TREATMENT OF LEAD-CONTAMINATED SOIL BY WETLAND PLANTS: THE HYDROPONIC AND BATCH STUDIES

KUNAPORN HOMYOG

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Thesis
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Kunaporn Homyog
.....
Miss Kunaporn Homyog
Candidate

.....
Assoc. Prof. Prayad Pokethitiyook, Ph.D.
Major-Advisor

M. Kruatrachue
.....
Prof. Maleeya Kruatrachue, Ph.D.
Co-Advisor

Rattanawat
.....
Assist. Prof. Rattanawat Chaiyarat, Ph.D.
Co-Advisor

B. Mahaisavariya
.....
Prof. Banchong Mahaisavariya, M.D. Ph.D.
Dean
Faculty of Graduate Studies

.....
Assoc. Prof. Prayad Pokethitiyook, Ph.D.
Chair
Doctor of Philosophy Program in Biology
Faculty of Science

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April 11, 2008

Kunaporn Homyog
.....
Miss Kunaporn Homyog
Candidate

Thanawan Panich-pat
.....
Assist. Prof. Thanawan Panich-pat, Ph.D.
Chair

.....
Assoc. Prof. Prayad Pokethitiyook, Ph.D.
Member

M. Kruatrachue
.....
Prof. Maleeya Kruatrachue, Ph.D.
Member

.....
Assist. Prof. Rattanawat Chaiyarat, Ph.D.
Member

B. Mahaisavariya
.....
Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof. Skorn Mongkolsuk, Ph.D.
Dean
Faculty of Science
Mahidol University

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Kunaporn Homyog

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KUNAPORN HOMYOG 4637815 SCBI/D

Ph.D. (BIOLOGY)

THESIS ADVISORS: PRAYAD POKETHITIYOOK, Ph.D., MALEEYA KRUATRACHUE, Ph.D., RATTANAWAT CHAIYARAT, Ph.D.

ABSTRACT

Seasonal and spatial variations in Pb concentrations in soils, plants, and Pb tolerant-plant species diversity, were studied in two different sampling sites at an open pit mine area, the pond site (PS) and land site (LS). Pb content in soil and plants was seasonally dependent. The lowest Pb concentrations in soils were found in July (0.6%) and highest in October (>11%). Most plants had the highest Pb content during May to October (wet season) and the lowest during November to April (dry season). Most plants were perennials with some annuals. Twelve species were common at both sampling sites. There were a total of 17 plant species that had Pb accumulation in shoots >1,000 mg/kg, however, only six species: *Ageratum conyzoides*, *Buddleja asiatica*, *Chromolaena odoratum*, *Conyza sumatrensis*, *Mimosa pudica* and *Sonchus arvensis*, showed a translocation factor >1.

Under a 15-day hydroponic condition, the accumulation efficiency of three metals (Pb, Cd, Zn) by *C. iria* and *C. flavidus* was compared. Pb was the metal which was most accumulated in the roots (38,000 mg/kg and 13,666.7 mg/kg in *C. iria* and *C. flavidus*, respectively). Glutathione (GSH) and phytochelatin (PC_2 and PC_3) synthesis was the response of plants to metal toxicity. The contents changes in *C. iria* were studied by varying concentration of Pb, Cd, and Zn in the solution for 8 days. Responds corresponded to Cd>Zn>Pb. The GSH content was detected in shoots>roots (1.92>0.82 mmol/mg), whereas the PC content was detected in roots>shoots (56.04>53.19 and 0.86>0.68 mmol/mg for PC_2 and PC_3, respectively). The maximum GSH and PC contents were detected between day 2 to day 4 and gradually decreased.

For batch study, *Cyperus iria* and *Typha angustifolia* grew well in the Pb contaminated soil for 3 months. New tillers and flowers were produced in *C. iria* but not in *T. angustifolia*. The highest Pb accumulation in roots of *C. iria* and *T. angustifolia* were 4,550 and 6,383 mg/kg DW, respectively. The shallow roots of *C. iria* were able to remove Pb from the surface soil, whereas the long deep-roots of *T. angustifolia* removed Pb from the subsurface soil. Although *C. iria* is not a hyperaccumulator plant, it has a good potential for use in the phytostabilization process in a constructed wetland system.

KEY WORDS: SEASONAL VARIATION/ ACCUMULATION/ GLUTATHIONE/ PHYTOCHELATIN/ PHYTOSTABILIZATION

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การบำบัดดินที่ปนเปื้อนด้วยสารตะกั่วโดยใช้พืชในพื้นที่ชุ่มน้ำ (TREATMENT OF LEAD-CONTAMINATED SOIL BY WETLAND PLANTS: THE HYDROPONIC AND BATCH STUDIES)

คุณาภรณ์ หอมยก 4637815 SCBI/D
ปรด. (ชีววิทยา)

คณะกรรมการควบคุมวิทยานิพนธ์: ประหยัด โภคฐิติยุกต์, Ph.D., มาลียา เครือตราชู, Ph.D., รัตนวัฒน์ ไชยรัตน์, Ph.D.

บทคัดย่อ

จากการศึกษาความผันแปรของฤดูกาลที่มีผลต่อความเข้มข้นของสารตะกั่วในดินพืชและความหลากหลายของพรรณพืชเป็นเวลา 1 ปีในเหมืองตะกั่วบ่องาม ประเทศไทย 2 บริเวณ คือ ขุมเหมืองที่ติดกับแหล่งน้ำและพื้นที่บนบกถัดจากขุมเหมืองขึ้นมาพบว่าฤดูกาลมีผลต่อความเข้มข้นของสารตะกั่วในดินโดยเดือนกรกฎาคมมีความเข้มข้นของตะกั่วต่ำที่สุด (0.6%) และเดือนตุลาคมมีความเข้มข้นตะกั่วสูงที่สุด(>11%)ในพืชส่วนมากจะสะสมตะกั่วสูงที่สุดในช่วงฤดูฝน(เดือนพฤษภาคม-ตุลาคม) และสะสมตะกั่วต่ำที่สุดในช่วงฤดูแล้ง(เดือนพฤศจิกายน-เมษายน) พืชที่พบส่วนใหญ่เป็นไม้ยืนต้นแต่มีบางชนิดเป็นไม้ล้มลุก มีพืช 12 ชนิดที่พบทั้งสองพื้นที่ที่ทำการศึกษาพืช 17 ชนิดที่สะสมตะกั่วในส่วนต้นมากกว่า 1,000 มก/กก แต่มีเพียง 6 ชนิดที่มีค่า TF มากกว่า 1 คือ *Ageratum conyzoides*, *Buddleja asiatica*, *Chromolaena odoratum*, *Conyza sumatrensis*, *Mimosa pudica* และ *Sonchus arvensis* ซึ่งพืชเหล่านี้เหมาะสำหรับนำไปบำบัดพื้นที่ที่มีการปนเปื้อนด้วยสารตะกั่ว

สำหรับการศึกษาประสิทธิภาพการสะสมสารตะกั่วในตะกอนดินจากเหมืองบ่องามระหว่าง *Cyperus iria* กับ *Typha angustifolia* พบว่าพืชทั้งสองชนิดสามารถเจริญเติบโตได้ดีโดยเฉพาะ *C. iria* ที่ออกดอกและแตกต้นใหม่ ตะกั่วสะสมมากที่สุดบริเวณราก (4,550 และ 6,383.33 มก/กก ของ *C. iria* และ *T. angustifolia* ตามลำดับ) รากที่แผ่กระจายแนวนอนของ *C. iria* สามารถดูดซับตะกั่วบริเวณผิวดินได้ดีขณะที่รากที่ยาวลึกลงของ *T. angustifolia* สามารถดูดซับตะกั่วในดินระดับที่ลึกลงไปได้ดีจากนั้นได้นำ *C. iria* มาศึกษาประสิทธิภาพการสะสมโลหะหนักสามชนิด (ตะกั่ว, แคดเมียม, สังกะสี) เทียบกับ *C. flavidus* ในสภาวะไร้ดินเป็นระยะเวลา 15 วัน พบว่าพืชทั้งสองชนิดสะสมตะกั่วได้ดีที่สุดเมื่อระยะเวลาเพิ่มขึ้นโดยสะสมมากที่สุดบริเวณราก 38,000 มก/กก ใน *C. iria* และ 13,666.7 มก/กก ใน *C. flavidus*

เนื่องจากกลูทาไทโอนและไฟโทคีลาตินที่สังเคราะห์ขึ้นในพืชตอบสนองต่อโลหะหนักที่พืชได้รับจึงได้ทำการศึกษาการเปลี่ยนแปลงปริมาณโปรตีนทั้งสองชนิดใน *C. iria* ที่ตอบสนองต่อตะกั่ว, แคดเมียม, สังกะสี เป็นเวลา 8 วัน พบว่าโปรตีนทั้งสองชนิดตอบสนองต่อแคดเมียมมากกว่าสังกะสีและตะกั่ว ตามลำดับ โดยกลูทาไทโอนสะสมบริเวณส่วนต้นมากกว่าในราก ขณะที่ไฟโทคีลาตินสะสมบริเวณรากมากกว่าส่วนต้น โปรตีนทั้งสองชนิดพบในปริมาณสูงสุด 2-4 วันแรกหลังจากได้รับโลหะหนัก จากการทดลองนี้จะเห็นว่าโปรตีนทั้งสองชนิดนี้มีระดับการตอบสนองต่อโลหะต่างชนิดได้ต่างกัน และจากการทดลองข้างต้นแม้ว่า *C. iria* จะไม่ใช่พืชที่สะสมโลหะหนักในส่วนต้นได้ดีแต่ก็มีประสิทธิภาพในการดูดซับโลหะหนักไว้บริเวณรากไม่ให้ถูกชะล้างไปปนเปื้อนในสิ่งแวดล้อมได้ดี

108 หน้า

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CONTENTS

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CONTENTS (CONT.)

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CONTENTS (CONT.)

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CONTENTS (CONT.)

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LIST OF TABLES

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LIST OF TABLES (CONT.)

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LIST OF FIGURES

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LIST OF FIGURES (CONT.)

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CHAPTER I
INTRODUCTION

Mining is a large global industry. More than 150 minerals are mined, each requiring different extraction and processing operations. Industry performance varies from responsible operations concerned to minimize impacts as far as possible to those that exhibit no concern at all. Surface mining is inevitably a major environmental disturbance since vegetation, top soil and underlying soil mantle have to be removed to gain access to the quantities of wastes produced more commonly exceed the volume of minerals (Samantaray *et al*., 1999). Baker *et al*. (1994) reported that metalliferous mining and processing, including the dumping wastes, usually produces the most severe cases of heavy metal pollution. Unlike organic pollutants, metals do not biodegrade and are generally not mobile, therefore their residence time in the soil is thousands of years. Metal uptake by plants depends upon metal bioavailability in the soil, and particularly on the supply from less plant-available fractions.

A few of higher plant species have adaptations that enable them to survive and to reproduce in soils heavily contaminated with heavy metals. They can be classified as metal-tolerant plants. Metal tolerance may result from two basic strategies: metal exclusion and metal accumulation (Baker, 1987). The exclusion strategy starts with comprising avoidance of metal uptake and restriction of metal transport to the shoots. These plants are used to revegetate bare soil areas. The accumulation strategy consists of strong concentration of metals in plant tissues (Dahmani-Muller *et al*., 2000).

Successful implementation of phytoremediation in the field depends on a significant quantity of metal being removed from the soil through plant uptake to effectively decrease the soil metal concentration. Several conditions must be met in order for phytoremediation to be effective. The availability of metals in the soil for root uptake is the first critical factor for metal uptake. Soils containing metal contaminants that cannot be solubilized or made available for plant uptake will limit

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the uptake and therefore the success of phytoremediation. Because some native hyperaccumulator plants from polluted regions have adapted to grow on polluted sites, it is possible to find and use them to revegetate degraded soils, either for extracting or stabilizing the elements (Gonzalez and Gonzalez-Chavez, 2006). In soil, the soil solution Pb concentrations represent a very small fraction of the total soil Pb. One of the major factors for the chelate-induced surge of Pb concentration in soil solution could be the chelation between Pb and the chelators in the soil solution that drives Pb desorption from soil to soil solution. This makes the Pb bioavailability to plants higher.

Selection of hyperaccumulator plants can be done by sampling plants from the abandoned mine site. The Bo Ngam lead mine area offers the suitable site for Pb hyperaccumulator selection. The mine ceased operation in 1996 and plants growing there have been adapted to the metalliferous soil for a long time. However, there were variation of plants during wet and dry seasons. Seasonal variation of plants can then be the important issue in plant selection.

Phytoremediation can be done on dry land and wetland. In wetland areas, metal contaminated sediments from open-pit mine can become health hazard if they were washed off to the streams where villagers rely themselves on this stream water. Hence, this needs to be remediated and constructed wetland can be the solution for. Constructed wetlands offer an unlimited potential for the phytoremediation of toxins and pollutants (Horne, 2000). Wetland plant species differ greatly in their abilities to accumulate and translocate metals (Greger and Kautsky, 1993; Rai *et al.*, 1995), so metal removal by wetland vegetation can be greatly enhanced by the selection of appropriate plant species (Fritioff and Greger, 2006). Primarily maximum uptake of metals in wetland is observed in roots. These roots have been reported to be the most beneficial for phytostabilization of the metal contaminants. Roots of the wetland plants play very important role in wastewater purification followed by stem and leaves. Plants that grow near the heavy metal contaminated areas showed good degree of heavy metal tolerance (Edroma 1974; Sheoran and Sheoran, 2005). *Cyperus* sp. is one of plants species which had the efficiency to accumulated heavy metals in their roots and was also found at the pond site of this open pit mine area. Two species of *Cyperus* sp. found in this area were *C. iria* and *C. flavidus*. Survey of plant species and the

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studies of their efficiency in metal accumulation were needed to be done in the laboratory under hydroponic condition.

The physical responses when plants exposed to heavy metals showed a variety of toxicity symptoms, such as reduced growth, chlorosis and darkening of the root system. The inhibition of root growth appears to result from metals induced inhibition of cell division of the root meristem (Eun *et al*., 2000). Moreover, metals also inhibit photosynthesis, alters the mineral nutrition and water balance, modifies hormone levels and affects the structure and permeability of the plasma membrane (Sharma and Dubey, 2005). These physiological symptoms indicate that heavy metal tolerance is one of the prerequisites of heavy metal hyperaccumulation in plants (Kramer, 1997). Leopold *et al*. (1999) discovered that heavy metals in tolerant plants are often chelated or precipitated inside the vacuoles indicating a transport of heavy metals through the cytoplasm. The mechanism of metal tolerance involve the chelation of heavy metal by specific organic molecules thereby lowering the intracellular concentration of potentially toxic free metal ions (Vogeli-Lange and Wagner, 1990).

To avoid toxicity plants have also been documented to catalyze redox reactions and alter the chemistry of metal ions. Glutathione (GSH) has been shown to act as an antioxidant in plant species, it was hypothesized that increased GSH synthesis might account for increased tolerance in *Thlaspi caerulescens* (Boominathan and Doran, 2003). Higher levels of GSH may contribute to metal detoxification either by direct binding or by synthesis of phytochelatins (PCs). De Vos *et al.* (1992) found that the depletion observed in GSH content may also be attributed to its consumption as substrate for PC synthesis. PC biosynthesis was induced by heavy metals such as Cd and As (Inouhe, 2005) and plays a role in homeostasis of heavy metals in plants. This is the mechanism that regulates the metal ion availability in plant cells (Zenk, 1996; Goldsbrough, 2000; Thomine *et al*., 2000). GSH and PC contents changing with changing in degree of metal exposure need to be monitored under the laboratory hydroponic condition. The preliminary tests had confirmed that *Cyperus iria* is a better candidate for Pb accumulation that *C. flavidus*. It was then chosen to be tested in the laboratory scale batch study along with *Typha angustifolia*.

There are several researches using wetland plants families Cyperaceae and Typhaceae for phytostabilization in mine tailing (Wright and Otte, 1999; Greger and

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Stoltz, 2002; Qu *et al*., 2003; Panich-Pat *et al*., 2004). Most of these plant species are grown easily since they are tolerant to heavy metal stress. *Cyperus iria* is the wetland plant Pb accumulator found in Bo Ngam lead mine and *Typha angustifolia* which can be found in the nearby area, is also reported to accumulate metals with high biomass (Panich-Pat *et al*., 2004). Both wetland plant species were then selected for the study in lead removal from lead contaminated wetland soil.

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OBJECTIVES

1) To investigate and compare seasonally the lead concentrations in plant and soil samples collected in land site and pond site areas at Bo Ngam lead mine in order to search for the potential lead hyperaccumulators and lead concentration changing in plant and soil samples.
2) To study the seasonal and spatial variations of plant community between the land and pond sites.
3) To compare the accumulation of lead, cadmium, zinc and plant growth in *Cyperus iria* and *Cyperus flavidus* under the laboratory condition.
4) To investigate glutathione and phytochelatin concentration changing of *Cyperus iria* under the hydroponic condition by varing lead, cadmium and zinc concentrations.
5) To compare the accumulation efficiency of lead in the sediment from Bo Ngam lead mine within the close system of constructed wetland between *Cyperus iria* and *Typha angustifolia*.

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CHAPTER II
LITERATURE REVIEW

2.1 Heavy Metals

2.1.1 Lead

2.1.1.1 Sources of lead

Lead (Pb; MW = 207.2) is a bluish-grey metal that occurs naturally in minute amounts within the Earth's crust. It has also been referred to as plumbum, lead metal, and pigment metal (Environment Writer, 2000). Frequent use in many industrial processes is the main reason for Pb contamination of the environment. There are a variety of industrial processes that involve the use of Pb such as mining, smelting, manufacture of pesticides and fertilizers, dumping of municipal sewage and the burning of fossil fuels that contain a lead additive. Many commercial products and materials also contain Pb including paints, ceramic glazes, television glass, ammunition, batteries, medical equipment (i.e., x-ray shields, fetal monitors), and electrical equipment (Meagher, 1998).

2.1.1.2 Forms of lead

Ionic lead (Pb^{2+}), lead oxides and hydroxides and lead-metal oxyanion complexes are the general forms of Pb that are released into the soil, groundwater and surface waters. The most stable forms of Pb are Pb^{2+} and lead-hydroxy complexes. Pb^{2+} is the most common and reactive form of Pb, forming mononuclear and polynuclear oxides and hydroxides (GWRTAC, 1997). The predominant insoluble Pb compounds are $Pb_3(PO_4)_2$, $Pb(NO_3)_2$ (form when the pH is above 6) and $PbOH^+$ (Raskin and Ensley, 2000). PbS is the most stable solid form within the soil matrix and forms under reducing conditions when increased concentrations of sulfide are present (GWRTAC, 1977).

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2.1.1.3 Lead and the Soil Matrix

Pb is found in all soils at varying concentrations (typical 5-30 mg/kg) (Rotard *et al*., 1995). Pb is very difficult to remove from the soil matrix. The transition metal resides within the upper 6-8 inches of soil where it is strongly bound through the processes of adsorption, ion exchange, precipitation, and complexation with sorbed organic matter (GWRTAC, 1997; Raskin and Ensley, 2000). Pb found within the soil can be classified into six general categories: ionic lead dissolved in soil water, exchangeable, carbonate, oxyhydroxide, organic or the precipitated fraction. All of these categories combined make up the total soil Pb content (Raskin and Ensley, 2000). Water soluble and exchangeable Pb are the only fractions readily available for uptake by plants. Oxyhydroxides, organic, carbonate, and precipitated forms of Pb are the most strongly bound to the soil (Chaney, 1998). All of the interactions that occur throughout the soil matrix are pH dependent. The soil pH has a significant effect on the mobility of Pb and other metals within the soil. The pH of soil generally ranges between 4.0-8.5. Under acidic conditions (pH<5.5), metal cations are more mobile, while anions tend to sorb to mineral surfaces (GWRTAC, 1997). Metals are more available to plant roots under these conditions; however, due to an increase in aluminum (Al) solubility, plant growth may be inhibited due to Al toxicity (Raskin and Ensley, 2000). The opposite occurs when basic conditions are present within the soil matrix. Anions are mobilized and cations are adsorbed to mineral surfaces or precipitate, decreasing the metal bioavailability for plant uptake (GWRTAC, 1997). The capacity of the soil to adsorb Pb increases with increasing pH, cation exchange capacity (CEC), organic carbon content, soil/water Eh (redox potential) and phosphate levels (USEPA, 1992).

2.1.1.4 Health effects

Pb has been listed as a potential carcinogen in the EPA Toxic Release Inventory (TRI) (Environment Writer, 2000). Inhalation and ingestion are the two routes of exposure, and the effects from both are the same. Pb accumulates in the body organs (i.e., brain), which may lead to poisoning (plumbism) or even death. The gastrointestinal tract, kidneys, and central nervous system are also affected by the presence of Pb. Children exposed to Pb are at risk for impaired development, lower

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IQ, shortened attention span, hyperactivity, and mental deterioration, with children under the age of six being at a more substantial risk. Adults usually experience decreased reaction time, loss of memory, nausea, insomnia, anorexia, and weakness of the joints when exposed to Pb (Environment Writer, 2000).

2.1.1.5 Phytoremediation of lead

Pb has extremely low solubility, as only 0.1% of soil Pb is available for extraction (Huang *et al*., 1997). The effort to phytoremediate Pb is concentrated on using soil amendments like EDTA to increase the available Pb uptake (Blaylock *et al*., 1997; Huang *et al*., 1997; Wu *et al*., 1999). Addition of chelators does increase the solubility and uptake but the amount of Pb transferred to shoots is still low in comparison to the amount of Pb in the soil, thus increases the likelihood that the mobilized Pb-EDTA will leach out of the soil and contaminate groundwater (Wu *et al*., 1999). The prospects for phytoremediation of Pb will depend on the development of novel systems for solublizing Pb and for transporting it to the leaves.

2.1.2 Cadmium

2.1.2.1 Source of cadmium

Cadmium (Cd; MW = 112.4), a group IIB transition element is a non-essential and toxic element without any metabolic significance. Cd pollution is increasing due to excessive mining, industrial usage and other anthropogenic activities (De, 1992; Prasad, 1995). Cd released into the environment tends to concentrate in soils and sediments, where it is potentially available to rooted plants. Cd is an analogue of Zn and it can be accepted in considerable amount by plants at the absence of Zn in soil, which causes suppression of their growth.

2.1.2.2 Form of cadmium

Cd occurs in natural waters in oxidation state +II as the cation Cd^{2+} and complexes of Cd^{2+}: $CdOH^{+}$ and $CdCO_3$ at high pH; $CdSO_4$ and $CdCl^{+}$ at lower pH and depending upon the Cd concentration. Cadmium salts with low solubility includes $Cd_3(PO_4)_2$, CdS, $Cd(OH)_2$, and $CdCO_3$. The solubility of these cadmium salts will increase with decreasing pH. Cd is found in all soils at low concentrations (typical 0.1-0.4 mg/kg) and in a variety of species, mainly as CdS and $CdCO_3$, but also as $Cd_3(PO_4)_2$. Frequent anthropogenic sources are metal (Zn) mining

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and smelting due to co-occurrence of CdS with the important zinc ore: ZnS. Phosphate fertilizers, atmospheric deposition and sewage sludges are other important sources of Cd in soils. Metal plating industries, PVC stabilizers, batteries and pigments are potential Cd sources. In soils, Cd will also be found binding to the cation exchange sites (clay minerals, iron oxyhydroxides and calcium carbonate minerals).

Overall, the primary mechanism for reduced availability of Cd in soil will be minerals and salts of low solubility and cation exchange, and the most important factor for release will be (acidic) exclusion from and/or dissolution of the cation exchange complexes, as well as acidic dissolution of Cd minerals and salts. Cd is known to bind to sulphydryl groups of membrane-bound proteins and destabilize the membrane system, by inducing the formation of disulphide links leading to distortion in structure and function of membrane ion channels and hence regulation of uptake/efflux of essential nutrients (Prasad, 1995).

2.1.2.3 Health effects

The available Cd thereby enters biogeochemical cycles, becomes bioconcentrated (Devi *et al*., 1996) and even affects human health (for example, the Itai-itai disease caused by Cd-contaminated rice in Japan) (Rivai *et al*., 1990). Cd has been classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC, 1993; Waalkes, 2000). The highest risk represents the accumulation of Cd in kidney, where the Cd concentration > 200 mg/kg brings about its dysfunction. Cd abundance results in humans and animals extraction of calcium from the bones, their demineralization, softening, shortening, deformation and their increased brittleness (Bujnov´a and Lesn´y, 2000)

2.1.2.4 Cadmium uptake in plants

Cd uptake is likely mediated through transporters or channels for other divalent ions (Cosio *et al*., 2004). Excess divalent cations in the media, such as Zn, can reduce Cd uptake in many plant species, including *Thlaspi caerulescens* Prayon (Lombi *et al*., 2001a, 2002). Significantly, divalent cations and Ca channel blockers had no effect on the Cd uptake of *T. caerulescens* Ganges, suggesting that this ecotype may have developed a novel Cd uptake system (Lombi *et al*., 2001a, 2002). Piñeros and Kochian (2003) demonstrated that *T. caerulescens* and *T. arvense*

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mesophyll cells exhibit different plasma membrane ion transport properties, but the differences cannot be directly linked to the differences in Zn and Cd accumulation.

Analysis of Cd/Zn transport capacity in leaf mesophyll protoplasts demonstrated that the constitutive transport capacity and affinity for these metals were indistinguishable in *Thlaspi caerulescens* Ganges, *Arabidopsis halleri*, and *T. caerulescens* Prayon; however, Cd accumulation increased in Ganges protoplasts but decreased in *A. halleri* protoplasts in conjunction with Cd pre-exposure (Cosio *et al*., 2004). Therefore, there may be multiple Cd transport systems in leaves.

2.1.2.5 Phytoremediation of cadmium

Ecotypes of *T. caerulescens* accumulate a wide range of Cd levels. The Ganges and Vivez ecotypes can accumulate up to 10,000 mg/kg Cd dry weight and 12,500 mg/kg Cd dry weight, respectively, without showing signs of toxicity; however, the Puy de Wolf and Prayon ecotypes only accumulate 2,300 mg/kg Cd dry weight and 4,800 mg/kg Cd dry weight, respectively (Lombi *et al*., 2000, 2001a, 2001b; Peer *et al*., 2003). Hyperaccumulation of Cd in *A. hallerii* has also been reported (Cosio *et al*., 2004; Küpper *et al*., 2000).

Pilot studies of Cd and Zn phytoremediation in the UK, agricultural soil, Cu toxicity limited the growth of *T. caerulescens*, demonstrating the need for plants that can tolerate to the toxic concentrations of multiple pollutants (Lombi *et al*., 2001b). The remediation potential of *T. caerulescens* is also limited by its small stature and biomass. In one study, even though *T. caerulescens* accumulated the highest concentrations of Cd and Zn in its leaves, *Brassica juncea* removed more Zn and equivalent amounts Cd due to its larger size (Ebbs *et al*., 1997). Transgenic approaches to either make *T. caerulescens* grow larger or to make *B. juncea* accumulate more Cd and Zn could make Cd phytoextraction feasible.

2.1.3 Zinc

2.1.3.1 Source of zinc

Zinc (Zn; MW = 65.4), a group IIB transition element, is one of the most essential microelements for the plant system. Zn plays a fundamental role in several of the critical cellular functions such as protein metabolism, gene expression, chromatin structure, photosynthetic carbon metabolism and indole acetic acid metabolism (Vallee and Falchuk, 1993; Prasad, 1995; Cakmak and Braun, 2001). Zn is

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an important component of many vital enzymes having a catalytic, co-catalytic or structural role, as well as being a structural stabilizer for proteins, membrane and DNA-binding proteins (Vallee and Falchuk, 1993), yet it is toxic at high concentrations.

2.1.3.2 Form of zinc

In natural waters, Zn will mainly be present as the cation Zn^{2+} but complexes with carbonates ($ZnCO_3$ and $ZnHCO_3^+$) and hydroxide (e.g.: $ZnOH^+$) may be present as well. The importance of organic complexes is not well described. Zn salts with limited solubility include $Zn_3(PO_4)_2$, ZnS, $Zn(OH)_2$, and $ZnCO_3$. $Zn_3(PO_4)_2$, $Zn(OH)_2$ and $ZnCO_3$ will exhibit increasing solubilities with decreasing pH. Zn is found in all soils at varying concentrations (typical 10-300 mg/kg). The natural occurrences are mainly ZnS. Frequent anthropogenic sources include mining and smelting, metal processing and plating, fertilizers and sludges. In soils, Zn will primarily be present bound to cation exchange sites of iron oxyhydroxides, clay minerals and organic matter. Reduced bioavailability will mainly be by ion exchange and to some degree by the presence of low solubility salts for alkaline soils. Release will be by (acidic) exclusion from and dissolution of ion exchange complexes.

2.1.3.3 Zinc and the Soil matrix

The Zn content of soils is largely dependent on the composition of the parent rock material. The average Zn concentration in soils is 50 mg/kg (Rotard *et al*., 1995). The total Zn content and their acceptability by plants is important to know for eventually artificial addition of elements into soils, not to exceed limit levels. Biologically accessible Zn is water-soluble and fractions solubilized by biological processes. The total amount of zinc in soils is distributed in several forms as follows:

- free ions Zn^{2+} and organo-zinc complexes in the soil solution,
- adsorbed and exchangeable Zn in the colloidal fraction of the soil (composed of clay particles humic compounds, Fe and Al hydroxides),
- Zn in secondary minerals and insoluble complexes in the solid phase of the soil.

The factors affecting availability of Zn in soils are total soil content, pH, climatic conditions, moisture regime and interaction between Zn and

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other macro- and micronutrients (Zn-P, Zn-Fe, Zn-Cu, Zn-N) at the soils (Bujnov´a and Lesn´y, 2000).

2.1.3.4 Health effects

Zn is an essential trace element for flora and fauna including humans. It is a component of numerous enzymes actuating in energy metabolism and genetic transcription. Zn deficiency results in humans and animals in appetence, severe growth depressions, skin lesions and sexual immaturity. For humans depression of immunocompetence and change of taste acuity is observed as an effect of Zn-deficiency. The recommended safe and adequate dietary intake for adults is as much as 15 mg/day. Zn content in general plays an important role in biochemical processes of plants as well. Zn^{2+} seems to be the predominating chemical form in which Zn is absorbed by plants as a functional, structural and regulatory cofactor of a large number of enzymes. The presence of Zn is unavoidable for activity of various dehydrogenases, aldolases, isomerases and polymerases (Bujnov´a and Lesn´y, 2000).

2.1.3.5 Zinc uptake in plants

Lasat *et al*. (1996) detected that the hyperaccumulator *T. caerulescens* and the non-accumulator *T. arvense* have the same affinity for Zn uptake, but the hyperaccumulator has a higher rate of uptake. Despite lower rates of uptake**,** the roots of *T. arvense* were found to accumulate substantially more Zn than in *T. caerulescens*. This difference is likely due to better transport to the leaves in the hyperaccumulator. *T. caerulescens* had five times more xylem sap Zn (Lasat *et al*., 1998) and ten times more Zn was translocated to the shoots in *T. caerulescens* than in *T. arvense* (Lasat *et al*., 1996). The leaf cells of the hyperaccumulator are able to accumulate more Zn when leaf sections are subjected to high Zn (1mM) conditions (Lasat *et al*., 1998). The molecular mechanisms of this increased uptake are unknown.

2.2 Bo Ngam Lead Mine

2.2.1 General Condition and Topography of Bo Ngam Mine Area

Kanchanaburi Province, 230 km. west of Bangkok, Thailand, is rich in minerals, especially there are the total reserves of 7.7 billion tons of Pb (Department of Primary Industries and Mines, 1998). There are several Pb mines in the area of Thong

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Pha Phoom District of that province that have been in operation for 40-60 years, several of which have been closed down due to the expiration of their concessions.

Bo Ngam Mine is located at Tambon Chalae, Amphoe Thong Pha Phoom, Kanchanaburi Province consisting of one plot of expired mining lease and one plot of valid mining lease of Phol and Son Co., Ltd. The total area is 574 rai. As a result of the Royal Decree in 1991, Thung Yai Naresuan Wildlife Sanctuary was expanded to the south covering some areas of the existing mineral lease. After land survey, the total area is only 418 rai. Currently, this area is classified as one of the northernmost potential Pb-Zn areas.

2.2.2 Activities in the Bo Ngam Mine

(1) Mining: Open pit mining method was performed in the pit. By using backhoe to excavate $PbCO_3$ ore found in the lateritic overburden of depth up to 5 m., it was transported to the washing plants nearby. Stripping ratio was approximately 10:1. Concentrate gained from washing process would be more than 25% Pb content. It would be upgraded to 60% Pb content at Klity processing plant before it was transported further for smelting to achieve Pb metal of 99.99% Pb content.

(2) Wastewater and tailing system: Sedimentation process within tailing pond is necessary for the separation of water and slime. Treated water would then be pumped back for reuse.

(3) Current activity: Mining operation ceased since 26 July 1996, due to the expiration of the concessions. Rehabilitation has been underway in the area.

2.2.3 Meteorology and Hydrology Studies

Climate at the area is under monsoon winds. Three seasons is expected: winter, summer and rainy. Variation of monthly rainfall is in the range of 6-235 mm. Rainfall over 89% of the yearly rainfall is between May-October. The heaviest rainfall is in September. For rainfall distribution analysis in the area, an average rainfall contour line map is drawn and in the range of approximately 1,000 to 1,500 mm. per year. Rainfall distribution depends on direction and duration of monsoon wind and topographical condition. Mountainous area at northwest side has a relatively high rainfall.

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2.2.4 Geology of Mineral Deposits

Mineral deposits in Thong Pha Phoom area of Kanchanaburi Province are Pb and Zn. Primary ore is PbS from hydrothermal process deposited in Ordovician limestone, secondary ore are $PbCO_3$ and $PbSO_4$, due to chemical alteration with host rock and oxidation process along contact zone with limestone and air by hydrothermal process.

2.2.5 Mining Engineering

$PbCO_3$ is the main ore product from this area. Open pit mining is employed. Overburden up to 5 m. depth is removed by bulldozer. Ore is removed by backhoe and dumped into a truck. A truck will carry the ore to a washing plant where waste is washed away back to tailing pond. Concentrate which contains more than 25% Pb will be trucked to Klity Processing Plant for secondary treatment.

2.2.6 Lead Contamination in Water and Soil

In general, it has been funnel that Pb contamination in water below the threshold limit. But contamination in soil is higher and above threshold limit. Besides, contamination covers a vast area, due to the fact that the area is a Pb deposit. Apart from Pb, other elements such as As, Cd and Hg have to be analyzed and observed due to hazardous effect of these elements to food chain.

From the survey, observed Pb solubility in various pH values. The result has turned out that under low pH condition, solubility is higher and euphoric acid has a higher soluble effect. It is expected that euphoric acid can be generated in natural condition where sulfide ores are found. This is the main cause of Pb contamination in natural water. In addition, secondary ores can be much more soluble than primary ores. Oxidation time and stockpiling period may affect solubility and contamination rate.

2.3 Phytoremediation

2.3.1 Phytoremediation processes

At present, many heavy metals constitute a global environmental hazard. For example, environmental pollution by Cd, arising mainly from mining and smelting, dispersal of sewage sludge and the use of phosphate fertilizers, is increasing. Surface mining is inevitably a major environmental disturbance since vegetation, top

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soil, and underlying soil mantle have to be removed (Samantaray *et al*., 1999). This mining process has destroyed the ecosystem, resulting in serious contamination problems arising from wind- and water-borne materials and loss of productive areas. Baker *et al*. (1994) reported that metalliferous mining and processing, including the dumping wastes, usually produces the most severe cases of heavy metal pollution. There is also a lack of organic matter and its associated nutrients such as nitrogen (N) in most degraded land materials. Unlike organic pollutants, metals do not biodegrade and are generally not mobile, therefore their residence time in the soil is thousands of years. Metal uptake by plants depends upon metal bioavailability in the soil. Availability to plants is governed by dynamic equilibriums involving these fractions, rather than by the total metal content (Lazaro *et al*., 2006). The use of microorganisms and plants for the decontamination of heavy metals has attracted growing attention because of several problems associated with pollutant removal using conventional methods.

Possible mechanisms that govern heavy metal tolerance in plant cells are:

1. Metal binding to the cell wall
2. Reduced transport across the cell membrane
3. Active efflux
4. Compartmentalization
5. Chelation

Apart from accumulating high levels of metal and translocating it to the harvestable segments of the plant, a plant suitable for phytoextraction should grow rapidly and reach a high biomass. The number of harvests requires plants that produce both a high biomass and accumulate at least 1-3% metal, by dry weight. Approaches to find metal-tolerant hyperaccumulating plants for phytoremediation involves searching for, and studying natural hyperaccumulators, or developing genetically engineered plants that possess these traits. The biological processes knowledge such as plant-microbe interactions, plant uptake, translocation mechanisms, tolerance mechanisms (compartmentation, degradation), and plant chelators involved in storage and transport can increase the efficiency of phytoremediation technologies.

Soil remediation is defined as a set of techniques for reducing the mobile and, in consequence, bioavailable fraction of contaminants in soils with the object of

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minimizing their transfer into food chains and groundwaters (Mench *et al*., 2000). Remediation can be achieved either by removal of the heavy metals or by preventing their spread to surrounding soil and groundwater. Phytoremediaton involves several steps: transfer of metals from the bulk soil to the roots surfaces, uptake into the roots and translocation to the shoots (Romeiro *et al*., 2006).

Under natural conditions metals accumulated in shoots are annually recycled to the soils. Topsoil is used to cover poor substrates and to provide improved growing conditions for plants. To maintain a good topsoil quality for any revegetation scheme, it supposed to have a suitable physical property, application of appropriate fertilizers, and inoculation of nitrogen-fixing bacteria and mycorrhiza would facilitate reconstruction of self-sustained ecosystems (Wong, 2003).

Phytoremediation may involve metal absorption from the soil and transportation to the harvestable parts of the plants (phytoextraction), absorption and concentration of toxic metals in plant roots from polluted effluents (rhizofiltration), and reduction of heavy metal mobility in the soil by plants (phytostabilization) (Salt *et al*., 1995, 1997). High root to shoot translocation of metals are vital characteristic for plants to be used in phytoextraction techniques (Lazaro *et al*., 2006). Metal-tolerant plants that do not accumulate metals in their shoots are selected for phytostabilization to minimize metals entering the food chain (Whiting *et al*., 2004).

Robinson *et al*. (2003) reported that plants pump water, solutes and organic matter from the surrounding medium as part of their natural physiological processes. This potential can be explored to stabilize, remove or breakdown contaminants in the soil

On mine spoils the first pioneers are metal accumulating plants, which slowly prepare the soils for the next colonizers. They increase soil organic content, improve microclimatic conditions and enhance the distribution of mycotrophic species (Gucwa and Turnau, 1998). A few of higher plant species may have adaptations that enable them to survive and to reproduce in soils heavily contaminated with heavy metals. They can be classified as metal-tolerant plants. Metal tolerance may result from two basic strategies: metal exclusion and metal accumulation (Baker, 1987). The exclusion strategy started with comprising avoidance of metal uptake and followed by restriction of metal transport to the shoots. These plants are used to revegetate bare

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soil areas. The accumulation strategy consists of high concentration of metals in plant tissues (Dahmani-Muller *et al*., 2000).

Owing to the fact that mine area contains relatively less top soil with low nutrient, the colonizing pioneer plant has to withstand drought condition and be able to adapt and grow in bad surrounding environment easy to acquire. Botanical survey and plant screenings are necessary because these could lead to the identification of plant species which have a potential for phytoremediation.

2.3.2 Biological processes affecting phytoremediation

The bioavailability of a pollutant is important for its remediation. Pollutant bioavailability depends on the chemical properties of the pollutant, soil properties, environmental conditions, and biological activity. Soils with small particle size (clay) hold more water than sandy soils, and have more binding sites for ions, especially cations (CEC). The concentration of organic matter (humus) in the soil is also positively correlated with CEC. Humus mainly consists of dead plant material, and plant cell walls have negatively charged groups that bind cations, as well as lignin that binds hydrophobic Bleuel *et al*. (2005) also supported that the availability of heavy metals is affected by pH, organic chelators, particles, presence of other heavy metals or anions, ionic strength, and temperature or light intensity.

Clemens *et al*. (1995) found that non-essential metals such as Cd or Pb are thought to enter plant cells by virtue of their chemical and physical similarity to plant nutrients like Ca, Mn, Fe or Zn, for example through Ca channels or broad-range metal transporters.

The plant vacuole is the transient storage compartment for these peptides (Zenk, 1996) Cd detoxification may require transport of the Cd-PC complexes into the vacuole. A transport system has been recently described for these complexes (Salt and Rauser, 1995). GSH-*S*-conjugates are also transported into the vacuole in an ATP-dependent manner. The Cd-PC complexes probably dissociate, and the metal-free peptide is subsequently degraded (Zenk, 1996).

2.3.3 Metals effects to plant metabolisms

Pb induced changes in lipid composition and potassium ion leakage in *Zea mays* (Malkowski *et al*., 2002). Pb ions are known to induce lipid peroxidation, decrease the level of saturated fatty acids and increase the content of unsaturated fatty

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acids of membranes in several plant species (Halliwell and Gutteridge, 1999). Pb is rapidly accumulated in roots if Pb is bioavailable in the plant growth media; however, only a small proportion of absorbed Pb is translocated to shoots (Huang and Cunningham, 1996). The retention of Pb in roots involves binding to the cell wall and extracellular precipitation, mainly in the form of $PbCO_3$ which is deposited in the cell wall. The cell wall and the vacuole together account for 96% of the Pb absorbed (Wierzbicka and Antosiewicz, 1993).

Baker and Brooks (1989) suggested that several metal-hyperaccumulator plant species have been identified to have genetic potential for successful phytoremediation of contaminated soils. Zn hyperaccumulation in plants could be controlled by various processes including stimulated root absorption and xylem transport as well as leaf cell uptake, which have been reported to be involved in Zn hyperaccumulation in *T. caerulescens* (Vazquez *et al*., 1994; Lasat *et al*., 1996, 1998; Yang *et al*., 2004). The castor bean plants were able to accumulate large quantities of Pb, especially in roots, demonstrating the high capacity for Pb absorption and accumulation of that organ. Similar results were obtained for *Carex rostrata*, *Eriophorum angustifolium* and *Phragmites australis* grown in hydroponics (Stoltz and Greger, 2002). A high capacity for Pb retention in the roots with restricted translocation to the shoots was reported for *Helianthus annus* L. (Romeiro, 2006), *Pinus radiata* (Jarvis and Leung, 2002), spinach (Tsen *et al*., 2002) and *Prosopis* spp. (Aldrich *et al*., 2004).

2.3.4 Plant uptake capacity to metals

Metal interactions in soils vary considerably with the nature of soil types. The phytoavailability of metals is determined by the nature of the metal species, their interaction with soil colloids, the soil characteristics and duration of contact with the surface binding these metals. Soil characteristics (eg. soil pH, clay, organic matter content and type, and moisture content) also determine availability to plants by controlling the speciation of the elements, temporary binding by particle surface (adsorption-desorption processes), precipitation reactions and availability in soil solution. Both the concentration of trace metals and their speciation vary significantly with the composition of soil solution and the amount of moisture present in the soils (Fotovat *et al*., 1997).

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The ability of plants to bioaccumulate metals and possibly other contaminants varies with both the nature of plant species and the nature of metal contaminants. Laboratory studies demonstrate that the capacity of plants to bioaccumulate metals varies extensively with the nature of metals as well as with plant types. Moreover, the differences in metal uptake may be attributed to both the markedly different binding capacity of soils for these metals and also to plant-root-metal interactions, which vary with metal types. The patterns of metal accumulation and distribution in the plant parts were significantly influenced by the metal, plant species and position

2.3.5 Plant hyperaccumulators

Metalliferous soils are often enriched in combinations of different heavy metals and therefore, local metallophyte populations often exhibit combined tolerance to different metals (Schat *et al*., 2000). Metallophytes are a group of plants that grow on mineralized areas and have developed mechanisms that allow them to resist metal concentrations which are toxic to most plants (Reeves and Baker, 2000). Some metallophytes are called hyperaccumulator plants. Baker and Brooks (1989) suggested that hyperaccumulator plants when grown in its natural habitat, should be contained > 100 mg/l Cd, >1000 mg/l Co, Cr, Cu, Ni, Pb or Se, or >10,000 mg/l Mn or Zn, on a dry biomass basis, in any aboveground tissue.

Normally, nonhyperaccumulator plants tend to store the absorbed heavy metals in the roots, whereas hyperaccumulator plants are capable of transporting most of the accumulated heavy metals to the shoots (Lasat *et al*., 1998). Hyperaccumulator plants show a stronger influx of heavy metals into the roots than do nonaccumulator species (Lasat *et al*., 1996), since nonaccumulator plants do not have strong metal detoxification mechanisms in the shoots, most of the metals are excluded from the shoot via root sequestration, most likely to protect the photosynthetic apparatus in leaf cells which is extremely sensitive to heavy metals (Küpper *et al*., 1999). On the other hand, hyperaccumulator plants very actively translocate heavy metals into the shoots, presumably because of the existence of heavy metal tolerance mechanisms operating in the shoot. Popular species for phytoextraction are Indian mustard and sunflower because of their fast growth, high biomass, and high tolerance and accumulation of metals and other inorganics. When choosing plant species for a certain site, it is

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advisable to include species that grow locally on or near the site. These species are competitive under the local conditions and, if they are growing on the site, can tolerate the pollutant (Pilon-Smiths, 2005).

Relatively few plants have been recorded to hyperaccumulate Pb. Some are aquatic and others are wetland and terrestrial. Among the aquatic plants, water hyacinth (*Eichhornia crassipes*) could accumulate an appreciable amount of Pb (25,800 mg/kg when treated with 8 mg/l of Pb) (Muramoto and Oki, 1983). The smartweed (*Polygonum hydropiperoides*) a wetland plant, could accumulate the highest Pb concentrations in both shoots (64 mg/kg) and roots (1,882 mg/kg) (Jin-Hong *et al*., 1999).

Grasses prevent wind erosion and lateral runoff with their dense root systems. However, grasses tend to not accumulate inorganic pollutants in their shoots as much as dicot species to minimize the exposure of wildlife to toxic elements. (Pilon-Smiths, 2005). Lombi *et al*. (2001b) reported that monocotyledon species usually more tolerant to metals than the dicotyledon species which the reason supported by Deram *et al*. (2006) discovered that monocotyledonous plants take up less Cd than dicotyledonous ones. Deram *et al*. (2006) selected *Arrhenatherum elatius*, a perennial grass as a study model for its ability to tolerate high concentrations of heavy metals in shoots. They found that *A. elatius* could be exploited for its ability to remove heavy metals from soil. It could be effective for phytoremediation operations when growing either in Co/Cu/Ni ore either in base-metal tailings rich in Pb, with some sort of amendment.

2.3.6 Plant accumulation and seasonal variation

Several field studies have found that metal accumulation may depend on seasonal variation. Martin and Couphtrey (1982) found the highest metal foliar levels during spring and the lowest during winter, whereas, Brekken and Steinnes (2004) indicated the highest metal contents (Cd, Cu, Ni, Pb, Sn, Zn) during autumn and relatively low levels during spring. They concluded that the lower metal concentrations in waters in the wet season may be due to a dilution effect by heavy rain in summer. However, Othman *et al*. (1997) studied Pb levels in roadside soils of Damascus city and found that Pb levels in the wet period was higher than in the dry period. Deram *et al*. (2006) found the significant seasonal variations of Zn and Cd

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concentrations in shoots of *A. elatius*. Shoot concentrations were high at the end of winter but decreasing until late spring and finally on the increase again in July. The recorded concentration decrease during spring is generally referred to as a dilution effect due to growth increase without an increase in translocation.

2.4 Phytotoxicity

The phytotoxicity is associated with phenomenon whereby a potentially harmful substance has accumulated in the plant tissue to a level affecting optimal growth and the development of the plant (Beckett and Davis, 1977). Positive confirmation of an incidence of metal toxicity requires that (Chang *et a*l., 1992):

1. plants have sustained injuries;
2. a potentially phytotoxic metal has accumulated in the plant tissue;
3. the observed abnormalities are not due to other disorders of plant growth;
4. the biochemical mechanisms that cause metal toxicity to be harmful to plants are observed during the course of growth.

In fact, all living organisms are dependent on certain essential metals (Cu, Zn, Fe, Ni etc.). They are needed in very small quantities as co-factors for enzymes or proteins. The non-essential metals include Cd, Hg or Pb. Depending on their concentration, both essential and non-essential metals act as cell toxins. The toxic action is based on the unspecific binding of the metals to important biomolecules and results in functional groups being blocked, essential metals being displaced, or the active form (conformation) of the biomolecules being changed (Mason and Jenkins, 1995).

For the physical responses, excessed Pb causes a variety of toxicity symptoms in plants, such as reduced growth, chlorosis and darkening of the root system. Inhibition of growth accompanied by chlorosis of young leaves when Co, Cr and Cu are administered in toxic doses to the plants (Hunter and Vergnano, 1953; Daniels *et al*., 1972). Inhibition of root growth from Pb-induced inhibition of cell division of the root meristem (Eun *et al*., 2000). Pb also inhibits photosynthesis, alters the mineral nutrition and water balance, modifies hormone levels and affects the structure and permeability of the plasma membrane (Sharma and Dubey, 2005).

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In a study on relative effectiveness of Mn, Cu, Zn, Co and Ni in barley (Agarwala *et al*., 1977), these heavy metals decreased catalase activity and reduced chlorophyll concentration associated with chlorosis due to excess Co^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+}. Luna *et al*. (1994) have observed the activity of catalase in oat is decreased due to direct effect of Cu-induced ROS on the enzyme protein under such conditions. In general, enzyme activities in plants increased at lower metal concentrations and decreased with increase in duration and concentration. The enzyme activity significantly declined at the highest concentration and duration.

2.4.1 Heavy metals transport in plants

Once metal ions have entered the root they can either be stored or exported to the shoot. Metal transport to the shoot probably takes place in the xylem. However, metals may redistribute in the shoot via the phloem. For metal ions to enter the xylem vessels they must first cross the casparian strip which divides the endodermis and the epidermis. To cross this strip of water impermeable cell wall metal ions must move symplastically, as apoplastic transport is blocked. It is therefore feasible that symplastic transport of metals within the endodermis is a rate limiting step in metal translocation to the shoot. Xylem cell walls have a high cation exchange capacity which would be expected to severely retard the movement of metal cations. Therefore, metal-chelate complexes, such as Cd-citrate should facilitate metal movement in the transpiration stream.

During metals transportation through the plant, they get bound largely on the cell walls near to the uptake site, which explains why most of the metals take up are commonly found in the roots (75-90%) and smaller amounts are distributed in the shoots (Prasad, 2004). Javis *et al*. (1976) examined the Cd distribution in 23 species and found that the higher up in the plant, the lower the Cd concentration, which decreased in the following order; fibrous roots>storage roots>stems>leaves.

2.4.2 Detoxification and chelation

The toxic heavy metal accumulation in soil would deteriorates crop growth and yield components, and threaten the agro-products security. There were significantly differences in the accumulation and tolerance to heavy metals among plant species and genotypes. The introduction of metal-binding proteins and peptides into plants to enhance metal tolerance and accumulation is a compelling strategy.

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These metal-binding peptides or proteins should be preferentially metal specific such that only the toxic metals are sequestered (for example Cd, Hg and Pb) and not essential trace metals such as Zn. The formation of phytochelatins (PC) in response to the stress caused by heavy metals was one of the truly adaptive responses occurred commonly in higher plants. In the heavy metal tolerant genotypes, there was a much higher accumulation of PC than the non-tolerant lines. Glutathione (GSH) was the substrate for the synthesis of PC, which chelated the metals. Krämer (2000) reported that the exposure of plants or plant cell cultures to metals results in the synthesis of PCs, small cysteine-rich oligopeptides that are non-translationally synthesized from GSH by PC synthase. The inactive toxic metal ions of metal-PC chelations were subsequently transported from cytosol to vacuole before they could poison the enzymes of life-supporting metabolic routes, and transiently stored in vacuole to reduce the heavy metal concentration in cytosol, thus, heavy metal detoxification was attained (Ortiz *et al*., 1995).

Plants defenses to metal toxicity may constitute different strategies. First is either the avoidance of metal entry into the cell via exclusion or binding of metal to cell wall andother ligands. Secondary defense system constitutes various antioxidants to combat increased production of reactive oxygen species (ROS) caused by metal. The tripeptide GSH (γ-Glu-Cys-Gly) is found in very high concentration within cell constituting major fraction of non-protein thiols (Mishra *et al*., 2006). GSH plays a key role in protecting membranes to damage by free radicals by trapping them in aqueous phase (Noctor and Foyer, 1998) and as a part of ascorbate–glutathione cycle.

2.4.3 Glutathiones and phytochelatins

Cations of heavy metals are often bound to soil particles in significant amounts because of soil cation exchange capacity. The binding affinity of cations also impedes cation movement in vascular plants, particularly in the negatively charged cells of the xylem. Chelation is generally meant as the process of a cation binding to a compound which results in a neutrally charged complex that can move more freely through a variety of substrates. PCs consisted of only the three amino acids:glutamine (Glu), cysteine (Cys) and glycine (Gly) with the Glu and Cys residues linked through a γ-carboxylamide bond. The PCs form a family of structures with increasing repetitions of the γ-Glu-Cys dipeptide followed by a terminal Gly; (γ-Glu-Cys)n-Gly, where n has

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been reported as being as high as 11, but is generally in the range of 2 to 5 (Fig. 2-1 and 2-2). The PCs are of the general form (γGlu-Cys)n-Gly with size ranging from 1.5-4 kDa. The tripeptide GSH (γ-Glu-Cys-Gly) is found in very high concentration within cell constituting major fraction of non-protein thiols. GSH is not only a precursor for PCs but also plays a key role in protecting membranes to damage by free radicals by trapping them in aqueous phase and as a part of ascorbate–glutathione cycle, participates in the detoxification of other pollutants and defense mechanisms against oxidative stress (Noctor and Foyer, 1998). Enzymes of ascorbate–glutathione play important role in combating the oxidative stress (Shigeoka *et al*., 2002). GSH metabolism involves many reactions where GSH is synthesized, degraded, conjugated or oxidized (Noctor *et al*., 1998). These relative contents of GSH and PCs are indirectly influenced in this way. However, there are numerous physiological, biochemical, and genetic studies have confirmed that GSH is the substrate for PCs biosynthesis. GSH contains sulfur, which is important in the stabilization of the complex via disulfide bonds. The in vivo experiments of Steffens (1990) also supported that exogenous GSH supports PCs synthesis. Enzyme PC synthase is activated by the heavy metal cations; PC synthesis can be observed within 5-15 minutes of exposure to excess metals which PC synthesis proceeds in the presence of cycloheximine until the GSH pool is depleted.

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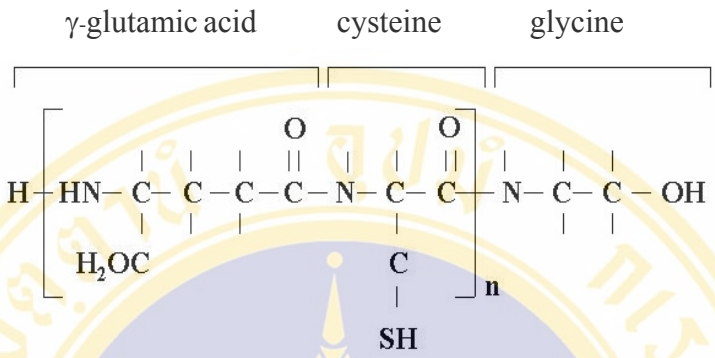

Figure 2-1 Phytochelatins (PCs) consisted of only the three amino acids: (γ-Glu-Cys)n-Gly.

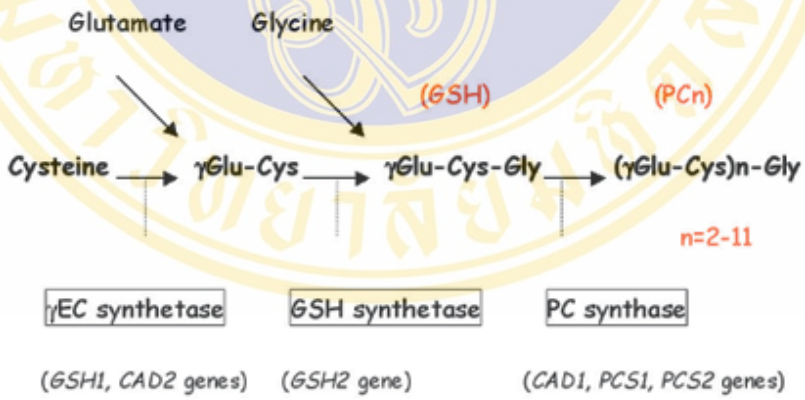

Figure 2-2 Biosynthesis of PCs in higher plants.

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2.4.3.1 Metal-phytochelatin complexes

The stability of the PC-Cd complex is enhanced by additional sulfur ions once the complex is in the vacuole. The experiment of Kneer and Zenk (1997) on the formation of Cd-PC complexes in plant cell cultures showed that sulfide appears to play a significant role in Cd-detoxification of many organisms by acid-labile sulphur (S^{2-}) found as ligand in Cd-PC complexes from many aquatic plants. Sun *et al*. (2005) also reported that PCs can detoxify metals through the formation of metal-PC complexes, presumably stored mostly in the vacuole (Ortiz *et al*., 1995). Beside metal-PC complexes, the buffer system in the plant cell comprises of organic acids, amino acids, phenolic compounds, metal carriers, and metal ligands of low molecular weight proteins, also has an important role in metal complexation and transport in the cell (Verkleij and Schat 1989; Ma *et al*. 1997).

2.4.3.2 Phytochelatin mechanism

The biosynthesis and turnover of metal-PC complexes obtained so far was shown by Peuke and Rennenberg (2005) (Fig. 2-3). Heavy metal ions enter the plant cell through the permeable cell wall. Catalytically inactive PC synthase is immediately activated by the presence of metal ion and uses GSH that is present in the cytosol. It is essential that PC synthase chelates and inactivates every toxic metal ion entering the cytosol before they can poison the enzymes of life-supporting metabolic routes. The metal-PC complex has been shown to protect sensitive metabolic enzymes and is eventually transported to the vacuole where it accumulates and is deposited. The metal can be bound to organic acids prevailing in the vacuole after which the metal-free PC is degraded and the individual amino acid can re-enter the cytosol (Zenk, 1996).

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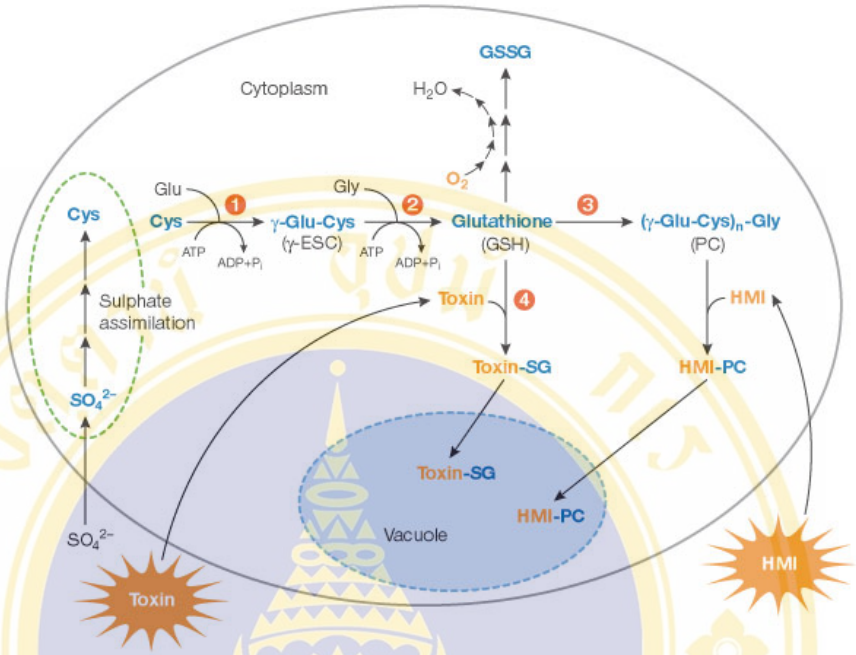

Figure 2-3 Mechanism of detoxification of heavy metals, organic pollutants and oxidative stress in plant cells by GSH. Cys; γ-Glu-Cys, γ-L-glutamyl-L-cysteine; γ-ECS, γ-glutamylcysteine synthetase; GSH, GSSG, oxidized GSH, PC, HMI, heavy metal ion; HMI-PC, heavy metal-PC complex; Toxin, xenobiotics; Toxin–SG, toxin–GSH conjugate. (1) γ-Glutamylcysteine synthetase; (2) GSH synthetase; (3) PC synthase; (4) GSH *S*-transferase (GST). (Peuke and Rennenberg, 2005).

Besides PCs synthesis, in certain plants (notably legumes) which can synthesize homoglutathione, in which β-alanine is substituted for glycine as the terminal amino acid, homophytochelatins are synthesized along with PCs in response to Cd (Klapheck *et al*., 1995). In maize and certain other species of the Poaceae, a third family of PCs has been found in which serine is the carboxy-terminal amino acid (Rauser and Meuwly, 1995). Some species of the family Poaceae synthesize PCs that contain glutamic acid at their C-terminal end (Zenk, 1996).

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2.5 Phytoremediation by wetland plants

2.5.1 Wetland plant characteristics

In general, remediation can be achieved either by removal of the heavy metals or by preventing their spread to surrounding soil and groundwater. The three main objectives for the successful *in situ* immobilization are as follows.

1. To stabilize the vegetation cover and limit metal uptake by crops.
2. To change the metal speciation in soil and thus minimize the possibility of surface and groundwater contamination.
3. To reduce the direct exposure of metal to leaching by rainwater with low pH.

Sorption, ion exchange, and precipitation can be used to convert soluble and preexisting potentially soluble solid phase forms to more geochemically stable solid phases, reducing the metal pool for root uptake (Mench *et al*., 2000).

There are several ways to evaluate the plant metal accumulation efficiency, one of them, transfer coefficients (concentration of metal in dried portion of plant relative to total concentration in the soil) is a convenient way of quantifying the relative differences in bioavailability of metals to plants. Metals such as Cu, Co, Cr, and Pb have low coefficients because they are usually strongly bound to sediment colloids. The discharge of heavy metals in wetlands may result in numerous physical, chemical and biological responses (Moore and Clements, 1984). Most responses depend upon physical and chemical characteristics of wetlands and the prevailing vegetation type. While sediments form primary sinks for heavy metals, in the anoxic zone, may contain very high concentrations of metals in a reduced state. As such, the bioavailability of the metals is low compared to terrestrial systems with oxidized soils. Different forms of metals have different availability: water-soluble metals and exchangeable metals are the most available, metals precipitated as inorganic compounds, metals complexed with large molecular weight humic materials and metals adsorbed to hydrous oxides are potentially available, and metals precipitated as insoluble sulfide and metals bound within the crystalline lattice of minerals are essentially unavailable (Gambrell, 1994). Because of these reducing conditions, the depth to which plant roots can penetrate is limited and this restricts the uptake of contaminants and rhizosphere actions to shallower levels.

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Many ores that are rich in metals consist of sulfide minerals that may form stable sulfide complexes with heavy metals. If sulfides interact with atmospheric oxygen and water, sulfuric acid is formed. Wetland plants can adapt to anaerobic environment in the substrate, they can cope with such situations by transporting oxygen within the tissue from the atmosphere through a lacunar system of intercellular airspaces or through parenchyma to underground organs for root respiration. Primarily maximum uptake of metals in wetland was observed in roots. These roots have been reported to be the most beneficial for phytostabilization of the metal contaminants. Roots of the wetlands play very important role in wastewater purification followed by stem and leaves (Sheoran and Sheoran, 2005). During the growing season, macrophytes communities can contain a substantial metal load which is released on senescence and death. Some macrophytes can tolerate high concentration of several metals in their body mass without showing negative effects on the growth.

In treatment wetlands, the mechanism for removal of metals is primarily immobilization of the sulfide for Cu, Fe, Mn, Zn, Cd. From the previous paragraph that wetlands are productive habitats with anoxic waterlogged soils. Under these conditions, decay of sulfur containing proteins and reduction of natural sulfate in the sediments produce sulfide. Therefore, most wetlands phytoremediation differs from terrestrial phytoremediation where the plants are used to extract and concentrate metals from contaminated soils. Sulfides of most metals are very stable under anoxic water-saturated conditions. In addition, the most abundant heavy metal, iron, forms plaques that are stable in reducing conditions (Horne, 2000).

Wetlands help to prevent the spread of heavy metal contamination from land to the aquatic environment since they are usually at the ecotone (boundary between land and open surface waters). The advantage of constructed wetlands is that they are stable being easy and cheap to construct and operate, therefore they are a suitable alternative for wastewater purification.

Typical species of emergent wetland macrophytes are the common reed (*Phragmites australis*), cattail (*Typha latifolia*), and bulrush (*Scirpus lacustris*). All species are morphologically adapted to growing in a water-logged sediment by virtue of large internal air spaces for transportation of oxygen to the roots and rhizomes (Brix, 1993).

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Salati (1987) studied on heavy metal uptake by water hyacinth (*Eichhornia crassipes*), found that it is a plant with good tolerance and high uptake of nutrients and heavy metals. Cattail and poplar are also used because they are tolerant, grow fast, and attain a high biomass. Pip and Stepaniuk (1992) reported that Zn and Cd were absorbed by *Cyperus esculentus* in oxidized sediments due to their abilities to absorb an tolerate heavy metals. Cheng *et al*. (2002) reported that constructed wetlands with well grown *Cyperus alternifolius* and *Vallarsia exaltata* is an effective tool in phytoremediation of Cd, Cu, Mn, Zn and Pb. They found that about one-third was absorbed predominantly by lateral roots while the rest was removed in the top layer of the sediment.

2.5.2 Wetland plants in phytoremediation

Neralla *et al.* (1999) grew plants in microcosms at a sub-surface flow constructed wetland to evaluate the importance of phytoremediation in improving water quality and the adaptability of different aquatic plants to grow in domestic wastewater from septic tanks. An initial screening of 20 plant species based on their ability to grow well in the wastewater, for experimentation on phytoremediation. *Typha* and *Cyperus* were the best performers whereas, *Cyperus alternifolius* seemed highly desirable because of its high water requirement, resistance to insects, ability to grow throughout the year, winter hardiness, absence of a period of dormancy and rapid recovery from frost damage.

Sao *et al*. (2007) grown *Cyperus rotundas* L. in Cd solution and Cd-Zn contaminated soil and found that it could accumulate Cd in both shoots and roots to a higher level than *Axonopus compressus*. These results might be because *C. rotundas* has tubers that effectively accumulate nutrient and metals (Baghour *et al.,* 2002) and *A. compressus* produced higher phytomass, therefore the value of Cd accumulated per gram dry weight was lower than in *C. rotundas*. The ability of *C. rotundas* and *A. compressus* to accumulate Cd depends on the Cd concentration and exposure time. Moreover, growth in Cd solution and Cd-Zn contaminated soil showed that the percentage of Cd accumulated in the root was higher than that in the leaf.

Buttler (2007) evaluate the biological removal and physical stabilization of RDX and TNT range contaminated soil with the addition of *Cyperus esculentus* (Yellow Nutsedge) grass. The initial concentrations for RDX and TNT were 94 mg/kg

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and 137 mg/kg, respectively. After 15 weeks, the results showed that vegetation was a factor in stabilizing RDX and TNT leachate concentrations and the biological degradation accounted for 73% RDX and 23% TNT removal. These decreases in mobility indicate that this technique may stabilize explosive concentrations in addition to increasing degradation

Stoltz and Greger (2002) reported that *Typha* sp. tolerated and enhanced levels of metals in its tissue without serious physiological damage. Metal concentrations are reported to increase in the following order: roots>rhizomes>non-green leaves>green leaves. Under contaminated conditions, the greater proportion of metal taken up by plants was retained in the root.

Lan *et al*. (1992) used *Typha latifolia* to treat wastewater from a Pb/Zn mine and found that it assimilated significant amounts of Pb and Zn, especially in its roots. Pb precipitate may have formed in or on plant roots with little translocation into aerial portions of the plant.

2.5.2.1 Cyperaceae (Sedge family)

2.5.2.1.1 General characteristic of plant in sedge family

Stems are usually solid and triangular in cross-section. Ligules are usually absent. Leaf is sheath closed. Inflorescence is usually subtended by one or more leaf-like involucral bracts. Spikelets are not usually subtended by bracts, individual florets usually subtended by one bract. Perianth is absent or represented by up to 6 scales or bristles. Pericarp and testa are usually free from one another and embryo are surrounded by the endosperm.

Few members of the sedge family hold economic importance as crop plants, but throughout the world these plants hold great regional importance in weaving mats, baskets, screens, and even sandals. Though not normally grown for crops sedges do hold economic importance to agriculture. A substantial amount of sedges are noxious weeds, invading crop fields in all climates of the world. These include species that invade rice paddies, grazing pastures, as well as others. Sedges do however have a considerable amount of ecological importance. They are of extreme importance to primary production as well as an integral part of the hydrologic cycle (Burmeister, 2000)

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2.5.2.1.2 Plant characteristic in genus *Cyperus* sp.

Seedling has leaves similar to those of mature plants but smaller. Stem base is slightly triangular. Midvein region is often pale. First 2-3 leaves emerge simultaneously and are folded lengthwise. Mature plant has erect stems, simple, glabrous and triangular in cross-section. Leaves are 3-ranked, mostly basal, glossy, glabrous and often creased lengthwise. Leaves lack ligules, auricles, and collar regions. Margins finely serrate. Sheaths are closed, membranous, pale green. Roots and underground structures of plants develop extensive systems of rhizomes, tubers, and roots. Rhizomes produce tubers and basal bulbs that bear aerial shoots. Tubers store starch and have several buds that produce rhizomes, which develop more basal bulbs and new plants. Tubers offer a mechanism for asexual reproduction, and they are the major dispersal unit that can survive extreme conditions. Roots often grow to greater soil depths than tubers or rhizomes. Rhizomes are slender, fleshy when young, covered with scales. Post-senescence characteristics, foliage dies back with cool temperatures in fall, but tubers survive and resprout the following spring. (http://www.gardenguides.com/plants/plantguides/grasses)

2.5.2.1.3 *Cyperus iria* L. and *Cyperus flavidus* Retz.

Kingdom *Plantae* – Plants
Subkingdom *Tracheobionta* -- Vascular plants
Superdivision *Spermatophyta* -- Seed plants
Division *Magnoliophyta* -- Flowering plants
Class *Liliopsida* – Monocotyledons
Subclass *Commelinidae*
Order *Cyperales*
Family *Cyperaceae* -- Sedge family
Genus *Cyperus* L. -- flatsedge
Species *Cyperus iria* L. -- ricefield flatsedge
Species *Cyperus flavidus* Retz. (Globe sedge)
(http://plants.usda.gov/index.html).

Habit; *C. iria* is an annuals (occasionally perennials) has 4-65 cm caespitose or rhizome creeping which is often stoloniferous, sometimes tuberiferous or with bulbils (Fig. 2-4a).

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Habit; *C. flavidus* is a perennial grass or grass-like plant, including grasses (Poaceae), sedges (Cyperaceae), rushes (Juncaceae), arrow-grasses (Juncaginaceae), and quillworts (*Isoetes*) family (Fig. 2-4b).

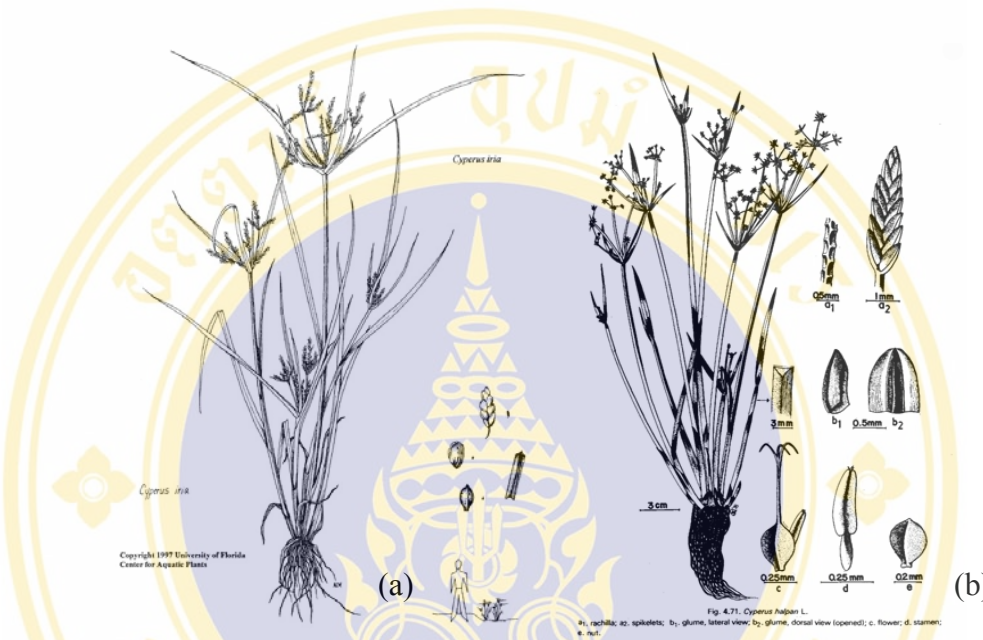

Figure 2-4 *Cyperus iria* (a) and *Cperus flavidus* (b)

2.5.2.2 Typhaceae (Cattail family)

2.5.2.2.1 General characteristic of plant in cattail family:

Cattails are aquatic or marsh herbs with creeping rootstocks long narrow leaves and tiny flowers crowded in terminal spikes. Spikes above bracts are which fall early (http://www.wildflower.org/plants).

Floral features are actinomorphic and unisexual/ monoecious. Inflorescence is a double spadix with female flowers on the bottom and male flowers at the apex. Each inflorescence of female and male flowers are subtended by a deciduous bract (spathe and borne on a long peduncle. Fruit feature is an acene or follicle, seed feature is present with endosperm. Vegetative features which habit is as a tall perennial herb in wet, marshy soils. Leaves are simple, entire, long and strap-shaped and alternate/basal. Sheathing is present at the base.
(http://botit.botany.wisc.edu/courses/systematics)

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Cattails are always found in or near water, in marshes, ponds, lakes and depressions. They are obligate wetland indicator plant species. Cattails tolerate perennial flooding, reduced soil conditions and moderate salinity. With influxes of nutrient or freshwater, cattails are aggressive invaders in both brackish salt marshes and freshwater wetlands.

Ecologically, cattails tend to invade native plant communities when hydrology, salinity, or fertility change. In this case they out compete native species, often becoming monotypic stands of dense cattails. Maintaining water flows into the wetland, reducing nutrient input and maintaining salinity in tidal marshes will help maintain desirable species composition. If cattails begin to invade, physical removal may be necessary. Other uses; wildlife, wetland restoration, wastewater tertiary treatment, edible (young shoots, base of stem, flower stalks, pollen, rhizomes), baskets, matting, bedding material, ceremonial bundles, caulking material, and cordage.
(http://www.gardenguides.com/plants/plantguides/grasses)

2.5.2.2.2 *Typha latifolia* (Cattail)

Kingdom *Plantae* – Plants
Subkingdom *Tracheobionta* – Vascular plants
Superdivision *Spermatophyta* – Seed plants
Division *Magnoliophyta* – Flowering plants
Class *Liliopsida* – Monocotyledons
Subclass *Commelinidae*
Order *Typhales*
Family *Typhaceae* - Cattail family
Genus *Typha* L. - cattail
Species *Typha angustifolia* L.- narrowleaf cattail

Habit is a vascular plant without significant woody tissue above or at the ground. Forbs and herbs may be annual, biennial, or perennial but always lack significant thickening by secondary woody growth and have perennating buds borne at or below the ground surface. In plants, graminoids are excluded but ferns, horsetails, lycopods, and whisk-ferns are included (Fig. 2-5) (http://plants.usda.gov/index.html).

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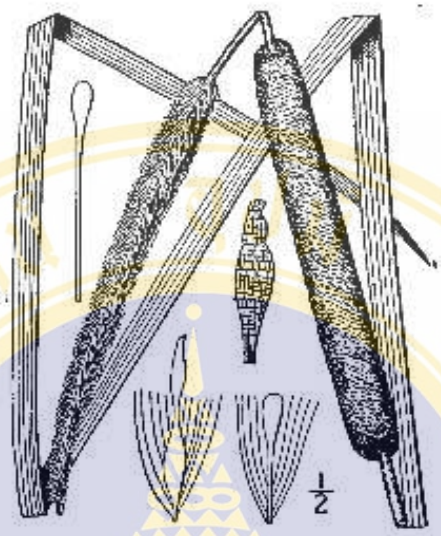

Figure 2-5 *Typha angustifolia*

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CHAPTER III
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plant materials

Cyperus iria and *C. flavidus* were collected from the open pit mine at Bo Ngam lead mine located in Thong-Pha-Phum District, Kanchanaburi Province, Thailand. Propagated in greenhouse for hydroponic study (heavy metal accumulation efficiency and GSH, PCs analysis).

Typha angustifolia were collected from the sedimentary pond at Mahidol University, Salaya Campus for constructed wetland batch study (Pb accumulation efficiency).

3.1.2 Media Preparation

3.1.3.1 Modified Hoagland's solution

Modified Hoagland's solution is the nutrient solution (Hoagland and Arnold, 1950) modified by changing the concentration of phosphate from 0.2 mM to 0.01 mM. The composition of the Hoagland's solution was described as follows:

1M KH_2PO_4	0.01	mL
1M KNO_3	1	mL
1M $Ca(NO_3)_2$	1	mL
1M $MgSO_4$	0.40	mL
Micronutrient	0.02	mL
5 ppm $FeCl_3 \cdot 6H_2O$	0.02	mL
1M KCl	0.0185	mL
Deionized water	1,000	mL

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The final pH of the solution was adjusted to 5.5 with HCl or NaOH.

3.1.3.2 Chemical standards

Glutathione reduced standard (Fluka)

Phytochelatin standard : PC_2, PC_3 (synthesized from ANASPEC)

3.1.3 Instruments

The instruments used in this study were as follows:

3.1.4.1 Centrifuge: Eppendorf 5417R

3.1.4.2 Flame Atomic Absorption Spectrophotometry (FAAS): SpectrAA 55B

3.1.4.3 Freeze dry: Supermodulyo freeze dryer

3.1.4.4 High Performance Liquid Chromatography (HPLC): Water 2690

3.1.4.5 Fume Cupboard

3.1.4.6 pH meter:

Hanna Instruments, model pH 211 micro process

3.1.4.7 Spectrophotometer: Aquarius, Cecil model CE 7200

3.1.4.8 Dry Block heater: Thermolyne

3.2 METHODS

3.2.1 Seasonal study

3.2.1.1 Site Description

The study area, Bo Ngam lead mine (47.8 km^2), was located in Klity village, Thong Pha Phum district, Kanchanaburi province, Thailand (north latitude 14°55'-14°60' and east longitude 98°55'- 98°60'; Fig.3-1). The average annual temperature is 26.7°C and the average annual rainfall is 1744.3 mm (Thai Meteorological Department). Bo Ngam lead mine was an open pit mine that ceased operation in 1996 and since 2005 was under the restoration project. Plant diversity in Bo Ngam lead mine is high and the secondary succession process in disturbed areas

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seems to be very fast. The rehabilitation project started in 2003, when several plants had started to colonize the bare mine spoiled land.

The mineral deposits in Thong Pha Phum area consist mainly of lead and zinc ores. The primary ore is lead sulfide and the secondary ores are lead carbonate (cerussite:$PbCO_3$) and lead sulfate due to chemical alteration with host rock and oxidation process along contact zone with limestone.

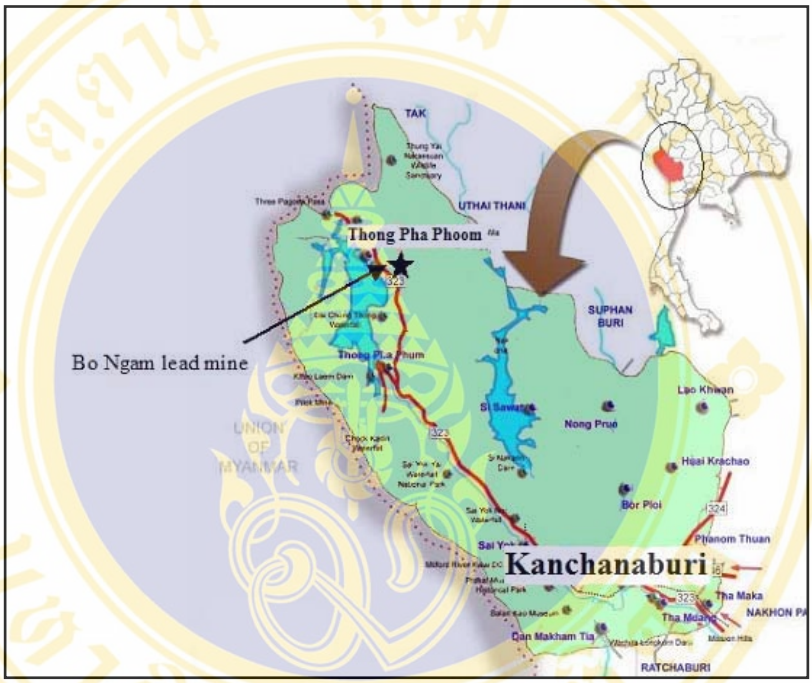

Figure 3-1 The map of Thailand showing Kanchanaburi province and the Bo Ngam lead mine study site.

3.2.1.2 Sampling sites

In order to compare the characteristics of the various plant communities, quadrat samples were set up in two different sampling sites at the open pit mine area; the pond site (PS) and land site (LS). The pond site was on the bank of the open pit pond where soil with lead carbonate was dug up for mining. The land site was about 500 m away from the pond on an approximately 30° slope. Two sampling plots of 10x10 m, one for each sampling site, were sampled at 3-month intervals (January, April, July, and October 2004). Each plot was divided into 100 quadrats (1x1m). Ten quadrats of each plot were randomly selected to study for plant diversity, evenness and frequency, and lead concentrations in plants and soil (Fig.3-2).

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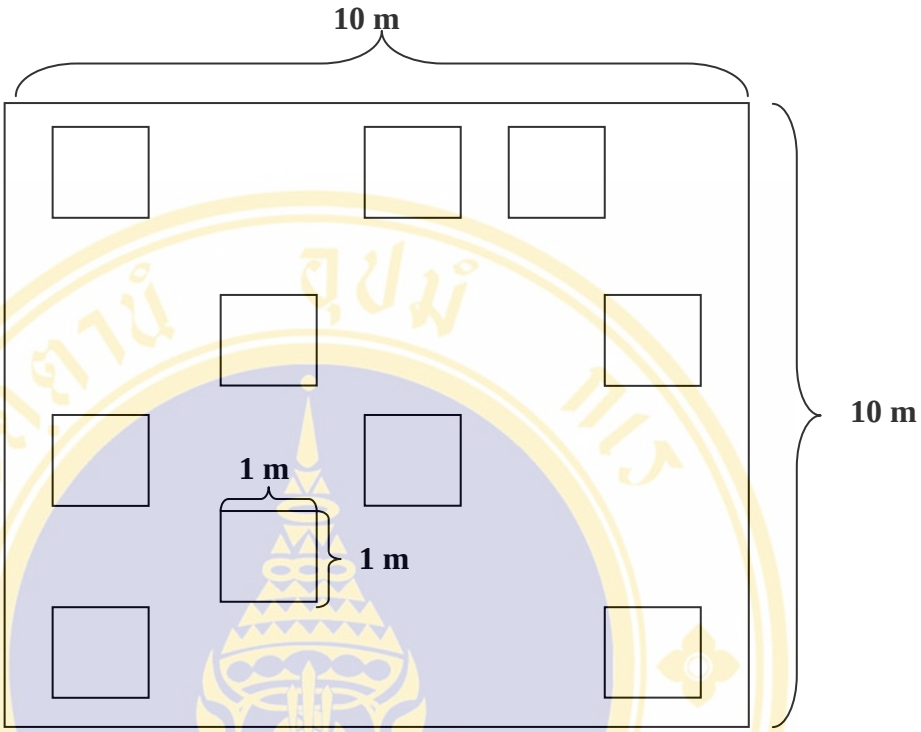

Figure 3-2 Sampling plot 10x10 m for each sampling site.

3.2.1.3 Soil sampling and analysis

At both sampling sites (PS and LS), soil samples around the plant roots were collected. After collection, they were dried at 60°C for 48 h, then ground into fine powder and sieved through a 0.28 mm nylon sieve. Soil samples were sent to the Department of Soil Science, Faculty of Agriculture, Kasetsart University for characterization of pH, electrical conductivity (EC), organic matter content, total N, available P, available K and texture. For analysis of soil lead content, 0.5 g of soil sample was digested with nitric acid (69% HNO_3:BDH) (APHA, 1998). After digestion, lead concentration was determined by a flame atomic absorption spectrophotometer (FAAS ; Varian SpectraA 55 B).

3.2.1.4 Plant sampling and analysis

Three individuals of each plant species were randomly collected within the 10 sampling quadrats of each sampling site. Plant samples for identification (whole young plants included flower) were kept in a plant press and classification

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followed that of Smitinand. Plant identification was confirmed by the Department of Botany, Kasetsart University, Bangkok, Thailand.

After collection, soil around the plant roots was separated and analyzed for lead concentration, while each plant sample was thoroughly washed with a solution of phosphate-free detergent for 15s followed by tap water for another 15s, rinsed with deionized water and separated into shoots and roots. Samples were dried in 60°C oven for 48 h and ground into fine powder before sieved through a nylon sieve. For lead analysis, each 0.5 g plant sample was digested with nitric acid (69% HNO_3:BDH) and lead concentration was determined by FAAS.

3.2.1.5 Translocation factor and Phytoextraction coefficient

Translocation factor (TF) was defined as the ratio of the heavy metals concentration in plant shoot to that in plant root. A translocation factor > 1 indicates preferential partitioning of metals to the shoot (Mattina *et al*., 2003).

Phytoextraction coefficient was defined as the ratio of lead concentration in plant shoot to lead concentration in the soil. The phytoextraction coefficient can be used to evaluate the ability of plant to accumulate the heavy metal (Rotkittikhun *et al*., 2006).

3.2.1.6 Plant community study

The Shannon-Wiener index was calculated to indicate the diversity of the plant community and to examine the variation in diversity throughout the one year.

Shannon-Wiener heterogeneity index (H′)

$$H' = - \Sigma p_i \ln p_i$$

where, p_i = the proportion of individuals found in the *i* th species

H′ indicates the relationship between species richness and the number of individuals (abundance) of each species for any given plant community. For normal communities it typically falls between 1.5 and 3.5 (Shannon and Weaver, 1949).

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3.2.1.7 Statistical analysis

Statistical analyses of the data were performed with an analysis of variance (One-Way ANOVA) to assess the seasonal variation of lead concentration in soil between two sampling sites, lead accumulation in shoots, roots, and soil of each plant species.

3.2.2 Hydroponic study

3.2.2.1 Heavy metal accumulation in plants

Plant samples (*Cyperus iria*, *Cyperus flavidus*) were collected and acclimatized in modified Hoagland's solution under pH 5.5 for one week. Treated plant samples with lead, cadmium and zinc by varying concentrations in modified Hoagland's solution, pH 5.5. The concentrations used for the three metals were:

0, 10 and 20 mg/L of $Pb(NO_3)_2$

0, 0.25 and 0.5 mg/L of $Cd(NO_3)_2$

0, 10 and 20 mg/L of $ZnSO_4$

Plant samples fresh weights were recorded before treatment. The solutions were changed every 3 days during the experiment period (15 days) and were filtered through 0.45 μm glass microfiber filter paper (Whatman no.42). Plant samples were thoroughly washed with deionized water and separated into shoots and roots. The samples were then oven dried at 60°C for 48 h. ground into fine powder. The concentrations of lead, cadmium and zinc in shoots and roots of plants were determined using 0.5g plant sample digested with nitric acid. The heavy metal concentrations in plants and solutions were measured by FAAS.

3.2.2.1.1 Relative Growth Rate (RGR)

RGR was calculated according to Hunt (1992);

$$RGR = \frac{[\ln(W_2) - \ln(W_1)]}{(t_1 - t_2)}$$

where, W_1 and W_2 are plant fresh weight (g) at time t_1 and t_2

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3.2.2.1.2 Percentage metal uptake

Percentage metal uptake was calculated from

$$\% \text{ uptake} = \frac{[(C_0 - C_1)]}{C_0} \times 100$$

where, C_0 and C_1 are initial and remaining concentrations of metal in medium (mg/l) (Abdel-Halim *et al*.,2003)

3.2.2.1.3 Bioaccumulation coefficient (BC)

BC was calculated according to Nanda-Kumar *et al*. (1995);

$$BC = \frac{\text{Heavy metal concentration in plant}}{\text{Heavy metal concentration in solution}}$$

3.2.2.2 Glutathione and Phytochelatin analysis

Cyperus iria was treated with lead, cadmium and zinc in modified Hoagland's solution, pH 5.5. The concentrations used for the three metals were: 20 mg/L of $Pb(NO_3)_2$, 0.5 mg/L of $Cd(NO_3)_2$, and 20 mg/L of $ZnSO_4$.

Glutathione and phytochelatin contents were analyzed on day 0, 1, 2, 4 and 8. The experimental period was 8 days.

3.2.2.2.1 Tissue preparation

Plant tissues were extracted followed the procedures of De Knecht *et al*. (1995). The 20 mg of lyophilized plant tissues were homogenized in 1 mL of ice-cold, 30mM Tris-HCl, 10mM β-mercaptoethanol and 100 mg of polyvinyl pyrrolidone, pH 8.0 at 0ºC, using mortar and pestle. The crude extract was centrifuged at 10,000x g for 10 min at 4ºC. After centrifuged 600 µL of the supernatant fluid was mixed with 200 µL of 5% (w/v) 5-sulphosalicylic acid (SSA) solution. The sample was filtered over 0.45 µm syringe filter and stored at -80ºC.

3.2.2.2.2 Derivatization

The supernatant was subjected to derivatization by SBD-F. The 200 µl of supernatant aliquot was derivatized in 12 µl of 10% TBP in DMF. A 10-min period was allowed for reduction after which 80 µl of 0.1M borate buffer containing 2.0 mM EDTA (pH 9.5), 16 µl of SBD-F solution and 8 µl of 1M NaOH

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were added. The reaction was allowed to proceed at 60°C for 60 min and then stopped by adding 20 µl of 5M HCl.

3.2.2.2.3 HPLC analysis

The HPLC analysis followed that of Tang *et al*. (2000). A Waters HPLC was used to measure thiolic compounds. This system was equipped with a separations module (Waters 2690) and scanning fluorescence detector (Waters 474). The system was controlled by Waters Millenium 32 software. The absorbance detector and the fluorescence detector were connected in series after the separation column. The thiol-SBD adducts after passing through a C_{18} guard column are separated using a reversed-phase C_{18} column (Waters Symmetry, 250 mm x 4.6 mm, particle size 5 µm). Mobile phase A contained 0.1% TFA in water and mobile phase B was acetonitrile, complementary to the TFA solution (A% = 100%-B%). A gradient elution using acetonitrile was performed for better analytical separation and column cleaning prior to subsequent injections. The elution profile was: 0-1 min, isocratic 10% B; 1-12 min, 10-15% B; 12-14 min, 15-100% B; 14-18 min, isocratic 100% B; 18-20 min, 100-10% B; 20-25 min, isocratic 10%B. In all steps, the gradients used are linear. The separation was carried out at room temperature using a flow rate of 1.0 ml/min. The fluorescence detector was set to 385 nm in the excitation and 515 nm in the emission modes. Reduced glutathione and phytochelatins were quantified comparing with the standard and the peak areas were integrated using Millenium[32] software.

3.2.3 Batch experiment on wetland plants

3.2.3.1 Plants preparation

The accumulation efficiency of Pb in 2 wetland plant species was compared:

- *Cyperus iria* from Bo Ngam lead mine, cultured in green house for 2 months. The beginning fresh weight, shoot and root lengths were recorded.
- *Typha angustifolia* from sedimentary pond at Mahidol University, Salaya Campus. The plant samples were thoroughly washed with tap water and rinsed with deionized water. The

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shoots were cut to 80 cm in length. The beginning fresh weight, shoot and root lengths were recorded.

3.2.3.2 Soils preparation

Pb contaminated soil from the open pit area of Bo Ngam lead mine was brought into the laboratory. The pH of the soil at open pit mine was adjusted to 5-6 by citric acid after air dried. The soil was mixed thoroughly with water and then air dried for another 2 weeks. Horticulture soil purchased from the store served as control.

The soil 300 kg were put in each glass aquarium (60 x 120 x 50 cm) until the height of 30 cm was attained. The aquarium was divided into 3 partitions; each partition was filled with 100 kg soil. There were a total of 6 aquaria for each plant species (3 for controls and 3 for treated groups).

3.2.3.3 Plants cultivation

For *C. iria*, 12 plants each were put in the aquarium with 4 plants/partition. For *T. angustifolia*, 6 plants each were put in the aquarium with 2 plants/partition. Each treatment was performed in triplicate. After the plants were put in, water was added to the aquarium to attain the height of 10 cm from the soil surface (27°C, light intensity = 20,000 Lux). The water level was kept constant during the experiment.

3.2.3.4 Data collection

Plant, soil, and water samples were collected every month for 3 months. Fresh weight, shoot and root lengths were recorded. The pHs of soil water in surface and soil surface were measured at the time of sample collection. Tiller production which defined as number of tiller per plant were also observed. The Pb concentrations in plant, soils and water samples were analyzed by FAAS.

3.2.3.5 Soil Sampling and analysis

Soil samples were collected at different levels (10, 20 and 30 cm from soil surface). After collection, they were dried at 60°C for 48 h, then ground into fine powder and sieved through a 0.28 mm nylon sieve. For analysis of soil lead content, 0.5 g of soil sample was digested with nitric acid (69% HNO_3:BDH). After digestion, lead concentration was determined by FAAS.

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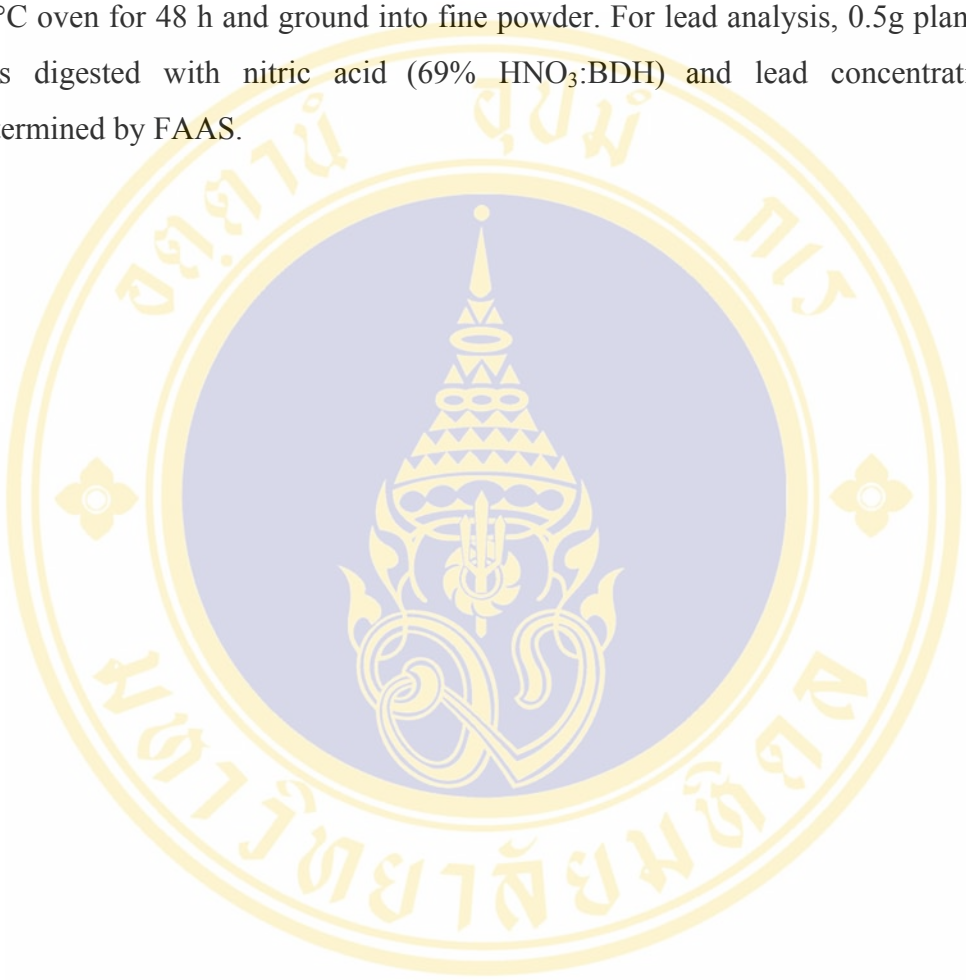
3.2.3.6 Plant Sampling and analysis

After collection, each plant sample was thoroughly washed with a solution of phosphate-free detergent for 15s followed by tap water for another 15s, rinsed with deionized water and separated into shoots and roots. Samples were dried in 60°C oven for 48 h and ground into fine powder. For lead analysis, 0.5g plant sample was digested with nitric acid (69% HNO_3:BDH) and lead concentration was determined by FAAS.



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CHAPTER IV
RESULTS

4.1 Seasonal study

4.1.1 Soil characterization and soil lead concentration

The soil textures of both sampling sites (pond site, PS and land site, LS) were sandy loam except for the subsurface soils at the pond site which were silt loam and loam. The soil pH was slightly higher at LS (7.7) than PS (6.8). The highest electrical conductivity (EC) values at PS and LS were 0.16 and 0.22 dS/m, respectively, and the lowest values were 0.15 and 0.2 dS/m, respectively. The percentage of total nitrogen from both sites did not differ (0.11-0.12), though the soil at the PS had more available phosphorus (7-14 ppm at PS, 2-3 ppm at LS). The organic matter (0.13-0.2) of soils from both sites was quite similar. Soil collected from the LS had higher Pb concentration (about 110,000 mg/kg) than that from the PS (about 12,000 mg/kg) (Table 4-1).

The relative humidity was stable, ranging from 60% in March to about 85% in July (Fig. 4-1). In general, there are 2 seasons in Thailand : dry and wet seasons. The dry season (with the average monthly rainfall of 10-50 mm and the relative humidity of 60-70 %) is from November to April. The wet season (with the average monthly rainfall of 300-400 mm and the relative humidity of 70-80%) is from May to October. Lowest Pb concentrations in soils from both sampling sites were found in July during the wet season (6,256.9 mg/kg from PS and 79,611.1 mg/kg from LS) and highest in October the end of wet season and during the dry season (8,086.8 mg/kg for PS and 111,722.2 mg/kg for LS) (Fig. 4-1). The fluctuation of Pb concentrations in soil at both sampling sites were inversely related to the monthly rainfall but directly affected to the relative humidity all year.

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4.1.2 Seasonal variation in plant diversity

A total of 22 species of plants, from 12 families were recorded on both study plots combined, including herbs, shrubs, and grasses (Table 4-2). The highest number of individual plant species was found in October, whereas the lowest was in April for both sites. The numbers of plant species were directly related to the monthly rainfall and the relative humidity. At PS, 17 plant species (10 families) were found, including some wetland and fern species. Ten species were found all year. At LS, 17 plant species (8 families) were found with 9 species present all year. Twelve species were common at both sampling sites (*Ageratum conyzoides*, *Buddleja asiatica*, *Conyza sumatrensis*, *Equisetum debile*, *Imperata cylindica*, *Mimosa pudica*, *Neyraudia reynaudiana*, *Paspalum conjugatum*, *Phragmites karka*, *Sonchus arvensis*, *Thysanolaena maxima*, and *Vigna umbellate*). *E. debile* had the higher number of individuals at both sites. Most of these plants were perennials (16 species) while the remainder were annuals (6 species).

The Shannon-Wiener diversity index (H′) (Fig. 4-2) was not significantly different all year for both sampling sites ($P>0.05$) except for April. In April, the diversity index at LS was lower than PS (2.27 and 2.64, respectively). The H′ value in January and April at both sampling sites rather lower than in July and October may be related to the monthly rainfall and the relative humidity in this area (Fig. 4-1). The highest H′ was found in October (2.75 and 2.67 at PS and LS, respectively).

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Table 4-1 Characteristics of soils from low (PS) and high lead concentration (LS) areas.

Sites	Depth	pH	EC[a] (dS/m)	Total N (%)	Available P (ppm)	Available K (ppm)	Total Pb (mg/kg DW)	Extractable Pb (mg/kg DW)	Soil Texture				OM[b] (%)
									% Sand	% Silt	% Clay	Texture	
PS	0-10 cm	6.8	0.15	0.12	14.00	20	11,345.2 ± 6,214.92	406.07 ± 12.36	60.33	32.33	7.33	SL	0.13
	10-40cm	6.77	0.16	0.11	7.33	20	14,113.4 ± 10,802.30	876.67 ± 8.33	37.67	49.67	12.67	SiL	0.13
LS	0-10 cm	7.67	0.20	0.11	3.00	20	100,820 ± 42,079.83	1,814.67±43.10	75.00	23.00	2.00	SL	0.17
	10-40 cm	7.77	0.22	0.12	2.67	20	117,700 ± 30,121.59	1,985.33±25.01	67.00	27.00	6.00	SL	0.20

EC[a] = Electrical conductivit

OM[b] = Organic matter

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Table 4-2 Seasonal variation in number of plant found for each species at pond site (PS) and land site (LS): A = Annual plant; P = Perennial plant

Family	Plant species	Duration	January		April		July		October	
			PS	LS	PS	LS	PS	LS	PS	LS
Buddlejaceae	*Buddleja asiatica*	P	2	8	0	4	0	9	2	6
Commelinaceae	*Commelina diffusa*	A	0	0	0	0	0	2	0	2
Asteraceae	*Ageratum conyzoides*	A	11	0	4	21	10	13	3	3
	Chromolaena odoratum	P	0	2	0	0	0	9	0	26
	Conyza sumatrensis	A	15	15	3	22	7	22	9	18
	Sonchus arvensis	P	21	22	31	21	35	88	14	62
Cyperaceae	*Cyperus iria*	A	76	0	69	0	72	0	66	0
	C. flavidus	P	0	0	0	0	12	0	9	0
Equisetaceae	*Equisetum debile*	P	77	153	162	250	237	196	212	277
Poaceae	*Crassocephalum crepidioides*	P	0	3	0	0	0	11	0	9
	Imperata cylindica	P	24	12	210	16	39	23	58	7
	Neyraudia reynaudiana	P	66	48	87	124	56	80	54	59
	Paspalum conjugatum	P	0	49	0	45	102	61	90	91
	Pennisetum polystachyon	P	0	0	0	4	0	1	0	3
	Phragmites karka	P	45	19	68	45	60	46	52	39
	Thysanolaena maxima	P	16	1	29	0	22	4	32	6
Mimosaceae	*Mimosa pudica*	P	6	18	27	49	26	90	22	89
Ophioglossaceae	*Ophioglossum* sp.	A	12	0	20	0	52	0	153	0
Papilionaceae	*Vigna umbellata*	A	2	1	76	0	24	1	28	1
Parkeriaceae	*Pityrogramma calomelanos*	P	5	0	6	0	9	0	7	0
Pteridaceae	*Pteris vittata*	P	8	0	7	0	4	0	4	0
Orchidaceae	*Eulophia* sp.	P	0	0	0	0	0	16	0	9

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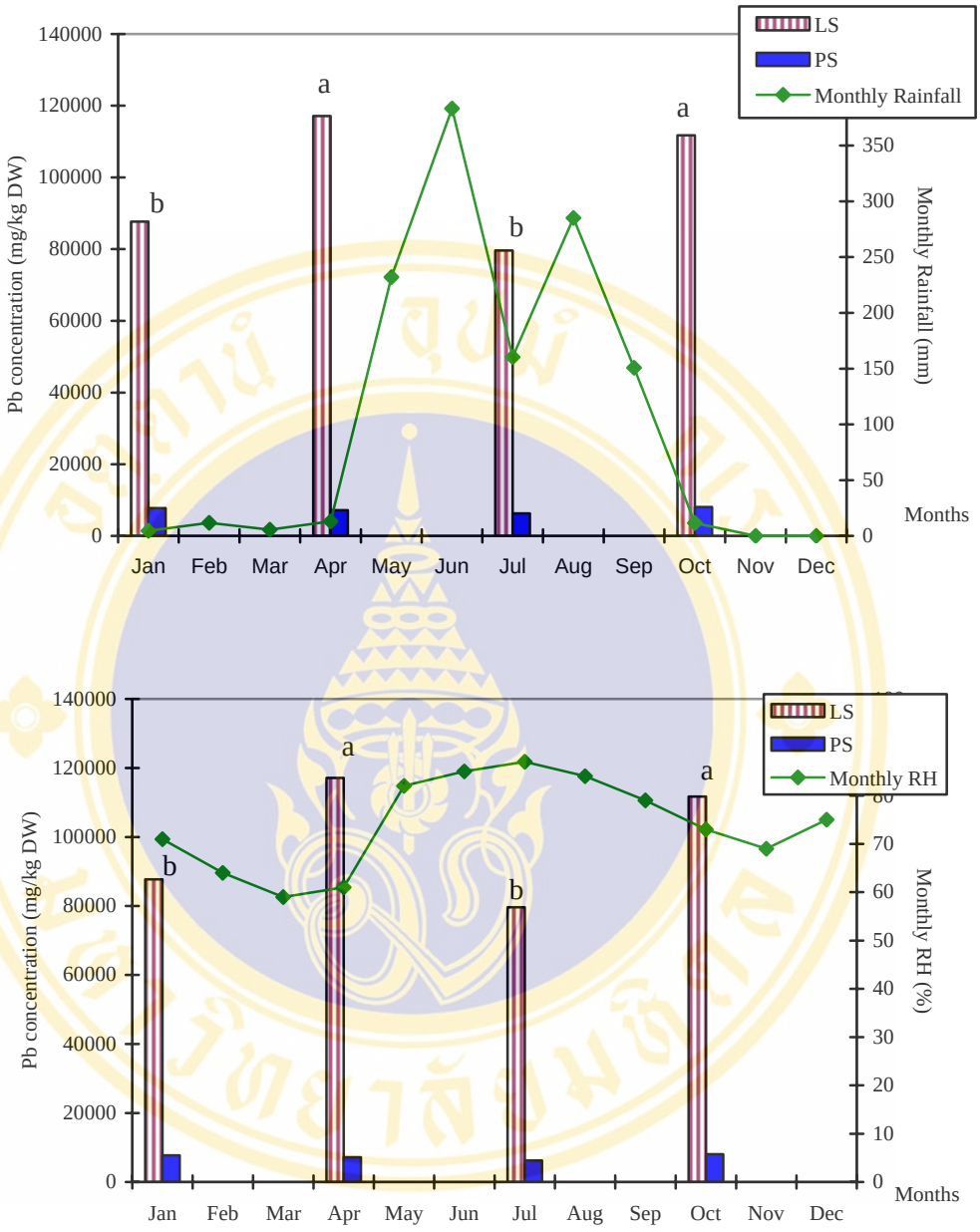

Figure 4-1 Seasonal variation in soil lead concentration in relation to monthly rainfall and relative humidity.

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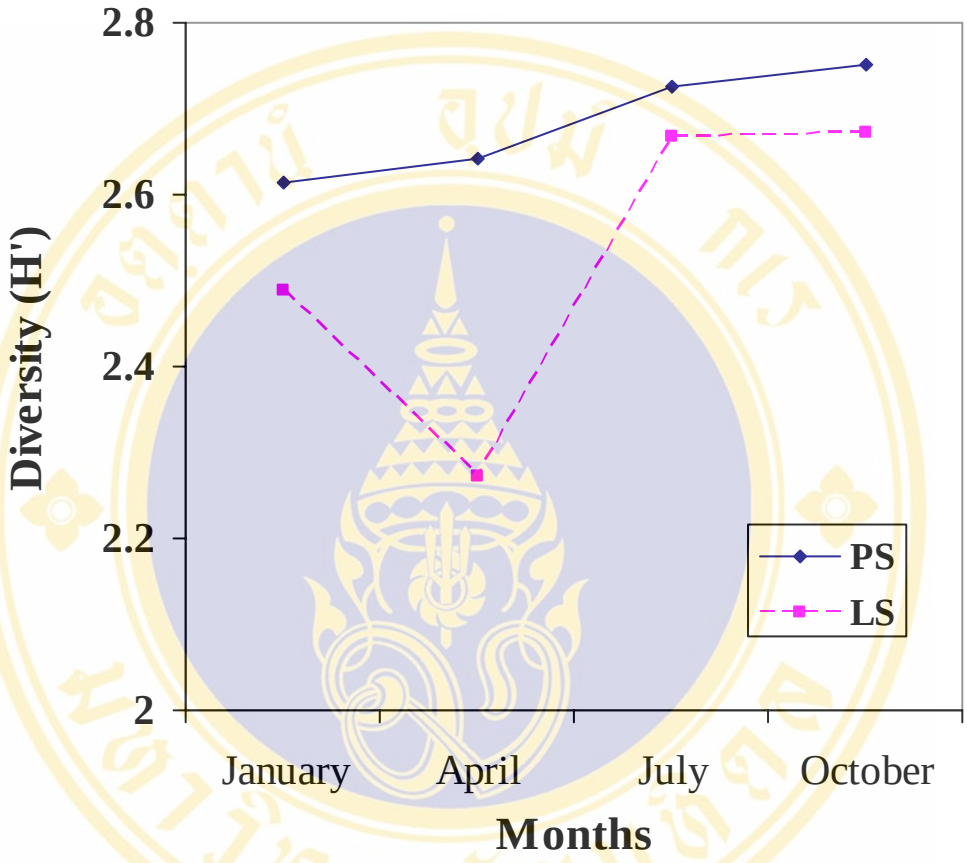

Figure 4-2 The changes of homogeneity index (H') during one year.

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4.1.3 Seasonal variation of lead accumulation in plants

The seasonal variation of Pb concentrations in plants from LS and PS was different even in the same species and time of collection (Table 4-3 to 4-5). The results clearly demonstrate the seasonal variation of plant accumulation. Thirteen plant species at both LS and PS could be found all year (Table 4-3 to 4-5). Pb concentration in whole plants varied between sampling times with remarkably high concentrations in the dry season (October to April) and reduction in the wet season. For example, 5,466.7 mg/kg DW in shoot of *I. cylindrica* at LS and 278.3 mg/kg DW in shoot of *N. reynaudiana* at PS during the dry season as compared to 296.7 mg/kg DW in shoot of *I. cylindrica* at LS and 41.7 mg/kg DW in shoot of *N. reynaudiana* at PS during the wet season (Table 4-3).

4.1.4 Choice of lead accumulator

The phytoextraction coefficient of plant species at both sampling sites varied between 0.01-0.12. The *B. asiatica* at PS had the highest phytoextraction coefficient of 0.12. When comparing the same plant species at PS and LS, the plant species at PS had higher phytoextraction coefficient than at LS (Table 4-6).

There were a total of 17 plant species that had Pb accumulation in shoot > 1,000 mg/kg at one or more sampling times (Table 4-2). Among these species, only six species showed a TF > 1, i.e., *A. conyzoides*, *B. asiatica*, *C. odoratum*, *C. sumatrensis*, *M. pudica*, and *S. arvensis* (Table 4-6). Thirteen of 17 perennial plant species and four out of six annual plant species had Pb accumulation in their shoots > 1,000 mg/kg and/or TF more than 1 (Table 4-2 and 4-6).

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Table 4-3 Seasonal variation of lead accumulation (mg/kg DW) (mean ± SD; n = 3) in plant shoot collected from land site (LS) and pond site (PS)

Plant species	Site	Duration	Lead accumulation in shoot (mg/kg DW)			
			January	April	July	October
A. conyzoides	LS	A	-	4850±3944	1375±566	-
	PS		400±57	346±168	738±210	-
B. asiatica	LS	P	1128±84b	858±188b	3023±1835a	1153±537b
	PS		293±97	-	-	730±680
C. odoratum	LS	P	1595±13	-	1983±404	2783±29
C. diffusa	LS	A	-	-	3533±2 872	1700±1083
C. sumatrensis	LS	A	1505±9a	7017±6605a	2700±854a	7033±3364a
	PS		509±269	-	1030±467	458±41
C. crepidioides	LS	P	2369±293	-	1292±342	688±445
	PS		-	-	-	62±8
C. flavidus	PS	P	-	-	128±92	192±19
C. iria	PS	A	208±43a	323±173a	223±153a	227±84a
E. debile	LS	P	1585±10b	6133±2 136a	497±141b	432±128b
	PS		160±40a	127±86a	208±102a	73±41a
Eulophia sp.	LS	P	-	-	3288±2 591	2050±300
I. cylindrica	LS	P	1395±351bc	5467±1436a	297±119c	1967±778b
	PS		182±104a	63±8b	33±28b	45±9b
M. pudica	LS	P	1542±14b	1087±1008b	2021±1308b	5267±1620a
	PS		193±59a	80±30b	118±36b	97±20b
N. reynaudiana	LS	P	612±172b	618±266b	674±138b	8033±4389a
	PS		130±9ab	278±163a	137±76ab	42±10b
Ophioglossum sp.	PS	A	-	576±217	682±195	963±159
P. conjugatum	LS	P	1068±469b	5400±3601a	1305±378b	3367±1985ab
	PS		-	-	160±35	1517±1294
P. polystachyon	LS	P	-	1600±229	-	412±182
P. karka	LS	P	695±365ab	643±270ab	203±60b	1030±467a
	PS		92±54b	333±231a	55±9b	95±27b
P. calomelanos	PS	P	400±40b	987±475a	287±203b	323±188b
P. vittata	PS	P	439±202a	377±65a	238±80a	275±103a
S. arvensis	LS	P	1592±6b	2900±2594ab	8967±6181a	7133±3116ab
	PS		520±232b	252 ±60b	218±180b	920±123a
T. maxima	LS	P	805±438	-	1827±1625	6017±5360
	PS		197±54a	213±19a	65±15b	92±24b
V. umbellata	LS	A	-	-	475±104	7467±6593
	PS		92±18	652±519	396±169	-

Means followed by a common letter in the same row for each metal are not significantly different from each other using LSD test (P > 0.05).

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Table 4-4 Seasonal variation of lead accumulation (mg/kg DW) (mean ± SD; n = 3) in plant root collected from LS and PS.

Plant species	Site	Duration	Lead accumulation in root (mg/kg DW)			
			January	April	July	October
A. conyzoides	LS	A	-	3204±697	2405±935	-
	PS		2125±1237	1944±1425	1250±250	-
B. asiatica	LS	P	1117±398b	3229±839a	1700±654b	1917±1077ab
	PS		575±136	-	-	1465±384
C. odoratum	LS	P	3708±1841	-	1404±609	806±222
C. diffusa	LS	A	-	-	23479±8071	5950±3054
C. sumatrensis	LS	A	2368±886b	10421±1736a	2746±1364b	4750±2179b
	PS		2400±736	-	2833±2 073	418±45
C. crepidioides	LS	P	8250±2833	-	1363±456	873±581
	PS		-	-	-	150±0
C. flavidus	PS	P	-	-	2361±743	3700±1735
C. iria	PS	A	3300±826a	4333±382a	1652±180b	3583±722a
E. debile	LS	P	8050±25a	41750±9331b	7542±1438a	8567±2725a
	PS		6366±3096a	4443±1164ab	1377±209b	3383±1651ab
Eulophia sp.	LS	P	-	-	5200±1998	11833±4980
I. cylindrica	LS	P	4708±2872b	14028±8461a	1400±922b	2358±101b
	PS		2458±1778a	1958±191ab	1048±356ab	237±187b
M. pudica	LS	P	7421±2040a	3778±440b	3269±1411b	5039±2055ab
	PS		917±217b	2583±1010a	375±139b	857±643b
N. reynaudiana	LS	P	7150±1301b	22375±6140a	13100±2020b	12950±6414b
	PS		813±249b	1667±181a	612±265bc	287±38c
Ophioglossum sp.	PS	A	-	1270±167	1581±181	2456±42
P. conjugatum	LS	P	4101±119b	24083±12475a	2583±236b	9550±5370b
	PS		-	-	1957±327	8833±4680
P. polystachyon	LS	P	-	23333±5481	-	1235±989
P. karka	LS	P	7850±284a	20972±12763a	7356±6614a	10778±6893a
	PS		1139±409b	7942±4692a	1208±539b	1285±452b
P. calomelanos	PS	P	6767±1038a	3386±1292bc	2356±830c	4861±1578ab
P. vittata	PS	P	3683±772a	2956±1267a	5520±3384a	4233±2599a
S. arvensis	LS	P	4392±2477a	2067±752a	4312.5±764a	4917±3547a
	PS		600±205a	500±250ab	283±63b	764±61a
T. maxima	LS	P	4414±3041	-	13517±11053	11183±8193
	PS		1142±597ab	1694±458a	340±123b	955±457ab
V. umbellata	LS	A	-	-	700±239	18400±2828
	PS		107±18	2575±1531	1088±589	-

Means followed by a common letter in the same row for each metal are not significantly different from each other using LSD test (P > 0.05).

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Table 4-5 Seasonal variation of lead concentration (mg/kg DW) (mean ± SD; n = 3) in soil around plant root system collected from LS and PS.

Plant species	Site	Duration	Lead concentration in soil (mg/kg DW)			
			January	April	July	October
A. conyzoides	LS	A	-	12333±5346	63500±29193	-
	PS		46500±13141	5883±2487	4700±2209	-
B. asiatica	LS	P	101111±16563a	4667±764b	74167±24111a	120000±46209b
	PS		6533±104	-	-	3833±1258
C. odoratum	LS	P	122167±15003	-	94333±17467	105167±23929
C. diffusa	LS	A	-	-	49333±2021	96000±49267
C. sumatrensis	LS	A	62500±9659b	14500±3905c	67833±17786b	112333±20251a
	PS		10917±775	-	32833±19504	6750±3733
C. crepidioides	LS	P	95833±3215	-	83167±15695	42333±9385
	PS		-	-	-	4667±2021
C. flavidus	PS	P	-	-	10667±7256	3333±878
C. iria	PS	A	15017±1969a	5267±448b	8000±4131b	3667±1283b
E. debile	LS	P	101167±30184a	13167±3329c	69167±7251ab	66500±15322b
	PS		4558±774bc	9233±2417a	6083±1665c	2667±764b
Eulophia sp.	LS	P	-	-	84000±26187	105000±40844
I. cylindrica	LS	P	89333±11251a	14667±7943b	91500±25239a	116500±39652a
	PS		6278±428a	4067±1069a	3750±1392a	4500±2500a
M. pudica	LS	P	82833±0c	9667±3055b	75667±18936c	131167±20251a
	PS		12983±10082a	11117±925a	2000±0a	12667±10531a
N. reynaudiana	LS	P	81500±6000b	157167±289a	83167±15695b	158500±4770a
	PS		3683±462b	7433±2570ab	10583±5364a	4000±3041b
Ophioglossum sp.	PS	A	-	9133±1390	8250±1572	10333±289
P. conjugatum	LS	P	77167±5008a	12500±3606b	77667±9005a	69667±22745a
	PS		-	-	2500±433	52583±13853
P. polystachyon	LS	P	-	7833±2887	-	88000±8231
P. karka	LS	P	74167±2843a	11500±2179b	80333±28108a	98500±11269a
	PS		7433±1450a	7100±1381a	3917±1465a	11500±8012a
P. calomelanos	PS	P	7467±407a	7867±2223a	7217±3467a	13000±8231a
P. vittata	PS	P	7217±225b	7867±2900b	6750±433b	39000±21868a
S. arvensis	LS	P	119333±4272a	7333±4042b	97000±19346a	132333±21050a
	PS		6550±150a	6700±781a	12250±9148a	8000±2598a
T. maxima	LS	P	83666±6110	-	80833±19763	159167±2887
	PS		5850±50b	5500±507b	3667±1809b	31500±15597a
V. umbellata	LS	A	-	-	77667±20642	98833±33168
	PS		6917±226	8117±3073	6167±1422	-

Means followed by a common letter in the same row for each metal are not significantly different from each other using LSD test ($P > 0.05$).

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Table 4-6 Plant species that showed phytoextraction coefficient, lead accumulation in shoot > 1,000 mg/kg and translocation factor >1 (underlined numbers).

Plant species	Site	Phytoextraction coefficient	Lead accumulation in shoot (mg/kg DW)				Translocation factor (TF)			
			Jan	Apr	Jul	Oct	Jan	Apr	Jul	Oct
A. conyzoides	LS	0.04	-	4850	1375	-	-	1.5	0.6	-
	PS	0.08	400	346	738	-	0.2	0.2	0.6	-
B. asiatica	LS	0.02	720	858	3023	1153	0.6	0.3	1.8	0.6
	PS	0.12	293	-	-	730	0.5	-	-	0.5
C. odoratum	LS	0.02	1595	-	1983	2783	0.4	-	1.4	3.5
	PS	-	-	-	-	-	-	-	-	-
C. diffusa	LS	0.05	-	-	3533	1700	-	-	0.2	0.3
	PS	-	-	-	-	-	-	-	-	-
C. sumatrensis	LS	0.05	1505	7017	2700	7033	0.6	0.7	1	1.5
	PS	0.05	509	-	1030	458	0.2	-	0.4	1.1
C. crepidioides	LS	0.02	2369	-	1292	688	0.3	-	1	0.8
	PS	0.01	-	-	-	62	-	-	-	0.4
E. debile	LS	0.03	1585	6133	497	432	0.2	0.2	0.1	0.1
	PS	0.03	160	127	208	73	0.03	0.03	0.2	0.02
Eulophia sp.	LS	0.03	-	-	3288	2050	-	-	0.6	0.2
	PS	-	-	-	-	-	-	-	-	-
I. cylindrica	LS	0.02	1395	5467	297	1967	0.3	0.4	0.2	0.8
	PS	0.02	182	63	33	45	0.1	0.03	0.03	0.2
M. pudica	LS	0.03	1542	1087	2021	5267	0.2	0.3	0.6	1.1
	PS	0.02	193	80	118	97	0.2	0.03	0.3	0.1
N. reynaudiana	LS	0.02	612	618	674	8033	0.1	0.03	0.1	0.6
	PS	0.02	130	278	137	42	0.2	0.2	0.2	0.2
P. conjugatum	LS	0.03	1068	5400	1305	3367	0.3	0.2	0.5	0.4
	PS	0.05	-	-	160	1517	-	-	0.1	0.2
P. polystachyon	LS	0.01	-	1600	-	412	-	0.1	-	0.3
	PS	-	-	-	-	-	-	-	-	-
P. karka	LS	0.01	695	643	203	1030	0.1	0.03	0.03	0.1
	PS	0.02	91.	333	55	95	0.1	0.04	0.1	0.1
S. arvensis	LS	0.06	1592	2900	8967	7133	0.4	1.4	2.1	1.5
	PS	0.06	520	252	218	920	0.9	0.5	0.8	1.2
T. maxima	LS	0.02	805	-	1827	6017	0.2	-	0.1	0.5
	PS	0.02	197	213	65	92	0.2	0.1	0.2	0.1
V. umbellata	LS	0.04	-	-	475	7467	-	-	0.7	0.4
	PS	0.06	92	652	396	-	0.9	0.3	0.4	-

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4.2 Hydroponic study

4.2.1 Plant growth

Pb, Cd and Zn did not have any effect on relative growth rate (RGR) in both plant samples when compared to control. Plants grew normally in the heavy metal solutions. The highest RGR were 103.8 % and 101.4 % as control of *C. iria* and *C. flavidus* when grown in 10 mg/L Pb solution, respectively. (Table 4-7 and 4-8). Moreover, some of *C. iria* produced small tillers in both control and treatment.

4.2.2 Metals accumulation in plants

The accumulations of Pb, Cd and Zn in both plant samples were metal concentration dependent. They were increased when the metal concentration in solution was increased especially in their roots (Table 4-7 and 4-8).

The highest Pb accumulation was 470 mg/kg DW in shoots of *C. flavidus* and 38,000 mg/kg DW in roots of *C. iria* treated with 20 mg/L Pb.

The highest Cd accumulation was 73.3 mg/kg DW in shoots and 520 mg/kg DW in roots of *C. flavidus* treated with 0.5 mg/L Cd, respectively.

The highest Zn accumulation was 888.3 mg/kg DW in shoots and 5,077.7 mg/kg DW in roots of *C. flavidus* treated with 20 mg/L Zn, respectively.

The metal accumulation efficiency in plants could be observed from BC value. The higher Cd and Zn concentration in the solutions, the lower BC value but the opposite result was observed in Pb solution. The highest BC of *C. iria* was 1,917.5 detected in 20 mg/L Pb, which was lower than *C. flavidus* which had the highest BC (2,990.8) in 20 mg/L Pb treatment (Table 4-7 and 4-8).

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Table 4-7 The metal accumulation, relative growth rate and bioaccumulation coefficient (BC) of *C. iria* exposed to Pb, Cd and Zn for 15 days.

Metal	Metal concentration (mg l^{-1})	Relative growth rate (% as control)	Metal content (mg kg^{-1} DW)		BC
			Shoot	Root	
Pb	0	98.7 ± 5.2	5.0 ± 5.0	16.7 ± 7.6	-
	10	103.8 ± 2.9	191.7 ± 127.1	14505.6 ± 2209.8	1469.7
	20	94.1 ± 3.4	350.0 ± 108.3	38000.0 ± 4993.8	1917.5
Cd	0	94.6 ± 9.5	1.0 ± 0.6	8.8 ± 4.3	-
	0.25	96.4 ± 8.1	17.8 ± 2.8	268.3 ± 15.3	1073.3
	0.5	93.4 ± 9.6	24.0 ± 3.8	323.3 ± 51.3	646.7
Zn	0	101.9 ± 7.9	44.5 ± 11.1	149.2 ± 47.0	-
	10	97.1 ± 7.4	275.0 ± 121.3	3262.0 ± 945.1	353.7
	20	99.4 ± 2.3	530.0 ± 140.8	4908.3 ± 535.6	271.9

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Table 4-8 The metal accumulation, relative growth rate and bioaccumulation coefficient (BC) of *C. flavidus* exposed to Pb, Cd and Zn for 15 days.

Metal	Metal concentration (mg l^{-1})	Relative growth rate (% as control)	Metal content (mg kg^{-1} DW)		BC
			Shoot	Root	
Pb	0	85.1 ± 12.7	8.3 ± 2.9	200.0 ± 72.6	-
	10	101.4 ± 4.5	760.0 ± 619.2	13666.7 ± 1200.4	1442.7
	20	99.6 ± 16.3	470.0 ± 220.2	29437.5 ± 7310.9	2990.8
Cd	0	85.8 ± 6.4	1.7 ± 0.4	2.1 ± 0.5	-
	0.25	89.3 ± 6.0	42.8 ± 22.8	345.0 ± 40.0	1380.0
	0.5	87.5 ± 3.7	73.3 ± 18.0	520.0 ± 162.6	1040.0
Zn	0	97.0 ± 15.2	80.5 ± 8.2	105.5 ± 22.1	-
	10	93.0 ± 7.5	820.8 ± 144.3	6534.0 ± 752.4	735.5
	20	94.1 ± 6.4	888.3 ± 102.6	5077.7 ± 3084.2	298.3

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Moreover, the potential of plants for phytoremediation is also based on the percentage uptake of each metal from the solution. The percentage uptake of Pb and Cd in both plant samples showed the same trend but the percentage uptake of Zn in *C.flavidus* was fluctuated between day 6 and 12. However, the overall percentage uptake of all metals in both plant samples were highest around the first 3 days (Pb > Cd > Zn) and decreased continuously until 15 days except the stable percentage uptake of 10 mg/L Pb in *C. flavidus* was quite stable (Figure 4-3 to 4-5).

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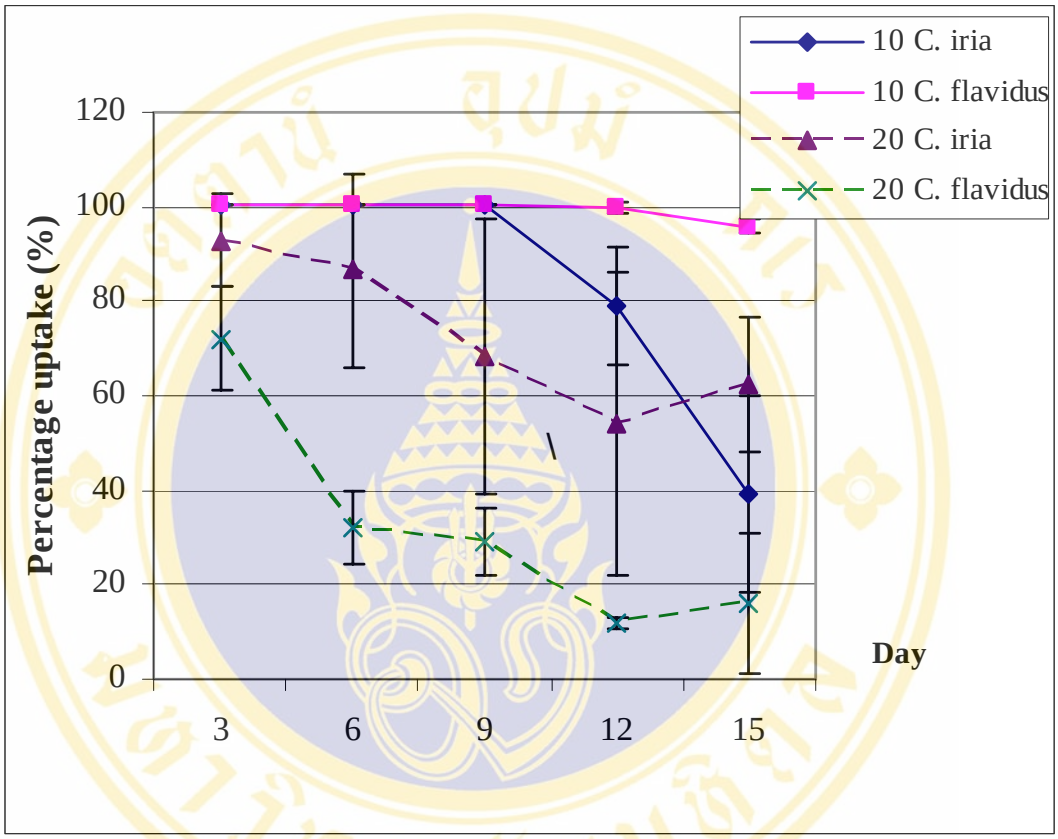

Figure 4-3 Percentage uptake of Pb compared between *C. iria* and *C. flavidus* when exposed to 10 and 20 mg/L Pb for 15 days.

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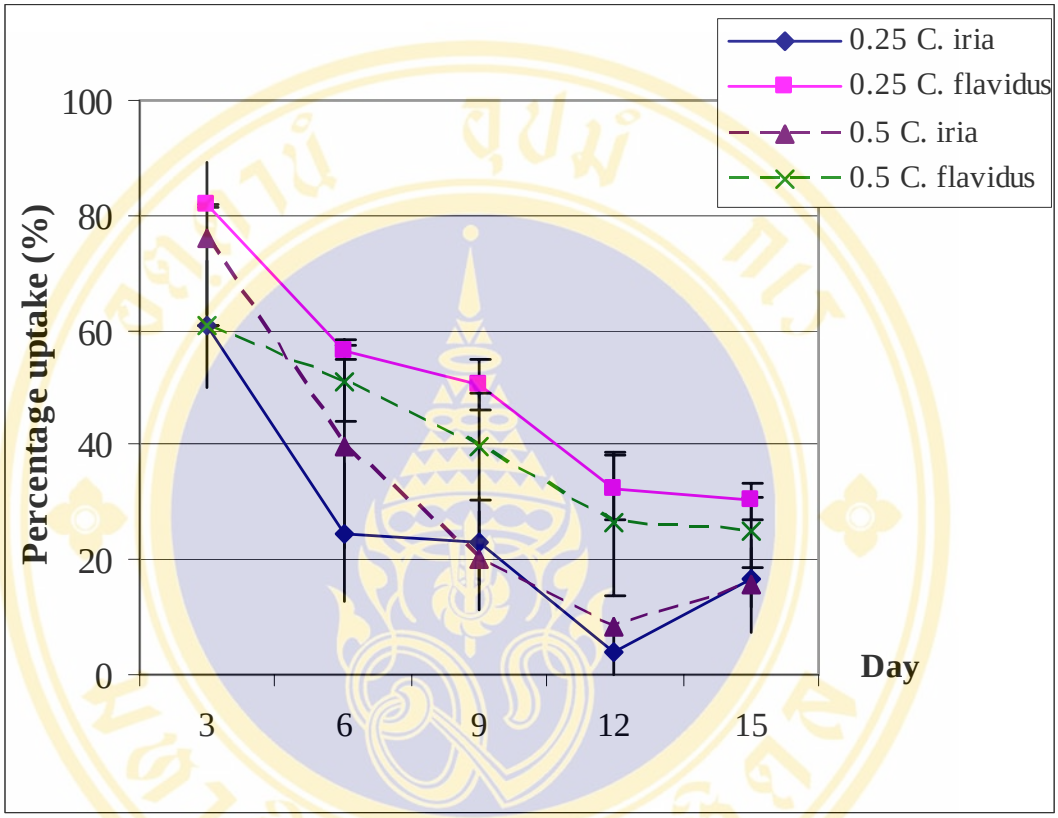

Figure 4-4 Percentage uptake of Cd compared between *C. iria* and *C. flavidus* when exposed to 0.25 and 0.5 mg/L Cd for 15 days.

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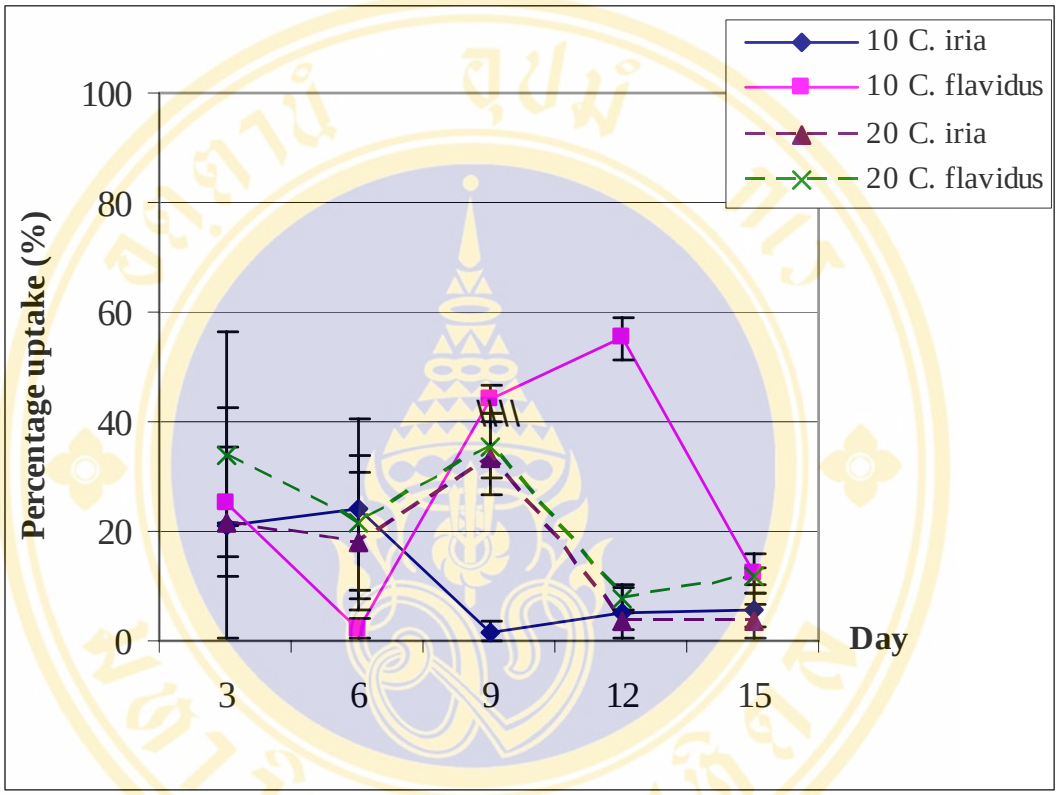

Figure 4-5 Percentage uptake of Zn compared between *C. iria* and *C. flavidus* when exposed to 10 and 20 mg/L Zn for 15 days.

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4.2.3 Plant tolerance mechanisms

The average GSH contents in shoots of *C. iria* were higher than those in roots during the experimental period (8 days) (Table 4-9, Fig. 4-6 and 4-7). GSH was detected in control plants and the response was in the following order Cd > Zn > Pb. The GSH contents in all groups were almost highest on day 2. The highest GSH contents could be detected in plant shoots treated with 20 mg/L Zn (1.92 mmol/mg DW) and plant roots treated with 0.5 mg/L Cd (0.82 mmol/mg DW) on day 2. The GSH contents in control plants and plants treated with 20 mg/L Pb were rather stable during the experimental period.

The average total PC_2 and PC_3 contents in roots were higher than those shoots and could be detected during day 1 to 8 of the experimental period. The PC_2 and PC_3 could not be detected in control plants and the response was in the following order Cd > Pb > Zn. These contents were increased on day 1 to day 4 (mostly highest on day 2) and gradually decreased until day 8. The highest PCs contents could be detected in plant roots treated with 0.5 mg/L Cd on day 2 (56.04 mmol/mg DW, and 0.86 mmol/mg DW for PC_2 and PC_3, respectively), whereas in plant shoots they could be detected in plants treated with 0.5 mg/L Cd on day 4 for PC_2 (53.19 mmol/mg DW) and day 2 for PC_3 (0.68 mmol/mg DW), respectively (Table 4-10 and 4-11; Fig. 4-8 to 4-11).

The GSH contents were detected in shoots > roots, whereas the PC_2 and PC_3 were detected in roots > shoots (PC_2 > PC_3). The PCs synthesized were highest in response to Cd solution especially in roots. However, there was no correlation between GSH and PCs contents changing during the experimental period.

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Table 4-9 Change in GSH contents in *C. iria* during the experimental period (8 days)

Day(s)	Control (mmol/kg DW)		Pb (mmol/kg DW)		Cd (mmol/kg DW)		Zn (mmol/kg DW)	
	shoot	root	shoot	root	shoot	root	shoot	root
0	0.15± 0.01	-	-	-	-	-	-	-
1	0.19 ± 0.06	0.16± 0.04	0.05± 0.04	-	0.92 ± 0.71	-	0.17 ± 0.1	-
2	0.21 ± 0.04	0.34± 0.09	0.09± 0.02	-	1.53 ± 0.19	-	1.92 ± 0.13	-
4	0.25 ± 0.03	0.45±0.14	0.76 ± 0.3	0.12 ± 0.11	-	0.82 ± 0.02	-	0.1 ± 0.08
8	0.27 ± 0.14	0.05±0.00	-	0.1 ± 0.04	-	-	1.45 ± 0.6	-

Table 4-10 Change in PC_2 contents in *C. iria* during the experimental period (8 days)

Day(s)	Control (mmol/kg DW)		Pb (mmol/kg DW)		Cd (mmol/kg DW)		Zn (mmol/kg DW)	
	shoot	root	shoot	root	shoot	root	shoot	root
0	-	-	-	-	-	-	-	-
1	-	-	5.94 ± 1.35	-	49.53 ± 1.93	35.09± 3.85		45.59 ± 9.03
2	-	-	8.68 ± 0.98	7.29 ± 0.33	50.54 ± 1.74	56.04 ± 3.3	50.54 ± 2.98	52.09 ± 3.71
4	-	-	9.19 ± 0.51	38.47 ± 2.75	53.19 ± 3.95	42.02 ± 0.98	45.59 + 9.03	50.54 ± 1.28
8	-	-	5.94 ± 1.35	12.62 ± 1.05	43.09 ± 1.94	40.29 ± 2.06	-	-

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Table 4-11 PC_3 contents changing in *C. iria* during the experimental period (8 days)

Day(s)	Control (mmol/kg DW)		Pb (mmol/kg DW)		Cd (mmol/kg DW)		Zn (mmol/kg DW)	
	shoot	root	shoot	root	shoot	root	shoot	root
0	-	-	-	-	-	-	-	-
1	-	-	0.02 ± 0.00	0.07 ± 0.01	0.02 ± 0.00	0.09 ± 0.04	0.06 ± 0.03	0.10 ± 0.04
2	-	-	0.11 ± 0.02	0.12 ± 0.04	0.68± 0.14	0.86± 0.01	0.14 ± 0.01	0.02 ± 0.00
4	-	-	0.1 ± 0.01	0.14 ± 0.00	0.48± 0.03	0.65 ± 0.02	0.48 ± 0.03	0.04 ± 0.01
8	-	-	0.04 ± 0.01	0.05± 0.01	0.11± 0.04	0.20 ± 0.09	-	-

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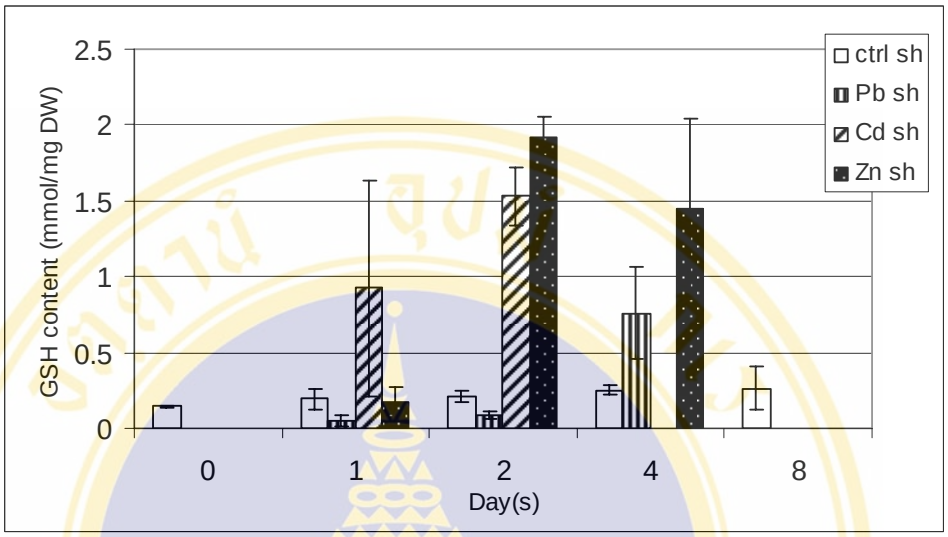

Figure 4-6 GSH contents changing in shoot of *C. iria* during the experimental period.

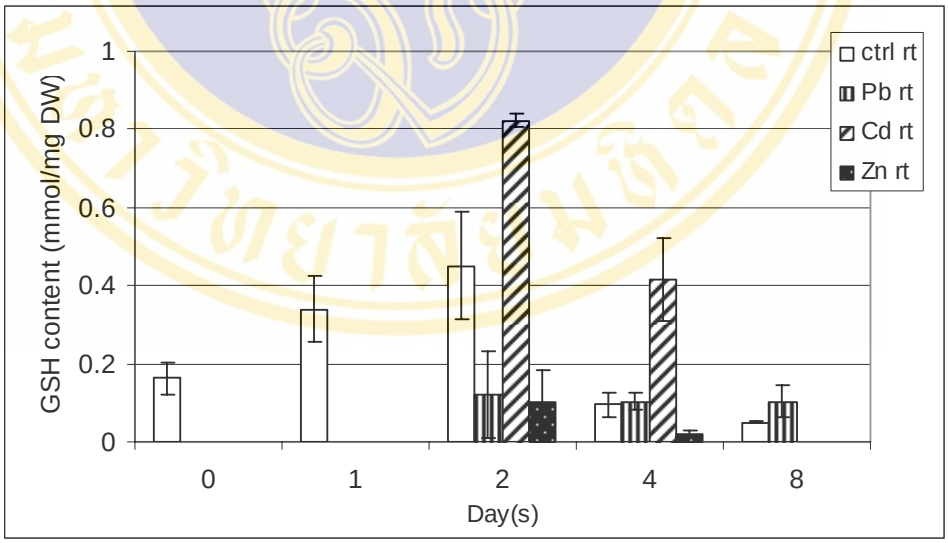

Figure 4-7 GSH contents changing in root of *C. iria* during the experimental period.

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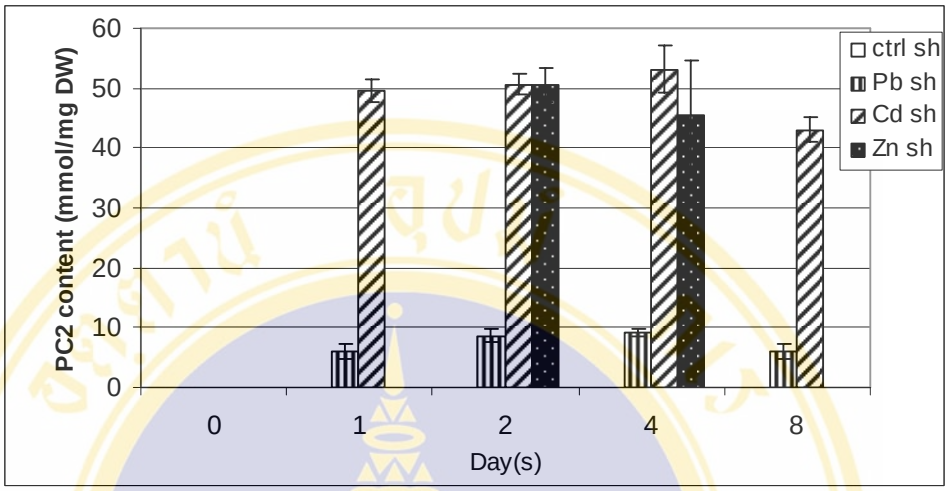

Figure 4-8 PC_2 contents changing in shoot of *C. iria* during the experimental period.

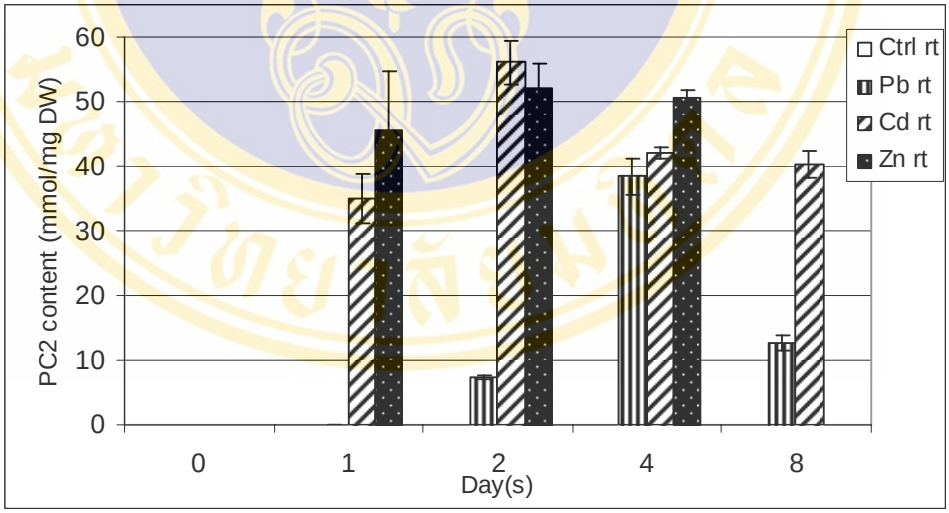

Figure 4-9 PC_2 contents changing in root of *C. iria* during the experimental period.

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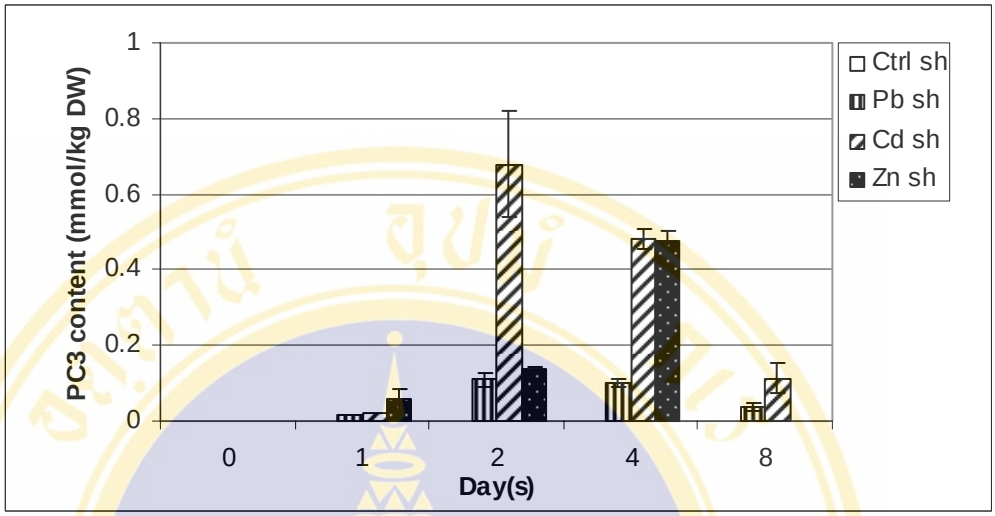

Figure 4-10 PC_3 contents changing in shoot of *C. iria* during the experimental period.

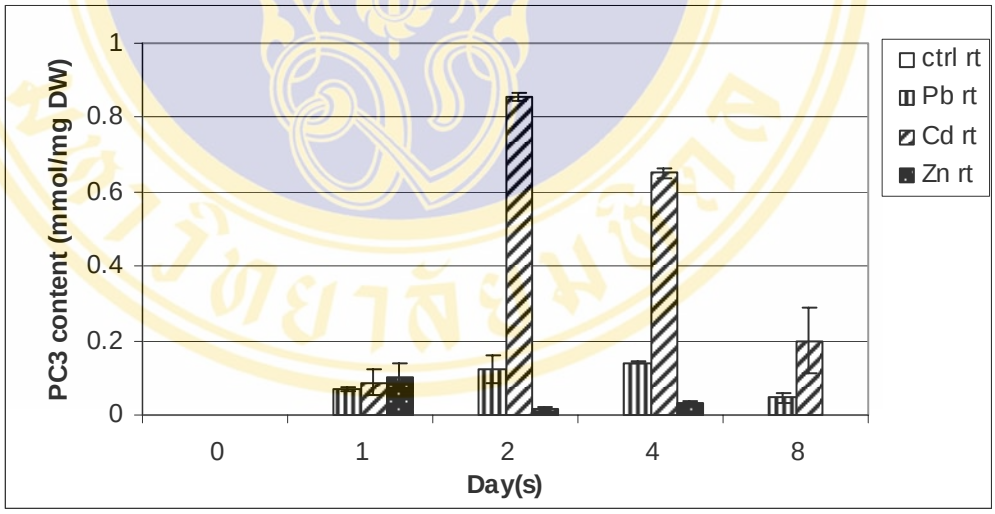

Figure 4-11 PC_3 contents changing in root of *C. iria* during the experimental period.

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4.3 Batch Experiment on wetland plants

4.3.1 Growth responses of plant samples

The growth of *C. iria* and *T. angustifolia* were presented in Tables 4-12 and 4-13. The shoot lengths were higher within 3 months in both plant samples especially the shoot lengths of *T. angustifolia* which grew rapidly. Whereas, the root lengths were longer in the control than treatment in both plant species after grown for 2 months (p value < 0.05).

The relative growth rate in both plant species were highest in the second month (0.39 in treatment of *C. iria* and 0.35 in control of *T. angustifolia*) and decreased in the third month. The growth of *T. angustifolia* was increased rapidly. There was no significant difference between control and treatment in the second month but the growth was decreased rapidly after grown in treatment for three months (Table 4-14 and Fig. 4-12).

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Table 4-12 Shoot and root length of *C. iria* grown in control and Pb contaminated soil for 3 months

Soil	Experimental period							
	Day 0		1 month		2 months		3 months	
	Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)
Control	57.93 ± 4.99	18.47 ± 1.25	66.38 ± 6.22	22.50 ± 7.93	60.78 ± 6.96	29.97 ± 10.97a	61.44 ± 11.51	26.49 ± 0.35a
Treatment	63.85 ± 3.87	20.43 ± 1.26	71.17 ± 12.08	22.25 ± 8.03	67.67 ± 3.33	15.82 ± 4.32b	68.50 ± 1.64	17.04 ± 1.66b

Table 4-13 Shoot and root length of *T. angustifolia* grown in control and Pb contaminated soil for 3 months

Soil	Experimental period							
	Day 0		1 month		2 months		3 months	
	Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)	Root (cm)
Control	80.00 ± 0	15.08 ± 3.09	116.75 ± 26.81	25.42 ± 7.02	166.83 ± 3.62	53.42 ± 8.92a	168.00 ± 29.44	38.33 ± 10.69a
Treatment	80.00 ± 0	17.86 ± 2.91	113.25 ± 31.05	20.17 ± 4.80	180.00 ± 76.37	21.75 ± 11.67b	190.50 ± 24.75	16.50 ± 2.12b

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Table 4-14 Relative growth rates of *C. iria* and *T. angustifolia*

Month(s)	Plant sample			
	C. iria		*T. angustifolia*	
	Control	Treatment	Control	Treatment
1	0.37	0.38	0.16	0.14
2	0.36	0.39	0.35	0.35
3	0.30	0.25	0.34	0.24

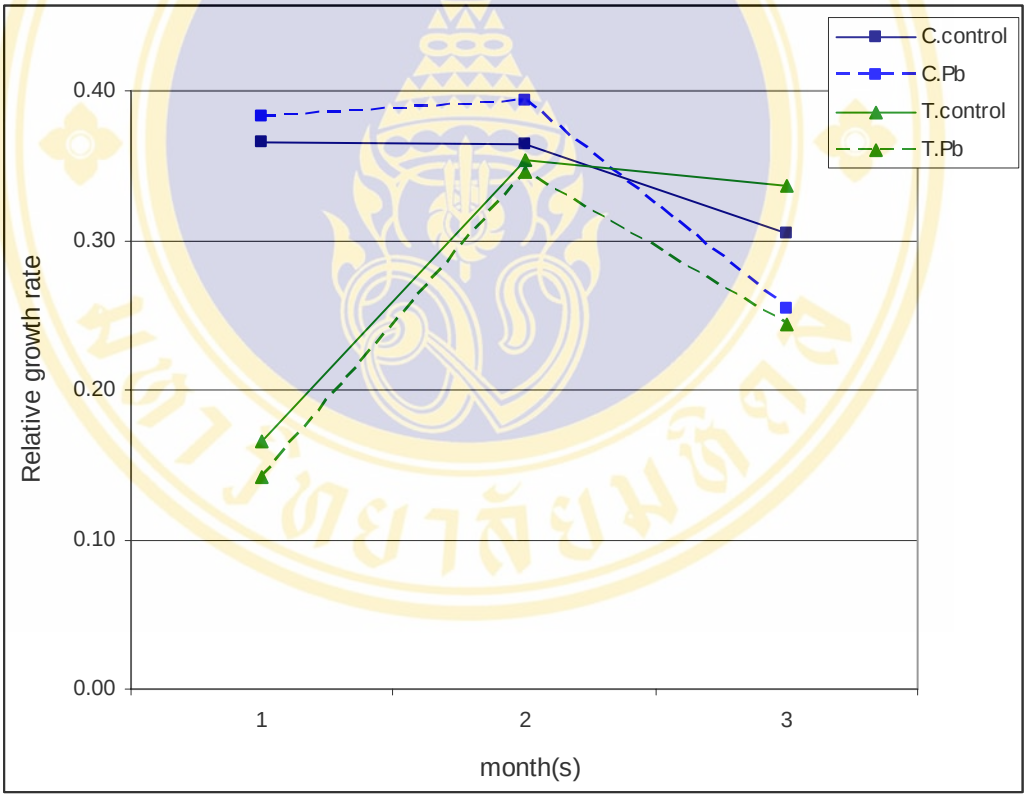

Figure 4-12 The relative growth rates of *C. iria* and *T. angustifolia* compared between control and Pb contaminated soil.

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4.2.2 Lead accumulation in plant

Table 4-15, 4-16 and Fig 4-13 and 4-14 showed Pb accumulation in plant samples. More Pb was accumulated in roots than shoots in both plant samples. The highest Pb accumulations in shoots of *C. iria* and *T. angustifolia* were 561.67 and 205 mg/kg DW in third month, respectively. The highest Pb accumulation in roots of *C. iria* and *T. angustifolia* were 4,550 and 6,383.33 mg/kg DW in first month, respectively. The Pb accumulation in shoots of *T. angustifolia* was decreased rapidly after 1 month of the experimental period.

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Table 4-15 Pb accumulation in plant tissues of *C. iria* for 3 months

Soil	1 month		2 months		3 months	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	8.33 ± 5.77	106.67 ± 81.45	15.50 ± 5.0	75.00 ± 63.84	16.67 ± 2.89	38.33 ± 10.41
Pb	275.56 ± 124.74b	4550.00 ± 973.40a	561.67 ± 137.51a	4472.00 ± 1051.10a	211.67 ± 25.66b	3888.00 ± 1160.62a

Table 4-16 Pb accumulation in plant tissues of *T. angustifolia* for 3 months

Soil	1 month		2 months		3 months	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	0.00 ± 0.00	9.44 ± 5.09	16.67 ± 10.41	3.33 ± 2.89	23.33 ± 20.21	11.67 ± 7.64
Pb	113.33 ± 75.72a	6383.33 ± 1290.67a	205.00 ± 101.49a	3116.67 ± 1069.27b	170.00 ± 77.78a	2225.00 ± 459.62b

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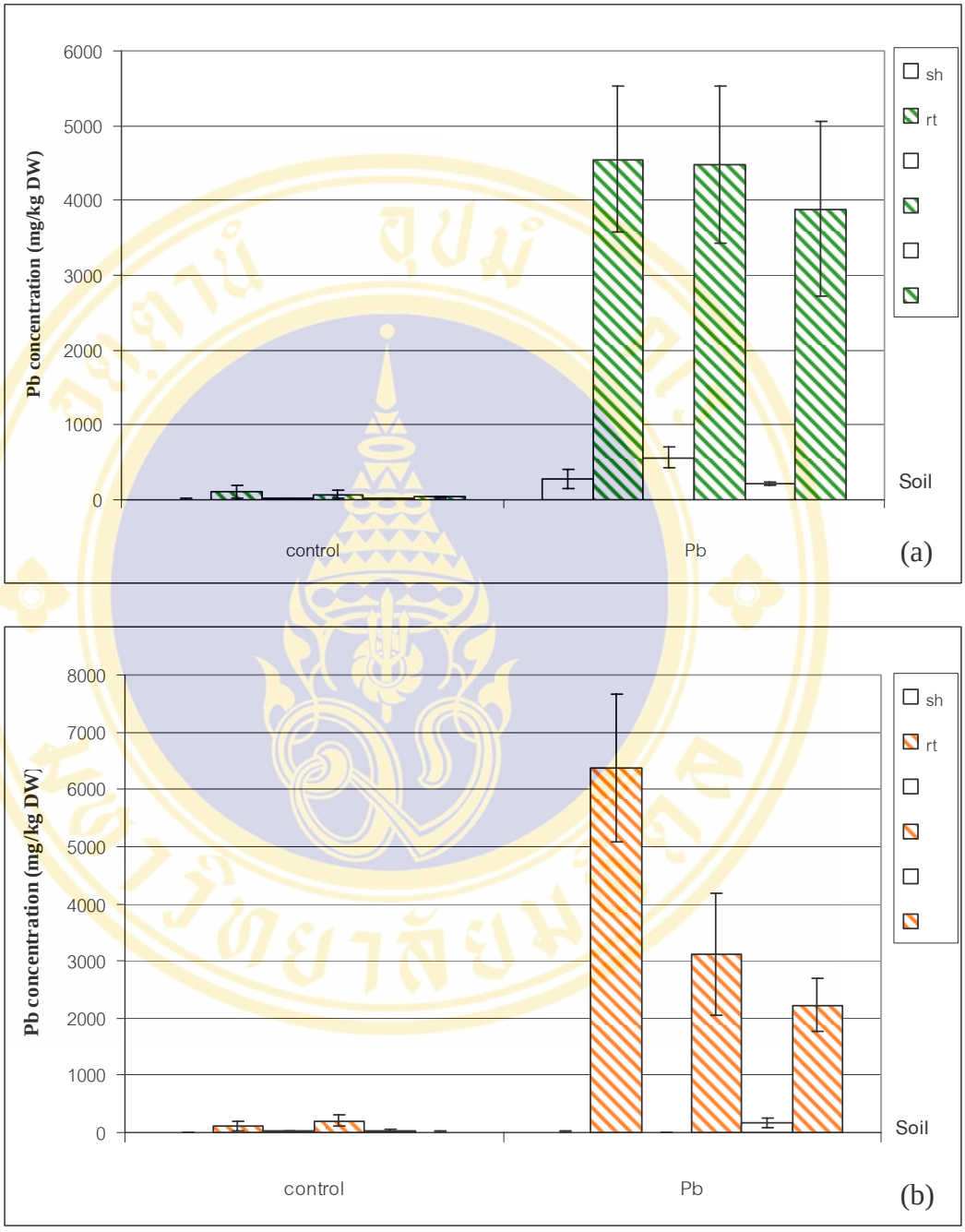

Figure 4-13 Pb accumulation in plant tissues compared between *C. iria* (a) and *T. angustifolia* (b).

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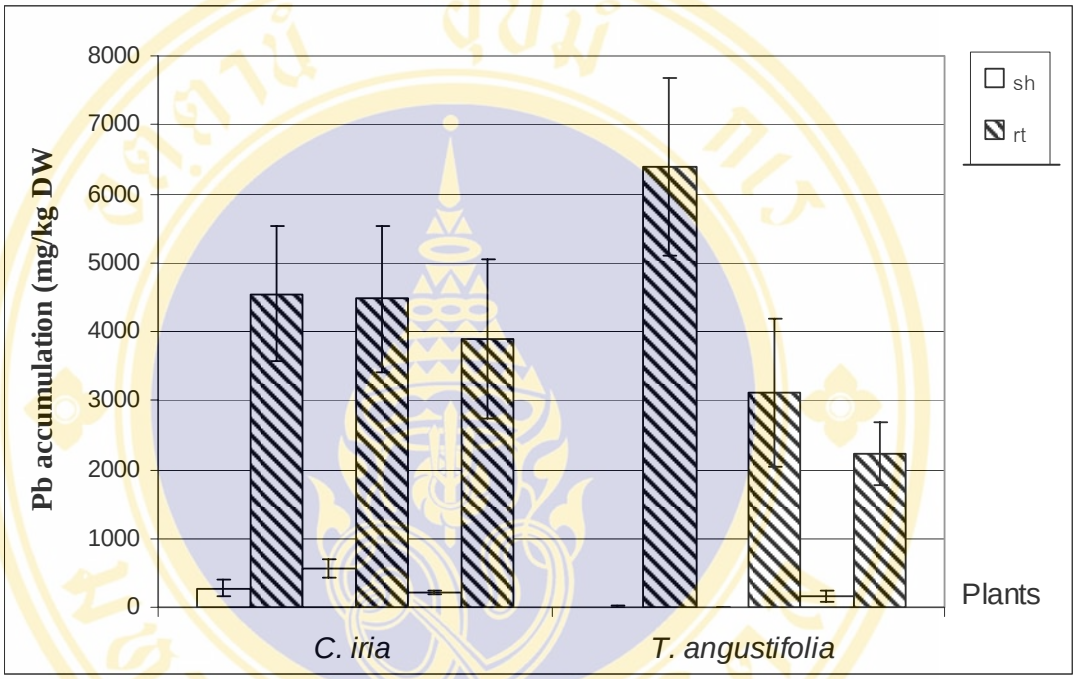

Figure 4-14 Pb accumulation in plant tissues compared between *C. iria* and *T. angustifolia* for 3 months.

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4.2.3 Lead concentration changing in soil

The soil characteristics of horticulture soil and Pb contaminated soil are presented in Table 4-17. The soil pH and total N between both soils were not different but the EC value, P, K, Ca, Mg and organic matter in Pb contaminated soil were very low. The soil texture of horticulture soil was loam whereas, the Pb contaminated soil was silty loam soil. The Pb concentrations in soil where *C. iria* were grown at different levels are shown in Table 4-18 and Fig.4-15. The average Pb concentrations in soil were 4,504.17 4,966.67 and 4,895.83 mg/kg DW for 10, 20, and 30 cm depth, respectively. Whereas, the Pb concentrations in soil where *T. angustifolia* were grown higher than were those in *C. iria* (Table 4-19 and Fig.4-15). The average Pb concentrations in soil were 4,579.17 4,366.67 and 4,175.0 mg/kg DW for 10, 20, and 30 cm depth, respectively. The Pb concentration in surface soil (0-10 cm) tended to be stable during the three-month study, whereas in subsurface soil (10-20, and 20-30 cm) Pb tended to be increased until reached highest level in the second month.

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Table 4-17 Characterizations of soils from horticulture soil (control) and the Bo Ngam lead mine area (treatment)

Soil	pH	Ec[a] (dS/m)	Total N (%)	Available P (ppm)	Available K (ppm)	Ca (ppm)	Mg (ppm)	Total Pb (mg/kg DW)	DTPA-Pb (mg/kg DW)	Soil Texture				OM[b] (%)
										% Sand	% Silt	% Clay	Texture	
Control	7.0	2.35	0.09	472	1000	6000	600	0.0	0.0	51	31	18	L	6.9
Pb	6.9	0.40	0.02	4.00	11	573	14	5,574	680	38	52	10	SiL	0.1

Table 4-18 Pb concentration in different soil depths (*C. iria* treatment)

Soil depth (cm)	Day 0	1 month	2 months	3 months
0 – 10	4616.67 ± 585.95	4433.33 ± 1575.07	4150.00 ± 576.63	4816.67 ± 189.30
10 - 20	4466.67 ± 520.42	4716. 67 ± 1266.23	5550.00 ± 1381.12	5133.33 ± 803.64
20 - 30	4566.67 ± 503.32	4500.00 ± 1040.43	5316.67 ± 1673.57	5200.00 ± 1775.53

Table 4-19 Pb concentration in different soil depths with (*T. angustifolia* treatment)

Soil depth (cm)	Day 0	1 month	2 months	3 months
0 – 10	4616.67 ± 585.95a,ab	4033.33 ± 340.34a,b	4983.33 ± 354.73a,a	4683.33 ± 361.71a,ab
10 - 20	4466.67 ± 520.42a,a	3916.67 ± 725.14a,a	4483.33 ± 375.28ab,a	4600.00 ± 304.14a,a
20 - 30	4566.67 ± 503.32a,a	3916.67 ± 125.83a,a	3883.33 ± 797.39b,a	4333.33 ± 548.48a,a

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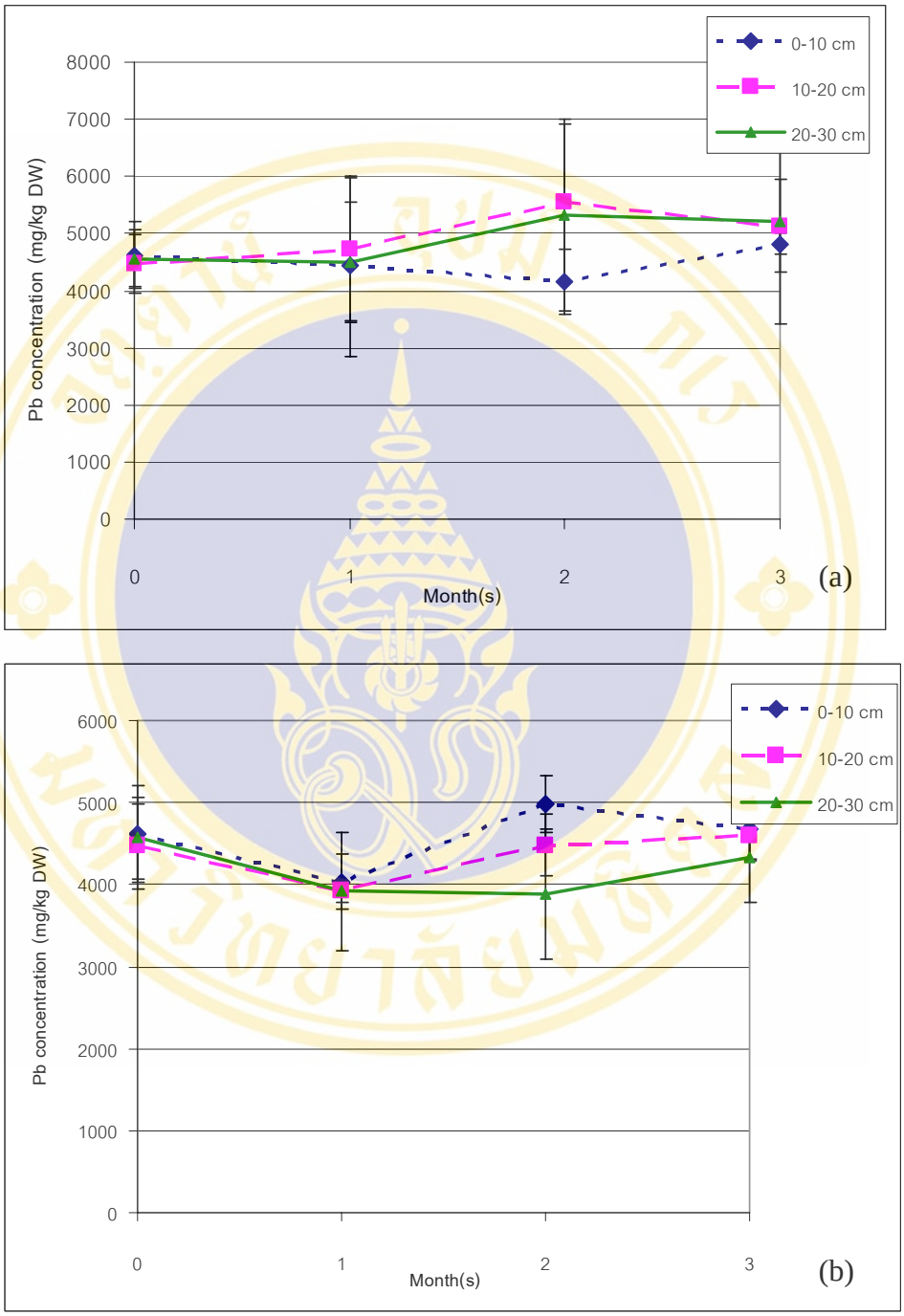

Figure 4-15 Pb concentration at different soil depths (a) with *C. iria* and (b) with *T. angustifolia*

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4.2.4 pH of surface soil, soil water at surface and plant productions

The pH value and plant productions were reported in Table 4-20. The pH of surface soil in control was lower than treatment in both plant species during three months. The pH of soil water at surface was higher than at the surface soil and was not significant different between control and treatment during three months.

There were few tillers of *C. iria* produced after cultured for 1 month. *C. iria* grown in control soil produced more tillers than those grown in Pb contaminated soil, while *T. angustifolia* grown in Pb contaminated soil did not have any tiller production. *C. iria* grown in Pb contaminated soil produced more flowers than those grown in control soil after 1 month of culture but decreased when the exposure time was increased. *T. angustifolia* did not produce any flowers in both control and Pb contaminated soil.

Table 4-20 The average pH of soil surface, pH of soil water at surface, tillers and flowers production of *C. iria* and *T. angustifolia* in control and Pb contaminated during the experimental time.

Month (s)	Soil types	Plants	Soil pH	pH of soil water in surface	Average tiller(s) production	Average flower(s) production
1	Control	*C. iria*	6.27	8.43	2.0	2
		T. angustifolia	6.47	8.23	-	-
	Pb	*C. iria*	7	8.30	1.89	2.5
		T. angustifolia	7	8.33	-	-
2	Control	*C. iria*	5.67	8.50	1.7	1.67
		T. angustifolia	5.87	8.47	2.0	-
	Pb	*C. iria*	6.93	8.57	2.63	2.5
		T. angustifolia	6.8	8.37	-	-
3	Control	*C. iria*	6.17	8.50	2.33	1.33
		T. angustifolia	5.3	8.53	2.6	-
	Pb	*C. iria*	7	8.53	2.44	1.56
		T. angustifolia	7	8.37	-	-

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CHAPTER V
DISCUSSION

5.1 Seasonal variation

The soil characteristics of both sampling sites were typical of mined degraded soils. They exhibited low nutrients, low organic matter and low EC values. These characters tend to inhibit soil-forming processes and plant growth (Wong, 2003).

Lead concentration in soil from LS was about ten times higher than that from PS. The PS was located near the open pit, where ore digging and transport were performed extensively. After the mine was closed down the pit was then filled with water, receiving run-off from the nearby slopes. The soluble Pb would have been washed into the pond and soil Pb content was consequently much lower, especially in the topsoil. However, the soil concentrations from both sites were both extremely high (10,000-100,000 mg/kg) as compared to other mining areas, for example, 100-12,139 mg/kg in soil from Lanping Pb-Zn mine area, China (Yanqun *et al*., 2004); 2,587-13,267 mg/kg from Pb-Zn mining area in Yunnan, China (Yanqun *et al*., 2005); 4,450 mg/kg in Au-Ag-Pb-Zn mine in Daduk, Korea (Lee *et al*., 2001); and 2335-5686 mg/kg from Pb-Zn mine tailing in Guangdong province of China (Shu *et al*., 2000).

In this study Pb content in soils was seasonally dependent. The lowest Pb concentration was found in July (0.6%) and the highest was in October (>11%). Similar seasonal variation in metal content was found elsewhere. Less mineral surface area was exposed and less water was available to flush weathering metals during winter months (August *et al*., 2002). Lee *et al*. (2001) reported on the seasonal variation on metals in stream sediments near Au-Ag-Pb-Zn mine. The results showed that relatively high concentrations of metals (Cd, Cu, Pb, Zn) were found in water and sediment samples in the dry season. Lower Pb concentration in soil in the wet season may be due to a dilution effect by heavy rain in the early wet season (May-June).

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The high Pb concentration of soil in dry season was considered as one of many factors which had effects on the plant diversity at LS. The plant diversity all year at PS was more stable than LS. This is likely due to the higher in water and moisture contents and the lower Pb concentration. Variations in Pb accumulation were more pronounced in plants collected from LS which contained soil with extremely high concentration of Pb. This indicates that plant growth period may be responsible for the higher uptake of Pb. This finding was related to that of Larsen and Schierup (1981) who found a sharp increase of Pb in leaves of *Phragmites australis* during and after the growth season. They found that leaves produced earlier in the growing season had higher concentration of Hg than leaves produced later. This is also similar to Cacador *et al*. (2000) who studied Zn, Pb, Cu and Cd accumulation in *Spartina maritima* and *Halimione portulacoides*. However, Gleason *et al*. (1979) had found that *Spartina alteriflora* accumulated Mn, Cu and Zn rapidly in their shoot part during spring then levels off. The decrease was attributed to a growth dilution effect due to an increase in biomass during growth (Deram *et al*., 2006).

Different metals and different plant species show different patterns of seasonal variation of metal accumulation. While several studies showed seasonal changes in metal contents in plants (such as Zn, Cu, Pb, Cr, Hg), others found no seasonal changes in these metals (Cacador *et al*., 2000; Gleason *et al*., 1979; Larsen and Schierup, 1981; Weis *et al*., 2003). Hence, it is very difficult to generalize about seasonal changes in metal levels since they appear to vary greatly with the metal and the species of plants studied (Weis and Weis, 2004). Most plant species found in the mine area were perennials which were good for phytostabilization since perennial plants can stabilize Pb in their shoots or roots for a longer time than annual plants which have a short life-cycle.

Considering that a translocation factor higher than 1 indicates a very efficient ability to transport metal from root to shoot (Baker *et al*., 1994; Baker and Whiting, 2002), then six plant species from this study could be considered Pb hyperaccumulators with an extremely high capacity to take up metals by roots, translocate and store them in the shoots (Baker *et al*., 2000). Rotkittikhun *et al*. (2006) also identified *A. conyzoides*, *B. asiatica*, *C. odorata*, *C. sumatrensis*, and *S. arvensis* as lead hyperaccumulator in the field study at Bo Ngam lead mine area.

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5.2 Hydroponic study

The metals accumulation study was carried out on hydroponics because in these conditions the entire metal pool was accessible to plants (Wojcik *et al*., 2005). This technique is a well-established procedure for the evaluation of plant tolerance to a variety of elements with a potential for phytotoxicity (Furlani and Clarck, 1981; Mcquattie and Schier, 1990; Alva and Chen, 1995). Normally, plants exposed to heavy metals always show the toxicity symptoms such as growth inhibition, biomass decreasing, leaf and root reduction (Singh *et al*., 1997; Romeiro *et al*., 2006). The inhibition of root growth may be due to a decrease in calcium in root tips, leading to a decrease in cell division or cell elongation (Haussling *et al*., 1988; Eun *et al*., 2000). Whereas, the inhibition of growth of shoot may be due to a decrease in photosynthesis, lack of mineral nutrition and water balance, changes hormonal status and affects membrane structure and permeability (Sharma and Dubey, 2005). However, *Cyperus* sp. in this study did not show any toxicity symptoms when exposed to Pb, Cd, and Zn. These plants were propagated from local plant species collected from Pb mine area. Therefore, they could be tolerant to metal stress condition. The plants that grow near the heavy metal contaminated areas showed some degree of heavy metal tolerance which is genetically determined and occurs through natural selection (Baker *et al*., 1987; Matagi *et al*., 1998).

In the past studies, Pb was found to rapidly accumulate in plant roots if Pb was bioavailable in the plant growth media. However, only a small proportion of absorbed Pb was translocated to shoots (Jones *et al*., 1973; Kumar *et al*., 1995; Romeiro *et al*., 2006; Tang *et al*., 2001). Similar results were obtained for *Carex rostrata*, *Eriophorum angustifolium*, *Phragmites australis*, *Helianthus annus* L, *Pinus radiata*, *Prosopis* sp., and spinach grown in hydroponics, a high capacity for Pb retention in the roots with restricted translocation to the shoots (Jarvis and Leung, 2002; Stoltz and Greger, 2002; Tsen *et al*., 2002; Aldrich *et al*., 2004; Romeiro, 2006). *Cyperus* sp. in this study was also able to accumulate metals in their roots higher than shoots. However, Papoyan *et al*. (2007) suggested that shoot metal accumulation could be stimulated by enhanced root metal influx and, presumably, enhanced xylem transport of these metals from the root to shoot.

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The lower rate of Pb, Cd, Zn removal and uptake to plant samples at longer duration were observed this may be attributed to saturation of metal binding sites as earlier observed in the Pb binding sites of *Pistia stratiotes* (Maine *et al*., 2001). Salt and Rauser (1995) reported that mechanism of tolerance or accumulation in some plants apparently involves binding potentially toxic metals at cell walls of roots and leaves, away from sensitive sites within the cell or storing them in a vacuolar compartment. The vacuole is the ultimate storage site for those heavy metal ions that happened to enter the cytosol of a given plant cell. The metal-PC complex is subsequently actively transported from the cytosol to the vacuole. The lower rate of metal uptake toward the longer time of exposure can also be due to the saturation of metal-PC complex in the vacuole as well.

In this study, the relationship between PCs-GSH derivatives and heavy metal concentrations, especially Cd, was detected. The GSH and PCs contents were heavy metal concentrations dependent. The trend of PCs-GSH derivatives increase with increasing metal concentration was similar to Wojcik *et al*. (2005). Their PCs-GSH derivatives level increased with increasing Cd concentration in the medium. Mishra *et al*. (2006) suggested that a high Pb accumulation in plant tissues may be attributed to its binding to cell wall and other different ligands such as GSH, PCs, etc.

The previous studies reported that Pb induced decline in GSH in *Vicia faba*, *Phaseolus vulgaris* (Piechalak *et al*., 2002), *Hydrilla* sp. and *Vallisneria* sp. (Gupta *et al*.,1995; 1998). Because the metal stressed plants need to maintain a high GSH/GSSG ratio besides induced GSH biosynthesis. This similar response was also found in this study which GSH of plant grew in heavy metals solution (Pb, Cd, and Zn) were detected in lower concentration than in the control. GSH was also decreased further when exposed to Pb, Cd, and Zn solutions for the long duration.

Mishra *et* al. (2006) detected that an increase in non-protein thiols (NP-SH) levels indicated plants' ability to tolerate the cellular metal load. Mehra *et al*. (2000) found that cysteine content increased with an increase in the metal concentrations. This increase in the level of thiols may be due to the stimulation of enzymes of sulfate reduction pathway (Noctor *et al*.,1998). Nocito *et al*. (2002) found that the entry of heavy metals such as Cd into plant stimulates sulfate absorption. Slight induction in GSH content and maintenance of high GSH/GSSG ratio at lower concentrations might

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be attributed to Pb induced synthesis of GSH (Piechalak *et al*., 2002). Higher levels of GSH may contribute to metal detoxification either by direct binding or by synthesis of PCs. The depletion observed in GSH content may also be attributed to its consumption as substrate for PC synthesis (De Vos *et al*., 1992). However, GSH in this study was not depleted when plants were exposed to heavy metals during the experimental period (8 days). The GSH contents in shoots were higher than roots because GSH was used as substrate for synthesis of PCs. Plant roots were exposed to heavy metals in higher concentration than shoots. Therefore, it was necessary to convert GSH into PCs at roots more than shoots (Zenk, 1996). GSH content was observed lower than that of the PC_2 and PC_3 contents which was similar to Friederich *et al*. (1998) who found that the ratio of GSH to Cd^{2+} as 4:1 was optimal for the enzymic conversion to the PCs.

The results in this study indicates that PCs contents were accumulated at the highest concentration during 2-4 days when plants were exposed in Pb, Cd and Zn solutions. This finding is similar to that of Srivastava *et al*. (2005) who found the induction in protein content in plants exposed to 10 µM Pb for shorter durations (2 days) and the protein content declined after 7 days. This induction at shorter duration is possible due to induction of stress proteins under metal exposure. In this study, the PCs contents in control plants could not be detected and at lower heavy metal concentration the level of PC_2 was higher than PC_3 similar to the study by Wojcik *et al*. (2005). They reported that the predominating form of PCs peptide derivative, (γ-Glu-Cys)n-Gly, were n=2 at lower concentrations of the metal, and the content of the derivatives n=3–4 increased when the metal concentration in the medium increase. Moreover, the synthesis rate of PC_2 was faster than the rate of its consumption as substrate for PC_3 (Wojcik *et al*., 2005).

The highest PCs synthesis of *C. iria* was detected when exposed to the highest Cd solution similarl to the previous studies (Kneer and Zenk, 1997; Le Faucheur *et al*., 2005). Besides PCs synthesis, Kramer (2000) found that the exposure of plants or plant cell cultures to metals results in the synthesis of small cysteine-rich oligopeptides that are non-translationally synthesized from glutathione by phytochelatin synthase. However, several studies reported that PCs were not involved in metal

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hyperaccumulation and tolerance in plants (De Knecht *et al*. 1992; 1994; Persans and Salt, 2000; Sun *et al*., 2005).

The study of Wojcik *et al*. (2005) showed that PCs production is not the primary mechanism of Cd-tolerance in *T. caerulescens* growing in the field as well as in plants cultivated under controlled conditions on soil in the greenhouse. This suggests that in plants exposed to chronic metal stress (long-term exposition to low metal concentrations) other mechanisms of Cd detoxification, such as binding to cell walls or complexing with organic acids predominate (Wagner, 1993).

5.3 Batch study of wetland plants

Both *C. iria* and *T. angustifolia* species, can grow in the Pb contaminated soil because they were heavy metal tolerant plants. *C. iria* produce flowers and generate new tillers, whereas *T. angustifolia* increased their biomass by shoot elongation and root lengths in both controlled and Pb contaminated soil. Both plant species have the highest relative growth rate in the second months and decreased in the third months due to the toxicity of Pb in soils.

The lower Pb removal from soils in both plant species can be due to the adsorption of metal ions to soil particles and organic matter by electrostatic attraction (Sheoran and Sheoran, 2005). Normally, soluble Pb concentrations in soil represent a very small fraction of the total soil Pb (< 0.1% of total soil Pb), therefore, the Pb availability to plants was limited. The Pb translocation from roots to shoots was less than 30% for the best Pb translocating plants (Huang and Cunningham, 1996). For these reasons there were two major limitations to the Pb phytoextraction; the low Pb bioavailability in soil and the poor Pb translocation from roots to shoots (Huang and Cunningham, 1996; Malone *et al*.,1974; Reeves and Brooks, 1983). Harrison and Laxen (1981) studied the absorption of Pb in several plants and found that translocation of Pb into the roots limited the quantities of Pb that move into the above-ground portions.

The pH of Pb contaminated soil was adjusted to 5.5-6 before plants were cultured. This is because soil pH is the limiting factor for Pb mobility in soil. It is reported that pH between 5 to 6 is the most suitable pH for Pb bioavailability to plants (Ross, 1994). This is because metal ions of Cu, Mn, Zn, and others are in

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exchangeable for at this pH (Sims and Patrick, 1978). Stoltz and Greger (2002) found that plants with higher root biomass achieve this suitable pH easier than plants with lower root biomass. The finding is corresponding to this study where *T. angustifolia* with higher root biomass accumulated Pb concentrations more than *C. iria* with lower root biomass. This study shows that there was a lower pH in control than in Pb contaminated soils because it was enriched in nutrients and organic matters. However, the change in water pH at soil water interface did not have any effects on plants Pb accumulation in this experiment.

For wetland plants, the greater proportion of metal taken up by plants was retained in the roots. Submerged rooted plants have some potential for the extraction of metals from water as well as sediments, while rootless plants extracted metals rapidly only from water (Cowgill, 1974). The study of Batty *et al*. (2003) discovered that root exudates can cause the precipitation of Fe, Mn, and Al plaques around the roots of the wetland plants. Although metals are taken up into the aerial parts of the plant, the majority are found concentrated in plaque deposits at the root surface. They reported that Pb concentration in root plaque of *Phragmites australis* was about 7,795 mg/kg DW. However, Ye *et al*. (1998) reported that Fe plaque is not a primary barrier to Pb uptake and translocation but the plaque may act as an effective Fe reservoir to increase Fe concentrations in active cells and then improve metal toxicity. This study yields similar results to Batty *et al*. (2003) where root plaque occurred in *T. angustifolia* resulted in high Pb accumulation in their roots but low translocated to their aerial parts.

There were several studies performed using *Typha* sp. and *Cyperus* sp. to remove metals from the sediments. Some studies found *T. angustifolia* and *T. latifolia* had the ability to accumulate and tolerate to high concentration of Pb, Cu, Ni, Pb, Zn, and Cd especially in their roots. (Taylor and Crowder, 1983; McNaughton *et al*., 1974; Panich-Pat *et al*., 2004; Shutes, 2000). The constructed wetlands with well grown *Cyperus alternifolius*, *C. papyrus* and *Vallarsia exaltata* were effective tools in phytoremediation of Cd, Cu, Mn, Zn and Pb in sediment and water (Cheng *et al*., 2002).

Edroma (1974) observed that shallow rooted plants tended to accumulate higher heavy metal concentrations than long deep-rooted plants. The results were

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similar to this study on *C. iria*, a shallow rooted plant, which can tolerate higher Pb contaminated soil than *T. angustifolia*, a long deep rooted plant.

By definitions, both plant species are classified as metal excluders because there are restrict uptake and transport of elements between root and shoot, maintaining low shoot levels over a wide range of external concentrations (Baker, 1981). In order to enhance the performance of constructed wetland systems, there is a need for further research into metal immobilization mechanisms, not only by the plants and substrate but in particular by microorganisms surrounded the root system. The collation of performance data from existing metal immobilization systems is also required to establish guidelines for the design.

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CHAPTER VI
CONCLUSION

From the field study, the patterns of metal accumulation and distribution in the plant parts were significantly influenced by kinds of metal, plant species and seasons. The different soil moisture content (between LS and PS) affected plant diversity, plant metal uptake and lead concentration in soil. Lead concentrations in soil and plants were seasonally dependent. Lead concentrations in plants differed even in the same species and same time of collection. Most plants had the highest lead concentrations during the wet season (May-September) and lowest during the dry season (October-April). Six herbaceous plant species showed a TF > 1; *A. conyzoides*, *B. asiatica*, *C. odoratum*, *C. sumatrensis*, *M. pudica*, and *S. arvensis*. However, if both phytoextraction coefficient and translocation factor concepts were both taken into account, *B. asiatica* will be the best candidate to be chosen for phytoremediation project.

The results of heavy metal accumulation under the hydroponic condition of *C. iria* and *C. flavidus* show that they grew well under Pb>Zn>Cd solutions, respectively. The heavy metal accumulation in their tissues especially in the roots was heavy metals dependent. The highest heavy metal accumulation and bioaccumulation efficient was found in Pb exposed condition when grown in 20 mg/L Pb for both plant samples. The response of plants when exposed to heavy metals was the change in GSH/PC contents. Their response to these three metals (Cd, Pb, and Zn) in this order: Cd>Pb>Zn. GSH was higher in shoots than roots but the opposite results could be detected for PC_2 and PC_3 contents which were higher in roots than in shoots. It can be concluded that GSH, PC_2 and PC_3 syntheses could be induced within 1-4 days after exposed to heavy metals.

For the constructed wetland batch experiment, *C. iria* and *T. angustifolia* have the efficiency to remove Pb from the mining soil by accumulating Pb in their roots but

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with low efficiency to translocate it to their shoots. Both plant species are classified as plant excluders because they are between restrict uptake and transport of elements to shoot maintaining low shoot levels over a wide range of external concentration (Baker, 1981). These plant species are Pb tolereant plants which can grow in Pb contaminated soil without any toxicity symptom. The pH of soil and water soil suface had not influence either on Pb uptake or removal from the Pb contaminated soil of *C. iria* and *T. angustifolia*. The Pb removal efficiency of *C. iria* and *T. angustifolia* from the soil were not different when considered from the total Pb concentration in soil but in the different soil levels *T. angustifolia* could remove Pb from the deeper soil better than *C. iria*. However, *C. iria* could grow in the Pb contaminated soil better than *T. angustifolia*, therefore, in a long run *C. iria* is more suitable to be selected for phytostabilization process.

In conclusion, this study was focused on plant metal accumulators, low metals transport to their shoots which suitable for phytostabilization process. It can be suggested that plant-based phytostabilization reduces the environmental risk by un- or sparsely-vegetated contaminated soil by the use of a combination of plants and soil amendments to establish a stable vegetation cover which may progressively reduce the soil labile metal pool. It can also be applied to the constructed wetland where metal ions washed off to the wetland area can be immobilized using *C. iria* and *T. angustifolia*. However, this technology does not achieve a clean up of the soil, but changes the mobility of potentially toxic elements by either reducing concentrations in the soil water and other freely exchangeable sites within the soil matrix or by reducing re-entrainment of toxic particulates following the development of a stable and permanent vegetation cover. Both processes alter the speciation of soil metals thus, reducing potential environmental impact.

In the future, the development of a stable and self-perpetuating ecosystem as a result of this type of treatment may be a further beneficial process, as in some circumstances, plant root activity may change metal speciation (changes in redox potential, secretion of protons, chelating agents); the microflora associated with their root systems may produce similar effects. The technique also has significant implications for polishing less contaminated soils (Vangronsveld, *et al*., 1997).

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BIOGRAPHY

NAME	Miss Kunaporn Homyog
DATE OF BIRTH	20 December 1977
PLACE OF BIRTH	Phayao province, Thailand
INSTITUTATIONS ATTENED	Chiangmai University, 1995-1999 Bachelor of Science (Biology) Mahidol University, 1999-2003 Master of Science (Environmental Biology) Mahidol University, 2003-2008 Doctor of Philosophy (Biology)
SCHOLARSHIP	Post-Graduate Education, Training and Research Program in Environmental Science, Technology and Management under High Education Development Project of the Commision on Higher Education, Ministry of Education.

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