts and compounds produced by the corroding iron metal. This is the most significant finding in this work, as the iron-bound Se in the iron metal modified soil columns could not be removed even with the nitric acid solution extraction in the highly regarded sequential extraction method of Martens and Suarez [37].

Flow-through column experiment

More important than the reductive sequestration of Se in soil is whether the Se will be reoxidized and resolubilized when weathered and exposed to infiltration waters. To that end an infiltration column experiment was conducted. The data from the flow-through

column experiments is shown in Fig. 7. These figures show the amount of Se extracted from the waste rock shale in μg Se/g sample as a function of pore volumes. The columns in order of mass of Se extracted were: ICW < IW < IC < I = CW < C < Cp < W. The amount of Se extracted was higher in the Cp and W columns than

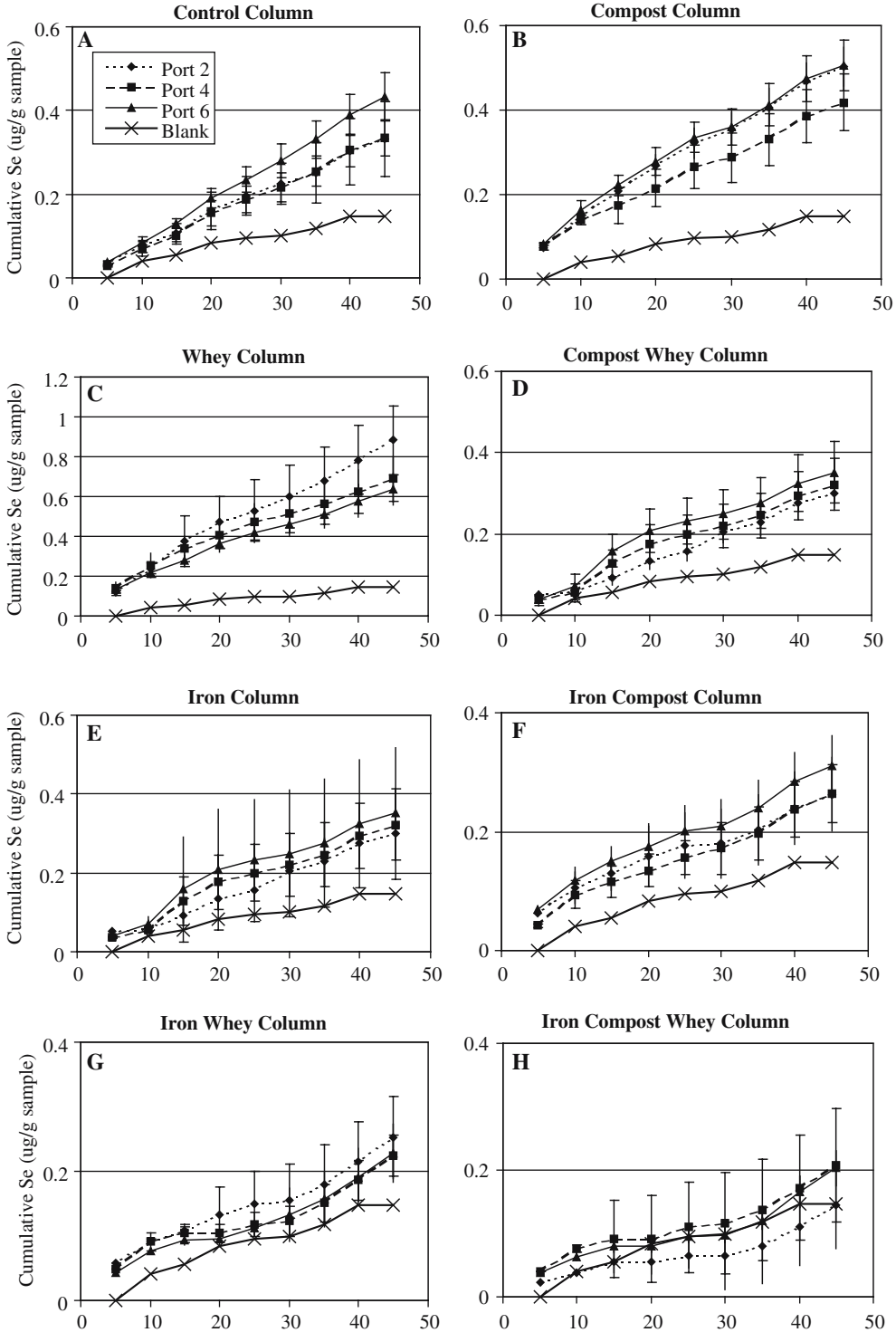

Fig. 7 Flow-through column experiment of seleniferous waste rock columns (error bars = 1 SD). *Individual lines* on the graphs correspond to ports along the length of the columns, *2* – top portion of the column, *4* – middle portion, and *6* – bottom portion. The *blank line*, which represents the flow-through of 4 g of washed sea sand, is the same line for all graphs and is a control that represents the cumulative error in the AFS Se analysis

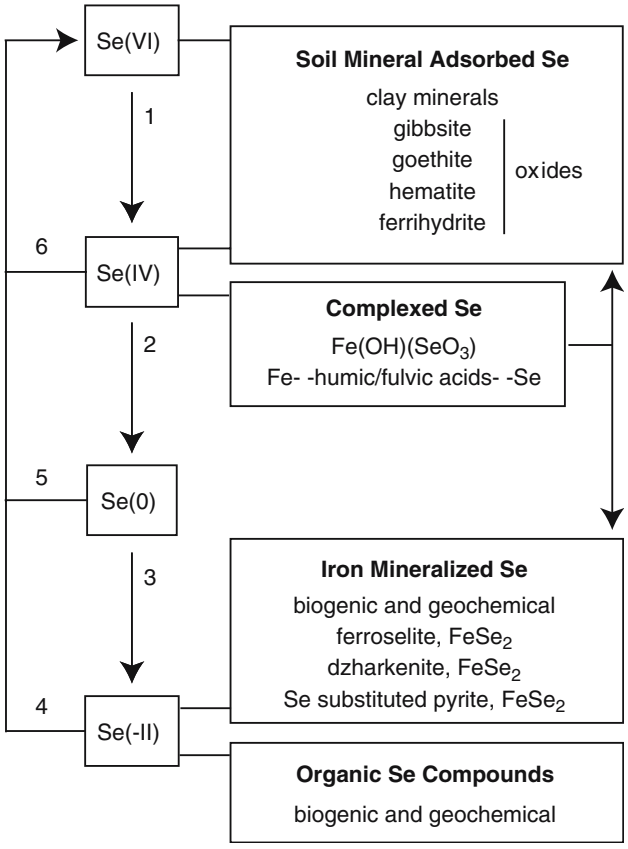

Fig. 8 Soil Se cycle where nutrient and iron amendment assists reductive mineralization and complexation. Reaction numbers as described in text

that extracted from the C column. The ICW column had the least amount of Se extracted, followed by the IW and IC columns. The data from the CW and I columns were very similar to one another and within one standard deviation of the control columns.

The column data, when combined with results from the sequential extractions, supports the hypothesis that oxidized Se can be reduced to an oxidation state, as Se(IV) or Se(-II), that then strongly binds to iron salts or compounds in the amended soil. The data suggest that iron is an important component for soil Se stabilization, as soils with bound selenite tend to have slow desorption of Se during water infiltration [16]. The data also indicate that microbial populations are very important in this transformation as demonstrated in comparing the iron amendment column results. There have been reports of green rust independently reducing selenate [42, 51]. If this were the only factor in reduction and binding of the Se, then the extracted Se would be consistent in all of the columns containing iron.

We observed in this system that addition of iron metal to the system essentially removed microbially or otherwise reduced Se from the biogeochemical cycle, thus reducing potential for mobilization. The soil cycle of Se described in this paper is shown schematically in Fig. 8. The microbial reduction of selenate [Se(VI)] to selenite [Se(IV)], Se(0), and selenide [Se(-II)] indicated by reaction steps 1–3 has been discussed previously [19, 53, 68]. However, these reduced forms can be reoxidized by microbial populations (reaction 5 [12]) or by infiltration waters (reactions 4 and 6 [67]). Selenite has been found to bind to soil but can also be removed by infiltration waters [13, 17, 45, 46]. Iron-bound Se is a relatively stable compound [7, 13, 24, 40, 53]. Iron-bound Se can be remobilized but only under highly oxidizing conditions [38] and in solutions with a high concentration of competing ions such as phosphate [24].

Conclusions

Iron-selenide and iron-selenite compounds are resistant to leaching by infiltration waters and thus coupling organic amendment and iron metal may be a preferred treatment technology for longer-term stabilization of Se in contaminated soil environments, especially in environments that are not exposed to surface weathering. Laboratory enrichments indicated that bacteria of the family Enterobacteriaceae were significant selenate reducers in the microbial populations of Se-contaminated sites. This work supports an approach for remediation of Se-impacted soils using accelerated microbial reduction via nutrient amendment in concert with an iron amendment to enhance more stable mineralization. Iron metal amendments enhance reducing conditions for support of anaerobic and facultative anaerobic organisms and provide a source of iron salts and iron compounds available for reaction with Se(IV) and Se(-II). The enhanced stabilization of Se in soils thus may require combining a microbial remediation step with an abiotic immobilization step to form a selenite or selenide mineralization cation such as ferrous or ferric iron. The optimum stabilization of Se was seen with acidic whey where selenate reduction was occurring in a lower pH environment that assists in minimizing passivation of the iron metal surface.

Acknowledgements This work was funded by J.R. Simplot Company and the U.S. Environmental Protection Agency (Grant # X970339010). Cornelia Sawatzky provided editorial assistance.

References

1. Alemi MH, Goldhamer DA, Grismer ME, Nielsen DR (1988) Elution of selenium from contaminated evaporation pond sediments. J Environ Qual 17:613–618

2. Alemi MH, Goldhamer DA, Nielsen DR (1988) Selenate transport in steady-state, water-saturated soil columns. J Environ Qual 17:608–613
3. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56:1919–1925
4. American Public Health Association (1995) Selenium. In: Eaton A, Clesceri L, Greenberg A (eds) Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC, pp 3–85–3–93
5. American Type Culture Collection (2000) ATCC medium: 1957 *Geobacter* medium. [Online] http://www.atcc.org/mediapdfs/1957.pdf. Accessed 16 Sep 2005
6. Anderson MS, Lakin HW, Beeson KC, Smith FF, Thacker E (1961) Selenium in agriculture. Agricultural Handbook 200. USDA, Washington, 65 pp
7. Balistrieri LS, Chao TT (1990) Adsorption of selenium by amorphous iron oxyhydroxide and manganese dioxide. Geochim Cosmochim Acta 54:739–751
8. Bautista EM, Alexander M (1972) Reduction of inorganic compounds by soil microorganisms. Soil Sci Soc Am Proc 36:918–920
9. Cummings DE, March AW, Bostick B, Spring S, Caccavo F Jr, Fendorf S, Rosenzweig RF (2000) Evidence for microbial Fe(III) reduction in anoxic, mining-impacted lake sediments (Lake Coeur d'Alene, Idaho). Appl Environ Microbiol 66:154–162
10. Czakis-Sulikowska D, Kaluzna-Czaplinska J (2000) Thermal properties of complexes of Mn(II), Fe(II), Co(II), Ni(II) with 2,2'-bipyridine or 4,4'-bipyridine and thiocyanates. J Therm Anal Calorim 62:821–830
11. Diez B, Pedros-Alio C, Marsh T, Massana R (2001) Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl Environ Microbiol 67:2942–2951
12. Dowdle PR, Oremland RS (1998) Microbial oxidation of elemental selenium in soil slurries and bacterial cultures. Environ Sci Technol 32:3749–3755
13. Elrashidi MA, Adriano DC, Workman SM, Linsay WL (1987) Chemical equilibria of selenium in soils: a theoretical development. Soil Sci 144:141–152
14. Environmental Protection Agency (1993) Selenium and compounds (CASRN 7782–49–2). [Online] http://www.epa.gov/iris/subst/0472.htm. Accessed 29 Apr 2003
15. Environmental Protection Agency (2002) Iris chemical files. [Online] http://www.epa.gov/iris/subst/index.html. Accessed 29 Apr 2003
16. Fio JL, Fujii R (1990) Selenium speciation methods and application to soil saturation extracts from San Joaquin Valley, California. Soil Sci Soc Am J 54:363–369
17. Fio JL, Fujii R, Deverel SJ (1991) Selenium mobility and distribution in irrigated and nonirrigated alluvial soils. Soil Sci Soc Am J 55:1313–1320
18. Frankenberger WT Jr, Karlson U (1994) Microbial volitilization of selenium from soils and sediments. In: Frankenberger WT Jr, Benson S (eds) Selenium in the environment. Marcel Dekker, New York, pp 369–387
19. Garbisu C, Ishii T, Leighton T, Buchanan BB (1996) Bacterial reduction of selenite to elemental selenium. Chem Geol 132:199–204
20. Geering HR, Cary EE, Jones LHP, Allaway WH (1968) Solubility and redox criteria for the possible forms of selenium in soils. Soil Sci Soc Am Proc 32:35–40
21. Gerrard TL, Telford JN, Williams HH (1974) Detection of selenium deposits in *Escherichia coli* by electron microscopy. J Bacteriol 119:1057–1060
22. Herbel M, Switzer-Blum J, Hoeft SH, Cohen SM, Arnold LL, Lisak J, Stolz JF, Oremland RS (2002) Dissimilatory arsenate reductase activity and arsenate-respiring bacteria in bovine rumen fluid, hamster feces, and the termite hindgut. FEMS Microbiol Ecol 41:59–67
23. Ike M, Takahashi K, Fujita T, Kashiwa M, Fujita M (2000) Selenate reduction by bacteria isolated from aquatic environment free from selenium contamination. Water Res 34:3019–3025
24. Jackson BP, Miller WP (2000) Effectiveness of phosphate and hydroxide for desorption of arsenic and selenium species from iron oxides. Soil Sci Soc Am J 64:1616–1622
25. Knight VV, Blakemore R (1998) Reduction of diverse electron acceptors by *Aeromonas hydrophila*. Arch Microbiol 169:239–248
26. Knotek-Smith HM (2003) Microbial bioremediation of selenium and cadmium. Ph.D. dissertation. University of Idaho, Moscow, ID
27. Knotek-Smith HM, Deobald LA, Ederer M, Crawford DL (2003) Cadmium stress studies: media development, enrichment, consortia analysis, and environmental relevance. Biometals 16:251–261
28. Knudsen AC, Gunter ME, Herring JR, Grauch RI (2001) Mineralogical characterization of strata of the Meade peak phosphatic shale member of the Permian phosphoria formation. United States Geological Survey, Denver, CO. Open file report 02–125:1–38
29. Lane D, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenic analyses. Proc Natl Acad Sci US 82:6955–6959
30. Lerman LS, Fischer SG, Hurley I, Silverstein K, Lumelsky N (1984) Sequence-determined DNA separations. Annu Rev Biophys Bioeng 13:399–423
31. Lipton DS, Davis R, Fujii R, Doner HE (1988) Selenium associated with hydrous oxides and organic matter in surface soils from the San Joaquin Valley, California. Trans Am Geophys Union 69:1181–1182
32. Long RHB, Benson SM, Tokunaga TK, Yee A (1990) Selenium immobilization in a pond sediment at Kesterson Reservoir. J Environ Qual Apr/June:302–311
33. Lortie L, Gould W, Rajan S, McCready R, Cheng K (1992) Reduction of selenate and selenite to elemental selenium by a *Pseudomonas stutzeri* isolate. Appl Environ Microbiol 58:4042–4044
34. Losi ME, Frankenberger WJ (1997) Reduction of selenium oxyanions by *Enterobacter cloacae* SLD1a-1: isolation and growth of the bacterium and Its expulsion of selenium particles. Appl Environ Microbiol 63:3079–3084
35. Losi ME, Frankenberger WT Jr (1998) Microbial oxidation and solubilization of precipitated elemental selenium. J Environ Qual 27:836–43
36. Mars JC, Crowley JK (2003) Mapping mine wastes and analyzing areas affected by selenium-rich water runoff in southeast Idaho using AVIRIS imagery and digital elevation data. Remote Sens Environ 84:422–436
37. Martens DA, Suarez DL (1997) Selenium speciation of soil/sediment determined with sequential extractions and hydride generation atomic absorption spectrophotometry. Environ Sci Technol 31:133–139
38. Masscheleyn PH, Delaune RD, Patrick WH Jr (1990) Transformations of selenium as affected by sedimentation oxidation-reduction potential and pH. Environ Sci Technol 24:91–96

39. Milne JB (1998) The uptake and metabolism of inorganic selenium species. In: Frankenberger WT Jr, Engberg RA (eds) Environmental chemistry of selenium. Marcel Dekker, Inc., New York, pp 459–477
40. Morrison SJ, Metzler DR, Dwyer BP (2002) Removal of As, Mn, Mo, Se, U, V and Zn from groundwater by zero-valent iron in a passive treatment cell: reaction progress modeling. J Contam Hydrol 56:99–116
41. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient get electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
42. Myneni SC, Tokunaga TK, Brown GE Jr (1997) Abiotic selenium redox transformations in the presence of Fe(II,III) oxides. Science 278:1106–1109
43. National Health Service for Scotland (2003) Selenium. [Online] http://www.griteu.demon.co.uk/index.htm. Accessed 29 Apr 2003
44. National Institutes of Health (2002) Basic local alignment search tool (BLAST®). [Online] http://www.ncbi.nih.gov/BLAST. Accessed 29 Apr 2003
45. Neal RH, Sposito G (1991) Selenium mobility in irrigated soil columns as affected by organic carbon amendment. J Environ Qual 20:808–814
46. Neal RH, Sposito G, Holtzclaw KM, Traina SJ (1987) Selenite adsorption on alluvial soils: I. soil composition and pH Effects. Soil Sci Soc Am J 51:1165
47. Oho T, Yamashita Y, Shimazaki Y, Kushiyama M, Koga T (2000) Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. Oral Microbiol Immunol 15:258–262
48. Oremland RS, Hollibaugh JT, Maest AS, Presser TS, Miller LG, Culbertson CW (1989) Selenate reduction to elemental selenium by anaerobic bacteria in sediments and culture: Biogeochemical significance of a novel, sulfate-independent respiration. Appl Environ Microbiol 55:2333–2343
49. Oremland R, Blum J, Culbertson C, Visscher P, Miller L, Dowdle P, Strohmaier F (1994) Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, strain SES-3. Appl Environ Microbiol 60:3011–3019
50. P S Analytical (2003) Millennium excalibur method for selenium in drinking, surface, ground, saline and industrial and domestic waste waters. PS Analytical LTD, Kent, England
51. Refait P, Simon L, Génin J-MR (2000) Reduction of selenate anions and anoxic formation of iron(II)-iron(III) hydroxyl-selenate green rust. Environ Sci Technol 34:819–825
52. Rybicki EP (2001) "Core Sample" PCR: A method to re-PCR unique bands from products of mixed size. [Online] http://www.uct.ac.za/microbiology/corepcr.htm. Accessed 19 Apr 2002
53. Ryser AL, Strawn DG, Marcus MA, Johnson-Maynard JL, Gunter ME, Möller G (2005) Micro-spectroscopic investigation of selenium-bearing minerals from the Western US Phosphate Resource Area. Geochem Trans 6:1–11
54. Schröder I, Rech S, Krafft T, Macy JM (1997) Purification and characterization of the selenate reductase from *Thauera selenatis*. J Biol Chem 272:23765–23768
55. Schwab AP, Su J, Wetzel S, Pekarek S, Banks MK (1999) Extraction of petroleum hydrocarbons from soil by mechanical shaking. Environ Sci Technol 33:1940–1945
56. Sharmasarkar S, Vance GF, Cassel-Sharmasarkar F (1998) Analysis and speciation of selenium ions in mine environments. Environ Geol 34:31–38
57. Stillings LL (2002) The importance of wetland processes in the removal of Se from mine drainage. In: Sources, Transport, Fate, and Toxicology of Trace Elements in the Environment II: A Tribute to Gunter Faure. Paper #197–1 of Geological Society of America Conference Proceedings, 30 Oct 2002
58. Stolz JF, Basu P, Oremland RS (2002) Microbial transformation of elements: the case of arsenic and selenium. Int Microbiol 5:201–207
59. Tetra Tech EM Inc. (2002) Final area wide human health and ecological risk assessment: Selenium project Southeast Idaho Phosphate Mining Resource Area. TetraTech EM Inc., Boise, ID. C023-AWI-00–01:1–165
60. Thompson-Eagle ET, Frankenberger WT Jr, Karlson U (1989) Volatilization of selenium by *Alternaria alternata*. Appl Environ Microbiol June:1406–1413
61. Tokunaga TK, Zawislanski PT, Johannis PW, Benson S, Lipton DS (1994) Field investigations of selenium speciation, transformation, and transport in soils from Kesterson Reservoir and Lahontan Valley. In: Frankenberger William T Jr, Benson S (eds) Selenium in the environment. Marcel Dekker, New York, pp 119–138
62. Torma A, Habashi F (1972) Oxidation of copper (II) selenide by *Thiobacillus ferroxidans*. Can J Microbiol 18:1780–1781
63. Tracy ML, Möller G (1990) Continuous flow injection vapor generation, inductively coupled argon plasma spectrometric analysis part 1: selenium. J Assoc Off Anal Chem 73:404–410
64. Turner RJ, Weiner JH, Taylor DE (1998) Selenium metabolism in *Escherichia coli*. Biometals 11:223–227
65. United States Department of Agriculture (2002) Elevated levels of selenium in waterways in the Caribou National Forest. [Online] http://www.fs.fed.us/r4/caribou/Caribou_main/Caribou/forest_plan/Deis/Chapter3/. Accessed 29 Apr 2003
66. Weaver R, Angel S, Bottomley P, Berdicek D, Smith S, Tabatabai A, Wollum A (1994) Methods of soil analysis part 2: microbiological and biochemical properties. Soil Science Society of America, Madison
67. Wright WG (1999) Oxidation and mobilization of selenium by nitrate in irrigation drainage. J Environ Qual 28:1182–1187
68. Zehr JP, Oremland RS (1987) Reduction of selenate to selenide by sulfate-respiring bacteria: Experiments with cell suspensions and estuarine sediments. Appl Environ Microbiol 53:1365–1369
69. Zhang Y, Moore JN (1997) Environmental conditions controlling selenium volatilization from a wetland system. Environ Sci Technol 31:511–517
70. Zhang Y, Moore JN, Frankenberger WT Jr (1999) Speciation of soluble selenium in agricultural drainage waters and aqueous soil-sediment extracts using hydride generation atomic absorption spectrometry. Environ Sci Technol 33:1652–1956