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공학박사 학위논문

**Enhancement of Phytoremediation for  
Cd and As through Facilitating  
Bioavailability by Rhizobacterial Activity  
and Associated Ecotoxicological  
Consideration**

근권 미생물에 의한 카드뮴과 비소의 생물학적  
이용성 향상을 통한 식물상 정화공법의 효율 증진  
및 생태독성학적 영향에 관한 연구

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정 슬 기

Enhancement of Phytoremediation for  
Cd and As through Facilitating Bioavailability by  
Rhizobacterial Activity and  
Associated Ecotoxicological Consideration

by

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Doctor of Philosophy

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August 2014

# ABSTRACT

## **Enhancement of Phytoremediation for Cd and As through Facilitating Bioavailability by Rhizobacterial Activity and Associated Ecotoxicological Consideration**

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Phytoremediation has demonstrated its beneficial use in the removal of heavy metals from polluted soil. Recently, many researches have focused on plant growth promoting rhizobacteria (PGPR) application to enhance metal removal efficiency during phytoremediation. In particular, phosphate-solubilizing bacteria (PSB) and siderophore-producing bacteria (SPB), kind of the PGPR, can solubilize nutrient such as phosphate and iron in soil and provide them for plant growth and these bacterial activities can affect phytoavailability of heavy metals in soils. This study is conducted to evaluate the effects of the PGPRs or its secretions inoculation on removal of heavy metals from soils in phytoremediation, and its environmental risk is investigated using ecological risk assessment during phytoremediation.

Firstly, phosphate-solubilizing bacteria (PSB), *Bacillus* sp. (i.e., a mixed culture of *B. aryabhatai* and *B. megaterium*) were used to promote plant growth

and enhance the phytoextractability of Cd from contaminated soils. This strain showed a potential for directly solubilizing phosphorous from soils more than ten-folds greater than the control without inoculation. The results of pot experiments revealed that inoculation with *Bacillus* sp. significantly increased the extent of Cd accumulation in *Brassica juncea* relative to the uninoculated control for eight weeks. The Cd accumulation by *B. juncea* increased up to 250%, when PSB was inoculated at the 8th week, while almost no further Cd uptake in the uninoculated soils was observed compared with initial soil (i.e., 0 week). The total dry weights of *B. juncea* significantly increased from 10.3 mg to 101.0 mg up to the 6<sup>th</sup> week, and 295.6 mg of total dry weight was observed at the 8<sup>th</sup> week, while it was 65.8 mg in the uninoculated soil. The change of the Cd speciation indicated that inoculation of *Bacillus* sp. as PSB increased the bioavailability of Cd and consequently enhanced its uptake by plants. This study was also conducted to investigate how the microbial community of indigenous soil bacteria is changed by PSB augmentation during phytoremediation. In initial Cd-contaminated soil, the major phyla were *Proteobacteria* (35%), *Actinobacteria* (38%) and *Firmicutes* (8%). While *Proteobacteria* were dominant at the 2<sup>th</sup> and 6<sup>th</sup> week (41 and 54%, respectively) in inoculated soil, *Firmicutes* dramatically increased in the eight week soil, contributing 63% of the sequences, and they mainly belonged to the *Bacilli* class (61%). For the uninoculated soil, the proportion of *α-Proteobacteria* increased after eight weeks (32%). Interestingly, *Actinobacteria* class, which was originally present in the soil (37%), seemed to disappear during phytoremediation, irrespective of whether PSB was inoculated or not. Cluster analysis and Principal component analysis revealed that the microbial community of eight-week inoculated soil was completely separated from the other soil samples, due to the

dramatic increase of *Bacillus aryabhatai*. These findings revealed that it took at least eight weeks for the inoculated *Bacillus* sp. to functionally adapt to the introduced soil, against competition with indigenous microorganisms in soil.

Siderophores are small molecular weight extracellular organic compound secreted by soil bacteria to ensure their iron nutrition by mobilizing iron (i.e.,  $\text{Fe}^{3+}$ ) from its mineral form. Since arsenic (As) in soil is mainly associated with iron oxides, they also play an important role in As mobilization through the dissolution of As-bearing iron oxides. This study focused on the interaction between siderophores and As bound to iron oxides and also evaluated the effect of siderophores on the removal of As from soil during phytoremediation. The ability of siderophores produced by *Pseudomonas aeruginosa* to release As bound to Fe-oxides and to relocate the absorbed As in the plants was investigated. Siderophores released Fe from ferrihydrite, and total Fe concentration was about 53.6  $\mu\text{mol}$ , which was more than that chelated by ethylenediaminetetraacetic acid (EDTA; i.e., 43.7  $\mu\text{mol}$ ). More importantly, about 1.79  $\mu\text{mol}$  of As was found to be associated with siderophores in aqueous phase when siderophores were used to release As from ferrihydrite. In contrast, As was not essentially detected in aqueous phase when EDTA was used, probably due to the readsorption of released As to ferrihydrite. Pot experiment show that, *Pteris cretica*, a known As hyperaccumulator, grown in the siderophore-amended soil showed about 3.7 times higher As uptake (5.62  $\text{mg-As}\cdot\text{g}^{-1}\text{-plant}$ ) than the plant grown in the EDTA-amended soil (1.51  $\text{mg-As}\cdot\text{g}^{-1}\text{-plant}$ ). In addition, As taken up by roots of *P. cretica* in the presence of siderophores seemed to be favorably translocated to shoots (i.e., stems and leaves). About 79% of total accumulated As were detected in the shoots in the presence of siderophores after ten weeks. Fluorescence microscopic analysis

confirmed that As in the roots was delivered to the leaves of *Pteris cretica* as siderophore-As complex.

Ecological risk by the hyperaccumulation of As in *Pteris cretica* during phytoremediation was evaluated at an old, abandoned As-contaminated site. Five receptor groups including terrestrial invertebrate, avian insectivore, small mammal, herbivore, and omnivore were selected as potentially affected ecological receptors. Soil and food ingestion were considered as major exposure pathways. Phytoremediation with only *Pteris cretica* and siderophore-applied phytoremediation to enhance As uptake by the plant were assessed. Ecological hazard index (EHI) values for only small mammal exceeded 1.0 at three week under both the phytoremediation condition due to its limited home range. The EHI value of mammalian herbivore, who mainly consume plant foliage, increased with the prolonged phytoremediation at normal phytoremediation condition. In contrast, when siderophores were applied the risk of mammalian herbivore greatly increased. The risk increased due to the facilitated translocation of As from roots to stems and leaves. Our results suggest that when phytoremediation strategy is considered for metals remediation, its ecological consequence should be taken into account to prevent the spread of accumulated metals through the food chain of ecological receptors such as fencing and netting. Uncertainties involved in the ecological risk assessment process were also discussed.

**Keywords:** plant growth promoting bacteria (PGPRs), phytoremediation, cadmium, arsenic, phosphate-solubilizing bacteria, siderophores, ecological risk assessment

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# CHAPTER 1

## INTRODUCTION

### 1.1 Background

Soil contamination with heavy metals such as cadmium (Cd) and arsenic (As) is becoming one of the most severe environmental issues, leading to pose a threat to food safety and a potential health risk. Moreover, the heavy metals cannot be degraded to harmless products and they are present in the environment indefinitely (Jiang et al., 2008; Ma et al., 2009a). To date, many methods have been developed to remediate heavy metal-contaminated soil, including a range of conventional physical and chemical engineering technologies that are often expensive and involve substantial excavation and transportation (Suresh and Ravishankar, 2004). These processes often employ stringent physic-chemical agents which have a significant negative impact on the ecosystem (Harvey et al., 2002; Lasat, 2002; Dasgupta, 2011). Recently, phytoremediation has been used as an emerging technology as a potential environment-friendly solution which uses plants to remove or immobilize contaminants. This emerging technology may offer a cost-effective, non-intrusive, and safe remediation to humans and environments, over conventional physical and chemical remediation technologies (Bert et al., 2009; Jadia and Fulekar, 2009; Zhang et al., 2010a).

There are a number of potential concerns pertaining to the use of phytoremediation in heavy metal-contaminated soil. Potential issues associated

with phytoremediation can be divided into three categories: efficiency, environmental concern, and economic concern. Among three concerns, the most important strategy is how to enhance the efficiency of phytoremediation. Most plants that could accumulate high concentrations of heavy metals (i.e., hyperaccumulator) are not suitable for field applications due to their small biomass and slow growth (Shen and Liu, 1998; Sheng et al., 2008). Moreover, the low bioavailability of heavy metals in soil may also limit the efficiency of phytoremediation (Alkorta et al., 2004; Sheng and Xia, 2006a; Jiang et al., 2008). To avoid these problems, the application of plant growth promoting rhizobacteria (PGPR) can be considered as important phytoremediation technologies for enhancing biomass production as well as bioavailability of heavy metals to plants in soil. Several mechanisms have explained how the PGPRs stimulate plant growth, which may include the growth hormones production, phosphate solubilization, nitrogen fixation and siderophore production (Kloepper, 1993; Compant et al., 2005; Chakraborty et al., 2006; Lugtenberg and Kamilova, 2009). Although the bioavailability of metals can be enhanced by chemical chelates such EDTA, these expensive compounds can increase the metal leaching risk and negative effects on soil fertility or soil structure (Blaylock et al., 1997; Kos and Lestan, 2004).

The environmental issues has been raised about how phytoremediation will impact the food chain through ecological receptors such as herbivores and omnivores that ingest the heavy metal accumulating plant biomass (Angel and Linacre, 2005; Pilon-Smits and Freeman, 2006). For example, will the heavy metals be spread through ecosystem during phytoremediation? Or phytoremediation contribute to the transport of heavy metals into ecosystem during/after phytoremediation? Early in the phytoremediation process, heavy metal

contents in plant biomass is low, and the ecotoxicity is rarely a concern. However, after growing, the high level of heavy metal contents accumulated in plant biomass can be potentially impact to ecosystem (Angel and Linacre, 2005). To address the environmental concern of the potential hazard phytoremediation poses to ecological receptors, an ecological risk assessment will likely be required (Henry, 2000). The ecological receptors that may expose to heavy metals through the direct ingestion of soil or plants grown on the heavy metal-contaminated soil or animals that have ingested plants grown on the heavy metal-contaminated soil during phytoremediation are all concern.

The application of phytoremediation to cleanup soil will likely be driven by its economic advantages over alternative remediation technologies. Phytoremediation involves costs related to the entire remedial process, from growing and harvesting the plants to disposing or recycling the metals in the plants (ITRC, 1997). However, the total cost of phytoremediation will mainly depend on the remedial duration which is determined by the rates of uptake from the soil and the number of crops which are needed to meet cleanup levels (Pilon-Smits, 2005). The remedial duration can be eventually shortened by increasing phytoremediation efficiency.

In the present study, two PGPRs (i.e., phosphate-solubilizing bacteria and siderophore-producing bacteria) were applied for the heavy metal (i.e., cadmium and arsenic) removal from soil to enhance both heavy metal bioavailability to plants in soil and the growth of the plants for a successful phytoremediation scheme. In addition, ecological risk associated with phytoremediation was assessed in this study. These findings may provide the useful understanding of plant-heavy metal-bacteria interactions for microbe-assisted phytoremediation.

## 1.2 Objectives

A primary objective of this study to understand the phytoremediation management strategy of heavy metal-contaminated soils considering its efficiency and environmental concerns are as follows:

- 1) Enhancement of the phytoremediation efficiency by rhizobacteria (e.g., PGPRs) focused on facilitating bioavailability of heavy metals to plants in heavy metal-contaminated soils
  - i. To investigate the effect of inoculation of a phosphate-solubilizing bacteria (PSB) on Cd uptake efficiency and its effect on the microbial community structure in soils during phytoremediation
  - ii. To investigate the beneficial effect of siderophores secreted by a siderophore-producing bacteria (SPB) in As-contaminated soil during phytoremediation
- 2) Evaluation of the environmental concerns (i.e., ecotoxicological concern) relation to phytoremediation by terrestrial ecological risk assessment

## 1.3 Dissertation structure

This dissertation consists of 6 chapters (Figure 1.1): Following this introduction in chapter 1 and chapter 2 presents the literature review. It includes the perspectives of rhizobacteria (PGPRs) in phytoremediation of heavy metal-contaminated soils, focusing the role of phosphate-solubilizing bacteria (PSB) and siderophore-producing bacteria (SPB), the current general framework of ecological

risk assessment. Chapter 3 and 4 describe the results from experiments to enhance the phytoremediation efficiency by rhizobacteria. Application of phosphate-solubilizing bacteria to Cd-contaminated soils for enhancing the phytoremediation efficiency and their impact on microbial community structure were investigated in chapter 3. And chapter 4 presents the enhancing the effectiveness of phytoremediation through siderophore-As complex formation in As-contaminated soils. Environmental concerns relation to phytoremediation by ecological risk assessment are discussed in chapter 5. Chapter 6 gives the summary of entire dissertation, conclusions and recommendations.

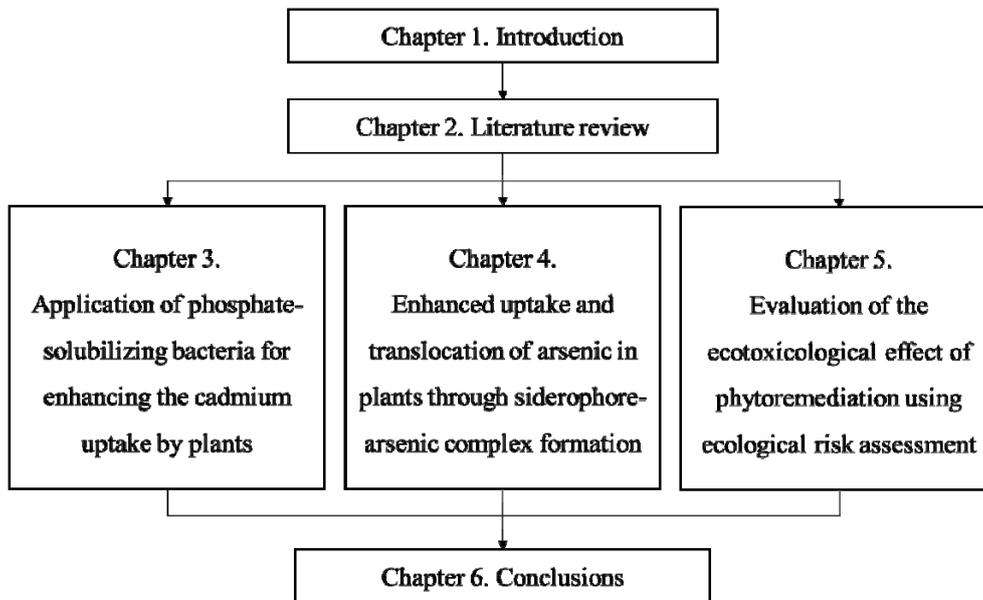


Figure 1.1 Structure of dissertation

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## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Bacterial-assisted phytoremediation**

Phytoremediation, an emerging cost-effective and green technology for decontamination of soils, can be defined as a biological process in which plants extract, stabilize and degrade contaminants in soils. Phytoremediation includes phytoextraction, phytostabilization, phytovolatilization, and phytodegradation (Wenzel, 2009). The success of phytoremediation depends on several factors including the potential of plants to yield high biomass and the ability to accumulate and translocate, and the heavy metal bioavailability to roots of plant in soils (Hooda, 2007; Sessitsch et al., 2013). Several chemical amendments, such as EDTA and limestone have been used to enhance the bioavailability of heavy metals to plants, thus increasing the efficiency of phytoremediation (Blaylock et al., 1997; Khan et al., 2000). However, these chemical compounds are not only phytotoxic but also negative effects on beneficial soil microorganisms or soil structures (Kos and Lestan, 2004; Ultra et al., 2005). For enhancing the effectiveness of phytoremediation, an alternative is to use plant species with a higher growth rate and a higher heavy metal accumulating capacity, such as hyperaccumulator. Another promising alternative could be applied the microbe-assisted process such as plant growth promoting rhizobacteria (PGPR), which is considered to be an important component of phytoremediation technology (Jing et al., 2007b).

PGPRs in rhizosphere in heavy-metal-contaminated soils could enhance the effectiveness of phytoremediation by two complementary ways: (1) indirect promotion of phytoremediation in which the microbes promote the plant biomass production, (2) direct promotion of phytoremediation in which microbes or secreted compounds by microbes change the bioavailability of heavy metal to plants, thus a higher concentrating it in plants. The various mechanisms how to microbe could improve heavy metal uptake by plants are summarized in Figure 2.1.

Generally, PGPRs are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like the facilitating resource acquisition such as nitrogen, phosphorus, iron, and essential minerals, phosphate solubilization, siderophores production, biological nitrogen fixation, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), modulating plant hormone levels, and decreasing the inhibitory effects of pathogen on plant growth (Bhattacharyya and Jha, 2012; Glick, 2012; Ahemad and Kibret, 2013). However, most of previous studies have focused only on plant growth promotion and enhancing the heavy metal uptake by plants, however, did not consider the change of chemical form or bioavailability of heavy metals in soils. Table 2.1 describes the examples of various heavy metal phytoremediation studies associated with plant-microbe interaction.

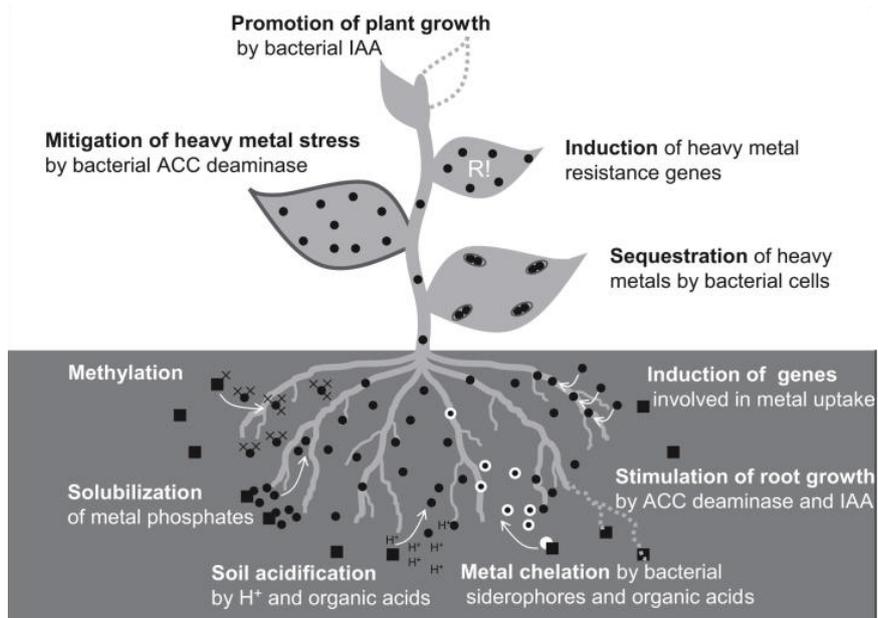


Figure 2.1 Microbe-plant interactions in rhizosphere (Pilon-Smiths, 2005)

Table 2. 1 Mechanisms and application of plant growth promoting rhizobacteria (PGPR) to phytoremediation

PGPRs	Plants	Effects	References
<i>Kluyvera ascorbata</i>	Canola	Increased biomass; ACC deaminase	(Burd et al., 1998)
<i>Enterobacter cloacae</i>	Canola	Increased biomass; ACC deaminase	(Nie et al., 2002)
Rhizosphere and endophytic bacteria	<i>Thlaspi goesingense</i>	ACC deaminase, siderophores	(Idris et al., 2004)
<i>Pseudomonas aspleni</i>	Canola	Increased biomass; IAA	(Reed and Glick, 2005)
<i>Variovorax paradoxus</i> , <i>Rhodococcus</i> sp., <i>Flavobacterium</i> sp.	Indian mustard	Increased root length; IAA, siderophores, ACC deaminase	(Belimov et al., 2005a)
<i>Pseudomonas</i> sp., <i>Bacillus</i> sp.	Indian mustard	Increased root and shoot length; IAA, siderophores, phosphate solubilization	(Rajkumar et al., 2006)
<i>Pseudomonas fluorescens</i>	Sunflower	Increased growth; mechanism unknown	(Shilev et al., 2006)
<i>Bacillus subtilis</i>	Indian mustard	Increased nickel uptake; IAA, phosphate solubilization	(Zaidi et al., 2006)
<i>Pseudomonas</i> sp., <i>Bacillus</i> sp.	Canola	Increased biomass and metal uptake; IAA	(Sheng and Xia, 2006b)
<i>Bacillus edaphicus</i>	Indian mustard	Increased biomass; IAA, siderophores, ACC deaminase	(Sheng et al., 2008)

<i>Pseudomonas</i> sp., <i>Bacillus</i> sp.	Tomato	Increased root length, above ground biomass and above ground metal; siderophores, IAA, ACC deaminase	(He et al., 2010)
<i>Achromobacter xylosoxidans</i>	Indian mustard	Increased root and shoot length and biomass; ACC deaminase, phosphate solubilization, IAA	(Sheng et al., 2008)
<i>Bacillus cereus</i> , <i>Candida</i> <i>Parapsilosis</i> <i>Bacillus</i> sp.	<i>Trifolium repens</i>  Sweet Sorghum, <i>P. acinosa</i> , <i>S. nigrum</i> L.	Increased biomass; IAA  Increased root biomass; IAA, siderophore, ACC deaminase	(Azcón et al., 2010)  (Luo et al., 2011a)
<i>Cellulosimicrobium cellulans</i>	Green chilli	Increased biomass; IAA, phosphate solubilization	(Chatterjee et al., 2009)
<i>Agrobacterium radiobacter</i> <i>Pseudomonas</i> sp.	<i>Populus deltoides</i> <i>Alyssum serpyllifolium</i> , <i>Brassica juncea</i>	Increased plant height and biomass; IAA, siderophore Increased biomass and metal uptake; IAA, siderophore, phosphate solubilization	(Wang et al., 2011a) (Ma et al., 2011a)
<i>Pseudomonas putida</i> , <i>Azospirillum</i> , <i>Azotobacter</i>	Artichoke ( <i>Cynara scolymus</i> )	Increased biomass; phosphate solubilization, nitrogen fixing	(Jahanian et al., 2012)

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## **2.2 Phosphate solubilization by phosphate-solubilizing bacteria (PSB)**

### 2.2.1 Phosphorus in soils

Phosphorus (P), the second important plant growth limiting nutrient after nitrogen, is applied to soil in the form of phosphatic fertilizers (Chen et al., 2006). Soil P exists in inorganic and organic forms. Organic P is held very tightly and is generally not available for plant uptake until the organic materials are decomposed and the phosphorus released via the mineralization process (<http://www.uaex.edu>). Mineralization, the breakdown of readily available organic P to inorganic solution P, occurs in most soils, but it is usually too slow to provide enough P for crop growth. The insoluble P is present as an inorganic mineral such as apatite or as one of several organic form including inositol phosphate and phosphotriesters (Glick, 2012; Ahemad and Kibret, 2013).

Although the total amount of P in the soil may be high, the amount of available forms of plants is generally low (only 0.1% of the total P is available to plants), because chemical fertilizer is immobilized rapidly and becomes unavailable to plants depending on soil pH and Al, Fe, and Ca content (Goldstein, 1995; Schachtman et al., 1998). Majority of soil P is found in the insoluble forms, while the plants can only absorb it in two soluble forms, the monobasic ( $\text{H}_2\text{PO}_4^-$ ) and the dibasic ( $\text{HPO}_4^{2-}$ ) ions (Bhattacharyya and Jha, 2012). Soil pH is the main property controlling inorganic P forms, although Al, Fe, and Ca content determine the amounts of these forms. In acidic soils, P can be dominantly adsorbed by Al/Fe

oxides and hydroxides, such as gibbsite, hematite, and goethite, while Ca compounds fix P in alkaline soils (Shen et al., 2011). The pKa for the dissociation of  $\text{H}_3\text{PO}_4$  into  $\text{H}_2\text{PO}_4^-$  and then into  $\text{HPO}_4^{2-}$  are 2.1 and 7.2, respectively. As a result, P availability is greatest at soil pH between 6 and 7 (Figure 2.2). Most studies on the pH dependence of P uptake in higher plants have found that uptake rates are highest between pH 5.0 and 6.0, where  $\text{H}_2\text{PO}_4^-$  dominates (Furihata et al., 1992; Schachtman et al., 1998).

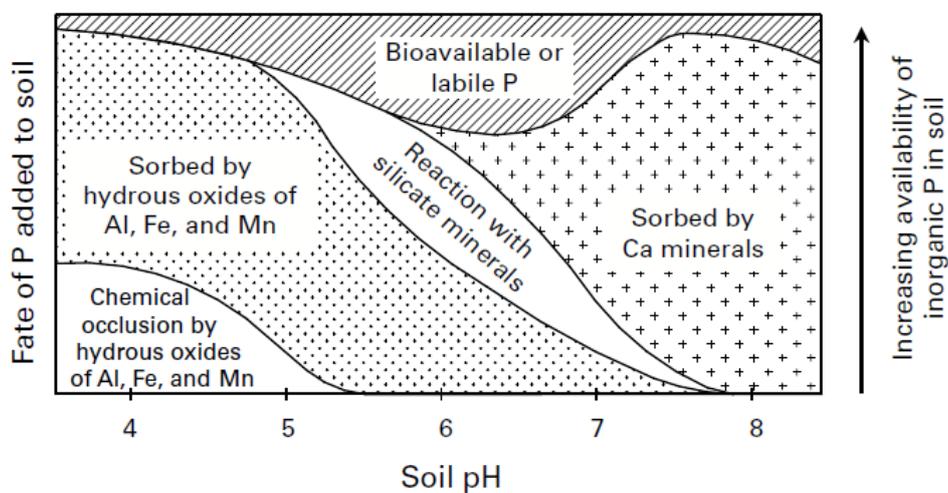


Figure 2.2 Representation of the fate of inorganic P according to soil pH (Source: Phosphorus and potassium in the soil from Plant & Soil Science eLibrary)

### 2.2.2 Phosphate solubilization by PSB

To overcome the P deficiency in soils, there are soil microorganisms, which have the capability to change the insoluble form of phosphorous into soluble one and they are known as the phosphate-solubilizing bacteria (PSB), as shown in Figure 2.3 (Hilda and Fraga, 1999). Phosphate solubilizing bacteria (PSB) have been used for the crop production since 1903. These bacteria play an important role in supplying phosphate to plants, in environment friendly (Awasthi et al., 2011). Bacterial genera like *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are reported as the most significant phosphate solubilizing bacteria (Bhattacharyya and Jha, 2012). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *B. aryabhatai*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains (Khan and Naqvi, 2009).

Phosphorus solubilizing activity is determined by the ability of microbes to secrete metabolites such as low molecular weight organic acids (Goldstein, 1995). These organic acids dissolve phosphatic minerals or chelate cationic partners of the phosphate ions ( $\text{PO}_4^{3-}$ ) through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, and consequently being converted to soluble forms (Sagoe et al., 1998; Khan et al., 2009). Organic acids, which solubilize phosphorous are mainly citric acid, lactic acid, gluconic acid, 2-ketogluconic acid, oxalic acid, tartaric acid and acetic acid (Goldstein, 1995). These organic acids are the source of biotical generated  $\text{H}^+$  ions, which are able to dissolve the mineral phosphate and to make it available for the plants (Bhattacharyya and Jha, 2012).

Several research reported that organic acid producing microorganisms such as PSB are able to enhance the solubilization of phosphatic rock (Gyaneshwar et al., 2002). The PSB strains exhibit inorganic P-solubilizing abilities ranging between 25–42  $\mu\text{g P mL}^{-1}$  and organic P mineralizing abilities between 8–18  $\mu\text{g P mL}^{-1}$  (Tao et al., 2008). *Pseudomonas striata* and *Bacillus polymyxa* solubilized 156 and 116  $\text{mg P L}^{-1}$ , respectively (Rodríguez and Fraga, 1999). *Pseudomonas fluorescens* solubilized 100  $\text{mg P L}^{-1}$  containing  $\text{Ca}_3(\text{PO}_4)_2$  or 92 and 51  $\text{mg P L}^{-1}$  containing  $\text{AlPO}_4$  and  $\text{FePO}_4$ , respectively (Henri et al., 2008). *Bacillus sp.* as one of PSB, could solubilize P up to 68.5 mg from  $\text{Ca}_3(\text{PO}_4)_2$ , 5.7 mg from  $\text{AlPO}_4$  and 22.1 mg from  $\text{FePO}_4$  on the 9<sup>th</sup> day and 13.8 mg from RP (rock phosphate) on the 12<sup>th</sup> day from inoculation, and the phosphate solubilization activities were decreased after that (Karunai Selvi et al., 2011). Park et al. (Park et al., 2011) also investigated that *Enterobacter sp.* as PSB solubilized 17.5% of RP (rock phosphate) in the growth medium.

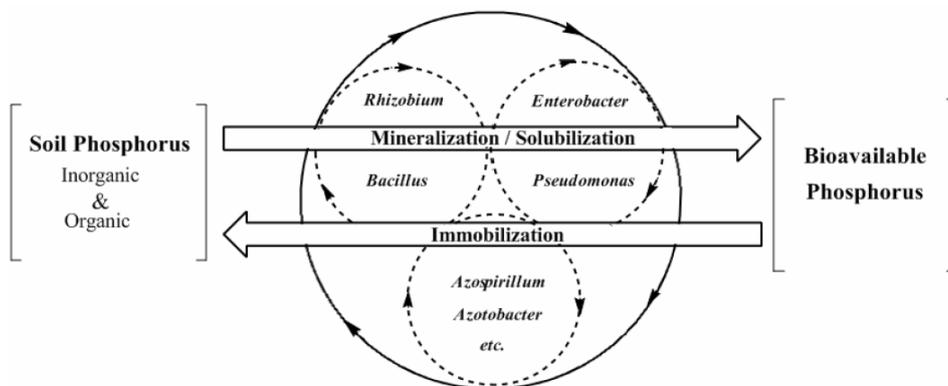


Figure 2.3 Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al., 2009)

### 2.2.3 Enhancement of phytoremediation efficiency by PSB

PSB, as one of plant growth promoting rhizobacteria (PGPRs), have the various beneficial effects on plants directly or indirectly. PSB directly enhance the plant growth and yield through solubilizing of insoluble phosphatic mineral to soluble form, thus providing P to plants (Ahemad and Kibret, 2013). PSB also produce phytohormone auxin such as IAA (indole-3-acetic acid), which are stimulated the plant growth, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene thereby increasing plant growth (Glick et al., 1998).

Moreover, organic acids, which are secreted by PSB, can either dissolve the phosphorous directly by lowering the pH of soil, which can help in ion exchange of  $\text{PO}_4^{2-}$  by acid ions or they can chelate heavy metal ions such as Ca, Al and Fe and release associated phosphorous with them. The pH of rhizosphere is lowered through biotical production of proton / bicarbonate release (anion/cation balance) and gaseous ( $\text{O}_2/\text{CO}_2$ ) exchanges (Khan et al., 2009). Lowering the soil pH can effect on heavy metal in soil, especially cationic metal. Generally, soil pH greatly influences adsorption and mobility of heavy metals in soils. For all cationic metals, the availability and mobility in soils increased with decreasing soil pH due to the chemical form in which cationic metal ions are present in soil solution (Zimmerman, 2010; Wuana and Okieimen, 2011).

Proposed mechanisms of PSB affected on phytoremediation efficiency by promoting plant growth as well as increasing the mobility of cationic metal ion in soils are shown in Figure 2.4.

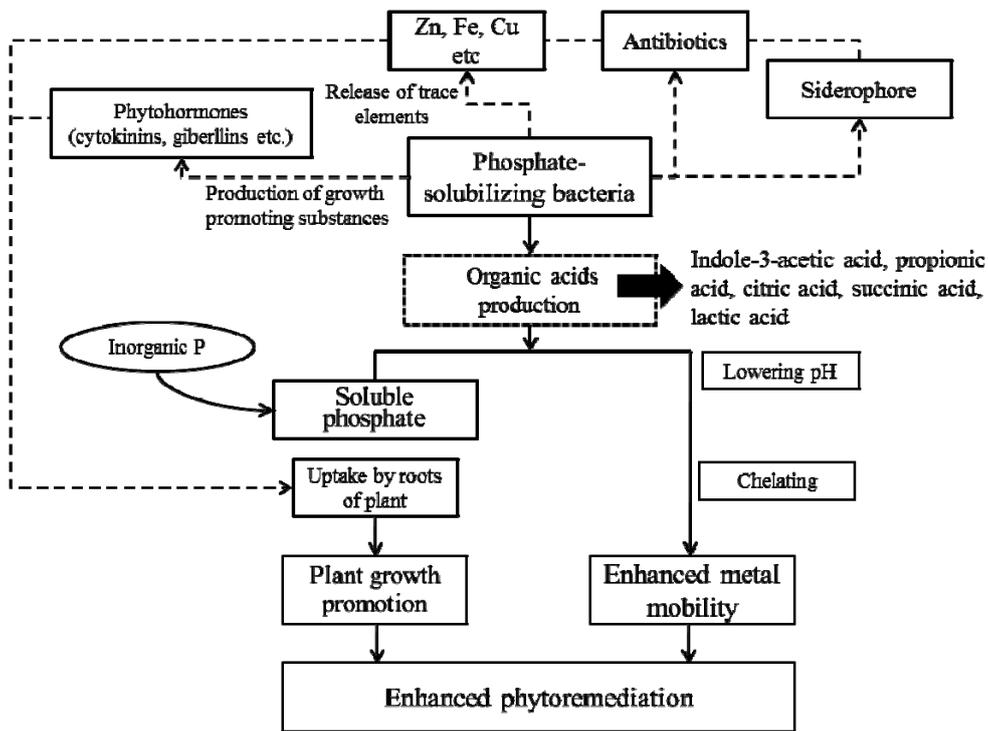


Figure 2.4 Mechanism of enhanced phytoremediation by phosphate-solubilizing bacteria (PSB)

## 2.3 Potential of siderophore-producing bacteria in phytoremediation

### 2.3.1 Iron, a limiting nutrient

Iron is one of the most abundant elements in soil, and is also essential plant nutrient (Rajkumar et al., 2012). In the aerobic environment, iron occurs principally as ferric ions ( $\text{Fe}^{3+}$ ) and is likely to form very insoluble mineral precipitates such as hematite, goethite, and ferrihydrite, thus, making it generally unavailable to plants or microorganisms in soil (Rajkumar et al., 2010). In contrast, Ferrous ions ( $\text{Fe}^{2+}$ ) is much higher water-soluble than  $\text{Fe}^{3+}$ , but exists only under anoxic environment. Minimal concentration of iron required for normal growth of plants and many microbe ranged from  $10^{-9}$  to  $10^{-4}$  M and  $10^{-5}$  to  $10^{-7}$  M (Schwab and Lindsay, 1983; Loper and Buyer, 1991). Nevertheless, the extreme insolubility of iron minerals ( $K_{sp} = 10^{-38}$ ), limits free iron at pH 7 under an aerobic environment to an equilibrium concentration of approximately  $10^{-18}$  M (Raymond and Carrano, 1979). Figure 2.5 represents that the bioavailable iron concentration in oxic soils is generally below the plant's requirement for normal growth (Marschner, 1995).

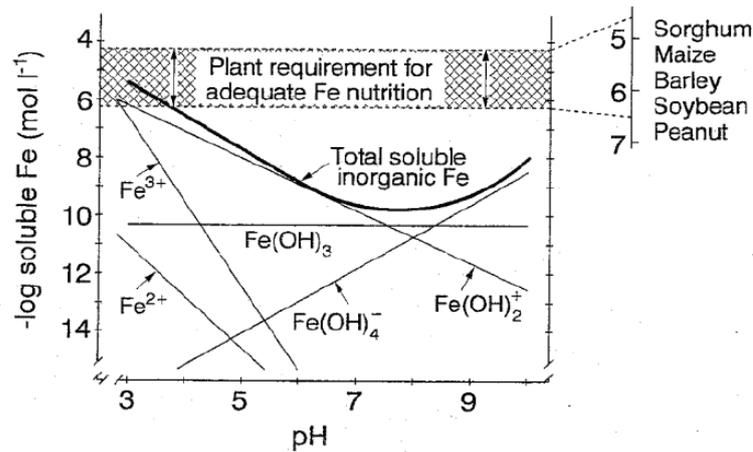


Figure 2.5 Solubility of iron with iron oxides in comparison to the requirement of iron for various plant species under aerobic environment

### 2.3.2 Iron mobilization by siderophore

Commonly, soil microbe acquire iron by the secretion of low-molecular mass (generally <1 kDa) iron chelator with high association constant for complexing iron, referred to as siderophore (Rajkumar et al., 2010). Siderophore generally can form 1:1 complexes with  $\text{Fe}^{3+}$ , which are then taken up by specific membrane receptor of bacteria, where the  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  and released from the siderophore into the cell (Jalal and van der Helm, 1991). The 1:1 stability constants ( $\log \beta$ ) of  $\text{Fe}^{3+}$ -siderophore complexes are between 23 and 52 compared to approximately 20 for the  $\text{Fe}^{3+}$ -EDTA complexes (Kraemer, 2004).

The iron acquisition of siderophore secreted by soil bacteria is illustrated in Figure 2.6 and can be divided into several processes following as:

- (1) Siderophore production by microorganism under iron deficiency environment, and its production is regulated by the bioavailable iron concentration.
- (2) Siderophore released into rhizosphere
- (3) Iron dissolution by complex formation: Siderophore attach on the insoluble iron mineral surface and thereby facilitate dissolution by coordinating the iron atom.
- (4) Uptake of the siderophore-iron complexes: The uptake of siderophore-iron complexes occurs by specific and sensitive translocator protein in the cell membrane (Marschner, 1995; Boukhalfa and Crumbliss, 2002).
- (5) Release of iron from the complexes: most propose mechanisms are discussed as a reduction of the coordinated iron from complexes. During reduction process, siderophore is may be destroyed or recycled.

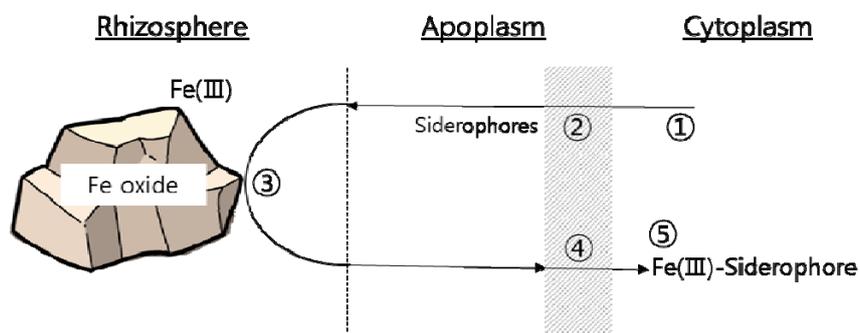


Figure 2.6 Scheme of iron acquisition by siderophores (reproduced from Marschner, 1995)

Siderophores are classified into three main groups based on the its functional group of the moieties donating the oxygen ligands for  $\text{Fe}^{3+}$  coordination, which are either of the catecholate, hydroxamate, or hydroxy-carboxylic acid group (Figure 2.7). Hydroxamate functional groups complex with  $\text{Fe}^{3+}$  by loss of a proton from the hydroxylamine (-NOH) group, and bidentate bonding with the carbonyl (C=O) and hydroxylamine (-NOH) groups, resulting in a five membered ring (Crumbliss, 1990). Catecholate groups also form five membered rings with iron through the phenolic oxygen after deprotonating in the neutral and alkaline pH range (Kraemer, 2004). For hydroxamate groups show pKa values ranged from 8 to 10, and catecholate siderophores have pKa values from 6.5 to 8 for the dissociation of the first hydrogen and 11.5 for the second hydrogen from the catecholic hydroxyl groups. The pKa values of carboxylates ranging from 3.5 to 5 make them efficient siderophores under lower-pH conditions at which catecholate and hydroxamate are still fully protonated. (Miethke and Marahiel, 2007).

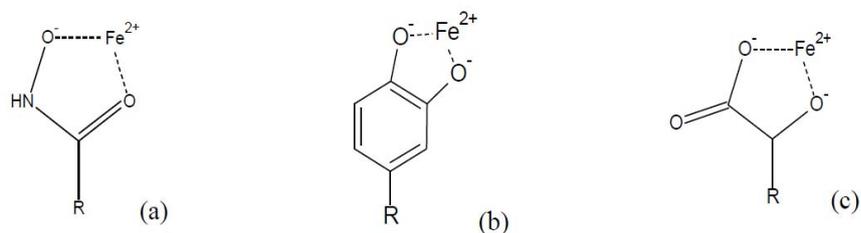
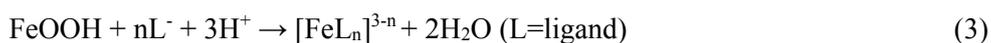
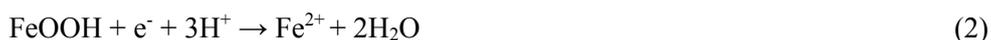


Figure 2.7 Siderophore structure: hydroxamate (a), catecholate (b), and hydroxy-carboxylic acid (c)

As discussed above, siderophore play an important role in increasing the iron oxide solubility by formation of soluble iron complexes. There are three principal reactions which iron oxide may release Fe into solution, referred as dissolution: protonation, reduction, and complexation. The respective reactions are as follows



The formation of surface complex by fast adsorption of protons (Equation 1; protonation-induced dissolution), electron donors (Equation 2; reduction or reductive dissolution), and ligands such as organic acid and siderophore (Equation 3; ligand-enhanced dissolution) leads to a polarizing and thereby to a weakening of the Fe-O bond, and then to the detachment of the Fe atom from iron oxide (Schwertmann, 1991).

### 2.3.3 Enhancement of phytoremediation efficiency by siderophores

Siderophore-producing bacteria (SPB) is capable of scavenging  $\text{Fe}^{3+}$  and making it available to plants in soils under iron-deficiency environment. Not only iron, siderophores also form stable complex with other metals (Neubauer et al., 2000; Ahemad and Kibret, 2013). An example of pyoverdine (pseudobactin), which is containing a partly cyclic 8-peptide chain composed of 2 D-serines, L-arginine, 2 L-ornithines, 2 L-threonines, and L-lysine, bound to a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline, attached to the chromophore is succinamide

(the R group in Figure 2.8), secreted by *Pseudomonas* bacteria forms complexes with many other metal ions, including:  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ga}^{3+}$ ,  $\text{In}^{3+}$ ,  $\text{Cr}^{3+}$ ,  $\text{UO}_2^{2+}$ ,  $\text{V}^{4+}$ ,  $\text{Th}^{4+}$ , and  $\text{Fe}^{2+}$  (Bouby et al., 1998; Baysse et al., 2000; del Olmo et al., 2003). In general, the overall equilibria of metal-ligand stability constants are expressed by a standard convention as  $\beta$ -values for the reaction:  $m\text{M} + l\text{L} + h\text{H} \rightarrow \text{M}_m\text{L}_l\text{H}_h$ , where M is metal, L is ligand, and H is proton(s) (Miethke and Marahiel, 2007). The metals studied and the respective stability constants ( $\log \beta$ ) were:  $\text{Fe}^{3+}$ , 29.6;  $\text{Cu}^{2+}$ , 22.3;  $\text{Zn}^{2+}$ , 19.8;  $\text{Mn}^{2+}$ , 17.3; and  $\text{Fe}^{2+}$ , 8.3 (Chen et al., 1994). Siderophore also showed high affinity for complexation with several metals in the following order:  $\text{Cd}^{2+}=\text{Ni}^{2+}=\text{Pb}^{2+}=\text{AsO}_4^{3-}=\text{AsO}_2^{1-} > \text{Mn}^{2+}=\text{Co}^{2+}=\text{Cu}^{2+}$ , and very weakly binds Al and Cr (Nair et al., 2007).

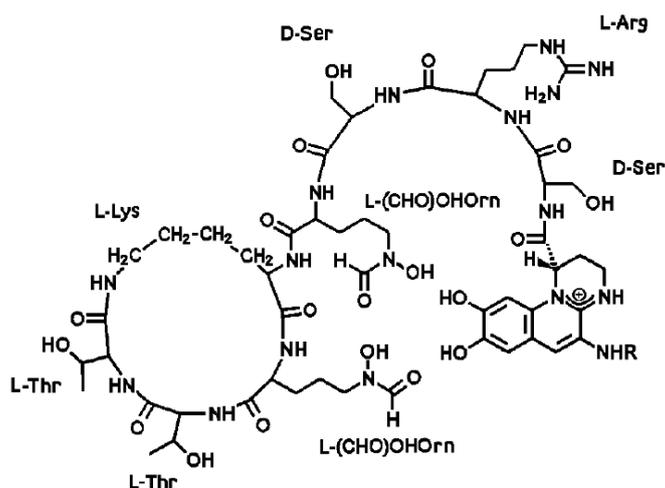


Figure 2.8 Pyoverdine structure from *Pseudomonas aeruginosa* ATCC 15692. R =  $\text{H}_2\text{NCOCH}_2\text{CH}_2\text{CONH}_2$  (Meyer et al., 1997)

Many studies demonstrated a significant role of siderophore in facilitating the release of adsorbed heavy metals into soils, thereby enhancing the uptake of heavy metals by plants. An example is the production of pyoverdin and pyochelin by rhizosphere bacteria *Pseudomonas aeruginosa*, which increase the concentrations of bioavailable Cr and Pb in the rhizosphere, thus making them available for maize uptake (Braud et al., 2009). The mobilization of U from U ore by inoculating three siderophore-producing bacteria, *Pseudomonas fluorescens*, *Shewanella putrefaciens* and *Pseudomonas stutzeri* was investigated (Kalinowski, 2004). Similarly, siderophores such as Desferrioxamine B and pyoverdine produced by *Streptomyces tendae* F4 significantly enhanced uptake of Cd by sunflower plant (Dimkpa et al., 2009). The secreted siderophore by *Pseudomonas aeruginosa* stimulated plant growth of Indian mustard and pumpkin (Sinha and Mukherjee, 2008), and siderophore-producing bacteria, *Pseudomonas fluorescens* Avm and *Rhizobium leguminosarum* bv *phaseoli* CPMex46, improved Cu and Fe translocation from root to shoot in Alfalfa (Carrillo-Castañeda et al., 2003).

## **2.4 Ecological risk assessment**

### 2.4.1 Definition and objectives

Risk is a measure of the probability that a hazard will cause harm to an individual, population or the natural environment under defined conditions of exposure to a contaminant. Ecological risk assessment is defined as a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (USEPA, 1992). The United States Environmental Protection Agency (EPA) is preparing supplemental guidance for its *“1998 Guidelines for Ecological Risk Assessment”* (USEPA, 1998) to address planning for ecological risk assessment.

Ecological risk assessments can help identify environmental problems, establish priorities, and provide a scientific basis for regulatory actions. The process can identify existing risks or forecast the risks of stressors not yet present in the environment (USEPA, 1992). The specific objectives of the process are as follows: (1) to identify and characterize the current and potential threats to the environment from a hazardous substance release; and (2) to identify cleanup levels that would protect those natural resources from risk.

#### 2.4.2 Framework of ecological risk assessment

The ecological risk assessment process is based on two major elements: characterization of effects and characterization of exposure (USEPA, 1998). The overall ecological risk assessment process is shown in figure 2.9. The three phases of risk assessment are enclosed by a dark solid line. Boxes outside this line identify critical activities that influence why and how a risk assessment is conducted and how it will be used.

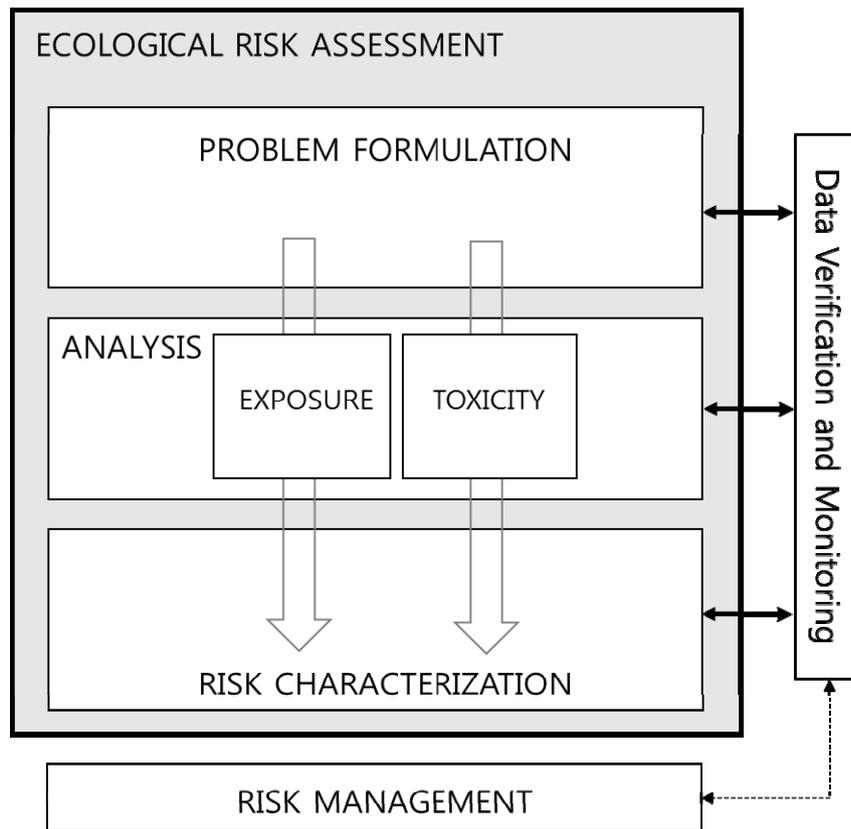


Figure 2.9 The framework for ecological risk assessment (reproduced USEPA, 1992)

Problem formulation, the first phase, is shown at the top. This process includes the integration of information on sources, stressors, ecological effect expected or observed, and ecosystem and receptor characteristics. Based on these information, whether exposure to stressors can cause an adverse effects or not was determined. The analysis phase of ecological risk assessment consists of characterization of ecological effects and characterization of exposure. The objective is to provide the ingredients necessary for determining or predicting ecological responses to stressors under exposure conditions of interest. Characterization of exposure evaluates the interaction of the stressor with the ecological receptors. The most

common approach to exposure analysis is to measure concentrations or amounts of a stressor and combine them with assumptions about contact and uptake. In addition, the exposure pathway should be describe to trace the paths of stressors from the source to the receptors in this phase. Stressors can be transported via many pathways such as by soil surface, surface water, groundwater, and food web etc. The final product of exposure analysis is an exposure profile. The exposure profile identifies the receptors and describe the applicable exposure pathways characteristics. For example, “How dose exposure occur?” or “How much exposure occurs?” or “When or where dose exposure occur?” or “What is the likelihood that exposure will occur?” To characterize ecological effects, the assessor describes the effects elicited by a stressor, links them to the assessment endpoints. During ecological response analysis, the relationship between the stressor and the ecological effects elicited is quantified. For example, an assessor may need a point estimate of an effect (such as an NOAEL and LC<sub>50</sub>) to compare with point estimates from other stressors.

Risk characterization is the final phase of ecological risk assessment and is the integration of the problem formulation, and analysis of predicted or observed adverse ecological effects related to the assessment endpoints. An uncertainty assessment is an integral part of ecological risk assessment. In the majority of assessments, data will not be available for all aspects of the characterization of exposure, and those data that are available may be of questionable or unknown quality. Typically, the assessor will have to rely on a number of assumptions with varying degrees of uncertainty associated with each.

Terminology related to ecological risk assessment is referenced in text box 2.1.

Text box 2.1 Terminology for ecological risk assessment (USEPA, 1992; USEPA, 1998)

<b>Term</b>	<b>Definition</b>
Ecological risk assessment	Evaluation of the likelihood of adverse effects on organisms, populations, and communities from chemicals present in the environment
Ecosystem	The biotic community and abiotic environment within a specified location in space and time
Source	An entity or action that releases to the environment or imposes on the environment a chemical, physical, or biological stressor or stressors
Stressor	Any physical, chemical, or biological entity that can induce an adverse response
Exposure	The contact or co-occurrence of a stressor with a receptor
Receptor	The receptor is the ecological entity exposed to the stressor
Characterization of ecological effects	A portion of the analysis phase of ecological risk assessment that evaluates the ability of a stressor(s) to cause adverse effects under a particular set of circumstances
Characterization of exposure	A portion of the analysis phase of ecological risk assessment that evaluates the interaction of the stressor with one or more ecological entities. Exposure can be

	expressed as co-occurrence or contact, depending on the stressor and ecological component involved
Endpoint	The biological or ecological entity or variable being measured or assessed
EC <sub>x</sub>	A statistically or graphically estimated concentration that is expected to cause one or more specified effects in x% (e.g. 50%) of a group of organisms under specified conditions
LC <sub>x</sub>	The concentration of a substance at which a lethal effect of magnitude x occurs. The x is usually 50% of the exposed population, in which case EC50 is known as the median lethal concentration
Lowest-observed-adverse-effect level (LOAEL)	The lowest level of a stressor evaluated in a test that causes statistically significant differences from the controls
No-observed-adverse-effect level (NOAEL)	The highest level of a stressor evaluated in a test that does not cause statistically significant differences from the controls
Risk characterization	A phase of ecological risk assessment that integrates the exposure and stressor response profiles to evaluate the likelihood of adverse ecological effects associated with exposure to a stressor

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# **CHAPTER 3**

## **APPLICATION OF PHOSPHATE-SOLUBILIZING BACTERIA FOR ENHANCING BIOAVAILABILITY OF CADMIUM FROM CONTAMINATED SOIL AND THEIR IMPACT ON MICROBIAL COMMUNITY STRUCTURE**

### **3.1 Introduction**

Cadmium (Cd) is one of the most toxic heavy metals in the environment, due to its high mobility and toxicity (Kusch et al., 2008; Moustakas et al., 2011). In particular, the accumulation of Cd in soils can pose a threat to food safety, crop growth, and potential health risks (Dell'Amico et al., 2005; Islam et al., 2007). Several studies have been demonstrated to remediate Cd from soil using phytoremediation (Chen et al., 2010; Luo et al., 2011b). However, small biomass as well as the low bioavailability of heavy metals in soils has limited the effectiveness of phytoremediation (Vara Prasad and de Oliveira Freitas, 2003; Sheng and Xia, 2006a; Lone et al., 2008).

To solve these problems, the application of plant growth promoting

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rhizobacteria (PGPR) has been considered as an enhanced phytoremediation technology (Compant et al., 2005; Lugtenberg and Kamilova, 2009). In particular, phosphate-solubilizing bacteria (PSB) are effective in promoting plant growth and biomass by releasing phosphorous (P) from inorganic and organic P pools through solubilization and mineralization (Hilda and Fraga, 1999; Jeong et al., 2012). In addition, these PSB activities strongly affect metal speciation and can increase the phytoavailability of metals in soils by means of phosphate enzyme and organic acid production, such as indole-3-acetic acid (IAA) (Chen et al., 2006; Vyas and Gulati, 2009).

The success of phytoremediation may not only depend on the plant itself but also on the survival and activity of introduced bacteria (PGPRs) in the soil (Ma et al., 2009b). A number of studies have demonstrated the importance of bacterial inoculation for plant survival in heavy metal-polluted environments. Studies frequently have observed the bioaugmented bacterial activity is temporary during bioremediation, and the number of exogenous microorganisms decrease shortly after addition to the field site (Ruberto et al., 2003; Terry et al., 2004). Although many studies on enhanced phytoremediation have focused on PGPR application during phytoremediation, only a few studies have addressed the survival and adaptation of inoculated bacteria, and their effect on the soil microbial community during phytoremediation (Jing et al., 2007a; İnceoğlu et al., 2011; Piromyou et al., 2011).

A number of fingerprinting molecular biology techniques, such as PCR-DGGE has been applied to investigate the soil microbial community structure (Sun et al., 2004; Rastogi and Sani, 2011). However, PCR-DGGE only allows an assessment of the dominant members of microbial communities in natural systems

(Muyzer et al., 1993), and the number of clones, which this effect holds true for the minority bacterial species in the microbial population remained unknown (İnceoğlu et al., 2011). Recently, pyrosequencing, a new DNA sequencing technology combined with 16S rDNA-based molecular methods, has emerged as the most powerful tool to analyze complex microbial communities in different ecosystems (Chun et al., 2010; Schneider and Riedel, 2010; Chang et al., 2011). This technique is based on the detection of pyrophosphate released during nucleotide incorporation, and provides a new way of dramatically reducing the costs associated with sequencing (Metzker, 2010; Roh et al., 2010).

In this study, PSB, *Bacillus* sp., was used for heavy metal removal from soils to enhance Cd bioavailability in soils as well as plant growth for a successful phytoremediation scheme. The hypothesis of this research is that inoculation of *Bacillus* sp. increases the solubilization of insoluble P compounds, thereby enhance plant growth. In addition, organic acid production during P solubilization may result in the increase of Cd bioavailability resulting from changing Cd speciation and mobility in soil. Although many phytoremediation researches have tried to enhance the efficiency of heavy metal uptake by plant or heavy metal immobilization using bacterial inoculation in heavy metal-contaminated soil (Park et al., 2011), there are little studies reported to examine the effect of PSB inoculation on Cd phytoremediation efficiency and Cd bioavailability. To date, no studies also have used pyrosequencing techniques to investigate the dynamics of microbial community by bacterial inoculation, during phytoremediation of Cd-contaminated soil.

The objective of this study was to investigate the effects of inoculation of *Bacillus* sp. on plant growth and Cd uptake efficiency by *Brassica juncea*. In

addition, the changes of bioavailable Cd-speciation over time in soils with PSB inoculation and its effects on Cd uptake were discussed. And we also compared the microbial community structure (i.e., composition and abundance) in soil with, and without inoculation of PSB during Cd- phytoextraction by *Brassica juncea* *Bacillus* using pyrosequencing technique during Cd phytoremediation, and investigated the survival and adaption of introduced PSB.

## 3.2 Materials and Methods

### 3.2.1 Soil characterization

Pristine surface soil (0-30 cm) was collected at Seoul National University in Korea. After the collected soil samples were air-dried, and ground to pass through a 2.0 mm sieve, the soil samples were artificially contaminated with an aqueous solution of cadmium chloride ( $\text{CdCl}_2$ ), to have the final concentration of about 40 mg-Cd/kg-soil. These prepared soil samples were aged for about 60 days. The soil was characterized for pH, organic matter (OM), texture, total nitrogen (T-N), total phosphorous (T-P), water soluble phosphorus and Cd concentration (Table 1). Soil pH was measured at 1:5 ratio of soil to water (SSSA, 1996). Organic matter content was determined following Walkley-Black method (Walkley and Black, 1934). Micro-kjeldahl procedure was used for total N in soil (Wicks and Firminger, 1942). Total phosphorus was determined by digestion with  $\text{H}_2\text{SO}_4\text{-HClO}_4$  method, and water soluble-P was extracted according to the Bray No.1 method in soil (SSSA, 1996). Total Cd concentration in soil was analyzed by EPA method 3052 (i.e., microwave assisted acid digestion).

Table 3.1 Physicochemical properties of the artificially contaminated soils used in this study

Soil properties		Unit	Values
	pH	-	5.78
	Organic Matter	%	6.89
Physical properties	Texture	-	Loamy sand
	Sand	%	76.7
	Silt	%	16.8
	Clay	%	6.48
Nitrogen	Total	%	0.11
Phosphorus	Total	mg·kg <sup>-1</sup>	413
	Water-soluble	mg·kg <sup>-1</sup>	32.35
Cadmium	Total	mg·kg <sup>-1</sup>	42.9 (±2.93)

### 3.2.2 Plants, bacterial strain and growth condition

The seeds of *Brassica juncea* which are well known as Cd hyper-accumulators (Fotiadis et al., 2009) were procured from the National Academy of Agricultural Science (NAAS, South Korea) and sown in pots containing Cd-contaminated soils. The PSB culture containing *Bacillus* species (i.e., *Bacillus aryabhatai* and *Bacillus megaterium*) (Chakraborty et al., 2006; Wu et al., 2007; Jeong et al., 2012; Lee et al., 2012; Meldau et al., 2012) were grown in the nutrient broth in a shaking incubator (160 rpm) at 30°C for 12 h. The cells in the exponential phase were harvested by centrifugation at 1000 g for 20 min, and washed three times with phosphate buffer saline, and re-centrifuged. Bacterial inoculums were prepared by resuspending the cell pellet in a 10 mL mineral salt basal medium (ca.  $10^8$  cells/mL). The mineral salt basal medium contained: 1,000 mg  $(\text{NH}_4)_2\text{SO}_4$ , 800 mg  $\text{K}_2\text{HPO}_4$ , 200 mg  $\text{KH}_2\text{PO}_4$ , 200 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 100 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5 mg  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 1 mg  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  in 1 L (pH 7 with 0.1 N HCl) (Mueller et al., 1989).

### 3.2.3 Phosphorus solubilization assay

The potential of *Bacillus* sp. for solubilizing phosphate from tricalcium phosphate (TCP) and the soils was quantitatively measured in 250 mL Wheaton bottles with 1 mL of *Bacillus* sp. at 30 °C for 13 d on a rotary shaker at 160 rpm. The P solubilization test from TCP was carried out in a 100 mL Pikovskaya medium (Pikovskaya, 1948) (in g): 10 glucose, 0.5  $(\text{NH}_4)_2\text{SO}_4$ , 0.1  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 yeast extract, 0.2 KCl, 0.2 NaCl, 0.002  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002 Mn  $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ , 5

$\text{Ca}_3(\text{PO}_4)_2$  and 1000 mL distilled water (pH 7). The P solubilization test from the soils was performed using 5 g soils in 100 mL nutrient broth (NB, 5 g peptone, 3 g beef extract, and 1000 mL distilled water; pH 7). The Pikovskaya medium and the NB broth without *Bacillus* sp. were used as controls. The culture samples were removed periodically from the bottles and filtered (0.4  $\mu\text{m}$ ). The solubilized phosphate (water soluble phosphorous) from TCP or soils in filtrate by *Bacillus* sp. was extracted using 20 mL of 0.03 N  $\text{NH}_4\text{F}$  and 0.025 N HCl (SSSA, 1996), and was analyzed using ion chromatography equipped with a CD20 conductivity detector and a Dionex IonPac AS14–4 mm column (DX-500, Dionex, USA). The experiment was conducted in triplicate.

#### 3.2.4 Pot experiment

A total of seven pots (10.0 cm diameter and 60.0 cm height as shown in Figure 3.1) were installed, to observe the PSB-enhanced Cd uptake by *B. juncea* in 300 g of the Cd-contaminated soils ( $42.9 \pm 2.93 \text{ mg-Cd/kg}$ ). Five pots were inoculated with the bacterial suspension (5 mL/pot), and re-inoculated periodically at the 1, 2, 4 and 6<sup>th</sup> week of the growth period, to maintain the bacterial activity in the soils. Two pots were not inoculated, as control. The plants were grown in a growth chamber (E15, Conviron Inc., Canada) under constant environmental conditions ( $25 \pm 1^\circ\text{C}$ , 18 h photoperiod), and the soils were watered with 50 mL tap water every day. The plants and soil samples were collected at the 1, 2, 4, 6 and 8<sup>th</sup> week from the inoculated pots, and the 0 and 8<sup>th</sup> week, for samples from the uninoculated pots as a control.

For the plant analysis, both plants in the inoculated and the uninoculated pots

were carefully removed from the soils; the roots and the shoots were then separated, and washed with distilled water to remove soil particles. The roots and shoots were oven-dried separately at 70°C for 72 h, and the total dry weight of the plants was measured. Oven-dried root and shoot samples were ground into fine powder and then digested with a solution of HNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>, and distilled H<sub>2</sub>O (9:1:1, v/v/v), using a microwave digester (MSP1000, CEM, USA), according to the EPA 3052 method for Cd analysis. After digestion, the volume of each sample was adjusted to 25 mL with distilled water.

When harvesting the plants, soils from each pot were taken at the same time, and air-dried at room temperature. The changes of Cd chemical forms in the soils over time were determined by extraction, using Tessier's sequential extraction method (Tessier et al., 1979). The concentrations of Cd in plants and soils were determined by Atomic Absorption Spectrometry (AAS, AA7000, Shimadzu, Japan).

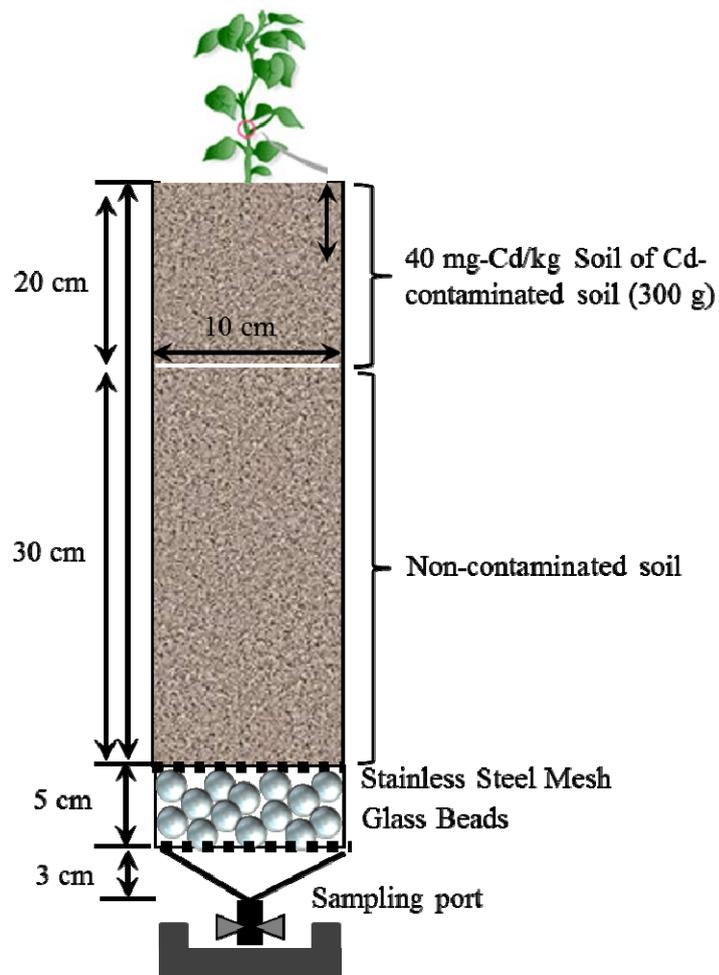


Figure 3.1 Schematic design of experimental pot used in this study

### 3.2.5 DNA extraction, PCR, and pyrosequencing

Bulk genomic DNA was extracted from 0.5 g of rizhosphere soil at the 2, 6 and 8<sup>th</sup> week for inoculated pots, and 0 and 8<sup>th</sup> week for uninoculated pots, using a FastDNA SPIN Kit for Soil (MP Biomedicals, USA). The extracted DNA was amplified, using primers targeting the V1 to V3 regions of 16S rRNA gene. For bacterial amplification, bar-coded primers of 9F (5'-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGC TCAG -3'; underlining sequence indicates the target region primer) and 541R (5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-ATTACCGCGGCTGCT GG-3'; 'X' indicates the unique barcode for each subject, <http://oklbb.ezbiocloud.net/content/1001>). The amplifications were carried out under the following condition, at 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final elongation at 72°C for 5 min. The PCR product was confirmed by using 2% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Equal concentrations of purified products were pooled together, and short fragments (non-target products) removed with an Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and product size were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA), using a DNA 7500 chip. Mixed amplicons were conducted by emulsion PCR, and then deposited on Picotiter plates. The sequencing was carried out at Chunlab, Inc. (Seoul, Korea), with GS Junior Sequencing system (Roche, Branford, CT, USA) according to the manufacturer's instructions.

### 3.2.6 Pyrosequencing processing

The sequencing reads from the different samples were sorted by unique barcodes. Primer sequences were removed from raw sequence reads. Only PCR amplicons that showed a significant match (expectation value of  $> e^{-5}$ ) to the database were selected for the bioinformatics analyses. For the taxonomic assignment of each pyrosequencing read, we used the EzTaxone database, which contains 16S rRNA gene sequences of type strains that have valid published names and representative species-level phylotypes of either cultured or uncultured entries in the database. Individual sequence reads were taxonomically assigned according to the following criteria ( $x$  = similarity): species ( $x \geq 97\%$ ), genus ( $97 > x \geq 94\%$ ), family ( $94 > x \geq 90\%$ ), order ( $90 > x \geq 85\%$ ), class ( $85 > x \geq 80\%$ ), and phylum ( $80 > x \geq 75\%$ ). If the similarity was below the cutoff point, the read was assigned to an unclassified group. Operational taxonomic units (OTUs) and rarefaction curves were generated using the CD-HIT program with an identity cut off of 97%.

### 3.2.7 Statistical community analysis

The diversity and species richness indices were calculated using the rRNA Database Project's pyrosequencing pipeline (<http://pryo.cme.msu.edu/>). The Shannon (Shannon and Weaver, 1949), and Simpson (Simpson, 1949) indices were calculated for each soil sample. For the variables of dominance hierarchy, relative abundance was calculated as the number of sequences, divided by the total number of sequences per sample (%). Genera making up less than 0.5% of the total composition in both libraries are defined as "others". Heat map and Cluster

analysis of microbial community profiles was performed using hierarchical clustering by an unweighted pair group with mathematical averages (UPGMA) using SPSS 20.0 (SPSS Inc, Chicago, IL) software. Sequences occupying less than 1% of the total sequences were removed from the subsequent statistical analysis. The display dendrogram was based on Fast Unifrac distance (Hamady et al., 2010) and UPMGA at the top of the Heat Map. These data were also used in application to Principal Component Analysis (PCA), to evaluate similarities between communities, using SPSS 20.0 (SPSS Inc, Chicago, IL) software.

### **3.3 Results**

#### 3.3.1 Potential of *Bacillus* sp. for solubilizing phosphorus in soils

Previous studies have observed the ability of *Bacillus* sp. to solubilize insoluble inorganic phosphate compounds such as TCP, dicalcium phosphate, hydroxyapatite, or rock phosphate (RP) (Cunningham and Kuiack, 1992; Chen et al., 2006), however, the phosphate solubilization activity of *Bacillus* sp. from soils has not been reported yet. Therefore, the ability of *Bacillus* sp. for solubilizing phosphate from insoluble phosphorous (i.e., TCP or soils) was investigated as the sole source of phosphate in this study. Inoculation with *Bacillus* sp. showed a significant increase of phosphate in the medium containing TCP compared with the uninoculated control and the phosphorous solubilization continued throughout the incubation period and the final phosphate concentration in TCP medium was 45.5 mgL<sup>-1</sup> (Figure 3.2(a)).

For soils, the maximum solubilization was achieved after 5 day (8.2 mg L<sup>-1</sup>)

while  $0.6 \text{ mg L}^{-1}$  was obtained from the soils under the uninoculated condition (Figure 3.2(b)). These results indicated that *Bacillus* sp. have the potential for solubilizing inorganic phosphorus from soils as well as TCP and the bacteria may have helped plant growth by the enhancement of the uptake of soil minerals such as P.

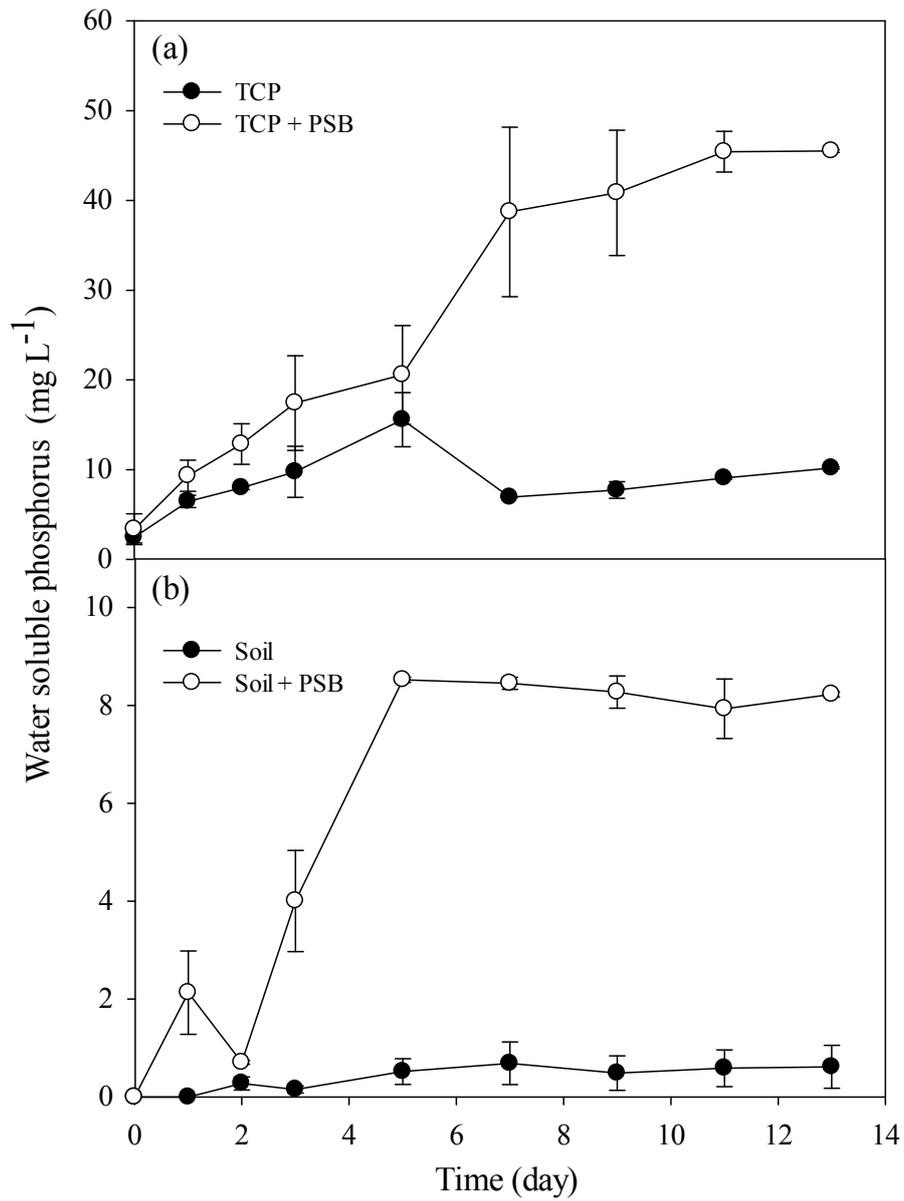


Figure 3.2 Solubilization of phosphorus from tricalcium phosphate (TCP) or soils by *Bacillus* sp., phosphate-solubilizing bacteria

### 3.3.2 Effect of PSB inoculation on Cd uptake by *Brassica juncea* and its change of phytoavailability

For soil with PSB inoculation, the total dry weights of *Brassica juncea* significantly increased from 10.3 mg to 101.0 mg up to the 6<sup>th</sup> week, and 295.6 mg of total dry weight was observed at the 8<sup>th</sup> week, while it was 65.8 mg in the uninoculated soil (Figure 3.3). The biomass of the above-ground tissues (i.e., stems and leaves) mainly increased by PSB inoculation after eight weeks. Also, a significant increase of accumulated Cd concentration in plants was observed in soils inoculated with PSB (Figure 3.4). The Cd accumulation by *B. juncea* increased up to 250%, when PSB was inoculated at the 8th week, while almost no further Cd uptake in the uninoculated soils was observed compared with initial soil (i.e., 0 week). Most of the extracted Cd was moved from root to shoot for eight weeks, even though Cd accumulation by the plant does not significantly increase, compared to the inoculation condition. Also, *B. juncea* seems to accumulate Cd effectively in their roots and stems by PSB inoculation. For the inoculated soils, the contents of Cd accumulation in roots and stems by *B. juncea* were 50 and 44% of total Cd concentrations after eight week, respectively. The water-soluble phosphorous in soil with PSB inoculation was greater by more than 50% after eight week compared with the uninoculated control (Figure 3.5).

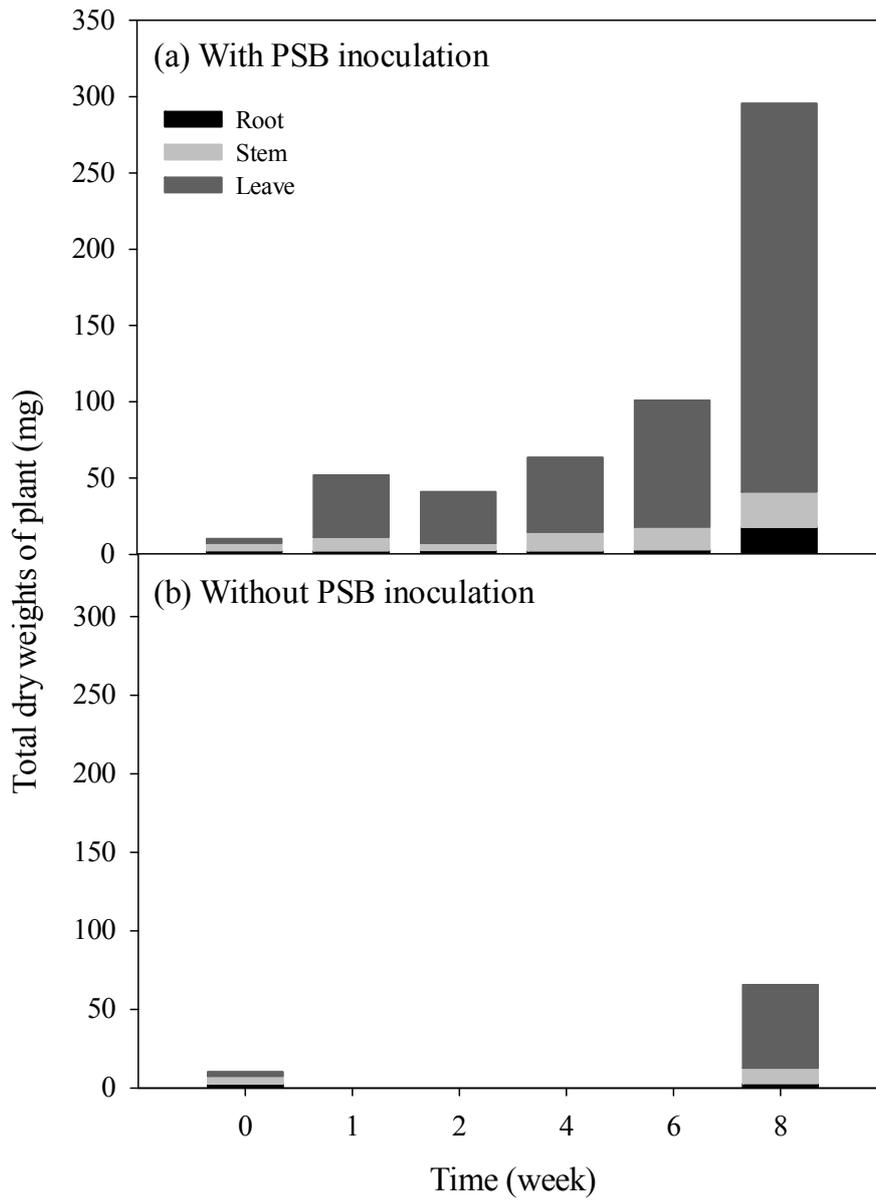


Figure 3.3 Total plant biomass of *Brassica juncea* in the PSB-inoculated and uninoculated soil with time

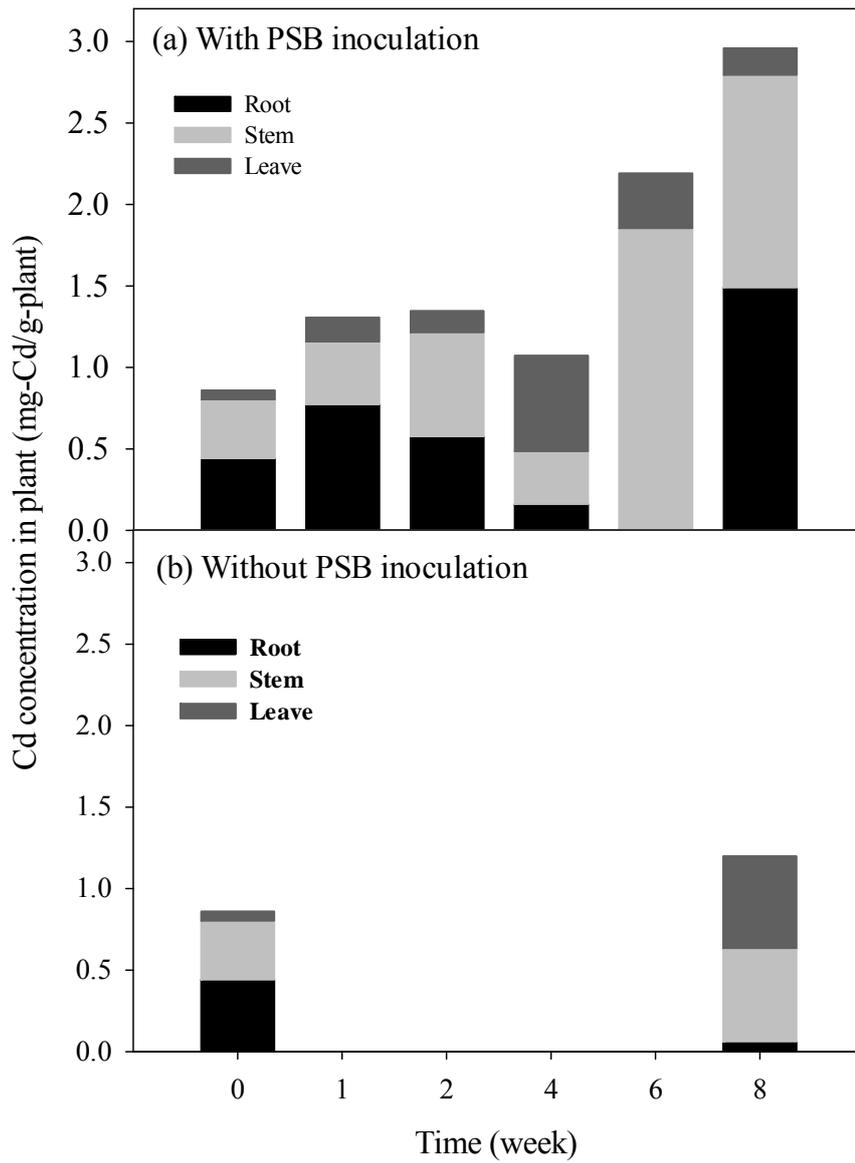


Figure 3.4 The cadmium (Cd) uptake by *Brassica juncea* in the PSB-inoculated and uninoculated soil with time

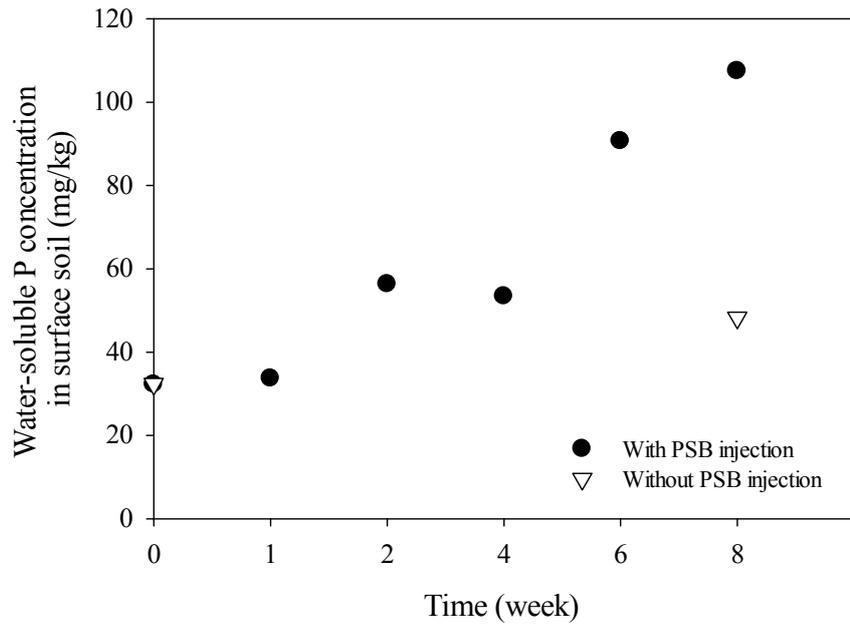


Figure 3.5 Influence of PSB inoculation on water-soluble phosphorous concentration in surface soil with time

The changes of Cd phytoavailability in soils (i.e., bioavailable fraction of Cd) resulting from PSB inoculation were tracked using Tessier's sequential extraction (Tessier et al., 1979) (Table 3.2). The Cd in initial soils before planting (aged for 60 days) mainly existed as exchangeable form (fraction 1), which was about 75% of the total Cd concentration, followed by 19% in the carbonates-bound form (fraction 2). The PSB inoculation resulted in an interesting change of chemical forms of Cd in soils; the ratio of the exchangeable metal form increased over time slightly, while the Cd chemical forms had changed little over time in the uninoculated soils. More importantly, the soil pH slightly decreased from 5.8 to 4.9 during the pot experiment in the inoculated soils (Figure 3.6(a)). This may result from the secretion of organic acid, such as IAA, by *Bacillus sp.* (Figure 3.6(b)), which led to acidification of the soil, or could have directly solubilized Cd from the soils. Also, concentrations of other organic acids (i.e., acetic acid and propionic acid) were detected in soil with PSB inoculation (data not shown).

Table 3.2 Changes of Cd speciation in soil using the five-step sequential extraction method by PSB inoculation with time

Ratio of Cd concentration in each chemical form (%)						
Time (week)	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Total
<b>(a) Cd concentration without PSB inoculation</b>						
0	74.68	18.61	4.77	1.41	0.53	100
8	75.75	17.60	5.52	0.84	0.29	100
<b>(b) Cd concentration with PSB inoculation</b>						
1	76.94	17.32	3.77	1.44	0.53	100
2	77.16	14.19	5.50	2.21	0.60	100
4	79.49	12.84	5.59	1.65	0.44	100
6	84.03	8.74	5.82	1.06	0.35	100
8	85.18	8.09	4.92	1.44	0.37	100

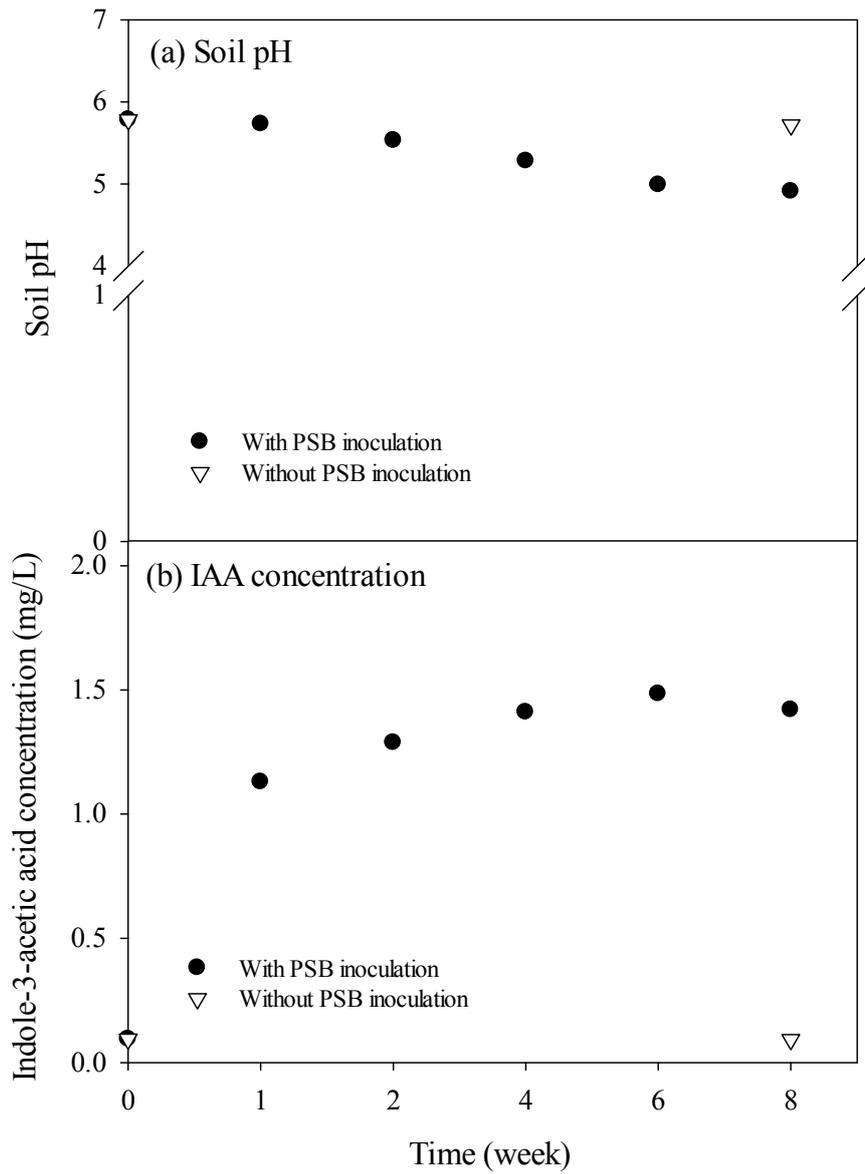


Figure 3.6 Influence of PSB inoculation on soil pH (a) and Indole-3-acetic acid (IAA, (b)) concentration in soils with time

### 3.3.3 Evolution of Microbial community structure and diversity by PSB inoculation

The 16S RNA gene survey produced a total of 10,235 sequences for five soil samples including three samples for inoculated soil (i.e., 2, 6, and 8<sup>th</sup> week) and two samples for uninoculated soil (i.e., 0 and 8<sup>th</sup> week). Total reads were obtained as 1055, 1708, 2027, 1291, and 4154 in each soil sample, respectively. In the sample-size-normalized subsamples, the average number of bacterial species ranged from 684 to 2,097, depending on the soil samples (Table 3.3).

The phylogenetic spectrum classification of sequences from the soil samples at the phylum and class level are summarized in Figure 3.7. The major phyla groups in initial soil (i.e., 0 week uninoculated soil) were *Proteobacteria* (34%), *Actinobacteria* (37%) and *Firmicutes* (7.7%). The remaining phylotypes were associated with *Bacteroidetes* (3.8%), *Armatimonadetes* (2.9%), *Acidobacteria* (2.7%), *Chloroflexi* (2.4%), *Verrucomicrobia* (1.5%), *Chlorobi* (1.4%), *Planctomycetes* (1.2%), *Cyanobacteria* (0.15%), *Gemmatimonadetes* (0.15%), *Spirochaetes* (0.15%), *Nitrospirae* (0.08%), *Fibrobacteres* (0.08%) and unknown (2.87%) bacterial group. While *Proteobacteria* were dominant at the 2<sup>nd</sup> and 6<sup>th</sup> week (40 and 53%, respectively) in inoculated soil, *Firmicutes* dramatically increased after eight weeks contributing 63% of the sequences, and they mainly belonged to the *Bacilli* class (62%) (Figure 3.7 (a) and (c)). For the uninoculated soil, the proportion of  $\alpha$ -*Proteobacteria* (19%) and  $\gamma$ -*Proteobacteria* (3%) increased to 32% and 5% of total bacteria after eight weeks, respectively.

Table 3.3 Species diversity and richness estimates of microbial community based on the pyrosequencing analysis

Samples	Total reads	No. of species observed	Shannon Index (H')	Simpson Index (D')
<b>(a) Soil samples with PSB inoculation</b>				
2 <sup>th</sup> week	1055	684	6.28	0.002
6 <sup>th</sup> week	1708	987	6.54	0.002
8 <sup>th</sup> week	2027	592	3.83	0.149
<b>(b) Soil samples without PSB inoculation</b>				
0 <sup>th</sup> week	1291	749	6.25	0.003
8 <sup>th</sup> week	4154	2097	7.13	0.002

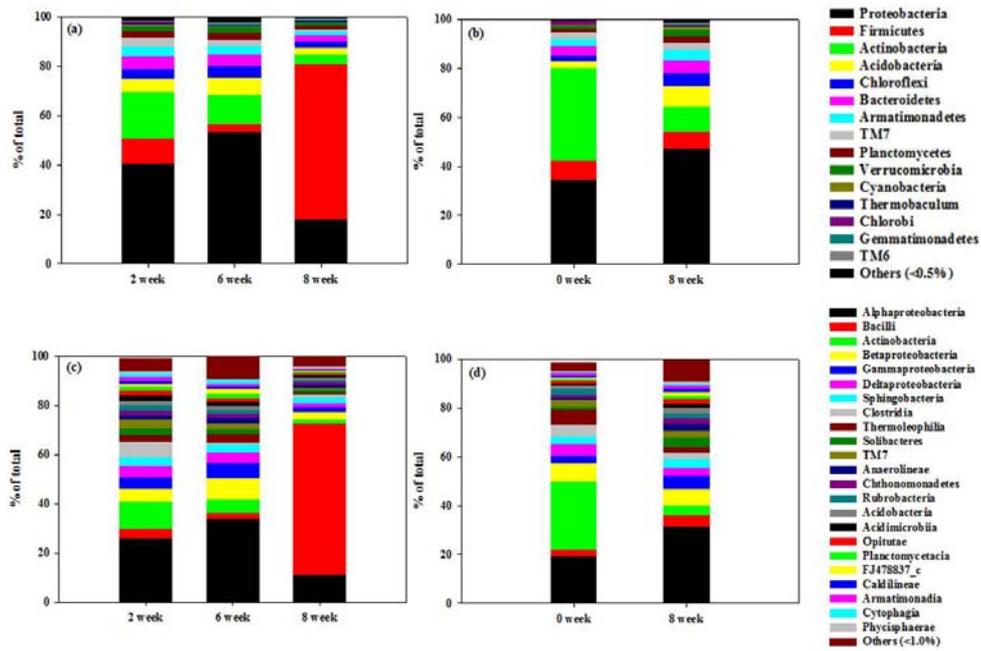


Figure 3.7 Phylogenetic classifications and distributions of the bacterial sequences detected in soils at phylum (a and b) and class level (c and d): The right (a and c) and left (b and d) panels shows the change of bacterial communities in the PSB-inoculated and uninoculated soils, respectively.

The analyses based on the genus level can allow us to further verify the functional evolution of the community (Figure 3.8). In the soil before the inoculation, *Nocardiodetes*, *Streptomyces*, *Amycolatopsis*, and *Pseudonocardia* (*Actinobacteria*) belonging to the nocardioform actinomycetes were found to be the predominant groups at the genus level. However, these populations, including *Mycobacterium* (*Actinobacteria*), significantly decreased over time. *Pseudolabrys* and *Devosia* ( $\alpha$ -*Proteobacteria*) were detected throughout the entire period in soil, irrespective of whether PSB was inoculated or not; however, *Pseudolabrys* seems likely to be more active in the uninoculated soil (6.2%) than inoculated soil (1.6%). Even its population increased until 6 weeks (4.7%), in the inoculated soil. *Prosthecomicrobium*, belonging to  $\alpha$ -*Proteobacteria*, became the predominant group over time in both conditions, except for inoculated soil at the 8th week. Interestingly, *Hyphomicrobium* ( $\alpha$ -*Proteobacteria*) and *Terrimonas* (*Sphingobacteria*) appeared over time, which was not detected in the initial Cd-contaminated soil. The relative abundance of *Burkholderia* which is one of the dominant  $\beta$ -*Proteobacteria* in this soil decreased over time in both conditions. *Bacillus\_g2* belonging to *Bacilli*, was remarkably increased in the inoculated soil at the 8th week. Species diversity and richness indices also clearly showed that the microbial community was significantly changed by PSB inoculation after eight week (Table 3.3). The Shannon index (H') decreased from 6.28 to 3.83 in the inoculated soil; however, it slightly increased from 6.25 to 7.13 in the uninoculated soil. The Simpson index (D') showed a clear difference between the inoculated and uninoculated soil after eight week.



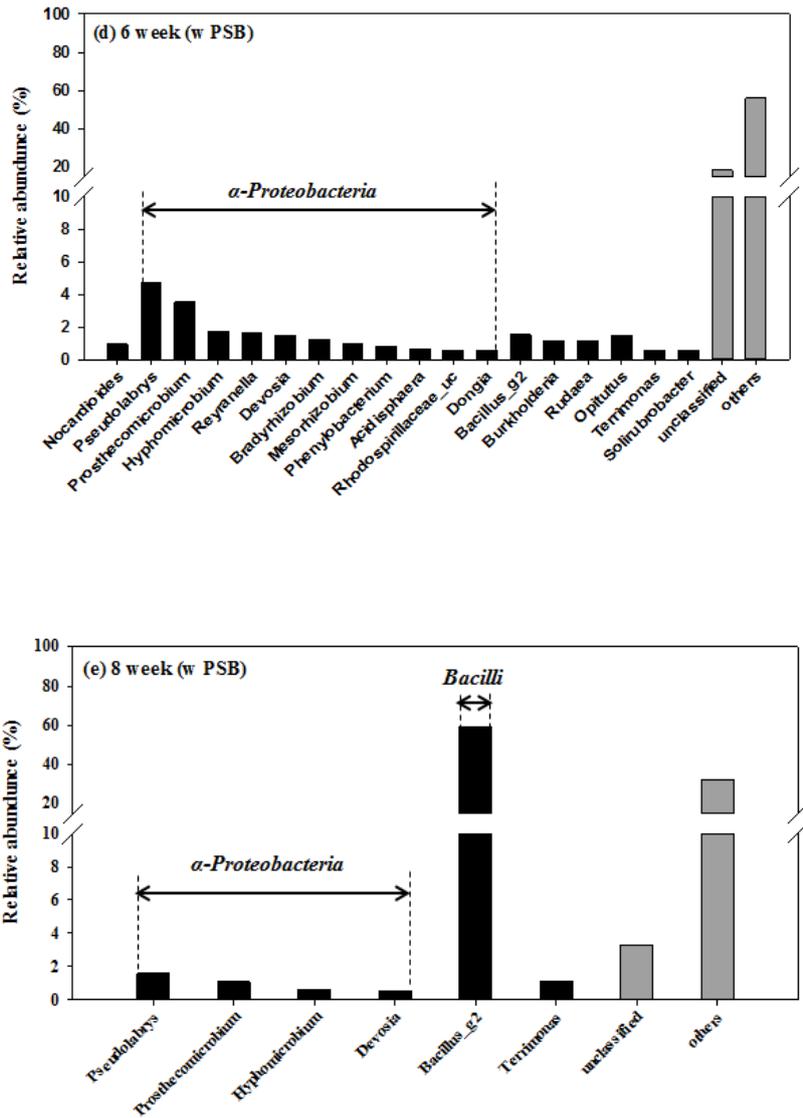


Figure 3.8 The relative abundances of the predominant phylogenetic groups at genus level. Relative abundance is defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Genera making up less than 0.5%

### 3.3.4 Survival and adaptation of inoculated PSB in Cd-contaminated soil

Heat map and hierarchical cluster analysis based on the relative abundance of OTUs at species level, revealed the eight-week inoculated soil was completely separated from the other soil, which resulted from dramatic increase of *B. aryabhatai* (Figure 3.9). The proportion of *B. aryabhatai* increased to 58%, and *B. megaterium* (0.5%) was only found in soils at eight week. Interestingly, other inoculated soils (i.e., two- and six-week soil) and eight-week uninoculated soil were grouped under the same subgroup, regardless of the different treatment. Principal Component Analysis (PCA) generated by Fast UniFrac, which shows 78% of the variance in the sequence data, was explained by the first two axes (PC1 and PC2) (Figure 3.10). The results revealed that the microbial diversity in the inoculated soil after 8 week formed distinct clusters and microbial diversity was clearly shifted depending on plant growth period for other samples. These results are similar to the trends in the phylogenetic classification, and rarefaction fraction results (Figure 3.11), where marked difference between microbial communities in was evident in the eight-week inoculated soil sample and other samples.

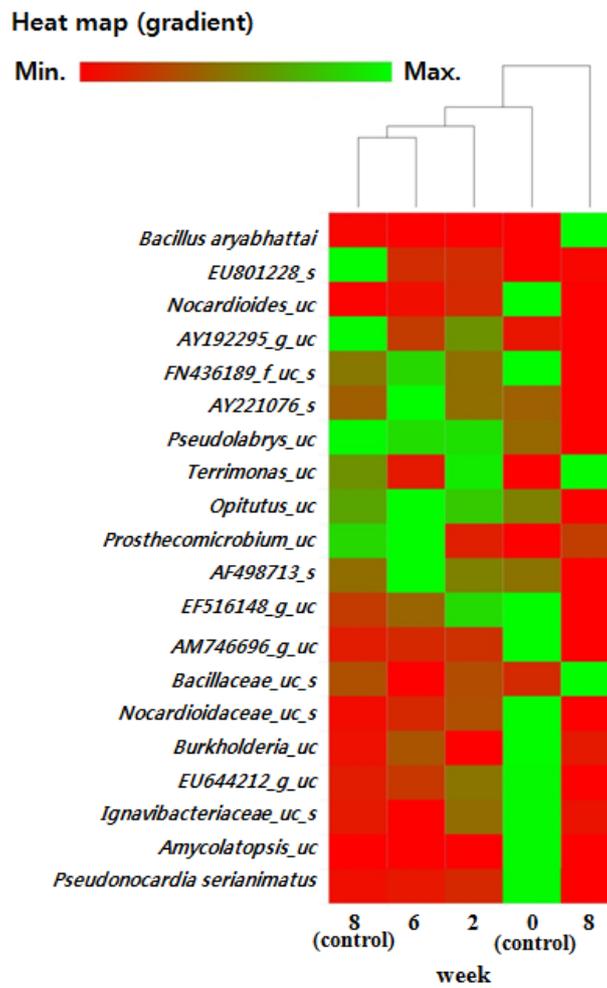


Figure 3.9 Clustering analysis for the bacterial community structures in the PSB-inoculated and uninoculated soil samples based on the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method

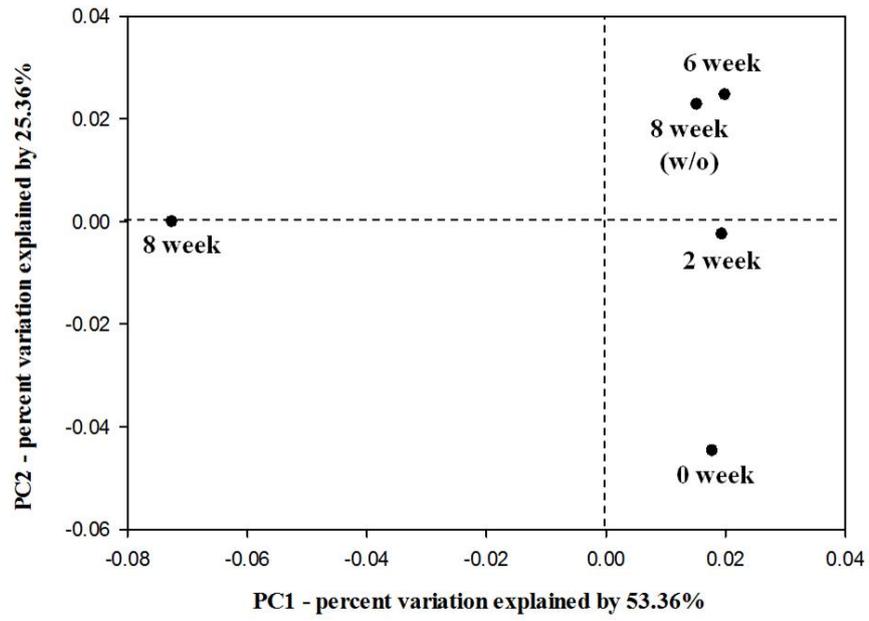


Figure 3.10 Principal Component Analysis (PCA) analysis of the bacterial community structures in the PSB-inoculated and uninoculated soil samples

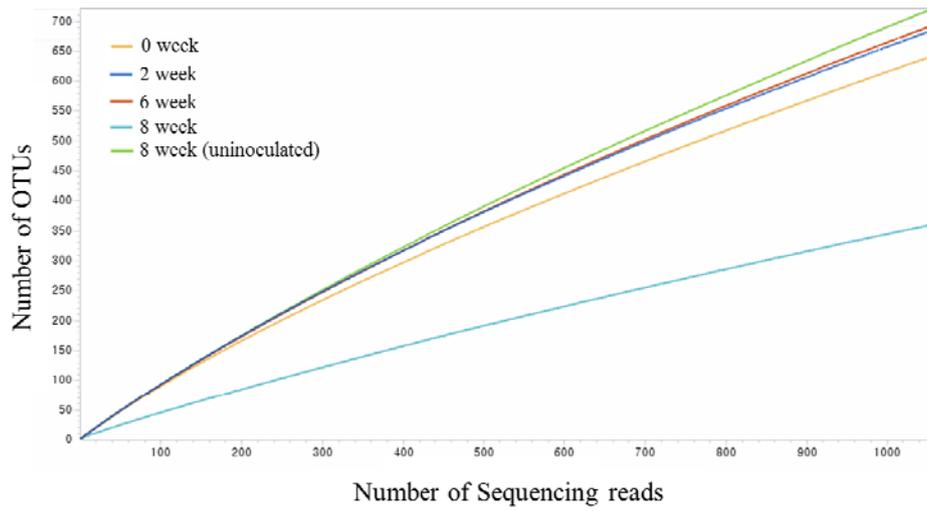


Figure 3.11 Rarefaction curves indicating the observed number of operational taxonomic unit (OTUs) in different soil samples: the X-axis shows the number of sequencing reads in each sample, while the Y-axis shows the numbers of operational taxonomic units (OTUs)

### 3.4 Discussion

Our study demonstrated that *Bacillus* sp. as phosphate solubilizing bacteria (PSB) has a potential for solubilizing phosphorous from soils. Several research reported that *Bacillus* sp. could solubilize P up to 68.5 mg from TCP, 5.7 mg from  $\text{AlPO}_4$  and 22.1 mg from  $\text{FePO}_4$  on the 9<sup>th</sup> day and 13.8 mg from RP on the 12<sup>th</sup> day from inoculation, and the phosphate solubilization activities were decreased after that (Karunai Selvi et al., 2011). Park et al. (2011) also investigated that *Enterobacter* sp. as PSB solubilized 17.5% of RP in the growth medium. It is generally known that the mechanism of mineral phosphate solubilization by the PSB strain is associated with the production of low molecular weight organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe et al., 1998). *B. megaterium* can produce organic acid such as propionic acid, citric acid, succinic acid, and lactic acid (Chen et al., 2006). In this study, idole-3-acetic acid significantly increased in soil over time and other organic acids such as lactic acid and succinic acid were also detected during the incubation (data not shown). These organic acids produced by PSB can lead to solubilize P in soil which can enhance plant growth providing nutrients. However, phosphate solubilization by PSB in soils is a complex phenomenon, which can depend on many factors such as nutritional, physiological, and growth conditions (Reyes et al., 2001).

Successful phytoextraction mainly depends on the plant growth (i.e., biomass) as well as the bioavailability of heavy metals in soils. Many studies indicated that the interaction between plant and microorganism could improve plant growth and metal bioavailability in the rhizosphere (Belimov et al., 2005b; Piromyou et al.,

2011). In this study, we used the mixed culture of *Bacillus* sp. (i.e., *B. aryabhatai* and *B. megaterium*,) as PSB, for improving the plant growth and bioavailability of Cd. These bacteria have been reported for their potential of phosphate solubilization from phosphate-based minerals (Chakraborty et al., 2006; Lee et al., 2012).

In this study, PSB application could effectively promote the plant growth and uptake of Cd by *B. juncea* resulting in change of the bioavailable Cd fraction (Figure. 3.3 and 3.4). The total dry weights, mainly the above-ground tissues (i.e., stems and leaves), of *B. juncea* in the inoculated soil significantly increased, compared to the control. Also, a significant increase of the ratio of the exchangeable Cd (i.e., phytoavailable Cd) was observed in soils inoculated with PSB, and consequently enhanced its uptake by *B. juncea*. These results indicated that the non-bioavailable and insoluble metal fractions were gradually solubilized by activities of the introduced PSB. Consequently, PSB increased the mobile fraction more such as the exchangeable Cd form secreted by organic acids in soils. In addition, these mechanisms increased the mobility, as well as the phytoavailability, of Cd in soils, and enhanced the total accumulation of Cd in plants. Although Cd mobility increased by PSB inoculation, the downward movement of Cd in the soil profile did not occur in this study. Because most Cd may be adsorbed to clay, hydrous metal oxide, and organic matter, or precipitated with carbonate ( $\text{CO}_3^{2-}$ ) and phosphate ( $\text{PO}_4^-$ ) in soil.

Our main question examined in this study is how the soil microbial community changes, by the interaction between introduced bacteria as PSB, and indigenous soil bacteria during phytoremediation. We were particularly interested in whether or not the introduced PSB successfully survive as PGPR in the rhizosphere soil

after inoculation, because the introduced bacterium, as successful inoculants, must be able to survive in the rhizosphere soil during competition with indigenous soil bacteria (Bashan and de-Bashan, 2010). The inoculated bacteria may not always successfully survive and persist in the rhizosphere (Trivedi et al., 2012). Sheng et al. (Sheng et al., 2012) reported that low survival of the introduced *Burkholderia* sp. GL 12 was observed on maize rhizosphere soils, while two other bacteria, (i.e., *B. megaterium* JL35 and *Sphingomonas* sp. YM22), were able to colonize in the rhizosphere soil.

In the current study, we adopted a pyrosequencing approach to monitor the change of microbial community during phytoremediation in Cd-contaminated soil. It may be inferred that the PCR-DGGE does not detect microorganisms present at a level lower than 1% of the total microbial population, and is less efficient in disclosing the evolution of microbial diversity (Felske et al., 1998). The results have revealed the clear impacts of PSB inoculation on the microbial community structure and composition after eight weeks (Figure 3.7). However, the composition of microbial community in soil samples was not significantly different by bacterial inoculation until six weeks. The changes of microbial community in soils until six weeks may be strongly affected by the plant development stage, which in turn can affect the rhizosphere communities over time (Yang and Crowley, 2000; Garbeva et al., 2004; Marschner et al., 2004) rather than the introduced bacteria.. Other studies also showed clear differences between bacterial communities on maize, in dependency of the growth stage over time (Baudoin et al., 2002). Also, Gyamfi et al. (Gyamfi et al., 2002) mentioned that the plant growth over time had a strong impact on the total bacterial community. In this study, the main change in microbial community by plant growth stage is that  $\alpha$ -

*Proteobacteria* increased over time, except for the 8-week inoculated soil, and the proportion of *Actinobacteria* significantly disappeared over time in both conditions (Figure 3.8).

The Shannon diversity index indicated that the microbial community is much more diverse in the uninoculated soil, than in the inoculated soil at the 8<sup>th</sup> week, although it seems to be diverse in the inoculated soil by six weeks. Likewise, the Simpson diversity index related to the species richness showed that the percentage of the species responsible for phytoavailability of Cd becomes more abundant and less diverse in the inoculated soil (Table 3.3).

Rhizobacteria having the characteristics of producing IAA, siderophore, and ACC deaminase can stimulate plant growth, and protect plants against heavy metal toxicity in heavy metal-contaminated soils (Madhaiyan et al., 2007; Glick, 2010; Rajkumar et al., 2010; Zhang et al., 2010b; Ma et al., 2011b). In the inoculated soil, *Prosthecomicrobium* species significantly increased until six weeks, which may be responsible for the dramatic increase of IAA in this rhizosphere soil (Figure 3.6). While the population of *Prosthecomicrobium*, *Pseudolabrys*, and *Devosia* species could be well colonized until 6 weeks in the inoculated soil they seem to compete with *Bacillus* sp. after that. However, the abundance of *Prosthecomicrobium* sp. increased in the uninoculated soil. These results indicated that the evolution and succession have occurred differently in the two different soils, through competitions among the species. Although *Burkholderia* sp. is known as Cd-resistant bacteria, which had been isolated from the root zone of Indian mustard (*Brassica juncea*) grown in Cd-contaminated soils in other studies (Belimov et al., 2005b), this bacteria group does not seem to be resistant to Cd-contaminated soil in our study at least for 8 weeks of plant growth (Figure 3.8). The most interesting

finding in this study is that the introduced bacteria (i.e., *Bacillus* sp.) as PSB in this study, was not detected as the most predominant population in the soil, until after 8 week of inoculation, even though they were periodically inoculated into the soil during the growth period (Figure 3.9). This means that the inoculated bacteria required sufficient time to demonstrate their ability to survive, and to exhibit their capability in soils. Their bacterial numbers (i.e., inoculum size) (Devliegher et al., 1995; Kumar et al., 2007) or adaptation time should be enough to compete with other indigenous bacteria commonly established in the rhizosphere (Rodríguez and Fraga, 1999), or to survive under Cd stress (Anyanwu et al., 2011). In addition, the toxic metal in soils also affects the growth and survival of introduced microorganisms (Anyanwu et al., 2011). These findings revealed that it took at least eight weeks for the inoculated *Bacillus species* to functionally adapt to the soil, against competition with indigenous microorganisms in soil.

### 3.5 Summary

The study demonstrated that the *Bacillus* sp. (i.e., a mixed culture of *B. aryabhatai* and *B. megaterium*) as PSB could promote plant growth, by providing soluble P during pot experiment, and the application of PSB in phytoremediation significantly enhanced the Cd uptake efficiency, by increasing the amount of bioavailable Cd in the soils. The analysis of microbial community change by inoculation through pyrosequencing, was significantly in agreement with the rate of plant growth promotion and Cd uptake by *Brassica juncea*. The introduced PSB seems to take time of at least 8 weeks to function by itself due to competition for limited resources between introduced and indigenous microorganisms in soils. The survival and adaptation of introduced bacteria corresponded directly with the plant growth and Cd uptake. These findings may provide the information on microbial processes and their activity of introduced bacteria in soil that is required for effective bacteria- associated phytoremediation.

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# **CHAPTER 4**

## **ENHANCED UPTAKE AND TRANSLOCATION OF ARSENIC IN PLANTS THROUGH SIDEROPHORE-ARSENIC COMPLEX FORMATION**

### **4.1 Introduction**

Since As is mostly present as an oxyanion form, it is tightly bound to amorphous and crystalline Fe-oxides in soil (Sharma and Sohn, 2009), and thus the fate of As in soil is mainly controlled by sorption-desorption, complexation-dissociation, and oxidation-reduction mechanisms onto Fe-(hydro)oxides (Wang and Mulligan, 2008; Casentini and Pettine, 2010). The dissolution of As-adsorbed/precipitated Fe-oxides is the main cause of As release to the soil environment (Fendorf et al., 2010). Such dissolved forms of As and some easily desorptive and labile forms of As are thought to be absorbed by plants. During the uptake, rhizospheric activity acts beneficially by promoting plant growth and enhancing desorption of As from soil (especially from Fe oxides) through bacterial secretions.

Plant growth promoting rhizobacteria (PGPR) is a useful group of bacteria whose activity eventually enhances phytoextraction efficiency by increasing biomass production as well as by providing plants with tolerance to heavy metals (Jing et al., 2007a; Glick, 2010). Generally, PGPR functions in three ways;

synthesizing particular compounds for plants, facilitating the uptake of certain nutrients from soil, and preventing the diseases in plants (Kloepper et al., 1980; Rajkumar et al., 2010). When iron is deficient for plant growth, *Pseudomonas aeruginosa*, a member of PGPR, produces an iron-chelating compound (i.e., siderophores) to provide iron for the plants by dissolving iron oxides present in the soil. Siderophores are low molecular compounds (400-1,000 Daltons) with high complex affinity for iron under iron-limited conditions. Three dissolution mechanisms of iron oxides are proposed, which includes protonation, complexation (ligand-enhanced mechanism), and reduction mechanisms and ligand-enhanced complexation is known to occur by siderophores (Schwertmann, 1991). Siderophores can also form a stable complex with other metals such as Al, Cd, Pb, Zn, and As (Cheah et al., 2003; Nair et al., 2007; Rajkumar et al., 2012). Nair et al. (Nair et al., 2007) reported that siderophores could accommodate several metal ions (e.g., Fe, Cd, Ni, Pb, Mn, Co, and As), especially showing a higher affinity for As than ethylenediaminetetraacetic acid (EDTA) and citric acid. Garcia-Sanchez et al. (García-Sánchez et al., 2005) observed that the presence of *Pseudomonas* sp. secreting siderophores could mobilize As adsorbed on Fe-oxides, and the released As was found in groundwater.

The fact that siderophores can dissolve iron oxides and form a complex with As suggests a useful application of the compounds in the remediation of As-contaminated soil, especially for phytoextraction. In this study, a beneficial effect of a siderophore-producing bacteria (SPB), *P. aeruginosa* was tested in As-contaminated soil during phytoextraction. As a test plant, *Pteris cretica* known as an As hyperaccumulator was used. Also, EDTA was used as a chemical iron-chelator to compare with siderophore treatment as a microbial iron-chelator.

Enhanced growth of plant biomass uptake of As were confirmed in a pot experiment. In addition, the lines of evidence showing the formation of the As-siderophore complex and its translocation from roots to stems and leaves in the plant are presented.

## **4.2 Materials and Methods**

### **4.2.1 Preparation of siderophores-containing inoculum**

*Pseudomonas aeruginosa* ATCC 15692 was obtained from Korean Collection for Type Culture (KCTC) and grown in an iron-depleted succinate medium for siderophore production in an incubating shaker (200 rpm) for 24 h at 37°C. The succinate medium contained (g/L): succinic acid, 4.0; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0; KH<sub>2</sub>PO<sub>4</sub>, 3.0; K<sub>2</sub>HPO<sub>4</sub>, 0.1; and MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2. The pH was adjusted to 7.0 with 0.1 N HCl. To remove traces of iron, the glassware used for the culture was cleaned with 6 M HCl and distilled water. The culture was centrifuged at 8,000 g for 20 min to remove bacterial cells, and the supernatant was collected and filtered through a 0.22-μm membrane filter. This cell-free supernatant, named siderophores-containing culture filtrate (SCF), was subsequently used as a siderophore solution for further experiment.

#### 4.2.2 Quantification of siderophore activity

To measure the siderophore activity in the cell-free culture supernatant, the Chrome Azurol S (CAS) assay was conducted with SCF, as described by Schwyn and Neilands (Schwyn and Neilands, 1987b). CAS assay is based on competitive exchange of  $\text{Fe}^{3+}$  between a strong chelator (i.e., siderophores in this study) and a weak iron binding chemical hexadecyltrimethylammonium bromide (HDTMA) which results in the color change of the CAS reagent from blue to orange, indicating the presence of siderophores. Briefly, 500  $\mu\text{L}$  of SCF was added to the same volume of CAS assay solution, and the mixture was incubated for 20 min at room temperature. The CAS assay solution contained 6 mL of 10 mM HDTMA, 1.5 mL of 1 mM  $\text{FeCl}_3$ , 7.5 mL of 2 mM CAS, 4.307 g of piperazine, and 6.25 mL of 12 M HCl, and was diluted to 100 mL with deionized water. The absorbance at 630 nm of each CAS solution-SCF mixture was determined to quantify siderophore activity, and the result was expressed as the OD ratio (Equation 1).

$$\text{Siderophore activity (\%)} = \frac{A_r - A_s}{A_r} \times 100 \quad (1)$$

, where  $A_r$  is the absorbance at 630 nm of the reference sample (CAS assay solution + succinate medium without cell growth) and  $A_s$  is the absorbance at 630 nm of the mixture of the CAS assay solution and SCF (Payne, 1994; Bholay, 2012).

#### 4.2.3 Fe-oxides dissolution and As release by SCF

A batch dissolution experiment was conducted using As-adsorbed ferrihydrite, a widespread Fe-oxide found in soil, to investigate the release of Fe and As by SCF. A chelating ligand EDTA was also tested for comparison. A two-line ferrihydrite (hereafter called ferrihydrite) was synthesized in the laboratory following the method of Cornell and Schwertmann (Schwertmann and M., 2000). Briefly, ferrihydrite was synthesized by dissolving 40 g of  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  in 500 mL distilled water and titrating with 330 mL of 1 M KOH with vigorous stirring to a pH 7–8. The precipitates were centrifuged and then the suspension was transferred to dialysis bag with a MWCO of 10,000 g/mol (Thermo Scientific Inc.). Dialysis against distilled water was performed for 3 days at room temperature to rapid removal of impurities, changing the water at least six times. The ferrihydrite suspensions were allowed to freeze dry, and then stored as a solid. To adsorb As onto ferrihydrite, sodium arsenate dibasic heptahydrate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ , 10 wt% of the synthetic ferrihydrite) was used.

3.0 g of As-adsorbed ferrihydrite was mixed with a 60 mL of SCF in a sterile 125-mL Wheaton bottle<sup>®</sup> at room temperature for 14 days with a slight agitation. The initial pH of the SCF solution was about 8.0, and its siderophore activity was approximately 86% (i.e., Siderophore activity is 100% when all the As adsorbed on HDTMA is detached by SCF). For comparison, 1 mM of EDTA in 60 mL of distilled water was also mixed with 3.0 g of As-adsorbed ferrihydrite. All experiments were performed in triplicates.

Aliquots were sampled from the bottle, and filtered. They were then acidified for chemical analyses including the pH of the ferrihydrite suspension, the

concentrations of released soluble Fe (i.e., total Fe and Fe<sup>2+</sup>), the concentration of released As, and the change of siderophore activity of SCF. The concentration of total Fe (i.e., Fe<sup>3+</sup> and Fe<sup>2+</sup>) and Fe<sup>2+</sup> in solution was determined by Ferrozine assay (Stookey, 1970; Viollier et al., 2000). Briefly, a 100 µL of the filtered solution (i.e., a mixture of ferrihydrite and SCF and mixture of ferrihydrite and EDTA) was added to a ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate) solution (i.e., 100 µL of ferrozine solution and 150 µL of 5 M ammonium acetate solution adjusted to pH 9.5 with ammonium hydroxide), and the absorbance at 562 nm was recorded. Ferrozine is a reagent specific to Fe<sup>2+</sup>, but the addition of a reducing agent can extend the method to determine total Fe concentration. After storing 100 µL of the filtered solution (mixture of ferrihydrite and SCF, and a mixture of ferrihydrite and EDTA) overnight at room temperature, it was mixed with 150 µL of hydroxylamine hydrochloride solution (1.4 M in 2 M HCl) for hydroxylamine to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup>. After 30 min, the total Fe concentration was determined by measuring the absorbance at 562 nm after the addition of ferrozine solution. The filtered solution was also employed for the analysis of As released from ferrihydrite. Released As concentration was determined by ICP-OES analysis (730-ES, Varian, USA).

#### 4.2.4 Pot experiment

Uncontaminated surface soil (0-30 cm) was collected from a small field area at Seoul National University, the Republic of Korea. The soil sample was air-dried and ground to pass through a 2.0-mm sieve, and the physicochemical properties of the soil were determined (Table 4.1). The soils were characterized for pH, organic matter (OM), texture, Fe oxide, Al oxide, and Mn oxide contents. Soil pH was measured at a 1:5 ratio of soil to water according to the Methods of Soil Analysis, Part 3 - Chemical Methods (SSSA, 1996). Organic matter content was determined following the Walkley-Black method (Walkley and Black, 1934). Three textural fractions, clay (0-2  $\mu\text{m}$ ), silt (2-50  $\mu\text{m}$ ), and sand (50-2,000  $\mu\text{m}$ ) were used to characterize the soil particle distribution of fine earth (< 2 mm) by classifying it into a soil texture class according to a soil texture triangle establishing by the U.S. Department of Agriculture (USDA). Total phosphorus was determined by digestion with  $\text{H}_2\text{SO}_4\text{-HClO}_4$  method, and water soluble-P was extracted according to the Bray No.1 method in soil (SSSA, 1996). Total As concentration in soil was analyzed by Wenzel's sequential extraction method. The oxide contents such as Fe, Al, and Mn oxides in soil were determined using the Dithionite-Citrate-Bicarbonate Method (DCB).

The soil was artificially contaminated with an aqueous solution of sodium arsenate dibasic heptahydrate ( $\text{Na}_2\text{HAsO}_4\cdot 7\text{H}_2\text{O}$ ) to give the final concentration of 90 mg-As $\cdot\text{kg}^{-1}$ -soil. A series of pots (9.0-cm diameter and 10.0-cm height) each containing 500 g of As-contaminated soil were prepared to investigate the effect of siderophores on As uptake by *Pteris cretica*. The plant grew for ten weeks in a growth chamber (E15, Conviron Inc., Canada) under the constant conditions (i.e.,

25±1°C and 18-h photoperiod). The pots were watered with tap water every day and SCF produced by *P. aeruginosa* was inoculated into the pots by approximately 80% of water holding capacity of the soil every two weeks. For comparison, EDTA solution (1 mM) was employed as a separate experiment. The plants were harvested after 3, 5, 7, and 10 weeks of growth, and the roots and the shoots (i.e., stems and leaves) were then collected and washed with distilled water to remove soil particles. All pot experiments were performed in triplicates.

Table 4.1 Physicochemical properties of the soil used in this study

Soil properties	Unit	Value
pH	-	5.41
Organic matter	%	2.19
Sand	%	82.56
Silt	%	3.32
Clay	%	14.12
Texture	-	Sandy loam
Total-P	mg·kg <sup>-1</sup>	247.0
Available-P	mg·kg <sup>-1</sup>	14.79
Fe-oxide	mg·kg <sup>-1</sup>	4002.17
Al-oxide	mg·kg <sup>-1</sup>	447.72
Mn-oxide	mg·kg <sup>-1</sup>	108.40
Total As	mg·kg <sup>-1</sup>	91.76

#### 4.2.5 Chemical analysis of plants and soils

The total biomass of the plants was determined after the shoots (i.e., stems and leaves) and the roots were oven-dried separately at 70°C for 24 h. The oven-dried roots and shoots samples were further ground into a fine powder for As content analysis. To extract As, the samples were digested with a solution of HNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>, and distilled H<sub>2</sub>O (9:1:1, v/v/v) using a microwave digester (MSP1000, CEM, USA) according to the US EPA 3052 method. After harvesting the plants, the soils in each pot were taken and air-dried at room temperature, and the chemical forms of As in the soils were also determined by Wenzel's sequential extraction method (Wenzel et al., 2001). The concentrations of As in the plants and the soils were determined by ICP-OES analysis (730-ES, Varian, USA).

After harvesting the plants, chlorophyll content was determined (Sharma et al., 2003). Briefly, fresh leaves were cut into small pieces followed by addition of 7 mL dimethyl sulphoxide (DMSO) per 100 mg leaf tissue, and incubated at 65 °C for 3 h. Each extract was made up to a volume of 10 ml with DMSO, and measured at 645 and 663 nm. Pure DMSO was used as blank. The amount of chlorophyll a, chlorophyll b and total chlorophyll in leaf tissue were calculated according to Hiscox and Israestam (Hiscox and Israelstam, 1979).

Siderophore-As complex in the plants were determined by Chrome Azurol S (CAS) assay in combination with fluorescent microscopy analysis. To recover siderophore-As complex from the plants, plant samples were digested with 12 M HCl adjusted to pH 3.0, and the acidified samples (25 mL) were extracted three times with 1/5 volume ethyl acetate (Tindale et al., 2000; Dhanya and Potty, 2007). The ethyl acetate layer was collected and the solvent was evaporated. The powder

obtained was then resuspended in distilled water and the resulting solution was applied for Chrome Azurol S (CAS) assay.

The plants were also taken for observing the cross-sectional images of the roots and the leaves by using a fluorescent microscope (DeltaVision RT, AppliedPrecision, USA) operated with a single wavelength laser beam at 480 nm. The wavelength was chosen to detect the yellow-green fluorescent siderophores produced by *P. aeruginosa* (Bar-Ness et al., 1992; Moore et al., 2006).

#### 4.2.6 Quality assurance and quality control (QA/QC)

For quality assurance and quality control (QA/QC), As analysis using the inductively coupled plasma optical emission spectrometry (ICP-OES) was performed three times consecutively for one sample and the average value was taken. ICP-OES operating condition are as following; 1200 W, generator power, 15 L/min, plasma gas flow, 0.7 L/min, nebulizer gas flow, 188.9 nm, wavelength of As. Multi-element calibration standard for As was used. The detection limit of ICP-OES for As was 0.006 mg/L. The recovery was found to be within 100±5%. The differences between specific pairs were identified by the student's t-test ( $p < 0.05$ ).

## 4.3 Results

### 4.3.1 Enhanced dissolution of As from ferrihydrite by siderophores

As a preliminary experiment, the ability of the SCF solution itself and the siderophores extracted to dissolve Fe-oxides and to further release As bound to Fe-oxides was determined. The siderophores that recovered from SCF by HCl-ethyl acetate extraction (Tindale et al., 2000; Dhanya and Potty, 2007) showed about 50-60% of Fe dissolution and As binding capacity compared to SCF solution itself during the batch dissolution experiment (Figure 4.1). This might be attributed to the instability of siderophores under acidic conditions. The pH of the extracted siderophore solution was about 4.2, and the siderophores could be easily oxidized under such conditions (Clark, 2004; McMillan et al., 2010). In contrast, the pH of SCF was about 8.0, which was likely to result in the reliable and stable activity of siderophores during the experiment. Thus, SCF solution was used as a siderophores-containing solution for further experiments. The pH changes of solution during experiment was shown in Figure 4.2.

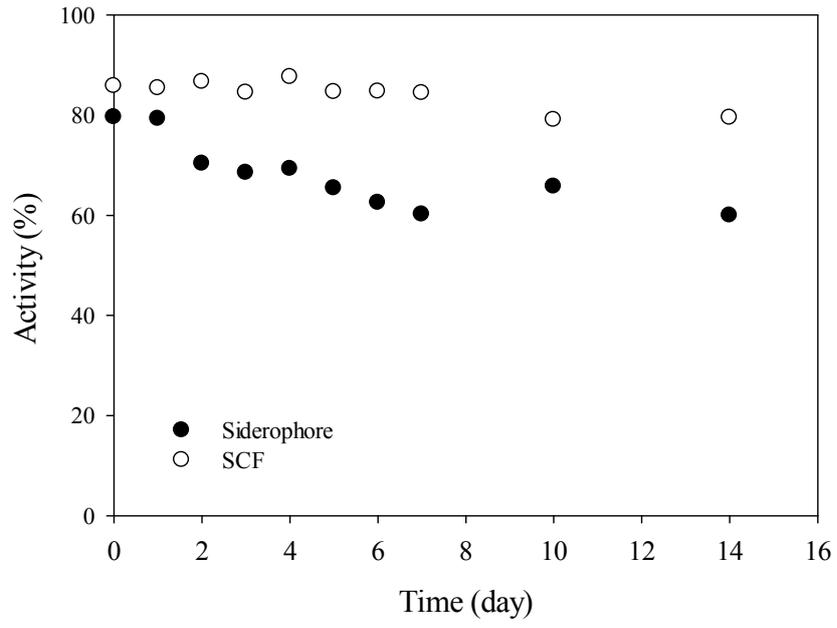


Figure 4.1 The changes of siderophore activity in SCF (siderophore-containing filtrate) and acidified extracted siderophore during the experiment

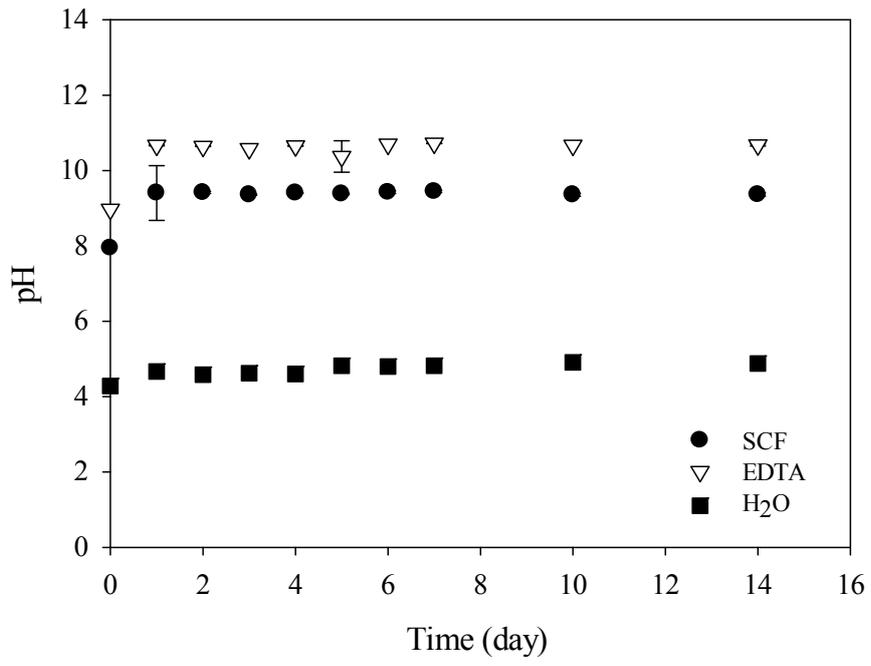
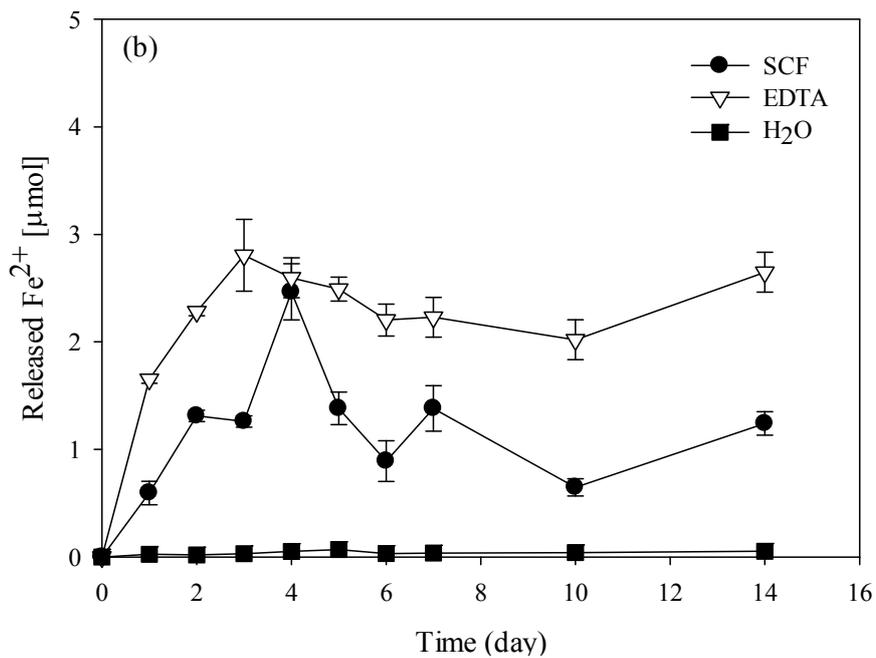
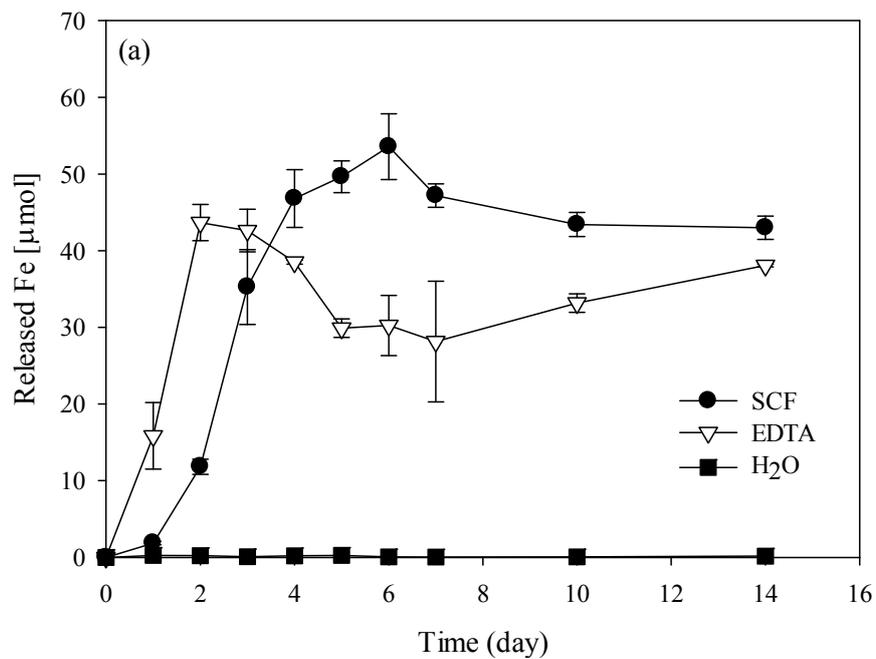


Figure 4.2 Effect of SCF (siderohore-containing filtrate), EDTA and distilled water as a control application on pH during the experiments

The release of As and Fe (i.e., total Fe and Fe<sup>2+</sup>) from ferrihydrite by SCF was determined, and the results were compared to the case when EDTA was used to dissolve ferrihydrite (Figure 4.3). As a reference, distilled water was used as a non-dissolving solvent. In the presence of either SCF or EDTA, the total Fe (i.e., sum of Fe<sup>3+</sup> and Fe<sup>2+</sup>) concentration continuously increased, showing the maximum of 53.6 μmol on the 6<sup>th</sup> day or 43.7 μmol on the 2<sup>nd</sup> day, respectively (Figure 4.3(a)). Apart from the maximum value, the initial release of total Fe by EDTA was more rapid than that by SCF, probably due to its higher complex stability constant. The complex stability constant for EDTA-Fe was estimated to be approximately 10<sup>25</sup> M<sup>-1</sup>, whereas the Fe stability constant of siderophores produced by *P. aeruginosa* was reported to be about 10<sup>5</sup>-10<sup>24</sup> M<sup>-1</sup> (Visca et al., 1992; Kraemer et al., 1999; Butler, 2010). In addition, the siderophore molecule can experience steric hindrance due to its molecular size (i.e., generally, < 1 kDa) (Cox and Adams, 1985; Morton et al., 2010), which limits its access to the surface of Fe-oxides (Liermann et al., 2000; Cervini-Silva and Sposito, 2002; Holland, 2011; Paris and Desboeufs, 2013). Contrary to total Fe concentration, Fe<sup>2+</sup> was only slightly (i.e., 0.59-2.81 μmol) released from ferrihydrite in both samples (Figure 4.3(b)). The highest Fe<sup>2+</sup> concentration released was 2.81 μmol on the 3<sup>rd</sup> day and 2.47 μmol on the 4<sup>th</sup> day by EDTA and SCF, respectively. These were 16-22-fold less than the total Fe concentration, indicating that reductive dissolution may not be the dominant mechanism in dismantling ferrihydrite.

More importantly, 1.11–1.79 μmol of As was released in the SCF-amended samples during the 14 days of the experiment, while less than 0.15 μmol of As was detected in the EDTA-amended samples (Figure 4.3(c)). This is probably due to the re-adsorption of the released As onto the available surfaces of ferrihydrite instead

of chelating As oxyanions. It is known that siderophores can form a complex with As, while EDTA cannot. A previous study also reported such re-adsorption of As on various mineral surfaces (Tabelin et al., 2010).



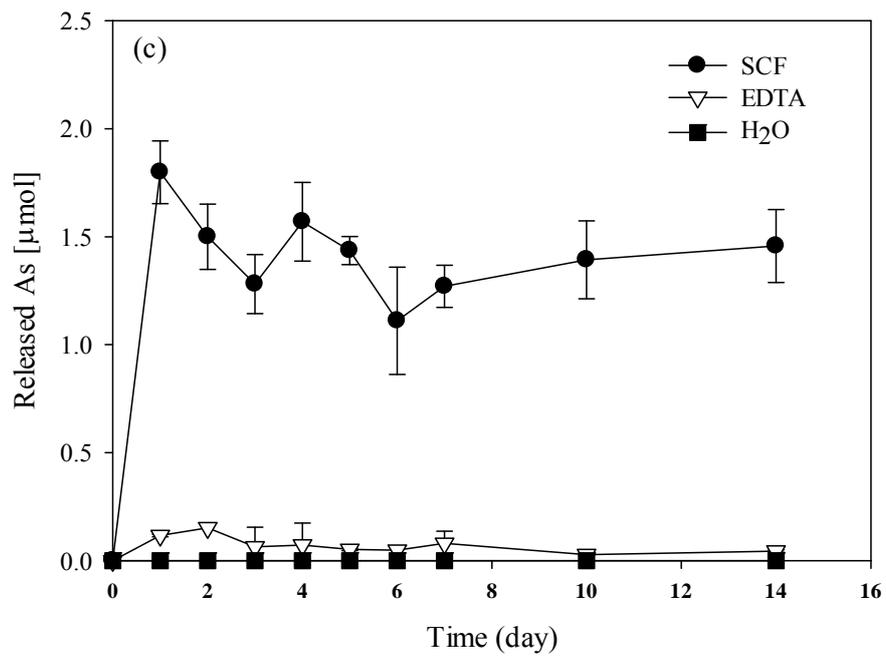


Figure 4.3 Ferrihydrite dissolution and As release from the oxide in the presence of SCF and EDTA. Distilled water was used as a control. SCF represents siderophores-containing culture filtrate produced by *P. aeruginosa*

#### 4.3.2 Biomass increase by siderophores

Enhanced growth of *P. cretica* was observed in the SCF-amended soil compared to the EDTA-amended and control soils. The soils were contaminated with As at a concentration of  $91.8 \pm 0.98$  mg/kg. The total biomass and its distribution of the biomass and chlorophyll content in each part of *P. cretica* (i.e., roots, stems, and leaves) are presented in Table 4.2 and 4.3. Chlorophyll a, chlorophyll b and total chlorophyll content was correlated with higher plant biomass. For the first three weeks, the plant biomass increased in all pot samples tested, but the biomass then decreased in the control and EDTA-amended pots afterward. Further biomass increase was observed only in the pots amended with SCF. The highest biomass of *P. cretica* grown in the SCF-amended pots was 5.83 g on the 5<sup>th</sup> week, which was about 85 and 146% higher than the control and EDTA-amended pots, respectively at the same time point. After ten weeks of pot experiment, the total biomass of *P. cretica* grown in the SCF-amended soils (4.90 g) was significantly ( $p < 0.05$ ) greater than the biomass of the other two treatments (1.70 and 1.92 g in the control and EDTA-amended pots, respectively). When EDTA was treated, the biomass did not significantly differ from the control samples, indicating the any significant inhibition of plant growth by EDTA amendment (1 mM) was not observed in our study. The distribution of biomass among roots, stems, and leaves was also determined. The root mass of *P. cretica* in the SCF-amended pots was the greatest showing a significant increase. The root mass after 10 weeks was approximately four times greater (3.74 g) than the initial mass (1.06 g). This remarkable increase in root mass is meaningful in the phytoextraction of As because the metal is known to be taken up and be

accumulated mainly in the roots (Moreno-Jiménez et al., 2008; Wang et al., 2008; Moreno-Jiménez et al., 2012).

Table 4.2 Total biomass (g) and its distribution of *Pteris cretica*

Time (week)	Roots	Stems	Leaves	Total
Control				
0	1.06	0.04	0.37	1.47
3	3.21	0.91	0.22	4.35
5	1.79	1.06	0.35	3.15
7	1.97	0.11	0.40	2.43
10	1.17	0.16	0.59	1.92
SCF				
0	1.06	0.04	0.32	1.47
3	2.81	0.84	0.18	3.82
5	3.44	1.75	0.64	5.83
7	3.69	0.95	0.72	5.35
10	3.74	0.55	0.61	4.90
EDTA				
0	1.06	0.04	0.32	1.47
3	2.60	0.89	0.22	3.72
5	1.21	0.90	0.26	2.37
7	1.45	0.04	0.16	1.66
10	1.51	0.05	0.14	1.70

Table 4.3 The changes of chlorophyll content in leave of *Pteris cretica*

Time (week)	Control	SCF	EDTA
0	1.55 (±0.819)	1.55 (±0.819)	1.55 (±0.819)
3	1.78 (±1.009)	1.24 (±0.653)	1.23 (±1.174)
5	1.19 (±0.255)	1.65 (±0.526)	0.91 (±0.453)
7	1.60 (±0.603)	1.97 (±0.850)	0.89 (±0.183)
10	1.00 (±0.157)	1.51 (±0.084)	0.85 (±0.329)

### 4.3.3 Changes of chemical forms of As in soil by siderophores

Changes of the chemical forms of As in the soils amended with SCF or EDTA were monitored during the pot experiment. When the pot experiment was started (i.e., 0 week in Table 4.2), the chemical forms of As in the soil was determined by using the Wenzel's five-step sequential extraction method (Wenzel et al., 2001), and the results of which are as follows; 3.5% of non-specifically bound As (fraction 1), 22.4% of specifically bound As (fraction 2), 51.9% of amorphous Fe-Al oxides bound As (fraction 3), 16.1% of crystalline Fe-Al oxides bound As (fraction 4), and 6.2% of residual As (fraction 5).

At the beginning of the pot experiment without SCF or EDTA, about 68% of the total As in the soil was found to be associated with Fe-Al oxides (i.e., fractions 3 and 4) and about 64.6% of As was still present as the same chemical forms after 10 weeks (Table 4.4). In contrast, the fractions associated with Fe-Al oxides decreased to 54.8 or 53.5% when SCF or EDTA, respectively, was added to the pot soils. Scrutinizing the As fractionation data indicated that the more weakly bound As fractions were newly formed by the amendment: After ten-week experiment, the non-specifically bound As (fraction 1) increased from 2.1 to 14.7% in the SCF-amended soil and specifically bound As (fraction 2) increased from 26.7 to 35.2% in the EDTA-amended soil. The fraction of residual As was nearly changed.

Table 4.4 Changes of As chemical form (%) in soil using the Wenzel's five-step sequential extraction method by addition with SCF, EDTA and distilled water as a control

Time (week)	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
<b>Control</b>					
0	3.5	22.4	51.9	16.1	6.2
3	3.0	25.3	49.1	16.8	5.9
5	2.6	26.3	45.5	18.2	7.4
7	2.6	30.3	44.0	16.9	6.2
10	2.1	26.7	47.0	17.6	6.7
<b>SCF</b>					
0	3.5	22.4	51.9	16.1	6.2
3	7.0	23.5	45.9	17.5	6.3
5	12.1	23.9	41.9	15.2	7.0
7	12.8	23.4	40.4	15.2	8.2
10	14.7	23.2	40.4	14.4	7.3
<b>EDTA</b>					
0	3.5	22.4	51.9	16.1	6.2
3	2.8	24.7	48.0	17.2	7.3
5	4.0	28.6	41.4	17.7	8.3
7	4.2	30.7	41.4	17.5	6.2
10	4.5	35.2	38.0	15.5	6.8

#### 4.3.4 Accumulation and distribution of As in plants by siderophores

The As concentration in *P. cretica* greatly increased with growth when the plant was grown in the SCF-amended soil ( $p < 0.05$ ) (Figure 4.4). After the ten-week pot experiments, the highest As concentration was observed in the SCF-amended samples (i.e.,  $5.62 \text{ mg-As}\cdot\text{g}^{-1}\text{-plant}$ ) whereas only  $1.76 \text{ mg-As}\cdot\text{g}^{-1}\text{-plant}$  was detected in the control samples. This is a significant difference, suggesting that the plant *P. cretica* is an As hyperaccumulator. When EDTA was treated, the As concentration taken up by the plant did not significantly differ from the control samples (i.e.,  $1.51 \text{ mg-As}\cdot\text{g}^{-1}\text{-plant}$ ), probably due to either the growth inhibition by EDTA or re-adsorption of the released As or both.

The distribution of As among the tissues of *P. cretica* was also determined (Figure 4.4). *P. cretica* grown in the SCF-amended soil accumulated As mainly in its roots up to 5 weeks, which can be expected because As is known to be dominantly accumulated in plant roots (Moreno-Jiménez et al., 2008; Wang et al., 2008; Moreno-Jiménez et al., 2012). Such trend was also observed with the control and EDTA-amended soils. In addition, at up to five weeks of incubation, the concentrations of As accumulated in the plants did not significantly differ among the three treatments. After seven weeks of growth, the As taken up by the plant increased considerably in the SCF-amended soil (i.e., 430% increase compared to three-week growth). More importantly, 58% of the total As present in the plant was found in the leaves. The percentage was further increased to 79% after ten weeks of growth, while for the As concentrations detected in the stems and leaves as well as the total As concentrations in the plant nearly changed in the control and EDTA-amended soils with time.

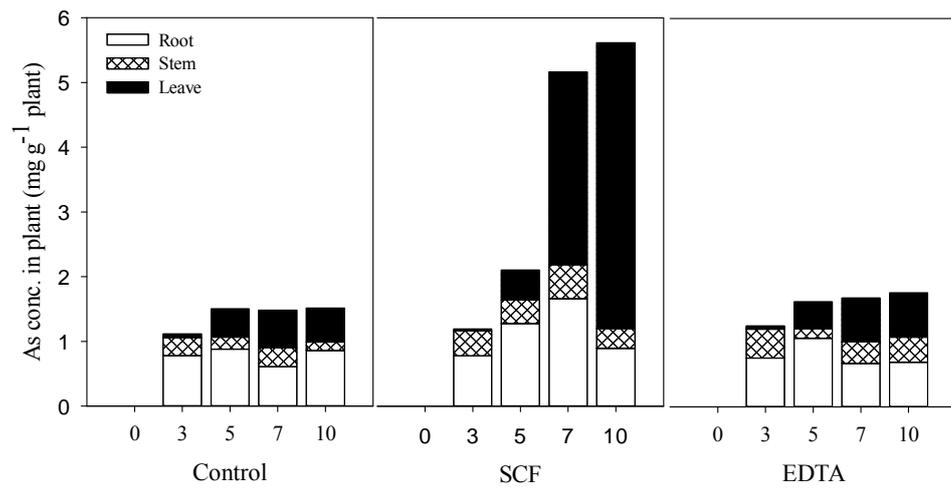


Figure 4.4 Effect of SCF and EDTA application on As uptake by *Pteris cretica* and As distribution among the plant tissues with time. Distilled water was used as a control

#### 4.3.5 Detection of siderophores-As complex in plants

Data for the As distribution in Table 4.2 indicate that As was transported to the leaves when *P. cretica* was grown in the SCF-amended soil. In addition to root mass increase in the presence of SCF (and the subsequent increased As uptake), this translocation of absorbed As is another important favorable effect of siderophore application. It is known that siderophores can form complex with As while EDTA cannot (Cheah et al., 2003; Nair et al., 2007). To verify the presence of the siderophore-As complex in the leaves of the plant tested, a CAS liquid assay was conducted. The original color of CAS solution is orange, and when hexadecyltrimethylammonium bromide (HDTMA) was present in the solution combines with As, its color turned into blue (Schwyn and Neilands, 1987a). In the case where a strong chelating agent is added to the solution and the As bound to HDTMA is transferred to the agent, such as siderophores, the solution color is changed back to orange. Using this chemical reaction, the presence of siderophore-As complex was identified and quantified and then expressed as the relative intensity in the roots, the stems, and the leaves of the plants grown in the three treatment samples. All relative intensities of *P. cretica* grown in the SCF-amended soil were higher than those in the other two treatments, and the difference became greater with time (Figure 4.5). The highest relative intensity was detected in the leaves of *P. cretica* grown in the SCF-amended soil.

Since siderophores are a fluorescence-emitting compound fluorescence microscopic analysis was conducted at 480 nm wavelength with the cross-sectional samples of the roots and the leaves of *P. cretica* grown in the SCF-amended soil. As shown in Figure 4.6, higher fluorescence signals were detected in the leaves

than in the roots, which further supported the presence of the siderophore-As complex. Little or no fluorescence was found in the tissue samples of *P. cretica* grown in the control and EDTA-amended soils.

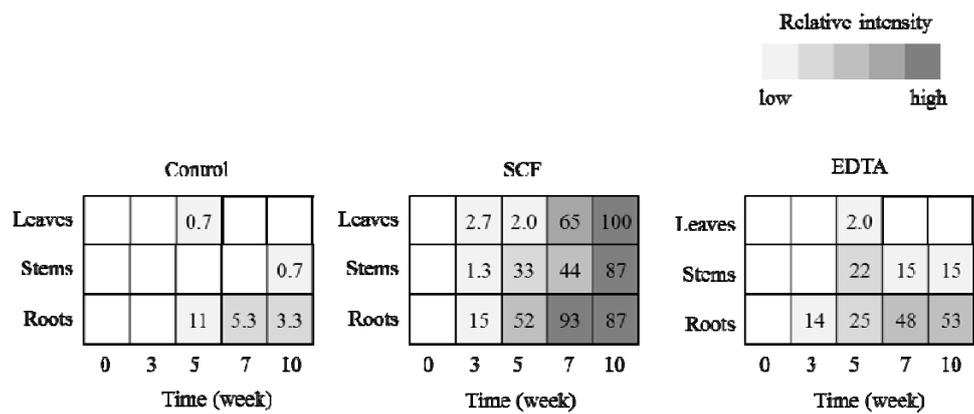


Figure 4.5 Detection of siderophore-As complex in *Pteris cretica* using CAS liquid assay. A higher relative intensity is obtained where more complex are present

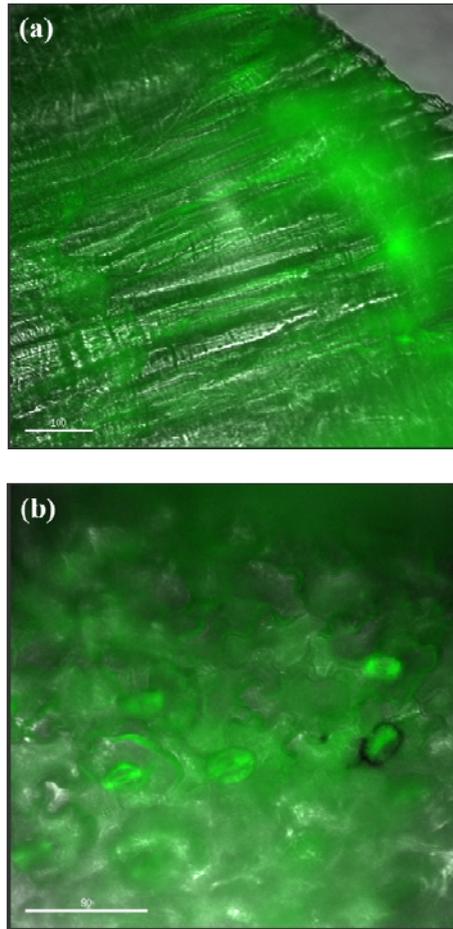


Figure 4.6 Cross-sectional images (x 160) of (a) leaves and (b) roots of *Pteris cretica* grown in SCF-amended soil observed by fluorescence microscope analysis. Green fluorescence represents the presence of siderophore-As complex

## 4.4 Discussion

Siderophores are produced by some rhizobacteria under an iron-deficient condition and *Pseudomonas aeruginosa* used in this study is a well-known siderophore-producing rhizobacterium. Siderophores can facilitate phytoextraction of As directly by increasing the release of As adsorbed in the soil (i.e., mainly to Fe oxides) and indirectly by promoting the growth of the plant biomass by supplying trace elements such as Fe (Jing et al., 2007a; Dimkpa et al., 2009; Rajkumar et al., 2010; Rajkumar et al., 2012). In the batch dissolution experiment, the As-adsorbed ferrihydrite was dissolved by siderophores produced by *P. aeruginosa* ATCC 15692, and consequently As and Fe were liberated. Also, the pot experiment for phytoextraction with the As hyperaccumulator *P. cretica* showed an increased As uptake as well as biomass growth when SCF (siderophores-containing culture filtrate) was amended. Weak organic acids secretion (e.g., lactic acid, acetic acid), Indole-3-acetic acid (IAA) and gibberellins (GA) might also be secreted to SCF by *P. aeruginosa*, suggesting that, along with these secretions, the siderophores also contributed to plant growth promotion.

Uptake ratio (i.e., calculated as the ratio of As concentration in plant to As concentration in the soil) clearly demonstrates the effect of siderophore application. For the first five weeks, uptake ratios did not significantly differ among the SCF-amended, the EDTA-amended, and the control samples, yielding 23.39, 18.35, and 16.10, respectively (Table 4.5). After five weeks, the uptake ratios did not change significantly in the control and the EDTA-amended soils. In the SCF-amended soil, however, the ratio increased to 56.61 and 63.31 after seven and ten week, respectively.

Table 4.5 Uptake ratio and translocation factor (TF) in *Pteris cretica* grown in soil addition with SCF, EDTA and distilled water as a control

Time (week)	Uptake ratio			Translocation factor (TF)		
	Control	SCF	EDTA	Control	SCF	EDTA
0	0.09	0.09	0.09	0.00	0.00	0.00
3	11.98	12.96	13.79	0.43	0.53	0.66
5	16.10	23.39	18.35	0.71	0.65	0.53
7	15.93	56.61	17.83	1.43	2.10	1.50
10	16.44	63.31	19.87	0.76	5.28	1.55

*P. cretica* has been widely used in many metal accumulation studies. A pot experiment study for screening As-hyperaccumulating plant demonstrated that *P. cretica* could accumulate 1.2–2.5 mg-As·g<sup>-1</sup>-plant by Meharg (Meharg, 2003). Wei and Chen reported much lower values of 0.14-0.69 mg-As·g<sup>-1</sup>-plant with the same plant (Wei and Chen, 2006). In our pot experiment, the same plant accumulated about 1.51 mg-As·g<sup>-1</sup>-plant of As in the control soil. However, when SCF was treated to *P. cretica*, the highest As concentration absorbed by the plant was 5.62 mg-As·g<sup>-1</sup>-plant, which is approximately four-fold greater than the control soil. Enhancement of As accumulation in other plants were also reported by the other researches when siderophores were applied. Kloepper et al (Kloepper et al., 1980). observed that crop plants such as potato, sugar beet, and radish increased up to 144% of As accumulation by the application of siderophores produced by *Pseudomonas* sp. Wang *et al.* also reported that As concentrations in the roots, the

stems, and the leaves of *Populus deltoids* were increased by 229, 113, and 291%, respectively with the siderophore-producing bacteria inoculation (Wang et al., 2011b). Recently, in a study with sunflower (*Helianthus annuus* L.), about 53% increase in As uptake by siderophore-producing rhizobacterium (*Alcaligenes* sp.) was reported (Cavalca et al., 2013).

The results showed that siderophores enhanced the release of As from soil, and the increased As in turn formed a complex with siderophores. A Siderophore molecule produced by *P. aeruginosa* is composed of three parts; a fluorescent hydroxyquinoline chromophore, an acyl side chain bound to the amino group of the chromophore, and a strain-specific peptide chain linked via an amide bond to the carboxyl group of the chromophore.(Varma) Functional groups in the siderophores can participate in the chelation of As oxyanions through polyvalent cation bridges and/or fast protonation of terminal amino groups (Debbie et al., 2004; Nair et al., 2007). The fact that the released As could be present as a siderophore-As complex indicated that As probably was absorbed by *P. cretica* as the complex forms (i.e., not as As oxyanions). In addition, the complex was transported to the leaves of the plant to a greater extent than As was present as oxyanions. This view was further supported by the translocation factors (Table 4.5), calculated as the ratio of the As concentration in the shoots (i.e., stems and leaves) to the roots (Kabata-Pendias and Pendias, 1984; Liu et al., 2008). The translocation factor of *P. cretica* was 5.28 at the 10<sup>th</sup> week in the SCF-amended soil, but only 1.55 and 0.76 was obtained in the EDTA-amended and the control soils, respectively. Data from the CAS liquid assay and fluorescent microscope analysis confirmed that As was present as the siderophore-As complex in *P. cretica* and most of the complex transported from the roots and existed in the leaves of the

plant. Although the mechanisms responsible have not been fully understood in our study, the formation of siderophore-As complex may facilitate As transport from roots to leaves of plants and by high complex stability and mobility while free As as oxyanions retain in roots with relatively low stability and high toxicity. Practically, such translocation of As by siderophores is a significant advantage in phytoremediation because the leaves could be removed, allowing additional green tissue production which enabled the plants to continuously eliminate and concentrate As from the contaminated soil (Baldwin and Butcher, 2007; Wang et al., 2011b).

The phytoavailable As was likely to be the As fractions that are non-specifically and/or specifically adsorbed to the solid phase including Fe/Al oxides, clay mineral, and organic matter (Matera et al., 2003; Wang and Mulligan, 2006; Qiu et al., 2010). In this context, the uptake of As by plants could be estimated to some extents by its chemical form in the soil. The results showed that the application of SCF changed the chemical forms of As in the soil. By introducing SCF or EDTA, the fractions of As bound to the Fe/Al oxides decreased to 54.8 and 53.5% from 64.6% (i.e., in the control soil), respectively. Interestingly, the introduction of SCF or EDTA resulted in the redistribution of the reduced fractions of As. In the SCF-amended soil, the non-specifically bound form of As increased to 14.7% whereas the fraction was only 2.1% in the control soil. However, in the EDTA-amended soil, the specifically bound form increased to 35.2% (i.e., 26.7% in the control soil). Since siderophores can chelate As, the released As is likely to be associated with siderophores, and the As in the siderophore-As complex was probably counted as the non-specifically bound form. In contrast, EDTA cannot chelate As, and thus the released As seemed to be re-adsorbed to the sorption sites

where available or with a higher sorption energy. The mechanisms of action of EDTA are likely to be limited to the dissolution of Fe from soil not form As complexes as siderophores do. This difference in the As relocation after the release from the soil probably yielded the difference in the amount of As absorbed by the plant in the SCF- or EDTA-amended soils.

Despite the large amount of literatures reporting the enhanced As uptake by rhizobacterial siderophores, few studies are available that show how the siderophores interact with the released As. Experimental data show that siderophores liberate As adsorbed onto ferrihydrite and form a complex with the released As. In addition, the siderophore-As complex are to be translocated from the roots to mainly the leaves. This study demonstrates the usefulness of siderophore-producing rhizobacteria not only for enhanced As uptake but also for practical maintenance during phytoremediation.

## 4.5 Summary

When a siderophore-producing bacterium, *Pseudomonas aeruginosa*, was introduced into As-contaminated soil, the release of As adsorbed to amorphous Fe-oxides (ferrihydrite) was greatly enhanced and the released As was transformed into forms that plants were easily able to uptake in soils (i.e., non-specifically bound forms). Once released, the As seemed to be present as siderophore-As complexes, which were absorbed in the roots and further transported to the stems and leaves. Practically, the leaves can be easily removed, allowing additional green tissue production which allowed for the plants to be continuously removed and for the concentrated As in plants from the contaminated soil.

#### Textbox 4.1 Estimation of phytoremediation duration

The top of 15 cm soil was used to estimate the phytoremediation duration, and the estimated time required to remove to worrisome level of arsenic in Korea (i.e., 25 mg-As/kg-soil) by *Pteris cretica* from As-contaminated soil was calculated. The time required was compared between two cases (i.e., phytoremediation with siderophore addition and without siderophore addition). To calculate As uptake on a plant dry weight basis, results from chapter 4 was used. However, only accumulated As in shoot (stems and leaves) by plants was considered in estimation of phytoremediation duration.

Area of studied site is 59 m<sup>2</sup> (7.7 m × 7.7 m), therefore the volume of top 15 cm soil is 8.85 m<sup>3</sup>. The density of the dry soil was 1.6 g/cm<sup>3</sup>. Therefore, the dry weight of soil for 15 cm soil of studied site is 14,160 kg (8.9 m<sup>3</sup> × 1.6 g/cm<sup>3</sup> × 10<sup>6</sup> cm<sup>3</sup>/m<sup>3</sup> × 10<sup>-3</sup> kg/g). As was contaminated of soils with 50.89 mg/kg (90<sup>th</sup> percentile concentration) in this studied site. The calculated total amount of As of the top 15 cm of soil of studied site is 720,602 mg (14,160 kg × 50.89 mg/kg). Assuming the planting interval as 20 cm, a total of 1475 of *Pteris cretica* was required. Based on the As accumulation in shoot of plants per 10 week, up to 2.89 and 0.32 mg-As/plant can be taken up by plants in soils either with the addition of siderophore or not. Therefore, plants treated with siderophore can remove 4,263 mg of As from soil per 10 weeks (2.89 mg/plant × 1475 plant), and 472 mg of As from soil per 10 week (0.32 mg plant × 1475 plant) can be removed from soil without addition of siderophore.

Meanwhile, the worrisome levels of As-contaminated soil in Korea was 25.0

mg/kg. Therefore, a total amount of As to be removed from soils of studied site was 366,602 mg ( $(50.89-25 \text{ mg/kg}) \times 14160 \text{ kg}$ ). The time to remove As from soil with siderophore treatment is estimated to be approximately 18 years. Whereas without siderophore treatment, the expected phytoremediation efficiency was 161 years.

One of the important limitations of phytoremediation is the time required to clean up a contaminated site (Salido et al., 2003). From estimation of phytoremediation duration results, it will probably be most successful depending on contaminants level when combined with other remediation techniques (O'Connor et al., 2003). Phytoremediation cost associated with planting, cultivating, and landfilling of plant material are included. However, the removal efficiency is greatly influenced economical consideration of phytoremediation. Thus, plant-microbe interactions in soils for enhancing the phytoremediation efficiency should be more focused.

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# **CHAPTER 5**

## **EVALUATION OF THE ENVIRONMENTAL EFFECT OF PHYTOREMEDIATION USING ECOLOGICAL RISK ASSESSMENT**

### **5.1 Introduction**

Many ecological issues need to be considered when developing a phytoremediation strategy for heavy-metal contaminated soils (Pilon-Smits and Freeman, 2006). Among them are the effect of phytoremediation on the local ecological relationships and food chain in ecosystem, the spreading or leaching of metal-chelating agent used for enhancing phytoremediation efficiency, and potential risk of the use of nonnative plant species to native plant species diversity in soil. Along with such concerns, heavy metals-accumulating plants may provide exposure pathways for toxic metals to enter the food chain and the resulting potential ecological risk needs to be carefully considered (Angel and Linacre, 2005; Robinson et al., 2006; Bianchi et al., 2011).

One of the paramount rules of phytoremediation is to avoid contaminant and metal-chelating agent dispersal. *In situ* application of chelating agents can cause groundwater pollution by uncontrolled metal dissolution and leaching (Vassilev et al., 2004). Wenzel et al. (2003) confirmed that chelating agents such as EDTA considerably increased metal liability in soil, but also observed enormously increased metal concentrations in the leachates collected below the root zone (Wenzel et al., 2003). However, a few studies have shown that the metal-chelating

agent has affect only on site (Meers et al., 2007). Moreover, the problems linked to the metal-chelating agent application may be overcome by using other chelating agents, such as EDDS (ethylene diamine disuccinate) and NTA (nitrilotriacetic acid) with a high degree of biodegradability (Vassilev et al., 2004; Bert et al., 2009). Drip irrigation also can be used to prevent further spreading and transfer into the ecosystem. Irrigation management including drip irrigation minimizes leaching as long as irrigation does not exceed plant water use (Gårdenäs et al., 2005). The problem related to the use of nonnative plant species can be solved by either only using native plant species or sterile exogenous plants (Henry, 2000).

To evaluate the potential risk to ecological receptors that ingest the heavy metals accumulating plant biomass such as herbivores and omnivores, an ecological risk assessment is likely required (Henry, 2000; Angel and Linacre, 2005). Ecological risk assessment is defined as a process that evaluates the likelihood that adverse effects may occur or are occurring, as a result of exposure to one or more stressors (USEPA, 1992). The process is used to systematically evaluate the information, assumptions, and uncertainties, in order to predict the relationships between stressors and ecological effects, in a way that is useful for environmental decision making (USEPA, 1998; Choi et al., 2011). In this study, we evaluated the terrestrial ecological risk during the phytoremediation of arsenic (As), and especially focused on whether or not an additional risk was expected by the application of an enhanced phytoremediation technique. Arsenic was a target compound and *Ptreis cretica* was used as a hyperaccumulating plant. Terrestrial invertebrate, avian insectivore (birds), small mammal, herbivore, and omnivore were considered as potential ecological receptors.

## 5.2 Site characterization

The study site was a former smelting area operated for over 60 years, located in the southwest coast of South Korea. Site investigation showed that the surface soil within 1.5 km radius from the refinery stack was contaminated with various heavy metals (i.e., As, Pb, Cu, Cd, Zn, Ni, Hg), but the main concern was As. Analyses about the soil property, land use, and the chemical forms of As revealed that the western part of the site along with the sea beach was suitable for phytoremediation (Figure 5.1). A small portion of the site (i.e., 7,500 m<sup>2</sup>) was selected for the test site of this study. The soil pH and organic matter content were 6.5 and 5.7%, respectively. The soil texture was sand containing 89.7, 3.0, and 7.3% of sand, silt, and clay contents, respectively.



Figure 5.1 Study area and its surroundings

### 5.3 Problem identification

A total of 16 soil samples were analyzed for As, Pb, and Cd, and 5 soil samples for Cu, Zn, Ni, and Hg. Soil concentrations of the metals were  $85.9 \pm 52.43$  mg As  $\text{kg}^{-1}$  soil,  $272.9 \pm 252.97$  mg Pb  $\text{kg}^{-1}$  soil,  $0.9 \pm 0.18$  mg Cd  $\text{kg}^{-1}$  soil,  $104.2 \pm 97.19$  mg Cu  $\text{kg}^{-1}$  soil,  $47.0 \pm 8.18$  mg Zn  $\text{kg}^{-1}$  soil,  $14.5 \pm 1.46$  mg Ni  $\text{kg}^{-1}$  soil, and  $0.09 \pm 0.02$  mg Hg  $\text{kg}^{-1}$  soil. Most of heavy metal concentrations found were much lower than the Korean soil standards except for As. The Korean soil standard of As of forest area for human is 40 mg As  $\text{kg}^{-1}$  soil. As a remediation practice, phytoremediation was considered and the above mentioned feasibility test of phytoremediation was performed. The data were used for the ecological risk assessment. For the initiation of risk calculation, a 95<sup>th</sup> percentile As concentration (i.e., 108.9 mg As  $\text{kg}^{-1}$  soil) was used following the determination of representative soil concentration procedure. Table 5.1 showed the physicochemical properties and concentrations of the target pollutant (As).

Table 5.1 physicochemical properties and concentrations of the target pollutant

Properties	Unit	Arsenic
Molecular weight	$\text{g mol}^{-1}$	74.82
Melting point	$^{\circ}\text{C}$	816.85
Density	$\text{g cm}^{-3}$	5.727

## 5.4 Receptor identification

The study area for the implementation of phytoremediation is a rural forest area, which is inhabited by a variety of animal and plant species. Ecological risk was calculated for five different ecological receptor groups; terrestrial invertebrate, avian insectivore (bird), small mammal, herbivore, and omnivore. Representative species for each group were selected based on the possibility of ecological habitation in the study area and the availability of ecological data for risk calculation. The selected species include earthworm (*Eisenia fetida*) for terrestrial invertebrate, shorebird (*Scolopax minor*) for avian insectivore, meadow vole (*Microtus pennsylvanicus*) for small mammal, water deer (*Hydropotes inermis argyropus*) for herbivore, and wild pig (*Sus scrofa*) for omnivore. The basic ecological characteristics of the target receptors are presented in Table 5.2.

Table 5.2 Basic ecological characteristics of the target organisms

Characteristics	Terrestrial invertebrates	Avian earthworms	Small mammal Shorebird Meadow vole	Herbivores Water deer	Omnivores Wild pig
Body weight (kg)	-	0.12-0.24	0.033- 0.065	9-14	100-300
Food composition	Soil organic matter	Insects	Foliage	Foliage	Insects, small mammal, and roots of plants
Home range (ha)	-	15-74	0.9	21	400

## 5.5 Characterization of exposure

A simplified exposure pathway based on the trophic level of each receptor group was proposed, as shown in Figure 5.2. It was hypothesized that soil ingestion was a valid exposure pathway for terrestrial invertebrate, avian insecticide, small mammal, herbivore, and omnivore and plant tissues were consumed (i.e., food ingestion) by small mammal, herbivore, and omnivore. Receptor group-specific pathways of exposure to soil contaminants for this ecological risk assessment are as follows; soil ingestion for terrestrial invertebrate, soil ingestion and food ingestion for avian insectivore, herbivore, and omnivore. Exposure pathways related to water ingestion were not considered because As was not detected in the surface water and pore water of the area. The concentration of As in soil during the phytoremediation was assumed to be constant (although the concentration should be decreased by plant uptake), being 108.9 mg As kg<sup>-1</sup> soil from which the As concentration accumulated in the lowest trophic level (i.e., terrestrial invertebrate) was estimated. The following regression equation was used to estimate the accumulated concentration of As in earthworm (Sample et al., 1999; USEPA, 2007).

$$\ln(C_{\text{invertebrate}}) = 0.706 \times \ln(C_s) - 1.421 \quad (1)$$

, where,  $C_{\text{invertebrate}}$  is the As concentration in earthworm (mg As kg<sup>-1</sup> DW), and  $C_s$  is the As concentration in soil (mg As kg<sup>-1</sup> soil).

The amounts of exposure for the higher trophic level receptors (i.e., shorebird, meadow vole, water deer, and wild pig) in each pathway were determined with the Eq. 2 using the species-specific ecological parameters such as ingestion rate, food

composition, and home range. Worst-case exposure scenarios were assumed. The absorbed fraction of As in each receptor assumed as 100% and *Pteris cretica* was the only plant source consumed by small mammal, herbivore, and omnivore.

Exposure dose (mg-As/kg-DW/day)

$$= ([\text{Soil}_j \times P_s \times \text{FIR} \times \text{AF}_{js}] + [\sum B_{ij} \times P_i \times \text{FIR} \times \text{Af}_{ij}]) \times \text{AUF} \quad (2)$$

, where,  $\text{soil}_j$  is the concentration of contaminant (j) in soil ( $\text{mg As kg}^{-1}$  soil),  $P_s$  is the soil ingestion as proportion of diet, FIR is the food ingestion rate ( $\text{kg food kg}^{-1} \text{BW day}^{-1}$ ),  $\text{AF}_{js}$  is the absorbed fraction of contaminant (j) from soil (s),  $B_{ij}$  is the concentration of contaminant (j) in biota type (j) ( $\text{mg kg}^{-1} \text{DW}$ ),  $P_i$  is the proportion of biota type (i) in diet,  $\text{Af}_{ij}$  is the absorbed fraction of contaminant (j) from biota type (i), and AUF is the area use factor, which is calculated by the site acreage ( $A_{\text{site}}$ )/home range of species (HR). When  $A_{\text{site}}/\text{HR}$  is over 1 it is considered to be 1. The  $\text{Af}_{ij}$  and  $\text{AF}_{js}$  are always considered to be 1.

For water deer (*Hydropotes inermis argyropus*) and wild pig (*Sus scrofa*), food ingestion rates (FIR) are not readily available in the literature. Hence, the following equations (Nagy, 1987) were used to derive FIR:

$$\text{For herbivores (water deer):} \quad \text{FIR} = 0.0875(\text{BW})^{0.727} \quad (3)$$

$$\text{For omnivores (wild pig):} \quad \text{FIR} = 0.0687(\text{BW})^{0.822} \quad (4)$$

For soil ingestion by wildlife, Beyer et al. (1994) reported soil consumption estimates for 28 wildlife species and found that soil ingestion by wildlife did not exceed 2.0% of total diet. In this study, 2.0% of soil ingestion ( $P_s$ ) was adapted for

conservative estimation. The data for home range (HR) were also available. HR for *Hydropotes inermis argyropus* and *Sus scrofa* was reported to be about 210,000 and 4,000,000 m<sup>2</sup>, respectively (Harestad and Bunnell, 1979; Lee, 2003). Water ingestion was not considered as an exposure pathway because As was not detected in surface water and pore water at the study site. The exposure parameters and assumptions for calculating exposure doses are summarized in Table 5.3. With the data, exposure doses for the five ecological receptor groups during the phytoremediation (including the siderophores application practice) were derived for corresponding exposure pathways and presented in Table 5.4.

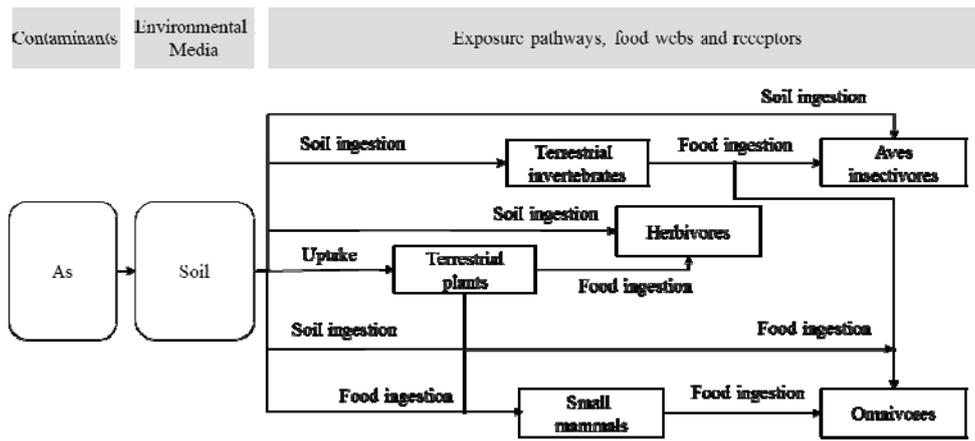


Figure 5.2 Simplified exposure pathways and food webs of the ecological receptors

Table 5.3 The exposure parameters and assumptions for calculating exposure doses for each organisms

Parameter	Unit	Value	Assumptions and sources
Terrestrial invertebrate			
Cs	mg As kg <sup>-1</sup>	108.9	95 <sup>th</sup> percentile
Avian insectivore			
Soil <sub>j</sub>	mg As kg <sup>-1</sup>	108.9	95 <sup>th</sup> percentile
B <sub>ij</sub>	mg As kg <sup>-1</sup>	-	Diet is assumed to be 100% earthworms
Ps	kg soil kg <sup>-1</sup> food	0.164	USEPA, 2007
Pi	kg food kg <sup>-1</sup> food	0.836	-
FIR	kg food kg <sup>-1</sup> BW day <sup>-1</sup>	0.214	USEPA, 2007
AUF	unitless	0.05	Animal Diversity Web
Small mammal			
Soil <sub>j</sub>	mg As kg <sup>-1</sup>	108.9	95 <sup>th</sup> percentile
B <sub>ij</sub>	mg As kg <sup>-1</sup>	-	Using the experimental data from Table 1 Diet is assumed to be 100% foliage
Ps	kg soil kg <sup>-1</sup> food	0.032	USEPA, 2007
Pi	kg food kg <sup>-1</sup> food	0.968	-
FIR	kg food kg <sup>-1</sup> BW day <sup>-1</sup>	0.0875	USEPA, 2007
AUF	unitless	0.833	USDA Forest service
Herbivore			

Soil <sub>j</sub>	mg As kg <sup>-1</sup>	108.9	95 <sup>th</sup> percentile
B <sub>ij</sub>	mg As kg <sup>-1</sup>	-	Using the experimental data from Table 1 Diet is assumed to be 100% foliage
Ps	kg soil kg <sup>-1</sup> food	0.02	Beyer et al., 1994
Pi	kg food kg <sup>-1</sup> food	0.98	-
FIR	kg food kg <sup>-1</sup> BW day <sup>-1</sup>	0.467	Nagy, 1987
AUF	unitless	0.036	Lee, 2003
Omnivore			
Soil <sub>j</sub>	mg As kg <sup>-1</sup>	108.9	95 <sup>th</sup> percentile
B <sub>ij</sub>	mg As kg <sup>-1</sup>	B <sub>11</sub> -	Plant roots Using the experimental data from Table 1
		B <sub>21</sub> -	Earthworm
		B <sub>31</sub> -	Small mammal
Ps	kg soil kg <sup>-1</sup> food	0.053	Harestad and Bunnell, 1979
Pi	kg soil kg <sup>-1</sup> food	P <sub>1</sub> 0.847	Plant roots
		P <sub>2</sub> 0.09	Earthworm
		P <sub>3</sub> 0.053	Small mammal
FIR	kg food kg <sup>-1</sup> BW day <sup>-1</sup>	5.351	Nagy, 1987
AUF	unitless	0.002	Harestad and Bunnell, 1979

Table 5.4 Calculated exposure dose of the target pollutants to the target organisms during phytoremediation

Receptors	Exposure pathway	Unit	Without siderophores			With siderophores		
			3 week	5 week	7 week	3 week	5 week	7 week
Terrestrial invertebrate	Earthworm ( <i>Eisenia fetida</i> )	Soil ingestion mg As kg <sup>-1</sup> DW	6.62	6.62	6.62	6.62	6.62	6.62
Avian insectivore	Shorebird ( <i>Scolopax minor</i> )	Soil ingestion mg As kg <sup>-1</sup>	0.19	0.19	0.19	0.19	0.19	0.19
		Food ingestion BW day <sup>-1</sup>	0.06	0.06	0.06	0.06	0.06	0.06
Small mammal	Meadow vole ( <i>Microtus pennsylvanicus</i> )	Soil ingestion mg As kg <sup>-1</sup>	0.25	0.25	0.25	0.25	0.25	0.25
		Food ingestion BW day <sup>-1</sup>	4.12	30.92	40.97	2.17	32.90	210.04
Herbivore	Water deer ( <i>Hydropotes inermis argyropus</i> )	Soil ingestion mg As kg <sup>-1</sup>	0.04	0.04	0.04	0.04	0.04	0.04
		Food ingestion BW day <sup>-1</sup>	0.95	7.15	9.48	0.50	7.61	48.60
Omnivore	Wild pig ( <i>Sus scrofa</i> )	Soil ingestion mg As kg <sup>-1</sup>	0.06	0.06	0.06	0.06	0.06	0.06
		Food ingestion BW day <sup>-1</sup>	6.63	7.48	5.19	6.62	10.85	14.16

## 5.6 Characterization of ecological effects

For avian insectivore, small mammal, and mammalian herbivore, USEPA presents conservative toxicological reference values (TRVs) of As (USEPA, 2005a; USEPA, 2005b). The mammalian omnivore is classified as terrestrial mammal like herbivore and thus the same TRV was provided by USEPA. However, a specific TRV for *Sus scrofa*, a representative species for mammalian omnivore used in this study, is available (Morrison and Chavez, 1983) and thus  $9.44 \text{ mg kg}^{-1} \text{ BW day}^{-1}$  was used. For soil invertebrate, USEPA does not provide the TRVs and thus the soil toxicological benchmark for As used in Korea (i.e.,  $20 \text{ mg As kg}^{-1} \text{ soil}$ ) was adapted (MOE, 2004). The determined TRVs for As are summarized in Table 5.5.

Table 5.5 Toxicological reference values (TRVs) of the target pollutants to the target organisms

Receptor group		TRVs	Unit	Reference
Terrestrial invertebrates	Earthworm ( <i>Eisenia fetida</i> )	20	mg-As/kg- DW	MOE (2004)
Avian insectivores	Shorebird ( <i>Scolopax minor</i> )	2.24	mg-As/kg- BW/day	USEPA, 2005a
Small mammal	Meadow vole ( <i>Microtus pennsylvanicus</i> )	1.04	mg-As/kg- BW/day	USEPA, 2005a
Herbivores	Water deer ( <i>Hydropotes inermis argyropus</i> )	1.66	mg-As/kg- BW/day	USEPA, 2005a
Omnivores	Wild pig ( <i>Sus scrofa</i> )	9.44	mg-As/kg- BW/day	Morrison and Chavez, 1983

## 5.7 Risk characterization

With the exposure dose ( $E_{A,i}$ ) and the toxicity reference value ( $TRV_A$ ) through exposure and toxicity assessment, the ecological hazard quotient ( $EHQ_{A,i}$ ) and the ecological hazard index ( $EHI_A$ ) of species A through exposure pathway i can be derived from the following equations:

$$EHQ_{A,i} = \frac{E_{A,i}}{TRV_A} \quad (5)$$

$$EHI = \sum_i EHQ_{A,i} \quad (6)$$

The possibility of risk is observed when the EHQ or the EHI value exceeds 1.0, meaning that the receptor is exposed to a contaminant at a dose exceeding the toxicological benchmark.

## 5.8 Results and Discussion

The ecological hazard quotient (EHQ) and ecological hazard index (EHI) of As to the target receptors under the phytoremediation conditions tested (i.e., normal vs. siderophore-applied) are presented in Table 5.6. All EHI values for small mammal *Microtus pennsylvanicus* exceeded 1.0 during phytoremediation regardless of siderophores application, suggesting the possibility of adverse health effect in small mammal at the test site due to the uptake of As-contaminated soil and food ingestion in their habitat. At normal phytoremediation condition, EHI values of all target receptors except for small mammal were below 1.0 at three week, meaning that no potential ecological risk was posed. However, the EHI value of mammalian herbivore (i.e., the EHI value of 0.60 at three week) increased with the prolonged phytoremediation, being 4.33 and 5.73 at five and seven week, respectively. When siderophores were applied to enhance phytoremediation efficiency the ecological risk of mammalian omnivore also increased. The EHI value of mammalian omnivore was 0.71 at three week, but the value increased to 1.16 and 1.51 at five and seven week, respectively. The same trend was observed in mammalian herbivore: EHI values increased from 0.32 at three week to 4.64 and 29.30 at five and seven week, respectively. For soil invertebrate, avian insectivore and mammalian omnivore, all EHI values were always less than 1.0 whether or not siderophores were added.

Because siderophores could enhance As uptake in plant and further translocate As from roots to shoot (i.e., stem and leaves) during the phytoremediation process, it is likely that the application of siderophores during phytoremediation affects the ecological risk in the receptors who mainly consume plant foliage. As a

consequence, higher ecological risk was observed in small mammal and herbivore with phytoremediation time and siderophores application. Data in Table 6 showed that food ingestion contributed more significantly to ecological risk than soil ingestion, especially in the two receptor groups. In contrast, the ecological risk in omnivore that consumes plant roots was slightly changed.

Home range is an important factor in determining ecological risk. The EHI values in small mammal were 4.21 and 2.33 in both conditions at three week (i.e., even before the translocation of As from roots to stems and leaves) due to its limited home range of the receptor (Table 2) and thus increased chance of ingestion of hyperaccumulated plant tissues. Whereas, herbivore (*Hydropotes inermis argyropus*) that has larger home range can simply move to other locations and thus may have a limited chance of exposure to the pollutant at the site.

Uncertainties exist at each step of the ecological risk assessment procedures. In the receptor identification process, we assumed the potentially representative species for small mammal (*Microtus pennsylvanicus*) and avian insectivore (*Scolopax minor*) considering the ecological environment and land use of the study area. Site-specific ecological survey at the study site would reduce such uncertainty. The bioavailability factor ( $AF_{ij}$ ) was assumed to be 1.0 (i.e., 100% of the ingested As was bioavailable) for the estimation of accumulated As concentration in avian and mammal, but this assumption definitely resulted in overestimation. Also, when the exposure dose through food ingestion was calculated, the food composition was assumed to be 100% of earthworm for *Scolopax minor* and 100% of As-absorbed plant (*Pteris cretica*) for *Microtus pennsylvanicus* and *Hydropotes inermis argyropus*. This reduced the site-specificity of the assessment and expected to overestimate ecological risk by food ingestion. Most of the toxicity reference

values (TRVs) were derived from the dose-response relationship of some frequently tested species in each receptor group including rats and mice for mammals and chickens for birds. This may also produce uncertainty. The most accurate means to reduce such uncertainties is to conduct an extensive investigation on the ecosystem of the site and to obtain species-specific data from the experiments on target species and site environment (USEPA, 1998).

Our assessment results suggest that phytoremediation which accumulates As in plant may pose ecological risk to small mammal and herbivore that consume the As metal-containing plant foliage at the study site, and the risk would be greater when an enhanced phytoremediation practice (i.e., siderophores addition in this case) is applied because of the translocation and thus higher accumulation of As. A sensitivity analysis to find the most affecting factors to ecotoxicological risk was performed, then showing the consumption ratio of hyperaccumulated plant tissue is critical factor in this study. If the consumption ratio of hyperaccumulated plant tissue reduced to less than 20% of total food consumption, there are almost no ecotoxicological risk through food chain in this study, especially the consumption of hyperaccumulated plant tissue.

Although heavy metals-accumulated plants are relatively unpalatable probably due to the high metal content (Angel and Linacre, 2005) the likelihood of significant consumption exists. Thus, some measures to reduce the ingestion of hyperaccumulated plant tissues may be needed during phytoremediation. Fencing, netting, deterrents such as periodic noise, and the planting of offensive plant species can be used to prevent receptors from coming in contact with hyperaccumulators (ITRC, 1997; Angel and Linacre, 2005). In addition, when phytoremediation strategy is considered for metals remediation, its ecological

consequence should be taken into account to prevent the spread of accumulated metals through the food chain of ecological receptors.

Table 5.6 Calculated ecological hazard quotients (EHQs) and indices (EHIs) of arsenic to the target organisms during phytoremediation

Receptors		Exposure pathway	Without siderophores			With siderophores		
			3 week	5 week	7 week	3 week	5 week	7 week
Terrestrial	Earthworm	Soil ingestion	0.33	0.33	0.33	0.33	0.33	0.33
invertebrate	( <i>Eisenia fetida</i> )	EHI	0.33	0.33	0.33	0.33	0.33	0.33
Avian insectivore	Shorebird ( <i>Scolopax minor</i> )	Soil ingestion	0.09	0.09	0.09	0.09	0.09	0.09
		Food ingestion	0.03	0.03	0.03	0.03	0.03	0.03
		EHI	0.12	0.12	0.12	0.12	0.12	0.12
Small mammal	Meadow vole ( <i>Microtus pennsylvanicus</i> )	Soil ingestion	0.24	0.24	0.24	0.24	0.24	0.24
		Food ingestion	3.96	29.73	39.40	2.09	31.63	201.96
		EHI	<b>4.21</b>	<b>29.97</b>	<b>39.64</b>	<b>2.33</b>	<b>31.88</b>	<b>202.21</b>
Herbivore	Water deer ( <i>Hydropotes inermis argyropus</i> )	Soil ingestion	0.02	0.02	0.02	0.02	0.02	0.02
		Food ingestion	0.57	4.31	5.71	0.30	4.59	29.28
		EHI	0.60	<b>4.33</b>	<b>5.73</b>	0.32	<b>4.61</b>	<b>29.30</b>
Omnivore	Wild pig ( <i>Sus scrofa</i> )	Soil ingestion	0.01	0.01	0.01	0.01	0.01	0.01
		Food ingestion	0.70	0.79	0.55	0.70	1.15	1.50
		EHI	0.71	0.80	0.56	0.71	<b>1.16</b>	<b>1.51</b>

## 5.9 Summary

The data showed that the potential risks of small mammal and mammalian herbivore to arsenic were significant during phytoremediation. Ecological risk of small mammal always exceed ecological benchmark (1.0) because of its small home range of the organisms compared to herbivores, and omnivores. The EHI value of mammalian herbivore, who mainly consume plant foliage, greatly increased when the phytoremediation efficiency was increased by the application of microbial culture containing siderophores. Such an additional potential risk was probably due to translocation of arsenic by siderophores from roots to shoots (i.e., stems and leaves) in plant tissue. Our results suggest that the field application of hyper-accumulating plants of heavy metals may result in ecological problems through food chain, especially in small mammal and mammalian herbivore. There were several uncertainties identified in the ecological risk assessment framework mainly due to the deficiency in exposure parameters and ecological and toxicological data of the species and chemicals studied. It suggests that a more exhaustive database and reasonable risk characterization tools for the case of limited information are required for a more site-specific and reliable assessment. Relatively little is known about environmental effects in implementation of phytoremediation. This study of the environmental consideration of phytoremediation may give some insight into the will result in a better understanding of the ecological relationships.

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## CHAPTER 6

### CONCLUSIONS

This dissertation has reported an interaction among plants-microbes-heavy metals, its application to phytoremediation for enhancing the removal efficiency, and also conducted the ecological risk assessment during phytoremediation for ecological consideration. Most previous studies about microbe assisted-phytoremediation have been based on screening the hyperaccumulator and plant growth promoting bacteria, and also investigated only its plant growth promoting effect for phytoremediation. However, this study have been focused on the changes of heavy metal bioavailability in rhizosphere, represented by chemical form, and resulting the enhancement of removal efficiency during phytoremediation. And ecological consideration of surrounding ecological receptor have never been reported during phytoremediation.

Successful phytoremediation mainly depends on the plant growth promotion as well as the bioavailability of heavy metals to plants in soils. Especially, microbe-assisted phytoremediation also was assessed the survival or adaptation of introduced bacteria in soils. In this study, two PGPRs (i.e., phosphate-solubilizing bacteria and siderophore-producing bacteria) were used to phytoremediation for the heavy metal (i.e., cadmium and arsenic) removal from soil. *Bacillus* sp. (i.e., a mixed culture of *B. aryabhattai* and *B. megaterium*) as phosphate-solubilizing bacteria and siderophore secreted by *Pseudomonas aeruginosa* as siderophore-producing bacteria can be investigated its ability. Inoculation with *Bacillus* sp. showed a significant increase of water-soluble phosphate in the medium containing

tricalcium phosphate (TCP) and soil. And when a siderophore secreted by *Pseudomonas aeruginosa* was introduced into Fe-oxide (i.e., ferrihydrite), siderophore can increase the release of iron from ferrihydrite by formation of siderophore-iron complexes. From these results, two PGPRs can transform phosphate and iron into easily plant-uptakable forms in soil, and thus promote plant growth by facilitating nutrient such as phosphorous and iron.

These two PGPRs can significantly enhance the Cd and As uptake efficiency by *Brassica juncea* and *Pteris cretica* from contaminated soils, respectively, probably due to changes of heavy metals chemical forms in soil. The introduction of *Bacillus sp.* as PSB can lead to solubilize P as well as increase the bioavailable Cd to plants (i.e., exchangeable fraction) by secreting the organic acids. Siderophore also can transform As into easily plant-uptakable forms in soil (i.e., non-specifically bound forms) by siderophore-As complex formation. Once released, the As seemed to be present as siderophore-As complexes, which were absorbed to roots and further transported to stems and leaves. These mechanisms increased the mobility, as well as the phytoavailability, of heavy metals in soils, and enhanced the total accumulation in plants. Another question is the soil microbial community changes by the interaction between introduced bacteria and indigenous soil bacteria during phytoremediation. In this study, the analysis of microbial community change by inoculation of *Bacillus sp.* through pyrosequencing, was seemed to take time of at least 8 weeks to function by itself, and the survival and adaptation of introduced bacteria corresponded directly with the plant growth and Cd uptake.

During phytoremediation of As-contaminated soils, ecological risk assessment identified the potential risk of mainly small mammal and mammalian herbivore

which consumed plant foliage. The uncertainties also identified mainly due to the deficiency in exposure parameters and ecological and toxicological data of the species and chemicals studied. Our results demonstrate that the field application of hyper-accumulating plants of heavy metals may result in ecological problems through food chain, thus its ecological consequence should be considered in phytoremediation strategy. It also suggests that a more exhaustive database and reasonable risk characterization tools for the case of limited information are required for a more site-specific and reliable assessment.

These findings in this study may provide the information on the useful understanding of plant-heavy metal-bacteria interactions as well as the microbial processes and their activity of introduced bacteria in soil that is required for effective bacteria-associated phytoremediation, and give some insight into the will result in a better understanding of the ecological relationships. Based on these results, the following schematic tree was recommended for bettering understanding of phytoremediation process integrating the efficiency and environmental concerns (Figure 6.1).

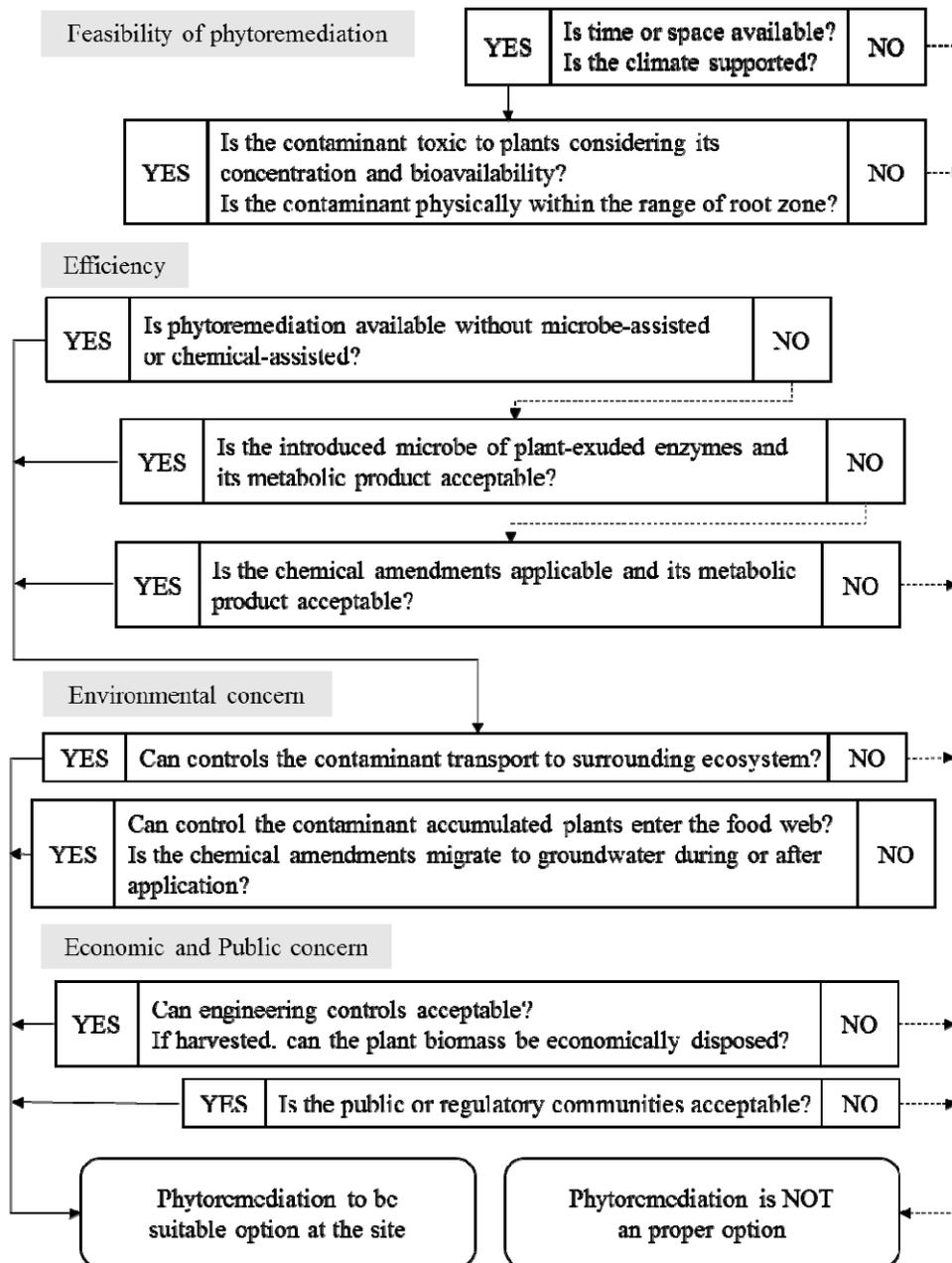


Figure 6.1 Schematic decision tree for phytoremediation integrating the efficiency, environmental, and economical concerns

## 국문초록

### 근권 미생물에 의한 카드뮴과 비소의 생물학적 이용성 향상을 통한 식물상 정화공법의 효율 증진 및 생태독성학적 영향에 관한 연구

최근 식물을 이용하여 토양 내 중금속을 제거하는 식물상 정화공법이 각광받으면서 근권 미생물 활성화에 의한 식물의 중금속 섭취 효율 향상에 대한 연구가 증가하고 있다. 특히, 중금속 섭취 효율 향상을 위해 식물 성장 촉진 미생물을 이용하여 토양으로부터 영양원소 섭취를 용이하도록 하여 식물 성장을 촉진시킬 뿐 아니라 중금속의 식물에의 이용성을 변화시켜 식물로의 중금속 섭취 효율을 증가시키고자 하는 연구가 활발하다. 본 연구에서는 식물 성장 촉진 미생물의 일종인 인산염 가용화 미생물과 사이드로포어 분비 미생물을 적용하여 카드뮴과 비소의 제거 효율 변화를 관찰하였다.

인산염 가용화 미생물로 사용한 *Bacillus* sp. 의 접종으로 인한 시간에 따른 식물 성장 촉진과 식물체 내 카드뮴 흡수 효율 향상 효과가 확인되었다. 식물체 (*Brassica juncea*, 갓)는 시간이 지남에 따라 생체량이 증가하였고, 8주 후에 미생물을 주입한 경우 295.6 mg, 주입하지 않은 경우에 65.8 mg으로 초기 생체량 (10.3 mg)에 비해 확연한 증가를 확인할 수 있었다. 또한, 식물체 내 카드뮴 농도는 초기 0.86 mg/g of plant에서 6주부터 점차 증가하기 시작하여 (2.20 mg/g of plant) 8주 후 미생물을

주입한 경우와 그렇지 않은 경우 각각 2.96과 1.20 mg/g of plant의 카드뮴이 검출되어 카드뮴 흡수 향상 효과를 확인할 수 있었다. 그리고 Tessier의 5단계 추출법을 사용하여 미생물 주입에 따른 카드뮴의 존재형태변화를 살펴본 결과 미생물 주입에 따라 상대적으로 생물학적 이용성이 높다고 사료되는 exchangeable phase 형태가 증가하였다. 이는 미생물이 유기산의 일종인 IAA (Indole-3-acetic acid)를 분비하여 토양 pH가 감소하였기 때문이라고 판단되며 실제로 토양 내 IAA농도의 증가와 이에 따른 pH의 감소를 확인하였다. 한편, 파이로시퀀싱을 이용하여 인산염 가용화 미생물 주입에 의한 미생물 군집 변화를 관찰한 결과 시간이 지남에 따라 Bacilli 강 (class)의 토양 내 우점율이 증가하였고 이는 주입된 미생물이 8주가 지난 후에 안정적으로 토양 내에 적응하였고, 토양에 인공적으로 주입한 미생물이 토착 미생물과 경쟁하여 생태적 지위를 가지고 본연의 역할을 수행하기까지 시간이 필요함을 의미한다.

식물 성장 촉진 미생물 중 하나인 사이드로포어 분비 미생물은 사이드로포어라는 철이온-특이결합화합물을 체외로 분비하여 철 결핍 환경에서 식물체로의 철 공급을 조절하여 식물생장을 촉진한다. 사이드로포어 분비 미생물로 *Pseudomonas aeruginosa*를 선정하여 사이드로포어만을 미생물이 제거된 상등액의 형태로 주입하였으며, 대표적인 비소 축적 식물인 큰 봉의 꼬리 (*Pteris cretica*)를 이용하여 실험을 수행하였다. 사이드로포어를 주입한 경우, 식재 후 5 주까지 지속적으로 생체량이 증가하여 최대 5.83 g의 생체량을 나타내어 초기

생체량 1.47 g에 비해 식물 생장이 촉진되었음을 알 수 있었다. 5 주 이후에는 생체량이 다소 감소하였지만 10 주 후에 4.90 g으로 나타나 대조군 (1.91 g)이나 EDTA 주입군 (1.70 g)에 비해 확연한 생체량 증가를 확인할 수 있었다. 한편, Wenzel의 5단계 추출법을 통한 존재형태 분석 결과, 대조군의 경우 토양 내 비소가 비특이적 결합, 특이적 결합, 비결정질 철산화물 결합 형태가 각각 2, 27, 47%의 비율로 존재하였다. 하지만 사이드로포어 주입으로 인해 대조군에 비해 비결정질 철산화물 결합 형태의 비율이 감소하고 (40%), 상대적으로 생물학적 이용성이 높다고 사료되는 비특이적 결합 형태의 비소의 비율이 증가하였다 (14.7%). 반면, EDTA 주입군의 경우 사이드로포어 주입의 경우와 마찬가지로 비결정질 철산화물 결합 형태의 비소의 비율이 감소하였지만 (38%), 특이적 결합 형태의 비소의 비율이 증가하였다 (35.2%). 결정질 철산화물 결합과 잔류형태의 비소의 비율은 모든 실험에서 큰 변화가 관찰되지 않았다. 이는 비결정질 철산화물과 결합한 비소가 사이드로포어와 EDTA에 의해 동일하게 용출되었지만, EDTA와 용출된 비소는 복합체 형성을 하지 못하여 용출된 비소의 재흡착이 이루어진 반면, 사이드로포어는 비소와의 복합체 형성을 통해 비특이적 결합형태의 비소로 존재하여 비소의 생물학적 이용성이 증가하였음을 의미한다. 실제로 식물체 내 비소 축적 농도를 확인한 결과, 사이드로포어 주입의 경우 식물체 내 비소 농도는 초기 0.008 mg/g of plant에서 지속적으로 증가하기 시작하여 10주 후 5.62 mg/g of plant의 비소가 축적되어, 대조군 (1.51 mg/g of plant)과 EDTA 주입군 (1.76 mg/g of

plant)에 비해 식물체의 비소 축적능이 월등히 증가하였음을 알 수 있었다. 또한, 주목할만한 점은 대조군과 EDTA 주입군의 경우 흡수된 비소가 대부분 뿌리 (39-70%)에 축적된 반면, 사이드로포어 주입군의 경우 시간이 지날수록 지상부 (줄기와 잎)로의 이동이 증가하여 10 주에는 식물체 내 흡수된 비소의 79%가 잎에 축적되었다. 이러한 식물체 내 흡수된 비소의 지상부로 이동은 사이드로포어-비소 복합체 형성에 의한 것으로 사료된다. CAS liquid assay 결과, 7, 10 주에 사이드로포어 주입 식물체의 잎에서 대조군과 EDTA 주입군에 비해 상대적인 흡광도가 매우 강하게 나타나 사이드로포어의 존재를 확인하였다. 또한, 상대적인 흡광도의 세기와 식물체 내 축적된 비소의 농도가 유사한 경향으로 나타나 흡수된 비소가 사이드로포어-비소 복합체의 형태일 가능성을 확인하였다. 식물체 내 사이드로포어는 형광 현미경을 통해서도 뿌리와 잎에서 사이드로포어 특유의 형광이 관찰되었다. CAS liquid assay와 형광 현미경 관찰 결과를 통해 사이드로포어-비소 복합체 형성 및 흡수뿐 아니라 흡수된 사이드로포어-복합체의 식물체 내 이동을 간접적으로 확인할 수 있었다.

식물상 정화공법이 수행되는 동안 중금속이 축적된 식물을 섭취함으로써 주변 생태계에 서식하는 수용체에 미치는 잠재적인 위해도를 확인하기 위해 토양생태위해성평가를 수행하였다. 평가 지역은 비소 오염 지역으로 사이드로포어가 적용된 고사리를 이용한 식물상 정화공법이 수행됨을 가정하고, 사이드로포어 적용 전, 후로 구분하여 평가를 실시하였다. 평가 대상 종으로는 토양 무척추 동물로 지렁이,

조류는 도요새, 작은 포유류로는 목초지 들쥐, 초식성 동물로 고라니, 잡식성 동물로 멧돼지를 선정하였다. 본 연구 결과, 사이드로포어 적용여부에 관계없이 모든 경우에 작은 포유류는 생태 위해 가능성이 있는 것으로 나타났다. 이는 서식지 면적이 다른 포유류에 비해 작아 중금속이 함유된 고사리를 섭취할 확률이 높기 때문이다. 주로 식물을 소비하는 초식성 동물의 경우 사이드로포어를 적용했을 때 생태 위해성이 급격히 증가하였는데 이는 사이드로포어에 의한 식물 뿌리에서 지상부로의 비소 이동 영향 때문으로 판단된다. 따라서 토양 정화공법으로 식물상 정화공법을 선정할 경우 중금속 함유 식물체에 의한 생태 영향을 반드시 고려해야 한다. 생태독성학적 위해성이 발생할 개연성이 있을 경우 생태계 수용체들의 중금속 함유 식물체로의 접근 제한을 위한 물리적인 조치가 정화설계 단계에서 반영되어야 한다. 본 토양생태위해성평가는 평가중에 대한 생태학적 자료의 부족에 기인한 많은 불확실성을 포함하고 있다. 따라서 보다 정확하고, 안전한 생태위해성평가를 수행하기 위해서 이에 대한 연구가 지속적으로 이루어져야 할 것이다.

**주요어:** 식물생장촉진미생물, 식물상 정화공법, 카드뮴, 비소  
인산염가용화미생물, 사이드로포어, 생태위해성평가

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