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Remediation of antimony-rich mine waters: Assessment of antimony removal and shifts in the microbial community of an onsite field-scale bioreactor☆

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ABSTRACT

An on-site field-scale bioreactor for passive treatment of antimony (Sb) contamination was installed downstream of an active Sb mine in Southwest China, and operated for one year (including a six month monitoring period). This bioreactor consisted of five treatment units, including one pre-aerobic cell, two aerobic cells, and two microaerobic cells. With the aerobic cells inoculated with indigenous mine water microflora, the bioreactor removed more than 90% of total soluble Sb and 80% of soluble antimonite (Sb(III)). An increase in pH and decrease of oxidation-reduction potential (Eh) was also observed along the flow direction. High-throughput sequencing of the small subunit ribosomal RNA (SSU rRNA) gene variable (V4) region revealed that taxonomically diverse microbial communities developed in the bioreactor. Metal (loid)-oxidizing bacteria including *Ferrovum*, *Thiomonas*, *Gallionella*, and *Leptospirillum*, were highly enriched in the bioreactor cells where the highest total Sb and Sb(III) removal occurred. Canonical correspondence analysis (CCA) indicated that a suite of *in situ* physicochemical parameters including pH and Eh were substantially correlated with the overall microbial communities. Based on an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree and PCoA (Principal Coordinates Analysis), the microbial composition of each cell was distinct, indicating these *in situ* physicochemical parameters had an effect in shaping the indigenous microbial communities. Overall, this study was the first to employ a field-scale bioreactor to treat Sb-rich mine water onsite and, moreover, the findings suggest the feasibility of the bioreactor in removing elevated Sb from mine waters.

1. Introduction

Antimony (Sb) is a naturally occurring metalloid widely used in industrial products such as lead-acid batteries and flame-retardants (Anderson, 2001). Sb and its compounds are suspected carcinogens (De Boeck et al., 2003) and have been listed as priority pollutants by the US Environment Protection Agency (USEPA, 1979). The rapid growth of Sb usage in industrial applications has raised great concerns about its environmental and health impacts. Sb has been investigated extensively in natural water systems (Filella et al., 2007). Different thresholds (from 5 to 20 μg/L) of Sb in drinking and agricultural water have been set by international health organizations (WHO, 2011). Sb can be released into natural water systems through natural and anthropogenic discharges such

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as mining activities and industrial emissions (He et al., 2012). China is the largest Sb producer in the world with more than 114 Sb mines accounting for approximately 90% of global Sb production (He et al., 2012). Massive discharge of Sb have been reported in many areas of China, posing a threat to ecosystems, habitats, species, and human health (He et al., 2012). Specifically, Sb-rich drainage from Sb mines holds great potential to contaminate local and regional ecosystems. Therefore, remediation of Sb-rich mine water is generally regarded as a priority issue for regulatory authorities.

Mine waters could be treated by utilizing various approaches, including abiotic and biotic treatments. Both approaches can be further classified into active and passive treatments (Johnson and Hallberg, 2005a). Active treatment requires continuous inputs of resources, but passive treatment requires relatively fewer inputs (Johnson and Hallberg, 2005a). Recently, passive bioreactor systems using indigenous acidophilic bacteria from acid mine drainage (AMD) have shown promise in treating metal contamination, due to the vast repertoire of microbial metabolic responses to metal(loid)s (Kalin et al., 2006; Kay et al., 2014; Sánchez-Andrea et al., 2015). These bioreactors demonstrated their capabilities to selectively precipitate and biotransform metal(loid)s from mine or metallurgical waste seepage. Therefore, indigenous microorganisms may play an important role in effective remediation of mine waters. Nevertheless, developing efficient bioreactors and consistent remediation strategies to treat mine waters requires an in depth understanding of how geochemical parameters affect the community structure and metabolic potentials.

We designed an onsite field-scale bioreactor to passively treat Sb mine contaminated water, and studied its performance and the development of microbial communities *in situ*. This bioreactor was constructed in a remote location in Southwest China; thus a low input passive system with minimal maintenance requirements was desired. The bioreactor system has been operating continuously to treat Sb-rich mine waters from an active upstream Sb mine that produces a 8–25 m^3/d of mine water with up to 7 mg/L soluble Sb. To the best of our knowledge, this is the first to explore the feasibility of a field-based bioreactor to remove Sb from mine waters. This innovative passive onsite treatment facility holds great potential to generalize to other contaminated mining sites, which are often remote, making routine operation and maintenance a challenge. Microorganisms are known to play a crucial role in metal removal and acid reduction in bioreactors treating mine waters (Fyson et al., 1996; Kalin and Chaves, 2003). However, to date, our understanding of the microbial community compositions and their dynamics in such water treatment system remains limited and the geochemical factors affecting the community composition are largely unknown. Thus, characterization of the indigenous microbial communities in each unit of the bioreactor can provide important knowledge to understand: 1) the relationship between microbial communities in the passive reactor and environmental factors within each unit; and 2) whether the microbial community composition impacts Sb removal or stabilization.

2. Materials and methods

2.1. Bioreactor description

The studied site is located at Banpo Sb mine, Guizhou province, Southwest China. This mining site is remote and thus routine operation and maintenance is a challenge. The field-scale bioreactor consists of a pre-aerobic precipitation unit (BP2), two aerobic units (BP3 and BP4), and two microaerobic units (BP5 and BP6) (Fig. 1). Three baffles were placed in each unit to direct the water flow. Mine waters from the mine portal (BP1) are pumped into BP2 and then flow by gravity through the reactor units to BP6 and ultimately, discharge to the environment. The pre-aerobic unit (BP2) was designed to partially oxidize Fe(II) and Sb(III) and induce partial settling. Its effluent was then directed into BP3, the first of two aerobic units (19 m^3 volume), followed by BP4 (18 m^3). Two microaerobic units, BP5 (18 m^3) and BP6 (19 m^3), followed the two aerobic units, were amended by carbon additives to promote the growth of anaerobic bacteria. Chicken litter was readily available at the study site, therefore it was selected as the organic matter additive in the microaerobic units. We tested the composition of chicken litter used in this research. It contains 21.8% C, 2% N, 0.17% S, and 18.36% total organic carbon (TOC). The C/N is 11.17. This bioreactor, which has been in continuous operation for over one year, occupied 80 m^2 in area and ~80 m^3 in volume. The treatment capacity is approximately 10 m^3/d with the retention time of 8 days.

It was expected that the most Sb will be removed at the pre-aerobic and two aerobic cells. The remaining Sb that could not be removed by oxidation and precipitation could be removed via precipitation with sulfide produced from anaerobic sulfate reduction. Our previous experiments tested the effect of chicken litter on sulfate and acidity reduction (see Fig. S1 for details). In the current study, because most Sb was removed in the aerobic cells, we did not examine the effect of strict anaerobic conditions in removing Sb.

2.2. Water parameters analysis

Due to the location (deep in high mountains) and the accessibility (unpaved road) to the mining area, we collected and analyzed the samples once a month. Water quality parameters including pH and Eh were measured at the site using a calibrated HACH HQ30d multi meter (HACH, Loveland, CO, USA). Major and trace elements including Sb and Fe in water samples were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Vista MPX, Varian, USA) and inductively coupled plasma mass spectrometry (ICP-MS) (Agilent, 7700x, Santa Clara, CA, USA), respectively, after acidification. The detection limits for Sb, calculated as the average of ten times the standard deviation of the ion counts obtained from the individual procedural reagent blanks (prepared in the same way as the sample decomposition), was 0.2 μg/L for water samples. Certified reference materials (SLRS-5) and internal standards (Rh at 500 μg/L) were used for quality control. Anions including F^-, Cl^-, SO_4^{2-}, and NO_3^- were measured by ion chromatography (Dionex, ICS-90, Sunnyvale, CA, USA). Aqueous Sb(III) was analyzed by hydride generation atomic fluorescence spectrometry (AFS-920, Beijing JitianCompany) in 1.5 mol/L HCl medium. 4% (m/v) citric acid was added to mask the undesired fluorescence emission signal (Fuentes et al., 2003).

2.3. Sediment sample collection and analysis

In October 2013, sediment samples were collected for high-throughput sequencing after nine months of operation. Sediments from different locations in the mine portal (BP1) and the pre-aerobic precipitation unit (BP2), which is essentially a shallow ditch, were collected by skimming the upper 1–2 cm sediment with a wide mouth container. Four reusable metal traps were placed at the bottom (2–3 m depth) of BP3 to BP6 and sediment was allowed to accumulate on these for analysis of the sediment microbial communities. Samples from each treatment unit and mine portal were placed in sterile 50-ml tubes, and immediately stored at −20 °C until further molecular analysis.

2.4. Measurement of metal(lloid)s in the sediments

To measure the concentrations of metals and metalloids in the sediment, sediment samples were air-dried and thoroughly ground

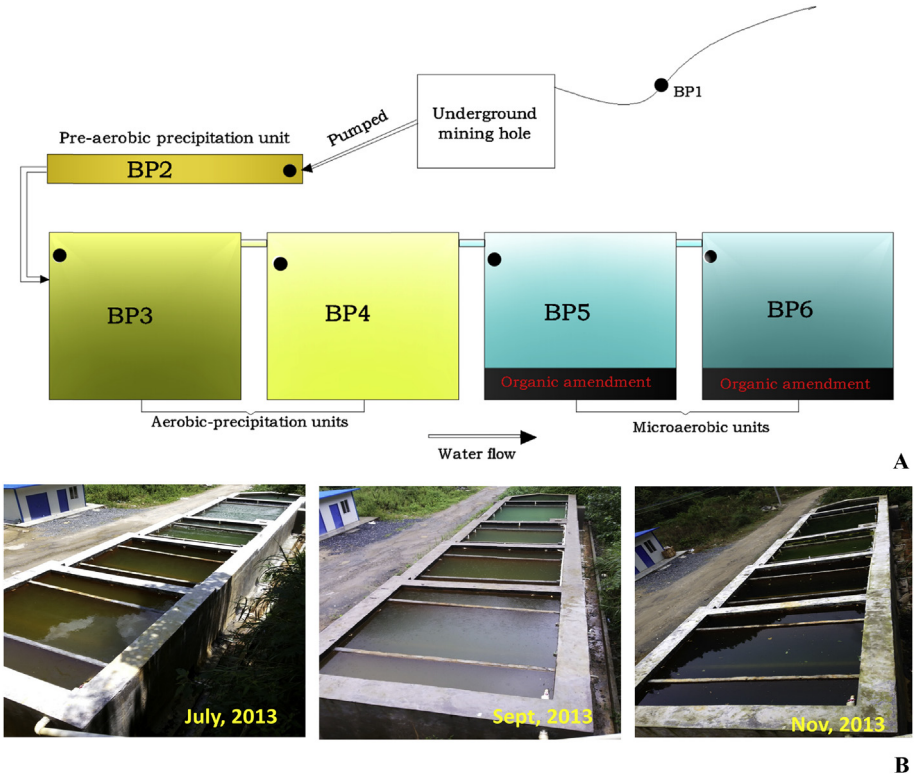

Fig. 1. A: Schematic diagram of the onsite field-scale bioreactor; B: Photographs of onsite bioreactor taken at three different time points (picture taken times are indicated in the pictures).

using a mortar and pestle before passing through a 200-mesh sieve. The sediment samples were fully digested with HF (Edgell, 1989) and the major and trace elements were then determined by ICP-OES (Vista MPX, Varian, USA) and inductively coupled plasma mass spectrometry (ICP-MS) (Agilent, 7700x, California, USA), respectively. The detection limits for Sb was 1 mg/kg for sediment samples. The certified reference materials and internal standards as mentioned above were used for accuracy testing. Standard reference material GBW07310 (Chinese National Standard) was used for analytical quality control. The measured total Sb concentration in GBW07310 was 6.46 ± 0.8 mg/kg, which is comparable to the certified value of 6.3 ± 0.9 mg/kg.

2.5. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectrometer (EDX)

SEM analysis was used to determine the presence and morphology of minerals in the sediments based on a protocol described previously (McBeth et al., 2013). SEM images were taken on a field-emission SEM (JSM-6460LV, JEOL, Tokyo, Japan) with an EDAX energy-dispersive X-ray spectrometer (EDAX-GENESIS, Mahwah, USA). The SEM was operated at 15 kV with a working distance of 10 mm. For energy-dispersive X-ray analysis, an accelerating voltage of 20 kV was used to obtain sufficient X-ray counts.

2.6. Illumina-derived reads analysis and statistical analysis

Total genomic DNA was extracted from the sediment sample using the FastDNA® spin kit (MP bio, Santa Ana, CA, USA) following the manufacture's protocol. The V4 region of the small subunit (SSU) rRNA gene was amplified using the 515f/806r primer set (Caporaso et al., 2011). SSU rRNA tag-encoded high throughput sequencing was carried out on the Illumina MiSeq platform at Novogene (Beijing, China). The reads were deposited into the NCBI short reads archive database under accession number of SRP064908. Additional details for analysis of sequencing reads and statistical analysis are provided in the supplementary materials.

3. Results

3.1. Performance of the onsite field-scale bioreactor

Water physicochemical parameters were continuously monitored for a six-month period, from July 2013 to January 2014. pH in the influent varied from 2.8 to 3, increasing substantially along the system, with pH in the effluent ranging from 5.6 to 9.3 (Fig. 2A). Eh was in a range of 467.1–539.7 mV in the influents (Fig. 2B), but decreased along the treatment system, ranging from 127 to 258.7 mV in the effluent. The decrease of Eh along the reactor indicated that the aquatic environments shifted from relatively oxidized to relatively reduced conditions. Other important physicochemical parameters of the mine water and sediments, such as major elements (e.g. Al, K, Mg, Na, and Ca) and anion (F^-, Cl^-,SO_4^{2-}, and NO_3^-) concentrations, were also determined in each unit and are presented in Tables S1 and S2. Sulfate, Mn, and Mg concentrations did not change substantially along the system, while a slight increase of Na, K, Ca, Sr, and Si were observed. The increase in these ions is likely dissolution from the concrete used to construct the system. Al is removed in the system due to the pH increase.

The concentrations of total soluble Fe (Fe_{tot}), Sb (Sb_{tot}), and Sb(III), were measured at the influent of BP2 as well as effluent of BP3 to BP6. More than 70% of Fe_{tot} was removed in BP2 and BP3 (Fig. S2), with removal increasing to more than 95% after October 2013. The removal of Sb_{tot} and Sb(III) occurred as well during the monitoring period. Sb_{tot} concentrations in the influent ranged from 1431 (July 2013) to 7753 μg/L (November 2013), while Sb_{tot} in the

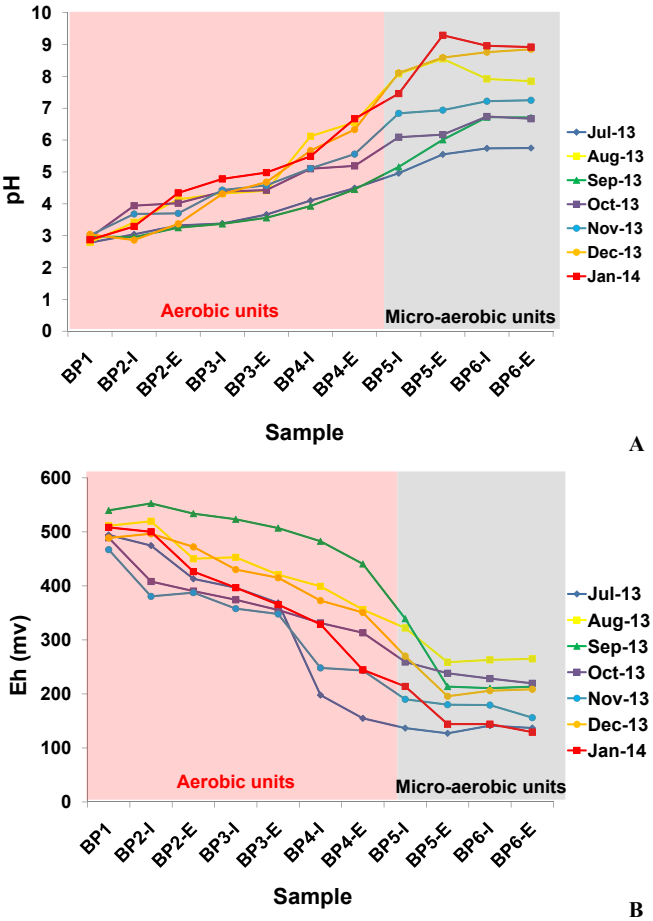

Fig. 2. Water physicochemical parameters from each monitoring point of the onsite field-scale bioreactor. (A), pH; (B), Eh. Acronyms: BPX-I: the influent of unit BPX (X could be 2–6); BPX-E: the effluent of unit BPX (X could be 2–6).

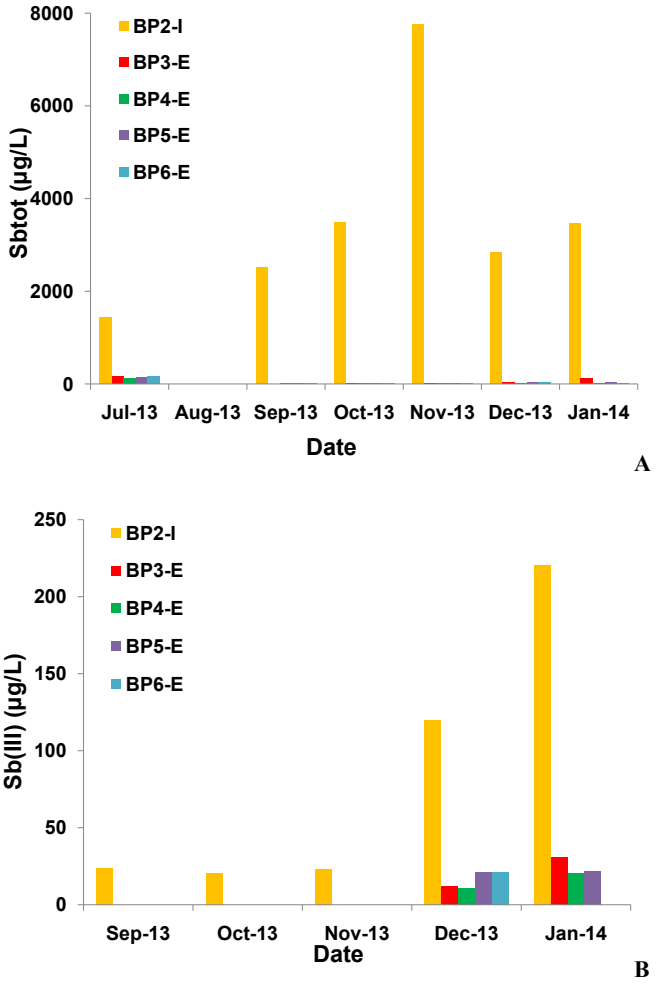

Fig. 3. The distribution of different soluble Sb species in each monitoring point. A: total soluble Sb; B: soluble Sb(III); Acronyms: BPX-I: the influent of unit BPX (X could be 2–6); BPX-E: the effluent of unit BPX (X could be 2–6).

effluent of BP6 ranged from 6.6 (September 2013) to 168.6 μg/L (July 2013) (Fig. 3A). More than 90% Sb_{tot} removal occurred in BP2 and BP3. In the influent, Sb(III) (81.5 ± 88.3 μg/L) accounted for 2.5 ± 2.9% of total Sb with the highest concentrations occurring in December 2013 (120 μg/L; 1.5% of Sb_{tot}) and January 2014 (220 μg/L; 7.7% of Sb_{tot}). In four of the five monitoring time points, no Sb(III) was detected in effluent of the bioreactor (>99.9% Sb(III) removal), while in January 2014, 82% of the Sb(III) was removed (note that Sb(III) was not measured in July and August 2013). The majority of Sb(III) removal (95.7 ± 6.8%) occurred in BP2 and BP3 (Fig. 3B). Total Sb in the sediments (Sb_{sed}) was measured in all sediments collected during October 2013. Sb_{sed} was high in treatment unit BP2 to BP4 but was relatively low in BP1, BP5, and BP6 (Table 1).

3.2. Microbial diversity and composition of sediments in different units

Altogether, 29 bacterial and archaeal phyla were recovered from the six samples. The majority of these reads were affiliated with known members of *Proteobacteria*. This phylum accounted for 55.9% of total reads (ranging from 46.3% to 65.4% in the six libraries) and was the most dominant phylum in all six samples (Fig. S3 and Table S3). *Cyanobacteria* was the second most abundant phylum, accounting for 12.8% of the total reads. This phylum was more abundant in microaerobic units (BP5 and BP6) than that in the other units. *Bacteroidetes* and *Firmicutes* ranked as the third and fourth most abundant phyla, accounting for 4.79% and 4.68% of the total reads, respectively. An UPGMA tree based on the shared phylogenetic distance between each sample indicated that distinct microbial communities developed within this bioreactor (Fig. 4). Among these microbial communities, the ones from the two microaerobic units, BP5 and BP6, grouped together.

Further comparison of the samples at the genus level was conducted to reveal the microbial community response to physicochemical conditions in each unit. PCoA (Fig. S4) at the OTU-level (97% sequence similarity) indicated that distinct microbial communities developed in each unit. Among them, microbial communities of BP5 and BP6 were closer to each other than to other samples. This taxonomic pattern was mainly driven by differences in the abundances of a few major genera (see heat map of Fig. S5 for detailed information). The mine portal (B1) was dominated by *Flavobacterium* (9.57%), but this genus accounted for a very low abundance in the bioreactor. In BP2, *Ferrovum* (11.8%), *Thiomonas* (5.4%), *Gallionella* (3.5%), and *Leptospirillum* (2.3%) demonstrated relatively higher abundances than other genera. Different genera were enriched in the two aerobic units. *Rhodanobacter* was dominant in BP3 (19.1%) but was present at very low relative abundance in other samples. *Halomonas* (9.1%) and *Ferrovum* (5.8%) were the most abundant genera in BP4. The most abundant genera in BP5 and BP6 were the same, including *Rhodobacter* (2.9% in BP5 and

Table 1
Concentrations of metal(loid)s in the sediment samples (mg/kg sediment).

Sample name	Sb	As	Cr	Mn	Ni	Cu	Zn	Se	Cd	Ba	Pb
BP1	629	69.1	190	2245	200	40.4	759	20.7	22.1	499	53.1
BP2	18,593	213	220	118	117	50.5	483	28.8	20.8	1010	66.5
BP3	17,241	330	243	134	82.3	86.0	555	24.5	20.5	428	325
BP4	20,629	345	183	175	88.6	59.2	591	28.5	21.8	508	265
BP5	8567	96.4	125	105	66.8	136	406	28.5	20.9	102	48.9
BP6	2377	221	171	218	127	168	655	46.5	21.1	100	55.8

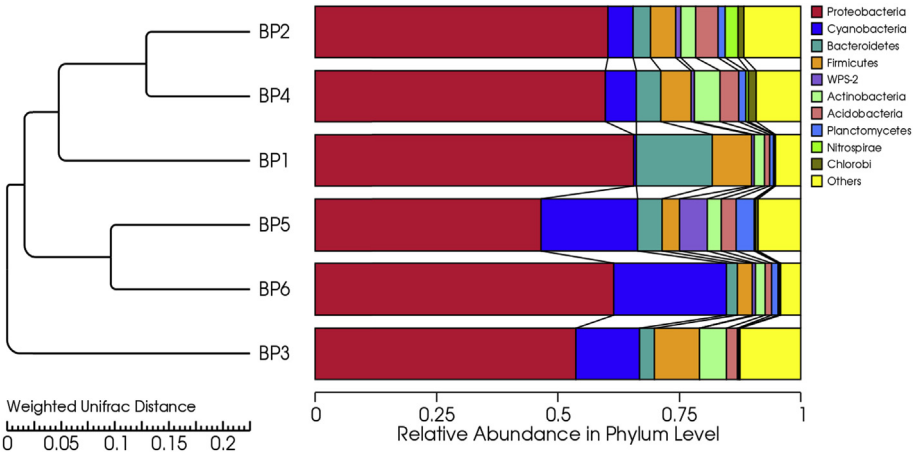

Fig. 4. The UPGMA tree showing clusters of microbial communities based on weighted UniFrac with 100% support at all nodes.

14.7% in BP6), *Methylosinus* (2.6% in BP5 and 3.6% in BP6), and *Thiomonas* (2.6% in BP5 and 3.3% in BP6). In addition, *Halomonas*, *Bdellovibrio*, *Novosphingobium*, and *Ferrovum* were also enriched in BP6. A profile clustering network analysis analyzed by a Cytoscape network highlighted the relative distribution and abundances of the 28 most abundant genera (Fig. 5). This profile showed that *Ferrovum*, *Rhodobacter*, *Rhodanobacter*, and *Escherichia* were in high abundance in at least one sequencing library based on the sizes of their corresponding nodes. *Ferrovum* was ubiquitous in all five libraries and was the most abundant genus in BP2. However, its abundance in other libraries was also relatively high. *Thiomonas* exhibited similar abundances in all five libraries. *Rhodobacter* and *Rhodanobacter* were the most abundant genera in BP3 while *Rhodobacter* was more abundant in BP6 than other units.

3.3. Effect of environmental parameters on the microbial community composition

Canonical correspondence analysis (CCA) was performed to discern possible linkages between the microbial communities and physicochemical parameters in each unit. Water and sediment physicochemical data measured in October 2013 when the biomass was sequenced was used for CCA. The first two axes explained 56.8% and 27.3% of the total variance, respectively. As indicated by the length of the environmental variables arrows in the CCA biplot, the strongest determinant for the microbial communities was pH (Fig. 6), which was negatively correlated with CCA axis 1. Eh, Sb_{sed}, and As_{sed} (arsenic concentrations in the sediments) were positively correlated with CCA axis 2. The magnitude of the vectors of these parameters indicated they were strongly correlated with the overall microbial communities. In particular, microbial communities within BP2 and BP4 were positively correlated with Eh and Sb_{sed} while BP5 and BP6 and microbial phylotypes of *Rhodobacter* and *Methylosinus* were positively correlated with pH.

4. Discussion

For the current study, a low-cost, minimal-maintenance bioreactor was constructed in an Sb mining area to evaluate its Sb removal capability. During the six-month monitoring period from July 2013 to January 2014, the bioreactor exhibited substantial decreases in dissolved Sb concentrations between the inlet and the outlet. Specifically, the majority of dissolved Sb_{tot}, Fe_{tot}, and Sb(III) were removed in the first two aerobic units (BP2 and BP3), indicating the importance of aerobic processes on the removal of dissolved metal(loid)s. In the second half of the monitoring period (September 2013 to January 2014), the bioreactor demonstrated significant Sb(III) removal with Sb(III) not detected in the effluent of the bioreactor at four of five time points. These Sb removals contrast with Sb concentrations in an adjacent river, which receives the same contaminated water as the bioreactor from the active Sb mine. Sb_{tot} concentrations in the river remained high, ranging from 3260 μg/L near the source to 1134 μg/L more than 2 km downstream just before its confluence with another river (Sun et al., 2016). This suggests that aqueous Sb is not effectively removed by the natural aquatic system, but our engineered system is effective in removing Sb from contaminated mine drainage.

It is reasonable to propose that the soluble Sb was precipitated in the sediments accumulated in the bioreactor. This hypothesis was supported by the fact that sediment from BP2-4, where the most Sb_{tot} removal occurred, demonstrated elevated Sb_{sed} (17,240–18,593 mg/kg) (Table 1). The remaining Sb was then precipitated in the downstream bioreactors, which was supported by the much lower Sb_{sed} (856–2376 mg/kg) in the sediments from the two microaerobic units (BP5 and BP6). Additional evidence of Sb precipitation was obtained using SEM-EDX analysis of the sediments. Sb-peaks with high intensity were observed in the sediment samples taken from BP2-BP6 (Fig. S6). It provided semi-quantitative evidence that high Sb content in the sediments likely precipitated from the soluble Sb in the influent. We also speculate

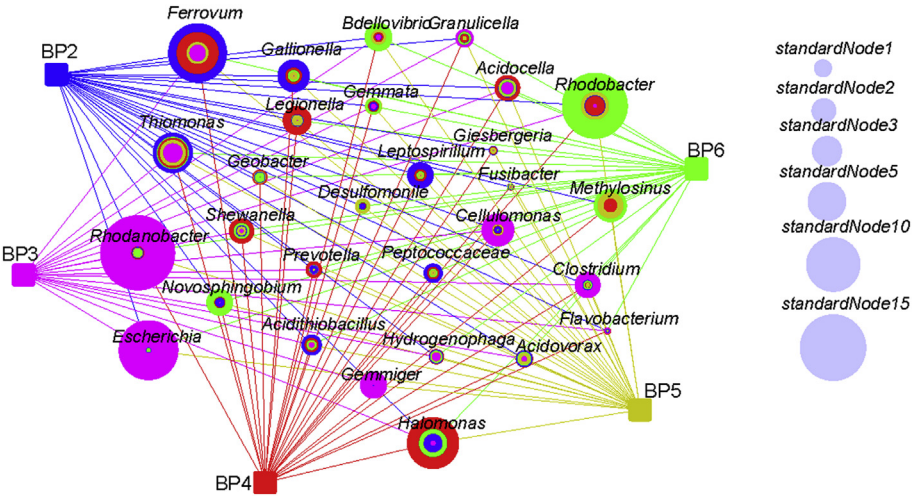

Fig. 5. Profile clustering Cytoscape network visualizing the 28 most abundant genera among the five bioreactor units (Note that microbial community from mine portal (BP1) was not included). Each unit was represented by a unique color. A comparative node (right of the figure) indicates the size of a node that would represent the relative abundance in a group. For example, standard node 1 shows that this size would represent 1% of the corresponding genus in a group. Standard node 15 shows that this size would represent 15% of the corresponding genus in a group. The combination of the size and the colors of the nodes represented the relative abundances of a genus in the bioreactor unit representing by the color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

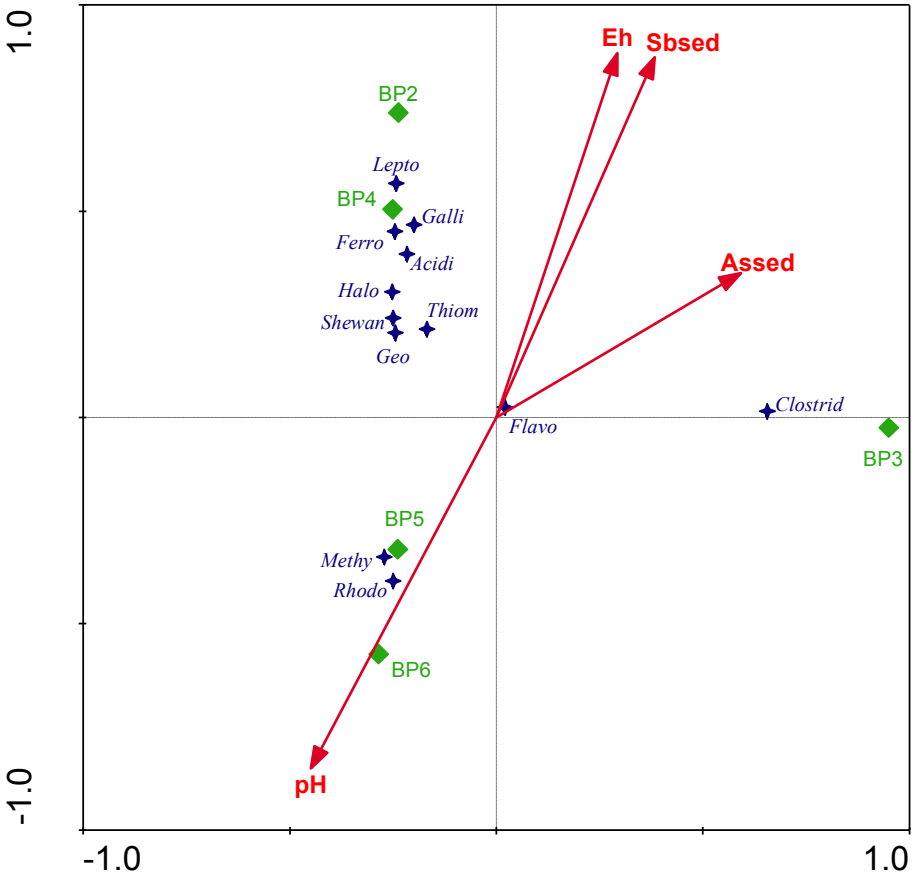

Fig. 6. Canonical correspondence analysis (CCA) of SSU rRNA gene data (relative abundances of dominant genera in each unit) and selected water and sediment physicochemical parameters. Arrows indicate the direction and magnitude of water and sediment parameters associated with bacterial community structures. Green diamond indicated the overall microbial community of each unit. Blue star indicated the dominant genera. Abbreviations of microbial phylotypes: *Lepto*, *Leptospirillum*; *Galli*, *Gallionella*; *Ferro*, *Ferrovum*; *Acidi*, *Acidithiobacillus*; *Thiom*, *Thiomonas*; *Geo*, *Geobacter*; *Clost*, *Clostridium*; *Methy*, *Methylosinus*; *Rhodo*, *Rhodobacter*; *Halo*, *Halomonas*; *Shewan*, *Shewanella*; *Flavo*, *Flavobacterium*. Abbreviation of physicochemical parameters: Sbsed, Sb concentrations in the sediments in each unit (measured in October 2013). Assed, As concentrations in the sediments in each unit (measured in October 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that Sb removal may be attributed to co-precipitation. For example, Sb may adsorb to Fe-oxyhydroxide produced either by chemical or biological oxidation. It is known that Sb species can be adsorbed by Fe-oxyhydroxide (Ashley et al., 2003): The more toxic Sb(III) can be strongly adsorbed on Fe-oxyhydroxide over a wide pH range and rapidly be oxidized to the less toxic Sb(V) (Belzile et al., 2001; Leuz et al., 2006) while the maximum adsorption of Sb(V) occurred below pH 7 (Leuz et al., 2006). Thus, low pH and relatively high Fe_{tot} in BP2 and BP3 are favorable for Sb(III) and Sb(V) adsorption. A co-occurrence of Sb and Fe peaks by SEM-EDX analysis in several sediment samples provides additional evidence for the co-precipitation hypothesis (Fig. S6).

This onsite bioreactor provides valuable practical data for treatment of Sb and possibly other metal(loid)s. In the current study, Sb_{tot} and Sb(III) removal were mostly observed in the first two aerobic units. In contrast, the third aerobic unit (BP4) may play an important role in increasing the pH and functioning as a buffer tank for further metal(loid) precipitation. As indicated by the elevated Sb_{sed} in BP4, it is speculated that this unit received some residual dissolved Sb from the upstream units and provided longer retention time for Sb precipitation. pH continued to increase in the two microaerobic units (partially due to the neutralization with chicken litter), suggesting a role for these units in acid reduction.

Environmental parameters impact the microbial composition and diversity on each unit. PCoA indicated that the microbial community at the mine portal was distinct from the microbial communities in the bioreactor. The differences among the microbial communities indicate that the microbial communities within the bioreactor are not simply successors of those in their adjacent surroundings (i.e. mine portal and contaminated river). The microbial communities even differed between units, demonstrating the effect of environmental factors in shaping the microbial communities. In addition, the microbial communities are most likely also driving those environmental changes themselves within the bioreactor. For instance, the shift of redox condition from oxidized environments to less oxidized environments caused by heterotrophic microbes may itself influence the distribution of microorganisms. Finally, the microbial communities of BP5 and BP6 suggest that the addition of chicken litter may introduce exogenous microorganisms (e.g. *Escherichia*) into these units, making them distinct from the other units (Fig. 4).

Microbial community composition and diversity are largely structured by specific physicochemical parameters in each unit. As indicated by CCA, a suite of physicochemical parameters, such as pH, Eh, and Sb_{sed} were strongly correlated with microbial communities. pH appears to be the most important environmental parameter based on the strength of the vector (Fig. 6). pH has been identified as an important factor in shaping microbial communities in AMD contaminated environments (Sun et al., 2015a, 2015b) and wastewater treatment plants (Wang et al., 2012). The increase of pH along the flow direction may drive the observed changes of microbial communities. For example, in the first two units with low pH (<4), more acidophilic bacteria such as *Ferrovum*, *Thiomonas*, *Leptospirillum*, *Acidithiobacillus*, and *Acidocella* occurred at high levels, while neutrophilic bacteria such as *Rhodobacter* and *Methylosinus* were more abundant in units with higher pH (BP5 and BP6). In addition to pH, microbial communities were also significantly linked to redox potential (Eh). It has been reported that Eh and pH are the major parameters that control the speciation of Sb in the environments (Ashley et al., 2003; Murciego et al., 2007; Nakamaru et al., 2006; Vink, 1996; Wilson et al., 2004; Xi et al., 2010). Sb will precipitate as different Sb-bearing minerals under various Eh and pH (Craw et al., 2003; Murciego et al., 2007; Wilson et al., 2004) with oxidizing conditions (high Eh) favoring higher Sb solubility (Ashley et al., 2003). Various Sb speciation may influence the distribution of microorganisms. Thus, this system represents a complicated interaction among microbial communities, physical/chemical conditions (Eh and pH), and Sb speciation (Sb mobility, solubility, and bioavailability).

Genus level analyses reveal the differences among the microbial communities in finer detail and allow inferences of the microbial functional development within the bioreactor. Although microorganisms were not isolated, we can infer their potential metabolic capabilities based on their known isolated relatives (Bond et al., 2000b). One of the notable findings of this study was that most Sb_{tot} and Sb(III) was removed in BP2 and BP3. In accordance with the removal of Sb species in these two units, diverse metal(loid)-oxidizing bacteria were detected in these units. For example, the most abundant genera in BP2, *Ferrovum* (11.8%), *Thiomonas* (5.4%), *Gallionella* (3.5%), and *Leptospirillum* (2.3%), contain members known to be Fe(II)- and As(III)-oxidizing bacteria (FeOB and AsOB, respectively). For instance, *Ferrovum* contains autotrophic acidophilic FeOB that are able to grow litho-autotrophically (Bruneel et al., 2011). *Ferrovum myxofaciens* was observed to oxidize Fe(II) in a mine water treatment plant (Hedrich et al., 2011a), acid streamers (Johnson et al., 2014), and a continuous flow reactor system (Hedrich and Johnson, 2012). *F. myxofaciens* were found to be abundant in an aerobic iron oxidation plant for mine water treatment (Tischler et al., 2014) and pilot plants for the treatment of acid mine waters (Heinzel et al., 2009). It has been reported that *F. myxofaciens* is able to produce copious amounts extracellular polymeric substances (EPS), which may provide a competitive advantage in such systems by facilitating attachment to surfaces and preventing washout (Rowe and Johnson, 2008). Thus, *Ferrovum* species seem to be important Fe(II) oxidizer in engineered systems treating mine waters.

Leptospirillum spp. are well-known acidophilic FeOB and are the dominant organisms in many commercial processes for the bio-oxidation of pyrite and related ores (Rawlings et al., 1999). *Leptospirillum* spp. are often abundant in strongly acidic AMD environments (e.g. pH < 2) (Bond et al., 2000a; García-Moyano et al., 2008), but they are seldom detected in AMD with pH > 2.5 (Brown et al., 2011; Tan et al., 2009). Some *Leptospirillum* strains have an arsenic resistance operon structure (Li et al., 2010; Tuffin et al., 2006), which may explain the enrichment of *Leptospirillum* spp. in this Sb-rich environment because Sb and As have similar chemistry and toxicity and frequently co-occur (Landrum et al., 2009; Majzlan et al., 2011; Telford et al., 2009). Unlike *Leptospirillum* spp., *Gallionella* spp. are neutrophilic FeOB detected in environments with moderate pH (Wang et al., 2009, 2011). However, the dominance of *Gallionella* spp. in a moderately acidic (pH 4.4) site has also been reported (Fabisch et al., 2013). *Thiomonas* are frequently detected in arsenic-contaminated environments and this genus contains important As(III)-oxidizing strains (Bryan et al., 2009; Duquesne et al., 2008; Johnson and Hallberg, 2005b). For instance, *Thiomonas arsenivorans* strain $b6^T$, which was isolated from an abandoned mine site, can grow chemoautotrophically on As(III), sulfur and thiosulfate, as well as heterotrophically on a variety of defined organic compounds (Battaglia-Brunet et al., 2006). Given the chemical similarities between As and Sb (Byrd, 1990), it is possible that *Thiomonas* spp. may be able to oxidize Sb(III).

Chemical oxidation of Fe(II) occurs very slowly at pH < 4 (Hedrich et al., 2011b; Ilbert and Bonnefoy, 2013), but FeOB have been shown to increase Fe(II) oxidation two to fivefold under these conditions both in pure cultures (Neubauer et al., 2002) and in microbial iron mats (Rentz et al., 2007). Given the low pH in BP2 and 3 (especially BP2), we propose that biotic Fe(II) oxidation occurred, based on both the significant Fe_{tot} removal and enrichment of FeOB in the bioreactor. Fe(II) oxidation can affect the mobility of Sb by adsorbing and precipitating Sb from the water

(Ashley et al., 2003). It is also possible that these iron-oxidizing or arsenic-oxidizing bacteria detected in this study are capable of respiring Sb. For instance, some FeOB have shown to transform other metal(loid)s, including oxidation of Cu^{+} to Cu^{2+} (Nielsen and Beck, 1972) and U^{4+} to U^{6+} (DiSpirito and Tuovinen, 1982). These observations collectively indicate the importance of metal(loid)-oxidizing bacteria in removing Sb.

With the shift from oxidized environments to less oxidized environments, as indicated by Eh, the microbial communities also shifted from clearly aerobic microbial communities to facultative and anaerobic assemblages. For example, a number of Fe(III)-reducing bacteria (FeRB) including *Shewanella*, *Geobacter*, and *Geothrix* were detected in the bioreactor, with higher relative abundances in less oxidized cells. *Shewanella,* a genus which contains species that are able to reduce Fe(III) and other metals (Heidelberg et al., 2002) was enriched in BP4 (2.05%), while *Geobacter* (another genus with members capable of Fe(III) reduction (Sung et al., 2006)) was present in relatively low abundance in all samples. The changes of redox state also facilitate the enrichment of a number of strictly anaerobic bacteria in less oxidized cells, namely, sulfate reducing bacteria (SRB). *Desulfomonile* (DeWeerd et al., 1990), *Peptococcaceae* (family, genus unclassified) (Alazard et al., 2010), and *Clostridium* (Bao et al., 2012) represented some of the SRB detected in this study. Among them, *Desulfomonile* and *Clostridium* were presented in relative high abundances in BP5 (0.96%) and BP3 (2.2%), respectively. Sulfate reduction could generate sulfide as a reaction product to precipitate metal(loid)s, including Sb (Johnson and Hallberg, 2005a). Wang et al. found that sulfides resulting from biological sulfate reduction were able to form precipitate such as Sb_2S_3 (Wang et al., 2013). Many FeRB and SRB are capable of respiring using other metal(loid)s as electron acceptors (Liu et al., 2002; Lovley et al., 1993). Some SRB can transform a wide variety of metalloids, transition metals, and actinides (Barton et al., 2015). *Shewanella* was able to reduce Fe, Mn, and As under different redox conditions (Fredrickson et al., 2002; Heidelberg et al., 2002; Saltikov et al., 2005). The metabolic versatility of SRB and FeRB suggests the possibility of direct enzymatic Sb(V) reduction. Furthermore, the relatively lower abundance of sulfate and metal-reducing bacteria than metal(loid)-oxidizing bacteria implies a prevalent oxidizing process along the bioreactor. The relatively high Eh and relatively lower abundance of sulfate and metal-reducing bacteria compared to metal(loid)-oxidizing bacteria suggest that the Fe(III), sulfate, and Sb(V) reduction may be relatively minor processes in the bioreactor. However, this low activity of enzymatic reduction may be beneficial for remediation of Sb because Sb(V) is significantly less toxic than Sb(III). (Filella et al., 2002).

Interestingly, distinct microbial communities developed in two microaerobic units (Fig. 4), possibly due to the addition of chicken litter. Both units harbored a high number of sequences related to genera *Rhodobacter* and *Methylosinus*. Furthermore, the enrichment of *Cyanobacteria*-related phylotypes in the bioreactor, especially in the microaerobic units was surprising since *Cyanobacteria* have not frequently been reported in mine-impacted environments (Mclean et al., 2007). The sporadic reports do suggest that *Cyanobacteria* are responsible for biotransformation of metal and metalloids in some mine-related habitats. For instance, removal of zinc and manganese by *Cyanobacteria* was reported in mine-contaminated environments (Bender et al., 1994; Phillips et al., 1995). In addition, *Cyanobacteria* could convert substantial amounts of Hg(II) into β-HgS (Anjana et al., 2007; Lefebvre et al., 2007; Phillips et al., 1995). It has also been reported that freshwater *Cyanobacteria* (*Microcystis*) are capable of biosorption of both Sb(III) and Sb(V), suggesting the potential ecological role of *Cyanobacteria* in removal of Sb (Sun et al., 2011; Wu et al., 2012). Another explanation for the enrichment of *Cyanobacteria* in the bioreactor is the attenuation of pH along the length of the reactor. *Cyanobacteria* have been reported to prefer neutral to slightly alkaline pH for optimum growth (Brock, 1973). A large fraction (98%) of the cyanobacterial SSU rRNA reads was identified as belonging to chloroplasts, particularly *Chlorophyta* and *Stramenopiles*. These reads, together with the non-chloroplast *Cyanobacterial* reads indicate that units BP5 and BP6 provided favorable environments for the growth of photosynthetic microbes. This would explain to some extent the difficulty in maintaining sufficiently low redox potential to sustain sulfate reduction in these units.

5. Conclusion

In summary, this study represents the first of its kind to document the field feasibility of remediating Sb-rich mine water using indigenous microbial communities in an onsite field-scale bioreactor. This low cost minimum maintenance onsite bioreactor system successfully removed soluble Sb_{tot} and Sb(III). The design and application of this technology can be generalized to other contaminated mining sites, which are often remote, making routine operation and maintenance a challenge. This bioreactor is not only intended to treat antimony-rich mine waters, but other polluted waters such as AMD. Illumina-sequencing revealed that distinct microbial communities had developed in each unit. Moreover, a wide diversity of microorganisms related to Fe(II)- and As(III)-oxidizing bacteria is thought to be involved in biogeochemical Sb cycling.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.05.008.

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