

CHAPTER III

METHODOLOGY

3.1 Apparatus, Instruments and Chemicals

3.1.1 Apparatus

- 1) *Chromolaena odorata* seedling obtained from Chachoengsao Province.
- 2) *Vetiveria zizanioides* seedling obtained from Pathumthani Province.
- 3) Uncontaminated soils used for pot experiment were excavated from Mae Ku, Mae Sot District, Tak Province, Thailand.
- 4) Contaminated soils used for pot experiment were excavated from Phra That Pha Daeng Sub-district, Mae Sot District, Tak Province.
- 5) Granular commercial fertilizer 15-15-15 NPK formulation
- 6) Plastic pots (Ø 12 inch) and plastic bag (Ø 14 inch)
- 7) Whatman filters paper No. 40 (Ø 110 mm.)
- 8) GF/C (Glass Micro Filter) filter paper (Ø 70 mm.)
- 9) Deionized water (DI)
- 10) Parafilm
- 11) Laboratory glass wares; beaker, cylinder, volumetric flask, pipette, watch glass, funnel, glass rod etc.
- 12) 2 mm diameter sieve
- 13) Planting equipment; hoe, shovel, watering can etc.
- 14) 60 ml plastic bottles
- 15) Zip-lock plastic bags

3.1.2 Instruments

The instruments used in this experiment are shown in Table 3.1.

Table 3.1 Instruments used in this research

Instruments	Model/ Brand
1) Atomic Absorption Spectrometer (AAS)	Analyst 800, Perkin Elmer
2) Microwave Digester	ETHOS SEL, MILESTONE
3) Hot Air Oven	ULE 500, MEMMERT
4) Hood	Wiwatsan
5) pH Meter	Sension 2, HACH
6) Analytical balance 4 digits	BP 221S, Sartorius
7) Analytical balance 1 digits	SBA61, SCALTEC
8) Vacuum pump	N035AN.18-IP20,
9) Shaker	OS-2, Green Seriker2

3.1.3 Chemicals

The chemicals for this experiment are shown in Table 3.2.

Table 3.2 Chemicals used in this research

Chemicals	Company/Country
1) Nitric acid (65% HNO ₃)	MERCK, Germany
2) Hydrochloric acid (37% HCl)	MERCK, Germany
3) Hydrogenperoxide (30% H ₂ O ₂)	MERCK, Germany
4) Diethylenetriamine-pentaacetic acid; DTPA (C ₁₄ H ₂₃ N ₃ O ₁₀)	Fluka, Switzerland
5) Triethanolamine (N(CH ₂ CH ₂ OH))	Unilab, Australia
6) Calcium chloride (CaCl ₂ .2H ₂ O)	Univar, Australia
7) Cadmium nitrate (Cd(NO ₃) ₃ .4H ₂ O)	Unilab, Australia
8) Zinc sulphate (ZnSO ₄ .7H ₂ O)	Univar, Australia
9) Lead (II) nitrate (Pb(NO ₃) ₂)	Unilab, Australia
10) Copper (II) sulphate (CuSO ₄ .5H ₂ O)	Univar, Australia

3.2 Soil preparation

Soil samples were excavated at surface to 30 cm depth from two areas; 1) uncontaminated soil at Mae Ku, Mae Sot District, Tak Province and 2) contaminated soil at Phra That Pha Daeng Sub-district, Mae Sot District, Tak Province, in the north of Thailand. The mapping site is shown in Appendix E.1. Soil was dried in the open air, crushed, passed through a 2 mm diameter sieve, mixed, and analyzed. Basic physical and chemical properties of the soil used in pot experiment were determined as presented in Table 3.3.

3.3 Experimental design and procedure

The pots for this study were 12 inch diameter pots each with a drainage hole. The plastic bags were placed under the pots to collect drainage water and this water was later poured back into the pots in order to prevent the loss of metals through leaching.

C. odorata and *V. zizanioides* seedlings were selected and pruned to 20 cm. height of stem, 8 cm. length of roots, and transplanted into pots that contained 5 kg of soil. The seedlings were potted into contaminated and uncontaminated soil. Then, the samples were maintained in a nursery for 3-4 weeks in order to observe their hardiness before adding heavy metals solution into the soil.

After that, the mixed heavy metal solution ($\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{Pb}(\text{NO}_3)_2$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was added into pots that contained uncontaminated soil. This was defined as synthetic soil as shown in Figure 3.1. Each heavy metal solution was added into the soil samples at the rate of 100 ppm. The calculations of heavy metals solution are shown in Appendix A. In addition, uncontaminated soil sample without plants and with no addition of heavy metals solution was studied for control purposes, and 400 milliliters water was applied in the control soil pot to serve as blank for metals uptake by plant.

Five hundred mL of water was applied to each pot every two days in the morning. Ten grams of 15-15-15 NPK fertilizer was also added to all pots every 30 days. The growth conditions such as height and symptoms of phytotoxicity manifested by the plants were recorded every 15 days.

Table 3.3 Basic physical and chemical of soils properties used in the experiment

Soil properties	Uncontaminated	Contaminated	Method
	soil	soil	
pH	6.38	7.38	pH meter
ORP (mS/cm)	236.40	200.50	pH Meter with ORP
Moisture Content (%)	6.35	9.89	Oven dried 105° C
Organic Matter (%)	1.38	3.26	Walkley and Black Method
CEC (meq/100g)	7.60	10.90	Ammonium Saturation and Distillation
Total Nitrogen (%)	0.07	0.16	Kjeldahl Method
Total Phosphorus (ppm)	415	439	Vanadomolybdate Method
Total Potassium (ppm)	74.00	118.00	Ammonia Acetate 1N pH 7 Extraction
Total Cd (mg/kg)	2.78	46.24	EPA 3052 Method
Total Zn (mg/kg)	36.95	2124.26	EPA 3052 Method
Total Pb (mg/kg)	16.42	87.96	EPA 3052 Method
Total Cu (mg/kg)	13.44	25.89	EPA 3052 Method
Sand (%)	55.40	32.80	Hydrometer Method
Silt (%)	24.60	35.00	Hydrometer Method
Clay (%)	20.00	32.30	Hydrometer Method
Soil Texture	Sandy Clay Loam	Clay Loam	Hydrometer Method

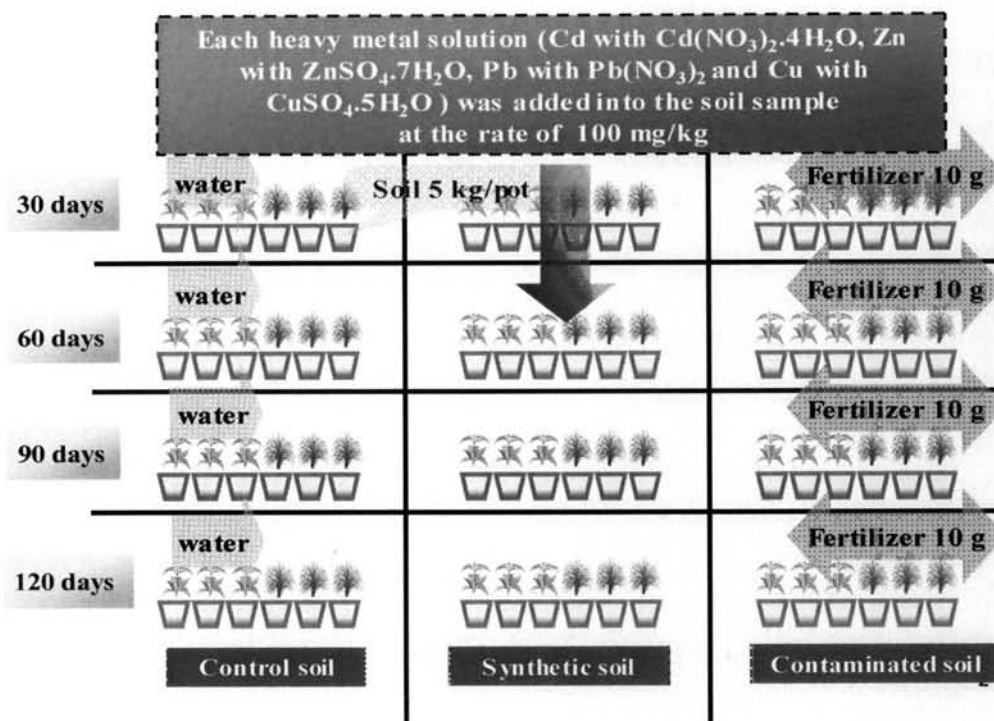


Figure 3.1 Illustrates the diagram of pot experiment design as well as how to sampling both soil and plants

3.4 Samples collection

3.4.1 Soil samples

Soil samples were collected at 30, 60, 90 and 120 days of the cultivation (see also Table 3.3). The soil from each sample pot was mixed and then was taken as soil samples to determine for pH and the heavy metals for three replications. The pH value in soil sample was determined by water-soil ratio (1:1 soil to water ratio) and measured with pH meter. The soil samples analyzed for total heavy metals and available heavy metals. For total heavy metals analysis, the samples were oven-dried at 70 °C for 72 hrs to get a constant weight and to determine dry matter yields. Then, crushed sample soils were passed through a 2 mm sieve and mixed to get homogenized form. For the determination of Cd, Zn, Pb and Cu available in soil, the process used was similar except the soil samples were dried in the open air for 72 hrs.

3.4.2 Plant samples

Same as soil samples, *C. odorata* and *V. zizanioides* samples were harvested at 30, 60, 90 and 120 days of cultivation. Samples were cleaned, washed with tap water twice and rinsed with deionized water. Then, the plant samples were cut into three parts: roots, stems and leaves. Samples were oven-dried at 70 °C for 72 hrs to get a constant weight, and dry matter yields were determined. Finally, the samples were ground with electric mill and thoroughly mixed to homogenize.

3.5 Samples analysis

3.5.1 Soil samples

The total heavy metal in soils was determined by the USEPA method 3052 (USEPA, 1996) (Appendix B.1.1). All samples were made up to 50 mL with deionized water and preserved at 4 °C until analysis. Available heavy metal in soil was estimated by diethylenetriamine-pentaacetic acid (DTPA) extraction method (Lindsay and Norvell, 1978) (Appendix B.2). Total heavy metal and available metal in solutions were analyzed by Flame Atomic Absorption Spectroscopy (FAAS) (Appendices E.4).

3.5.2 Plant samples

Total heavy metal in roots, stems and leaves of *C. odorata* and *V. zizanioides* were determined by the USEPA method 3052 (USEPA, 1996) (Appendix B.1.2 and B.1.3). After digestion, all samples were made up to 25 mL by addition of deionized water and stored at 4 °C until analysis. The digested solutions were analyzed by Furnace Atomic Absorption Spectrophotometer (FAAS).

3.6 Data and statistic analysis

The relative growth rate (RGR) was calculated by this formula:

$$\text{RGR} = [\text{Ln}(W_2) - \text{Ln}(W_1)] / (t_2 - t_1)$$

Where: W_1 and W_2 are plant fresh weight (g) at time t_1 and t_2 .

Analysis of variance (ANOVA) was performed by using Statistical Package for the Social Sciences (SPSS) program analyzing heavy metals (Cd, Zn, Cu, and Pb) accumulation in soil, whole plant and separate parts of plant with significant level at $p < 0.05$. Another analysis was done using Duncan's New Multiple Range Test, to analyze the different total concentrations of heavy metals in the soil and plants at the different harvesting times. Also, this test was used to obtain the mean values of heavy metals uptake that were not significantly different among themselves.

The research methodology of the study is shown in Figure 3.2

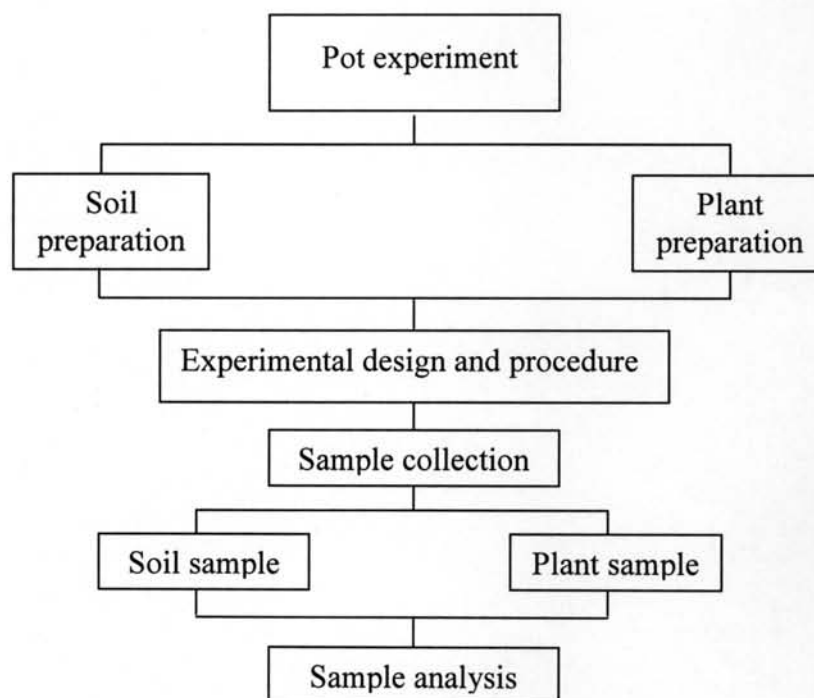


Figure 3.2 Schematic diagram of experimental metric