CHAPTER THREE

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MATERIALS AND METHODS

3.1 Introduction

The data in this thesis was obtained from laboratory and greenhouse experiments carried out to investigate and model the fate and chemical fractionation of cadmium, lead, nickel and zinc added to three Malaysian soils. The experimental and analytical materials and procedures used for the soil and plant analysis in the present study are described in this chapter.

3.2 Experimental soils:

Three soil samples were used in the laboratory experiments; two of them were collected from the University of Malaya at different locations, while the third one was from Damansara Utama, Petaling Jaya. Soil I and soil II are classified as sandy loam soils, while soil III was classified as sand clay loam classified with higher percentage of clay content. The soils were air-dried and ground to pass through 2.00-mm stainless steel sieve. Soil physical, chemical and mineralogical properties of the soils as well as the background level of heavy metals were determined using appropriate analytical instrumental techniques and their related recommended procedures. Table 3.1 presents the basic physical and chemical properties of the three experimental soils.

3.2.1 Soil pH

The soil pH was determined on the fresh air dried samples using Knick digital pH meter on a suspension of soil in water, 1 M KCl after one hour of intermittent shaking and overnight stand.

3.2.2 Organic matter content

Organic matter content was obtained by multiplying the amount of organic carbon with a factor of 1.724 expressed as percentage of the air-dried soils. The total organic carbon was determined using the Walkley and Black titration method as described in the manual of the Soil Science Department, Universiti Putra Malaysia (UPM). Briefly one gram of each soil sample was placed into an Erlenmeyer flask (500 ml) and 10 ml of Potassium dichromate ($K_2Cr_2O_4$) and 20ml of concentrated sulfuric acid were added to the soil and shacked for a moment. 200 ml of distilled water and 3-5 ml phosphoric acid and 17 drops of diphenylamine were then added. The contents were shaken and the resulting solution was titrated with ferrous ammonium sulfate ($FeSo_4 (NH_4)_2 SO_4.6H_2O$).

3.2.3 Cation exchange capacity

The cation exchange capacity (CEC) was determined by the continuous leaching of 10 g of each soil sample with 1 M ammonium acetate at pH 7.0 for 6 hours in a batch system (Blakemore et al., 1987). The leachate was used to measure the basic cations; the exchangeable potassium and sodium were measured using Coring 400 Flame photometer, while the exchangeable calcium and magnesium were measured using Perkin Elmer 370 atomic absorption spectrophotometer. The ammonium in the leachate was then determined by an auto-analyzer and estimated to give the cation exchange capacity.

3.2.4 Total phosphorous

The total phosphorous was determined using spectrophotometer. The soil extract was prepared as described by Chapman and Praff, (1961).

3.2.5 Iron and aluminum

Iron content was measured using atomic absorption spectrophotometer (Perkin Elmer 370 model). The iron was extracted using dithionate citrated bicarbonate method of Mehra and Jackson (1960). Aluminum was extracted by 1 M KCl. The Aluminum was determined calorimetrically by the Hsu method (1963) using a spectrophotometer (Spectronic 88 model) at wavelength of 530 nm

3.2.6 Soil physical properties

The particle size distribution was determined by successive sedimentation. The moisture retention was determined using pressure plate apparatus.

3.2.7 Soil mineralogical composition

X-ray diffraction analysis was carried out to identify the mineral composition of the experimental soils with a Phillips X-Ray Diffractometer. The interpretations of the X-ray patterns were made following the standard guide for mineral identification (Berry 1974). See Appendix A

3.2.8 Background levels of heavy metals in soil

In order to understand the polluting contribution of the added material, the natural background levels of such metals must be known. Levels of heavy metals compared with reference Soil sample So-2 (See Appendix B) were determined by x-Ray fluorescence spectrometer model Philips PW 1480/10. Cd and Co, because of their apparent low concentration, were determined using ICP-MS after 1.0 g of the soil have been digested in 5ml of HNO_3, 2.5 ml $HClO_4$ and 1ml HCl and heated at 130° C for 2 hours. The digest was then diluted to 10ml with double distilled water. Table 3.2 shows the mean of the total concentration of heavy metals in the three experimental soils.

Table 3.1: Physical and chemical properties of the experimental soils

Soil Typ	pH	Organic C (%)	CEC (cmol/kg)	Total P (%)	Fe (%)	Al (%)	Sand (%)	Silt (%)	Clay (%)
Soil I	5.5	0.76	03.24	0.291	1.51	2.20	80.00	7.10	12.19
Soil II	6.1	0.75	03.76	0.246	1.03	1.93	70.00	11.70	18.20
Soil III	7.3	0.86	04.67	0.135	2.40	3.50	63.60	3.80	32.60

Table 3.2: Background levels of heavy metals in soils

Soil Type	Heavy metal concentration ((μg/g))								
	As	Cd	Co	Cr	Cu	Pb	Ni	Sr	Zn
Soil I	6	0.06	0.15	16.00	10.00	125.00	56.20	27.00	93.00
Soil II	7	0.04	0.11	14.30	10.20	179.00	50.10	25.00	68.00
Soil III	12	0.01	0.14	19.60	08.80	197.00	64.00	30.00	54.00

3.3Laboratory experiments

Laboratory long-term incubation experiments were conducted for cadmium (Cd), lead (Pb), nickel (Ni) and zinc (Zn). The elements in the present study were selected due to their association with battery industry and their potential hazard for human health.

3.3.1 The added metals

Cd, Pb, Ni and Zn in the nitrates forms were used in the experiments at four application rates 5, 10, 20 and 40 mmol/kg soil.

3.3.2The incubation procedure

The three soils were amended with cadmium, lead, nickel and zinc in the form of Cd^{++}, Pb^{++}, Ni^{++} and Zn^{++} nitrate salts respectively at rates from 5, 10, 20 and 40 mmol metal/Kg soil (oven-dry weight basis). Incubated in plastic bags at room temperature for three, six, nine, twelve and fifteen months respectively. The soils were treated with the metal nitrates on the 4th March 1999. From the amended soils, soil solutions and solid and solution phases were extracted after three months. The first set of extraction data for soil solution and solid content of the metals was collected on the 4th June 1999. The successive data sets were collected at three-month interval. The last set of extraction data was collected on 4th June 2000.

3.3.3 Determination of total metals content

The soil samples were air-dried at room temperature for seven days, screened through a 2mm sieve and then digested with 60% perchloric acid, 40% hydrochloric acid, conc. nitric acid and conc. sulphuric acid according to Page et al., (1986) method. The total contents of cadmium, lead, nickel and zinc were then determined using Inductively Coupled Plasma Mass Spectrometer (ICP-MS).

3.3.4 Soil solution extraction procedure

The amended soils were air-dried and ground to pass through 2.00-mm stainless steel sieve; soil solution was extracted from 100 g soil, which was rewetted to its field capacity with distilled water. The moisten soils were then equilibrated for one day in plastic containers (Menzies and Bell 1988) and soil solution was extracted by centrifuging at 2000 rpm for two hours. The solution obtained was filtered through 0.22 nm, thereafter, were kept in cold storage prior to instrumental analysis. The solution contents of Cd, Pb, Ni and Zn were measured by ICP-MS

3.3.5 Metals adsorption procedure

Soil samples were prepared and dispersed in distilled water. Known volumes of Cd, Pb, Ni and Zn solution were added to produce final concentrations of heavy metals in the reaction flasks in the range from 5-40 mmol/kg soil. The flasks were shaken at different 5-7 hours and the solid phase separated by filtration through a 0.45-mm membrane filter. The quantity of metal adsorbed onto the soil solid was phase calculated by the difference between the amount of metal added and the final soluble concentration. The filtrates were analyzed for heavy metals concentration using the ICP-MS. (Jingsheng, 1987; Kwanshen 1984)

3.3.6 Sequential extraction procedure

The solid phase distribution of metals was examined with following sequential extraction procedure. Soluble and exchangeable metals were determined by shaking duplicate 2.5g sample with 25ml of 1 M NH_4OAc (pH 7) for 2 hours in 50ml polycarbonate centrifuge tubes. The suspensions were centrifuged, filtered and analyzed. The soils were resuspended in 25ml of 0.125 M $Cu(OAc)_2$ and shaken for 16 hours. The suspensions were centrifuged filtered and analyzed to determine the complexed metals. The soils were then resuspended in 25 ml of 1 M HNO_3 and allowed to react with frequent mixing with a glass rod for 24 hours. The supernatant solutions were filtered and analyzed to determine the strongly adsorbed and precipitated metals. The filtrates were analyzed for Cd, Pb, Ni and Zn by ICP-MS with background correction of the metals using blank samples made up from the extracting solutions. All equilibrations were done at room temperature (Soon and Bates, 1982).

3.3.7 Soil solution speciation model Visual MINTEQA

Visual MINTEQ is a Windows version of MINTEQA2 ver 4.0, which was released by the United States Environmental Protection Agency (USEPA) in 1999 (See Appendix C). MINTEQA2 ver 4.0 is a chemical equilibrium model for the calculation of metal speciation, solubility equilibria etc. for natural solutions. MINTEQA2 is a DOS computer program designed by the USEPA to determine the activities and speciation of many soil constituents using a database of equilibrium constants. The program consists of two parts, the first, called PRODEFA, serves as a front end to create an input file that describes the system. The second part, MINRUN, runs the MINTEQA2 speciation models and creates an output file. The database from MINTEQA2 was last updated in 1991. MINTEQA2 is capable of computing equilibrium among the dissolved, adsorbed and solid phases in an environmental setting. MINTEQA2 includes an extensive database of reliable thermodynamic data that is accessible to PRODEFA2, an interactive pre-processor program that was executed prior to MINTEQA2 for the purpose of creating the required MINTEQA2 input files. MINTEQA2 and PRODEFA2 version 3.0 of the USEPA (1991) was used in this study to calculate equilibrium values of different chemical species of the added metals in the soil solution. The input data used in MINTEQA2 consists of the total dissolved concentrations of metals at equilibrium, pH, other relevant soil properties and the reliable preloaded database of thermodynamic data. MINTEQA2 specifies as equilibrium values.

3.4 Greenhouse experiments

Greenhouse experiments were conducted at the Department of Botany, University of Malaya greenhouse to study the plant up take of cadmium, lead, nickel and zinc from soils amended with the four metals at 0, 5, 10, 20, 40 mmol/kg using a sand clay loam (soil III), the physical and chemical properties of the experimental soil were shown in table 3.1. The soil was air-dried, ground to pass 2.00mm sieve. Plastic pot of 20.0 cm in diameter and 16.0 cm deep were used as experimental units each pot was filled with 5kg soil

3.4.1 Metals amendments

Four concentrations (0, 5, 10 and 20 mmol/kg) of cadmium, lead, nickel and zinc nitrates were applied to the soil beginning at three, six, nine, twelve and fifteen months respectively before planting. Metal nutrients solutions were applied and mixed to the soil to ensure evenly distribution and prevent the possible precipitation of the metal by the soil components. The soils were frequently wetted to their field capacity by distilled water and the excess soil moisture draining from perforations at the bottom of each pot was trapped in a plastic saucer placed below each pot to prevent the leaching from the soil and the cross contamination among pots. Each treatment received basal doses of nitrogen, phosphorous and potassium (NPK) one week before each sowing. The sources of NPK are urea, triple super phosphate and potassium sulphate, respectively.

3.5.2 Experimental plant

Lettuce (*Lactuce sativa*) was used as test crop. Lettuce was grown under the natural daylight. Four seeds of lettuce per pot were sown and then thinned to two per pot after germination. Deionized water was used for irrigation throughout each experimental period. The crop was harvested six weeks after each sowing.

3.5.3 Experimental design

There is a separate experiment for each of the above-mentioned metals each of which consists of four levels of the named metal and three replicates in a Completely Randomized Design (CRD)

3.5.4 Heavy metal analysis of plant samples

Heavy metals contents of plant tissue were extracted using nitric acid-hydrogen peroxide procedures (USEPA, 1990) with slight modifications. Briefly, 40 ml of 50% aqueous nitric acid were added to a representative 1- to 2-gram sample of ground plant tissue. The acidified sample was heated to 95C, refluxed for 15 minutes without boiling and then allowed to cool. Another 10 ml of 50% aqueous nitric acid were added and the sample was again heated and refluxed for 30 minutes. The heated sample was allowed to cool, then completely oxidized in 5 ml concentrated nitric acid. The oxidized solution was allowed to evaporate to approximately 5 ml without boiling. To initiate the peroxide reaction, 2 ml of deionized, distilled water and 3 ml of 30% hydrogen peroxide were added to the concentrated digestate and then were heated until effervescence subsided. Another 7 ml of 30% hydrogen peroxide were added continuously in 1 ml aliquots as the digestate was again heated. The digestate was heated until effervescence was minimal and its volume reduced to approximately 5 ml. After cooling, the final digestate was diluted to about 100 ml with deionized, distilled water. The digestate was filtered through a filter paper (Whatman No. 1) and the final volume was adjusted to 100 ml deionized distilled water (USEPA, 1990). Metals content of each sample were quantified using inductively coupled argon plasma spectroscopy and expressed as μg metal/gram dry weight of plant tissue.

3.5.5 Statistical analysis

Statistical analysis for the data collected from the laboratory experiments and the greenhouse trails for the four metals separately has been carried out using computer program SPSS Version 10.