Rhizosphere bacterial community PCR-DGGE profiles and metal speciation in shooting range soils

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Abstract
Chemical immobilization in conjunction with vegetation coverage is a practical remediation technology for metal contaminated soils. This paper evaluated metal solubility and bacterial community structure in a highly contaminated shooting range soil treated with a phosphorus amendment (AP) and plant growth. The low dose of amendment addition decreased the SPLP-Pb concentration to 2.1 mg/L. However, the high dose treatment increased Pb solubility more than that of Control soil. The soils treated with the amendment along with plant growth (Plant + AP) had a lower SPLP-Pb concentration than the soils treated solely with the amendment. The soils with the amendment addition had a decreased number of bands in their DGGE profile, and the detrimental effect of amendment appeared more significant for the high dosage than in low dosage. According to the DNA sequence identification, the band corresponding to *Bacillus sp*., and *Rhizobium sp*. that dominated in the Control and Plant soils was not observed in the soil with the high dose of amendment.

Key Words
Microbial activity, heavy metals, lead, antimony, phytoremediation.

Introduction
In situ chemical immobilization is a practical remediation technology for metal-contaminated soils because of its capability to reduce cost and environmental impacts. We have suggested a new immobilization technology (chemically-enhanced phytostabilization) that can be applied along with a vegetation cover as a form of phytostabilization (Hashimoto *et al.* 2009). For development and evaluation of chemically-enhanced phytostabilization technology for shooting ranges, it is necessary to investigate how the plant rhizosphere affects speciation and solubility of preexisting heavy metals in the soil. In addition to mineralogical and chemical perspectives of heavy metals, the technology should be assessed with respect to remediation of soil biological functions in the rhizosphere soil. This paper evaluated metal solubility and bacterial community structure in a highly contaminated shooting range soil treated with a phosphorus amendment and plant growth.

Methods
Experiment setup
Ryegrass (*Lolium perenne* L.) was grown with a modified a split-root system described elsewhere (Perez *et al.* 2007). The split-root system was designed to isolate rhizosphere and non-rhizosphere soils. Two PVC tubes with a 14 cm internal diameter and 10 cm length formed lower and upper sections, both of which contained contaminated soils collected from a shooting range in Central Japan. These sections were connected by a central compartment with 32 PVC tubes each with 10 mm internal diameter and 10 cm in length. The tubes of central compartment were filled with a 0.45 mm sieved soil and treated with 2% and $4\%_{(w/w)}$ of hydroxyapatite (AP_{low} and AP_{high}). The tubes without amendment but allowing root entry were also prepared (Plant). The soil tubes where roots were blocked from entry using a 10 µm nylon filter served as non-rhizosphere soils (Control).

Heavy metal analysis in soils
Synthetic Precipitation Leaching Procedure (SPLP, EPA Method 1312) was used to determine extractable Pb and Sb in the soil. Twenty (20) mL SPLP solution (H_2SO_4/HNO_3 = 60/40% wt.) was added to 1.0 g soil, and the mixture was equilibrated for 24 hours on a shaker. The supernatant passed through a paper filter was analyzed by ICP-AES. Equilibrium modeling was performed by a thermodynamic code, Visual MINTEQ ver. 2.61 (KTH, Stockholm, Sweden) to compute the saturation indices (SI) of Pb precipitates that may control Pb solubility in liquid phases. The SI represents the degree of saturation with respect to a specific Pb

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solid phase is defined as SI = log IAP − log K_{sp} where IAP is the ion activity product and K_{sp} is the solubility product constant. If $-1 < SI < 0$, the solution is saturated with respect to the solid; if $SI < -1$, the solution is undersaturated with respect to the solid; and if $SI > 0$, the solution is supersaturated with respect to the solid. The extraction procedure of soil solution and following elemental analysis were the modifications of previous study (Hashimoto *et al.* 2008).

Soil DGGE analysis
Nucleic acid was extracted directly from duplicate 0.5-1.0g composite samples. PCR amplification of the 16S rDNA fragments prior to DGGE was performed as described by Muyzer *et al.* (1993). DGGE was performed by using a D-Code 16/16-cm gel system with a 1.5-mm gel width (Bio-Rad, Hercules, Calif.) maintained at a constant temperature of 60°C in 7 liters of 1× TAE buffer. The central 1-mm 2 portions of strong DGGE bands were excised with a razor blade and soaked in 50 µl of purified water overnight. Purified DNA was sequenced with an ABI-Prism model 373 automatic sequencer. Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information and the Sequence Match facility of the Ribosomal Database Project.

Results
Soil characterization
The Pb-contaminated soil had a sandy clay loam texture based on the USDA soil textural classification system. The soil had a relatively high proportion of organic matter (8.6%) since the shooting range is located in a forested area. The soil had a pH value of 5.9. Total concentration of Pb and Sb in the soil was 29600 and 50 mg/kg, respectively. An XRD analysis revealed that cerussite ($PbCO_3$) was a predominant Pb mineral in the soil.

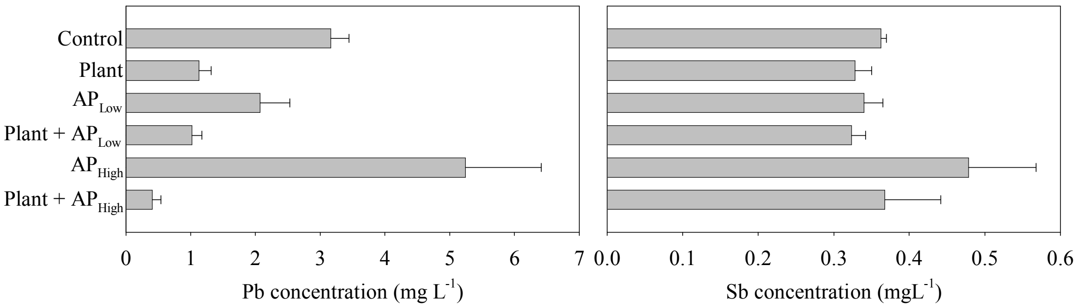

Figure 1. Concentrations of SPLP extracted Pb and Sb in the soils treated with and without amendment and plant

Pb and Sb solubility
The SPLP-Pb concentration for the Control soil was 3.2 mg/L (Figure 1). The low dose amendment decreased the SPLP-Pb concentration to 2.1 mg/L. As expected, the decreased Pb solubility resulted in transformation of pre-existing cerussite into pyromorphite. However, the high dose amendment increased Pb solubility more than that of Control soil. The soils treated with the amendment along with plant growth (Plant + AP) had a lower SPLP-Pb concentration than the soils treated solely with the amendment (AP_{low} and AP_{high}). The rhizosphere processes including proton and organic acid secretions may enhance the dissolution of soil Pb and amendment P and induce their reformation as pyromorphite precipitates. Solubility of Sb was not attenuated by the amendment rather appeared to be increased by the high dose amendment (AP_{high}). The dissolution of Sb was attributable to anion exchange processes between soil Sb and amendment P. Our result suggests that a high dose of apatite application increased both Pb and Sb solubility.

Aqueous Pb speciation
The SI values of solubility controlling phases were calculated to examine the Pb solubility in elutes among the treatments (Table 1). Over 98% of total dissolved Pb in all treatments was present as organically complexed forms. Chloropyromorphite [$Pb_5(PO_4)_3Cl$] had SI values of over -1.0 in the Control, Plant, AP_{low} and AP_{high} treatments, indicating a possible saturation in solution. The SI value of the combination treatments with amendment and plant growth indicated that chloropyromorphite appeared to be undersaturated in solution. Increased DOC concentration observed in these solutions accelerated the Pb-organic complex which may block the formation of chloropyromorphite.

Table 1. Saturation index values of minerals in the soils treated with plant and different levels of amendments (AP).

Minerals	Control	Plant	AP_{low}	Plant+AP_{low}	AP_{high}	Plant+AP_{high}
$CaCO_3$	-1.6	-2.0	-0.9	-1.1	-0.9	-0.5
$Ca_5(PO_4)_3OH$	-4.7	-6.9	-0.4	-3.3	0.0	-0.8
$PbCO_3$	-1.8	-2.1	-2.1	-2.1	-2.0	-2.7
$Pb_3(CO_3)_2(OH)_2$	-5.6	-6.4	-6.5	-6.6	-6.3	-8.4
$Pb_5(PO_4)_3OH$	-10.9	-12.1	-11.6	-13.6	-10.5	-17.1
$Pb_5(PO_4)_3Cl$	0.0	-0.9	-1.0	-2.8	0.0	-6.5
PbO	-7.3	-7.6	-7.6	-7.6	-7.5	-8.2
$Pb(OH)_2$	-2.6	-2.8	-2.9	-2.9	-2.8	-3.5

Soil DGGE analysis

Apparent differences in the bacterial community among the treatments were observed in the DGGE profiles (Figure 2). Plant growth in the contaminated soil appeared to enrich bacterial populations since the general band profile exhibited in the Plant soil was brighter than the Control soil. The soils with the amendment addition decreased the number of bands in their DGGE profile, and the detrimental effect appeared more significantly in AP_{high} than in AP_{low} soils. However, the number of bands lost in the solo application of amendment was recovered in the soil treated with amendment along with plant growth. According to the DNA sequence identification, the band corresponding to *Bacillus sp*., and *Rhizobium sp*. dominated in the Control and Plant soils was not observed in the soil with the high dose of amendment.

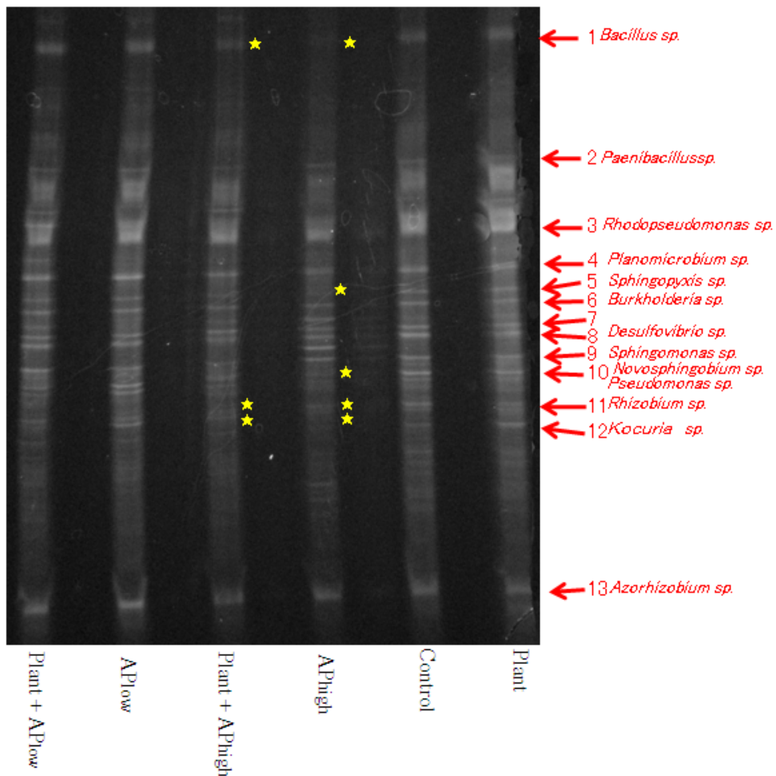

Figure 2. DGGE profiles and DNA sequence identification from the soils treated with and without amendment and plant.

Discussion and summary

Solubility of soil Pb was attenuated by the amendment application. When the amendment was added, Pb immobilization was more enhanced in the rhizosphere than the non-rhizosphere soils. Previous studies identified the formation of phosphate-bound Pb precipitates at the root surface (Cotter-Howells *et al.* 1999). Original Pb species in the soil may be transformed via rhizosphere processes that have a catalysing role for chloropyromorphite formations, although the exact mechanism remains unclear. A significant decrease of number of band in the DGGE profile was found in the AP_{high} soils where solubility of Pb and Sb increased by the high dose of amendment. The detrimental impact of amendment on soil bacterial community may be attributed to the increased metal toxicity and salinity levels which were induced by the high application dosage. Plant growth in combination with the amendment even at high dosage attenuated Pb solubility and generally conserved bacterial community found in the Control soil. Our study suggests that phytostabilization assisted with the amendment enhance Pb immobilization and maintain bacterial community even in a highly contaminated shooting range soil.

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