Bio-reduction of Selenium Oxyanions in the Presence of Nitrate in Neutral pH Coal Mining Influenced Water

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Abstract

Biological treatment to remove dissolved selenium from coalmine-influenced water is inhibited by co-contaminants, especially nitrate. Consortia of bacteria capable of reducing selenate and nitrate were enriched from two sites impacted by coalmine waste seepage. Enrichments from a natural vegetated marsh removed both dissolved selenium and nitrate simultaneously, whereas tailings pond sediment enrichments reduced total dissolved selenium more effectively in the presence of nitrate but only once all nitrate was removed. When inoculated into actual mine water, the enriched tailings pond bacteria removed 40% selenium, whereas natural marsh enrichment bacteria achieved less selenium removal (10%).

Keywords: coalmining, selenium, nitrate, biological treatment

Introduction

Selenium is found in most coal waste rock (Lussier et al. 2003) and oxidative weathering can release the selenium into aquatic environments, together with other contaminants such as nitrate (by product of explosives used in mining) and sulfate. Selenium tends to biomagnify up the food chain leading to deleterious environmental effects (Janz et al., 2010). Active and semi-passive bioreactors have been developed for the removal of dissolved selenium from mine water (Tan et al., 2016). The success of these is dependent on seeding them with bacterial communities that can transform dissolved selenium in the presence of other contaminants to the less bioavailable elemental selenium. Nitrate is a competing anion often found in coalmine-affected water known to inhibit selenium reduction in previous attempts to biologically treat these waters (Steinberg et al. 1992). This study investigated the use of enrichments of bacterial consortia resident in sediments of ponds impacted by coalmine seepage, to remove total dissolved selenium in the presence of nitrate. We enriched bacteria from the sediments of both a vegetated natural marsh and a non-vegetated inactive tailings pond. Our results showed that the bacterial consortium enriched from the marsh reduced selenate and nitrate simultaneously in growth media. The tailings pond sediment bacteria reduced selenium more effectively in the presence of nitrate, but only once all nitrate had been consumed. Both enrichments were tested for their ability to remove selenium from actual mine waste seepage water. After transfer to the mine water both enrichment consortia changed in composition and comprised similar taxonomic groups. However the enrichment bacteria from the inactive tailings pond sediment were more efficient at removing selenate from the mine water than those from the marsh.

Materials and Methods

Preparation of selenate reducing enrichments

Sediment samples were collected from the water sediment interface from two aquatic environments located on two mine sites: A natural vegetated marsh (marsh) and a non-vegetated inactive tailings pond (pond). Measurements with an YSI Sonde model 600QS and probes indicated that, at sampling time, the sediments were anaerobic (dissolved oxygen < 2 mg/L), the pH was circum-neutral (≈7) and the temperature was 10-12oC. All samples were stored in a cooler with ice whilst en route to the laboratory for further experiments.

Organisms were enriched from the sedi-

ments in two types of growth media: one with selenate as the sole electron acceptor (GM1) and the second with both selenate and nitrate as electron acceptors (GM2). The enrichment medium was prepared according to (Stams et al. 1992), omitting selenite and adding 1 mg-Se-as-selenate/L instead, and using phosphate buffer instead of carbonate. Lactate was used as the carbon source and was added at a concentration of 600 mg/L. For the growth medium containing nitrate and selenate, 50 mg-N-as-nitrate/L was added also. Each growth medium was dispensed into two duplicate autoclaved 250 ml culture bottles for each sediment containing 25 ml of sediment slurry, which were then filled to the brim to remove any headspace. Thereafter, the bottles were sealed with butyl septa caps before subjected to static incubation at 30°C. Samples were collected at time intervals 0, 4, 8, 12, 24, 48 and 72 hr for analysis of total nitrite- plus nitrate-N (APHA method No. 4500 –NO_3^- E) and total dissolved selenium (ICP-MS at ALS Laboratory, Burnaby, BC). After 72 hours, culture bottles were decanted and 25 ml of biomass was transferred into freshly prepared growth medium and the culturing procedure repeated for three passages.

Removal of total dissolved selenium from actual mine water using the enrichments

Enrichments from the growth medium containing both selenate and nitrate were tested for their capability to remove dissolved selenium from actual mine water using triplicate batch culture bottles for five passages of three days each. The mine water was filtered through 0.45-µm nitrate cellulose membrane (GE Healthcare Life Sciences, USA) to remove suspended materials, including microorganisms. The actual mine water had the following chemical composition, total dissolved selenium (0.354 mg/L) and total nitrite-plus nitrate-N (54.9 mg/L), sulfate (2030 mg/L), sodium (14.4 mg/L), chloride (19 mg/L), calcium (404 mg/L), magnesium (390 mg/L) and CaCO3 (2610 mg/L). The major trace elements in the mine water were nickel (0.0327 mg/L) and strontium (0.250 mg/L). It was supplemented with phosphate buffer (K2HPO4 1 g/L / KH2PO4 0.2 g/L), 0.3 g/L ammonium chloride, and 600 mg/L lactate as essential nutrients. The pH of the nutrient amended mine water remained near neutral (6.8 – 7.04) throughout the duration of the experiment. Samples were collected daily for total dissolved selenium, total nitrate- plus nitrite-N and soluble chemical oxygen demand analyses, and for DNA extraction for bacterial population composition identification.

Bacterial community analysis

DNA was extracted using the FastDNA® SPIN kit for soil (MoBio Laboratories Inc. USA) according to the manufacturer's protocol. Sequencing and bioinformatics methods were the same as reported in Subedi et al. (2017). Read counts in each sample were normalized to 20,000 reads each.

Results and Discussion

Total dissolved selenium reduction in the presence and absence of nitrate

When sediments from the marsh were inoculated into growth medium containing selenate as the only electron acceptor, total dissolved selenium concentrations decreased with tiwme reaching almost complete removal within 48 hr (Figure 1a) indicating that these sediments contained bacteria capable of using selenate as an electron acceptor. No lag period was observed suggesting that the sediment bacteria were already adapted to grow on selenate. When the same sediments were inoculated into growth medium containing both selenate and nitrate, reduction in soluble selenium proceeded similarly with concomitant reduction in nitrate. Thus, the marsh sediments contained microorganisms capable of reducing selenate and nitrate simultaneously. In contrast, when equivalent amounts of sediment from the pond were inoculated in selenate-only growth medium, dissolved selenium was reduced more slowly (Figure 1b) suggesting that these sediments contained fewer or less capable selenate reducing microorganisms. In the growth medium with both selenate and nitrate, there was a lag period before dissolved selenium concentrations decreased, the duration of which was approximately the same as the time taken for complete denitrification. After this lag period, the rate of reduction in dissolved selenium was faster than that observed in the selenate-only growth medium. Thus, selenate

reduction by the pond microorganisms was apparently stimulated by presence of nitrate. The results indicate that some selenate reducing bacteria from the marsh might be using a specific selenate reductase enzyme that is not competitively inhibited by the presence of nitrate, which resulted in simultaneous reduction of both nitrate and selenate. Whereas, the selenate reducing bacteria in the pond sediments that were enriched might have been denitrifying bacteria that increased in concentration first due to the presence of nitrate and then when nitrate was depleted, the non-specific nitrate reductases metabolized selenate. The bacteria enriched in the selenate plus nitrate growth medium from these two sediments represent two potentially different mechanisms of selenate reduction that were tested for their efficacy in removing dissolved selenium from actual coalmine affected water sourced from a mine site.

Total dissolved selenium removal from actual mine water containing nitrate and sulfate as well as selenium oxyanions

When inoculated into nutrient-amended mine water, the enrichment consortia were less efficient at reducing selenate compared to when grown in medium over the same period of time (3 days) (Table 1 & Figure 1). Unfortunately no data for total dissolved selenium were available for the first two passages. The extents of total dissolved selenium reduction increased with passaging, reaching in the final passage 8 ± 1% for the marsh enrichment inoculated mine water cultures and 40 ± 15 % for the pond enrichment inoculated mine water cultures. It is not known if these extents would continue to increase with further passaging as the microorganisms continue to adapt to the chemical environment of the nutrient-amended mine water. These results indicate that the microorganisms from the pond were more capable of reducing selenate in the mine water either due to their greater abundance or the metabolic traits of the species in that enrichment. It was not possible to measure the exact number or concentration of microorganisms in each enrichment that were inoculated into the mine water. Therefore, it is possible that there were more microorganisms in the pond enrichment cultures than in the marsh enrichment cultures at the beginning of the experiment. Similarly, although the same volume of suspended microorganisms was transferred into each new passage, the concentrations of microorganisms in that volume might have been different. The microorganism concentrations in these cultures were too low to capture with total volatile suspended solids analysis. Presence of particulates interfered with cell counts

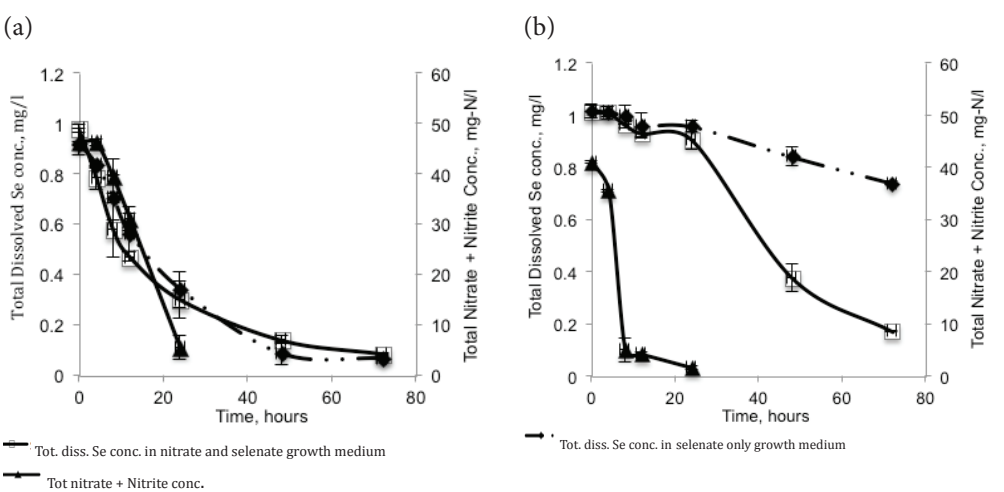

Figure 1: *Total dissolved selenium (closed diamonds and triangles) and nitrate plus nitrite concentrations (mg/L) (closed triangles) versus time (in hours) measured in the selenate-only (black diamonds) and selenate plus nitrate (open squares and closed diamonds) growth media for sediment sourced from a) the natural vegetated marsh b) the inactive tailings pond. Data points are averages from duplicate culture bottles. Error bars represent the standard deviation.*

Marsh	Passage1	Passage 2	Passage 3	Passage 4	Passage 5
Tot. diss. Se reduction (%)	n.d	n.d	3±2	8±2	8±1
Tot. NO2- + NO3-N reduction (%)	86±2	66±3	84±2	68±18	86±2
Δ SCOD concn. (mg/L)	1546 – 1337	1281 – 1186	1090 – 810	873-720	1407-956
Pond					
Tot. diss. Se reduction (%)	n.d	n.d	10±5	19±9	40±15
Tot. NO2- + NO3-N reduction (%)	88±1	86±8	91±1	87±4	78±1
Δ SCOD concn (mg/L)	1586-1373	1290-1055	1027-866	845-738	1467-1272

n.d not determined

Table 1: *Total dissolved selenium, total nitrite- plus nitrate-N reduction (%) and soluble chemical oxygen demand concentrations change (mg/L) measured over each passage for the mine water cultures inoculated with enriched marsh and pond microorganisms, respectively*

using flow cytometry.

One possible reason for less than expected dissolved selenium removal might be because the concentrations of active microorganisms inoculated into the mine water were low and more time would be required for them consume all of the selenate. However, presence of active microorganisms was confirmed by almost complete denitrification in the first passage (Table 1). Another possible factor that could have contributed to lower than expected dissolved selenium reduction might have been the availability of carbon source. However, this was not the case since soluble chemical oxygen demand (SCOD) concentrations suggested that not all carbon source was consumed (Table 1). Thus, contrary to the observations made in the growth medium enrichment experiments, nitrate might be inhibiting selenate reduction and removal of total dissolved selenium in the nutrient-amended mine water. It is possible that the microbial population composition of the enrichments changed when they were inoculated into the mine water due to differences in the chemical environment of the mine water compared to that in the growth medium. Indeed, tracking of the microbial community composition in the nutrient-amended mine water cultures indicated shifts in relative abundance of certain OTUs (species) with increasing adaption of the microbial community to the mine water environment. In fact, nine of the 20 dominant species in the pond enrichments declined in relative abundance, as did 14 out of 20 species from the marsh enrichments, with increasing passaging. A few dominant species continued to thrive in the mine water and some rare species not detected in the enrichments became dominant in the mine water microbial communities. For instance, a *Sulfurospirillum* species (OTU1) that was present in both enrichments increased in relative abundance in the nutrient-amended mine water to become the most dominant (Figure 2). A *Veillonella* species (OTU3) not detected in either enrichment, became dominant in most of the nutrient-amended mine water cultures. *Paracoccus* (OTU4) and *Macellibacteroides* (OTU6) dominated the nutrient-amended mine water cultures inoculated with the pond enrichments, but were absent from or rare in the marsh enrichment inoculated cultures. *Sulfurospirillum* species are members of the Epsilonproteobacteria and have been reported to grow on substrates such as selenate, as well as nitrate and S compounds (Goris & Diekert 2016), which explains its dominance of the nutrient-amended mine water cultures. *Veillonella* species are denitrifying bacteria and one species, *V. atypica*, produced elemental Se from reduction of selenite (Pearce et al. 2009). *Paracoccus* are well known denitrifiers. One species, *P. denitrificans*, was found to

reduce selenite but not selenate. But when it was combined with a selenate-reducing bacterium (a *Pseudomonas* species) both selenate and nitrate reduction occurred simultaneously (Morita et al. 2007). Thus, a mixture of microorganisms performing different roles can contribute to simultaneous removal of multiple pollutants. The role of *Macellibacteroides* (OTU6) in the cultures is less clear since there is only one species that has been characterized and it was reported not to be able of using nitrate or S species as electron acceptors, and was a fermentative bacterium metabolizing sugars (Jabari et al. 2012). Taken together these observations suggest that both enrichment cultures had species with the potential to reduce selenate, with more of the pond enrichment bacteria persisting in the mine water.

The mine water contained high concentrations of some salts other than dissolved selenium and nitrate that might have influenced the metabolism of bacteria in the enrichments. For example, sulfate concentration in the mine water was 2030 mg/L. Even though sulfate is a less thermodynamically favourable electron acceptor than nitrate and selenate or selenite, its presence may have influenced the microbial community structure. Additionally, mine water was missing the essential minerals iron and molybdenum, which are required for enzymes involved in selenate and nitrate reduction (Sabaty et al. 2001). Additionally, the high concentrations of Mg^{2+} and Ca^{2+} in the mine water might have caused precipitation of the essential micro and macronutrients inhibiting microbial activity, as was seen in another study (Fernandez-Nava et al. 2008).

Conclusions

Bacteria with the capability to reduce both selenium oxyanions and nitrate can be enriched from native communities present in anaerobic sediments of both a natural marsh and tailings pond impacted by coalmine waste rock seepage. Even though species with the capability to reduce selenate survived when enriched bacteria from these two sites were inoculated into actual coalmine-affected water, the extents of total dissolved selenium removal were lower than those observed in

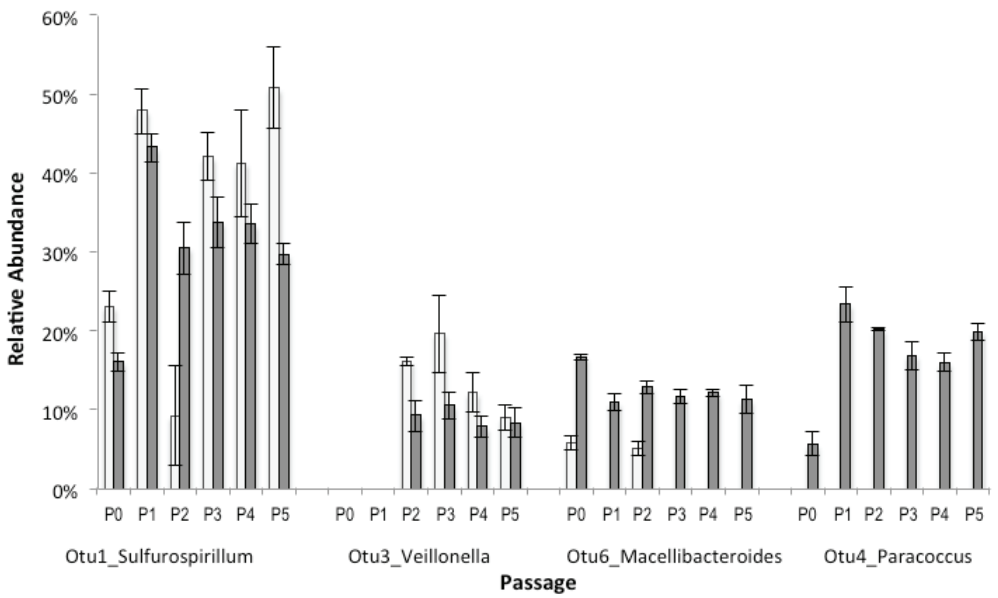

Figure 2: *Percentage relative abundance of selected dominant species (OTUs) and their genus-level taxonomic classification versus passage (P) in the nutrient-amended mine water cultures inoculated with enrichments from the (a) marsh, white filled with black border and (b) pond, grey filled with black border. Error bars represent standard deviations from the mean of measurements from triplicate culture bottles. P0 refers to samples taken at time zero at the start of the experiment.*

growth medium. Possibly other constituents of the coalmine-affected water negatively affected the selenium reducing bacteria or essential nutrients for selenium removal were missing from the coalmine-affected water although sufficient carbon source was provided.

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References

APHA, AWWA and WEF (2017). Standard method for the examination of water and waste water 23rd edition. Edited by Baird, R.B., Eaton, A.D., and Rice, E.W. American Public Health Association, NW, Washington, 5.21-5.22.

Goris T, Diekert G (2016). The Genus *Sulfurospirillum*, in: Organohalide-Respiring Bacteria. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 209–234.

Fernández-Nava Y, Marañón E, Soons J, Castrillón L (2008). Denitrification of wastewater containing high nitrate and calcium concentrations. Bioresour. Technol. 99, 7976–7981.

Jabari L, Gannoun H, Cayol J-L, Hedi A, Sakamoto M, Falsen E, Ohkuma M, Hamdi M, Fauque G, Ollivier B, Fardeau M-L (2012). Macellibacteroides fermentans gen. nov., sp. nov., a member of the family Porphyromonadaceae isolated from an upflow anaerobic filter treating abattoir wastewaters. Int. J. Syst. Evol. Microbiol. 62, 2522–2527.

Janz DM, DeForest DK, Brooks (2010). Selenium Toxicity to Aquatic Organisms, in: Chapman, P.M., Adams, W.J., Brookes, M.L., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlenforf, H.M., Presser, T.S., Shaw, D.P. (Eds.), Ecological Assessment of Selenium in the Aquatic Environment. Society of Environmental Toxicology and Chemistry, pp. 141–231.

Lussier C, Veiga V, Baldwin S (2003). The geochemistry of selenium associated with coal waste in the Elk Valley, Canada. Environ geology; 44; 905-913.

Morita M, Uemoto H, Watanabe A (2007. Reduction of selenium oxyanions in wastewater using two bacterial strains. Eng. Life Sci. 7, 235–240.

Pearce CI, Patrick RA, Law N, Charnock JM, Coker VS, Fellowes JW, Oremland RS, Lloyd JR (2009). Investigating different mechanisms for biogenic selenite transformations: *Geobacter sulfurreducens, Shewanella oneidensis* and *Veillonella atypical*. Environ Technol 30: 1313-1326.

Sabaty M, Avazeri C, Pignol D, Vermeglio, A (2001). Characterization of the reduction of selenate and Tellurite by nitrate reductases. Applied Microbioloby 67 (11) 5122-5126.

Stams AJM, Grolle KCF, Frijters CTM Van Lier JB (1992). Enrichment of thermophilic propionate-oxidizing bacteria in syntrophy with Methanobacterium thermoautotrophicum or methanobacterium thermoformicium. Appl. Environ. Microbiol. 58 (1), 346-352.

Steinberg NA, Blum JS, Hochstein L, Oremland RS (1992). Nitrate is a preferred electron acceptor for growth of freshwater selenate-respiring bacteria. Appl. Environ Microbiol 58, 426-428.

Subedi G, Taylor J, Hatam I, Baldwin SA (2017). Simultaneous selenate reduction and denitrification by a consortium of enriched mine site bacteria. Chemosphere 183, 536–545.

Tan CL, Nancharaiah YV, van Hullebusch ED, Lens PNL (2016). Selenium: environmental significance, pollution, and biological treatment technologies. Biotechnology Adv. 34; 886 – 907.