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Tel.: +32(0)11 26 81 11

Risk based assessment of *in situ* remediation strategies for metal contaminated soils:
metal mobility, phytoavailability and phytotoxicity

Ann Ruttens

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Risk based assessment of *in situ* remediation strategies for metal contaminated soils: metal mobility, phytoavailability and phytotoxicity

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Ann RUTTENS

Promotor: prof. dr. J. VANGRONSVELD
Copromotor: prof. dr. J.V. COLPAERT

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Ann
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Contents

Abbreviations

General

AAS	atomic absorption spectroscopy
ATP	adenosine tri-phosphate
BCR	community bureau of reference
C	compost
CA(H)	cyclonic ashes (type H)
CEC	cation exchange capacity
DOC	dissolved organic carbon
DW	dry weight
EC	electric conductivity
E.C.	enzyme commission
EDTA	ethylenediaminetetraaceticacid
EXAFS	extended X-ray absorption fine structure
e^-	electron
FIA	flame injection analyser
FIAM	free ion activity model
FW	fresh weight
ICP	induced coupled plasmaspectrometry
LMWOA	low molecular weight organic acids
M_{exch}	exchangeable soil metal concentration (0.1M $Ca(NO_3)_2$ extract), with M referring to the various metals
OECD	organization for economic cooperation and development
OM	(soil) organic matter
PBET	physiologically based extraction test (Brown and Chaney, 1999)
PC	phytochelatin
PI	phytotoxicity index
pK	-log(equilibrium constant)
PZC	point of zero charge
ROS	reactive oxygen species
SE	standard error
sp.	species
SS	steel shots
WHC	water holding capacity
w/w	weight/weight
XRD	X-ray diffraction
XRF	X-ray fluorescence

Enzymes and related compounds

AOX	alternative oxidases	
APX	ascorbate peroxidase	(E.C. 1.11.1.11)
CAT	catalase	(E.C. 1.11.1.6)
DHA	dehrydroascorbate	
DHAR	dehydroascorbate reductase	(E.C. 1.8.5.1)
FD	ferredoxin	
FNR	ferredoxin NADPH reductase	(E.C. 1.18.1.2)
G6PDH	glucose-6-phosphate dehydrogenase	(E.C. 1.1.1.49)
GlDH	glutamate dehydrogenase	(E.C. 1.4.1.2)
GLR	glutaredoxin	
GPOD	guaiacol peroxidase	(E.C. 1.11.1.7)
GPX	glutathione peroxidase	(E.C. 1.11.1.9)
GR	glutathione reductase	(E.C. 1.6.4.2)
GSH	reduced glutathione	
GSSG	glutathione disulfphide (oxidized GSH)	
GST	glutathione-S-transferase	(E.C. 2.5.1.18)
ICDH	isocitrate dehydrogenase	(E.C. 1.1.1.42)
LOX	lipoxygenase	(E.C. 1.13.1.11)
MDHA	monodehydroascorbate	
MDHAR	MDHA reductase	(E.C. 1.6.5.4)
ME	malic enzyme	(E.C. 1.1.1.40)
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate)	(oxidized form)
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)	(reduced form)
POD	peroxidase (general)	(E.C. 1.11.1.7)
PrxR	peroxiredoxin	(E.C. 1.11.1.15)
SOD	superoxide dismutase	(E.C. 1.15.1.1)
SPOD	syringaldazine peroxidase	(E.C. 1.11.1.7)
TRX	thioredoxin	

Abstract

Soil contamination with anthropogenic contaminants has become a growing concern is our industrialized world. This concern relies primarily on the potential adverse effects of contaminants on human health and ecosystems (food chain transfer, inhalation of wind blown dust, impacts on groundwater quality,...), and is likely accentuated by an increasing need for land, to meet the demands of worlds ever-increasing population. *In situ* immobilization of metals, by means of metal immobilizing soil amendments, whether or not in combination with phytostabilization approaches, has been proposed as a low cost and environmentally sound alternative to current remediation options for metal contaminated soils. Even if these techniques can not be considered as real clean-up methods, because they do not affect the total soil metal content, they can reduce the inherent risk associated with a contaminated site based on a reduction of the soil mobile and bioavailable metal fraction. As a result, food and feed can be grown more safely, or on more heavily contaminated sites a vegetation cover can develop which stabilizes the soil (reduction of wind erosion, water runoff and metal leaching to the groundwater). These techniques can complement or replace civil engineering techniques when budgetary and/or scale constraints do not allow the complete clean-up of a site.

An example of a large-scale metal contamination can be found in the north east of Belgium (Campine region; Dutch: de Kempen). In this region, at the Dutch-Belgian boundary, an estimated area of at least 280km^2 (at the Belgian side of the border) is contaminated with metals (mainly Zn, Cd, Pb) due to the historical presence of several zinc smelters. Because it is virtually impossible to apply conventional clean-up methods all over the area, the Flemish government has expressed a fundamental interest in potential applications of alternative remediation technologies, including *in situ* metal immobilization.

The objectives of the present work were: (1) to asses the potential of several metal immobilizing soil treatments to reduce the risks associated with the soil contamination in the Kempen on the long term, and (2) to optimize a phytotoxicity test (PlantTox) aiming at an accurate detection of soil phytotoxicity (detection of soil phytotoxicity, both prior to and after metal immobilization, is an important aspect of phytostabilization studies).

To meet these objectives the **first experimental part of this work** evaluates and compares the durability and metal immobilization efficiency of several inorganic soil treatments, being: lime (LIME), cyclonic ashes (CA), steel shots (SS), and the combined application of cyclonic ashes + steel shots (CA+SS). Potential benefits of combination with an organic treatment (compost, C) were also addressed. Amendment induced reductions in metal phytotoxicity, plant metal accumulation, exchangeable/soluble soil metal fractions and metal leaching were evaluated using soil columns (Ø 25cm, height 1 m) or outdoor-placed lysimeters (base 50x50 cm; height 70 cm).

In a rainfall simulation experiment in soil columns, CA showed a more durable immobilization compared to LIME during a 26-years simulation period. The soil used in this experiment was collected at a former agricultural field contaminated with metals due to atmospheric depositions from a nearby zinc smelter (Balen). In order to distinguish between pH-induced metal immobilization processes on the one hand, and the potential presence of additional sorption mechanisms (in case of CA) on the other hand, both treatments were compared at a similar start pH. While soil pH in both treatments decreased in parallel during the simulation period, the evolution in metal mobility and phytoavailability showed a clearly distinct pattern. In the LIME treatment, reductions in exchangeable soil metal fractions, plant metal accumulation and soil pore water concentrations relative to the untreated soil, were much smaller at the end of the simulation period than at the start. The only exception was found for Pb in soil pore water. During the first weeks of the experiment an increase of the soluble Pb concentration was observed in the LIME treatment compared to the untreated soil; this LIME-induced Pb mobilization disappeared at later moments. In contrast with the observed reductions in metal immobilization by LIME in the course of time, the metal immobilizing effect of CA was maintained much better during the 26-years simulation period. Moreover the initial mobilization of Pb was not observed in the CA treatment. At the end of the simulation period plant accumulation of Zn, Cd and Pb in the CA treatment was still a factor 2.6, 2.0 and 1.4 lower compared to results obtained in the untreated soil, while in the LIME treatment the reduction factors were not higher than 1.4 at that moment. Metal immobilization in a combined CA+SS treatment was in general stronger than in the individual treatments.

The potential benefit of the application of an organic soil amendment in combination with the inorganic products, was evaluated in a lysimeter set-up, under natural environmental conditions. An industrially contaminated soil, originating from an old zinc smelter site in Overpelt, was treated with compost (C), C+CA, C+CA+SS or CA+SS and effects on metal phytotoxicity, plant availability of metals and metal leaching were measured during a 1-year experimental period. Addition of compost alone, reduced soil phytotoxicity to bean seedlings (*Phaseolus vulgaris)* cultivated on the soils, as illustrated by a reduction in 'Phytotoxicity Index' (on a scale from 1 to 4) from 3.5 to 2.8 after this treatment. Soil treatment with compost also reduced metal accumulation in grasses (*Agrostis capillaris*), which were colonizing the lysimeters spontaneously: mean Zn, Cd and Pb concentrations dropped respectively from 623 to 135, from 6.2 to 1.3 and from 10.7 to <6 mg kg^{-1} dry weight. Stronger reductions in soil phytotoxicity and metal accumulation were obtained when compost was combined with the inorganic soil amendments. Best results were obtained when using C+CA+SS. This treatment reduced metal accumulation in grasses to values obtained in uncontaminated soils, led to the highest diversity of spontaneously colonizing plants, and to the lowest phytotoxic responses in bean plants. The soil ‘Phytotoxicity Index’ after this treatment was 1.7. All treatments decreased Zn and Cd leaching. The strongest reductions were found after CA+SS and C+CA+SS treatments (Zn: 99.0% and 99.2% reduction respectively; Cd: 97.2% and 98.3% reduction respectively). In addition to reduced metal concentrations in the leachate, the improved water-holding capacity of the soils (and as a result a reduced amount of leaching water) also contributed to the effect. Total Cu and Pb leaching from the lysimeters increased after C treatment (17 and more than 30 times for Cu and Pb respectively) and C+CA treatment (4.4 and more than 3.7 times for Cu and Pb respectively). After soil treatment with C+CA+SS or CA+SS Cu leaching was not increased. Because Pb concentrations in the untreated percolate were below the detection limit of our measurements, it is unclear whether or not the slightly increased Pb concentrations after these treatments could be fully compensated by the reduced amount of leachate. Our results demonstrate that attention should be paid to Cu and Pb leaching when organic matter additions are considered for phytostabilization of metal contaminated soils. Based on the first year evaluation, C+CA+SS was concluded to be an efficient treatment for the amendment assisted phytostabilization of Overpelt soil.

The **second experimental part** of this thesis aimed to contribute to the refinement of a phytotoxicity test with bean plants (*Phaseolus vulgaris cv. Limburge vroege*) in order to improve its applicability as a bioassay. More in particular the study aimed to gain insight in the frequent ranges of the test endpoints in non-toxic conditions and to contribute to the determination of the most suited reference level or reference soil (negative control) for the test when used as a bioassay in ecotoxicological risk assessment of contaminated soils. The so-called PlantTox test is based on the analysis of growth parameters and biochemical plant responses, in particular enzyme activities that are directly or indirectly involved in the antioxidative defence systems of plants. A series of 25 potential reference soils with various textures and with different land use and cropping history were collected throughout Flanders (Belgium). The samples were collected in areas with expected low levels of metal deposition. An artificial soil, prepared according to the prescriptions of the Organization for Economic Cooperation and Development (OECD) and consisting of 70% sand + 20% kaolinite clay + 10% *Spaghnum* peat, was also included in the test series. Physico-chemical soil characteristics (pH, cation exchange capacity (CEC), conductivity (EC), organic matter content (OM), total and exchangeable metal contents of Zn, Cd, Cu, Pb, Ni, Al, Fe and Mn) were determined and the phytotoxicity test with bean plants (PlantTox) was performed as described by Van Assche and Clijsters (1990a). When very acid soils were excluded, endpoints of the PlantTox test showed a rather limited range in response variation, over the complete series of potential reference soils. Therefore it was proposed as an option to use only one reference soil in the PantTox test in screening experiments for soil phytotoxicity. OECD standard soil was proposed as a candidate general reference soil in the test, at least for soils with pH>4. A soil toxicity classification system was developed based on the use of this standard soil as a negative control in the PlantTox test, and taking the 90th or 10th percentile, respectively, of enzymatic and growth responses in the reference dataset as upper/lower limit of the first phytotoxicity class (i.e. non toxic). Additional toxicity classes were added based on the extent of effects found in toxic conditions. Multiple regression analysis relating plant responses to physicochemical soil parameters, delivered significant models for all enzymes measured, with R^2 ranging from 0.83 to 0.99. Soil pH, conductivity and exchangeable levels of Zn, Cu, Al, Fe or Mn were present as predictor variables in the regression equations. No significant models could be fitted for any of the growth responses measured. This observation suggests that growth responses, in contrast to enzyme capacities, are to a large extent determined by other soil factors than those investigated in our study. Available soil levels of macronutrients (N, P, K) and some physical or biological soil factors are possible candidates. Application of the models potentially allows the use of a flexible reference level in the PlantTox test, adapted to each individual soil. However, due to the relatively large prediction limits of the models compared to the total range of response variation in non-toxic conditions, practical benefits of this ‘model approach’ could not (yet) be obtained. In a last chapter, OECD standard soils, that had been prepared with constituents of different commercial origins were found to show varying enzymatic responses. These results raise questions about the general suitability of this standard soil as negative control in the PlantTox test (and in other tests?). We concluded that the term OECD standard soil should be interpreted with care because its composition, by definition, is not completely standardized.

Samenvatting

In onze geïndustrialiseerde samenleving is de verontreiniging van bodems met allerhande contaminanten (zoals bevoorbeeld metalen) een toenemende bekommernis. Deze bekommernis heeft vooral te maken met de mogelijk nadelige effecten van contaminanten op de menselijke gezondheid en op natuurlijke ecosystemen (transfer in de voedselketen, inademing van stof, aantasting van de grondwaterkwaliteit,...). De groeiende vraag naar bruikbare oppervlakten, om te kunnen voldoen aan de noden van een alsmaar uitbreidende wereldbevolking, speelt daarin wellicht een versterkende rol. Een mogelijke optie voor de remediëring van metaalverontreinigde bodems is de *in situ* immobilisatie van metalen, met behulp van metaalimmobiliserende bodemadditieven, eventueel in combinatie met fytostabilisatie. Dergelijke remediatiemethoden kunnen niet beschouwd worden als echte zuiveringstechnieken, omdat ze het totaal metaalgehalte van de bodem niet verlagen, maar ze zijn wel in staat om de risico's van een bodemverontreiniging te verminderen omdat ze zorgen voor een reductie van de mobiele en biobeschikbare metaalfractie in de bodem. In landbouwgebieden kan dit ervoor zorgen dat voedsel- en voedergewassen weer veilig kunnen geteeld worden, of op sterker verontreinigde, onbegroeide sites kan zich hierdoor een vegetatiedek ontwikkelen dat de bodem stabiliseert (vermindering van stofopwaai, van watererosie en van doorsijpeling van metalen naar het grondwater). Deze technieken kunnen daarom een nuttige aanvulling, of zelfs een vervanging, zijn van de klassieke ingenieurstechnieken indien budgetbeperkingen of de grootschaligheid van een contaminatie de volledige decontaminatie van een site onmogelijk maken.

Een voorbeeld metaalverontreiniging op grote schaal, is te vinden in het noordoosten van België (De Kempen). In deze streek, aan de Belgisch-Nederlandse grens, is de bodem in een gebied van naar schatting 280 km ^{2}

(alleen al aan de Belgische kant van de grens) aangerijkt met metalen (vooral Zn, Cd en Pb) ten gevolge van de historische aanwezigheid van de non-ferro industrie. Omdat het quasi onmogelijk is de bodems in de hele regio te saneren met behulp van klassieke saneringsmethoden (zoals afgraven, storten, *ex situ* purificatie,...), heeft de Vlaamse overheid een gegronde interesse uitgesproken in de mogelijke toepassingen van alternatieve remediatietechnieken, waaronder metaalimmobilisatie.

Het huidige werk had tot doel om: (1) de potenties van verschillende metaalimmobiliserende bodemadditieven te evalueren om de risico's van de metaalverontreiniging in de Kempen op lange termijn te reduceren, en (2) een fytotoxiciteitstest te optimaliseren met het oog op een accurate detectie van bodemfytotoxiciteit; dit laatste is van belang met het oog op fytostabilisatie toepassingen.

Om deze objectieven te bereiken werd in het eerste experimentele deel van dit werk de duurzaamheid en het metaalimmobiliserend vermogen van verschillende anorganiche bodemadditieven geëvalueerd en vergeleken: namelijk kalk, cycloonassen (CA; en gemodificeerd aluminosilicaat), steel shots (SS, een sterk Fe-houdend materiaal) en de combinatie van cycloonassen met steel shots (CA+SS). Tevens werd aandacht besteed aan een mogelijk gunstige effect van de toevoeging van een organische behandeling (compost). Reducties van fytotoxiciteit, van metaalaccumulatie in planten, van de oplosbare of uitwisselbare metaalfractie in de bodem, en van de doorsijpeling van metalen, werden geëvalueerd met behulp van bodemkolommen (Ø 25 cm, hoogte 1m) enerzijds, en van lysimeters (grondoppervlakte 50x50 cm; hoogte 70 cm), anderzijds.

In een neerslagsimulatie experiment in bodemkolommen vertoonde CA een meer duurzaam immobilisatie effect in vergelijking met KALK gedurende een simulatie periode van 26 jaar. De bodem voor het experiment was afkomstig van een voormalige akker, verontreinigd met metalen ten gevolge van atmosferische depositie afkomstig van een naburige zinkfabriek (Balen). Om een onderscheid te kunnen maken tussen een pH-geïnduceerde immobilisatie van metalen, en de mogelijke aanwezigheid van bijkomende sorptiemechanismen (voor CA) werden beide behandelingen vergeleken bij een zelfde initiële pH. Alhoewel de pH van de bodem in beide behandelingen in parallel afnam gedurende de simulatieperiode, vertoonde de evolutie van metaalmobiliteit en plantbeschikbaarheid duidelijk verschillende patronen. In de KALK behandeling werd op het einde van het experiment een veel geringere reductie vastgesteld van de uitwisselbare metaalfractie, van de plantaccumulatie en van de metaalconcentratie in het poriewater, in vergelijking met de onbehandelde bodem, tegenover het effect bij de start. Een uitzondering werd vastgesteld voor Pb waar de KALK behandeling gedurende de eerst weken na behandeling leidde tot een sterk verhoogde concentratie in het poriewater. Deze door KALK geïnduceerd mobilisatie van Pb was niet meer aanwezig op latere ogenblikken van de simulatie periode. In tegenstelling tot de waargenomen afname van het immobiliserend effect van KALK in de loop van de tijd, bleef het effect van CA veel beter behouden gedurende de 26 gesimuleerde jaren. Bovendien werd de initiële mobilisatie van Pb niet vastgesteld in de CA behandeling. Op het einde van simulatieperiode was de accumulatie van Zn, Cd en Pb in planten nog steeds een factor 2.6, 2.0 en 1.4 lager in vergelijking met de resultaten bekomen op onbehandelde bodem, terwijl voor KALK en SS de reductie factor op dat moment voor geen enkel element meer was dan 1.4. De gecombineerde CA+SS behandeling vertoonde in het algemeen een sterker effect dvan de individuele behandelingen.

In een lysimeter experiment, uitgevoerd onder natuurlijke klimaatsomstandigheden, werd het mogelijk gunstig effect van de toepassing van een organisch bodemverbeteringsmiddel in combinatie met de anorganische behandelingen geëvalueerd. Een industiëel gecontamineerde bodem, afkomstig van een voormalige zinksmelter site in Overpelt, werd behandeld met compost

(C), C+CA, C+CA+SS en CA+SS en de invloed van de verschillen de behandelingen op metaal fytotoxiciteit, plant beschikbaarheid van metalen en uitspoeling werd gemeten tijdens een 1-jaar durende experimentele periode. De resultaten toonden aan dat toevoeging van compost alleen in staat was om de bodemfytotoxiciteit (voor bonenplanten, *Phaseolus vulgaris*) te reduceren. De 'Fytotoxicteits Index' van de bodem (op een schaal van 1 tot 4) nam af van 3.5 tot 2.8 bij deze behandeling. Toevoeging van compost aan de bodem verlaagde ook de metaalaccumulatie in grassen (*Agrostis capillaris*) die spontaan gekiemd waren op de lysimeters: Zn, Cd en Pb concentratie in de grassen verlaagde respectievelijk van 623 tot 135, van 6.2 tot 1.3 en van 10.7 tot <6 mg kg^{-1} droge stof. Sterkere reducties in bodem fytotoxiciteit en metaal accumulatie in planten werden bekomen wanneer compost gecombineerd werd met de anorganische additieven. De beste resultaten werden bekomen in de C+CA+SS behandeling. Deze behandeling reduceerde metaalconcentraties in het gras tot waarden gelijk aan deze bekomen op niet verontreinigde bodems, ze leidde tot de hoogste diversiteit aan spontaan koloniserende plantensoorten, en tot de laagste fytotoxische respons in bonenplanten. De 'Fytotoxiciteits Index' van de bodem na deze behandeling was 1.7. Verder werd in alle behandelingen een verlaging van de doorsijpeling van Zn en Cd vastgesteld. De sterkste reducties werden gevonden in de CA+SS en C+CA+SS behandelingen (Zn: respectievelijk 99.0% en 99.2% reductie; Cd: respectievelijk 97.2 en 98.3% reductie). Het effect werd niet alleen veroorzaakt door de verlaging van de metaalconcentraties in het percolerend water, maar ook door het verbeterd waterhoudend vermogen van de bodem (waardoor minder water percoleerde). De totale uitspoeling van Cu en Pb in de lysimeters nam toe na compost behandeling (voor Cu: 17 keer meer dan in de onbehandelde; voor Pb: >30 keer meer) en na C+CA behandeling (voor Cu: 4 keer meer dan in de onbehandelde; voor Pb: >3.7 keer meer). Na bodembehandeling met C+CA+SS of CA+SS werd geen verhoging van de Cu uitspoeling vastgesteld. De Pb concentraties waren in het percolatiewater van de onbehandelde lysimeters beneden de detectielimiet van de metingen waardoor het niet helemaal duidelijk is of de schijnbaar lichte toename van de Pb concentraties in de laatste genoemde wel of niet volledig kon gecompenseerd worden door de verminderde hoeveelheid percolatiewater. Onze resultaten tonen aan dat het nodig is om een mogelijk verhoogde doorsijpeling van Cu en Pb niet uit het oog te verliezen, wanneer toevoegingen van organisch materiaal overwogen worden bij fytostabilisatie van metaalverontreinigde bodems. Algemeen bleek op basis van deze 1-jarige evaluatie, dat C+CA+SS een geschikte behandeling was voor de met additieven ondersteunde fytostabilisatie van de industriële bodem uit Overpelt.

Het tweede experimentele luik van deze thesis had als doel een bijdrage te leveren aan de optimalisatie van een fytotoxiciteitstest met bonenplanten *(Phaseolus vulgaris*) om de toepasbaarheid ervan te verhogen voor de bepaling van bodemfytotoxiciteit. Er werd beoogd een inzicht te verweren in de typische range van de eindpunten van de test in niet-toxische omstandigheden, om zo bij te dragen tot de bepaling van de meest geschikte referentiebodem of het meest geschikt referentieniveau (negative controle), wanneer de test wordt toegepast voor de ecotoxicologische risico-evaluatie van verontreinigde bodems. De zogenaamde ‘PlantTox’ test is gebaseerd op de analyse van groeiparamters en biochemische plantresponsen, met name de activiteit van enzymen die direct of indirect betrokken zijn in het anti-oxidatief verdedigingssysteem van de plant. Een reeks van 25 potentiële referentiebodems, met verschillende bodemtextuur en afkomstig van lokaties met een verschillend bodemgebruik, werd verzameld doorheen de verschillende geografische streken van Vlaanderen, op plaatsen waar geen verhoogde depositie van metalen verwacht werd. Een artificiële bodem, bereid volgens de voorschriften van de Organisatie voor Economische Samenwerking en Ontwikkeling (OECD), en bestaande uit 70% sand, 20% kaoliniet klei en 10% turf, werd ook opgenomen in de bodemreeks. Een aantal fysicochemische bodemeigenschappen (pH, cationenuitwisselingscapaciteit, conductiviteit, organische stof gehalte, en totale en uitwisselbare gehalten van Zn, Cd, Cu, Pb, Ni, Al, Mn, Fe) werden bepaald en vervolgens werd de PlantTox test uitgevoerd zoals beschreven door Van Assche en Clijsters (1990). Wanneer geen rekening werd gehouden met de zeer zure bodems, dan vertoonden de eindpunten van de PlantTox test een vrij beperkte range van responsvariaties over de volledige reeks van potentiële referentiebodems. Daarom werd het gebruik van slecht 1 referentiebodem in screeningsexperimenten voor bodemtoxiciteit voorgesteld als een mogelijke optie voor de PlantTox test. De OECD standaard bodem werd voorgesteld als potentieel algemeen bruikbare referentiebodem, tenminste voor bodems met een pH>4. Er werd een bodemfytotoxiciteits-klassifikatiesysteem ontwikkeld gebaseerd op het gebruik van deze standaardbodem als negative controle in the PlantTox test, waarbij respectievelijk het 90ste en 10de percentiel van de enzymatische en van de groeiresponsen bekomen in de referentiedataset, werd gehanteerd als boven-/ benedenlimiet van de eerste toxiciteitsklasse (niet toxisch). Bijkomende toxiciteitsklassen (klasse 2 tot 4) werden toegevoegd op basis van de omvang van effecten waargenomen in toxische condities (literatuur data). Met behulp van multiple regressieanalysen, waarin plantresponsen werden gerelateerd aan fysicochemische bodemparameters, werden significante modellen gevonden voor alle gemeten enzymen, met R^2 waarden variërend van 0.83 tot 0.99. Bodem pH, conductiviteit en uitwisselbare gehalten van Zn, Cu, Al, Fe of Mn waren aanwezig als predictorvariabelen in de regressievergelijkingen. Voor de groeiresponsen konden geen significante modellen gevonden worden. Deze vaststelling suggereert dat de gemeten groeiresponsen, in tegenstelling tot de enzymatische responsen, in grote mate bepaald worden door bodemfactoren die niet in deze studie gemeten werden. Mogelijke factoren zijn o.a. de beschikbare gehalten aan macronutriënten (N,P,K) and bepaalde fysische of biologische bodemparameters. De toepassing van deze modellen laat potentieel toe om een bodem-speciek referentieniveau te berekenen aangepast aan de eigenschappen van elke individuele bodem. Omwille van de vrij brede predictieintervallen rond de modellen echter, in vergelijking met de volledige range in responsvariatie zoals vastgesteld in de reeks van potientiële referentiebodems in deze studie, kan (nog) geen praktisch voordeel bereikt worden met deze modelbenadering. In een laatste hoofdstuk werden verschillen in enzymatische responsen waargenomen in OECD standaardbodems die bereid werden met bestanddelen (zand, turf, klei) van verschillende commerciële origines. Deze resultaten doen vragen rijzen bij de algemene geschiktheid van de OECD standaard bodem als negatieve controle in de PlantTox test en mogelijk ook in andere testen. De term 'OECD standaardbodem' dient met de nodige voorzichtigheid geïnterpreteerd te worden, vermits zijn samenstelling, bij definitie, niet volledig gestandardiseerd is.

Part I

Introduction

1. BACKGROUND AND OBJECTIVES

Trace metals occur as natural constituents of the Earth's crust, and are ever-present elements in soils, natural waters and living organisms. Some of these elements (e.g. Cu, Zn, ...) have been demonstrated to be essential to living organisms, while others (e.g. Cd, Pb, ...) are not known to have any biological function, but all of them become toxic when present at very high levels in the environment (Adriano, 2001).
The contamination of soil, sediment and water resources with toxic levels of trace metals is an unfortunate side effect of industrialization. The primary sources of contamination have been aerial deposition from burning of fossil fuels and mining and smelting activities. Additional contamination has been caused by motorized traffic, agricultural activities (pesticides, wood preservatives, impurities in fertilizers, manure from intensive animal production, especially pigs and poultry,...), chemical industries (batteries, pigments and paints, medical products,...), electricity and electronics (cables, connectors, semi-conductors, ...), metallurgical industries, military training and sport shooting, waste disposal and sewage sludge (Alloway, 1995).

These metals become of environmental concern when their concentrations in soil, sediment or water are shown to affect human health and/or ecosystems. At the most severely contaminated sites, indigenous plant and animal species have declined or disappeared leaving the sites biologically barren. At many of these more heavily contaminated sites there is often a significant risk of off-site migration of the contaminated soils due to wind- and water erosion, and leaching of contaminants into the groundwater. Such spread of the pollution increases the likelihood of organism exposure and toxic effects. In some areas agricultural production can become problematic due to phytotoxicity or human health effects.

Remediation of metal contaminated sites is classically based on engineering approaches such as excavation and landfilling of contaminated soil, *ex situ* purification treatments using soil washing techniques, solidification, vitrification or physical barriers (caps) (Vangronsveld and Cunningham, 1998). Due to the high costs of these treatments (for estimations of costs see Cunningham and Berti, 1999; Vassilev et al., 2004) in particular when the polluted areas are large, and due to secondary problems such as the difficulty to find suitable soil for replacement in case of excavation and changes in physico-chemical and biological characteristics of the treated substratum (drastically reducing its further potential to be used as a normal soil) in case of *ex situ* purification (Vangronsveld and Cunningham., 1998) remediation programs progress slowly and many sites remain untreated. There is a clear need for remediation alternatives which are cost-effective, environmentally sound and equally protective of human health and the environment.

The focus of much recent experimental work has been directed towards this end developing techniques that exploit biological (plant or microorganisms) and/or chemical (use of metal immobilizing soil amendments) processes to remediate metal contaminated sites (Kamnev and van der Lelie, 2000; Vassilev et al., 2004). Strategies of this nature are classified under the generic heading of ‘soft’ remediation techniques.

Examples of ‘soft’ remediation options include *in situ* metal immobilization and phytostabilization. The term ‘ *in situ* metal immobilization’ refers to the use of metal immobilizing agents to reduce the ‘biovailability’ of metals in soils, bioavailability being defined as that fraction of the total metal content that can interact with the biological target (Vangronsveld and Cunningham, 1998). Due to their nature, metal immobilizing soil amendments generally are able to enhance geochemical processes such as precipitation, sorption, ion exchange, and redox reactions which is the basis of their action (Mench et al., 2000). Subsequently, food and feed can be grown more safely, and on more heavily contaminated sites, a vegetation cover can develop to stabilize the soil. Different types of soil additives have been investigated in the context of *in situ* metal immobilization. They include liming materials, organic matter, P-rich products, aluminosilicates (clays and related substances), Fe- and Mn oxides or Fe- and Mn-rich materials (see Mench at al., 2000 and references therein; Brown et al., 2004 and references therein). In general preference is given to cheap materials such as natural minerals or industrial by-products. Phytostabilization describes metal inactivation processes as a result of revegetation. For highly contaminated, bare or scarcely vegetated areas phytostabilization will only be possible in combination with *in situ* immobilization techniques (amendment assisted phytostabilization). Both techniques also have the potential to reduce leaching of metals to the groundwater. A potential obstacle of these techniques as remediation options is that only the mobile or bioavailable fraction of the contaminants will be treated. From ecological, toxicological and health point of view, however, this should be the most important consideration (van der Lelie et al., 2001).

An example of a large-scale metal contamination can be found in the north east of Belgium (Campine region; Dutch: de Kempen). Due to the historical presence of several zinc smelters, large areas of land in this region are contaminated with metals (mainly Zn, Cd, Pb), including industrial sites and farmland. Because of the difficulty to apply conventional clean-up methods all over the area, the Flemish government has expressed a fundamental interest in potential applications of alternative remediation technologies, including *in situ* metal immobilization (Dua and van Geel, 2002).

Objectives

The objectives of this thesis are:

(1) To investigate the possibilities of *in situ* metal immobilization and phytostabilization (by means of cyclonic ashes, steel shots, and a combination of cyclonic ashes and steel shots) as a risk-based remediation option for metal contaminated soils in the Kempen.

(2) To optimize a phytoxicity test (PlantTox), aiming at an accurate detection of soil phytotoxicity (which is important both prior to and after metal immobilization in highly contaminated soils).

Thesis outline

This work starts with a literature review (**PART I**) situating key aspects related to the role and toxicity of metals in human life, their behavior in soils and their toxicity to plants.

The first experimental part **(PART II**) focuses on *in situ* metal immobilization and phytostabilization. Column experiments, simulating long term behavior of soil amendments, and lysimeters, placed under natural climatologic conditions, are used in the experimental set-up. Attention is focused on reductions in metal phytotoxicity, plant metal accumulation and metal leaching. This first part is organized in three chapters. Because durability of a soil treatment is a key issue in metal immobilization studies Chapter 1 evaluates and compares the long term behavior of lime with other inorganic metal immobilizing soil amendments (cyclonic ashes, steel shots and the combination of cyclonic ashes and steel shots). Lime is widely regarded as a key ameliorant in agriculture for decreasing metal mobility and availability, but regular lime applications are essential to avoid soil acidification and a concomitant loss of the immobilization effect. Other materials, having a more persistent remediation effect, are required for durable *in situ* applications (Hamon et al., 2002). In Chapter 2 and Chapter 3 the effect of combination of the inorganic soil amendment with compost will be evaluated. At industrially contaminated sites, a lack of organic matter, a limited water holding capacity (WHC), and a low availability of nutrients may be limiting factors for plant growth in addition to potential phytotoxicity. It is well known that soil fertility and WHC can be ameliorated by compost addition (Brady, 1984).

Detection of metal phytotoxicity, both prior to and after *in situ* immobilization, is an important aspect of phytostabilization studies. Therefore, the second experimental part of this work (**PART III)** concentrates on the optimization of a phytotoxicity test (PlantTox) (Van Assche and Clijsters, 1990a, Vangronsveld and Clijsters, 1992). In this test, growth parameters and biochemical responses of bean seedlings (*Phaseolus vulgaris* cv. Limburgse vroege*),* in particular the capacity of so-called 'stress enzymes' are measured. By investigating plant responses on a series of potential reference soils (Chapter 4) we aimed to gain insight in the common range of test endpoints in uncontaminated soils, and in the soil characteristics that influence the endpoints of the test in non-toxic conditions. The objective of this part of the work is to contribute to the determination of the most suited reference level or reference soil (negative control) for use in the test. In Chapter 5 plant responses are compared between different OECD standard soils, prepared according to the prescriptions of OECD guideline 207 (OECD, 1984b), but with different (commercial) origins of their constituents. This standard soil was proposed as a potentially suited negative control for the PlantTox test in the previous chapter.

In **PART IV** main conclusions of the experimental work are summarized and research perspectives are formulated.

2. METALS IN HUMAN LIFE

2.1 Anthropogenic use of metals

The oldest signs of anthropogenic use of metals (first copper, and also some gold and silver) date from the end of the Neolithicum, ca. 8000 - 4.000 years ago. Ever since than, human beings made systematic and organized use of natural raw materials (Merian, 1991). Metal mineral mining and processing experienced an exponential growth during the last century. Today, the numerous applications of all different kinds of metals have become indispensable in our daily lives. The main metal production originates from ore extraction and processing, but an increasing amount of metals is also produced trough recycling (Merian, 1991).

2.1.1 Anthropogenic use of zinc

Zinc is the fourth most used metal in the world after Fe, Al and Cu. The world production of Zn in 2005 was about 10 million tons a year. A little more than 2% of this amount was produced in Belgium (ILZSG, 2006). The main market of Zn is in galvanization, which accounts for 47% of modern day demand (Figure 1). Coating of steel with Zn gives added protection against corrosion of building structures, vehicles, machinery,…. Zinc is often used in alloys: brass and bronze account for 19% of its use and other alloys are responsible for an additional 14%. The ability to diecast complicated components makes zinc indispensable in a multitude of industrial and household products. The use of Zn in chemicals accounts for 9% of the Zn use. Organozinc compounds are used as fungicides (e.g. in carbamate compounds) or antiseptics (e.g. Zn bacitracine, Zn salicilate,…) (Merian, 1991). Zn soaps are water repellent and are used as lubricants as well as for the water proofing of textiles, papers and concrete. ZnO is used in floor coverings, plastics, printing inks, or in pharmaceutical applications (powders and ointments) for burns and skin infections (Merian, 1991). It is also used in the manufacture of paints and rubber products. Zinc sulfide serves as a white pigment and is used in making luminous dials, X-ray and TV screens and fluorescent lights. Eight percent (8 %) of Zn goes to semi-manufacturing applications which include for example Zn sheets used in construction applications (roofs, facades,...). Other divers uses represent 3% of the market (toys, Zn-air batteries, ...).

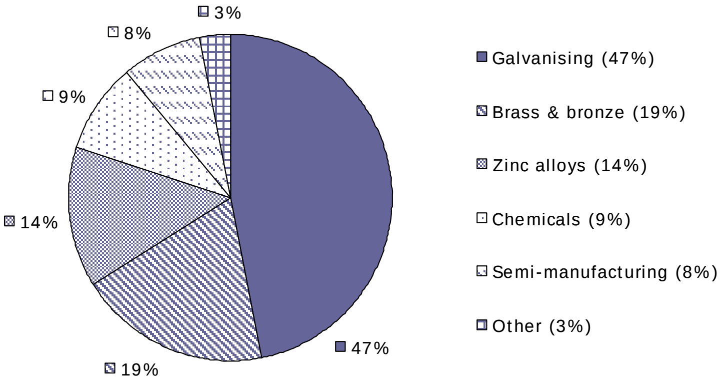

Figure 1 End uses of zinc in the world[1].

2.1.2 Anthropogenic use of copper

Worldwide ca. 16 million tons Cu was produced in 2005. Literature data about the Belgian production are very variable: ICSG (2006) reported a production of ±100 000 tons in 2005, while VMM (2001) mentioned of Flemisch Cu production of 844 306 tons Cu in 1998. The differences may be due to a difference in definitions. Copper can be used in its pure form or in Cu alloys, such as bronze (Cu-Sn), brasse (Cu-Zn), cupronickel or monel (Cu-Ni),... Also derived Cu compounds have interesting applications. The demand for Cu comes mainly from

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[1] source: http://www.ilzg.org/statistics

electrical and electronic applications (48%) (Figure 2). Copper wires are used worldwide in electric circuits. Cu also is the dominant and favored material in connectors. Since their invention, electron tubes (in TV and computer monitors, in microwave ovens,....) have depended on copper (and copper alloys) for their internal components. Wave-guides containing Cu, to carry radio- and television signals, are 30% to 40% more efficient than their aluminum counterparts. Even communication between computers can occur on existing copper lines.

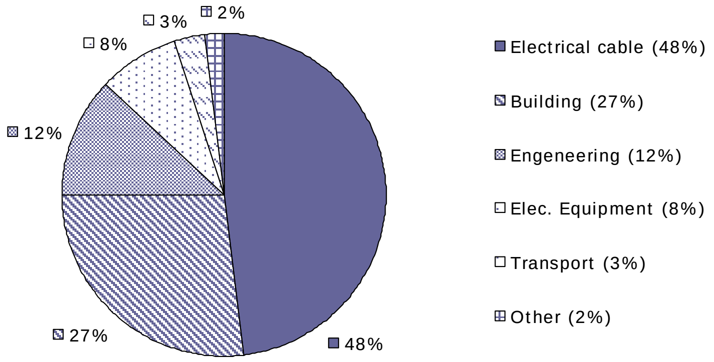

Figure 2 End uses of copper in the world[2].

The construction sector is the second largest user of Cu (27%): roofing, roof gutters and drainage, plumbing tubes and air-conditioning systems are important construction applications. Interesting to mention is the use of copper doorknobs in hospitals to reduce the transfer of diseases due to its biostatic properties. Numerous engineering applications come on the third place (12%), followed by all types of electrical equipment (8%). Also the transport sector is responsible for a considerable Cu use (3%): regardless the huge amount of Cu wires in automobiles (a luxury car contains about one mile in length of Cu wires), Cu is used in automotive radiators and engine hardware, railroad tracks are made of Cu alloys etc. Minor, but interesting, uses (2%) of Cu include Cu in

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[2] Source: http://www.eurocopper.org

cookware, musical instruments, coins, statues, ... Also Cu derivates have various applications: e.g Cu chromates in pigments or as catalysts, Cu sulfate in agriculture as fungicide or pesticide, and also in food supplements or fertilizers. The latter is related to the essential role of Cu for life (Paragraph 2.2.2).

2.1.3 Anthropogenic use of cadmium

In the year 2000, worldwide Cd production was about 20 000 ton year^{-1} (Buckingham and Plachy, 2004). Cadmium consumption and production are declining fairly steeply in recent years due to environmental concerns. At present, there is no Cd metal production anymore in Belgium. The main use of Cd is as active electrode material in Ni-Cd batteries (79%) (Figure 3), 11% of Cd (as Cd sulphide or Cd sulphoselenide) is used in pigments (for plastic, ceramic, glass), and 2% of the Cd (as organic Cd salts) serves as stabilizer in PVC (protection against heath and light). Similar to Zn, Cd can also be used to coat steel or other metals (7%). Minor uses (1%) include the use of cadmiumsulfide and cadmiumtelluride in solar cells and in some electronic devices and the use of Cd in alloys.

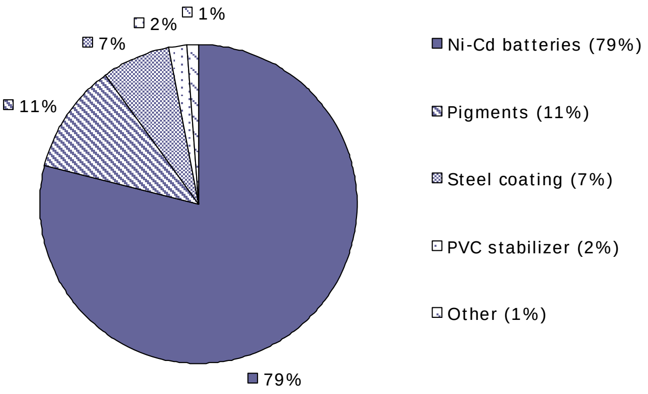

Figure 3 End uses of cadmium in the world[3].

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[3] source: http://www.cadmium.org

2.1.4 Anthropogenic use of lead

Around 7.5 million tonnes of lead were produced in the world in 2005. Belgium contributed with about 1% to this total production (ILZSG, 2006). The principal consumption of lead (71%) is for rechargeable lead-acid batteries, which are used in vehicles, emergency energy systems (hospitals), industrial batteries in computers, etc. Lead is used in pigments and other compounds in the glass and plastic industries (12%). Rolled extrusions account for 7% of the lead use (application in construction sector) and cable sheeting for 3%. The high density makes Pb also useful for ammunition purposes (6%). Minor applications include the use of Pb in alloys, the use of lead derivates such as PbO in glass of TVs and computer screens to protect users from radiation, Pb in crystal,.... (1%).

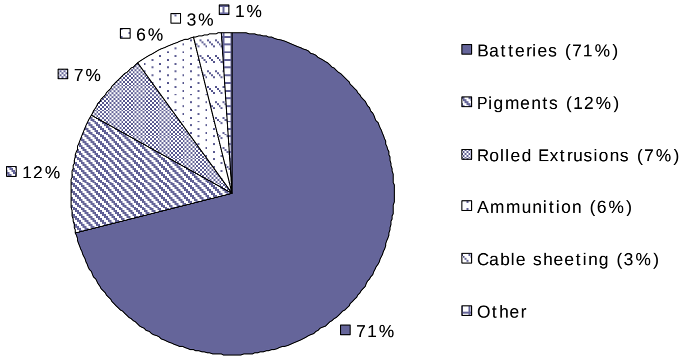

Figure 4 End uses of lead in the world[4].

2.2 Metals and human health

2.2.1 Exposure routes

Main routes of human exposure to Zn, Cu, Cd and Pb are trough ingestion (food chain transfer, dust), inhalation, or by dermal contact (Adriano, 2001). The relative importance of the exposure routes varies depending on the metal and

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[4] source: http://www.ilzg.org/statistics

the conditions (e.g. occupational exposure, contamination level of the area, smoking ,...).

Zn and Cu enter the body almost exclusively by food. Human Cd exposure occurs trough ingestion (food and dust) or inhalation. Exposure via inhalation occurs primarily under occupational settings or in smokers (tobacco contains high Cd concentrations, which is inhaled when smoking). In the rest of the population ingestion is the main exposure route (Järup et al., 1998). In contaminated areas Cd-rich dust may contribute significantly to Cd exposure (Hogervorst et al., 2006).

Like Cd, Pb enters the body mainly via inhalation and ingestion (food, dust), inhalation being primarily caused by occupational sources. The most important route in the general population is ingestion of food and dust. The latter is of particular importance in young children due to hand-to-mouth behavior. In children attention has to be paid also to potential ingestion of Pb from paints (Adriano, 2001). Moreover, children are more sensitive to lead compared to adults because they absorb and retain more lead in proportion to their body weight (Mushak et al., 1989).

2.2.2 Essentiality versus toxicity of metals

Among trace elements essential as well as non-essential elements exist. In general an element is considered essential if a reduction in its total daily intake below some minimal level consistently induces symptoms of deficiency, and its subsequent augmentation prevents and reverses metabolic changes. This definition can be applied to all organisms, but elements essential for one organism are not necessarily essential in other organisms (Adriano, 2001). More specific definitions, and criteria for essentiality, were given by Hewitt and Smith (1974) (plants) and by Underwood (1977) (animals). Among the metals in this study Zn and Cu are essential for humans and plants, while Cd and Pb are considered non-essential elements for these organisms. Both essential and non-essential trace elements, can become toxic when they are taken up in excessive amounts by living organisms. This is described excellently by Adriano (2001): 'Some elements are paradoxally intriguing! Too little of them may not be enough for proper nutrition but a little bit more might be too much! This indicates that some elements may serve as a nutrient or a toxin.'

2.2.2.1 Zinc and copper: two essential elements

Zinc and copper are trace elements indispensable for (human) life. The metabolic functions of zinc are mainly based on its strong tendency to form tetrahedral complexes with N-, and particularly S-ligands. The most important and best-known role for Zn is as a structural co-factor in metallo-proteins (Jiang et al., 2004). Hundreds of zinc proteins possessing one or more Zn-stabilized motives have been identified (Cox et al., 2000). The Zn-finger family of proteins is involved in regulation of gene-expression. Zinc nutrition aids wound healing trough a family of zinc dependent endopeptidases (Jiang et al., 2004). In a large number of enzymes Zn is an integral part of the enzyme structure and can play a (co-)catalytic role. In addition several types of cells secrete Zn ions. In brains, synaptically released Zn seems to plays a role in neural signal transmission (Jiang et al., 2004).

Similar structural and (co)-catalytic roles of Zn in metalloproteins in humans, are also found in plants (Marschner, 1995).

In contrast with Zn, Cu is a redox active element and its oxidation state is easily changed ($Cu^{+} \Leftrightarrow Cu^{2+}$). One of the principal functions of Cu in life is its participation in enzymatically bound metal in redox reactions (Marschner, 1995). In some proteins Cu act as a structural element (Yruela, 2005). In humans, copper containing enzymes are e.g involved in the formation of callogen (the fibrous protein in bone, tendons and other connective tissues) and protective coverings of nerves. In plants, copper containing proteins are devided into three groups (Sandman and Böger, 1983): (1) blue copper proteins without oxidase activity, which function in one-electron transfer reactions (e.g. plastocyanin); the blue color arises from a ligand-to-metal charge transfer absorption band around 600 nm, (2) non-blue proteins which represent oxidases and oxidize monophenols to diphenols (3) multi-copper proteins containing at least four Cu atoms per molecule and which also act as oxidases (e.g. ascorbate oxidase).

Zinc and copper are present in a wide variety of vegetables, fruits, grains,... as well as in drinking water. It is assumed that almost no risk for toxicity of these elements exists in humans (Merian, 1991). Accumulation of these elements is lethal to plants well before concentrations become harmful to man. In contrast, Cd and Pb concentrations in plants can reach levels that can become harmful upon human consumption, even when plants do not yet experience toxicity.

Biotoxicity induced trough metal exposure is the consequence of (bio)chemical reactions that disrupt biological processes e.g by displacing an essential trace element ions in biomolecules, blocking the functional groups of biomolecules, or modifying the active configuration of such molecules (Adriano, 2001). Also oxidative stress (i.e. toxicity caused by reactive oxygen species) has often been linked with metal toxicity

The following paragraph briefly summarizes toxic effects in humans caused by Cd en Pb, the two non-essential metals in this study. Toxic effects of metals on plants are discussed in more detail in Paragraph 4 of this introduction.

2.2.2.2 Cadmium and lead: two non-essential elements

Human exposure to Cd and Pb is of much greater concern compared to Zn and Cu. Excessive exposure to Cd (in non-occupational populations) has been linked mainly with renal disturbances. The kidney is considered to be the critical organ for long-term Cd exposure and the organ that first reveals signs of toxicity. Cadmium induced proteinuria occurs as an early sign of renal dysfunction. Cadmium accumulates in the body, in particular in the kidney and to a lesser degree in liver (Adriano, 2001). Together these two organs account for 50% of the total Cd accumulation. The elimination of Cd is very slow with a biological half-life of 10-30 years (Staessen et al., 1999). Cadmium exposure has also been associated with an increased bone fragility, which is likely related to its interference with the metabolism of calcium, Vitamine D and collagen (Järup et al., 1998; Staessen et al., 1999). There is no current epidemiologic or experimental evidence that Cd exposure via food may be associated with an increased risk of cancer, but it may enhance the occurance of lung and possibly prostate cancers in workers exposed to high airborne Cd concentrations (Lauwerys, 1989). In rat, Cd was found carcinogenic both after inhalation and after ingestion. Cd is classified as human carcinogenetic (IARC, 1993). Recently, Nawrot et al. (2006) found a significant association (not necessarily causal in origin) between the risk of cancer (mainly lung cancers) and the environmental exposure to Cd (measured by 24 h urinary Cd excretion) in the Kempen area.

Lead in the human body is in first instance concentrated in the blood and later translocated to bone and teeth. The latter can be explained by similarities in physico-chemical properties between Ca en Pb (Howard, 1998). Some accumulation also occurs in soft tissues (kidney, liver, brain) (Adriano, 2001). Lead has a half-life in blood of approximately 25 days, in soft tissues about 40 days and in the non-labile portion of bone, more than 25 years. The most sensitive target of Pb poisoning in humans is the nervous system (Adriano, 2001). It has been shown that lead can impair the mental development of young children; even relatively low levels of exposure can cause a measurable reduction in IQ (Lenphear et al., 2005). Other toxic effects of Pb are high blood pressure, dysfunctions in the renal system and adverse effects on bone growth (the latter through decreased vitamine D metabolism). Lead inhibits several enzymes, for example ALA(aminolevulinic acid)-dehydratase, which is crucial in the normal production of heme. This can result in Pb-induced anemia. Very high levels of Pb exposure may reduce reproductive health (sperm count, mobility, morphology; fetus development) (Adriano, 2001).

Several studies on occupational exposed people have shown an increased risk of cancers (lung, brain, gastro-intestinal). Results of such studies are often not conclusive because individuals were also exposed to other substances. Based on results in rats and mice, Pb is regarded as potential carcinogen to humans (IARC, 2004).

A summary of exposure routes and accumulation of metals in the human body is presented in Figure 5.

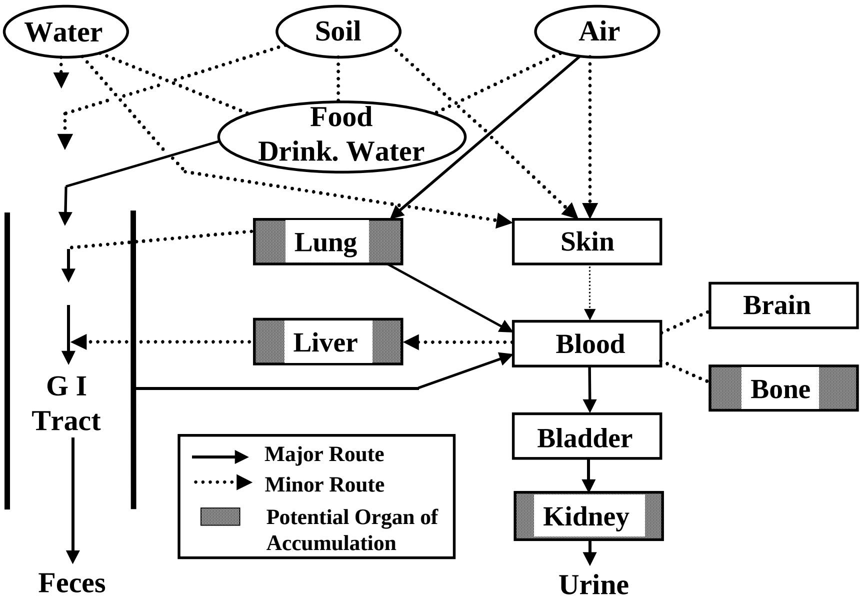

Figure 5 Exposure routes and potential sites of accumulation of metals in humans (Source: Adriano, 2001).

3. METALS IN SOILS

3.1 About (heavy) metals, trace elements and Type-B metal ions

Metals can be distinguished from other chemical elements by their physical characteristics (high electrical and thermal conductivity, gloss, malleability), which can to a large extent be explained by their chemical properties, mainly their tendency to cede electrons (Cambier and Mench, 1998).

The metals investigated in this study (Zn, Cu, Cd, Pb) are sometimes referred to as 'heavy metals'. Heavy metals are in general defined as metallic elements with a high specific mass, but the level of the lower limit differs from source to source (> 3 g cm^{-3}; > 5 g cm^{-3} , > 7 g cm^{-3}, ...). In some definitions other specifications are added such as atomic weight, atomic number,.... The variety in definitions makes it a very poorly defined term (Duffus, 2002). In environmental studies the term 'heavy metals' in often incorrectly used to refer generally to potentially toxic trace elements (including non-metallic elements such as As, Se,...) and some authors argue against the use of this term (Duffus, 2002; Hodson, 2004). For the elements in this study (Zn, Cu, Cd, Pb), the term, 'heavy metals' is correct whatever definition that is applied, but, because of the controversies related to it, the term will not be employed here.

The metals under consideration (Zn, Cd, Cu, Pb) can also be referred to as trace elements or trace metals. Chemical elements are conventionally divided into two groups: macro-elements and micro-elements, the latter also named 'trace elements'.

In 'Earth sciences' macro-elements are the eight abundant rock-forming elements (O, Si, Al, Fe, Na, Mg, Ca and K) making up together more then 90% of the Earths crust. The other natural occurring elements are considered as trace elements, each accounting for <0.1% of the total. In 'Life sciences' trace elements are defined as those elements present in living organisms in concentrations below 0.01% or <100 mg kg^{-1} (Adriano, 2001). Macro-elements are C, O, H, N, P, S, Mg, Ca and K, present in concentrations which depend the organism under investigation. The term 'trace elements' has no relationship with certain functions or element characteristics. Among trace elements, metals (e.g Zn, Cu) as well as non-metallic elements (e.g. As, Se) exist. A trace element with metallic properties is called a trace metal.

Metals can also be classified based on their distinct chemical characteristics (Nieboer and Richardson, 1980). Metals in (aerated) soils and in organisms generally occur in a positive oxidation state, they act as Lewis acids and are capable of accepting electrons. This characteristic provides metals with the ability to form complex compounds with organic or inorganic ligands. Metals can be classified based on electronegativity and the ligand affinity of their ions as presented in Table 2. Type-A metal cations preferentially interact with ligands having oxygen as donor atom. In contrast, type B-metal ions coordinate preferentially with bases containing S or N as donor atoms. The properties are not absolute, but gradually varying qualities. In the classification scheme a transition group exists between the type-A and type-B group.

Table 1 Classification of metal ions in different groups according to their ligand affinity (Nieboer and Richardson, 1980).

Category	Ion
Class A	Li^{+}, Na^{+}, K^{+}, Cs^{+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Mg^{2+}, Be^{2+}, Al^{3+}
Borderline	Mn^{2+}, V^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Sn^{2+}, Pb^{2+}, Cr^{3+}, Fe^{3+}, As^{3+}, Sn^{3+}, Sb^{4+}
Class B	Ag^{+}, Au^{+}, Tl^{+}, Hg^{2+}, Bi^{2+}

Since this classification has a biological, toxicological and environmental relevance, several authors promote its use in environmental reports (Duffus, 2002; Hodson, 2004). In most existing environmental studies, however, little or no attention is paid to this classification.
For convenience we decided to use the very general term 'metals' throughout this study. Occasionally 'trace metals' will be used interchangeably with 'metals'.

3.2 Background levels and clean-up values for metals in soils

The Flemisch legislation (Belgisch Staatsblad, 1996) provides background concentrations for metals in soils, as well as clean-up values. Background concentrations are total concentrations of metals in soils found in areas where metal concentrations are not (or only minimally) enriched by human activities. They were defined as the 90th percentile of soil samples collected in 'background' areas. Clean-up values refer to the total metal concentrations in soils above which the official procedure of 'descriptive soil investigation' and possibly remediation needs to be initiated. In this context a distinction is made between 'recent' contaminations on the one hand (these are contaminations that originated after the soil sanitation decree of 22/02/95 came into effect) and 'historic' contaminations on the other hand. While recent contaminations need to be remediated when clean-up values are exceeded, remediation of historical contaminations is only mandatory if a 'descriptive soil investigation' has indicated a serious risk of the contamination.

Background levels and clean-up values for Zn, Cu, Cd and Pb in soils according to the Flemish regulation (Belgisch Staatsblad, 1996) are presented in Table 2. The levels presented are valid for a standard soil with 10% clay and 2% organic matter. A formula exists to calculate specific concentrations for each given soil, depending on its clay and organic matter content. This will slightly adjust the values indicated in Table 2. Clean-up values are in addition dependent on the 'land use' of the soil. Basically 5 different 'land use types' can be distinguished I. Nature, II. Agriculture, III. Residential area, IV. Recreational area, V. Industry (Table 2). Fore more information regarding the soil specific calculations and the different land use types we refer to Belgisch Staatsblad (1996).

Table 2 Background concentrations and clean up values for Zn, Cu, Cd and Pb (mg kg $^{-1}$ D.W.) in soils according to the Flemish regulation for a standard soil with 10% clay and 2% organic matter, and in function of the 'landuse' of the soil (Belgisch Staatsblad, 1996)

mg kg $^{-1}$ DW	I*	II*	III*	IV*	V*
Zn	600	600	1000	1000	3000
Cd	2	2	6	15	30
Cu	200	200	400	500	800
Pb	200	200	700	1500	2500

* I. Nature, II. Agriculture, III. Residential area, IV. Recreational area, V. Industry

3.3 Metal retention and metal availability in soils

3.3.1 The concept of bioavailability and phytoavailability

A scientific consensus exists indicating that the total content of metals and other contaminants in contaminated soils does not reflect the risks associated with a contamination (Meers et al., 2006b). Of the three environmental compartments being water, air and soil, the quality of soil, in terms of environmental impact, is the most difficult to determine in a quantitative way. This is mainly due to the heterogeneity in the composition of soils, affecting the behavior and toxicity of compounds (NAP, 1990).

The direct risk posed by soil contaminants depends on their 'bioavailability'. Some care has to be taken when using the term 'bioavailability' because it may have different meanings to different disciples (Adriano, 2001). For sediment associated contaminants 'bioavailability' was defined as 'the fraction of the total contaminant in the interstitial water and on sediment particles that is available for ‘bioaccumulation’; whereas bioaccumulation is described as ‘the accumulation of contaminants via all routes available to the organism’ (Landrum and Robbins, 1990). This definition could be extrapolated to soil associated contaminations (Vig et al., 2003). Pijnenburg et al (1997) proposed to treat ‘bioavailability’ as a dynamic process in two separate phases with (a) ‘environmental bioavailability’ defined as a physico-chemically determined fraction and (b) ‘toxicological bioavailability’ as a measure of physiologically induced effects. Correspondingly, Caussy et al (2003) point out that some authors differentiate between ‘external bioavailability’, which largely depends on the ability of metals to be dissolved and released from media’ and ‘internal bioavailability’ which reflects the ability to reach target tissues and exert toxicological effects. In this context the term bioavailability, as defined by Landrum and Robbins (1990), and as it will be used in this study, mainly refers to ‘external bioavailability’ or ‘environmental bioavailability’.

Three main factors are influencing metal ‘bioavailability’ in soils: (1) the metal species present, (2) the physical, chemical and biological characteristics of the soil and (3) the target organism itself (Adriano, 2001). Some other factors such as climatic conditions may also be of influence. Due to a wide variety in characteristics of target-organisms, including the capability of certain organisms to alter their environment (e.g. exudation of protons, siderophores, organic acids by plant roots) the bioavailable fraction is species dependant (Ernst, 1998a). Even within one species differences can be found (Ernst, 1998b; Schat et al., 1996). Soil and organism properties may additionally interfere with each other leading to different organism influences in different environments. The diversity of these factors and their reciprocal influences make of the easy concept of ‘bioavailability’ a very complicated reality. Meyer et al. (2002) stated correctly that ‘bioavailability’ describes a general qualitative concept, but that the ‘bioavailable fraction’ is hard to define quantitatively. The bioavailability of metals to plants is often described as phytoavailability (Song et al., 2004).

Paragraph 3.3.3 will extend on some major factors controlling metal phytoavailability in soils. The role of soil factors in metal phytoavailability can only be understood if basic insights are obtained in the composition of some important soil constituents and in the geochemical processes controlling the distribution of metals between the soil solid phase and the soil solution. Therefore these topics will first be introduced in Paragraph 3.3.2.

3.3.2 Important soil constituents and geochemical processes controlling the distribution of metals between the soil solid phase and the soil solution

3.3.2.1 Soil constituents relevant to metal interactions

The soil is a complex heterogeneous medium consisting of 3 phases: a solid phase (with mineral material and organic matter), aqueous solution and a gaseous phase (Brady, 1984). The minerals present are usually weathering (chemically decomposing) rock fragments and secondary minerals such as phyllosilicates, oxides of Fe, Al and Mn (including hydrous oxides and oxyhydroxides), and sometimes precipitates such as carbonates and phosphates. The organic matter comprises living organisms (mesofauna and microorganisms), dead plant material (litter) and humus formed by the action of microorganisms on plant litter. These solid components are usually clustered together in the form of aggregates, thus creating a system of interconnected voids (pores) of various size filled with either gases or aqueous solution.

The inorganic as well as organic soil constituents are variable in size and composition. The finest particles, the smallest of them being colloidal in size, are the result of a more advanced weathering of rocks or a more advanced decay of plant litter. While the coarse fraction of the soil may be more important from the viewpoint of soil physics, the fine materials are the most active soil fraction from a chemical point of view.

The mineral colloidal fraction consists almost exclusively of clay minerals (kaolinite, illite, vermiculite,...) with small amounts of oxides (Fe, Mn); the organic colloidal fraction is represented by humus. These particles have a large surface area per unit weight and are characterized by a surface charge to which water and ions are attracted.

Plants depend for their nutritional demand on mineral uptake from the soil. This uptake predominantly occurs directly from the soil solution. The chemical make-up of the soil solution is dynamic and is, from physico-chemical point of view, influenced by multiphase equilibria involving the soil solid phase and the aqueous phase (Adriano, 2001) (Figure 6).

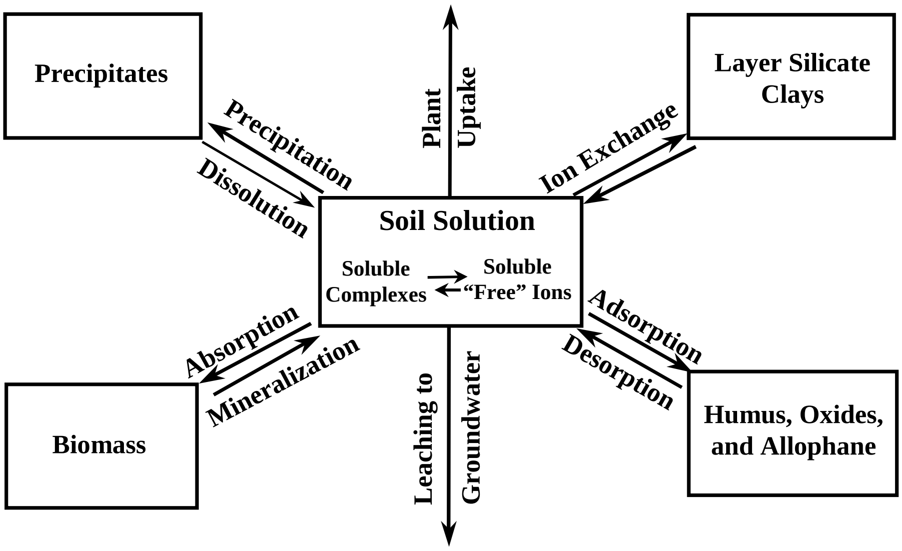

Figure 6 Schematic presentation of key interactive processes in the soil system affecting the partitioning of trace metals between the solid and aqueous phases (Adriano, 2001).

Geochemical processes, controlling this distribution of metals between the soil solid phase and the soil solution, are described in the next paragraph (3.3.2.2). This information will contribute not only to a basic understanding of the role of some soil factors in metal bioavailability, but also to an understanding of the choice of some substances (e.g. iron and manganese oxides, aluminosilicates,...) as metal immobilizing soil additives (paragraph 3.3.5).

3.3.2.2 Geochemical processes controlling the distribution of metals between the soil solution and the soil solid phases

Clay minerals, hydrated metal oxides and organic matter are considered to be the most important constituents contributing to the sorption of trace metals in soils. 'Sorption' is a general term, which can be used if the specific process leading to the loss of a solute from aqueous solution is not known. On closer examination different sorption processes can be distinguished. Their general principles are discussed below mainly based on information given by Brady (1984), Alloway (1995) and Adriano (2001). Additional references are added in the text. It must be realized that the binding mechanisms of metals in the soil are complex and are not yet, if ever, completely understood.

Sorption processes related to mineral soil constituents

Cation exchange or non-specific adsorption

Solid phase soil particles often carry a negative permanent charge, which arises from isomorphous substitutions. The overall electroneutrality of the system is maintained by the presence of cations in close proximity to the solid surface. The sorbed cations may be more or less easily replaced by other cations, maintaining the electroneutrality of the system. The release of a cation held by a solid soil particle, and the accompanying adsorption of one or more cations from the soil solution is termed cation exchange. This non-specific adsorption on mineral particles is governed by electrostatic attractions and is mainly pH independent. pH dependent charges on mineral soil particles only contribute to a limited extent, if at all, to the cation exchange process. The cation exchange capacity (CEC) is an expression of the number of cation adsorption sites per unit weight of the soil or soil constituent. It is defined as the sum total of exchangeable cations that a soil/soil constituent can adsorb, and is expressed in cmol/kg oven-dry soil (1cmol/kg = 1milli-equivalent/100 gram, the latter unit also being used frequently). The approximate CEC (cmol/kg) of some common clay minerals is: montmorillonite: 100; illite: 30; and kaolinite: 8. Fe and Al oxides have almost no CEC. Among Mn oxides, only monoclinic birnessite exhibits CEC properties (300 meq/100g), but this mineral is not frequent in soils because it is unstable and transforms to hexagonal birnessite at neutral pH. For many soils in the temperate region, the average CEC of the clay fraction as a whole might be in the vicinity of 50 meq/100g (Foth and Turk, 1972). The CEC of the sand and silt fractions is omitted in the approximation of the CEC since it is so small. The total exchange capacity of the soil is the total number of exchange sites of both mineral and organic colloids.

The practical implication of this cation exchange is that the cations hold in the soil on cation exchangeable sites can enter the soil solution and may therefore become available for uptake by plants. Alternatively, cation exchange makes it possible that ions, brought into the system via the solution phase, are temporarily retained by the soil. In such a case cations that were held originally by the solid phase are exchanged and released into the soil solution.

Chemisorption or specific adsorption

This sorption mechanism results from the breaking of the bulk structure of solids at their surface. Dangling chemical bonds on particle edges generates a pH-dependent variable charge, the magnitude of which much depends on the surface area of the solid. Metal cations may form covalent bonds with surface ligands of the sorbent. It results in metal ions being adsorbed to a far greater extent than would be expected uniquely from the CEC of a soil, and ions sorbed by specific adsorption processes are inherently far less reversibly released in the environment than the ions sorbed by simple ionic exchange. Brümmer (1986) showed that the sorptive capacities of amorphous Fe and Al oxides for Zn were 7 and 26 times greater, respectively, than their CECs at pH 7.6.

The dissociation reaction of the surface hydroxyl groups is pH dependent, increasing at high pH and decreasing at low pH, hence the term pH dependent charge (Schindler and Sposito, 1991).
The protonation reaction of the surfaces at high pH is written as:

$$>S\text{-}OH + H^{+} \Leftrightarrow >S\text{-}OH^{2+}$$

The deprotonation at high pH is shown as:

$$>S\text{-}OH \Leftrightarrow >S\text{-}O^{-} + H^{+}$$

Dissociated surface hydroxyl can serve as a Lewis bases towards metal cations (Lewis acids). The chemisorption reaction of a metal ion M^{2+} on the surface can therefore be written as:

$$>S\text{-}OH + M^{2+} \Leftrightarrow >S\text{-}O\text{-}M^{+} + H^{+}$$

or:

$$2>S\text{-}OH + M^{2+} \Leftrightarrow >(SO)_{2}\ M + 2\ H^{+}$$

where S is the metal of the adsorbing surface. As the pH is raised from an acid value, chemisorption is favoured.

Metal sorption is dependent on characteristics of the metal-ion. As an illustration metal sorption of different metals on hematite (McKenzie, 1980) is presented in Figure 7. There seem to be chemical parallels between bonding of a metal ion with a surface O^{2-} or OH^{-}, and coordination of the same metal ion with OH^{-} in solution (Brümmer, 1986). This is made apparent by comparing the previous reaction scheme to the hydrolysis reaction for the metal M^{2+}:

$$H\text{-}OH + M^{2+} \Leftrightarrow H\text{-}O\text{-}M^{+} + H^{+}$$

The pK value of the previous reaction characterizes the general adsorption behaviour of the different metal cations (increasing specific sorption with decreasing pK) (with $pK_{Cd}=10.1 < pK_{Ni}=9.9 < pK_{Co}=9.7 < pK_{Zn}=9.0 << pK_{Cu}= pK_{Pb} =7.7 < pK_{Hg}=3.4$. In soils other factors or properties may change the selectivity of sorption (see also Paragraph 3.3.3.2).

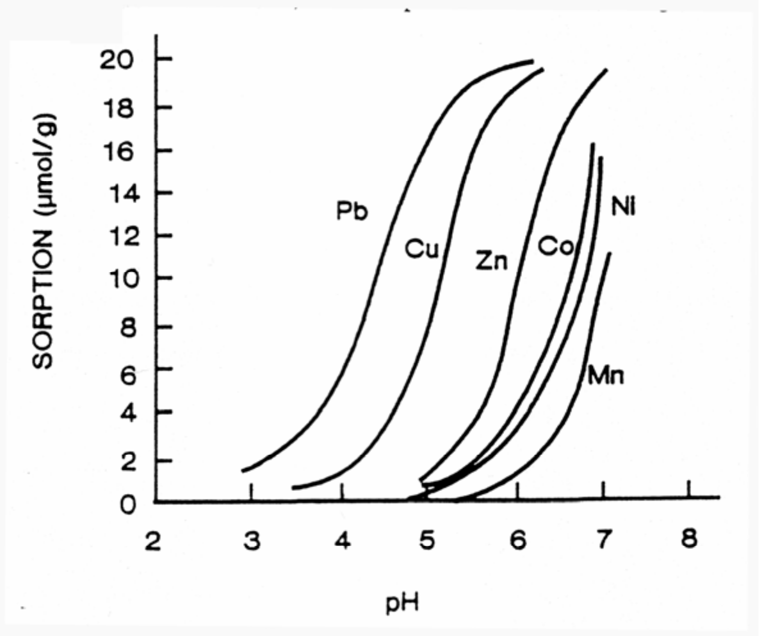

Figure 7 Metal cation adsorption versus pH on hematite (Fe_2O_3) (McKenzie, 1980).

Each type of mineral shows different sorption characteristics. All soil minerals are characterized by a point of zero charge (PZC), this is the pH value where they have no net negative charge (negative and positive charges in equilibrium). When pH is below the PZC the mineral has a net positive charge and at higher pH a net negative charge. In minerals with a low PZC specific sorption starts already at lower pH (Stumm, 1992).

The most important soil minerals involved in chemisorption reactions are poorly crystallized oxides (Al, Fe, Mn) and layer silicates (edges sites only).

An alternative approach to adsorption processes

An alternative approach to describe adsorption reactions in soils was introduced by Sposito (1984). According to this approach adsorption reactions of metals in soils can be described as complexation reactions with surface functional groups, to create surface metal complexes. The surface complexes can be of two types: (1) inner-sphere complexes (IS), in which no molecule of the bathing solvent (water) is interposed between the surface groups and the ion or molecule it binds, and (2) outer-sphere complexes (OS), in which at least one molecule of the solvent comes between the functional group and the ion.

The approach of outer-sphere and inner-sphere complexes is different from that of CEC and chemisorption. IS and OS can exist in case of a CEC mechanism as well as in case of chemisorption. Without giving a complete description of the theory, this paragraph only aimed at introducing these notions as an alternative approach.

Absorption (diffusion of metals into soil minerals)

In addition of being adsorbed on mineral surfaces, heavy metal ions may subsequently diffuse into minerals such as goethite, Mn oxides, illites, smectites and some other minerals (Gerth and Brümmer, 1983; Brümmer, 1986). The maximum relative diffusion rates in goethite decrease in the order Ni<Zn<Cd, and can be related to the ionic radii of these metals (0.69, 0.74, 0.97 Å, respectively). It was stated that the 'binding' of metals by goethite comprises three different steps: first, surface adsorption; second, diffusion of metals into goethite particles and third, fixation at positions within the mineral particles. This phenomenon may be responsible for the decrease of the metal mobility in a soil on the long term.

Precipitation, (surface)precipitation and co-precipitation

When the solubility product of a compound in the soil is reached, metals can precipitate in a fairly insoluble form (carbonates, phosphates, hydroxides, sulphides,... Homogeneous nucleation and precipitation (in the absence of foreign surfaces) cannot occur until the solubility product of the solid has been exceeded to a certain degree. In most soils the occurrence of such precipitation reactions is rather limited.

The presence of other mineral surfaces may reduce the extent of super-saturation necessary for precipitation to an extent determined by the similarity of lattice dimensions (McBride, 1991). The effect is termed heterogeneous nucleation and is probably the most important process of crystal formation in soil systems. The mineral reduces the energy barrier for the nuclei of the new crystals to form from solution by providing a sterical similar, yet chemically foreign, surface for nucleation. The energy barrier arises from the fact that the small crystallites, which must initially form in the crystallization process, are more soluble than large crystals because the higher interfacial energy between small crystals and solution. Heterogeneous nucleation may result in the formation of a coating on the surface of a pre-existing solid phase (surface precipitation). Evidence for a chemisorption-precipitation continuum is seen f.i. for Zn^{2+}, Mn^{2+} and Cd^{2+} sorption on $CaCO_3$ (McBride, 1991).

At last, precipitation may also involve the formation of a solid mixture either by inclusion or by co-precipitation. Co-precipitation is defined as the simultaneous precipitation of a chemical agent in conjunction with other elements by any mechanism and at any rate (Alloway, 1995). The types of mixed solids commonly formed include clay minerals, hydrous Fe and Mn oxides and calcite (Table 3).

Table 3 Trace metals normally found co-precipitated with secondary minerals in soils (Alloway, 1995).

Mineral	Co-precipitated metals
Fe oxides	Mn, Ni, Cu, Zn, Mo
Mn oxides	Fe, Co, Ni, Zn, Pb
Ca Carbonates	Mn, Fe, Co, Cd
Clay minerals	Ni, Co, Zn, Cu, Pb, Mn, Fe

Regardless the variations mentioned above, the essential characteristic of precipitation is the development of a solid phase whose molecular ordering is intrinsically three-dimensional. An adsorption mechanism on the contrary involves only two-dimensional molecular arrangements on a surface.

A schematic presentation of the different sorption processes on mineral soil surfaces is presented in Figure 8.

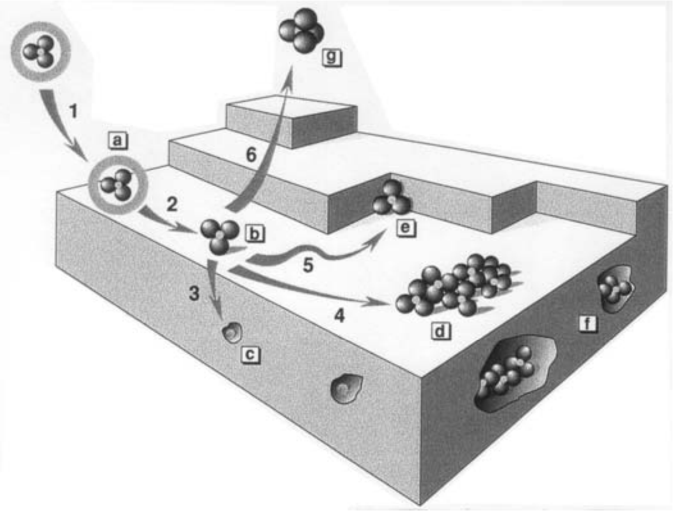

Figure 8 Summary of the sorption processes on mineral surfaces (according to Charlet and Manceau, 1993; Hargé, 1997): (1) adsorption of an ion via formation of an outer-sphere complex (a); (2) loss of hydration water and formation of an inner-sphere complex (b); (3) lattice diffusion and isomorphic substitution within the mineral lattice (c); (4) and (5) rapid lateral diffusion and formation of either a surface polymer (d), or adsorption on a ledge (which maximizes the number of bonds to the atom) (e). Upon particle growth, surface polymers end up embedded in the lattice structure (f); finally, the adsorbed ion can diffuse back in solution, either as a result of dynamic equilibrium or as a product of surface redox reactions (g).

Sorption processes related to humic substances

As already mentioned humic substances have a pH dependent negative charge due to the dissociation of their functional groups. Similarly to mineral soil particles humic substances can therefore retain metals by specific adsorption processes (formation of covalent bonds), but also by non-specific electrostatic interactions (CEC). In case of specific adsorption, complexes are formed involving one or several functional groups (formation of mono-dentate or multi-dentate complexes). Chelation is the reaction between a metal ion and a complexing agent, characterized by the formation of more than one bond between the metal and the functional groups of the complexing agent that results in the formation of a ring structure incorporating the metal ion. For the bulk of the metal binding the COOH and COH groups are of importance (De Wit, 1992).

General mechanisms of reactions of metals with fulvic or humic acids are illustrated in Figure 9 (Huang, 1997). A divalent metal ion is taken as an example. These reactions are likely to proceed by one or more, or simultaneously all four of the reaction mechanisms. In reaction (1), one COOH group reacts with one metal ion to form a mono-dentate complex. Equation (2) describes a reaction with one COOH and one adjacent OH group reacting simultaneously with the metal ion to form a bi-dentate complex or chelate. In reaction (3), two adjacent COOH groups interact simultaneously with the metal ion to form a chelate. Reaction (4) shows a situation in which the metal ion M^{n+} is linked to the organic molecule, in addition to bonding by electrostatic attraction, also through hydrogen bonding to a C=O group via a water molecule in its primary hydration shell.

Low-molecular-weight organic ligands, not necessarily humic in origin, can form soluble complexes with metals and prevent them from being adsorbed or precipitated.

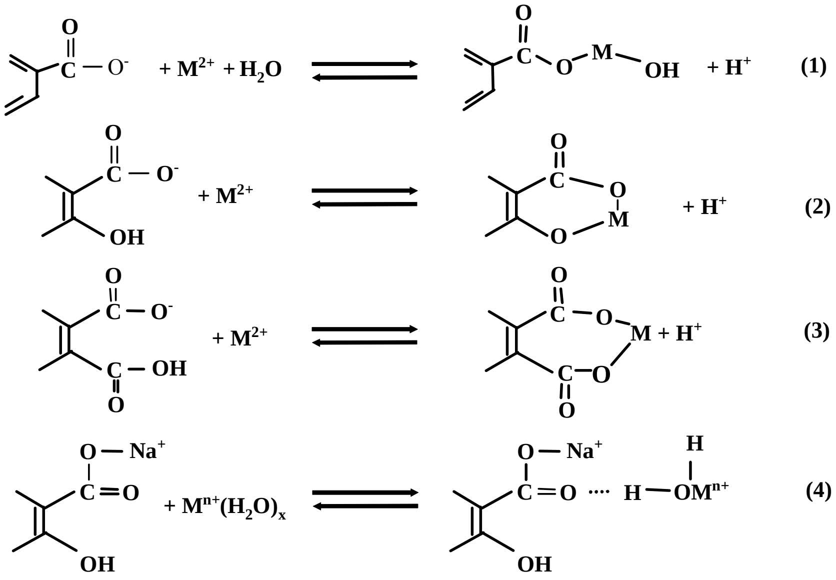

Figure 9 General mechanisms (postulated) of reaction of fulvic and humic acids with metals (according to Huang, 1997).

Weathering of minerals and mineralization of organic matter

In contrast to the sorption processes discussed above, weathering of soil minerals and mineralization of organic matter can affect the distribution of metals between the solid phase and the soil solution in the opposite direction. Weathering refers to the desintegration and alternation of rocks and minerals by physical, biological and chemical process. When primary minerals are weathered to form secondary minerals, trace elements bound in the crystal lattices, such as Ca, Mg, Fe, Mn, Zn, Cu can be solubilized and become available for plant uptake (Adriano, 2001). Correspondingly, mineralization of organic matter may lead to a release of complexed metals. In addition to meteorologic agents, living organisms have an important role in weathering (e.g root activities) and organic decomposition processes (e.g micro-organisms) (Gobran et al., 1999).

3.3.3 Factors influencing metal phytoavailability in soils

3.3.3.1 Soil factors

Numerous studies on metal sorption by soils have indicated that specific soil compounds (organic matter, clay minerals, Fe-, Mn- and Al oxides) and specific soil properties (CEC, pH, redox status,..) are important factors determining metal mobility and bioavailability in soils (Martinez and McBride, 1999; Sauvé et al., 2000; Stumm, 1992; Zachara, 1992). Since the CEC of soils is largely dependent on the amount and type of clay, organic matter and the oxides of Fe, Al and Mn, the CEC can be considered as an indicator of soil compounds that may limit the solubility of metals (Adriano, 2001) (Paragraph 3.3.2.1). Soil pH and redox potential can be viewed as primary driving factors influencing geochemical processes discussed in paragraph 3.3.2.2 (Adriano, 2001).

Generally soil pH is considered to be the most important parameter affecting the solubility of metal in soils. Higher pH values tend to reduce metal bioavailability. Summarized, the following effects of pH are potentially observed:

- influence on the reactivity of surfaces: with increasing pH, pH dependent sites (on minerals as well as on organic matter) are increasingly deprotonated and become accessible for metals (Berti and Cunningham, 1998; Stumm, 1992).
- influence on the speciation of metals: with increasing pH higher concentrations of OH-, HCO_3^- or CO_3^{2-} and of organic ligands are present. As a result, respectively insoluble or soluble metal phases can be formed (Adriano, 2001; Sauvé et al., 1998). In calcareous soils, free $CaCO_3$ can adsorb or precipitate metal ions depending on the metal concentrations (McBride, 1994). Formation of metal-hydroxy complexes may increase the strength of metal sorption (Bolan, 2003).

- influence on the stability of mineral soil constituents: when pH decreases minerals can dissolve (weathering) and thereby free metals that are present on their surface or in their structure (Adriano, 2001).

Furthermore, soil redox status plays a determining role in metal bioavailability. Under reducing conditions (e.g flooded soils) reduction of sulfates can result in the formation of insoluble metal sulfides (e.g. CdS, CuS,...); these sulfides become soluble again under oxidizing conditions. If the reducing conditions cause a dissolution of Mn-, Fe- or Al-hydroxides, metals adsorbed or co-precipitated with these hydroxides will come in solution (Alloway, 1995). These processes however do not take place in well-aerated soils.

The composition of the soil solution also influences metal mobility and bioavailability. The presence of organic and inorganic ligands in the soil solution can increase metal solubility, by the formation of soluble complexes (Doner, 1978; Christensen et al., 1996). The effect of these soluble complexes on plant metal uptake seems to vary depending on the metal and the complex. According to the FIAM (Free Ion Activity Model), which postulates that that the uptake of metals by an organisms is proportional to their free ion activities in (soil) solution, these soluble complexes do not contribute to metal uptake (Sauvé et al, 1996, 1998). For higher plants deviations form the FIAM have repeatedly been reported. Smolders and McLaughlin (1996a, 1996b) for example reported that Cd accumulation in *Beta vulgaris* was not only correlated with the activity of Cd^{2+} in solution but also with the activity of $CdCl_n^{2-n}$ complexes. The authors suggested that either the complex was available for plant uptake, either the Cl- ions led to an increased Cd uptake due to an increased diffusion of Cd to plant roots. Uptake of metal-ligand complexes has been observed e.g for Pb-EDTA (Sarret et al., 2001) and can certainly can occur when low molecular weight metabolites are involved. Recently, Degryse et al. (2006) showed evidence that plant uptake of Cd, at small free ion concentrations, is rate-limited by diffusion of the free ion to the root surface. When an ion becomes depleted near the membrane surface, metal complexes can dissociate, and enhance the diffusional flux, in turn increasing plant metal uptake. In their study, Cd complexes increased Cd uptake in spinach, with an extent dependent on the dissociation behavior of the complexes (higher uptake for fast dissociating complexes). These findings may explain previously reported departures from the FIAM (e.g enhanced Cd uptake in presence of Cl-ions).

An indirect effect of the composition of the soil solution on metal bioavailability is the competition of metals for root absorption on the one hand and fixation by the solid phase on the other hand. Christensen (1987) for example has shown that fixation of Cd by the soil solid phase was inhibited in the presence of Ca^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, and Zn^{2+}.

3.3.3.2 Other factors

Type and speciation of the element

In paragraph 1.4.2.2.2 it was mentioned that certain physico-chemical properties of elements have some bearing on the biogeochemical behavior of metals (e.g. electronegativity and ionic potential). Electronegativity influences the order in which trace metals sorb onto soil constituents. Stronger covalent bonds can be expected with the more electronegative metals. For some divalent metals the bonding preference would be: Cu>Ni>Co>Pb>Cd>Zn>Mg. On the basis of electrostatic attraction, the strength of bonding is influenced by the ionic potential producing a different pattern (charge/radius ratio) (Adriano, 2001).

Furthermore, chemical speciation influences the reactivity and mobility of metals in the environment. For example automotive exhaust contain Pb in more readily soluble form (PbClBr and $PbSO_4$) compared to Pb oxides species (PbO_x, $PbO{\cdot}PbSO_4$) present after smelter operations (Davis et al., 1992). Pb oxide species in turn are more soluble than e.g. galena (PbS) associated with ore and mining activities (Ruby et al., 1999). Some metals have a different mobility and toxicity depending on their oxidation state. For example hexavalent Cr is much more mobile and toxic than trivalent Cr (Adriano, 2001).

Biological factors

Last but not least, biological factors influence metal phytoavailability. A focal point in this context is the micro-ecosystem surrounding the plant roots: the rhizosphere. This micro-ecosystem is created by plant roots and microbial associations and is characterized by physical, chemical and biological conditions that differ from the bulk soil (Wenzel et al., 1999). The population of micro-organisms in de rhizosphere seems to be a few orders of magnitude higher than in the bulk soil (Anderson, 1993). Both plant roots and microbes exude inorganic and organic compounds with various functional groups possessing acidifying, chelating and/or reductive power. These compounds can trigger a range of reactions resulting in an altered state (phase), speciation, and solubility of chemical elements. Mench and Martin (1991) for example have shown that root exudates of *Nicotiana* sp. increased Cd solubility and availability. On the other hand some root exudates may reduce metal bioavailability (Hall, 2002).

Relevant in this context is also the strategy plants use to mobilize scarcely mobile essential trace elements (e.g. Fe) in the soil. Dicotyledons and non-grass monocotyledons are so called strategy-I plants. They mobilize metals in the rhizosphere by proton release, by the exudation of organics and phenolics and by reductase systems, which transform ferric into ferrous ion. Ferrous ions released from chelates are transported into the cytoplasm via transport proteins (Kochian, 1991; Grusak et al., 1999). Grasses and cereals have evolved an alternative method for acquiring Fe from insoluble sources and are called strategy-II plants (Marschner and Römhled, 1994). This method involves the release of low-molecular weight Fe(III)-chelating compounds, commonly known as phytosiderophores, which solubilize Fe(III) in the rhizosphere. The metal siderophore complex is recognized for uptake by specific carrier proteins (Chaney, 1987; Grussak et al., 1999). Siderophore production is also found in some rhizobacteria (Kloepper et al., 1980). Altough phytosiderophore release is considered primarily being directed towards Fe nutrition, most siderophores also show a lower but significant affinity for heavy metals (van der Lelie, 1998). Siderophores released by plants or rhizobacteria may therefore affect metal phytoavailability. Another potential role of rhizobacteria in metal phytoavailability can be found in bacterial metal tolerance mechanisms, reducing phytoavailable metal concentrations, such as the czc efflux system of *Alcaligenes eutrophus* CH34 (van der Lelie, 1998; Nies, 1999). In addition to bacteria, also fungi, and in particular mycorrhizal fungi affect metal phytoavailability. Proposed mechanisms are presented in paragraph 1.6.1 when discussing the potential role of mycorrhizal fungi in plant metal tolerance. Furthermore, the role of soil invertebrates in metal mobility and phytoavailability may not be underestimated. Bioturbation of the soil by soil fauna, redistributes contaminants in the soil profile (Adriano, 2001), and feeding activities (e.g of earthworms) may influence metal speciation (Indeherberg et al., 1998). Some invertebrates play a role in the regulation of soil bacterial compositions and densities, thereby also influencing bacterial mediated processes (Blair et al., 1995). In general, all living organisms immobilize, to an extent influenced by their own metal tolerance mechanisms, metals in their tissues.

3.3.4 Estimating metal phytoavailability

3.3.4.1. Chemical extractions

Specific chemical agents can theoretically remove metals associated with different soil fractions. Chemical extractions allow to speciate metals into pools, which differ in mobility, behaviour and susceptibility for interaction with biological receptors. A wide range of chemical extraction methods has been proposed and evaluated, trying to reflect phytoavailability of metals in soils (Beckett, 1983; Lebourg and Sterckeman, 1996, Houba et al., 1996, Davidson et al., 1998, Wang et al., 2003; Meers et al., 2006a). The single extraction methods that have been studied can be divided into a few main groups:

-soil solution sampling or the use of water extracts:

Since plants rely on the soil solution for nutrient uptake, estimates of the composition of the soil solution may provide information on metal phytoavailability. Different methods have been proposed to obtain information about this most labile soil metal fraction, based on displacement (water extractions), centrifugation or suction methods (Blaylock et al., 1997; Meers et al., 2006a). According to the Free Ion Activity Model (Sauvé et al., 1996, 1999) free metal ions in solution are even more important than total solution concentrations in estimating metal phytoavailability. But exceptions on the FIAM have been reported (Degryse et al., 2006).

In this thesis we used soil moisture samplers (Eijkelkamp, 10 cm MOM-type) to obtain insight in the effect of soil amendments on the mobile soil metal fraction. The samplers consist of a porous filter tube, which can be inserted in the soil and on which suction is applied by means of a syringe. The filter is a synthetic polymer with a pore diameter of 0.1 µm and excludes colloidal material from the extract.

-extractions with neutral salt solutions to determine exchangeable soil metals: Neutral salt solutions are supposed to mirror the soil solution and to exchange their cations with easily exchangeable metals on the soil solid phase. These loosely adsorbed metals are considered as labile and available for plant uptake (Häni and Gupta, 1985; Kabata-Pendias, 1993). A variety of salty extractants has been investigated including 0.1 M $NaNO_3$, which is used in the Swiss legislative framework (Bo, 1986; Aten and Gupta, 1996), 0.1M $CaCl_2$ used in the Dutch legislation (Novozamsky et al., 1993; Houba et al., 2000; Pueyo, 2004), and 0.1 M $Ca(NO_3)_2$ frequently used for the assessment of metal immobilizing soil treatments (Boisson et al., 1999a; Geebelen et al., 2006).

Because the content of metals in solution plus the 'weakly absorbed' content (i.e. the exchangeable pool) generally provides a good measure of the plant available amount of metals (Adriano, 2001) we selected 0.1 M $Ca(NO_3)_2$ for evaluation of exchangeable metal fractions. Advantages of using $Ca(NO_3)_2$ over the more commonly used $CaCl_2$ can be found in the fact that chlorides may form stabile soluble complexes with certain heavy metals, which may induce release of metals in surplus of the targeted loosely adsorbed soil fraction (Gupta and Aten, 1993; Häni, 1996; Boisson et al., 1999a).

-extractions with organic acids:

In the first step of the BCR sequential extraction, which is presently proposed by the Standards, Measurements and Testing program of the European Commission as an alternative to the various Tessier-based methods (Tessier, 1979; Rauret et al., 1999), acetic acid (0.11M, L:S 40:1) is used as weak complexing extractant (Davidson et al., 1998; Geebelen et al., 2003). The acidic effect additionally induces dissolution of hydroxides and carbonates. The approach is based on the consideration that acetic acid is one of the most abundant Light Molecular Weight Organic Acid (LMWOA) in the rhizosphere of many plants (Meers et al, 2006a).

The use of 1 M NH_4OAc as extractant (L:S 20:1, 1h) has been adopted in French legislation (French standard NF X 31-108; Brun *et al.*, 2001). Whereas acetate simulates complexing behavior of root exudates, $NH4^+$ is capable of desorbing the exchangeable soil fraction. This solution can be buffered at around pH 7 to avoid carbonate dissolution (Baker *et al.*, 1994) or at acidity levels corresponding to relevant values in the rhizosphere (Yu *et al.*, 2004).
A similar route was investigated by Wang *et al.* (2003), who propose a 10 mmol LMWOA extraction solution, consisting of acetic acid, formic acid, citric acid and malic acid at a ratio of 2:2:1:1 to estimate metal phytoavailability. When correlations were compared between plant uptake and extractable levels with (i) the first step of the BCR protocol or (ii) the LMWOA based extraction solution, better results were obtained with the 10 mM LMWOA solution.

-*extractions with chelating agents* :
A mixed EDTA - NH_4OAc was first introduced by Lakanen and Ervio (1971). Less aggressive variants of this protocol, buffered at higher pH, and with lower chelate concentrations have been proposed and are currently still in use (Lindsay and Norvell, 1978).

Extraction liquids discussed above have shown, at least on some occasions, satisfying relationships with plant metal accumulation, but no single all-encompassing chemical extraction method is found to predict metal phytoavailability over a wide range of species and soil conditions (Adriano, 2001). A difficulty related the use of chemical extractions for the determination of metal phytoavailability, is that we do not know exactly which soils fraction(s) plants or other organisms are exposed to and are removing metals from (Sauvé, 1999), and that this fraction may be species (or even cultivar) dependent (Ernst, 1998). In addition interactions between elements and/or other compounds may alter the phytoavailability and phytotoxicity of a certain environment.

3.3.4.2. Biological test systems to detect soil metal phytotoxicity

Higher plants are an essential part of a healthy and balanced ecosystem. They produce oxygen and organic substances on which most other life forms depend. Plants provide food and nesting habitat for insects, invertebrates, birds, mammals, etc. They have an essential role in the processes of nutrient cycling and soil stabilization. Toxic effects on plants may have severe consequences for wildlife (Wang and Freemark, 1995) and the whole ecosystem. Because of the difficulty to find chemical extraction methods well predicting metal phytoavailability over a wide range of soil conditions, bioassays, which directly assess the toxic effect of metals on a plant species, are complementary and indispensable tools to assess the potential phytotoxicity of contaminated sites. In bioassays, interactions among elements are integrated in the observed effects. The tests offer the additional advantage that toxic effects caused by unexpected compounds, which were not assessed in chemical analysis, may become apparent (Meers et al., 2006b).

The oldest and most frequently used application of phytotoxicity tests occur in risk assessments of new or existing chemicals, which are required by several regulatory agencies such as the Organization for Economic Cooperation and Development (OECD) in Europe, by the Environmental Protection Agency (US-EPA) and the US Food and Drug Administration (US-FDA) in the United States and by the International Organization for Standardization (ISO) (OECD, 1984a; US-EPA 1996a, 1996b; ISO, 1993, 1995).

Currently three terrestrial phytotoxicity tests are recognized by these regulatory agencies in the context of evaluation and classification of new or existing chemicals: a seed germination test, root elongation and an early seedling growth test (Wang and Freemark, 1995; Fletcher, 1991). The test species can be selected from lists, which differ among agencies. Each agency has its specific requirements about the plant families that need to be represented (Fletcher, 1991). The requirement of tests with different plant species, belonging to a number of different families, offers the potential advantage that a range of sensitivities can be covered. Only limited information is available about the accuracy of the recognized tests, when applied as bioassays.

According to Gong et al. (2001) there is a need to refine and adjust existing phytotoxicity tests to make them applicable as bioassays to assess the toxicity of contaminated soils. It is generally recognized that nutrient levels may to a large extent determine plant growth responses. The dependence of plant growth on edaphic factors complicates to a large extent interpretations of growth tests when used as a bioassay (Wang et al., 1995). In particular, an improved insight in the main soil properties that influence test results on reference soils and an additional check of the soil sample to be assessed and the reference soil for these properties before the test start may increase the accuracy of test results. The choice of an appropriate reference soil, is extremely important to obtain a reliable interpretation of test results. Selection of an unsuited reference soil will easily lead to a misinterpretation of test results and to false positive or false negative conclusions about the toxicity of the substrate (Frische, 2003).

Alternative phytotoxicity endpoints are described in literature, including e.g chlorophyll fluorescence (Hulsen et al., 2002; Krugh and Miles, 1996; Marwood et al., 2001), enzyme capacities (Byl and Cline, 1991; Van Assche and Clijsters, 1988, 1990a) and fatty acid compositions (Verdoni et al., 2001). None of these endpoints are currently adopted by regulatory agencies in recognized tests. Alternative toxicity tests, however, could be of great value to improve the accuracy of toxicity assessments of contaminated soils. As far as we know, data on the influence of edaphic factors on these biochemical responses have not yet been published.

In this context, the present study contributes to the optimization of a phytotoxicity test with *Phaseolus vulgaris* (Van Assche and Clijsters, 1990a; Vangronsveld and Clijsters, 1992). The test is based on the analysis of growth parameters and biochemical plant responses, in particular enzyme activities that are directly or indirectly involved in the antioxidative defence systems of plants, including guaiacol peroxidase (GPOD; E.C.: 1.11.1.7), malic enzyme (ME; E.C.: 1.1.1.40), glutamate dehydrogenase (GlDH; E.C.: 1.4.1.2) and iso-citrate dehydrogenase (ICDH; E.C.: 1.1.1.42). More information on the background of this test in given in paragraph 4.4.

3.3.5 Metal immobilizing soil amendments

Different types of soil amendments have been investigated for their capacity to immobilize heavy metals. Their selection has been based on the knowledge of general soil constituents and processes contributing to metal retention in reference conditions (paragraph 3.3.2). They include:

- liming materials (Albesel and Cottenie, 1985; Bolan, 2003; Chlopecka and Adriano, 1996; Mench et al., 1997; Pierzynski an Schwab, 1993, Hamon, 2002; Lombi 2002a, 2002b),
- various types of organic matter (Baste et al., 2001; Berti and Cunningham, 1997; Brown et al., 2003a, 2003b, 2004; McBride and Martinez, 2000, Shuman et al., 2002),
- natural and synthetic zeolites (Chlopecka and Adriano, 1997; Gworek, 1992; Oste et al., 2002; Rebedea and Lepp, 1995),
- clays and related substances such as cyclonic ashes (CA, 'beringite') and coal fly ashes (Alvarez-Ayuso and Garcia-Sanchez, 2003; Badora et al., 1998; Boisson et al., 1998; Boisson et al., 1999b; Bouwman and Vangronsveld, 2004; Geebelen et al., 2003; Lock and Janssen, 2003; Lotenbach, et al., 1998; Mench et al., 1994, 2003, 2006; Shende et al.,

1994; Sims and Boswell., 1978; Vangronsveld et al., 1995a, 1995b, 1996),

- Fe- and/or Mn oxides or Fe- and or Mn-rich materials such as steel shots (SS) and bauxite residue (red muds) (Boisson et al., 1998; Boisson et al., 1999a,b; Mench et al., 1994a,b, 1997, 2003, 2006; Lombi et al., 2002a,b; Hettiarachchi et al., 2000, Hettiarachchi and Pyrzynski, 2002).
- P-rich compounds such as phosphate rock, synthetic apatite, phosphoric acid or phosphate salts (Basta et al., 2001; Boisson et al., 1999a; Brown et al. 2004; Chlopecka and Adriano, 1997; Geebelen et al., 2003; Hettiarachchi et al., 2001; Laperche et al., 1997; Ma and Roa, 1997; Ruby et al., 1994; Ryan et al. 2001; Yang et al. 2001).

The most frequently used methods to evaluate reductions in metal bioavailability (mobility) by these products are: reductions is exchangeable metal contents ($CaCl_2$, $Ca(NO_3)_2$), sequential extractions, plant metal uptake, phytotoxicity tests, bacterial biosensors, microbial biomass,…. Some researchers have put a lot of additional efforts in investigating effects of P-additions (in different forms) on availability of soil Pb to humans (gastro-intestinal; PBET-test).

Making a comparison of the efficiency of the different additives throughout the numerous immobilization studies mentioned above, to come to a selection of 'the best additive' or to a ranging of additives in order of increasing immobilizing capacity, is not evident or even impossible. The use of different evaluation methods by different research groups e.g. to estimate exchangeable metal fractions, or the use of different plant species to evaluate reductions in crop metal uptake contribute to the difficulty. Moreover the remedial action of an additive seems to be soil dependent (Geebelen et al., 2003; Lombi et al., 2002a, 2002b). Several factors including soil pH, the nature and extent of the metal pollution, the presence of other ions in solution,... may influence the extent of metal immobilization in a certain soil. This means that an additive, which proved to be very successful in one type of soil, may be less efficient in another soil. This soil type dependence of the immobilization process has to be taken into account when developing remediation options for a specific site.

When one has to make decisions about the suitability of a product for *in situ* applications, durability of the effect is an important parameter in addition to the short-term effects as discussed above. Even if the duration of a satisfactory immobilization treatment depends on several parameters such as land use, treatments costs and acceptable risks (Mench et al., 2003), it is generally recognized that the stronger the metal binding, and the longer the proven durability of a treatment, the higher the chance that decision makers will allow to use it. Generally valuable information about the chances on a long-term immobilization is obtained when the mechanism responsible for the immobilization process can be determined. For some products (e.g. phosphates) this action mechanism is well understood. Due to the formation of pyromorphite, which is the most stable Pb-mineral under a wide range of geochemical conditions, P-addition is believed to be very promising for reduction of human health risks associated with Pb contaminated soils (see Brown et al, 2004 and references therein). Among all treatments studied this may be, at least from chemical point of view, the strongest proven form of metal immobilization. Detailed information on metal immobilization mechanisms is not available for most other amendments that have been considered. Detection limits of physical techniques such as XRD (X-ray diffraction), XRF (X-ray fluorescence) or EXAFS (Extended X-ray absorption fine structure) often seem to be too high to identify metal bearing phases in treated contaminated soils. Information about expected long-term effects can be obtained from chemical methods (acid extraction and isotopic dilution methods), or from long-term field experiments (Hamon et al., 2002; Lombi et al., 2002a, 2002b; Geebelen et al., 2006; Vangronsveld et al., 1995a, 1996, 1999). Results obtained from both types of studies, however, were inconsistent regarding the potential durability cyclonic ashes as a metal immobilizing soil treatment. This thesis, therefore aimed to gain additional insight in the long-term behavior of this treatment by means of a rainfall simulation experiment in soil columns (Chapter 1). Other treatments, included in the experiment for the purpose of comparison, were: lime, steel shots and cyclonic ashes + steel shots. The following paragraph describes cyclonic ashes (CA) and steel shots (SS) a metal immobilizing soil treatments. Most of the information was summarized based on reports of Mench et al. (1998) and Vangronsveld et al. (1999).

3.3.5.1 Cyclonic ashes

Cyclonic ashes are modified aluminosilicates originating from the fluidized bed burning of coal refuse (mine pile material). The first cyclonic ashes evaluated for *in situ* immobilization purposes originated from the fluidized bed situated at the former coal mine in Beringen (north east of Belgium) and are alternatively known as 'beringite'. Mine pile material from the coal mine was burnt in the fluidized bed, the primary purpose of the burning being the recuperation of energy left in the coal refuse. The burnt material contained about 30 % coal; the remaining fraction was inorganic and mainly consistsed of schists. Minerals present in the schists were: quartz, illite, kaolinite, chlorite, calcite ($CaCO_3$), dolomite ($(Ca,Mg)CO_3$), anhydrite ($CaSO_4$), siderite ($FeCO_3$) and pyrite (FeS_2) (De Boodt, 1991). Illite was the most dominant clay present. The schists were burned by heating in an electronically guided fluidized bed oven at ca. 800°C. During heating, schists undergo partial breakdown and recrystallization. An X-ray diffraction pattern made after heating showed the disappearance of peaks for kaolinite, chlorite and pyrite (De Boodt, 1991). A new crystalline mineral called ettringite ($6CaO. Al_2O_3.3SO_4.31H_2O$) was formed and the formation of minerals of the pyroaurite and hydrotalcite families was also postulated (De

Boodt, 1991). By heating at the high temperature, the swelling and shrinking characteristics (in presence or absence of water) were almost eliminated, although the lamellar structure was conserved, resulting in an improved accessibility of the inner surfaces for metal immobilization processes.

By use of air suction (air current) most of the particles with a median diameter of less than 0.2 mm (including the clay fraction) can be separated in a cyclone; these are the so-called cyclonic ashes (about 25 % of the total ash fraction). A limited amount of these small particles (about 5 % of the total remaining fraction, fly ashes) pass this cyclone. The main fraction of the ashes (about 70 %) is collected at the bottom of the oven (bottom ashes).

The pH of the cyclonic ashes is strongly alkaline (± 11). The high pH can be explained by the presence of MgO and CaO which are formed during the heating of $CaCO_3$ and $(Ca,Mg)CO_3$ minerals present in the schists. The oxides form hydroxides ($Ca(OH)_2$ and $Mg(OH)_2$) when they come in contact with water. The formation of these hydroxides also is responsible for the high buffering capacity. Additional quantities of lime were added during the heating process aiming at the reduction of sulphur emissions.

The cation exchange capacity of the cyclonic ashes was found to be about 20 meq/100g. A mean specific surface of ± 20 m^2 /g was measured (De Boodt, 1991).

De Boodt (1991) suggests that the high metal immobilizing capacity of cyclonic ashes is based on ion exchange, chemical precipitation, and crystal growth. Based on evolution of pH, results of selective and sequential extractions and plant availability of the metals in long-term time course simulation and field experiments a three step sorption process is hypothesized (Vangronsveld et al., 1999): (a) an initial rapid first step (hours) representing adsorption onto highly accessible sites on the surface of the modified clay and on binding sites of the original soil components freed due to a 'liming effect' (presence of ($Ca(OH)_2$ and $Mg(OH)_2$), followed by (b) a slower type (days) of sorption characteristic for modified surfaces, i.e. coprecipitation associated with Al, Fe and Mn oxides and (c) on the longer term (years) crystal growth and metal diffusion into the mineral surface. This last step should be responsible for the permanent decrease observed for the metal extractability by chemicals and availability for plants.

Improvement of metal binding by cyclonic ashes can be obtained by addition of aluminum salt during the heating process (De Boodt, 1991). This results in surface coating by aluminium-hydroxides allowing increased chemical adsorption of the metals. This 'ameliorated' product however was not intensively tested in soils since (a) the efficiency to the original cyclonic ashes was satisfactory and (b) the increase in cost as the result of this treatment.

Cyclonic ashes from Beringen (beringite) have been successfully applied to reduce metal mobility and phytotoxicity in contaminated soils (Vangronsveld and Clijsters, 1992; amendement A5 in their study corresponds to beringite). In a field experiment at an abandoned barren zinc smelter site, compost+ cyclonic ashes (C+CA) was used as a metal immobilizing soil treatment (Vangronsveld et al., 1995a; 1999). A healthy and sustainable vegetation cover established, even at sites with metal contents as high as 13250 mg kg^{-1} Zn (sandy soil; pH-H_2O = 5.5) (Vangronsveld et al. 1999, Bouwman et al., 2001). Twelve years after treatment the nematode fauna is still improving (Bouwman and Vangronsveld, 2004), available metal fractions are very low and no signs of phytotoxicity are observed (Ruttens et al., unpublished results).

The production of beringite ceased in 1995 as the fluidized bed in Beringen (Belgium) closed down. The encouraging results achieved with beringite both in the lab and in the field convinced the Public Waste Agency of the Flemish Region [Openbare Afvalstoffenmaatschappij voor het Vlaams Gewest (OVAM)] that *in situ* metal immobilization using cheap industrial by products could be a realistic and cost-effective strategy to reduce environmental and health risks posed by contaminated soils in the Kempen. The 'Studycouncil Alternative Soil Remediation' (Studiesyndikaat Alternatieve Bodemsanering) was founded as a collaboration between OVAM, Umicore (non ferrous industrial company) and the Centre for Environmental Sciences (Hasselt University) with the aim of examining cost-effective remediation techniques for lowering risks associated with contaminated sites in the Kempen. A screening of the coal refuse burning facilities throughout Europe resulted in the selection of cyclonic ashes from a Spanish coal burning plant showing reasonable similarities with beringite (Devoldere et al., 1999). The burnt mother material, as well as the burning conditions, were among the burning facilities explored, the most similar to the conditions in Beringen. These ashes, further referred to as CAH (cyclonic ashes type H), together with series of other soil amendments have been screened for their short-term metal immobilizing capacities (Ruttens et al., 2003). Based on promising short term results obtained with these ashes, it was decided to evaluate its long-term efficiency. The composition of CAH and the cyclonic ashes from Beringen (beringite), in terms of oxide composition, is shown in Table 4.

Table 4 Composition of beringite (CA) and alternative cyclonic ashes (CAH), on oxide basis (in %), measured by means of XRF (X-Ray Fluorescence) (Devoldere et al., 1999)

	CA-Beringite	CAH
SiO_2	51.0	38.5
Al_2O_3	31.0	24.6
CaO	4.1	7.6
Fe_2O_3	6.6	4.3
K_2O	3.5	2.6
MgO	1.6	1.2

3.3.5.2 Steel shots

Steel shots are an industrial material containing mainly iron (97% α-Fe) and native impurities such as Mn (0.6 to 1%), Si (0.8 to 1.2%), C (0.8 to 1.2%) and Cr (0.2 to 0.5%). Steel shots contain little Cd (3.6×10^{-6} %), Zn (0.01 %) and Ni (0.074 %). They are used for shaping metal surfaces and are available in various particle sizes (Mench et al., 1998). They corrode in soil, resulting in the formation of several Fe and Mn oxides (Sappin-Didier, 1995). Release of iron or manganese by steel shots is an alternative for application of Fe and Mn oxides to immobilize metals in contaminated soils. The oxidation process of steel shots seems to play a crucial role in the metal immobilization process since the Fe oxides (maghemite, magnetite and lepidocrocite) applied as such to the same soil did not reduce metal mobility. The newly formed oxides may coat soil particles developing a large surface for reaction with trace elements. It is hypothesized that metals may co-precipitate with the newly formed Fe oxides (Mench et al., 1998). Also Mn present in the steel shots (at 0.6-1%) may contribute to the immobilization process since specific adsorption of metals is known to occur on Mn oxide surfaces (McKenzie 1980, Fu et al., 1991). McKenzie (1980) showed that the adsorption of Pb to Mn oxides was 40 times greater then sorption on Fe oxides and was less reversible. By means of EXAFS-studies (Extended X-ray Absorption Fine Structure), Manceau et al. (1996) showed the formation of phyllomanganates when SS was added to the soil.

Several studies have indicated that steel shots are an effective treatment to reduce metal mobility in contaminated soils and its uptake in crops (Mench et al. 1994a,b, 1997, 2003; 2006, Sappin-Didier et al., 1997). PBET-tests showed a decreseased bioaccessibility of Cd(-30%) and Pb(-35%) after soil treatment with SS (Mench et al., 2006). More than in the case of CA, results of chemical techniques (acidification) suggest a durable metal immobilization when Fe- or Mn- rich amendments are applied to contaminated soils (Lombi et al., 2002b, 2003; Hettiarachi et al., 2000).

3.3.5.3 Combination of cyclonic ashes and steel shots

The combination of CA + SS was investigated first by Boisson et al. (1999a). Based on extractions with $Ca(NO_3)_2$ the combined treatment showed to be more efficient compared to each of the compounds applied separately. Other studies (Geebelen et al., 2003; Mench et al., 2003, 2006) have confirmed the high metal immobilizing capacity of the treatment. In particular for soils contaminated with a mixture of metals and arsenic, the combined treatment may be very suited, due to the strong arsenic immobilizing properties of SS (Mench et al., 2006). The metal immobilizing potential of this combined treatment was further explored in this work (Chapters 1, 2, 3).

4. METALS IN PLANTS

4.1 Mechanisms of metal homeostasis and metal tolerance

In order to maintain the concentration of essential metals within physiological limits and to minimize detrimental effects of non-essential metals, plants, like all other organisms, have evolved a complex network of homeostatic mechanisms that serve to control uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001). The main components of metal homeostasis are transport, chelation and sequestration processes. Their regulated activities aim to avoid the build up of excess metal levels in the cytosol, and thus to prevent the onset of toxicity symptoms. These mechanisms result in a basic metal tolerance (Hall, 2002).

Some plant species and genotypes posses increased levels of metal tolerance. Tolerance to metals in plants may be defined as the ability to survive in a soil that is too toxic to other plants, and is manifested by an interaction between a genotype and its environment (Macnair et al., 2000). Mechanisms that are involved in metal tolerance may largely correspond to the ones that are employed in general metal homeostasis in plants. Tolerant plants seem to show enhanced avoidance and homeostatic mechanisms to prevent the onset of stress (Hall, 2002).

Tolerance mechanisms can be divided in (a) those operating at the plant level, and (b) cellular tolerance mechanisms (Ernst, 1992). A summary of the most important mechanisms that might be involved in metal tolerance is presented in Figure 10.

4.1.1 Metal tolerance at the plant level

At the plant level, avoidance strategies are involved in increased metal tolerance. Differences in the distribution of metals over root and shoot have been frequently reported (Ernst et al., 1992). Reduction of root to shoot metal translocation may protect sensitive and essential metabolic processes in aerial plant organs (e.g. photosynthesis) (Verkleij and Prast, 1989, De Knecht et al., 1992). Allocation of metals into old leaves creates the possibility to remove metals via natural shedding. (Ernst, 1992). Root exudates can form complexes with metals thereby reducing metal uptake or converting them into less toxic species (Hall, 2002; Salt et al., 2000; Horst et al., 1982; Ma et al., 2001). The range of exuded compounds, however, is large and depending on their characteristics root exudates may also enhance the uptake of certain elements (Mench and Martin, 1991; Rengel et al., 1998).

Increased metal tolerance can also be the result of infection with mycorrhizal fungi (Hall, 2002; Jentschke and Goldbold, 2000; Leyval et al., 1997). However, the mechanisms involved in conferring this increased tolerance have proved difficult to resolve. They may be quite diverse and show considerable species and metal specificity. In relation to the role of ectomycorrhizal fungi most mechanisms that have been proposed involve various exclusion processes that restrict metal movement to the host root (Hall, 2002). They include reduced access to the apoplast due to the hydrophobicity of the fungal sheet (Ashford et al., 1989), extracellular binding trough adsorption of metals onto external mycelium (Colpaert et al., 1992), hyphal slime (Denny and Ridge, 1995) or cell wall components (Galli et al., 1994), chelation by fungal exudates (Jones, 1998; Ahonen-Jonnarath et al., 2000) or absorption of metals by the fungal sheet (Martin et al., 1994; Courbot et al., 2004). Since the nutritional state of a plant may have a pronounced influence on the susceptibility to metal toxicity, it is likely that the improved nutrition of mycorrhizal plants contributes to their higher metal tolerance (Jentschke and Goldbold, 2000). According to Meharg

(2003) there are only few examples where the presence of mycorrhizal fungi clearly alleviates metal toxicity of the host. Recently, Adriaessen et al (2004, 2005) have shown that a zinc adapted *Suillus bovinus* strain much better survived zinc stress, and significantly improved the nutrient status and growth of the host plant (*Pinus sylvestris*) when exposed to high zinc concentrations, compared to a zinc sensitive isolate of the same fungus. It is still unclear whether the mechanism of tolerance involved, is the binding of metal either internally or externally, or an altered uptake of nutrients (Adriaenssen et al., 2005).

There are fewer reports on the role played by arbuscular mycorrhizal fungi or ericoid mycorrhizal fungi in metal tolerance. Lux and Cumming (2001) showed that Tulip-poplar (*Liriodendron tuliperifera*) colonized by arbuscular mycrorrhizal fungi was less Al sensitive than in its non-myccorrhizal state. Similarly in ericoid mycorrhiza, colonization increased growth and survival of *Calluna vulgaris* at excess Zn concentrations and enabled plant growth at concentrations that completely inhibited growth of uncolonized plants (Bradley et al., 1982).

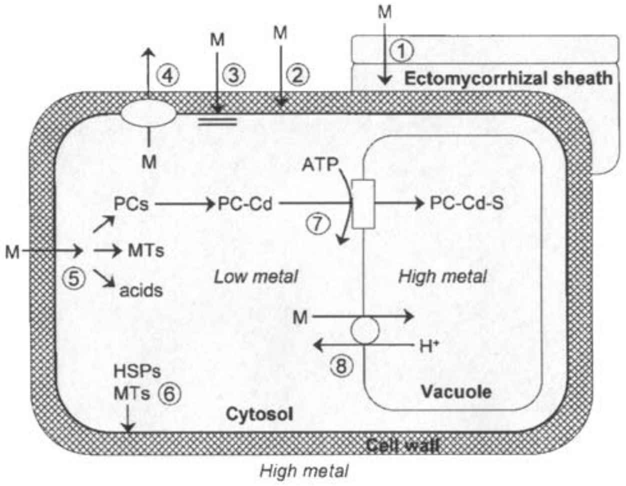

Figure 10 Summary of potential mechanisms available for metal detoxification and tolerance in higher plants (Hall, 2002).

4.1.2 Metal tolerance at the cellular level

Precipitation or bio-sorption of metals onto cell walls has been proposed as an avoidance mechanism operating at the cellular level (Ernst et al., 1992). Once the metal has crossed the cell wall, various mechanisms are available to protect the symplast and to reduce free metal concentrations in the cytosol (Clemens, 2001). They include restricted influx (De Vos et al., 1991), active efflux (Bringeze et al., 1999) and intracellular chelation and/or compartimentalization into the vacuole (Coleman, 1997; Vekleij et al., 1998), away from metabolic processes. Intracellular metal ligands are for example phytochelatins, metallothioneins, organic acids and polyphosphates (Wang et al., 1992). These chelators contribute to metal detoxification by buffering cytosolic free metal concentrations (Figure 10).

Next to avoidance and detoxification strategies, as discussed above, plants possess mechanisms that may reduce toxic effects, or induce signals that create the plants ability to react to the environmental stress factor (Paragraph 4.3.3) (Clijsters et al., 1999; Foyer and Noctor, 2005).

4.2 Metal phytotoxicity

Stunted growth, leaf epinasty and chlorosis are visual symptoms of strong metal phytotoxicity (Lepp, 1981; Balsberg Pahlsson, 1989). At the physiological level disturbances of many important processes have been reported upon increased metal exposure, including photosynthesis (Ciscato et al., 1997; Krupa and Baszynski, 1995), respiration (Burzynski and Klobus, 2004; Koeppe and Miller, 1970), transpiration and water status (Barceló and Poschenrieder, 1990; Haag-Kerwer et al., 1999), plasma membrane permeability (Cuypers et al., 2005; De Vos et al., 1989; Murphy et al., 1999; Skórzyńska-Polit et al. 2006) and nutrient uptake and translocation (Burzynski 1987; Hernández et al., 1996; Huang, 1997). Also inhibitions of cell division have been reported (Eun et al., 2000) and changes in gene expresssion are observed (Foyer and Noctor, 2003; Scandalios, 2005). Reviews about toxic effects of metals on plants are provided for example by Rout and Das (2003) for Zn, by Yruela (2005) for Cu, by Benavides et al. (2005) for Cd, and by Sharma and Dubey (2005) for Pb. Generally the various toxic effects of trace metals on diverse aspects of the plant metabolism, can be related to three main characteristics of metals (Vangronsveld and Clijsters, 1994; Schützendübel and Polle, 2002):

- their capability to interact with ligand groups of biomolecules (e.g proteins or nucleic acids),
- the possible substitution of an essential metal by the toxic metal in metalloenzymes or other metalloproteins,
- their capability to induce directly or indirectly oxidative stress (and to activate a sequence of signals in response to the environmental stress factor; Foyer and Noctor, 2005).

Whereas the first two characteristics of metals generally lead to inhibitions of enzymes or other proteins, the induction of oxidative stress by metals is often accompanied with an increase in enzyme capacities (Van Assche and Clijsters, 1990b) (Parapgraph 4.3.3.2). In the following paragraphs the three effect mechanisms mentioned above are illustrated with some examples.

4.2.1 Interaction of metals with ligand groups of enzyme proteins or other biomolecules

Metals are known to demonstrate a relatively high affinity for sulphydryl and carboxyl groups, depending on the physicochemical properties of the cation (see Paragraph 3.1; Nieboer and Richardson, 1980). Interaction of metals with SH-groups of proteins has been suggested to be involved in several metal induced effects, e.g. inhibition of chlorophyll biosynthesis, reduction of CO_2 fixation and alteration of plasma membrane permeability (Vangronsveld and Clijsters, 1994).

The metal sensitivity of chlorophyll biosynthesis has been linked to interaction of metals with sulphydryl groups of important enzymes in the biosynthesis pathway, such as δ-aminolaevulinic acid (ALA)-dehydratase (Prassad and Prassad, 1987) and protochlorophyllide reductase (Griffiths, 1975; Stobart et al., 1985). Several studies indicate that ATP-ases of the plasmalemma, responsible for electrogenic pumping and involved in the cellular permeability barrier, are very sensitive to various sulphydryl reagents including metals (Beffagna et al., 1979; Kennedy and Gonsalves, 1989). Blocking SH-groups inhibits the activity of over 100 currently known enzymes (Seregin and Ivanov, 2001).

Also the numerous nucleophilic centres in nucleic acids are favourite binding sites of metal ions. Induction of crosslinks between both DNA strands, single-strand DNA breaks and chelation or formation of complexes between DNA and metals have been reported (Gebhart and Rossman, 1991). Similar reactions can be expected between metals and RNA.

4.2.2 Substitution of an essential metal by the toxic metal in metallo-enzymes or other metallo-proteins

Inhibition of the photosynthetic electron transport by Zn and Cd has been described in green algae as well as in higher plants. Photosystem II was found to be sensitive to these metals in the alga *Euglena viridis* (De Filippis et al., 1981) and in *Lycopersicon esculentum* (Baszinsky et al., 1980) and *Phaseolus vulgaris* (Van Assche and Clijsters, 1983). In the three species the water splitting enzyme was the site of action. Mn ions are essential for an efficient water-splitting activity. Baszinsky et al (1980) reported the restoration of the cadmium-inhibited electron transport by additional application of manganese. A sharp increase in the thylakoid bound Zn/Mn ratio after Zn intoxication of *Phaseolus vulgaris* (Van Assche and Clijsters, 1986a) suggested a partial substitution of Mn by Zn in the water splitting enzyme, causing inhibition of protolysis of water.

Zinc inhibition of ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco), the key enzyme of the Calvin cycle, could be explained by substitution of Mg by Zn in the active ternary Rubisco-CO_2-metal complex (Van Assche and Clijsters, 1986b). Küpper et al. (1998) showed a substitution of Mg in chlorophyll by several metals. The metal substituted chlorophyll species exhibited a lower quantum yield, a lowered electron transport capacity and an unstable first excitation state, explaining the observed decrease in photosynthetic rates. These results were confirmed by Krupa and Moniak (1998).

4.3 Metal induced oxidative stress and oxidative signaling

4.3.1 Reactive oxygen species in normal plant metabolism

Reactive oxygen species (ROS) are partially reduced forms of atmospheric oxygen (${}^3O_2$) (Figure 11). They typically result from, either the excitation of O_2 to form singlet oxygen (${}^1O_2$), or from the transfer of one, two or three electrons from O_2 to form, respectively, a superoxide radical ($O_2^{\circ -}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical (°OH) (Mittler, 2002).

$$O_2 \xLeftrightarrow{+e^-} O_2^{-\circ} \xLeftrightarrow{+e^-,\ +2H^+} H_2O_2 \xLeftrightarrow{+e^-,\ +H^+} {}^\circ OH(+\ H_2O) \xLeftrightarrow{+e^-,\ +H^+} 2H_2O$$

Figure 11 Interconversion of O_2 and water, showing intermediate reactive oxygen species (adapted from Foyer and Noctor, 2000).

These ROS can be very toxic since they attack several cell constituents, such as proteins, carbohydrates, nucleic acids, and lipids (Winston, 1990). At the biomembrane level, lipid peroxidation is a very important destructive reaction (Vangronsveld and Clijsters, 1994; Skórzyńska-Polit et al., 2006). Ever since the introduction of molecular oxygen into our atmosphere by 0_2-evolving photosynthetic organisms about 2.7 billion years ago, ROS have been considered the unwelcome companions of aerobic life (Halliwell and Gutteridge, 1989). In recent years, however, a new role for ROS has been identified: the control and regulation of biological processes such as growth, cell cycle, programmed cell death, hormone signaling, biotic and abiotic stress responses and development. ROS may act as intermediate signaling molecules to regulate the expression of genes (Mittler, 2002; Mittler et al., 2004; Schützendübel and Polle, 2002). The use of ROS as signaling molecules suggests that, during the course of evolution, plants were able to achieve a high level of control over ROS toxicity.

Many potential sources of reactive oxygen species exist in plants under normal conditions. In chloroplasts, chlorophyll pigments associated with the electron transport system are the primary sources of singlet oxygen ($^10_2$). The superoxide radical (0_2^-°) is produced at any location where an electron transfer chain is present. In chloroplasts the formation of 0_2^-°is mainly caused by electron transfer from ferredoxin to oxygen (Asada, 1996; Arora et al., 2002) ($20_2 + 2Fd_{red} \rightarrow 2\ 0_2^-° + 2\ Fd_{ox}$) . Hydrogen peroxide ($H_20_2$) is formed trough dismutation of 0_2^-°. The latter occurs spontaneously ($2\ 0_2^-° + 2H^+ \rightarrow H_20_2 + 0_2$) but the velocity of the reaction is greatly increased by the enzyme Super Oxide Dismutase (SOD). The well known reactivity of H_20_2 is not due to its reactivity *per se*, but requires the presence of a metal as reductant to form the highly reactive °0H radical, which is the strongest oxidizing agent known. Already in the late nineteenth century (1899) Fenton described the oxidizing potential of H_20_2 mixed with ferrous salt. Thirty-five years later (1934) Haber and Weiss identified °0H as the oxidizing species in this reaction (Figure 12, equation 1) (Halliwell and Gutteridge, 2002). In biological systems the availability of ferrous ions limits the rate of reaction, but the recycling of iron from the ferric to the ferrous form by a reducing agent (Figure 12, equation 2) (e.g. 0_2^-° or organic radicals) can maintain an ongoing Fenton reaction, resulting in a continued production of very reactive °OH radicals (Arora et al., 2002). The overall reaction is presented in equation 3 (Figure 12).

(Equation 1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\circ}OH + OH^{-}$ (Fenton reaction)

(Equation 2) $Fe^{3+} + O_2^{-\circ} \rightarrow O_2 + Fe^{2+}$

(Equation 3) $H_2O_2 + O_2^{-\circ} \xrightarrow{(Fe^{2+}/Fe^{3+})} O_2 + {}^{\circ}OH + OH^{-}$ (Haber-Weiss reaction)

Figure 12 Sub-reactions and overall reaction describing the production of hydroxyl radicals by H_2O_2 in the presence of Fe^{2+} and a reducing agent (e.g. the superoxide radical).

In mitochondria over-reduction of the electron transport chain is the main source of $O_2^{-\circ}$-production (Möller, 2001; Mittler et al., 2004). Superoxide radicals and H_2O_2 are also produced in plant peroxysomes (Del Rio et al., 2002).

Next to the ROS sources discussed above, which can be considered as unintentional by-products of plant metabolism, there is increasing evidence that ROS in plants, even under normal conditions, are generated intentionally to guide certain metabolic processes. Apoplastic ROS generated by NADPH oxidases for example regulate developmental processes in *Arabidopsis thaliana*. *AtrbohD* and *AtrbohF* genes (=*Arabidopsis thaliana* respiratory burst oxidase homolog) are expressed in guard cells and play an important role in the ABA signaling during stomatal closure (Bright et al., 2006). Furthermore *Atrboh*C regulates cell expansion during root hair formation (Torres and Dangl, 2005). Such intentionally produced ROS are also considered to play an important role in the plants ability to react to environmental stress factors (Paragraph 4.3.3.1).

4.3.2 Antioxidative defence mechanisms and regulation of ROS levels

In normal conditions, production of reactive oxygen species is low and their level is controlled by different antioxidative protection systems. These systems, including enzymatic and non-enzymatic reactions, have the potential to control the cascades of uncontrolled oxidation in the cell under normal conditions or when stimulated by any stress condition (Gratão et al., 2005). They help to regulate the level of ROS in order to balance oxidative stress and oxidative signaling (Foyer and Noctor, 2005). The steady state level of ROS in the different cellular compartments is thus determined by an interplay between different ROS-producing and ROS-scavenging mechanisms (Pargraph 4.3.2). These are controlled by a ROS-signal-transduction pathway and constitute the basic ROS-cycle (Mitler et al., 2004)(Figure 13).

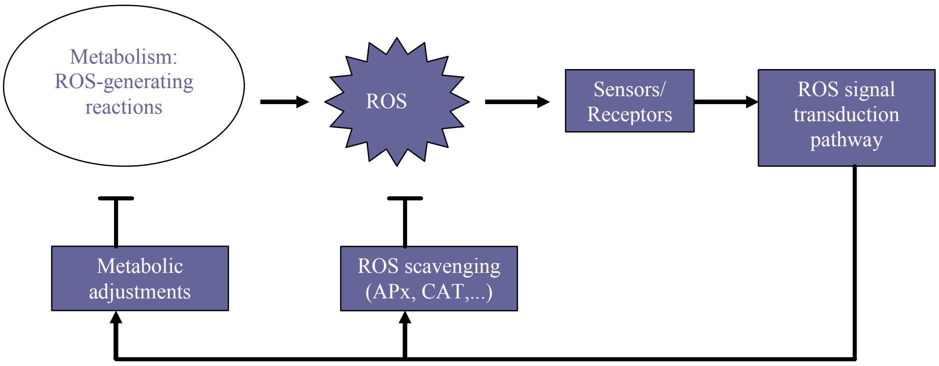

Figure 13 The basic ROS-cycle; this cycle modulates ROS levels during normal metabolism.

Important components of the plant antioxidative system are summarized below.

Non-enzymatic detoxification mechanisms

Substrates that function as reductants can serve as antioxidants in the protection against oxidative damage. Molecules involved in the non-enzymatic antioxidant responses include e.g. α-tocopherol (vitamine E), carotenoids, glutathione and ascorbate. Their respective action mechanisms can be summarized as follows:

- *α -tocopherol (vitamine E):* The antioxidant properties of this membrane bound (chloroplast) antioxidant result from its ability to quench both singlet oxygen and peroxides. Its most dominant effect may be the breaking of radical chain reactions by trapping peroxyl radicals. (Fryer, 1992; Munné-Bosh, 2005).
- *Carotenoïds:* In terms of antioxidant properties carotenoids can protect cells from oxidative damage in different ways: (1). by reacting with lipid peroxidation products to terminate chain reactions (cfr. α -tocopherol) (Burton and Ingold, 1984), (2). by scavenging singlet oxygen (Mathis and Kleo, 1973), (3). by reacting with excited chlorophyll molecules to prevent formation of singlet oxygen. Excess energy from carotenoids is dissipated as heat. Carotenoids are located in plastids of both photosynthetic and non-photosynthetic plant tissues (Raven et al., 1999).
- *Ascorbate (vitamine C):* As a direct antioxidant ascorbate can react with the $O_2^{-\circ}$ and with H_2O_2. The reaction with hydrogen peroxide is catalysed by ascorbate peroxidase (Asada, 1992) (Figure x). Ascorbate also plays an indirect role by regeneration of oxidized α -tocopherol (Smirnoff, 1996). It occurs in the chloroplast, mitochondria, peroxysomes, the vacuole, the cytosol, and in the apoplast.
- *Glutathione*: Glutathione is a tripeptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphydryl group of cysteine (Rennenberg and Brunhold, 1994). It can react chemically with singlet oxygen, superoxide anion and hydroxyl radicals and therefore function directly as a scavenger. Indirectly it plays a role in the recycling of ascorbate from its oxidized to its reduced form by the enzyme dehydroascorbate reductase (Loewus, 1988; Figure 14). In addition GSH may stabilize

membrane structures by removing acylperoxides formed during lipid peroxidation reactions (Price et al., 1990). Through the action of glutathione-S-transferase (GST, EC 2.5.1.18) GSH can reduce peroxides (Gratão et al., 2005). At the subcellular level, GSH concentrations are the highest in the chloroplast, but significant quantities also accumulate in the cytosol (Noctor et al., 2002)

Enzymatic detoxification mechanisms

Enzymatic detoxification mechanisms for ROS, operating in plant cells, were reviewed excellently by Mittler et al. (2004), and Gratão et al. (2005).

Major ROS-scavenging enzymes of plants include (Mittler, 2004):

- superoxide dismutase (SOD, EC 1.15.1.1),
- ascorbate peroxidase (APX),
- catalase (CAT, EC 1.11.1.6),
- glutathione peroxidase (GPX, EC 1.11.1.9)
- peroxiredoxines (PrxR)
-

Additional enzymes of importance in ROS detoxification are (Gratão et al, 2005):

- glutathione-S-transferase (GST, EC 2.5.1.18)
- guaiacol peroxidase (GPOD, 1.11.1.7)
- enzymes of the ascorbate-glutathione pathway : in this pathway (Figure 14, upper right) peroxides are detoxified by APX; the oxidized forms (monodehydroascorbate, MDHA or dehydroascorbate, DHA) are recycled to ascorbate using respectively monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and reducing equivalents of NADPH or dehydroascorbate reductase (DHAR, EC 1.8.5.1) and reducing equivalents of glutathione. Glutathione in turn is reduced by glutathione reductase (GR) with NADPH as electron donor (Foyer and Halliwell, 1976; Foyer, 1993).

Superoxide dismutases are considered to have a primary role in ROS defence, dismutating O_2^{-}° to H_2O_2 and O_2 (O_2^{-}°+ $O_2^{-\circ}$+ $2H^+ \rightarrow H_2O_2 + O_2$). By this reaction SOD influences concentrations of O_2^{-}° and H_2O_2, the two substrates of the Haber-Weiss reaction (Figure 12, equation 3), and has an important role in preventing the formation of the very toxic °OH. APX and CAT subsequently detoxify H_2O_2 according to the following reactions (CAT: $2H_2O_2 \rightarrow 2H_2O + O_{2;}$; APX: H_2O_2 + 2 ascorbate→ 2 monodehydroascorbate +$2H_2O$). Glutathione peroxidase can also metabolise H_2O_2 and peroxyredoxines can reduce a broad range of toxic peroxides (Finkemeier et al., 2005). Gluthatione-S-transferases constitute a complex family of proteins. The primary role of many GSTs is conjugation, either of xenobiotics or of intermediates and secondary metabolites. Certain GSTs play a role as peroxidases or in regenerating ascorbate from dehydroascorbate (Foyer and Noctor, 2005). Guaiacol peroxidases deliver physiologically active reaction products in the cell wall (involvement in lignification process) (Mittler, 2004).

An overview of the different detoxification pathways and their localization is presented in Figure 14. The various scavenging enzymes can be found in almost every subcellular compartment. In thylakoids of the chloroplast the water-water cycle detoxifies O_2^{-}° and H_2O_2, and alternative oxidases (AOX) reduce the production rate of O_2^{-}°. In some plants Fe-SOD might replace Cu-Zn SOD in chloroplasts (Figure 14, top left). ROS that escape this cycle and/or are produced in stroma undergo detoxification by SOD and the stromal ascorbate-glutathione cycle. Peroxiredoxin (PrxR) and glutathione peroxidase (GPX) are also involved in H_2O_2 removal in the stroma (Figure 14, top right). ROS produced in peroxysomes during photorespiration, fatty acid oxidation or other reactions are decomposed by SOD, catalase (CAT) and ascorbate peroxidase (APX) (Figure 14, middle right). SOD and other components of the ascorbate-glutathione pathway are also present in mitochondria. In addition, AOX prevents oxidative damage in mitochondria (Figure 14, bottom right). In principle, the cyctosol contains the same set of enzymes found in the stroma (Figure 14, bottom left). The enzymatic compounds responsible for ROS detoxification in the apoplast and cell wall are only partially known, and may include ascorbate peroxidase (APX) and guaiacol peroxidases (GPOD) (Gratão et al., 2005). ROS scavenging pathways at the vacuole are unknown. Although the pathways in the different compartments are mostly separated from each other, H_2O_2 can easily diffuse through membranes and antioxidants such as glutathione and ascorbate (reduced or oxidized) can be transported between different compartments (Mittler et al., 2004).

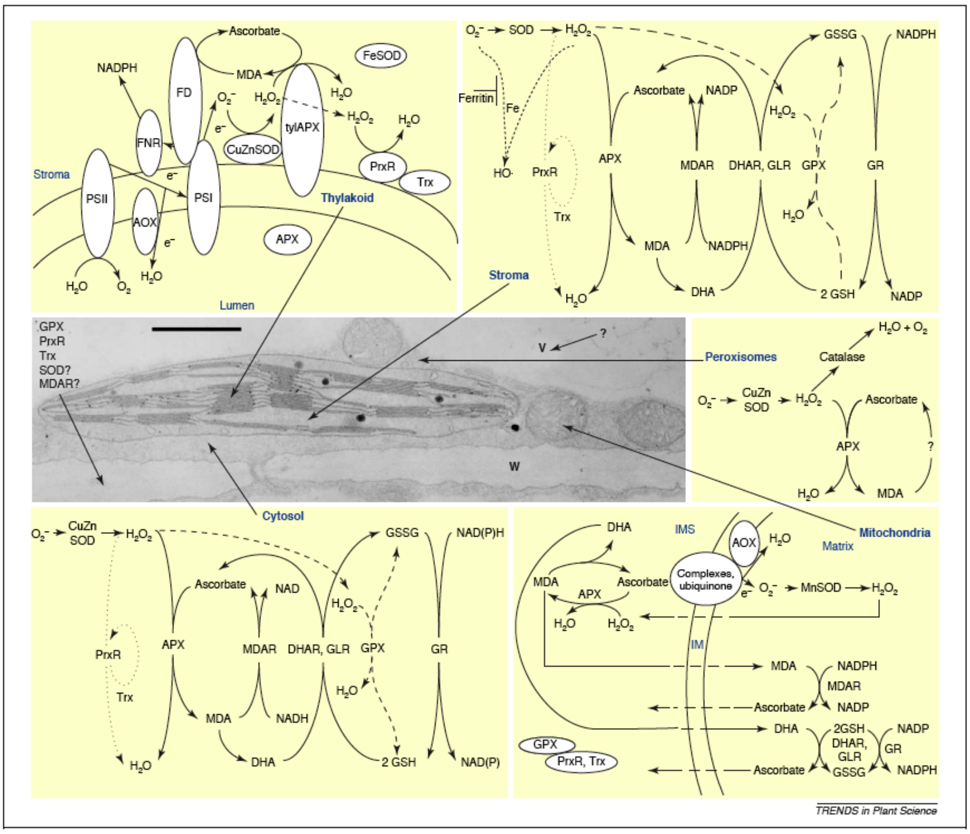

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Figure 14 Localization of reactive oxygen scavenging pathways in plant cells. A transmission electron micrograph of a portion of a plant cell is used to demonstrate the different cellular compartments and their physical separation (source: Mittler et al., 2004). Abbreviations: PSI: photosystem I; PSII: photosystem II; V: vacuole; W: cell wall; for enzyme abbreviations see page iv).

The antioxidant systems in plants described above are functioning in concert, in order to minimize ROS damage as much as possible, but not to eliminate the ROS completely (Mittler, 2002).

4.3.3 Oxidative stress

4.3.3.1 The oxidative stress paradigm

Many stresses including metals, drought stress, salt stress, heat shock, ozone, ultraviolet radiation, pathogen attack, etc. enhance the production of reactive oxygen species (Mittler, 2002). Situations which provoke enhanced ROS production have, for long time, been categorized under the heading of 'oxidative stress' (Foyer and Noctor, 2005).

Transition metals such as copper (and iron) are able to perform one-electron transfer reactions and therefore can directly generate reactive oxygen species. In particular because of their possible involvement in Fenton-like reactions (Figure 12, equation 1) and the generation of hydroxyl radicals, they are considered to be strong inducers of oxidative stress (Koppenol, 1994; Weckx and Clijsters, 1996). Interestingly, the increased occurance of activated oxygen and symptoms of oxidative injury have also been observed in plants exposed to metals, such as zinc, cadmium, and lead, which do not belong to the group of transition metals (Schützendübel and Polle, 2002). These indirect effects have been attributed to (1) interactions with the ascorbate-glutathione cycle (Paragraph 4.3.2) including depletion of GSH and inhibition of antioxidant enzymes (Schützendübel and Polle, 2002), (2) to electron leakage e.g. in the photosynthetic or mitochondrial electron transport as a result of metal binding to functional groups of proteins or substitution of essential elements in metallo-enzymes (Van Assche and Clijsters, 1986b; Gwzódz et al., 1997) or (3) to enzymatically induced lipid peroxidation (e.g lipoxygenase) (Weckx and Clijsters, 1997, Skórzyńska-Polit, 2006).

In conditions that limit CO_2 fixation such as drought, salt and temperature, production of ROS in the chloroplast is found to be enhanced. In C3 plants, limiting CO_2 conditions can activate the photorespiratory pathway. As part of this pathway, H_2O_2 is generated in peroxysomes by the enzymatic activity of glycolate oxidase (Mittler et al., 2004).

Oxidative stress, in itself, is a negative term implying a harmful process. Oxidative stress can originate when there is a strong imbalance between ROS production and ROS scavenging. More and more evidence, however, suggest that in many cases enhanced oxidation has a positive role (Foyer and Noctor, 2005). ROS can be actively produced both under normal conditions and upon stress exposure. At the apoplast NADPH-oxidase, pH-dependent cell wall peroxidases, oxalate oxidases and amine oxidases have been proposed to generate ROS (Mittler et al., 2004). These are tightly regulated and participate in the production of ROS during processes such as programmed cell death, stress reponses, pathogen defence (Mittler, 2002). Lipoxygenases (LOX) are induced upon exposure to redox active as well as non redox active metal (Weckx and Clijsters, 1997; Skórzyska-Polit et al., 2006). Upon stress exposure, these ROS will activate a cascade of signals and induce the plants ability to react to an environmental stress factor (Foyer and Noctor, 2005).

4.3.3.2 Metal induced enzyme inductions in plants

In response to many stress factors, previously described as being involved in the generation of 'oxidative stress' (Paragraph 4.3.3.1) changes in the activity of a number of enzymes have been reported in numerous studies (see also Table 5). The affected enzymes can be divided in two groups (Clijsters et al., 1999; Mittler et al., 2004): (1) enzymes directly or indirectly involved in ROS detoxification (Figure 14), and (2) $NAD(P)^+$ reducing enzymes and GlDH. Both of these enzyme groups will be further referred to as 'stress enzymes'.

It has been hypothesized that induction of these ‘stress enzymes’ can be considered as defence mechanism against metal toxicity, activated when mechanisms of metal homeostasis and tolerance, as discussed in Paragraph 4.1, are incapable of keeping cellular metal levels below toxic concentrations (Clijsters et al., 1999). The first group of enzymes, involved in ROS detoxification, was discussed already in Paragraph 4.3.2. The second enzyme group includes $NAD(P)^+$ reducing enzymes, involved in or related to the Krebs cycle, such as isocitrate dehdrogenese (ICDH), malic enzyme (ME) and glutamate dehydrogenase (GlDH) (Clijsters et al., 1999; Mathys, 1975; Van Assche et al., 1988). Ernst (1980) suggested that the capacity of these enzymes is possibly stimulated to compensate for the decrease in ATP and NAD(P)H production, normally provided by the metal sensitive photosynthetic reactions. This could be particularly important to provide the cell with sufficient reducing power, to maintain an ongoing ascorbate-glutathione cycle (Clijsters et al, 1999). The NAD(P)H dependent conversion of α-ketoglutarate in glutamate by the enzyme glutamate dehydrogenase, plays an important role in amino acid metabolism. Induction of GlDH capacity, which has been observed after metal exposure (Van Assche et al., 1988), may therefore have a role in the *de novo* protein synthesis (Vangronsveld et al., 1994).

The interpretation of the inductions of the ‘stress enzymes’ as defence mechansims against oxidative stress remains controversial (Chaoui and El Farjani, 2005). In a literature survey, Schützendübel and Polle (2002) were not able to find evidence that oxidative stress could be alleviated by increased levels of antioxidative systems. In the light of the recent evolutions, in which enhanced oxidation is not longer considered as an unavoidable harmful process, but rather as an essential component of a repertoire of signals that plants use to make appropriate adjustments to gene expression and cell structure in response to environmental and developmental cues (Foyer and Noctor, 2005), it

Table 5 Increase in activities of antioxidative enzymes in different plant species and plant organs exposed to metals (for enzyme abbreviations see page iv).

Metal	Plant species	Enzymes	Plant organ	Reference
Cadmium	*Phaseolus vulgaris*	ME; ICDH; GDH; G6PDH; POD	Leaves	Van Assche *et al.* (1988)
	Silene italica	ME; ICDH; GDH; G6PDH	Shoots	Mattioni *et al.* (1997)
	Phaseolus vulgaris	APX, GPOD, GR, CAT	Roots, Leaves	Chaoui *et al.* (1997)
	Zea mays	POD	Leaves	Lagriffoul *et al.* (1998)
	Phaseolus vulgaris	POD, APX, GR, ICDH, ME	Leaves	Smeets *et al.* (2005)
	Zea mays	SOD, POD	Leaves, Roots	Rellan-Alvarez *et al.* (2006)
Copper	*Phaseolus vulgaris*	APX; GR; DHAR; MDHAR	Roots	Gupta *et al.* (1999)
	Phaseolus vulgaris	APX; SOD, CAT	Leaves	Weckx and Clijsters (1996)
	Phaseolus vulgaris	APX; GR; DHAR; MDHAR	Leaves	Cuypers *et al.* (1999, 2000)
	Helianthus annuus	CAT; POD; SOD	Roots, Leaves	García *et al.* (1999)
	Zea mays	ME; ICDH; GDH; G6PDH; POD	Roots, Leaves	Mocquot *et al.* (1996)
	Arabidopsis thaliana	LOX	Leaves	Skorzynska – Polit (2006)
Lead	*Phaseolus vulgaris*	APX; DHAR; GPOD; GR; SOD; SPOD	Roots, Leaves	Geebelen *et al.* (2002)
	Macrotyloma	SOD, CAT, POD, GR, GST	Roots, Leaves	Reddy *et al.* (2005)
Zinc	*Phaseolus vulgaris*	ME; ICDH; GDH; G6PDH; POD	Leaves	Van Assche *et al.* (1988)
	Phaseolus vulgaris	APX; CAT; LOX	Leaves	Weckx and Clijsters (1997)
	Phaseolus vulgaris	APX; GR; DHAR; MDHAR	Leaves	Cuypers *et al.* (1999, 2001)
	Brassica juncea	APX; CAT; DHAR; GR; MDHAR; POD; SOD	Shoots	Prasad *et al.* (1999)
	Triticum aestivum	CAT, GPOD, APX	Leaves	Zhao *et al.* (2005)

may be necessary to re-evaluate the suggested 'defence role' of enzyme inductions in a broader context. Certainly the increased activities of ROS quenching enzymes may not eliminate all ROS, because this would interfere with their signaling role.

Whatever the exact role and sequence of this wide variety in enzymatic responses, it is clear that they are related to the stress metabolism of the plant. Our interest in these enzyme inductions in the context of the present work is related to their potential use as biomarker for (metal) phytotoxicity (Paragraph 4.4). A large amount of literature, describing effects of metals and other toxic agents on the 'stress enzymes' mentioned, can be found. Depending on the plant species used, the organ considered, the type and concentration of the toxicant and the time after application, increases or decreases of antioxidative enzymes and metabolites were found (Schützendübel and Polle, 2002). Some examples of enzyme inductions as a result of metal exposure are presented in Table 5. Attention was focused on inductions (not on inhibitions) because they have been the basis for development of a phytotoxicity test, which is subject of the second experimental part of this work (see also paragraph 4.4).

4.4 Enzymatic changes in plants as diagnostic criteria for the evaluation of phytotoxicity of metal contaminated soils

As discussed in Paragraph 4.3.3, increased levels of metal exposure may increase the production of ROS and induce the capacities of so-called 'stress enzymes' (Table 5). Studies with bean plants (*Phaseolus vulgaris cv Limburge vroege)*, have shown that the extent of enzyme induction was function of the amount of metals assimilated by the plant (Van Assche et al., 1988). These quantitative relations in combination with metal specific changes in the iso-peroxidase pattern (Van Assche and Clijsters, 1990b) have been the basis for use of enzymatic parameters as responses in a phytotoxicity test for the evaluation of the phytototoxicity of metal contaminated soils (Van Assche and Clijsters, 1990a; Vangronsveld and Clijsters, 1994). The test integrates enzyme inductions (GPOD, ME, ICDH, GlDH), with data on the iso-POD pattern and morphological parameters (shoot length, primary leaf area and root weight) in *Phaseolus vulgaris* (cv. Limburgse vroege). In short, bean plants are cultivated on the soil-samples to be assessed, under controlled environmental conditions (the latter is important to avoid other stress factors that may influence the endpoints of the test). Two weeks after sowing, morphological parameters (shoot length, primary leaf area, root weight) are measured and samples of primary leaves and roots are stored at –70°C for (iso)enzyme analysis. In addition, samples of primary leaves are taken for elemental analysis. Enzymes are measured spectrophotometrically and anionic peroxidases are separated on a 7.5-16% gradient slab gel (Van Assche et al., 1988). After running, the gels are stained enzymatically to detect different iso-forms of POD (Van Assche and Clijsters, 1990a).

The values obtained for each parameter can be 'transposed' into phytotoxicity classes based on the % reduction (morphological parameter) or % increase (plant enzymes) compared to plants grown on the non-contaminated reference soil (Table 6). Each parameter thus is used to classify the substrate in a given phytotoxicity class (class 1= not toxic, class 2= slightly toxic, class 3= moderately toxic, class 4= highly toxic). The 'Phytotoxicity Index' is obtained as an average of the toxicity class number obtained for each parameter separately (Van Assche and Clijsters, 1990a; Vangronsveld and Clijsters, 1992).

Over the last decade the main use of the test has been the evaluation of the reduction of soil phytotoxicity after application of metal immobilizing soil additives such as, Fe- en Mn oxides, steel shots, cyclonic ashes, hydroxyapatite, zeolite, bentonite (Geebelen et al., 2002a; Mench et al., 1994; Vangronsveld et al., 1995, 1996). Experimental evidence (Geebelen et al., 2002; Vangronsveld et al., 1995a, 1995b) suggests that plant responses in the test are subject to soil influences, other than metal toxicity. Optimization of the phytotoxicity test in this regard will be the subject of part III of this study (Chapter 4, Chapter 5).

Table 6 Classification of soils into phytotoxcity classes based on results of the PlantTox test (Vangronsveld and Clijsters, 1992). Classification depends on the magnitude of the effect. Values are expressed as percentage of the response obtained on a reference soil (negative control).

	Class 1 (not toxic)	Class 2 (slightly toxic)	Class 3 (moderately toxic)	Class 4 (strongly toxic)
Growth responses				
Shoot length	>85	85-70	70-50	<50
Leaf area	>85	85-70	70-50	<50
Root weight	>85	85-70	70-50	<50
Enzymes in leaves				
GPOD	<150	150-325	325-500	>500
ME, ICDH	<125	125-175	175-250	>250
Enzymes in Roots				
GPOD, ME, GlDH	<125	125-175	175-250	>250

Part I
References

Adriaensen, K., van der Lelie, D., Van Laere, J., Vangronsveld, J., Colpaert, J., 2004. A zinc adapted fungus protect pines from zinc stress. New Phytol. 161: 549-555.

Adriaensen, K., Vangronsveld, J., Colpaert, J.V., 2005. Zinc tolerant *Suillus bovinus* improves growth of Zn-exposed *Pinus sylvestris* seedlings. Mycorrhiza. *In press*.

Adriano, D.C., 2001. Trace elements in terrestrial environments: biogeochemistry, bioavailability and risks of metals. Springer-Verlag, New York, USA.

Ahonen-Jonnarath, U., Van Hees, P.A.W., Lundstrom, U.S., Finlay, R.D., 2000. Organic acids produced by mycorrhizal Pinus sylvestris exposed to elevated aluminium and heavy metal concentrations. New Phytol. 146: 557-567.

Albesel, N., Cottenie, A., 1985. Heavy metals uptake from contaminated soils as affected by peat, lime and chelate. Soil Sci. Soc. Am. J. 48: 386-390.

Alloway, B.J., 1995. Heavy metals in soils. Blackie Academic and Professional, London. UK.

Álvarez-Ayuso, E., García Sánchez, A., 2003. Palygorskite as a feasible amendment to stabilize heavy metal polluted soils. Environ. Pollut. 125: 337-344.

Andersen, T.A., Guthrei, E.A., Walton, B.T., 1993. Bioremediation in the rhizosphere: plant roots and associated microorganisms clean contaminated soils. Environ. Sci. Technol. 27: 2630-2636.

Arora, A., Sairam, R.K. and Srivastava, G.C., 2002. Oxidative stress and antioxidative system in plants. Curr. Sci. 82: 1227-1238.

Asada, K., 1996. Radical production and scavenging in the chloroplasts. *In*: Photosynthesis and the Environment. Ed. Baker, N.R., Kluwer Academic Publishers, Dordrecht, The Netherlands.

Ashford, A.E., Allaway, W.G., Peterson, C.A., Cairney, J.W.G., 1989. Nutrient transfer and the fungus root interface. Austr. J. Plant Physiol. 16: 85-97.

Aten, C.F., Gupta, S.K., 1996. On heavy metals in soil; rationalization of extractions by dilute salt solutions, comparison of the extracted concentrations with uptake by ryegrass and lettuce, and the possible influence of pyrophosphate on plant uptake. Sci. Total Environ. 178: 45-53.

Badora, A., Furrer, G., Grunwald, A., Schulin, R., 1998. Immobilization of zinc and cadmium in polluted soils by polynuclear Al-13 and Al-montmorillonite. J. Soil Contam. 7: 573-588.

Baker, A.J.M., Reeves, R.D., Hajar, A.S.M., 1994. Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens*. New Phytol. 127: 61-68.

Balsberg Pahlsson, A.M. 1989. Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants-a literature review. Water, Air and Soil Pollut. 47: 287-319.

Barceló, J., Poschenrieder, C., 1990. Plant water relations as affected by heavy metal stress: a review. J. Plant Nutr. 13: 1-37.

Basta, N.T., Gradwohl, R., Snethen, K.L., Schroder, J.L., 2001. Chemical immobilization of lead, zinc and cadmium in smelter-contaminated soils using biosolids and phosphate rock. J. Environ. Qual. 30: 1222-1230.

Baszinsky, T., Wajda, L., Krol, M., Wolinska, D., Krupa, Z., Tukendorf, A., 1980. Photosynthetic activities of cadmium-treated tomato plants. Physiol. Plant. 48: 365-370.

Becket, P.H.T., Warr, E., Davis, R.D., 1983. Cu and Zn in soils treated with sewage sludge: their 'extractability' to reagents compared to their 'availability' to plants. Plant Soil. 70: 3-14.

Beffanga, N., Marré, E., Cocucci, S.M., 1979. Cation-activated ATPase activity of plasmalemma-enriched membrane preparations from maize coleoptiles. Planta 146: 387-391.

Belgisch Staatsblad, 1996. Decree of the Flemish government of 5 march 1996 concerning the Flemish regulation on soil remediation (Vlarebo). Belgisch Staatsblad - Moniteur Belge, 27.03.1996. (modified on repeated occasions, latest change BS 5.08.2004).

Benavides, M.P., GAllego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in plants. Braz. J. Plant Physiol. 17(1): 21-34.

Berti, W.R., Cunningham, S.D., 1997. In-place inactivation of Pb in Pb–contaminated soils. Environ. Sci. Technol. 31: 1359-1364.

Berti, W.R., Cunningham, S.D., Cooper, E.M., 1998. Case studies in the field – In-place inactivation and phytorestoration of Pb contaminated sites. *In:* Metal contaminated soils*: in situ* inactivation and phytorestoration. Vangronsveld, J., Cunningham, S.D. (eds.). Springer, New York, USA.

Blair, J.M., Parmelee, R.W., Lavelle, P., 1995. Influences of earthworms on biogeochemistry. *In*: Earthworm Ecology and Biogeography in North America. Hendrix, P. (ed.). CRC Press, Boca Raton, FL, USA.

Blaylock, M.J., Salt, D.E., Dushenkov, S., Zakhrova, O., Gussman, C., Kapulnik, Y., Ensley, B.D., Raskin, I., 1997. Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ. Sci. Technol. 13: 860-865.

Bo, V.S., 1986. Verordnung über schadstoffgehalt im boden. Swiss ordenance on pollutants in soils. Nr. 814.12, Publ. Eidg. Drucksachen und Materialzentrale (EDMZ), Bern, Switzerland.

Boisson, J., Mench, M., Sapin-Didier, V., Solda, P., Vangronsveld, J., 1998. Short-term immobilization of Cd and Ni by beringite and steel shots application to long term sludged plots. Agronomie. 18: 347-359.

Boisson, J., Mench, M., Vangronsveld, J. *et al.*, 1999a. Immobilization of trace metals and arsenic by different soil additives: evaluation by means of chemical extractions. Commun. Soil Sci. Plant Anal. 30: 365-387.Zn

Boisson, J., Ruttens, A., Mench, M., Vangronsveld, J., 1999b. Evaluation of hydroxyapatite as a metal immobilizing soil additive for the remediation of polluted soils. Part 1. Influence of hydroxyapatite on metal exchangeability in soil, plant growth and plant element concentrations. Environ. Pollut. 104: 225-233.

Bolan, N.S., Adriano, D.C., Mani, P.A., Duraisamy, A., 2003. Immobilization and phytoavailability of cadmium in variable charge soils. II Effect of lime addition. Plant Soil. 251: 187-198.

Bouwman, L.A., Vangronsveld, J., 2004. Rehabilitation of the nematode fauna in a phytostabilized heavily zinc-contaminated, sandy soil. J. Soils Sediments. 4: 17-23.

Bradley, T., Burt, A.J., Read, D.J., 1982. The biology of mycorrhiza in the Ericaceae. The role of mycorrhizal infection in heavy metal resistance. New Phytol. 91: 197-209.

Brady, N.C., 1984. The nature and properties of soils. Macmillan Publishing Company, New York, USA.

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., Neill, S.J., 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J. 45: 113-122.

Bringezu, K., Lichtenberger, O., Leopold, I., Neumann, D., 1999. Heavy metal tolerance of *Silene vulgaris*. J. Plant Physiol. 154: 536-546.

Brown, S.L., Chaney, R.L., Hallfrish, J., Ryan, J.A., Berti, W.R., 2004. *In situ* soil treatments to reduce the phyto- and bioavailability of lead, zinc and cadmium. J. Environ. Qual. 33: 522-531.

Brown, S.L., Chaney, R.L., Hallfrish, J.G., Xue, Q., 2003a. Effect of biosolid processing on the availability of lead in an urban soil. J. Environ. Qual. 32: 100-108.

Brown, S.L., Henry, C.L., Chaney, R.L., Compton, H., De Volder, P.S. 2003b. Using municipal biosolids in combination with other residuals to restore metal-contaminated mining area's. Plant Soil 249: 203-215.

Brümmer, G.W., 1986. Heavy metal species, mobility and availability in soils. *In*: The importance of chemical speciation in environmental processes. Bernard, M. et al. (eds). Springer-Verlag, Berlin. Germany.

Brun, L.A., Maillet, J., Hinsinger, P., Pepin, M., 2001. Evaluation of copper availability to plants in copper-contaminated vineyard soils. Environ. Pollut. 111: 293-302.

Buckingham, D.A., Plachy, J., 2004. Cd statistics. United States Geological Survey (USGS), Reston, Virginia, USA.

Burton, G.W., en Ingold, K.U., 1984. β-carotene: an unusual type of lipid antioxidant. Science 224: 569-573.

Burzynski, M., 1987. The influence of lead and cadmium on the absorption and distribution of potassium, calcium, magnesium and iron in cucumber seedlings. Acta Physiol. Plant. 9: 229-238.

Burzynski, M., Klobus, G., 2004. Changes in photosynthetic parameters in cucumber leaves under Cu, Cd and Pb stress. Photosynthetica 42 (4): 505-510.

Byl, T.D., Klaine, S.J., 1991. Peroxidase Activity as an indicator of Sublethal Stress in the Aquatic Plant *Hydrilla Verticillata* (Royle). *In:* Plants for Toxicity Assessment. Second Volume, ASTM STP 115. Gorusch, J.W. et al (eds.). American Society for Testing and Materials, Philadelphia. USA.

Cakmak, I., Horst, W.J., 1991. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean *(Glycine max)*. Physiol. Plant. 83: 463-468.

Callahan, C.A., Menzie, C.A., Burmaster, D.E., Wilborn, D.C., Ernst, T., 1991. Environ. Toxicol. Chem. 10: 817-826.

Cambier, P., Mench, M., 1998. Contamination des sols par de métaux lourds et autres elements traces. *In:* Sol, interface fragile. INRA editions, Paris. France.

Caussy, D., Gochfield, M., Gurzau, E., Neagu, C., Ruedel, H., 2003. Lessons from case studies of metals: investigating exposure, bioavailability, and risk. Ecotoxicol. Environ. Saf. 56: 45-51.

Chaney, R.L., 1987. Complexity of iron nutrition: lessons for plant soil interaction research. J. Plant Nutr. 10: 963-994.

Chaoui, A., El Ferjani, E., 2005. Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. C.R. Biol. 328: 23-31.

Chaoui, A., Mazhyoudi, S., Ghorbal, M.H., El Ferjani E., 1997. Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). Plant Sci. 127: 139-147.

Charlet, L., Manceau, A., 1993. Structure, formation and reactivity of hydrous oxide particles: insight from X-ray absorption spectroscopy. *In*: Environmental particles. Buffle, J., Van Leeuwen, H. (eds.). Lewis Pubishers; Chelsea, MI, USA.

Chlopecka, A., Adriano, D.C., 1997. Influence of zeolite, apatite and Fe-oxide on Cd and Pb uptake by crops. Sci. Total Environ. 207: 195-206.

Christensen, J.B., Jensen, D.L., Christensen, T.H., 1996. Effect of dissolved organic carbon on the mobility of cadmium, nickel and zinc in leachate polluted groundwater. Water Res. 30: 3037-3049.

Christensen, T.H., 1987. Cadmium soil sorption at low concentrations: V. Evidence for competition by other heavy metals. Water Air Soil Pollut. 34: 293-303.

Ciscato, M., Valcke, R., Van Loven, K., Clijsters, H., Navari-Izzo, F., 1997. Effect of *in vivo* copper treatment on the photosynthetic apparatus of two *Triticum durum* cultivars with different stress sensitivity. Physiol. Plant. 100: 901-908.

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212: 475-486.

Clijsters, H., Cuypers, A., Vangronsveld, J., 1999. Physiological responses to heavy metals in higher plants; defence against oxidative stress. Z. Naturforsch. 54: 730-734.

Coleman, J.O.D., Blake-Kalff, M.M.A., Davies, T.G.E., 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. Trends Plant Sci. 2: 144-151.

Colpaert J.V., Van Assche, J.A., 1992. Zinc toxicity in ectomycorrhizal *Pinus sylvestris*. Plant Soil 143: 201-211.

Courbot, M., Diez, L., Ruototlo, R., Chalot, M., Leroy, P., 2004. Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Appl. Environ. Microb. 70: 7413-7417.

Cox, E.H., McLendon, G.L., 2000. Zn- dependent protein folding. Curr. Opin. Chem. Biol. 4(2): 162-165.

Cunningham, S.D., Berti, W.R., 1999. Phytoextraction and phytostabilisation: technological, economic, and regulatory consideratins of the soil-lead issue. *In*: Phytoremediation of contaminated soil and water. Terry, N., Banuelos, G. (eds.). Ann Arbor Press-CRC Press, Boca Raton, FL, USA.

Cuypers, A., Koistinen, K.M., Kokko, H., Kärenlampi, S., Auriola, S., Vangronsveld, J., 2005. Copper stress affects several PR-10 proteins and two putative chloroplast-related proteins in bean (*Phaseolus vulgaris* L.). J. Plant Physiol. 162: 383-392.

Cuypers, A., Vangronsveld ,J., Clijsters, H., 2001. The redox status of plants cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of *Phaseolus vulgaris*. Plant Physiol. Biochem. 39: 557-664.

Cuypers, A., Vangronsveld, J., Clijsters, H., 1999. The chemical behaviour of heavy metals plays a prominent role in the induction of oxidative stress. Free Rad. Res. 31: 39-43.

Cuypers, A., Vangronsveld, J., Clijsters, H., 2000. Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of *Phaseolus vulgaris* L. seedlings during the early stages of metal assimilation. Physiol. Plant. 110: 512-517.

Davidson, C.M., Duncan, A.L., Littlejohn, D., Ure, A.M., Garden, L.M., 1998. A critical evaluation of the three-stage BCR sequential extraction procedure to assess the potential mobility and toxicity of heavy metals in industrially-contaminated land. Anal. Chim. Acta. 363: 45-55.

Davis, A., Ruby, M.V. & Bergstrom, P.D., 1992. Bioavailability of arsenic and lead in soils from Butte, Montana mining district. Environ. Sci. Technol. 26(3): 461-468.

De Boodt, M.F., 1991. Application of the sorption theory to eliminate heavy metals from waste waters and contaminated soils. *In*: Interactions at the soil colloid - soil solution interface. Bolt GH et al. (eds.). NATO ASI Series, Series E: Applied Sciences. 190. Kluwer Academic Publishers, Dordrecht, The Netherlands.

De Filippis, L.F., Hampp, R., Ziegler, H., 1981. The effects of sublethal concentrations of zinc, cadmium and mercury on Euglena. Respiration, photosynthesis and photochemical activities. Arch. Microbiol. 128: 407-411.

De Knecht, J.A., Koevoets, P.L.M., Verkleij, J.A.C., Ernst, W.H.O., 1992. Evidence against a role for phytochelatins in naturally selected increased cadmium tolerance in *Silene vulgaris* (Moench) Garcke. New Phytol. 122: 681-688.

De Vos, C.H.R., Schat, H., 1991. Free radicals and heavy metal tolerance. *In*: Ecological responses to environmental stresses. Rozema, J., Verkleij, J.A.C. (eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands.

De Vos, C.H.R., Schat, H., De Waal, M.A.M., Vooijs, R., Ernst, W.H.O., 1991. Increased resistance to copper-induced damage of the roots cell plasmalemma in copper tolerant *Silene cucubalus*. Physiol. Plant. 82: 523-528.

De Vos, C.H.R., Vooijs, H. Schat, H. and Ernst, W.H.O., 1989. Copper induced damage to the permeability barrier in roots of *Silene Cucubalis*. J. Plant Physiol. 135: 165-169.

De Wit, J.C.M., 1992. Proton and Metal Ion Binding to Humic substances. PhD thesis. Wageningen Agricultural University. The Netherlands.

Degryse, F., Smolders, E., Mercxk, R., 2006. Labile cadmium complexes increase availability to plants. Environ. Sci. Technol. 40: 830-836.

del Rio, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., Gomez, M., Barroso, J.B., 2002. Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. J. Exp. Bot. 53: 1255-1272.

Denny, H.J., Ridge, I., 1995. Fungal slime and its role in the mycorrhizal amelioration of zinc toxicity to higher plants. New Phytol. 130: 251-257.

Devoldere, K, Dreesen, R., Laethem, B., Corbisier, P., Diels L., 1999. Mineralogische en technische evaluatie van aan Beringen-cycloonas analoge producten. Studie in opdracht van Studiesyndicaat Alternatieve Bodemsanering. Uitgevoerd door VITO, Mol, Belgium.

Doner,H.E., 1978. Chloride as a factor in mobilities of Ni(II), Cu(II) and Cd(II) in soil. Soil Sci. Soc. Am. J. 42: 882-885.

Dorn, P.B., Vipond, T.E., Salanitro, J.P., Wisniewski, H.L., 1998. Assessment of the acute toxicity of crude oils in soils using earthworms, microtox and plants. Chemosphere 37: 845-860.

Dua, V., Van Geel, P.L.B.A., 2002. Declaration of intention (Flanders-the Netherlands) related to soil contamination in the Kempen area (translated from Dutch). Document signed by the Flemisch and Dutch governments.

Duffus, J.H, 2002. Heavy metals: a meaningless term? Pure Appl. Chem. 74: 793-807.

Elstner, E.F., Wagner, G.A., Schutz, W., 1988. Activated oxygen in green plants in relation to stress situations. Curr. Topics Plant Biochem. Physiol. 7: 159-187.

Engbersen, J.F.J., de Groot, A.E., 1992. Inleiding in de bio-organische chemie, Pudoc, Wageningen, The Netherlands.

Ernst, W.H.O., 1980. Biochemical aspects of cadmium in plants. *In*: Cadmium in the environment, Part I. Nriagu, J.O. (ed.). John Wiley & Sons, New York, USA.

Ernst, W.H.O., 1998a. Effects of heavy metals in plants at the cellular and organismic level. *In:* Ecotoxicology. Schüürmann, G., Market, B. (eds.). John Wiley & Sons, New York, USA.

Ernst, W.H.O., 1998b. The origin and ecology of contaminated, stabilized and non-pristine soils. *In*: Metal contaminated soils: *in situ* inactivation and phytorestoration. Vangronsveld J., Cunningham S. (eds.). Springer-Verlag, New York, USA.

Ernst, W.H.O., Verkleij, J.A.C., Schat, H., 1992. Metal tolerance in plants. Acta Bot. Neerl. 41: 229-248.

Eun, S.O., Youn, H.S., Lee, Y., 2000. Lead disturbs microtubule organization in the root mersitem of *Zea mays*. Physiol. Plant. 110: 357-365.

Finkemeier, I., Goodman, M., Lankemeyer, P., Kandlbinder, A., Sweetlove, L.J., Dietz, K.J., 2005. The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of *Arabidopsis thaliana* under stress. J. Biol. Chem. 280: 12168-12180.

Fletcher, J., 1991. Keynote speech: A Brief Overview of Plant Toxicity Testing. *In*: Plants for Toxicity Assessment. Second Volume. Gorusch, J.W. et al. (eds.). ASTM STP 115. American Society for Testing and Materials, Philadelphia, USA.

Foth, H.D., Turk, L.M., 1972. Fundamentals of soil science. John Wiley & Sons Inc., New York, USA.

Foyer, C., Noctor, G., 2003. Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol. Plant. 119: 355-364.

Foyer, C.H., 1993. Ascorbic acid. *In* Antioxidants in higher plants. Alscher, R.G., Hess, J.L. (eds.). CRC Press, Florida, USA

Foyer, C.H., Graham, N., 2000. Oxygen processing in photosynthesis: regulation and signaling. New Phytol. 146: 359-388.

Foyer, C.H., Noctor, G., 2005. Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28: 1056-1071.

Frische, T., 2003. Ecotoxicological evaluation of *in situ* bioremediation of soils contaminated by the explosive 2,4,6 trinitrotoluene (TNT). Environ. Pollut. 121: 103-113.

Fryer, M.J., 1992. The antioxidant effects of thylakoid vitamin E (α-tocopherol). Plant, Cell Environ. 15: 381-392.

Fu, G., Allen, H.E., Cowan, C.E., 1991. Adsorption of cadmium and copper by manganese oxide. Soil Sci. 152: 72-81.

Galli, U., Shuepp, H., Brunold, C., 1994. Heavy-metal binding by mycorrhizal fungi. Physiol. Plant. 92: 364-368.

Garcia, A., Baquedano, F.J., Navarro, P., Castillo, F.J., 1999. Oxidative stress induced by copper in sunflower plants. Free Rad. Res. 31: 45-50.

Garcia-Miragaya, J., Page, A.L., 1976. Influence of ionic strength and inorganic complex formation on the sorption of trace amounts of Cd by montmorillonite. Soil Sci. Soc. Am. J. 40: 658-663.

Gebhart, E., Rossman, T.G., 1991. Mutagenicity, carcinogenicity and teratogenicity. *In*: Metals and their compounds in the environment. Merian, E. (ed.). VCH Verlagsgesellschaft, Weinheim, Germany.

Geebelen, W., Sappin-Didier, V., Ruttens, A., Yperman, J., Carleer, R., Bongué-Boma, K., Mench, M., Vangronsveld, J., 2006. Evaluation of cyclonic ash and commercial Na-silicates for heavy metal immobilisation purposes in contaminated soils in Flanders. Environ. Pollut. *In Press*.

Geebelen, W., Adriano, D.C., Van der Lelie, D., Mench, M., Carleer, R., Clijsters, H., Vangronsveld, J., 2003. Selected bioavailability assays to test the efficacy of amendment-induced immobilization of lead in soils. Plant Soil. 249: 217-228.

Geebelen, W., Vangronsveld, J., Adriano, D.C., Van Poucke, L.C., Clijsters, H., 2002. Effects of Pb-EDTA and EDTA on oxidative stress reactions and mineral uptake in *Phaseolus vulgaris*. Physiol. Plant. 115: 377-384.

Gerth, J., Brümmer, G., 1983. Adsorption und festlegung von nickel, zink und cadmium durch goethit (-FeOOH). Fres. Z. Anal. Chem. 316: 616-620.

Gobran, G.R., Clegg, S., Courchesne, F., 1999. The rhizosphere and trace element acquisition in soils. *In*: Fate and transport of heavy metals in the vadose zone. Selim, H.M., Iskandar I.K. (eds.). CRC Press, Boca Raton, FL, USA.

Gong, P., Wilke, B.M., Strozzi, E., Fleischmann, S., 2001. Evaluation and refinement of a continuous seed germination and early seedling growth test for the use in the ecotoxicological assessment of soils. Chemosphere. 44 (3): 491-500.

Gratão P.L., Polle, A., Lea P.J. and Azevedo A., 2005. Making the life of heavy metal-stressed plants a little easier. Funct. Plant Biol. 32: 481-494.

Griffiths, W.T., 1975. Characterization of terminal stage of chlorophyll(ide) synthesis in etioplast membrane preparations. Biochem. J. 152: 623-635.

Grusak, M.A., Pearson, J.N., Marentes, E., 1999. The physiology of micronutrient homeostasis in field crops. Field Crops Res. 60: 41-56.

Gupta, M., Cuypers, A., Vangronsveld, J., Clijsters, H. 1999. Copper affects the enzymes of the ascorbate-glutathione cycle and its related metabolites in the roots of *Phaseolus vulgaris*. Physiol. Plant. 106: 262-267.

Gupta, S.K., Aten, C.F., 1993. Comparison and evaluation of extraction media and their suitability in a simple-model to predict the biological relevance of heavy-metal concentrations in contaminated soils. Int. J. Environ. Anal. Chem. 51: 25-46.

Gworek, B., 1992. Inactivation of cadmium in contaminated soils using synthetic zeolites. Environ. Pollut. 75: 269-271.

Gwózdz, E.A., Przymusinski, R., Rucinska, R., Deckert, H., 1997. Plant cell responses to heavy metals: molecular and physiological aspects. Acta Physiol. Plant. 19: 459-465.

Haag-Kerwer, A., Schäfer, H.J., Heiss, S., Walter, C., Rausch, T., 1999. Cadmium exposure in *Brassica juncea* causes a decline in transpiration rate and leaf expansion without effect on photosynthesis. J. Exp. Bot. 50: 1827-1835.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53: 1-11.

Halliwell, B., Foyer, C.H., 1976. Ascorbic acid, metal ions and the superoxide radical. Biochem. J. 155: 697-700.

Halliwell, B., Gutteridge, J.M.C., 2002. Free Radicals in Biology and Medicine. Oxford University Press, NY, USA.

Hamon, R.E., McLaughin, M.J., Cozens, G., 2002. Mechanisms of attenuation of metal availability in in situ remediation treatments. Environ. Sci. Technol. 36: 3991-3996.

Häni, H., 1996. Soil analysis as a tool to predict effects on the environment. Commun. Soil Sci. Plant Anal. 27: 289-306.

Häni, H., Gupta, S., 1985. Reasons to use neutral salt solution to assess the metal impact on plants and soils. *In*: Chemical methods for assessing bioavailable metals in sludges and soils. Leschber, R., et al. (eds.). Elsevier, New York, USA.

Hargé, J.C., 1997. Spéciation comparée du zinc, du plomb et du manganèse dans des sols contaminés. PhD thesis, Université Joseph Fourier, Grenoble, France.

Hernández L.E., Cárpena-Ruiz, R., Garate, A., 1992. Alternations in the mineral nutrition of pea seedlings exposed to cadmium. J. Plant Nutr. 19: 1581-1598.

Hettiarachchi, G.M., Pierzynski, G.M., 2002. *In situ* stabilization of soil lead using phosphorus and manganese oxide: influence on plant growth. J. Environ. Qual. 31: 564-572.

Hettiarachchi, G.M., Pierzynski, G.M., Ransom, M.D., 2000. *In situ* stabilization of soil lead using phosphorus and manganese oxide. Environ. Sci. Technol. 34: 4614-4619.

Hettiarachchi, G.M., Pierzynski, G.M., Ransom, M.D., 2001. *In situ* stabilization of soil lead using phosphorus. J. Environ. Qual. 30: 1214-1221.

Hewitt, E.J., Smith, T.A., 1974. Plant mineral nutrition. English Univ. Press, London, UK.

Hodson, E.H., 2004. Heavy metals - geochemical bogeyman? Environ. Pollut. 129: 341-343.

Hogervorst, J., Plusquin, M., Vangronsveld, J., Nawrot, T., Cuypers, A., Van Hecke, E., Roels, H.A., Carleer, R., Staessen, J.A., 2006. House dust as possible route of environmental exposure to cadmium and lead in the adult general population. Environ. Res. *In Press*.

Houba, V.J.G., Lexmond, T.M., Novozamsky, I., Van der Lee, J.J., 1996. State of the art and future developments in soil analysis for bioavailability assessment. Sci. Total Environ. 178: 21-28.

Houba, V.J.G., Temminghoff, E.J.M., Gaikhorst, G.A., Van Vank, W., 2000. Soil analysis procedures using 0,01 M calcium chloride as extraction reagent. Commun. Soil Sci. Plant Anal. 31: 1299-1396.

Howard, H., 1998. Bone lead as a new biological marker of lead dose: recent findings and implication for public health. Environ. Health Perspect. 106: 961-967.

Huang, P.M., 1997. Environmental impact of metal-organic interactions. *In*: Biogeochemistry of trace metals. Adriano et al. (eds.). Science Reviews, Northwood, USA.

Hulsen, K., Top, E.M., Höffe, M., 2002. Chlorophyll fluorescence to monitor microbial degradation of linuron applied in low concentrations to bean plants. New Phytol. 154: 821-829.

IARC, 1993. Cadmium and cadmium compounds: summary and evaluation. Vol. 58. International Agency for Research on Cancer. Lyon, France.

IARC, 2004. Inorganic and organic lead compounds. Monographs on the evaluation of carcinogenic risks to humans. Vol. 87. International Agency for Research on Cancer. Lyon, France.

ICSG, 2006. Copper Bulletin, May 2006. International Copper Study Group. Lisbon, Portugal.

ILZSG, 2006. Lead and zinc statistics. Monthly bulletin of the International Lead and Zinc Study Group. Vol 46 n°2, February, 2006. ILZSG, Lisbon, Portugal.

Indeherberg, M.B.M., De Vocht, A.J.P., Van Gestel, C.A.M., 1998. Biological interactions: Effects on the use of soil invertebrates in relation to soil contamination and *in situ* soil reclamation. *In*: Metal contaminated soils*: in situ* inactivation and phytorestoration. Vangronsveld, J., Cunningham, S. (eds.). Springer-Verlag, New York, USA.

ISO, 1993. International Organization for Standardization. Soil Quality. Determination of the effect of Pollutants on soil Flora. Part 1: Method for the Measurement of Inhibition of Root Growth. ISO 11269-1.

ISO, 1995. International Organization for Standardization. Soil Quality. Determination of the effect of Pollutants on soil Flora. Part 2: Effects of chemicals on the Emergence and Growth of Higher Plants. ISO 11269-2.

Järup, L., Berglund, M., Elinder, C.G., Nordberg, G., Vahter, M., 1998. Health effects of cadmium exposure-a review of the literature and risk estimate. Scand. J. Work Environ. Health. 24: 1-51.

Jentschke, G., Goldbold, D.L., 2000. Metal toxicity and ectomycorrhizas. Physiol. Plant. 109: 107-116.

Jiang, P., Guo, Z., 2004. Fluorescent detection of zinc in biological systems: recent developments on the design of chemosensors and biosensors. Coordination chemistry reviews. 248: 20-229.

Jones, D.L., 1998. Organic acids in the rhizosphere – a critical review. Plant Soil 205: 25-44.

Kabata-Pendias, A., 1993. Behavioural properties of trace metals in soils. Appl. Geochem. Supplementary Issue. 2: 3-9.

Kamnev, A.A., van der Lelie, D., 2000. Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. Biosci. Rep. 20: 239-258.

Kennedy, C.D., Gonsalves, F.A.N., 1989. The action of divalent Zn, Cd, Hg, Cu and Pb ions on the ATPase activity of plasma membrane fraction isolated from roots of Zea mays. J. Exp. Bot. 38: 800-817.

Kloepper, J.W., Leong, J., Teintze, M. et al., 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature. 286: 885-886.

Kochian, L.V., 1991. Mechanisms of micronutrient uptake and translocation in plants. *In*: Micronutrients in Agriculture. Mortvedt, J.J. et al. (eds.). Soil Science Society of America, Madison, WI, USA.

Koeppe, D.E., Miller, R.J., 1970. Lead effects on corn mitochondrial respiration. Science 167: 1376-1378.

Koppenol, W.H., 1994. Chemistry of iron and copper in radical reactions. *In*: Free radical damage and its control. New comprehensive biochemistry 28. Rice-Evans, C.A., Burdon, R.H. (eds.). Elsevier, Amsterdam, The Netherlands.

Krugh, B.W., Miles, D., 1996. Monitoring the effects of five "nonherbicidal" pesiticide chemicals on terrestrial plants using chlorophyll fluorescence. Environ. Toxicol. Chem, 15/4: 495-500.

Krupa, Z., Baszynski, T., 1995. Some aspects of heavy metals toxicity towards photosynthetic apparatus – direct and indirect effects on light and dark reactions. Acta Physiol. Plant. 7: 55-64.

Küpper, H, Küpper, F., Spiller, M., 1998. *In situ* detection of heavy metal substituted chlorophylls in water plants. Photosynthesis Res. 58: 123-133.

Lagriffoul, A., Mocquot, B., Mench, M., Vangronsveld, J., 1998. Cadmium toxicity effects on growth, mineral and chlorophyll contents, and activities of stress related enzymes in young maize plants (*Zea mays* L.). Plant Soil 200: 241-250.

Lakanen, E., Ervio, R., 1971. A comparison of eight extractants for the determination of plant available micronutrients in soils. Acta Agr. Fenn. 123: 223-232.

Landrum, P.F., Robbins, J.A., 1990. Bioavailibility of sediment- associated contaminants to benthic invertebrates. *In*: Sediments: chemistry and toxicology of in-place pollutants. Bando R. et al. (eds.). Lewis Publishers, Chelsea, MI, USA.

Laperche, V., Logan, T.J., Gaddam, P., Traina, S.J., 1997. Effect of apatite amendments on plant uptake of lead from contaminated soil. Environ. Sci. Technol. 31: 2745-2753.

Lauwerys, R., 1989. Metals - epidemiological and experimental evidence for carcinogenicity. Arch. Toxicol. 13: 21-27.

Lebourg, A., Sterckeman, T., 1996. Intérêt de différents réactifs d'extraction chimique pour l' évaluation de la biodisponibilité des métaux en trace du sol. Agronomie. 16: 201-215.

Lenphear B.P., Hormung, R., Khoury, J., Yolton, K., Baghurst, P., Bellinger, D.C., Canfield, R.L., Dietrich, K.N., Roberts, R., 2005. Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. Environ. Health Perspect. 113: 894-899.

Lepp, N.W., 1981. Effect of heavy metal pollution on plants. Vol. 1. Effects of trace metals on plant function, vol. 2. Metals in the environment, Applied Science Publishers, Oxford, UK.

Leyval, C., Turnau, K., Haselwandter, K., 1997. Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7: 139-153.

Lindsay, W.L., Norvell, W.A., 1978. Development of a DPTA soil test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J. 42: 421-428.

Lock, K., Janssen, C.R., 2003. Effect of new soil metal immobilizing agents on metal toxicity to terrestrial invertebrates. Environ. Pollut. 121: 123-127.

Loewus, F.A. 1988. Ascorbic acid and its metabolic products. *In*: The biogeochemistry of plants. Vol. 14. Preiss, J. (ed.). Academic Press, New York, USA.

Lombi, E., Hamon, R.E., McGrath, S.P., McLaughlin, M.J., 2003. Lability of Cd, Cu and Zn in polluted soils treated with lime, beringite and red mud and identification of a non-labile colloidal fraction using isotopic dilution techniques. Environ. Sci. Technol. 28: 1219-1228.

Lombi, E., Zhao, F., Wieshammer, H., Zhang, G., McGrath, S.P., 2002b. *In situ* fixation of metals in soils using bauxite residue: Chemical assessment. Environ. Pollut. 118: 445-452.

Lombi, E., Zhao, F., Wieshammer, H., Zhang, G., McGrath, S.P., 2002a. *In situ* fixation of metals in soils using bauxite residue: Biological effects. Environ. Pollut. 118: 435-443.

Lotenbach, B., Krebs, R., Furrer, G., Gupta, S., Schulin, R., 1998. Immobilization of cadmium and zinc in soil by Al-montmorillonite and gravel sludge. Eur. J. Soil. Sci. 49: 141-148.

Lux, H.B., Cumming, J.R., 2001. Mycorrhizae confer aluminium resistance to tulip-poplar seedlings. Canadian Journal of Forest Research-Revue Canadienne de Recherche Forestière 31: 694-702.

Ma, L.Q., Roa, G.N., 1997. Effects of phosphate rock on sequential chemical extraction of lead in contaminated soils. J. Environ. Qual. 26: 788-794.

Macnair, M.R., Tilstone, G.H., Smith, S.E., 2000. The genetics of metal tolerance and accumulation in higher plants. *In*: Phytoremediation of contaminated soil and water Terry, N., Banuelos, G. (eds.). CRC Press, Boca Raton, FL, USA.

Manceau, A., Boisset, M.C., Sarret, G., Hazemann, J.L., Mench, M., Cambier, P., Prost, R., 1996. Direct determination of lead speciation in contaminated soils by EXAFS spectroscopy. Environ. Sci. Technol. 30: 1540-1552.

Marschner, H., 1995. Mineral nutrition of higher plants. Academic Press, London, UK.

Marschner, H., Römheld, V., 1994. Strategies of plants for acquisition of iron. Plant Soil 165: 261-274.

Martin,F., Rubini, P., Cote, R., Kottke, I., 1994. Aluminum polyphosphate complexes in the mycorrhizal basidiomycete *Laccaria bicolor* – a Al-27 nuclear-magnetic-resonance study. Planta 194: 241-246.

Marwood, C.A., Solomon, K.R., Greenberg, B.M., 2001. Chlorophyll fluorescence as a bioindicator of effects on growth in aquatic macrophytes from mixtures of polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 20: 890-891.

Mathis, P., Kleo, J., 1973. The triplet state of β-carotene and of analogous polyenes of different length. Photochem. Photobiol. 18: 343-346.

Mathys, W., 1975. Enzymes of heavy metal-resistant and non-resistant populations of *Silene cucubalus* and their interactions with some heavy metals *in vitro* and *in vivo*. Physiol. Plant. 33: 161-165.

Mattioni, C., Gabbrielli, R., Vangronsveld, J., Clijsters, H., 1997. Nickel and cadmium toxicity and enzymatic activity in Ni-tolerant and non-tolerant populations of *Silene italica* Pers. J. Plant Physiol. 150: 173-177.

McBride, M., 1994. Environmental chemistry of soils. Oxford Univ. Press. New York, USA.

McBride, M.B., 1991. Processes of heavy metal and transition metal sorption by soil minerals. *In*: Interactions at the soil-colloid-soil solution interface. NATO ASI Series, Series E: Applied Sciences. Bolt, G.H. et al. (eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands.

McBride, M.B., Martinez, C.E., 2000. Copper phytotoxicity in a contaminated soil: Remediation tests with adsorptive materials. Environ. Sci. Technol. 34: 4386-4391.

McKenzie, R.M.,1980. The adsorption of lead and other heavy metals on oxides of manganese and iron. Australian Journal of Soil Research. 18:61-73.

Meers, E., Du Laing, G., Vanbroekhoven, K., Geebelen, W., Vandegehuchte, M., Samson, R., Diels, L., Ruttens A., Vangronsveld, J., Tack, F.M.G., Verloo, M.G., 2006a. Comparison of Cd extractability from soils and sediments by commonly used single extraction protocols. Geoderma. *In press*.

Meers, E., Ruttens, A., Geebelen, W., Vangronsveld, J., Samson, R., Vanbroekhoven, K., Vandegehuchte, M., Diels, L., Tack, F.M.G., 2006b. Potential Use of the Plant Antioxidant Network for Environmental Exposure Assessment of Heavy Metals in Soils. Environ. Mon. Assess. *In Press*.

Meharg, A.A., 2003. The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. Mycol. Res. 107: 1253-1265.

Mench M.J., Vangronsveld, J., Didier, V., Clijsters, H., 1994. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. Environ. Pollut. 86: 279-286.

Mench, M., Amans, V., Sappin-Didier, V., Fargues, S. et al., 1997. A study of additives to reduce the availability of Pb in soil to plants. *In*: Remediation of soils contaminated with metals. Iskandar, I.K., Adriano D.C. (eds). Science Reviews, Northwood, USA.

Mench, M., Bussière, S., Boisson, J., Castaing, E., Vangronsveld, J., Ruttens, A., De Koe, T., Assunçao, A., Manceau, A., 2003. Progress in remediation and revegetation of the barren Jales gold mine spoil after in situ treatments. Plant Soil 249: 187-202.

Mench, M., Martin, E., 1991. Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L, *Nicotiana tabacum* L, and *Nicotiana rustica* L. Plant Soil 132: 187-196.

Mench, M., Vangronsveld, J., Beckx, C., Ruttens, A., 2006. Progress in assisted natural remediation of an arsenic contaminated agricultural soil. Environ. Pollut. *In Press*.

Mench, M., Vangronsveld, J., Didier, V., Clijsters, H., 1994b. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. Environ. Pollut. 86: 279-286.

Mench, M., Vangronsveld, J., Lepp, N., Edwards, R., 1998. Physico-chemical aspects and efficiency of trace element immobilization by soil amendments. 151-182 *In:* Metal contaminated soils: *in situ* inactivation and phytorestoration of. Vangronsveld, J., Cunningham, S. (eds.). Springer-Verlag, New York, USA.

Mench, M.J., Vangronsveld, J., Clijsters, H., Lepp, N.W., Edwards, R., 2000. *In situ* metal immobilisation and phytostabilisation of contaminated soils. *In*: Phytoremediation of contaminated soils and water. Terry, N., Banuelos, G. (eds.). Lewis Publ., CRC Press, Boca Raton, FL, USA.

Merian, E., 1991. Metals and their compounds in the environments. Occurance, analysis, and biological relevance. VCH Verlagsgeselschaft mbH, Weinheim, Germany.

Meyer, J.S., 2002. The utility of the terms 'biovailability' and 'bioavailable fraction' for metals. Mar. Environ. Res. 53: 417-423.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410.

Mittler, R., Vanderauwera S., Gollery, M., Van Breusegm F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9: 490-498.

Mocquot, B., Vangronsveld, J., Clijsters, H., Mench, M., 1996. Copper toxicity in young maize (*Zea mays* L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities. Plant Soil 182: 287-300.

Möller, I.M., 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Ann. Rev. Plant Physiol. Plant Mol. Biol 52: 561-591.

Munné-Bosh, S., 2005. The role of α-tocopherol in plant stress tolerance. J. Plant Pysiol. 162: 743-748.

Murphy, A.S., Eisinger, W.R., Shaff, J.E., Kochian, L.V., Taiz, L., 1999. Early copper-induced leakage of K^+ from *Arabidopsis* seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol. 121: 1375-1382.

Mushak, P.J.M., Davis, A.F., Crocetti, A.F., Grant, L.D., 1989. Prenatal and postnatal effects of low level lead exposure: integrated summary of a report to the U.S. Congress on Childhood Lead Poisoning. Environ. Res. 50: 11-36.

National Academy Press, 1990. Ground water and soil contamination remediation: towards compatible science, policy and public perception. NAP, Washington, D.C., USA.

Nawrot, T., Plusquin, M., Hogervorst, J., Roels, H.A., Celis, H., Thijs, L., Vangronsveld, J., Van Hecke, E., Staessen, J.A., 2006. Environmental exposure to cadmium and risk of cancer. Lancet Oncol. 7(2): 119-126.

Nieboer, E., Richardson, D.H.S., 1980. The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. Environ. Pollut. 1: 3-26.

Nies, D.H., 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. 51: 730-750.

Noctor, G., Gomez, L., Vanacker, H., Foyer, C.H., 2002. Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. J. Exp. Bot. 53: 1283-1304.

Novozamsky, I., Lexmond, T.M., Houba, V.J.G., 1993. A single extraction procedure of soil for evaluation of uptake of some metals by plants. Int. J. Environ. Anal. Chem. 51: 47-58.

OECD, 1984a. OECD Guidelines for Testing of Chemicals, N° 208, Terrestrial Plants, Growth Test. OECD publications. Organization for Economic Cooperation and Development. France. Paris.

OECD, 1984b. OECD Guidelines for Testing of Chemicals, N° 207, Earthworm toxicity test. OECD publications. Organization for Economic Cooperation and Development. France. Paris.

Oste, L.A., Lexmond, T.M., Van Riemsdijk, W.H., 2002. Metal immobilization using synthetic zeolites. J. Environ. Qual. 31: 813-821.

Paschke, M.W., Redente, E.F., Levy, D.B., 2000. Zinc toxicity thresholds for important reclamation grass species of the western Unites States. Environ. Toxicol. Chem. 19(11): 2751-2756.

Pierzynski, G.M., Schwab, A.P., 1993. Bioavailability of zinc, cadmium, and lead in a metal contaminated alluvial soil. J. Environ. Qual. 22: 247-254.

Pijnenburg, W.J.G.M., Posthuma, L., Eijsackers, H.P.J., Allen, H.E., 1997. A conceptual framework for implementation of biovailability in Dutch field soils for the Oligochaete *Enchytraeus crypticus*. Ecotoxicol. Environ. Saf. 43: 170-186.

Prassad, D.D.K., Prassad, A.R.K., 1987. Altered delta-aminolevulinic-acid metabolism by lead and mercury in germinating seedlings of Bajra (*Pennisetum thypoideum*) J. Plant Physiol.127: 241-249.

Price, A., Luca, P.W., Lea, P., 1990. Age dependent damage and glutathione metabolism in ozone fumigated barley: a leaf section approach. J. Exp. Bot. 41: 1309-1317.

Pueyo, M., Lopez-Sanchez, J.F., Rauret, G., 2004. Assessment of $CaCl_2$, $NaNO_3$ and NH_4NO_3 extraction procedures for the study of Cd, Cu, Pb and Zn extractability in contaminated soils. Anal. Chim. Acta. 504: 217-226.

Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Quevauviller, P., 1999. Improvement of the BCR three-step sequential extraction procedure prior to the certification of new sediment and soil reference materials. J. Environ. Monit. 1: 57-61.

Raven, P.H., Evert, R.F., Eichhorn, S.E., 1999. Biology of plants. W.H. Freeman and Compagny. Worth Publishers Inc., New York, USA.

Rebedea, I., Lepp, N.W., 1995. The use of synthetic zeolites to reduce plant metal uptake and phytotoxicity in two polluted soils. Environ. Geochem. Health. Special Issue 16: 81-88.

Reddy, A.M., Kumar, S.G., Jyothsnakumari,G., Thimmanaik; S., Sudhakar, C., 2005. Lead induced changes in antioxidant metabolism of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) and bengalgram (*Cicer arietinum* L.). Chemosphere 60: 97-104.

Rellan-Alvarez, R., Ortega-Villasante, C., Alvarez-Fernandez, A., del Campo, F.F., Hernandez, L.E., 2006. Stress responses of *Zea mays* to cadmium and mercury. Plant Soil 279: 41-50.

Rengel, Z., Römheld, V., Marschner, H., 1998. Uptake of zinc and iron by wheat genotypes differing in tolerance to zinc deficiency. J. Plant Physiol. 152: 433-438.

Rennenberg, H., Brunhold, Chr., 1994. Significance of glutathione metabolism in plants under stress. Progr. Bot. 55: 142-155.

Rout, G.R., Das, P., 2003. Effect of metal toxicity on plant growth and metabolism: I. zinc. Agronomie 23: 3-11.

Ruby, M.V., Nicholson, A. 1994. *In situ* formation of lead phosphates in soils as a method to immobilize lead. Environ. Sci. Technol. 28: 646-654.

Ruby, M.V., Schoof, R., Eberle, S., Sellstone, C.M., 1996. Estimation of lead and arsenic biovailability using a physiologically based extraction test. Environ. Sci. Technol. 33(21): 3697-3705.

Ruby, M.V., Schoof, R., Brattin,W., Goldade, M., Post, G., Harnois, M., Mosby, D.E., Casteel, S.W., Berti, W., Carpenter, M., Edwards, D., Cragin, D., Chappel; W., 1999. Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. Environ. Sci. Technol. 33 (21): 3697-3705.

Ruttens, A., Geebelen, W., Vangronsveld, J., 2003. Screening van bodemadditieven in de context van metaalimmobilisatie: samenvatting van de huidige resultaten en situering van de resterende onderzoeksnoden. Studie in opdracht van 'Studiesyndicaat Alternatieve Bodemsanering'. Uitgevoerd door LUC, Diepenbeek, Belgium.

Ryan, J.A., Zhang, P., Hesterbe, D., Chou, J., Sayers, D.E., 2001. Formation of chloropyromorphite in a lead-contaminated soil amended with hydroxyapatite. Environ. Sci. Technol. 35: 3798-3803.

Salt, D.E., Kato, N., Krämer, U., Smith, R.D., Raskin, I., 2000. The role of root exudates in nickel hyperaccumulation and tolerance in accumulator and nonaccumulator species of *Thlaspi*. *In*: Phytoremediation of contaminated soil and water. Terry, N., Banuelos, G. (eds.). CRC Press, Boca Raton, FL, USA.

Sandmann, G., Böger, P., 1983. The enzymatological function of heavy metals and their role in electron transfer processes in plants. *In:* Encyclopedia of plant physiology. New series. 15A. Läuchli, A., Bielski, R.L. (eds.). Springer-Verlag, New York, USA.

Sappin-Didier, V., 1995. Utilisation de composés inorganiques pour diminuer les flux de métaux dans deux agrosystèmes pollués: étude des mécanismes impliqués par l'emploi d'un composé du fer. PhD thesis. Analytical Chemistry and Environment, Bordeaux I University, France.

Sappin-Didier, V., Mench, M., Gomez, A., Lambrot, C., 1997. Use of inorganic amendments for reducing metal bioavailability to ryegrass and tobacco in contaminated soils. *In*: Remediation of soils contaminated with metals. Iskandar, A., Adriano, D.C. (eds.). Science Reviews, Northwood, USA.

Sarret, G., Vangronsveld, J., Manceau, A., Musso, M., D'Haen, J., Menthonnex, J.-J., Hazeman, J.-L., 2001. Accumulation forms of Zn and Pb in Phaseolus vulgaris in the prescence and absence of EDTA. Environ. Sci. Technol. 35: 2854-2859.

Sauvé, S., 1999. Free ion activity model and chemical determinations of metal bioavailability in contaminated soils. Document of a workshop in Vienna, Austria.

Sauvé, S., Cook, N., Hendershot, W.H., McBride M.B., 1996. Linking plant tissue concentrations and soil copper pools in urban contaminated soils. Environ. Pollut. 94: 153-157.

Sauvé, S., Martinez, C.E., McBride, M;, Henderschot, W., 2000. Adsorption of free lead (Pb^{2+}) by pedogenic oxides, ferrihydrite, and leaf compost. Soil Sci. Am. J. 64: 595-599.

Sauvé, S., McBride, M., Hendershot, W., 1998. Soil solution speciation of lead (II): effect of organic matter and pH. Soil Sci. Soc. Am. J. 62:618-621.

Scandalios, J.G., 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz. J. Med. Biol. Res. 38: 995-1014.

Schat, H., Vooijs, R., Kuiper, E., 1996. Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. Evolution 50: 1888-1895.

Schindler, P.W., Sposito, G., 1991. Surface complexation at (hydr)oxide surfaces. *In*: Interactions at the soil-colloid-soil solution interface. Bolt, G.H. et al. (eds.). NATO ASI Series, Series E: Applied Sciences. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schützendübel, A, Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53: 1351-1365.

Seregin, I.V., Ivanov, V.B., 2001. Physiological aspects of cadmium and lead toxic effects on higher plants. Russ. J. Plant Physiol. 48: 523-544.

Sharma, P., Dubey, R.S., 2005. Lead toxicity in plants. Braz. J. Plant. Physiol. 17(1): 35-52.

Shende, A., Juwarkar, A.S., Dara, S.S., 1994. Use of fly ash in reducing heavy metal toxicity to plants. Resour. Conserv. Recycl. 12: 221-228.

Shuman, L.M., Dudka, S., Das, K., 2002. Cadmium forms and plant availability in compost-amended soil. Commun. Soil Sci. Plant Anal. 33: 737-748.

Sims, T., Boswell, F.C., 1978. Effect of bentonite on plant availability of sludge-borne heavy metals in soil. J. Environ. Qual. 7: 501-505.

Skórzyńska-Polit E, Pawlikowska-Pawlega, B., Szczuka E., Drzkiewicz, M., Krupa, Z., 2006. The activity and localization of lipoxygenases in *Arabidopsis thaliana* under cadmium and coppers stresses. Plant Growth Regul. 48: 29-39.

Smeets, K., Cuypers, A., Lambrechts, A., Semane, B., Hoet, P., Van Laere, A., Vangronsveld, J., 2005. Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd-application. Plant Physiol. Biochem. 43: 437-444.

Smirnoff, N., Pallanca, J.E., 1996. Ascorbate metabolism in relation to oxidative stress. Biochem. Soc. Trans. 24: 472-478.

Smolders, E., McLaughlin, M.J., 1996a. Effect of Cl on Cd uptake by Swiss chard in unbuffered and chelator buffered nutrient solutions. Plant Soil. 179: 57-64.

Smolders, E., McLaughlin, M.J., 1996b. Influence of Chloride on Cd availability to Swiss chard: a resin buffred solution culture system. Soil Sci. Soc. Am. J. 60: 1443-1447.

Song, J., Zhao, F.I., McGrath, S.P., Zhang, H., 2004. Copper uptake by *Elsholtzia splendens* and *Silene vulgaris* and assessment of copper phytoavailability in contaminated soils. Environ. Pollut. 128: 307-315.

Sposito, G., 1984. The surface chemistry of soils. Oxford Univ. Press, New York, USA.

Staessen, J.A., Roels, H.A., Emerlianov, T., Thijs, L., Vangronsveld, J., Fagard, R., 1999. Environmental exposure to cadmium, forearm density, and risk of fractures: prospective population study. Lancet. 353: 1140-1144.

Stobart, A.K., Griffiths, W.T., Ameen-Bukhari, I., Sherwood, R.P., 1985. The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley Physiol. Plant. 63: 293-298.

Stumm, W., 1992. Chemistry of the solid-water interface. Processes at the mineral-water and particle-water interface in natural systems. John Wiley & Sons, New York, USA.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51: 844-851.

Torres, M.A., Dangl, J.L., 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol. 8: 397-403.

U.S. EPA, 1996a. Ecological Effects Test Guidelines. Terrestrial Plant Toxicity, Seedling Emergence. U.S. Environmental Protection Agency. EPA 712-C-96-153. OPPTS 850.4100.

U.S. EPA, 1996b. Ecological Effects Test Guidelines. Terrestrial Plant Toxicity, Seed Germination/Root Elongation Toxicity Test. U.S. Environmental Protection Agency. EPA 712-C-96-154. OPPTS 850.4200.

Underwood, E.J., 1977. Trace elements in human and animal nutrition. Academic Press, New York, USA.

Van Assche, F., Cardinaels, D., Clijsters, H., 1988. Induction of enzyme capacity in plants as a result of heavy metal toxicity: dose-response relations in *Phaseolus vulgaris* L., treated with zinc and cadmium. Environ. Pollut. 52: 103-115.

Van Assche, F., Clijsters, H., 1983. Multiple effects of heavy metal toxicity on photosynthesis. *In*: Effects of Stress on Photosynthesis. Marcelle, R. et al. (eds.). Martinus Nijhoff/Dr W. Kunk Publishers, Den Haag, The Netherlands.

Van Assche, F., Clijsters, H., 1986a. Inhibition of photosynthesis in *Phaseolus vulgaris* by treatment with toxic concentrations of zinc – effects on eletron-transport and photophosphorylation. Physiol. Plant. 66: 717-721.

Van Assche, F., Clijsters, H., 1986b. Inhibition of photosynthesis in *Phaseolus vulgaris* by treatment with toxic concentration of zinc – effect on ribulose-1,5-bisphosphate carboxylase oxygenase. J. Plant Physiol. 125:355-360.

Van Assche, F., Clijsters, H., 1990a. A Biological test system for the evaluation of the phytotoxicity of metal-contaminated soils. Environ. Pollut. 66: 157-172.

Van Assche, F., Clijsters, H., 1990b. Effects of metals on enzyme activity in plants. Plant Cell Environ. 13: 195-206.

van der Lelie, D., 1998. Biological Interactions: The Role of Soil Bacteria in the Bioremediation of Heavy Metal-Polluted Soils. *In*: Metal contaminated soils: i*n situ* inactivation and phytorestoration. Vangronsveld, J., Cunningham, S. (eds.). Springer-Verlag, New York, USA.

van der Lelie, D., Schwitzguébel, J.P., Glass, D.J., Vangronsveld, J., Baker, A., 2001. Assessing phytoremediation's progress in the United States and Europe. Environ. Sci. Technol. 35: 447A-452A.

Vangronsveld, J. and Clijsters, H., 1994. Toxic effects of metals. *In*: Plants and the chemical elements. Farago, M.E. (ed.). VCH Verlagsgesellschaft, Weinheim, Germany.

Vangronsveld, J., Clijsters, H., 1992. A biological test system for the evaluation of metal phytotoxicity and immobilization by additives in metal contaminated soils. *In:* Metal compounds in environment and life, 4. Interrelation between chemistry and biology. Merian, E., Haerdi, W. (eds.). Science Reviews Inc., Wilmington, USA.

Vangronsveld, J., Colpaert, J., Van Tichelen, K. 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environ. Pollut. 94: 131-140.

Vangronsveld, J., Cunningham, S., 1998. Introduction to the concepts. *In:* Metal contaminated soil: *in situ* inactivation and phytorestoration. Vangronsveld J., Cunningham S. (eds.). Springer-Verlag, New York, USA.

Vangronsveld, J., Ruttens, A., Clijsters, H., 1999. The use of cyclonic ashes of fluidized bed burning of coal mine refuse for long-term immobilization of metals in soils. *In*: Biogeochemistry of coal and coal combustion byproducts. Sajwan et al. (eds.). Plenum Academic/Plenum Publishers, New York, USA.

Vangronsveld, J., Stercks, J., Van Assche, F., Clijsters, H., 1995b. Rehabilitation studies on an old non-ferrous waste dumping ground: effects of revegetation and metal immobilization by beringite. J. Geochem. Expl. 52: 221-229.

Vangronsveld, J., Van Assche, F., Clijsters, H., 1995a. Reclamation of a bare industrial area contaminated by non-ferrous metals: *in situ* metal immobilization and revegetation. Environ. Pollut. 87: 51-59.

Vassilev, A., Schwitzguébel, J.-P., Thewys, T., van der Lelie, D., Vangronsveld, J., 2004. The use of plants for remediation of metal-contaminated soils. Scientific WorldJournal. 4: 9-34.

Verdoni, N., Mench, M., Bessoule, J.J., Vangronsveld, J., 2001. Fatty acid composition of tomato leaves as biomarker of metal-contaminated soils. Environ. Toxicol. Chem. 20: 382-388.

Verkleij J.A.C., Prast, J.E., 1989. Cadmium tolerance and co-tolerance in Silene vulgaris (Moench) Gareke(Silene cucubalus (L.). New Phytol. 111: 637-645.

Verkleij, J.A.C., Koevoets, P.L.M., Blake-Kalff, M.M.A., Chardonnes, A.N., 1998. Evidence for an important role of the tonoplast in the mechanism of naturally selected zinc tolerance in *Silene vulgaris*. J. Plant Physiol. 153: 188-191.

Vig, K., Megharaj, M., Sethunathan, N., Naidu, R., 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. Adv. Environ. Res. 8: 121-135.

Walter, A., Römheld, V., Marschner, H., 1994. Is the release of phytosiderophores in zinc-deficient wheat plants a response to impaired iron utilization? Physiol. Plant. 92: 493-500.

Wang, J., Evangelou, B.P., Nielsen, M.T., Wagner, G.J., 1992. Computer-simulated evaluation of possible mechanisms for sequestering metal ion activity in plant vacuoles. II. Zinc. Plant Physiol. 99: 621-626.

Wang, W., Freemark, K., 1995. The use of plants for environmental monitoring and assessment .Ecotoxicol. Environ. Saf. 3: 289-301.

Wang, W.-S., Shan, W.-Q., Wen, B., Zhang, S.-Z., 2003. Relationship between the extractable metals from soils and metals taken up by maize roots and shoots. Chemosphere. 53: 523-530.

Weckx, J.E.J., Clijsters, H., 1996. Oxidative damage and defence mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of cpooer. Physiol. Plant. 96: 506-512.

Weckx, J.E.J., Clijsters, H., 1997. Zinc phytotoxicity induces oxidative stress in primary leaves of *Phaseolus vulgaris*. Plant Physiol. Biochem. 35: 405-410.

Wenzel, W.W., Lombi, E., Adriano, D.C., 1999. Biogeochemical processes in the rhizosphere: role in phytoremediation of metal polluted soils. *In*: Heavy metal stress in plants: from molecules to ecosystems. Prasad, M.N.V., Hagemeyer, J. (eds.). Springer, New York.

Winston, GW, 1990 Physiochemical basis for free radical formation in cells: production and defenses. *In:* Responses in plants: adaptation and acclimation mechanisms. Alscher, R.G., Cumming, J.R. (eds.). Wiley-Liss Inc., Wilmington, DE, USA.

Yang, J., Mosby, D., Casteel, S.W., Blanchar, R.W. 2001. Lead immobilization using phosphoric acid in a smelter contaminated urban soil. Environ. Sci. Technol. 35: 3553-3559.

Yruela, I., 2005. Copper in plants. Braz. J. Plant Physiol. 17: 145-156.

Yu, S., He, Z.L., Huang, C.Y., Chen, G.C., Calvert, D.V., 2004. Copper fractionation and extractability in two contaminated variable soils. Geoderma. 123: 163-175.

Zachara, J.M., Smith, S.C., Resch, C.T., Cowan, C.E., 1992. Cadmium sorption to soil separates containing layer silicates and iron and aluminum oxides. Soil Sci. Soc. Am. J. 56: 1074-1084.

Part II

In situ metal immobilization and phytostabilization

CHAPTER 1

Long-term sustainability of metal immobilization by soil additives: cyclonic ashes vs. lime

A. Ruttens, W. Geebelen, M. Mench, R. Carleer and J. Vangronsveld.

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Abstract

A rainfall simulation experiment, based on water leaching in soil columns, was used to gain insight in the long-term metal immobilization behavior of cyclonic ashes - type H (CAH), steel shots (SS) and CAH+SS compared to LIME during a 26-years simulation period. An equal soil pH in the CAH, CAH+SS and LIME treatments at the start of the experiment was provided, to create optimal conditions for the detection, if present, of metal immobilization mechanisms differing from a pH-effect in the CA and CA+SS treatments. Exchangeable soil metal concentrations and plant metal accumulation in soil taken from the 0-25 cm top layer of the columns were evaluated at the start and at the end of the experiment. At regular intervals during the simulation period metal concentrations were determined in soil pore water sampled at a depth of 10 cm and 25 cm from the surface of the soil columns. While soil pH in LIME, CAH and CAH+SS treatments decreased in parallel during the simulation period, the evolution in metal mobility and phytoavailability showed a clearly distinct pattern. A strong reduction in metal immobilizing efficiency in the LIME treatment at the end of the simulation period compared to the start moment, was much less pronounced, or even absent, in the CAH and CAH+SS treatments. Moreover a strong LIME induced increase in Pb pore water concentrations during the first weeks of the experiment, was not present in the CAH treatment. After 26 simulated years plant accumulation of Zn, Cd and Pb after CAH treatment was

still a factor 2.6, 2.0 and 1.4 lower compared to results obtained in the untreated soil. The effect of CAH+SS on Zn and Cd bioavailability was even stronger. Lime and SS reached a maximum reduction factor of 1.4 at that moment. These results do not support former literature data based on acid extractions, or isotopic dilution techniques performed under acidification which suggested that the action mechanism of CAH is the same as LIME. However, they are supported by long term field observations and results of isotopic dilution techniques performed at soil pH.

1.1 Introduction

In the north east of Belgium (Campine area; Dutch: de Kempen) zinc smelters have been active since the end of the 19th century. As a result, large areas in the region are contaminated with metals (mainly Zn, Cd, Pb). In addition to the presence of highly polluted locations in or close to the smelter sites, a diffuse contamination of agricultural land and residential areas exist in the surroundings due to atmospheric metal depositions. By estimation an area of 280km^2 has soil Cd concentrations higher than 1 mg kg^{-1}, together with a few hundreds mg kg^{-1} Zn and Pb (Ide, 1992), Background metal levels in these soils are in the range 0.1-0.5 mg Cd kg^{-1}, 25-70 mg Zn kg^{-1}, and 5-40 mg Pb kg^{-1} (De Temmerman et al., 2003). The soils in large parts of the region have a typical sandy texture and low pH which result in a relatively high soil metal mobility. Emissions have been drastically reduced since the 1970's when a total shift was made from pyrometallurgical to hydrometallurgical process technologies. Historic soil contamination however leads to a continued metal exposure of people and ecosystems in the area (Hogervorst et al., 2006; Nawrot et al., 2006; Staessen et al., 1999). Recently, several local vegetable harvests cultivated for food industry (carrots, scorzenera) have been confiscated by the Belgian Federal

Agency for Food Safety (FAVV) because Cd concentrations in the crops were above consumption limits.

Remediation of metal contaminated land is classically based on engineering approaches such as excavation and landfilling of contaminated soil, *ex situ* purification treatments using soil washing techniques, solidification, vitrification or fixation using physical barriers (caps) (Vangronsveld and Cunningham, 1998). Due to the high costs of these treatments (for cost information see Cunningham et al., 1995, Berti and Cunningham, 1997) in particular when the polluted areas are large, and due to secondary problems such as the difficulty to find suitable soil for replacement in case of excavation, and changes in physico-chemical and biological characteristics of the treated substratum (drastically reducing its further potential to be used as a normal soil) in case of *ex situ* purification (Vangronsveld and Cunningham, 1998) remediation programs world wide often progress slowly and many sites remain untreated. There is a clear need for remediation alternatives which are less expensive and equally protective of human health and the environment.

In situ immobilization of metals at contaminated sites, by means of metal immobilizing soil additives, has been proposed as an attractive, low cost, remediation alternative (Vassilev et al., 2004). Due to their nature, metal immobilizing soil amendments generally are able to enhance geochemical processes such as precipitation, sorption, ion exchange, and redox reactions, which result in a reduced metal mobility and bioavailability (Mench et al., 2000). Subsequently food and feed can be grown more safely, or on more heavily polluted sites a vegetation cover can develop which stabilizes the soil (reduction of wind- and water erosion and reduction of metal leaching to the groundwater). *In situ* immobilization therefore is potentially suited to reduce metal exposure routes in agricultural areas as well as on industrial sites. A potential obstacle of *in situ* immobilization as a remediation method is that only the bioavailable fraction of the contaminants will be treated. From ecological, toxicological and health point of view this should be the most important consideration, but present environmental regulations in many countries are still based on total metal concentrations (van der Lelie et al., 2001). Because of the large dimensions of the contamination in the Kempen area, and the difficulty to apply conventional clean-up methods all over the area, the Flemish government has expressed a fundamental interest in potential applications of alternative remediation technologies, including metal immobilization (Dua and van Geel, 2002).

Liming is the oldest and likely most widely adopted metal immobilizing soil treatment. Its primary incentive is the suppression of Al and Mn phytotoxicity in acidic arable soils, but it can also limit the mobility and plant uptake of other metals (Pierzynski and Schwab, 1993; Chlopechka and Adriano, 1996; Mench at al., 1997). Several reasons have been attributed to the lime-induced immobilization of metals (Bolan et al., 2003; Hammon et al., 2002): increase in negative charges on soil components; formation of hydroxy metal species with strong sorption behavior; precipitation of metals as hydroxides or carbonates; and sequestration due to enhanced microbial activity. A disadvantage associated with liming in the context of *in situ* metal immobilization is the gradual disappearance of its effect in the course of time due to dissolution and leaching of the liming agent, possibly accelerated by acid depositions. This temporary character makes liming inapplicable for phytostabilization purposes, and offers little guarantees for a permanently safe crop production in metal contaminated agricultural areas. Moreover, a stronger immobilization effect than the one obtained by liming may be desirable.

Even if the duration of a satisfactory immobilization treatment is dependent on several parameters, such as, land use, treatments costs and acceptable risks (Mench et al., 2003), the longer the proven durability of a treatment and the stronger the metal binding, the higher the chance that decision makers will be convinced to use it.

Mineral soil amendments (other than lime) that have been studied most intensively for metal immobilization purposes include: P-rich compounds, aluminosilicates (including zeolites, clays and related substances), Fe-rich materials and Mn oxides (see Mench at al., 2000 and references therein; Brown et al., 2004 and references therein). Valuable information about the potential durability of a treatment is obtained when the mechanism responsible for the immobilization process can be determined. Among metal immobilizing soil amendments the best-documented action mechanism is formation of the very stable pyromorphite after addition of P-compounds to Pb-contaminated soils (Ryan et al., 2001). For other soil amendments sorption mechanisms mainly remain hypothetic. Physical (X-ray based) techniques have not (yet) been able to reveal detailed information on metal retention in the treated soils. To estimate the potential durability of most treatments chemical techniques or field observations have been used (see below).

Field experiments the Kempen, have demonstrated that application of cyclonic ashes (CA) from Beringen (Belgium) (formerly called 'Beringite'; a modified alumino-silicate, originating form the fluidized bed burning of coal refuse) resulted in a successful and long term metal immobilization at a former zinc smelter site (Vangronsveld et al., 1995a, 1996). A healthy and sustainable vegetation cover, regenerating by vegetative means and by seeds, established at the formerly barren site with Zn concentrations as high as 13250 mg Zn kg^{-1} (sandy soil; pH-H_2O = 5.5); 12 years after treatment metal mobility continued to be low, no signs of phytotoxicity were observed (Ruttens et al., unpublished results) and nematode fauna was still improving (Bouwman and Vangronsveld, 2004). Due to the closure of the fluidized bed at the former coalmine in Beringen, the cyclonic ashes from Beringen are not longer available. Alternative ashes (cyclonic ashes - type H, CAH), resembling the ones from Beringen (and selected based on similarities in parent material and oven conditions), are currently under investigation (Geebelen et al., 2006).

The long-term results of cyclonic ashes mentioned above are in contrast with results of acid extractions and isotopic dilution experiments performed upon acidification, which suggest that the active mechanism of cyclonic ashes does not differ from that of lime (irreversible fixation at constant pH) (Geebelen et al., 2006; Lombi et al., 2002, 2003). When soil solution was acidified to a pH of 5, isotopic exchange studies showed that reductions in metal exchangeability induced by cyclonic ashes disappeared to a similar extent as reductions induced by lime (Geebelen et al., 2006; Lombi et al., 2003).

On the other hand, the isotopic dilution technique, when performed at soil pH, revealed differences in the speed of Cd exchange between soil solution and the soil solid phase in soils treated with cyclonic ashes compared to lime treated soils (Geebelen et al., 2006). This observation demonstrates a different strength in metal binding of both amendments and the existence of additional action mechanisms in case of cyclonic ashes, which, however, clearly disappear under conditions of strong acidification. The long term effectivity of cyclonic ashes observed in field conditions raise the question whether or not the additional immobilization mechanisms shown by Geebelen et al. (2006) may be sufficient to obtain a difference in durability of the effect of cyclonic ashes compared to lime in field conditions, where acidification is generally slow and mild in comparison with the conditions imposed in laboratory experiments cited.

The aim of the present study was therefore to gain additional insight in the long-term immobilization behavior of the alternative ashes (CAH) in comparison with lime. To this end a rainfall simulation experiment was performed using soil columns. Metal mobility and bioavailability in untreated and treated soils were evaluated at different moment throughout the simulation period. Exchangeable soil metal concentrations, plant metal uptake (*Lolium perenne*), soil pore water metal concentrations, and pH evolutions were measured. In addition to the CAH and LIME treatments, steel shots (SS) (Mench et al. 1998) and a combined treatment of CAH+SS were included in the comparison. Steel shots are an industrial material intended for shaping metal surfaces prior to coating. They consist mainly of iron (Fe°) and contain native impurities such as Mn. Several studies have reported an improved efficiency of the combined application of CAH+SS compared to the single treatments (Boisson et al., 1999b; Ruttens et al., 2006a,b) (Chapter 2, Chapter 3). Summarizing, the following treatments were applied for comparison of their long-term effects: LIME, CAH, SS, CAH+SS.

1.2 Materials and Methods

1.2.1 Collection and treatment of soil samples

The soil for the experiment (1000 kg in total) was collected from the top-soil layer (0-25 cm) of a former agricultural field in Balen (Belgium), situated 500 m north east from a zinc smelter site. The sandy soil (86% sand, 9% silt, 5% clay; determined according to Meers et al., 2006) is contaminated with metals due to atmospheric depositions from the zinc smelter. Total metal concentrations (*aqua regia*) are: 226±35 mg Zn kg^{-1}, 6.3±0.4 mg Cd kg^{-1}, 240±12 mg Pb kg^{-1}, and pH-KCl=4.3±0.2. Cadmium and Pb concentrations in the soil are above Flemish clean-up values for agricultural soils, which are set at 2 mg Cd kg^{-1} and 200 mg Pb kg^{-1} (Belgisch Staatsblad, 1996). Amendments were added to the soil using a concrete mixer to obtain the following treatments (3 replicates per treatment): untreated soil [UNT], cyclonic ashes [CAH, 5% (w/w)], steel shots [SS, 1% (w/w)], CAH 5% + SS 1%, and LIME [0.43 % (w/w)]. Application rates of CAH and SS were chosen based on previous studies showing an optimal effect at theses doses (Mench et al., 1998; Vangronsveld; unpublished results). A preliminary experiment, was performed to examine which LIME dose led to the same pH as the CAH treatment (based on a calibration line with increasing lime doses). The use of similar pH levels for both treatments seemed the best option to allow the detection (if present) of mechanisms other than pH increase in the CAH treatments. The lime used was a seaweed lime (maerl) (DCM, Grobbendonk, Belgium)[acid binding value=50, grain size <0.16mm (90%)].

1.2.2 Construction and functionality of soil columns

PVC columns with a diameter of 25 cm and a total height of 1 m were filled with 45 kg treated or untreated soil (3 columns/treatment; soil moisture content 20% w/w). After an equilibration period of 4 weeks, a yearly amount of rainfall of 600 mm was simulated each week by dropping distilled water on the top of the columns (Figure 1.1). One experimental week therefore corresponded to one simulated year. The total experimental period lasted 26 weeks (26 simulated years).

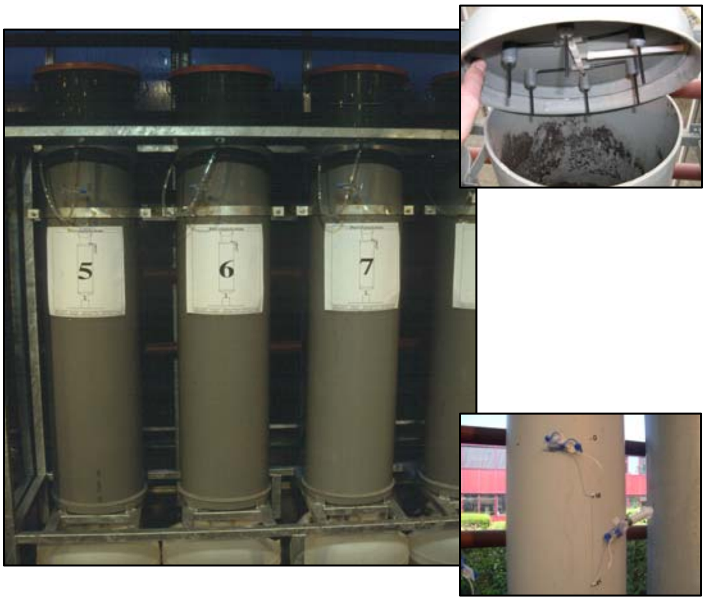


Figure 1.1 Picture of soil columns with a detail of the rainfall simulation system and pore water samplers.

At the start of the experiment, 2 kg soil of each column was set aside for determination of soil parameters (paragraph 1.2.3) and plant metal accumulation (paragraph 1.2.4). At the end of the 26 years simulation period soil and plant measurements were repeated on samples collected from the upper soil layer (25 cm) of the columns. At a soil depth of 10 cm and 25 cm rhizon pore water samplers (Eijkelkamp Agrisearch, MOM type, 10 cm) penetrated the columns. Water samples were collected from these samplers and analysed for metals after the second week and later on every 4 weeks. Induced Coupled Plasmaspectrometry (ICP, Perkin Elmer, Optima 3000 DV) or graphite-furnace Atomic Absorption Spectroscopy (AAS, Perkin Elmer, 5100) were used for the measurements depending on the element and the concentration.

1.2.3 Soil characteristics

Total metal concentrations (*aqua regia*) were determined upon digestion of 500 mg air-dried soil in 4 ml *aqua regia* using a microwave (Milestone, 1200 MEGA). Exchangeable soil metal contents of the untreated and treated soils were determined using extractions with $Ca(NO_3)_2$ (0.1 M, soil solution ratio 1:5, 2 h shaking at 65 rpm, filtration through Whatman filter paper n°40). Soil pH (in 1M KCl) was determined in a 10:25 soil:solution ratio after shaking for 2h (65 rpm) and after filtration through a paper filter (Whatman n°1). A standard pH meter (Radiometer, PHM82) and glass electrode (Hamilton, LiqPlast) was used for the measurements after calibration with pH 4.0 and pH 7.0 buffer solutions.

1.2.4 Plant metal accumulation

Lolium perenne L. (cv. Vigor) was cultivated on untreated and treated soil at the start and at the end of the experimental period to evaluate evolutions in plant metal accumulation. Seeds were sown in 500 ml polyethylene pots (1 g seeds/pot; 3 pots/treatment) and plants were grown for 5 weeks in a greenhouse at 20°C. Soils were watered daily with distilled water to maintain the initial soil moisture content (20% w/w), by adjusting their weight on a balance (450 g/pot). At harvest, above ground biomass was cut at 1 cm above the soil surface and plant weight was recorded per pot. Plants were washed (3 times) with distilled water and dried in the oven (60°C). Aliquots of 150 mg dry plant material were wet digested in hot HNO_3 (70-71%). Blanks and certified reference material (trace elements in spinach, n° 1570a of the National Institute of Standards and Technology, U.S Department of Commerce) were included for quality control of the data. Metals in the extracts were analyzed by Induced Coupled Plasmaspectrometry (ICP, Perkin Elmer, Optima 3000 DV).

1.2.5 Statistical analysis

Two-way factor ANOVA was used to detect effects of treatment, time and possible interactions effects, using the proc GLM statement is SAS (SAS, 2004). Normality of residuals as well as their heteroscedasticity was confirmed graphically. Results of formal tests were used as an additional check. Tukey multiple comparison procedure was used to separate the means.

1.3 Results

1.3.1 Soil pH

At the start of the experiment all treatments led to an increase in soil pH compared to the untreated soil (Figure 1.2). The effect was the strongest for LIME, CAH and CAH+SS treatments, of which the resulting pH did not differ significantly (±2.5 units increase). A smaller pH-effect was observed when SS was applied alone (1 unit increase). While the pH of the untreated soil did not change significantly between both moments, the pH of the treated soils decreased significantly. Decreasing trends were similar for the lime, CAH and CAH+SS (±1.5 à 2 units decrease) and the resulting pHs were still equal at the end of the 26 weeks simulation period. Soil pH of the SS treatment slightly decreased in time and at the end of the experiment the pH did not differ anymore from the untreated soil.

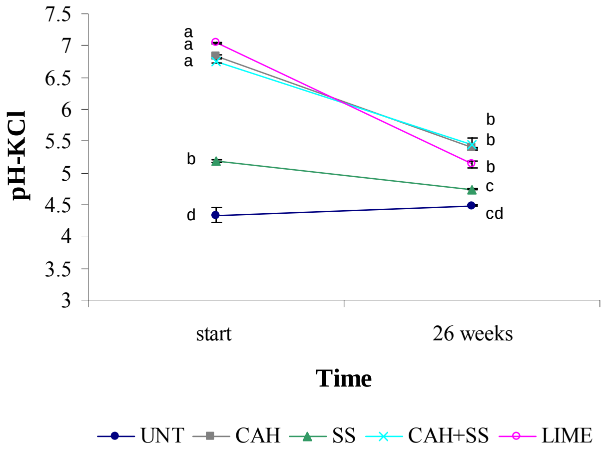


Figure 1.2 Soil pH of the 0-25 cm top-soil layer at the start and at the end of the simulation experiment. Data followed by a different letter differ significantly at the 95% level (Tukey multiple comparison test). Data indicate mean ± standard error of 3 replicates**.**

1.3.2 Exchangeable soil metal contents

All treatments led to a significant decrease in exchangeable Zn and Cd concentrations compared to the untreated soils, both at the start and at the end of the experiment. The resulting concentrations were in the order CAH+SS<CAH<LIME<SS (LIME=SS for Zn after 26 weeks) (Figure 1.2). Increases in exchangeable metal concentrations were observed for all treatments with increasing simulation time. However, after 26 simulated years CAH and CAH+SS still reduced exchangeable Zn concentrations respectively with a factor of ±4 and ±6 compared to the untreated soil. Concentrations after LIME and SS treatment on the contrary were only a factor 1.5 lower compared to concentrations in the untreated soil. Reductions in exchangeable Cd were slightly smaller than reductions in exchangeable Zn. Exchangeable Pb concentrations at the start of the experiment were 3.6±0.01 mg kg^{-1} in the untreated soils and reduced to values below detection limit (<0.5 mg Pb kg^{-1}) in all treated soils (data not presented). At the end of the experiment exchangeable Pb in the untreated soil was still the same but detectable Pb levels were observed in the SS and the LIME treatment (1.1 and 1.0 mg Pb kg^{-1} respectively) illustrating a reduced Pb immobilization in these treatments. In the CAH and CAH+SS treatment exchangeable Pb remained below detection limit at the end the experiment. Exchangeable soil metal levels after soil treatment thus seemed to follow the same trends for all metals (Zn, Cd, Pb).

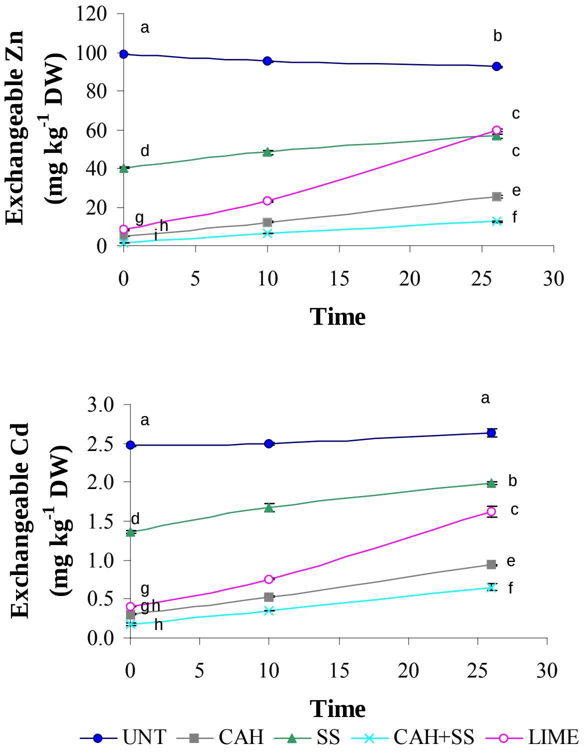


Figure 1.3 $Ca(NO_3)_2$ exchangeable Zn and Cd contents measured in the 0-25 cm top-soil layer of the soil columns at the start and the end of the experiment (26 simulated years) and an intermediate moment (10 simulated years). Data indicate mean ± standard error of 3 replicates. A two-factor ANOVA was performed to compare start and end-moment ($p_{interaction}$ < 0.001). Data followed by a different letter differ significantly at the 95% level (Tukey multiple comparison test). DW=dry weight, UNT=untreated, CAH=cyclonic ashes, SS= steel shots.

1.3.3 Plant metal accumulation

Corresponding to the results of the $Ca(NO_3)_2$ extractions, significant reductions in plant metal accumulation were observed after all treatments (Figure 1.1.3).

At the start of the experiment the reductions in Zn and Cd accumulation were the strongest for LIME=CAH=CAH+SS (no significant differences among these treatments). The effect of SS was smaller, and at the end of the simulation period Cd accumulation in these plants was the same as in plants grown on the untreated soil. Zn and Cd plant accumulation in SS and LIME treatments showed a strong increase after 26 simulated years compared to the start of the experiment. In contrast, no change in Zn and Cd accumulation was observed in CAH and CAH+SS treatments. These additives were able to maintain a low plant metal uptake even after a simulated period of 26 years. In week 26 plant Zn concentrations were a factor 2.6 (for CAH) and 3.6 (for CAH+SS) lower than in the UNT, while LIME and SS only reached a reduction factor of 1.4. Final reductions in plant Cd concentration were a factor 2 and 3 for CAH and CAH+SS respectively. In plants grown on the untreated soil Zn and Cd accumulation slightly reduced between both moments.

In comparison with Zn and Cd, plant Pb accumulation demonstrated different effects of time and amendments. At the start of the experiment, SS and CAH resulted in a similar, but weak, reduction of plant Pb accumulation (factor 1.4 reduction compared to UNT). Reductions by LIME and CAH+SS were stronger (factor 2.2) but did not reach the efficiency observed for Zn and Cd. Despite the relatively strong effect of LIME on plant Pb accumulation at the start, Pb concentration in plant of the LIME treatment increased in week 26 relative to the other amendments. At the end of the simulation period plant Pb concentrations in the LIME, CAH and SS treatment were not significantly lower than in the UNT. Only CAH+SS was able to maintain a reasonable reduction in Pb accumulation throughout the experiment (± factor 2.2 reduction). With the exception of LIME, Pb accumulation in all treatments, including UNT, was lower after 26 weeks than at the start of the experiment. This effect may be caused by a speciation change of Pb in the course of time.

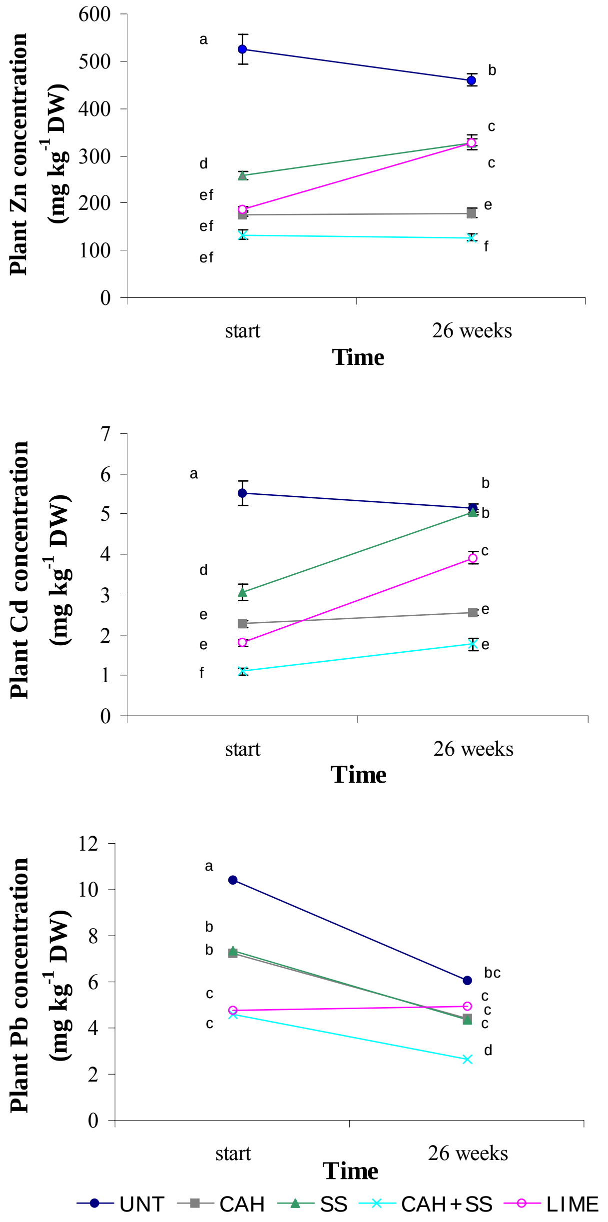


Figure 1.4 Accumulation of Zn, Cd and Pb in *Lolium perenne* (cv. Vigor) grown for 5 weeks in untreated and treated soils from the soil columns and the start of the experiment and after a simulation period of 26 years (0-25 cm top-soil layer). Data indicate mean ± standard error of 3 replicates. Data followed by a different letter differ significantly at the 95% level (Tukey multiple comparison test). DW= dry weight, UNT= untreated, CAH=cyclonic ashes, SS= steel shots.

1.3.4 Leachate metal concentrations

Zinc and Cd concentrations in the soil pore water collected at 10 cm and 25 cm soil depth in the columns showed a 'cyclic' pattern. For all treatments the highest Zn and Cd concentrations were observed after 10 weeks and 22 weeks of leaching, while the curves were at their minimum after 6 and 14 weeks (Figure 1.5). The cyclic variations were the strongest in the untreated soil. In order to allow a statistical analysis of treatment and time effects in the presence of these fluctuating metal concentrations, a two factor ANOVA analysis followed by the Tukey multiple comparison procedure, was performed at the two moments were metal concentrations reached a maximum, i.e after 10 weeks and 22 weeks of water percolation. All treatments led to a significant reduction in Zn and Cd pore water concentrations compared to the UNT, at both depths. At the first (statistically) evaluated moment (10 weeks) Zn and Cd concentrations at a depth of 25 cm were in the order UNT > SS $\geq$ LIME > CAH > CAH+SS. Reductions were the strongest after CAH+SS treatment. Whereas Zn and Cd concentrations after CAH+SS and CAH did not change between moments, an increase of their concentration was found in the LIME and SS treatment. This time trend did not change the relative order of soil treatments, but increased the difference between LIME and SS on the one hand and CAH and CAH+SS on the other hand. Also in the untreated soil Zn and Cd mobility significantly increased between both moments. Similar trends were observed at 10 cm, but due to slightly higher standard errors at this depth, less significant differences were observed among treatments at a fixed moment (Figure 1.5).

Zn concentrations after CAH+SS, the best treatment in this study, dropped at all moments below the level of our detection limit (<50 µg L^{-1}). At a depth of 25 cm (=standard treatment depth *in situ*) Zn concentrations in week 10 after CAH+SS treatment were >9.8 times lower than concentrations in the untreated. The reduction increased to >11.0 times after 22 weeks. Cd pore water concentrations at the same depth reduced with a factor 8.9 and 12.8 compared to the untreated in week 10 and week 22 respectively. In contrast, reduction factor induced by the LIME only ranged from 4.6 to 3.8, and from 2.6 to 2.0, for Zn and Cd respectively. CAH showed an intermediate behavior, with reductions ranging from a factor 7.2 to 9.4 for Zn and from 3.9 to 5.8 for Cd.

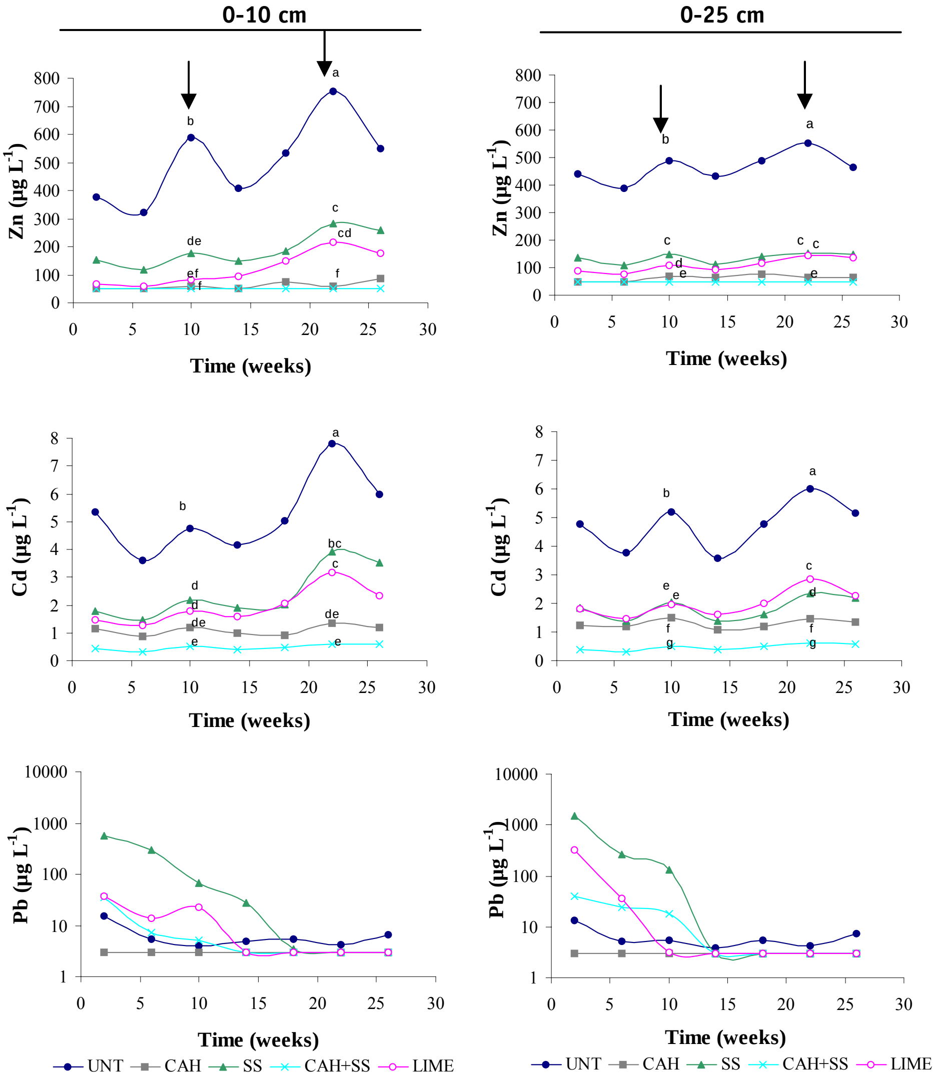


Figure 1.5 Zn, Cd and Pb concentrations in soil pore water collected at different moments on a depth of 10 cm (left graphs) and 25 cm (right graphs) in the soil columns. CAH= cyclonic ashes, SS= steel shots. Arrows indicate moments of statistical comparisons (two-factor ANOVA, $p_{interaction}<0.001$). Data followed by the same letter are not statistical significantly different at the 95% level (Tukey multiple comparison test). Values below dl (<50µg Zn L^{-1}, <3µg Pb L^{-1}) are presented as the value of the dl.

In contrast with reductions in Zn and Cd mobility, Pb mobility during the first 14 weeks of the experiment increased after all treatments (except CAH) at 25 cm soil depth (Figure 1.5). In the water samples collected at 10 cm depth, increases in Pb mobility during this period were smaller (lime, SS) or even absent (CAH+SS). During the last weeks of the experiment Pb concentrations after all treatments were below the level of the untreated soil, and even below detection limit (<3µg L^{-1}).

1.4 Discussion

1.4.1 Soil pH

As a general rule, soil pH is considered to drop with one unit over a period of 10 years in field conditions, but specific trends are soil and location dependent. Römkens and de Vries (1995) reported a top-soil pH decrease from 6 to 4 within 20 to 30 years after land use conversion to forest in the Netherlands. The pH decrease in the lime treatment observed in the present experiment (2 units decrease after a simulated rainfall period of 26 years; Figure 1.2), is well in line with these results. The time scale of the simulation experiment, at least from this point of view, therefore seems to correspond to real-life conditions.

1.4.2 Exchangeable soil metal contents and plant metal accumulation

Corresponding to the decrease in pH of the LIME treatment with increasing simulation time (Figure 1.2), a reduction in metal retention was observed as illustrated by exchangeable metal concentrations as well as plant metal accumulation (Figure 1.3, Figure 1.4). This agrees with general expectations because the metal immobilizing effect of LIME entirely results from its high pH (Paragraph 1.1). In contrast, despite a similar pH decrease in CAH treated soil, a strong metal immobilization in this treatment was maintained till the end of the simulation period (Figure 1.3, 1.4). This strongly suggests that mechanisms, different from a 'liming-effect, are involved in metal binding by CAH in this metal contaminated acid sandy soil. These results are in contrast with previous studies, using aced extraction and isotopic dilution techniques, reporting that the active immobilization mechanism of cyclonic ashes should be the same as lime (Geebelen et al., 2006; Lombi et al., 2002)

Without being able to unravel all underlying immobilization mechanisms, we hypothesize that in addition to the liming effect the following mechanisms are likely responsible for the observed metal retention:

1. addition of clay minerals to the sandy soil increases the metal sorption capacity of the soil (clay size particles have a large surface available for sorption reactions); cation exchange and specific adsorption reactions are likely to occur.
2. the newly introduced clay particles may act as surfaces for precipitation of Fe, Al or Mn oxides, which in turn increase the sorption capacity of the soil. Moreover metals may co-precipitate with these oxides (Alloway, 1995). In particular in acid soils where Fe, Al and Mn tend to be very mobile (Chapter 4) this phenomenon might be important.
3. interactions between new or existing mineral surfaces and organic material, in turn influencing metal sorption behavior, can not be ruled out.

These hypotheses suggest that the specific characteristics of this soil (sandy texture + acid pH) assisted an efficient action of the cyclonic ashes. A different immobilization potential may be observed in soils with different physico-chemical characteristics. Differences in amendment efficiencies among soil types have been reported previously (Geebelen et al., 2003; Lombi et al., 2002).

Next to soil characteristics, other factors may also contribute to the observed differences in immobilization behavior of cyclonic ashes among studies. A factor possibly of importance for example is a differential stabilization time of the amendments in the soil. Lombi et al. (2002) reported a stabilization period of 1 month prior to analysis, while the present study integrates effects over at least 7 months (1 month stabilization time + 6 months during experiment). The difference in immobilization behavior between CAH and LIME in isotopic dilution experiments performed at soil pH (Geebelen et al., 2006) was found in soils that were left equilibrating for 6 months (Geebelen et al., personnel communication). Several studies have demonstrated that metal sorption on soil constituents (silicates, oxides, carbonates, organic matter,...) displays an ageing effect which usually causes metal solubility and extractability to decrease with time (Swift and McLaren, 1991). Metal diffusion into minerals (such as goethite, Mn oxides, illites, smectites, ...) has been reported (Brummer, 1986; Alloway, 1995). Therefore, short-term sorption experiments tend to over-estimate the ultimate solubility of metals in soils (McBride et al., 1997). A possible involvement of diffusion reactions in the observed metal immobilization behavior of CAH in this study may be suggested by the differential retention of Zn and Cd (weaker retention of Cd). Brümmer (1986) reported decreases in the maximum relative diffusion rate of Ni, Zn and Cd in goethite, with increasing ionic radius of the elements. If a similar tendency is true for other minerals, the higher ionic radius of Cd (0.97 Å) compared to Zn (0.74 Å) may explain the stronger retention of Zn compared to Cd. Also in soils only a limited fixation of Cd is often observed (Smolders et al., 1999). In previous studies with cyclonic ashes, results from chemical extractions argued against the existence of such metal diffusion phenomena. Acid digestions (Lombi et al., 2002) or other acid extractions (Lombi et al., 2002; Geebelen et al., 2003; 2006) always eliminated metal binding in cyclonic ashes treated soils.

Another factor possibly contributing to differences in immobilization behavior observed among studies, is the origin of the cyclonic ashes. In column simulation studies, similar to the ones in the present study, but performed with cyclonic ashes from Beringen (Vangronsveld et al, 1999) $Ca(NO_3)_2$ exchangeable soil Zn and Cd contents in CA treated soils did not change between the start and the end of the experimental period, which already suggests a more durable metal retention by the cyclonic ashes from Beringen compared to the CAH used in this study. Correspondingly, differences can be expected between the cyclonic ashes used by Lombi et al. (2002) and the one in the present study.

Whatever the ultimate action mechanism, our results clearly demonstrate that under conditions of mild acidification (pH-KCl>5.5), representative for field conditions, application of CAH to a metal contaminated acid sandy soil resulted in a more durable metal immobilization compared to LIME. These results are in line with the results obtained by Geebelen et al. (2006) using isotopic exchange kinetics at soil pH.

Soil treatment with SS initially led to the weakest reductions in exchangeable metal content and plant metal concentrations in comparison with the other amendments. This can at least partly be explained by the limited pH effect of this treatment (Figure 1.1), which makes its action completely dependent on other sorption mechanisms. Surprising, however, is the observed reduction in immobilization by steel shots with increasing simulation time (Figure 1.3, Figure 1.4). Due to the formation of Fe- and Mn oxides upon SS addition to the soil, which are, in turn, responsible for metal sorption and potential co-precipitation of metals (Mench et al., 1998), application of Fe-bearing materials is expected to result in a durable immobilization (Lombi et al., 2002). On the other hand oxides are not particularly strong adsorbers of metals in the acid pH range (Mc Bride et al., 1997) which may explain the limited efficiency of SS in the present soil (pH-KCl 4.3). This observation again illustrates the soil type dependency of immobilization results.

Across all tests the combination of CAH+SS was the most efficient treatment. Potential mechanisms explaining the strong metal immobilizing capacity of this treatment were discussed in Chapter 2 (Ruttens et al., 2006a).

1.4.3 Leacheate metal concentrations

Cyclic patterns in Zn and Cd mobility as demonstrated in Figure 1.5, were also reported by Ahlberg et al. (2006) for several metals, including Zn and Cd. In their study, plastic columns (30 cm diameter) were placed outside under natural climatologic conditions to study leaching of metals in sewage sludge. The authors found no correlations between leachate metal concentrations and temperature, precipitation or pH of precipitation, nor a correlation with the size of the released particles. Our results confirm the presence of such cyclic behavior of Zn and Cd mobility in a smelter contaminated soil. For Pb no such cyclic pattern was observed in either of the studies.

Reductions of Zn and Cd concentrations in soil pore water showed similar trends among treatments as observed for $Ca(NO_3)_2$ exchangeable contents and plant accumulation. The increased Pb concentrations in soil pore water at the start of the experiment in LIME, SS and CAH+SS treatment, on the contrary, were not reflected in plant Pb accumulation. In case of the LIME treatment, we assume that the raise in pH led to increased DOC concentrations (Dissolved Organic Carbon), and thereby induced the presence of soluble organo-Pb complexes. Increases in DOC upon increases of soil pH have been reported regularly, and complexation reactions with organic matter are well known to control the solubility of Pb (Sauvé et al., 1998). These complexes do not appear to be available for plant uptake. A strong Pb sorption by CAH likely explains why no increases in Pb mobility were observed in this treatment. In case of SS and

CAH+SS, a high mobile fraction of Pb originating from the steel shots themselves may have contributed to the effect (Ruttens et al., 2006a,b) (Chapter 2).

1.5 Conclusions

In a column experiment, simulating 26 years of rainfall, application of CAH resulted in a more durable metal immobilization compared to LIME. Soil pH was similar in both treatments and reduced in parallel with increasing simulation time, but a strong increase in time of metal mobility and bioavailability present in the LIME treatment, was not observed for CAH. Moreover, even in the first weeks after application, reductions in exchangeable Zn and Cd fractions and reductions in soil pore water concentrations were stronger in the CAH treatment compared to the LIME treatment. A mobilization of Pb observed in the LIME treatment during the first weeks of the experiment was not present after CAH application.

When steel shots were applied in combination with the ashes, the strongest reductions in metal mobility and bioavailability were observed, while the durability of the effect was similar to CAH alone. Previous studies have illustrated and discussed the advantages of the combined CA+SS treatment (Ruttens et al., 2006a,b ; Mench et al., 2003, 2006) (Chapter 2).

CHAPTER 2

Phytostabilization of a metal contaminated sandy soil: I. Influence of compost and/or inorganic metal immobilizing soil amendments on phytotoxicity and plant availability of metals

A. Ruttens, M. Mench., J.V. Colpaert, J. Boisson , R. Carleer and J. Vangronsveld.

Environmental Pollution. Article *In Press*.

Abstract

In a lysimeter set-up, compost addition to an industrial contaminated soil slightly reduced phytotoxicity to bean seedlings. The 'Phytotoxicity Index' (on a scale from 1 to 4) decreased from 3.5 to 2.8. The same treatment also reduced metal accumulation in grasses: mean Zn, Cd and Pb concentrations decreased respectively from 623 to 135, from 6.2 to 1.3 and from 10.7 to <6 mg kg^{-1} dry weight. When combined with inorganic metal immobilizing amendments, compost had a beneficial effect on plant responses additional to the inorganic amendments alone. Best results were obtained when using compost (C) + cyclonic ashes (CA) + steel shots (SS). The 'Phytotoxicity Index' decreased to 1.7, the highest diversity of spontaneously colonizing plants occurred, and metal accumulation in grasses reduced to values for uncontaminated soils. Based on the first year evaluation, C+CA+SS showed to be an efficient treatment for amendment assisted phytostabilization of the contaminated Overpelt soil.

2.1 Introduction

Metal-contaminated soils contribute to human and animal metal exposure, through food chain transfer, inhalation of wind blown dust, or direct ingestion of soil (Pierzynski, 1997). At the most severely contaminated sites, where biodiversity is extremely reduced, and plant growth is strongly inhibited, there is a significant risk of off-site migration of the contaminated soil and leaching of contaminants into the groundwater (Vangronsveld and Cunningham, 1998). This dispersion of metals increases the likelihood of human and animal exposure. In response to a growing concern for human health and environmental quality, many technologies have been developed to treat and remediate metal-contaminated soils, in an attempt to replace or complement the very expensive and invasive excavation and soil washing scenarios (Cunningham et al., 1995). One of the remediation options gaining considerable interest over the last decade is the *in situ* immobilization of metals using metal immobilizing agents (van der Lelie et al., 2001; Vangronsveld and Cunningham, 1998). In contrast to the more classical remediation techniques such as solidification, vitrification, or soil washing (Iskandar and Adriano, 1997), in situ immobilization techniques aim to reduce contaminant exposure, without being destructive to structure and biological activity of soils. Moreover no by-products are generated. *In situ* metal immobilization can be used in combination with phytostabilization approaches. Sorption or precipitation reactions induced by the soil amendments decrease the concentration of contaminant mobile pools in the amended soil. A corresponding reduction in the plant available metal fraction allows revegetation and ecosystem restoration on heavily contaminated sites. The vegetation reduces or even prevents the dispersion of the contamination through wind and water erosion, and improves the aesthetic value of formerly bare areas (Vangronsveld and Cunningham, 1998). The vegetation itself may contribute to metal immobilization processes through biological activities and the production of organic matter (Bouwman, 2004).

Cyclonic ashes are described as modified aluminosilicates originating from the fluidized bed burning of coal refuse. The ashes used in this study, are alternatively known as 'beringite' and were produced at the former coal mine in Beringen (Belgium) (Vangronsveld et al., 1999). These ashes have been successfully applied to reduce metal mobility and phytotoxicity in metal contaminated soils in greenhouse and field experiments (Vangronsveld and Clijsters, 1992; Vangronsveld et al., 1995a). A healthy and sustainable vegetation cover established at an abandoned barren zinc smelter site with soil metal contents up to 13250 mg Zn kg^{-1} (sandy soil; pH-H_2O = 5.5) (Bouwman et al., 2001; Vangronsveld et al. 1999). Twelve years after treatment the nematode fauna was still improving (Bouwman and Vangronsveld, 2004); metal availability remained low, and no increase of phytotoxicity was observed (Ruttens et al., unpublished results). Several other studies have indicated that steel shots are an effective treatment to reduce metal mobility and its accumulation in crops (Mench et al. 1994, 1997, 2003; Sappin Didier et al., 1997). Steel shots (iron grit) are an industrial, iron bearing material (97% α-Fe) intended for shaping surfaces (Mench et al., 1998). Basically the working mechanism of steel shots is based on Fe and Mn release, followed by the formation of Fe and Mn oxides, and sorption and/or co-precipitation reactions of metals with these oxides; the action mechanism of cyclonic ashes is based on alkaline properties, sorption on clay particles and (co-)precipitation reactions (Boisson et al. 1999b; Mench et al., 1998).

The present study evaluates the potential of the combined application of cyclonic ashes and steel shots (CA+SS) to phytostabilize a contaminated substrate located at an old Zn smelter site in Overpelt (Belgium). Since 1993, only secondary Zn production occurred at this plant. Boisson et al. (1999b) have shown, based on chemical extractions with $Ca(NO_3)_2$, that the combined use of cyclonic ashes and steel shots increased the metal-immobilizing capacity of these amendments compared to the application of each of the compounds separately. These authors investigated 6 contaminated soils of different origin, among them also a soil from the site in Overpelt, which is subject of the present study (OV1). The combination of CA (5% w/w) + SS (1% w/w) generally reduced exchangeable fractions of Zn, Cu, and Ni more than treatment with phosphates applied as hydroxyapatite (HA 5% w/w). Cadmium and Pb exchangeability on the contrary were reduced more by HA (5%) than by CA (5%) + SS (1%). For all these elements, CA (5%) + SS (1%) was more efficient than HA (1%). The CA+SS treatment had the advantage that it also diminished water extractable As concentrations, whereas this concentration was strongly increased after HA treatment. Meanwhile other studies have confirmed the effectiveness of the combined CA+SS treatment (Geebelen et al., 2003; Mench et al., 2003), which is particularly promising to remediate mixed arsenic and metal contaminated soils. The use of combinations of treatments has been shown to be effective also in other studies (Brown et al., 2004; Hettiarachchi and Pierzynski, 2002; Hettiarachchi et al., 2000).

The soil in Overpelt has a sandy texture and is, like many other industrial sites, characterized by a low fertility and a low water-holding capacity (WHC), which may hamper revegetation success. Soil fertility and WHC can be ameliorated by compost addition (Brady, 1984). Organic matter additions may also increase soil pH and add exchange capacity which both contribute to a decrease of metal availability. Different types of organic matter have been evaluated for their capacity to immobilize metals (Basta et al., 2001; Berti and Cunningham, 1997; Brown et al., 2003a, 2003b; McBride and Martinez, 2000; Shuman et al., 2002).

On the other hand, organic matter additions can increase the amount of soluble organic ligands and eventually lead to the opposite effect (Martinez and Mc. Bride, 1999; Shuman, 2002). The objective of the present study was to investigate the success or failure of the CA+SS treatment on a semi-field scale and to evaluate the effect of a combined treatment with compost and mineral soil amendments. The C+CA treatment was added because it has resulted in a successful and sustainable revegetation at another site in the same contaminated area (Bouwman et al., 2001; Bouwman and Vangronsveld, 2004; Vangronsveld et al., 1995a, 1996). The experiments were conducted outdoors using lysimeters, allowing us to study plant responses and metal leaching. The results on metal leaching are reported in Chapter 3.

2.2 Materials and Methods

2.2.1 Soil sampling and soil treatment

Six thousand kg of a metal-contaminated sandy soil were collected on a barren area at the periphery of an old Zn smelter site in the North East of Belgium (Overpelt). Soil was sieved (1 cm), thoroughly homogenized, and subsamples of the mixture (325 kg each) were treated with soil additives using a concrete mixer. The following soil treatments were studied (w/w %): 1. untreated polluted soil (UNT); 2. compost (5%) (C), 3. compost (5%) + cyclonic ashes (5%) (C+CA), 4. compost (5%) + cyclonic ashes (5%) + steel shots (1%) (C+CA+SS), 5. cyclonic ashes (5%) + steel shots (1%) (CA+SS). Each treatment was available in 3 replicates (3 separate lysimeters).

The compost was a commercial product (Intercompost, Bilzen, Belgium) produced of organic domestic wastes and garden debris. Element compositions and pH of C, CA, and SS are presented in Table 2.1.

Table 2.1 Total element concentrations (*aqua regia*) and pH of cyclonic ashes (CA), steel shots (SS) and compost (C).

	Cyclonic ashes (CA)	Steel shots (SS)	Compost (C)
Zn mg kg^{-1}	153	79	347
Cd mg kg^{-1}	2.3	60	1.9
Cu mg kg^{-1}	64	834	74
Pb mg kg^{-1}	35	75	356
Ni mg kg^{-1}	35	591	10
Cr mg kg^{-1}	32	1289	23
Fe g kg^{-1}	13.6	922	-
Mn g kg^{-1}	0.85	7.7	-
SiO_2 %	51	-	-
Al_2O_3 %	31	-	-
CaO %	4.14	-	2.84
K_2O %	2.56	-	1.10
MgO	1.44	-	0.57
pH (H_2O)	9.3	6.1	8.5

-= not determined

2.2.2 Construction of lysimeters

The lysimeters were poly-ethylene tubs (surface area: 0.25 m^2, height: 0.7 m), surrounded by a 0.25-m compensation zone (Figure 2.1). This compensation zone was filled with the same soil as the main lysimeter, and aimed at a compensation of potential water loss near the lysimeter borders due to splashing raindrops. We estimated the presence of this zone of particular importance for the second part of our study, investigating effects of soil amendments on lysimeter leachates (Chapter 3, Ruttens et al., 2006b). A gravel layer (5 cm) was placed on the bottom of the tubs to improve water drainage. A synthetic cloth (Agratex) was used to separate soil and gravel layers. The lysimeters were placed outdoors in a completely randomized design under natural climatologic conditions (Diepenbeek, Belgium) (see Chapter 3, Ruttens et al., 2006b).

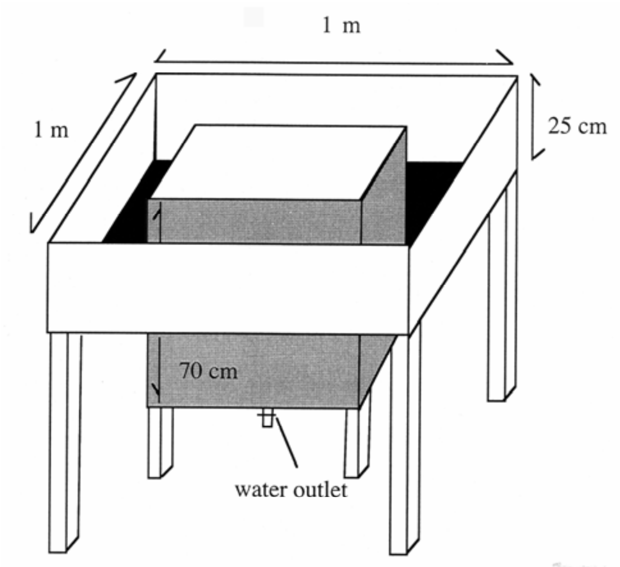


Figure 2.1 Schematic presentation of a lysimeter.

2.2.3 Total and exchangeable soil metal contents and soil pH

Total metal concentrations (*aqua regia*) in the homogenized soil sample were determined prior to soil treatment by digestion of 500 mg air-dried soil in 4 ml *aqua regia* using a microwave (Milestone, 1200 MEGA).

Exchangeable soil metal contents of the untreated and treated soils were determined using extractions with $Ca(NO_3)_2$ (0.1 M, soil solution ratio 1/5, 2 h shaking at 65 rpm, filtration through Whatman filter paper n°40) 13 weeks and 47 weeks after soil treatment. Metals were analysed by flame Atomic Absorption Spectroscopy (AAS, Perkin Elmer 1100 B). On the same soil samples pH-H20 was determined in a 10:25 air-dried soil:water ratio (Millipore, Milli-Q Water purification System) after shaking for 24h (65 rpm) and after filtration through a paper filter (Whatman n°1).

2.2.4 Determination of soil phytotoxicity

Thirteen weeks and 47 weeks after soil treatment, the potential soil phytotoxicity, and its reduction following the application of soil amendments, was assessed system using dwarf beans (*Phaseolus vulgaris* cv. Limburgse vroege) as bio-indicator species (Vangronsveld and Clijsters, 1992). The reference soil used in this study was the same sandy garden soil as the one used by Vangronsveld and Clijsters (1992).

After 1 day vernalization and 4h imbibition, beans were sown in 400 ml polyethylene pots (4 plants/pot, 4 pots per treatment), and plants were grown under controlled environmental conditions (22 °C, 65% relative humidity, 12h light, photosynthetically active radiation: 150 µmol m^{-2} s^{-1}). Fourteen days after sowing, morphological parameters (shoot length, primary leaf area, root weight) were measured for each plant. Per pot samples of primary leaves were taken for elemental analysis, and samples (1 g fresh weight, FW) of primary leaves and roots were frozen in liquid nitrogen and stored at –70°C for (iso)enzyme analysis.

Within 3 weeks after harvest, frozen plant tissue was homogenized with a Polytron PT 3000 homogenizer in ice cold Tris-HCl buffer (0.1 M, pH 7.8) containing 1mM EDTA, 1mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (5 ml buffer g FW^{-1}). The homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 20000xg (4°C). Subsequently the capacity (i.e. the potential activity measured *in vitro* under non-limiting conditions of substrate and coenzyme) of the following enzymes was measured spectrophotometrically (Shimadzu UV-1602) at 25°C in the supernatant as described by Van Assche et al. (1988): guajacol peroxidase (GPOD; E.C. 1.11.1.7), malic enzyme (ME; E.C. 1.1.1.40), glutamate dehydrogenase (GlDH; E.C. 1.4.4.2) and iso-citrate dehydrogenase (ICDH; E.C. 1.1.1.42). Enzyme capacity was expressed in milli-Units (mU) per gram FW. These enzymes are related to the plants stress responses (Van Assche and Clijsters, 1990b) and will be further referred to as 'stress enzymes'.

According to the system presented by Vangronsveld and Clijsters (1992), the values obtained for each parameter were 'transposed' into phytotoxicity classes based on the % reduction (morphological parameter) or % increase (plant enzymes) compared to plants grown on the non contaminated reference soil. Each parameter thus was used to classify the substrate in a given phytotoxicity class (class 1: not toxic, class 2: slightly toxic, class 3: moderately toxic, class 4: highly toxic). The 'Phytotoxicity Index' was obtained as average of the toxicity class number obtained for each parameter separately.

After extraction of root and leaf tissue equal protein amounts (leaves: 65 µg, roots: 10 µg) were subjected to non-denaturating polyacryl amide gel electrophoresis. Anionic peroxidases were separated on a 7.5 to 16% gradient slab gel (10mA per gel; 1.5 h separation time). After running, the gels were stained enzymatically to detect different iso-forms of plant peroxidases (POD). In the enzymatic reaction H_2O_2 is used as a substrate and is reduced by POD. A redoxindicator (tretramethylbenzidine) changes in colour and becomes insoluble after oxidation in this reaction. Metal specific changes in the iso-peroxidases pattern in leaves and roots of bean plants have been reported (Van Assche and Clijsters, 1990a, 1990b). Results of the gels were interpreted qualitatively based on presence and intensity of new bands.

2.2.5 Plant metal concentrations

Plant material was washed with distilled water (3 times), dried at 50°C and grounded using a sample mill (Retch-MM 2000). Portions of 200 mg dry weight (DW) were wet digested with 3 ml of HNO_3 (69-70%) using a microwave oven (Milestone 1200 MEGA). Metals were analysed using atomic absorption spectroscopy (AAS). Depending on the concentration and the element, flame (Perkin Elmer, 1100 B) or graphite furnace (Perkin Elmer, 5100) was used.

2.2.6 Statistical analysis

Because of the small samples sizes (n=3), a non-parametric statistical test (Kruskall-Wallis) followed by a multiple comparison procedure was used to calculate statistical significance of differences among treatments ($p<0.05$) (Conover, 1980).

2.3 Results and Discussion

2.3.1 Characteristics of the contaminated soil

The soil is a sandy, acid soil with low nutrient content (Table 2.2). High total soil metal levels in comparison to background values for uncontaminated Belgian sandy soils (De Temmerman et al., 2003), and the absence of vegetation in the field illustrate the contaminated character of the soil (Table 2.2).

Table 2.2 Physico-chemical soil characteristics in the homogenised soil sample (Overpelt) and background ranges for Belgian sandy soils (De Temmerman et al., 2003).

		Overpelt (contaminated)	Normal range (background)
Sand*	g kg^{-1}	902	
Silt*	g kg^{-1}	81	
Clay*	g kg^{-1}	17	
P_2O_5*	g kg^{-1}	0.011	
Organic C*	g kg^{-1}	7.43	
Organic N*	g kg^{-1}	0.33	
pH-H_2O		5.7	
Total metal content (*aqua regia*)			
Zn	mg kg^{-1}	1620±118	25-70
Cd	mg kg^{-1}	60±5	0.1-0.5
Cu	mg kg^{-1}	319±22	3-15
Pb	mg kg^{-1}	756±45	5-40

* parameter measured according to Boisson et al. (1999b)

2.3.2 Soil pH

At both evaluation moments all treatments caused a pH increase in comparison with the untreated soil (Table 2.3). This effect can be attributed to the alkalinity of CA and the compost used (Table 2.1). The increase in pH following soil treatment undoubtedly contributes to the observed reductions in metal bioavailability (Figure 2.3, Figure 2.5). Nevertheless other immobilization mechanisms also play an important role as illustrated by the fact that C+CA and C+CA+SS caused the same pH level 13 weeks after treatment but led to different reductions in exchangeable Zn and Cd contents (Table 2.4). In addition, exchangeable metal fractions (Zn, Cd, Cu, Pb) after CA+SS treatment at the same moment were significantly lower than after C+CA, while the pH after the first treatment was the lowest. Mechanisms supposed to contribute to metal immobilization by CA and SS, were discussed by Mench et al. (1998, 2000), and Boisson et al. (1999b).

Table 2.3 pH-H_20 of the untreated and treated soils taken from the lysimeters respectively 13 weeks and 47 weeks after soil treatment.

Soil treatment	pH-H_20	
	Week 13	Week 47
UNT	6.3 (a)	6.3 (a)
C	7.0 (b)	7.0 (b)
C+CA	7.5 (d)	7.2 (c)
C+CA+SS	7.4 (d)	7.4 (d)
CA+SS	7.2 (c)	7.2 (c)

At each evaluation moment, statistical significantly different values ($p<0.05$) are followed by different letters.

2.3.3 $Ca(NO_3)_2$ exchangeable soil metals

$Ca(NO_3)_2$ extractable fractions of Zn, Cd, Cu, and Pb in lysimeter soils were determined 13 and 47 weeks after treatment (Table 2.4). Neutral salt solutions, such as 0.1 M $Ca(NO_3)_2$, are supposed to mirror the soil solution and to exchange their cations with easily exchangeable metals on the soil solid- phase (Houba, 1996). These loosely adsorbed metals are considered as labile and available for plant uptake (Kabata-Pendias, 1993). Therefore 0.1M $Ca(NO_3)_2$-extractions can be used for a rapid evaluation of amendment induced changes in easily available metals. Advantages of using $Ca(NO_3)_2$ over the more commonly used $CaCl_2$ were discussed by Boisson et al. (1999b). In Table 4 the absolute figures (mg kg^{-1} DW) and the relative reductions in exchangeable metal contents (% of the untreated soil) are presented.

Table 2.4 $Ca(NO_3)_2$ extractable amount of Zn, Cd, Cu and Pb in soils taken from the lysimeters, respectively 13 weeks and 47 weeks after soil treatment.

Metal	Soil treatment	Week 13		Week 47	
		mg kg^{-1} DW	% reduction	mg kg^{-1} DW	% reduction
Zn	UNT	615 (a)	-	406 (a)	-
	C	341 (b)	45	237 (b)	42
	C+CA	111 (c)	82	65 (c)	84
	C+CA+SS	57 (e)	91	36 (d)	91
	CA+SS	77 (d)	87	55 (c)	86
Cd	UNT	27.9 (a)	-	20.0 (a)	-
	C	20.4 (b)	27	11.6 (b)	42
	C+CA	9.2 (c)	67	4.8 (c)	76
	C+CA+SS	5.2 (d)	81	3.1 (d)	84
	CA+SS	7.6 (c)	73	5.2 (c)	74
Cu	UNT	5.1 (a)	-	3.6 (a)	-
	C	2.8 (b)	45	1.4 (b)	61
	C+CA	2.0 (c)	61	1.0 (c)	72
	C+CA+SS	1.7 (c)	67	0.9 (c)	75
	CA+SS	1.1 (d)	78	0.9 (c)	75
Pb	UNT	20.7 (a)	-	11.9 (a)	-
	C	10.8 (b)	48	5.1 (b)	57
	C+CA	8.0 (c)	61	3.3 (c)	72
	C+CA+SS	7.5 (c)	64	3.0 (cd)	75
	CA+SS	4.1 (d)	81	2.8 (d)	76

% reduction= result expressed as percentage reduction compared to the untreated soil. At each evaluation moment, statistical significantly different values ($p<0.05$) are followed by different letters.

All treatments, including C alone, reduced the extractable metal fractions. The effect of C was always lower than the effect of the other treatments (Table 2.4). For Zn and Cd, the strongest reductions were observed after C+CA+SS treatment (91% reduction of Zn, 81-84% reduction of Cd). At both moments the addition of C to the CA+SS treatment led to an extra reduction of exchangeable Zn and Cd compared to the same treatment without C (87-86% reduction of Zn, 73-74% reduction of Cd). In contrast, C+CA+SS led to higher (13 weeks) or equal (47 weeks) exchangeable Cu and Pb fractions than CA+SS. We assume that this result was due to the formation of soluble organic Cu and Pb complexes in the presence of C (Chapter 3). The difference in Cu and Pb extractability between C+CA+SS and CA+SS disappeared 47 weeks after treatment. This observation suggests that these complexes are mainly present during the first weeks after compost addition, and decrease later on due to e.g. leaching, microbial degradation or sorption reactions. Its also may explain the increasing reductions in exchangeable Cu and Pb fractions in the course of time for all treatments that include C (Table 2.4).

2.3.4 Soil phytotoxicity

Poor growth and high capacities of 'stress enzymes' in plant tissues are symptoms of strong phytotoxicity (Van Assche and Clijsters, 1990b; Van Assche et al., 1988). In table 2.5, responses of bean plants grown on the untreated and treated soils 47 weeks after treatment are summarized by means of phytotoxicity classes (Van Assche and Clijsters, 1990a; Vangronsveld and Clijsters, 1992). Plant responses 13 at weeks after treatment were basically the same (results not shown).

On the untreated soil, growth of bean plants was strongly inhibited and very strong enzyme inductions were observed, resulting for most parameters in phytotoxicity class 4 (=strongly toxic).

Table 2.5 Evaluation of the phytotoxicity of the untreated and treated soils at the second evaluation moment (week 47), using a phytotoxicity test with bean plants (*Phaseolus vulgaris*).

	Morphological parameters			Enzyme capacities leaves			Enzyme capacities roots			PI
	Shoot length	Leaf area	Root weight	GPOD	ME	ICDH	GPOD	ME	GlDH	
Reference	1	1	1	1	1	1	1	1	1	**1**
UNT	3	4	4	4	4	4	3	2	4	**3.5**
C	3	4	4	2	4	4	1	1	2	**2.8**
C+CA	2	4	3	2	3	3	1	1	1	**2.2**
C+CA+SS	1	3	2	1	2	3	1	1	1	**1.7**
CA+SS	2	4	3	2	3	3	1	1	1	**2.2**

GPOD= guaiacol peroxidase, ME= malic enzyme, ICDH= isocitrate dehydrogenase, GlDH= glutamate dehydrogenase. UNT=untreated, C=compost, CA= cyclonic ashes, SS=steel shots, SL= shoot length, LA= leaf area, RW=root weight. Results are presented by means of phytotoxicity classes based on the % reduction in growth or % increase in enzyme capacity compared to the control plants (according to Vangronsveld and Clijsters, 1992); class 1=not toxic; class 2= slightly toxic; class 3= moderately toxic; class 4= highly toxic. PI= 'Phytotoxicity Index' = mean of individual phytotoxicity classes.

After addition of C alone enzyme capacities in roots were restored to reference levels, but growth remained poor, and enzyme capacities in leaves still were high, leading to a mean phytotoxicity class of 2.8. The strongest reductions in phytotoxicity were observed after addition of C+CA+SS (mean phytotoxicity class 1.7). None of the treatments could eliminate completely phytotoxic responses. However, it must be mentioned that the phytotoxicity classes mainly reflect relative differences among treatments and may not necessarily be considered as absolute values. Their levels by definition depend on plant responses obtained on the reference soil used. Leaf area and GPOD capacity in both leaves and roots are presented in Figure 2.2. Statistical differences between treatments indicated on the graph are consistent with differences in toxicity classes.

Metal concentrations in the bean leaves are presented in Figure 2.3. Insufficient plant material was harvested from the untreated soil for this measurement. The strongest phytotoxic responses in the C treated soil correspond to the highest Zn and Cd concentrations in bean leaves. The higher toxicity class of CA+SS in comparison with C+CA+SS is supported by the higher Zn and Cd concentrations in the leaves of plants grown on the CA+SS treated soil. Differences in leaf Cu concentration across the treatments showed a trend similar to that of Zn and Cd, but were not significant. Pb concentrations in leaves were below detection limit (<6 mg.kg^{-1} DW) for all treatments.

Several studies have shown that differential patterns of iso-enzymes in plants can be linked to plant stress (Lee et al., 2000, 2001). Van Assche and Clijsters (1990a, 1990b) reported changes in the pattern of iso-POD in bean as a result of metal exposure. In primary leaves zinc induced the appearance of two specific anionic iso-POD bands (D-bands) that were not present in the control, while the iso-POD pattern of roots was not affected. Cadmium induced the same two bands in primary leaves and roots, plus an additional E-band in roots. Copper only changed iso-POD pattern in roots with effects similar to these of Cd.

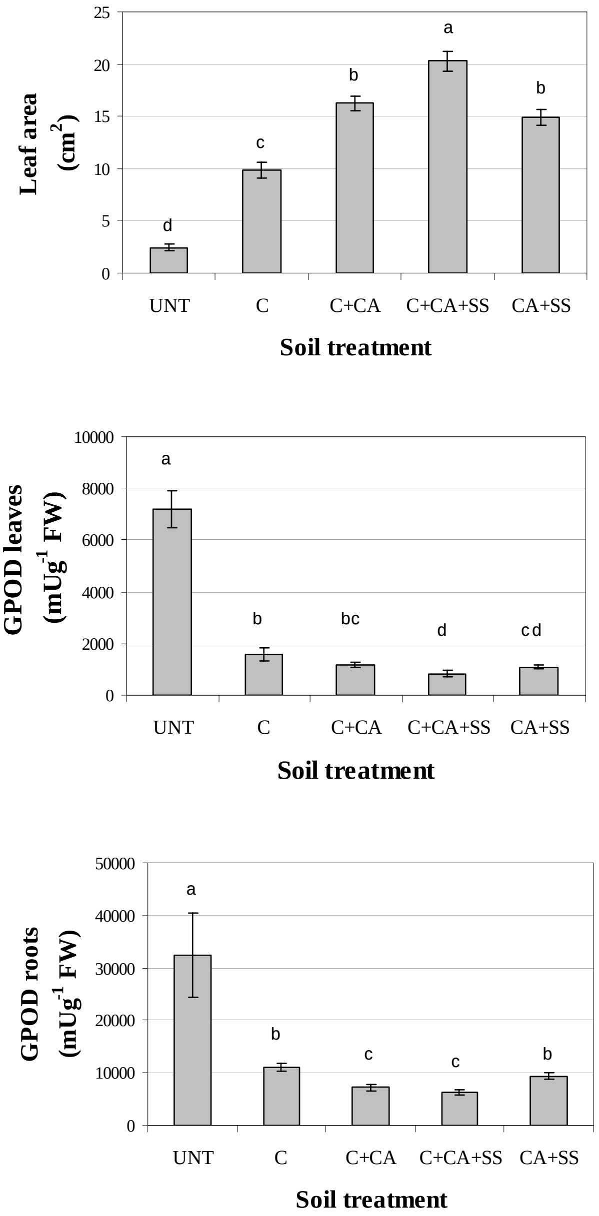


Figure 2.2 Leaf area (cm^2), GPOD capacity in leaves (mU g^{-1} Fresh Weight, FW) and GPOD capacity in roots (mU g^{-1} Fresh Weight, FW) of 14 days old *Phaseolus vulgaris* plants grown on the untreated and treated soils 47 weeks after soil treatment. C= compost, CA= cyclonic ashes, SS= steel shots. Bars indicate mean ± standard error. Letters above the bars refer to statistical significances between treatments ($p<0.05$).

The iso-POD pattern in roots of plants grown on the untreated Overpelt soil clearly showed the presence of new D-bands and E-bands (Figure 2.4a), which are likely the result of Cd toxicity. Copper toxicity is unlikely to be involved since no differences in Cu concentrations in the bean leaves were observed between different treatments (Figure 2.3) whereas toxic responses clearly differed (Table 2.5, Figure 2.2). Moreover Cu concentrations in grasses grown on the untreated soil did not differ from background values (see below). The latter was also observed for Pb. In all treatments the iso-POD pattern in roots was restored to the control pattern, suggesting elimination of toxicity in roots. In leaves of plants grown on the untreated soil, no change in iso-POD signal was detected (Figure 2.4b). Since growth responses clearly indicate a very strong phytotoxicity of this soil, the absence of changes in iso-POD pattern most likely is due to a serious loss of physiological functionality of the plants. Similar observations on very toxic soils were made earlier (Vangronsveld et al., unpublished results). D-bands in leaves were strongly present after C addition, but disappeared in C+CA and C+CA+SS treatments. CA+SS showed a weak induction of D-bands in the leaves. These iso-POD patterns in leaves correspond well to the results of Zn and Cd concentrations in leaves (Figure 2.3). After addition of C alone, Zn and Cd concentrations in bean leaves remained high. The normal iso-POD pattern in the roots after all soil treatments, suggest that Zn is likely the element responsible for toxic responses in the leaves after C and CA+SS treatments. Further reductions in Zn (and Cd) uptake after C+CA and C+CA+SS treatments, restored the iso-POD pattern in leaves to the reference pattern.

Changes in metal concentrations in leaves and morphological and/or biochemical parameters were in general consistent with changes in metal exposure assessed by $Ca(NO_3)_2$ extractions.

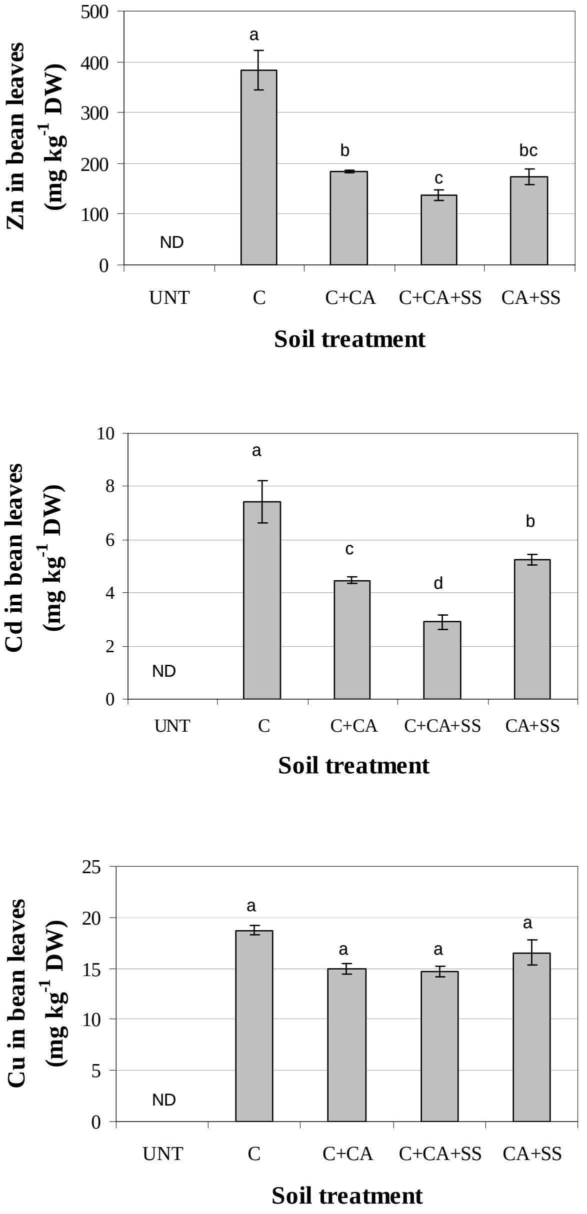
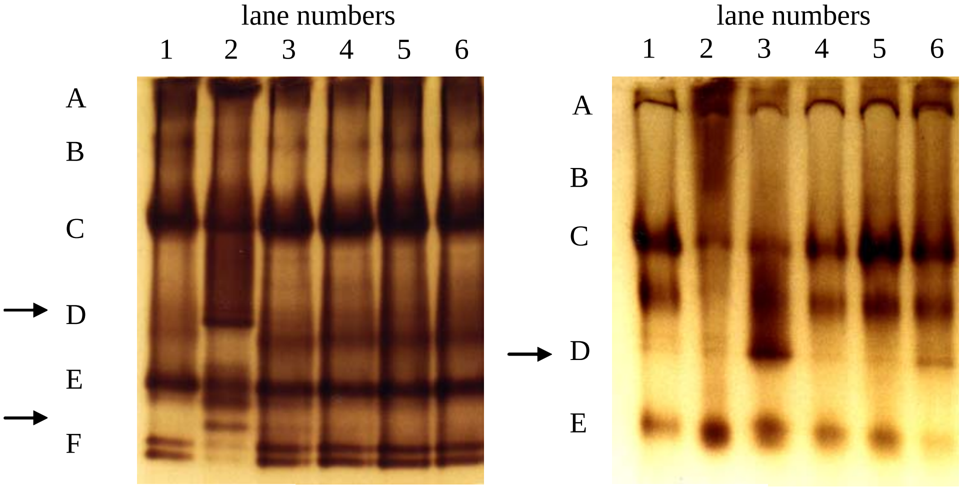


Figure 2.3 Metal concentrations (Zn, Cd, Cu) in leaves of 14 days old *Phaseolus vulgaris* plants grown on the untreated and treated soils 47 weeks after soil treatment. C= compost, CA= cyclonic ashes, SS= steel shots. Bars indicate mean ± standard error. Letters above the bars refer to statistical significances between treatments ($p<0.05$). ND= not determined: insufficient plant material was available on the untreated soil for this measurement. Pb in plants is below detection limit for all soil treatments (<6 mg kg^{-1} DW).



a. roots b. leaves

Figure 2.4 Pattern of anionic iso-peroxidases in roots (a) and leaves (b) of 14 days old *Phaseolus vulgaris* plants grown on the untreated and treated soil 47 weeks after soil treatment. Letters indicate the different iso-POD bands. Arrows indicate metal induced D-and E-bands. lane 1: reference, lane 2=UNT, lane 3=C, lane 4=C+CA, lane 5=C+CA+SS, lane 6= CA+SS (C= compost, CA= cyclonic ashes, SS= steel shots).

All data indicated that C+CA+SS was the best treatment for restoring plant growth, limiting root exposure and metal uptake and keeping the stress responses of plants, as assessed by enzymatic changes, on a low level.

The increased efficiency of C+CA+SS compared to C+CA can be related to the fact that the addition of this extra compound in the soil induces extra metal immobilizing potential. The use of a mixture of products generally offers the advantage that metals can be immobilized by different mechanisms and on different binding sites. Two additional hypotheses explain the high efficiency of the combination of CA and SS:

- an increased efficiency of metal binding on Fe or Mn oxides due to the high pH induced by the presence of CA;
- an increased precipitation of Fe-oxides (and co-precipitation of metals) on clay particles introduced in the soil by CA addition.

2.3.5 Natural colonization of plant species on the lysimeters

During the experimental period plants started colonizing the lysimeters spontaneously (Table 2.6). In the untreated soil, only *Agrostis capillaris* was growing, whereas after addition of C, C+CA and C+CA+SS the number of plant species increased up to 2, 5 and 6 respectively. For these treatments this observation corroborates the results of $Ca(NO_3)_2$ extractions and of the phytotoxicity test: the number of plant species able to grow on each of the lysimeters corresponds to the metal immobilizing capacity of the additives. Surprisingly, despite the strong reductions in $Ca(NO_3)_2$-extractable metals after CA+SS addition, only 1 species colonized this soil. This illustrates that besides phytotoxicity, other factors such as the low nutrient content and limited WHC of the sandy soil determine the colonization potential.

Table 2.6 Natural colonization of plant species on the lysimeters

Soil treatment	Number of plant species	Plant species
UNT	1	*Agrostis capillaris* (1 individual)
C	2	*Agrostis capillaris* *Cerastium fontanum*
C+CA	5	*Agrostis capillaris* *Cerastium fontanum* *Sonchus asper* *Poa annua* *Chenopodium album*
C+CA+SS	6	*Agrostis capillaris* *Cerastium fontanum* *Sonchus asper* *Claytonia perfoliata* *Tanacetum vulgare* *Barbarea sp.*
CA+SS	1	*Agrostis capillaris*

Since *Agrostis capillaris* was growing on all lysimeters, metal concentrations (Zn, Cd, Cu, Pb) were determined in the aerial parts of this species (Figure 2.5).

Trends in metal uptake in *A. capillaris* were similar to those observed in bean plants. The lowest concentrations of Zn, Cd, and Cu in the grasses were found with the C+CA+SS treatment. With regard to Zn and Cd, plant metal accumulation corresponds to the results of $Ca(NO_3)_2$ extractions, however not for Cu. Even though C addition to CA+SS tended to increase Cu extractability, Cu accumulation in grasses was significantly reduced. Apparently, the soluble Cu complexes formed after C addition, which are reflected in the extraction results, did not increase Cu concentration in the shoots of *A. capillaris*. Bean plants did not show significant differences in Cu uptake between treatments, however general trends were similar (Figure 2.3). Lead content in the grasses was 10.2 mg kg^{-1} DW in the untreated soil and decreased to values <6 mg kg^{-1} DW after treatment. Background metal concentrations in grasses range within the following values (mg kg^{-1} DW): 20-80 Zn, 0.05-0.5 Cd, 2-20 Cu and 2-6 Pb (Kabata-Pendias and Pendias, 1984). Zinc and Cd concentrations in *A. capillaris* grown on the untreated soil strongly exceeded these values, but after C+CA+SS treatment these concentrations were restored to background values (Zn: 52 mg kg^{-1} DW) or close to background (Cd: 0.8 mg kg^{-1} DW). Cu and Pb concentrations in grasses were at the upper limit of the normal range when grown on the untreated soil, but decreased after soil treatment**.** C+CA+SS was the only treatment that reduced Cd concentrations in the grasses below the Belgian limit concentration for animal feed crops, which is 1.1 mg kg^{-1} DW (Belgisch Staatsblad, 1999). Limit values for Pb according to this decree are 45 mg kg^{-1} DW; this concentration was never exceeded, not even on the untreated soil.

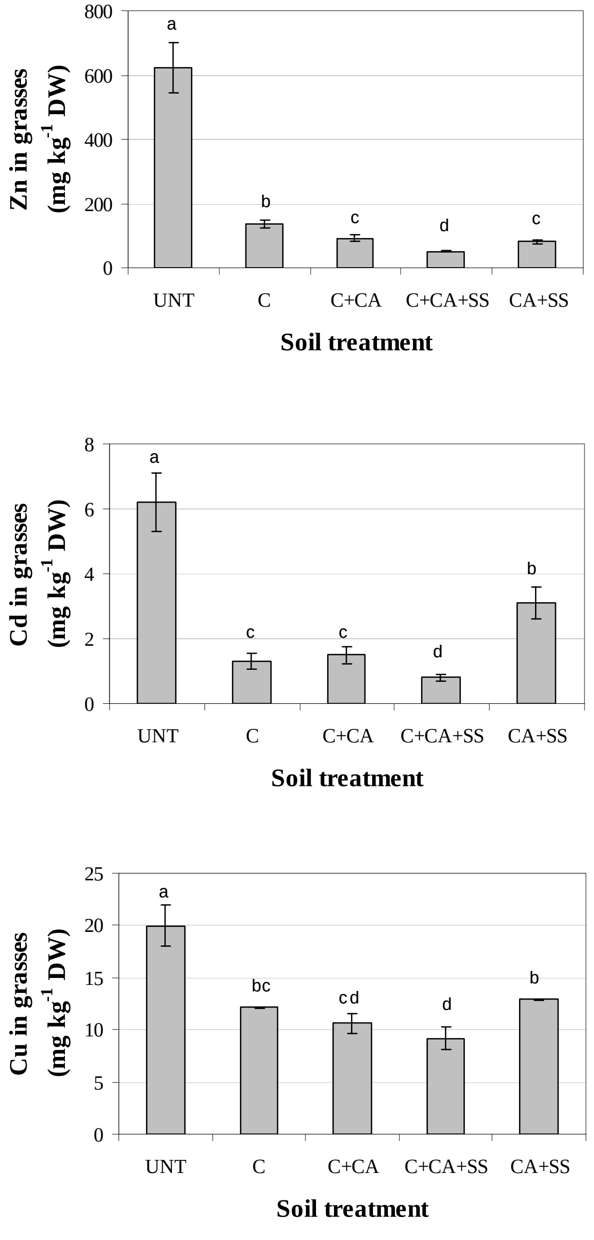


Figure 2.5 Metal concentrations (Zn, Cd, Cu) in aerial parts of *Agrostis capillaris* colonizing spontaneously the untreated and treated lysimeters. C= compost, CA= cyclonic ashes, SS= steel shots. Bars indicate mean ± standard error. Letters above the bars refer to statistical significances between treatments ($p<0.05$). Pb in plants was below detection limit after soil treatments (<6 mg Pb kg^{-1}).

Decrease in soil phytotoxicity following amendment supply and subsequent establishment of a vegetation cover may contribute to a decreased percolation of water and metals (Vangronsveld et al., 1995b). A second part of this study deals with effects on metal percolation (Chapter 3)(Ruttens et al., 2006b), but the effect of a completely closed vegetation cover could not yet be evaluated because plants only progressively colonized the lysimeters during the first year of this study.

2.4 Conclusions

- Soil treatment with C alone reduced metal mobility and plant availability compared to the untreated soil; however, soil phytotoxicity remained high after this treatment, resulting in a limited diversity of plant species. Zn and Cd-concentrations in aerial parts of *Agrostis capillaris* plants, germinating spontaneously on the C treated soil, were well above background values. Stronger reductions in soil phytotoxicity and metal accumulation were obtained when compost was combined with the inorganic soil amendments.
- One year after treatment C+CA+SS displayed the highest efficiency, and was concluded to be an effective treatment for the assisted phytostabilization of the Overpelt soil. This amendment led to:
  -the lowest phytotoxic responses and lowest metal concentrations in *Phaseolus vulgaris,*
  -the highest diversity of spontaneously colonizing plant species,
  -a reduction of metal concentrations in spontaneously colonizing grasses *(Agrostis capillaris)* to background values.
- When CA+SS was applied without C, plant responses were less improved:
  -stronger phytotoxic responses were observed in *Phaseolus vulgaris,*
  -only one species (*Agrostis capillaris*) was spontaneously colonizing the lysimeters.

- Soil treatment with C+CA showed plant responses intermediate between the C and C+CA+SS treatments.

CHAPTER 3

Phytostabilization of a metal contaminated sandy soil: II. Influence of compost and/or inorganic metal immobilizing soil amendments on metal leaching

A. Ruttens, J.V. Colpaert, M. Mench, J. Boisson, R. Carleer and J. Vangronsveld.

Environmental Pollution. Article *In Press*.

Abstract

A lysimeter approach (under natural climatologic conditions) was used to evaluate the effect of four metal immobilizing soil treatments [compost (C), compost + cyclonic ashes (C+CA), compost + cyclonic ashes + steel shots (C+CA+SS)) and cyclonic ashes + steel shots (CA+SS)] on metal leaching through an industrially contaminated soil. All treatments decreased Zn and Cd leaching. Strongest reductions occurred after CA+SS and C+CA+SS treatments (Zn: -99.0% and -99.2% respectively; Cd: -97.2% and -98.3% respectively). Copper and Pb leaching increased after C (17 and >30 times for Cu and Pb respectively) and C+CA treatment (4.4 and >3.7 times for Cu and Pb respectively). C+CA+SS or CA+SS addition did not increase Cu leaching; the effect on Pb leaching was not completely clear. Our results demonstrate that attention should be paid to Cu and Pb leaching when organic matter additions are considered for phytostabilization of metal contaminated soils.

3.1 Introduction

Soil, sediment and water resources contaminated with toxic levels of trace metals are found all over the world. In most countries, guideline values for metal contaminated soils are still based on total concentrations. However there is a tendency towards the development of new approaches based on site specific risk assessment, in which the necessity for remediation is linked to human health and/or ecological risks associated with the contaminated site. *In situ* immobilization of the pollution by strong immobilizing agents could be an attractive remediation option when the new legislative frameworks become more accepted. Low cost, non-invasive technologies are indeed urgently required to remediate large areas of metal contaminated soils. The fundamental premise of the immobilization technique is to reduce the 'bioavailability' of metals in the soil, bioavailability being defined as that fraction of the total metal content that can interact with the biological target (Vangronsveld and Cunningham, 1998). Subsequently, food and feed can be grown more safely, and on more heavily contaminated sites, a vegetation cover can develop to stabilize the soil. A combination of contaminant immobilization and revegetation thus may limit the environmental impact of contaminated areas.

Different types of soil amendments, including various types of organic matter, natural and synthetic zeolites, clays and related substances, Fe-rich materials, Mn oxides, and P-rich compounds, have been tested as metal immobilizing soil amendments (for a review see Mench et al., 1998). The efficiency of the substances has been evaluated mainly by chemical extractions ($CaCl_2$, $Ca(NO_3)_2$), sequential extractions, Toxicity Characteristic Leaching Procedure-TCLP) and biotests with plants or bacteria. Some researchers have put additional efforts in investigating (estimating) effects of P additions (in different forms) on availability of soil Pb to humans (simulation of gastro-intestinal conditions) (Brown et al., 2004; Hettiarachchi et al., 2000; Hetiarachchi and Pierzynski, 2002; Ruby and Nicholson, 1994; Ryan et al., 2004; Yang et al. 2001). From the numerous papers on *in situ* metal immobilization and phytostabilization it is not possible to identify one single soil additive as 'the best' option for field applications. Different tests often lead to different conclusions (Brown et al., 2004; Geebelen et al. 2003) and results are soil dependent (Geebelen et al., 2003; Lombi et al., 2002). It is clear that a whole set of tests and analysis (both chemical and biological) is needed for well-supported decisions, necessarily taking into account the strength of metal binding and the expected durability of the treatment in field conditions. Aspects such as actual and future land use, legislation, acceptable risks, economic aspects, etc. will influence the selection of the most appropriate treatment. Across all studies only limited attention has been paid to the leaching of metals in relation to the application of metal immobilizing soil amendments. Reductions of metal leaching could be an important advantage of *in situ* immobilization treatments, and may play an important role in groundwater protection and reduction of metal dispersion.

With column studies Álvarez-Ayuso and García Sánchez (2003) found reductions in Zn (52%), Cd (66%), Cu (59%) and Pb (50%) leaching after application of Palygorskite to a metal contaminated soil. Some soil treatments, however, may increase metal or As leaching. Oste et al. (2002) found an increase in Zn and Cd leaching after zeolite application (in Na-form) to a metal contaminated soil. Lead and As leaching increased after compost addition to a gold mine spoil (Mench et al., 2003). Basta et al. (2004) found next to strong reductions in Zn, Cd and Pb elution that diammonium phosphate treatment also increased As release.

In the present study a lysimeter approach was used to evaluate and compare the effect of four soil treatments [compost (C), compost + cyclonic ashes (C+CA), compost + cyclonic ashes + steel shots (C+CA+SS) and cyclonic ashes + steel shots (CA+SS)], on the percolation of metals in an industrially contaminated soil originating from a site in Overpelt (Belgium). During a one-year experimental period the total amount of leaching water, the metal concentrations in the leachates, and the total amount of leached metals were determined for untreated and treated soils. The semi-field lysimeters allow collection of results under natural climatologic conditions but avoid the heterogeneity at the field site. Plant responses on the same lysimeters have been reported in Chapter 2 (Ruttens et al., 2006a). The results of the latter study illustrated that addition of C alone could reduce metal uptake but not eliminate soil phytotoxicity. When used in combination with the mineral soil amendments, the addition of C had an extra ameliorating effect on plant responses compared to the inorganic amendment alone.

3.2 Materials and Methods

3.2.1 Soil sampling and soil treatment

Six thousand kg of a metal-contaminated sandy soil were collected at a former Zn smelter site in the North East of Belgium (Overpelt). Subsamples of the mixture (325 kg each) were treated as follows (% w/w): 1. untreated polluted soil (UNT); 2. compost (5%) (C), 3. compost (5%) + cyclonic ashes (5%) (C+CA), 4. compost (5%) + cyclonic ashes (5%) + steel shots (1%) (C+CA+SS), 5. cyclonic ashes (5%) + steel shots (1%) (CA+SS). Each treatment was produced in three replicates (3 separate lysimeters). For more details about soil treatment and products see Chapter 2 (Ruttens et al., 2006a).

3.2.2 Construction of lysimeters

The lysimeters were polyethylene tubs (surface area: 0.25 m^2, height: 0.7 m), surrounded by a 0.2 m compensation zone (Chapter 2, Figure 2.1)(Ruttens et al., 2006a) and placed outdoors in a completely randomized design under natural climatologic conditions (Diepenbeek, Belgium). Data on local temperature and precipitation during the experimental period are presented in Figure 3.1.

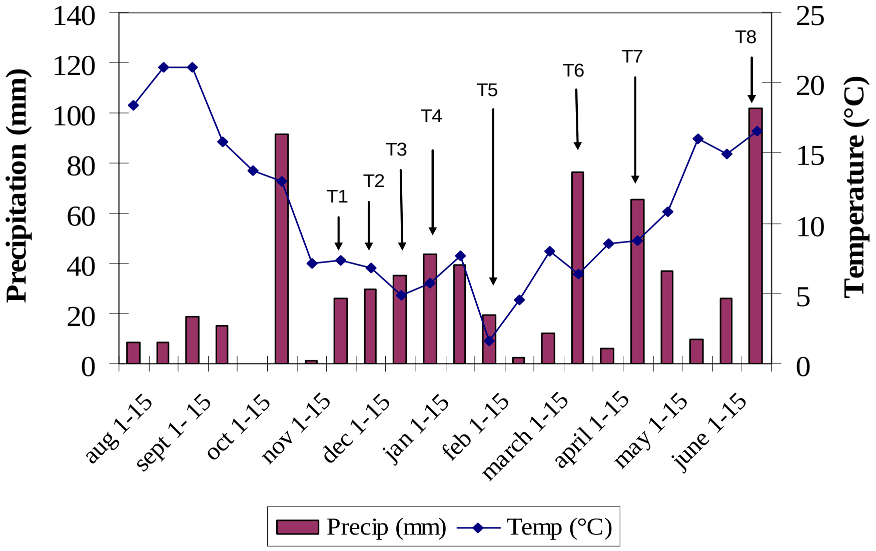


Figure 3.1 Average temperature (Diepenbeek, Belgium) and precipitation (Bilzen, Belgium) over 2-week intervals during the experimental period (KMI, 1998), with indication of the sampling moments (T1-T8).

3.2.3 Collection and analysis of percolation water

Leachate was collected in 30-L vessels pre-rinsed with HCl (10%). During a one-year period, water was sampled with intervals ranging from several weeks to several months depending on the amount of precipitation (Figure 3.1). In total, eight sampling moments were obtained (T1-T8). Concentrated HNO_3 (70-71%) (20 ml per 30-L vessel) was used to acidify the percolate to keep metals in solution. At each sampling time (T1-T8), the amount of percolation water and the concentration of metals in the percolate were determined for each lysimeter. Samples for metal analysis were collected in glass tubes, pre-rinsed with HCl (10%) and stored at 4°C. No further treatment of the water samples occurred prior to analysis. Metals were analysed by induced coupled plasmaspectrometry (ICP, Perkin Elmer, Optima 3000 DV).

3.2.4 Statistical analysis

Because of the small number of replicates (n=3), a non-parametric statistical test (Kruskall-Wallis) followed by a multiple comparison procedure was used to calculate statistical significance of differences between treatments ($p<0.05$) (Conover, 1980).

3.3 Results and Discussion

3.3.1 Total metal concentrations in the contaminated soil

Mean total metal concentrations in the homogenized soil sample were 1620 mg Zn kg^{-1}, 60 mg Cd kg^{-1}, 319 mg Cu kg^{-1}, 756 mg Pb kg^{-1}. The acid (pH-$H_2$0 5.7) sandy soil had low nutrient contents (Chapter 2, Tabel 2.2) (Ruttens et al. 2006a). The high total soil metal levels in comparison to background values for uncontaminated Belgian sandy soils (De Temmerman et al., 2003) and the absence of vegetation in the field, demonstrate the contaminated character of the soil.

3.3.2 Metal percolation

The cumulative amounts of metals (mg) (Zn, Cd, Cu, Pb) leached from the soils are presented as a function of the total amount of leachate (Figure 3.2a, b). These metal quantities were obtained by multiplying at each sampling moment the metal concentration in the percolate (mg L^{-1}) and the percolate volume (L). Each data point represents the mean of the three replicates.

Zn and Cd percolation

All soil treatments (C, C+CA, C+CA+SS, CA+SS) resulted in an immediate and overall reduction of the amount of percolated Zn and Cd (Figure 3.2a). At the end of the experimental period, 10.2 g Zn had leached in average from the untreated soil, corresponding to 4.5% of the total amount of Zn present in the lysimeter. Total Zn leaching was reduced by 86.0%, 95.3%, 99.2% and 99.0% in the C, C+CA, C+CA+SS, and CA+SS treatments respectively. Total Cd leaching from the UNT amounted up to an average of 218 mg Cd or 2.6% of the total Cd amount in the lysimeter and was lowered by 83.8%, 93.9%, 98.3% and 97.2% by the respective soil treatments. Treatment efficiency to decrease Zn and Cd leaching followed the order: C+CA+SS = CA+SS > C+CA > C. Differences between treatments were all significant ($p<0.05$), except for C+CA+SS and CA+SS.

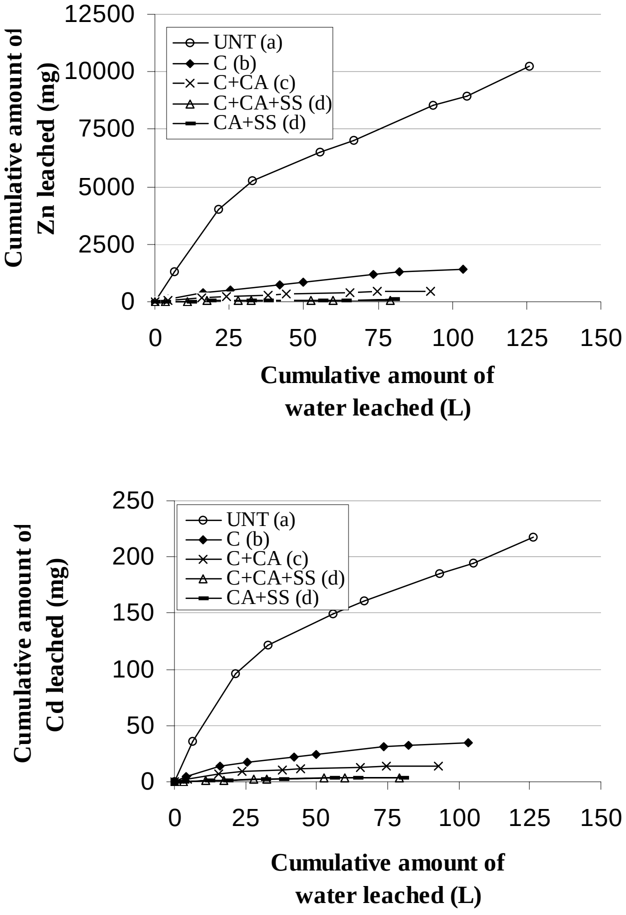
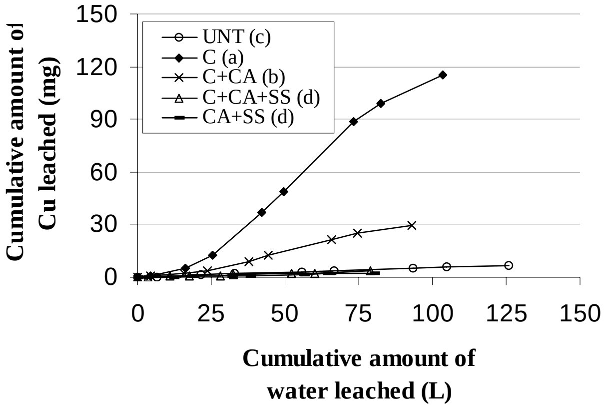


Figure 3.2a Total amount of Zn and Cd percolated from the untreated and treated soils, as a function of the total amount of percolated water during the 1-year experimental period. Values are means of three replicates. Treatments followed by a different letter are statistical significantly different ($p<0.05$) based on endpoint analysis.



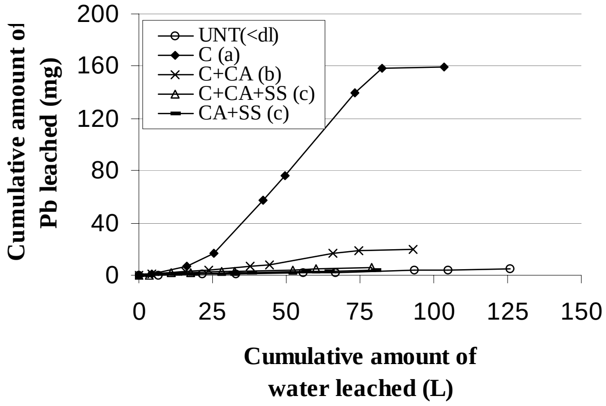


Figure 3.2b Total amount of Cu and Pb percolated from the untreated and treated soils, as a function of the total amount of percolated water during the 1-year experimental period. Values are means of three replicates. Treatments followed by a different letter are statistical significantly different ($p<0.05$) based on endpoint analysis. Pb concentrations < dl were presented using the value of the dl=0.05 mg L^{-1}.

The overall effect of the amendments on Zn and Cd percolation resulted from a reduction in metal concentration in the percolate and from a reduced amount of percolating water (Figure 3.2a). In Figures 3.2a,b the slope of the curves between two sampling points corresponds to the average metal concentrations in the percolate during that period.

Zinc and Cd concentrations in the percolate at three moments (T1, T4, T8 = 17, 24 and 47 weeks) are presented in Table 3.1a. The first (T1), the last (T8) and an intermediate (T4) sampling moment were selected to illustrate the observed evolution in concentrations in the course of time: in all soils, Zn and Cd concentrations were the highest during the first months after the set up of the lysimeters. The same effect was observed in $Ca(NO_3)_2$ extractions (Chapter 2) (Ruttens et al, 2006a). This high initial metal mobility is likely caused by soil disturbance during the collection of soil samples and their homogenization. Increases in metal mobility after soil manipulation were observed also in previous experiments (Vangronsveld et al., 1995b). Different processes can potentially contribute to the increased metal mobility, such as destruction of soil aggregates influencing (de)sorption processes, and oxidation of soil functional groups changing the structure of compounds and their metal binding capacity (e.g oxidation of C=C bonds of soil organic matter) (Huang, 1997; Wenzel and Blum, 1997). Increased amounts of dissolved organic matter resulting from oxidation reactions and capable of complexing metals, may also play a role in this context. Soil equilibria, temporarily disturbed in advantage of the mobile fraction, reach a new equilibrium in the course of time.

Treatment of the polluted soil with C, C+CA, C+CA+SS or CA+SS in average decreased Zn concentration in leachates (average T1-T8) by 82.1%, 93.3%, 98.6% and 98.3% respectively. The same treatments reduced the leachate Cd concentration by 79.2%, 91.4%, 97.0% and 95.4% respectively (Table 3.1a). Differences in concentrations between the treatments were significant ($p<0.05$) at all 8 sampling moments, except C+CA+SS was never statistically different from CA+SS. When ranked according to efficiency the treatments followed the same order (C+CA+SS = CA+SS > C+CA > C) for metal concentrations and percolated amounts of metal.

Table 3.1 Average concentrations of (a) Zn, Cd and (b) Cu, Pb in the percolate in week 17, 25 and 47 after soil treatment.

(a)	Zn (mg L^{-1})			Cd (mg L^{-1})		
	T1	T4	T8	T1	T4	T8
	week 17	week 25	week 47	week 17	week 25	week 47
UNT	200 ± 16	56.7 ± 7.8	58.3 ± 1.2	5.44 ± 0.39	1.24 ± 0.20	1.08 ± 0.06
	(a)	(a)	(a)	(a)	(a)	(a)
C	25.6 ± 3.9	13.4 ± 2.2	5.9 ± 2.0	1.09 ± 0.16	0.32 ± 0.05	0.12 ± 0.03
	(b)	(b)	(b)	(b)	(b)	(b)
C+CA	7.7 ± 0.8	5.7 ± 0.3	1.7 ± 0.5	0.39 ± 0.06	0.14 ± 0.01	0.040 ± 0.006
	(c)	(c)	(c)	(c)	(c)	(c)
C+CA+SS	1.4 ± 0.2	1.1 ± 0.5	0.6 ± 0.2	0.09 ± 0.05	0.07 ±0.03	0.013 ± 0.003
	(d)	(d)	(d)	(d)	(d)	(d)
CA+SS	1.8 ± 0.3	1.1 ±0.3	0.7 ± 0.1	0.10 ± 0.03	0.08 ±0.02	0.035 ± 0.010
	(d)	(d)	(d)	(d)	(d)	(c)

(b)	Cu (mg L^{-1})			Pb (mg L^{-1})		
	T1	T4	T8	T1	T4	T8
	week 17	week 25	week 47	week 17	week 25	week 47
UNT	0.059 ± 0.005	0.057 ± 0.005	0.043±0.003	<0.05	<0.05	<0.05
	(c)	(c)	(c)			
C	0.21 ± 0.05	1.45 ± 0.29	0.76 ± 0.18	0.18 ± 0.08	2.45 ± 0.70	<0.05
	(a)	(a)	(a)	(a)	(a)	
C+CA	0.14 ± 0.02	0.40 ± 0.08	0.25 ± 0.04	0.19 ± 0.01	0.25 ± 0.07	<0.05
	(b)	(b)	(b)	(a)	(b)	
C+CA+SS	0.033 ± 0.004	0.034 ± 0.002	0.057±0.02	0.14 ± 0.01	0.09 ± 0.02	<0.05
	(c)	(c)	(c)	(a)	(c)	
CA+SS	0.045 ± 0.008	0.028 ± 0.002	0.025±0.01	0.13 ± 0.02	0.09 ± 0.01	<0.05
	(c)	(c)	(c)	(a)	(c)	

C= compost, CA= cyclonic ashes, SS= steel shots. Values are means ± SE of three replicates. At each evaluation moment statistical significantly different values ($p<0.05$) are followed by a different letter

Soil remediation can be considered successful when regulatory authorities pass it as ‘fit for purpose’ (Mench et al., 2003). Zn and Cd concentrations in the leachate were compared to Flemish intervention values for groundwater clean up, *i.e.* 0.5 mg Zn L^{-1} and 0.005 mg Cd L^{-1} (Belgisch Staatsblad, 1996).

Of course, such comparisons must be interpreted carefully because many factors will influence metal concentrations in the percolating water before groundwater layers are reached. Also dilution effects in the receiving groundwater flow need to be considered when evaluating the importance of the percolating metal concentrations. According to our opinion, however, the intervention values can be used as a guideline to estimate the importance of the effects observed. In the leachate of the untreated soil, the Zn concentration exceeded the intervention value for groundwater remediation 400 times at T1 and 117 times at T8. Cadmium concentrations were between 1000 times too high (T1) and 200 times too high (T8) compared to its intervention value (Table 3.1a). After all treatments mean Zn and Cd concentrations remained higher than the intervention values at all moments, but the 95% confidence interval around the mean of the C+CA+SS treatment often came close to or even included the intervention values.

Additives significantly affected the amount of water leaching through the soils (Figure 3.3). Compared with the UNT, water leaching came significantly down by 27%, 38% and 37% in the C+CA, C+CA+SS and CA+SS treatments. The observed reductions can be attributed to an increased water-holding capacity of the soil (WHC) following treatment. It is well known that the WHC of a sandy soil can be increased by compost or clay addition (Brady, 1984). This results in lower percolation rates, which in turn lead to an increased water evaporation from the soil and therefore to lower percolation volumes. The reduction of the amount of leachate only plays a minor role in the overall effect on metal leaching. The contribution of both factors to the total reduction in metal percolation is summarized in Table 3.2. However, the influence of soil treatments on the quantity of leachate can be soil-type dependent. In previous experiments, Vangronsveld *et al.* (1995b) found an increase in the amount of leachate after C+CA addition to a contaminated loamy substrate.

Nevertheless, the increase in water percolation in that study did not eliminate the positive effect of the treatment on metal percolation, since it was overcompensated by strong reductions of Zn and Cd concentrations in the percolate.

Table 3.2 Relative contributions of decreases in metal concentration and decreases in the amount of percolation water, to the total reduction in the Zn and Cd percolation.

Metal	Soil treatment	Total reduction in metal percolation	Contribution due to reduced metal concentrations in the percolate	Contribution due to reduced water percolation
Zn	C	86.0%	82.1%	3.9%
	C+CA	95.3%	93.3%	2.1%
	C+CA+SS	99.2%	98.6%	0.6%
	CA+SS	99.0%	98.3%	0.7%
Cd	C	83.8%	79.2%	4.6%
	C+CA	93.9%	91.4%	2.5%
	C+CA+SS	98.3%	97.0%	1.3%
	CA+SS	97.2%	95.4%	1.8%

C= compost, CA= cyclonic ashes, SS= steel shots.

Cu and Pb percolation

In comparison with Zn and Cd, leaching of Cu and Pb from the UNT was very low. At the end of the experimental period only 0.015% of the total Cu amount in the lysimeter and 0.006% of the total Pb amount had leached out of the soil. In contrast with the overall reductions of Zn and Cd percolation, the addition of C or C+CA to the contaminated soil increased Cu and Pb percolation (Figure 3.2b). At the end of the experimental period the total amounts of Cu percolated from C- and C+CA-treated soils were respectively 17 times and 4.4 times higher than the amount percolated from the UNT. For Pb, approximately 30-fold and 3.7-fold increases were observed after the same treatments. Very accurate calculations of the increases however were not possible due to several observations below detection limit (<0.05 mg L^{-1}).

Since the amount of water percolation was reduced by all treatments (Figure 3.3) the observed increases in Cu and Pb leaching must be attributed to a concentration effect. Only the CA+SS and C+CA+SS treatments were effective in maintaining the Cu percolation at the same level as the UNT. Because of the lack of exact values for Pb concentrations in the UNT, it is unclear whether or not the observed increases in Pb concentrations after these treatments are fully compensated by the reduced leachate amount. The mechanisms of Cu and Pb mobilization were not determined, but their mobility may have been enhanced by complexation with soluble organic ligands. Addition of the alkaline and Ca-rich cyclonic ashes to a similar metal contaminated soil did not directly influence dissolved organic carbon (DOC) concentrations (Oste *et al,*. 2002). It is therefore suggested that soluble (and/or colloidal) organic molecules, originating from the compost addition at the start of the experiment, have complexed and mobilized Cu and Pb. Stability constants of metallo-organic complexes, although quite variable depending on pH and other properties of the medium, are generally higher for Cu and Pb than those for Zn and Cd (Mc Bride and Martinez, 2000; Kabata-Pendias and Pendias, 1984) which can explain the differential leaching behaviour of these elements. The Cu and Pb 'mobilization effect' became gradually stronger during the first months of the experiment, but after 47 weeks, leaching of these elements dropped drastically (Table 3.1b). Exhaustion of the initial pool of soluble organic compounds is suggested to be responsible for this. The increased mobility of Cu was partly compensated by CA and fully compensated by addition of CA+SS at all moments. For Pb, both treatments (C+CA and C+CA+SS) showed an incomplete compensation potential at T1 and T4. At T8, Pb concentrations after all treatments (including C alone) were restored to a low level (<0.05 mg L^{-1}). Even in the CA+SS treatment without compost a slight increase in Pb concentration was observed at T1 and T4. This eventually could be related to the relatively high Pb concentration in SS (Chapter 2) (Ruttens et al., 2006a).

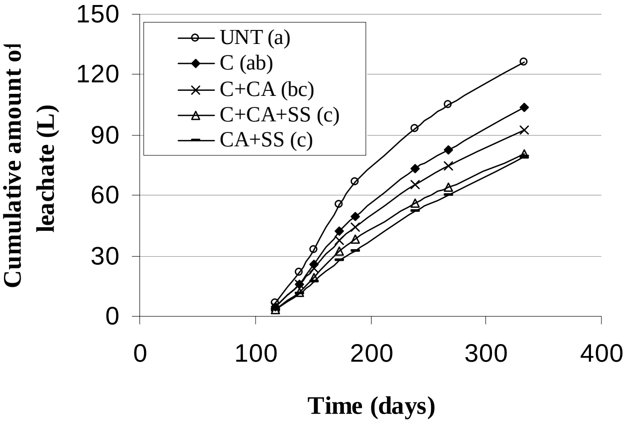


Figure 3.3 Influence of the soil treatments on the amount of water percolating trough the soil. Values are means of three replicates. Treatments followed by a different letter are statistical significantly different ($p<0.05$) based on endpoint analysis.

In the UNT percolate the Cu concentration was below the Flemish intervention value for groundwater clean up (0.1 mg L^{-1}) (Belgisch Staatsblad, 1996). In the percolates of the treated soils, only CA+SS and C+CA+SS kept Cu concentrations below the intervention value at all moments. The Pb concentration in the UNT was below our detection limit (<0.05 mg L^{-1}), but this detection limit was higher than the intervention value (0.02 mg L^{-1}). With exception of T8 (where Pb concentrations dropped below the detection limit for all treatments), the Pb intervention value was clearly exceeded after all treatments. The strongest effect was present in the C treatment at T4 (Table 3.1b).

Surprisingly, the observed increases of Cu and Pb concentrations in the percolate after C and C+CA addition were not reflected in the results of $Ca(NO_3)_2$ extractions (Chapter 2) (Ruttens et al., 2006a). Exchangeable fractions of these elements decreased in all treatments, including C alone. Cu and Pb in the percolate may be associated mainly with a colloidal fraction, which precipitates under the conditions of high ionic strength in the $Ca(NO_3)_2$ extract. When investigating metal speciation in landfill-leachate polluted groundwater, Jensen *et al.* (1999) also found that 85% to 95% of Cu and 96% to 99% of Pb were associated with the colloidal fraction. On the other hand, at T1 higher exchangeable Cu and Pb fractions were observed after C+CA+SS addition than after CA+SS; consequently we assume that not all colloidal metal organic complexes precipitated in the extract. Compost addition in combination with CA+SS led to an extra reduction in exchangeable Zn and Cd fractions compared to the same treatment without compost, and this effect was in contrast with the leaching data where both treatments did not differ significantly. Therefore, also for these elements, compost addition may result in a limited leachable colloidal fraction.

In any case, these data indicate that caution must be paid when organic matter additions are considered in the context of phytostabilization projects of metal contaminated soils. Although C addition reduced the percolation of Zn and Cd, which are highly mobile contaminants in the present soil, it at least temporarily increased the mobility of less available metals like Cu and Pb. The combined CA+SS addition, overall, was able to compensate for these increases, whereas, at least for Cu, CA was not (Figure 3.2b).

Further monitoring of the lysimeters will deliver more information on the influence of a closed vegetation cover on metal leaching and on the long-term sustainability of the treatments.

3.4 Conclusions

- After C treatment, Zn and Cd percolation during the first year decreased respectively 86.0% and 83.8%, but Cu and Pb percolation increased respectively 17 and more than 30 times compared to the UNT. Copper and Pb concentrations in the leachate of the C treated soil raised to values far above Flemish limit values for groundwater remediation.

- C+CA+SS treatment displayed the best efficiency for the amendment assisted phytostabilization of the Overpelt soil. In addition to strong reductions in Zn and Cd percolation (99.2% and 98.3% respectively) this treatment allowed the addition of C to improve soil characteristics, without overall increases of Cu leaching. When the same treatment was applied without compost, leaching data were similar but, as shown by Ruttens et al. (2006a), plant responses were less positive. The total effect on Pb leaching after these treatments could not be estimated exactly because the concentration in the UNT percolate was below detection limit.
- In the combined C+CA treatment, CA was not able to fully compensate the increases in Cu leaching caused by C. Plant responses after this treatment were intermediate between the C and C+CA+SS treatments (Chapter 2) (Ruttens et al., 2006a).
- These results generally show that attention should be paid to Cu and Pb mobility when organic matter additions are considered for phytostabilization of metal contaminated soils.

PART II
References

Ahlberg, G., Gustafsson, O., Wedel, P., 2006. Leaching of metals from sewage sludge during one year and their relationship to particle size. Environ. Pollut. *In Press.*

Alloway, B.J., 1995. Heavy metals in soils. Blackie Academic and Professional, London, U.K.

Álvarez-Ayuso, E., García Sánchez, A., 2003. Palygorskite as a feasible amendment to stabilize heavy metal polluted soils. Environ. Pollut. 125: 337-344.

Basta, N.T., Gradwohl, R., Snethen, K.L., Schroder, J.L., 2001. Chemical immobilization of lead, zinc and cadmium in smelter-contaminated soils using biosolids and phosphate rock. J. Environ. Qual. 30: 1222-1230.

Basta, N.T., McGowen, S.L., 2004. Evaluation of chemical immobilization treatments for reducing heavy metal transport in a smelter-contaminated soil. Environ. Pollut. 127: 73-82.

Belgisch Staatsblad, 1996. Decree of the Flemish government of 5 march 1996 concerning the Flemish regulation on soil remediation (Vlarebo). Belgisch Staatsblad - Moniteur Belge, 27.03.1996. (modified on repeated occasions, latest change BS 5.08.2004).

Belgisch Staatsblad, 1999. Belgian Royal Decree of 08.02.1999 concerning the trade and use of products for animal nutrition. Belgisch Staatsblad-Moniteur Belge 21.04.1999.

Berti, W.R., Cunningham, S.D., 1997. In place inactivation of Pb in Pb contaminated soils. Environ. Sci. Technol. 31: 1359-1364.

Boisson, J., Mench, M., Vangronsveld, J., Ruttens, A., Kopponen, P., De Koe, T., 1999b. Immobilization of trace metals and arsenic by different soil additives: evaluation by means of chemical extractions. Commun. Soil Sci. Plant Anal. 30: 365-387.

Boisson, J., Ruttens, A., Mench, M., Vangronsveld, J., 1999a. Evaluation of hydroxyapatite as a metal immobilizing soil additive for the remediation of polluted soils. Part 1. Influence of hydroxyapatite on metal exchangeability in soil, plant growth and plant element concentrations. Environ. Pollut., 104: 225-233.

Bolan, N.S., Adriano, D.C., Mani, P.A., Duraisamy, A., 2003. Immobilization and phytoavailability of cadmium in variable charge soils. II Effect of lime addition. Plant Soil 251: 187-198.

Bouwman, L.A., Bloem, J., Römkens, P.F.A.M., Boon, G.T., Vangronsveld, J., 2001. Beneficial effects of the growth of metal tolerant grasses on biological and chemical parameters in copper and zinc contaminated sandy soils. Minerva Biotec 13: 19-26.

Bouwman, L.A., Vangronsveld, J., 2004. Rehabilitation of the nematode fauna in a phytostabilized heavily zinc-contaminated, sandy soil. J. Soils Sediments 4: 17-23.

Brady, N.C., 1984. The nature and properties of soils. Macmillan, New York, USA.

Brown, S.L., Chaney, R.L., Hallfrish, J., Ryan, J.A., Berti, W.R., 2004. *In situ* soil treatments to reduce the phyto- and bioavailability of lead, zinc and cadmium. J. Environ. Qual. 33: 522-531.

Brown, S.L., Chaney, R.L., Hallfrish, J.G., Xue, Q., 2003a. Effect of biosolid processing on the availability of lead in an urban soil. J. Env. Qual. 32: 100-108.

Brown, S.L., Henry, C.L., Chaney, R.L., Compton, H., De Volder, P.S., 2003b. Using municipal biosolids in combination with other residuals to restore metal-contaminated mining area's. Plant Soil 249: 203-215.

Brümmer, G.W., 1986. Heavy metal species, mobility and availability in soils. *In*: The importance of chemical speciation in environmental processes. Bernard M et al. (eds). Springer-Verlag, Berlin. Germany.

Chlopecka, A., Adriano, D.C., 1997. Influence of zeolite, apatite and Fe-oxide on Cd and Pb uptake by crops. Sci. Total Environ. 207: 195-206.

Conover, W.J., 1980. Practical nonparametric statistics. Wiley, New York, USA.

Cunningham, S.D., Berti, W.R., Huang, J.W., 1995. Phytoremediation of contaminated soils. Trends Biotechnol. 13: 393-397.

De Temmerman, L., Vanongeval, L., Boon, W., Hoenig, M., Geypens, M., 2003. Heavy metal content of arable soils in northern Belgium. Water, Air Soil Pollut. 148: 61-76.

Dua, V., Van Geel, P.L.B.A., 2002. Declaration of intention (Flanders-the Netherlands) related to soil contamination in the Kempen area (translated from Dutch). Document signed by the Flemish and Dutch governments.

Geebelen, W., Adriano, D.C., van der Lelie, D., Mench, M., Carleer, R., Clijsters, H., Vangronsveld J., 2003. Selected bioavailability assays to test the efficacy of amendment-induced immobilization of lead in soils. Plant Soil 249: 217-228.

Geebelen, W., Sappin-Didier, V., Ruttens, A., Yperman, J., Carleer, R., Bongué-Boma Kwèlè, Mench, M., Vangronsveld, J., 2006. Evaluation of cyclonic ash and commercial Na-silicates for heavy metal immobilization purposes in contaminated soils in Flanders. Environ. Pollut. *In Press*.

Hamon, R.E., McLaughlin, M.J., Cozens, G., 2002. Mechanisms of attenuation of metal availability in in situ remediation treatments. Environ. Sci. Technol. 36: 3991-3996.

Hettiarachchi, G.M., Pierzynski, G.M., 2002. *In situ* stabilization of soil lead using phosphorus and manganese oxide: influence on plant growth. J. Environ. Qual. 31: 564-572.

Hettiarachchi, G.M., Pierzynski, G.M., Ransom, M.D., 2000. *In situ* stabilization of soil lead using phosphorus and manganese oxide. Environ. Sci. Technol: 34: 4614-4619.

Hogervorst, J., Plusquin, M., Vangronsveld, J., Nawrot, T., Cuypers, A., Van Hecke, E., Roels, H.A., Carleer, R., Staessen, J.A., 2006. House dust as possible route of environmental exposure to cadmium and lead in the adult general population. Environ. Res. *In Press*.

Houba, V.J.G., Lexmond, T.M., Novozamsky, I., Van der Lee, J.J., 1996. State of the art and future developments in soil analysis for bioavailability assessment. Sci. Total Environ. 178: 21-28.

Huang PM (1997). Environmental impact of metal-organic interactions. *In*: Biogeochemistry of trace metals. Adriano D.C. Chen Z., Yang S, Iskandar I.K. (eds.). Science Reviews, Northwood, USA.

Ide, G., 1992. Cadmium in de Belgische bodem: een situatieschets. Bodem 3: 119-121.

Jensen, D.I., Ledin, A., Christensen, T.H., 1999. Speciation of heavy metals in landfill-leachate polluted groundwater. Water Res. 33: 2642-2650.

Iskandar, I.K., Adriano, D.C., 1997. Remediation of soils contaminated with metals: a review of current practices in the USA. *In:* Remediation of soils contaminated with metals. Iskandar I.K., Adriano D.C. (eds.). Science Reviews, Northwood, USA.

Kabata-Pendias, A., 1993. Behavioural properties of trace metals in soils. Applied Ecochemistry 2: 3-9.

Kabata-Pendias, A., Pendias, H., 1984. Trace elements in soils and plants. CRC Press. Boca Raton, FL, USA.

KMI (1998). Meteorological data provided by 'Koninklijk Meteorologisch Instituut van België' (Royal Meteorological Institute of Belgium). Brussels. Belgium.

Lee, D.H., Kim, Y.S., Lee, C.B., 2001. The inductive response of the antioxidant enzymes by salt stress in the rice (*Oryza sativa L.*). J. Plant Physiol. 158: 737-745.

Lee, D.H., Lee, C.B., 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Science 159: 75-85.

Lombi, E., Hamon, R.E., McGrath, S.P., McLaughlin, M.J., 2003. Lability of Cd, Cu and Zn in polluted soils treated with lime, beringite and red mud and identification of a non-labile colloidal fraction using isotopic dilution techniques. Environ. Sci. Technol. 28: 1219-1228.

Lombi, E., Zhao, F., Wieshammer, H., Zhang, G., McGrath, S.P., 2002. *In situ* fixation of metals in soils using bauxite residue: Chemical assessment. Environ. Pollut. 118:445-452.

Martinez, C.E., Mc Bride, M.B., 1999. Dissolved and labile concentrations of Cd, Cu, Pb and Zn in aged ferrihydrite-organic matter systems. Environ. Sci. Technol. 32: 743-748.

Mc Bride, M., Sauvé, S., Henderschot, W., 1997. Solubility control of Cu, Zn, Cd and Pb in contaminated soils. European Journal of Soil Sciences 48: 337-346.

McBride, M.B., Martinez, C.E., 2000. Copper phytotoxicity in a contaminated soil: Remediation tests with adsorptive materials. Environ. Sci. Technol. 34: 4386-4391.

Meers, E., Vandecasteele, B., Ruttens, A., Vangronsveld, J., Tack, F.M.G., 2006. Comparative screening of five willow species for phytoextraction of heavy metals. J. Exp. Environ. Bot. *In Press*.

Mench, M., Amans, V., Arrouays, D., Sappin-Didier, V., Fargues, S., Gomez, A., Löffler, M., Masson, P., 1997. A study of additives to reduce the availability of Pb in soil to plants. *In*: Remediation of soils contaminated with metals. Iskandar I.K., Adriano D.C. (eds), Science Reviews, Northwood, USA.

Mench, M., Vangronsveld, J., Beckx, C., Ruttens, A., 2006. Progress in assisted natural remediation of an arsenic contaminated agricultural soil. Environ. Pollut. *In Press*.

Mench, M., Bussière, S., Boisson, J., Castaing, E., Vangronsveld, J., Ruttens, A., De Koe, T., Assunçao, A., Manceau, A., 2003. Progress in remediation and revegetation of the barren Jales gold mine spoil after in situ treatments. Plant Soil 249: 187-202.

Mench, M., Vangronsveld, J., Didier, V., Clijsters, H., 1994. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. Environ. Pollut. 86: 279-286.

Mench, M., Vangronsveld, J., Lepp, N., Edwards, R., 1998. Physico-chemical aspects and efficiency of trace element immobilization by soil amendments. *In*: Metal contaminated soils: *in situ* inactivation and phytorestoration. Vangronsveld, J., Cunningham, S. (eds.). Springer-Verlag, New York, USA.

Mench, M., Vangonsveld, J., Clijsters, H., Lepp, N.W., Edwards, R., 2000. *In situ* metal immobilization and phytostabilisation of contaminated soils. *In*: Phytoremediation of contaminated soils and water. Terry, N., Banuelos, G. (eds.). Lewis Publishers, CRC Press, Boca Raton, FL., USA.

Nawrot, T., Plusquin, M., Hogervorst, J., Roels, H.A., Celis, H., Thijs, L., Vangronsveld, J., Van Hecke, E., Staessen, J.A., 2006. Environmental exposure to cadmium and risk of cancer. Lancet Oncol. 7(2): 119-126.

Oste, L.A., Lexmond, T.M., Van Riemsdijk, W.H., 2002. Metal immobilization using synthetic zeolites. J. Environ. Qual. 31: 813-821.

Pierzynski, G.M., 1997. Strategies for remediating trace-element contaminated sites. In: Remediation of soils contaminated with metals. Adriano, D.C. (ed.). Science Reviews, Northwood, USA.

Pierzynski, G.M., Schwab, A.P., 1993. Bioavailability of zinc, cadmium, and lead in a metal contaminated alluvial soil. J. Environ. Qual. 22: 247-254.

Römkens, P.F., de Vries, W., 1995. Acidification and metal mobilization: effects of land use changes on Cd mobility. *In*: Acid rain research: do we have enough answers? Hey, G.J., Erisman, J.W. (eds.). Elsevier Science, Amsterdam, The Netherlands.

Ruby, M.V., Nicholson, A., 1994. In situ formation of lead phosphates in soils as a method to immobilize lead. Environ. Sci. Technol. 28:646-654.

Ruttens, A., Colpaert, J., Mench, M., Boisson, J., Carleer, R., Vangronsveld, J., 2006b. Phytostabilization of a metal contaminated sandy soil. II: Influence of compost and/or inorganic metal immobilizing soil additives on metal leaching. Environ. Pollut. *In Press*.

Ruttens, A., Mench, M., Colpaert, J., Boisson, J., Carleer, R., Vangronsveld, J., 2006a. Phytostabilization of a metal contaminated sandy soil. I: Influence of compost and/or inorganic metal immobilizing soil additives on phytotoxicity and plant availability of metals. Environ. Pollut. *In Press*.

Ryan, J.A., Scheckel, K., Berti, B.R., Brown, S.L., Casteel, S.W., Chaney, R.L., Hallfrisch, J., Doolan, M., Grevatt, P., Maddaloni, M., Moseby, D., 2004. Reducing children's risk to soil lead: in place inactivation and natural ecological restoration technologies-summary of a field experiment. Environ. Sci. Technol. 38: 18A-24A.

Ryan, J.A., Zhang, P., Hesterberg, J., Chou, J., Sayers, D.E., 2001. Formation of chloropyromorphite in a lead-contaminated soil amended with hydroxyapatite. Environ. Sci. Technol. 35: 3798-3803.

Sappin Didier, V., Mench, M., Gomez, A.N., Lambrot, C., 1997. Use of inorganic amendments for reducing metal bioavailability to ryegrass and tobacco in contaminated soils. *In*: Remediation of soils contaminated with metals. Iskandar I.K., Adriano D.C. (eds.). Science Reviews, Northwood, USA.

SAS Institute, 2004. SAS/STAT User's guide, version 9. SAS Institute Inc., Cary, NC, USA.

Sauvé, S., Mc Bride, M., Henderschot, W., 1998. Soi Solution speciation of lead (II): Effects of organic matter and pH. Soil Sci. Soc. Am. J. 62: 618-621.

Shuman, L.M., Dudka, S., Das., K, 2002. Cadmium forms and plant availability in compost-amended soil. Commun. Soil Sci. Plant Anal. 33: 737-748.

Smolders, E., Brans, K., Földi, A., Merckx, R., 1999. Cadmium fixation I soils measured by isotopic dilution. Soil Sci. Am. J. 63: 78-85.

Staessen, J.A., Roels, H.A., Emerlianov, T., Thijs, L., Vangronsveld, J., Fagard, R., 1999. Environmental exposure to cadmium, forearm density, and risk of fractures: prospective population study. Lancet. 353: 1140-1144.

Swift, R.S., McLaren, R.G., 1991. Micronutrient adsorption by soils and soil colloids. *In*: Bolt, G.H. et al. (eds). Interactions at the soil colloid – soil solution interface. Kluwer, Dordrecht, The Netherlands.

Van Assche, F., Cardinaels, C., Clijsters, H., 1988. Induction of enzyme capacity in plants as a result of heavy metal toxicity: dose response relations in *Phaseolus vulgaris* L., treated with Zn and Cd. Environ. Pollut. 52: 103-115.

Van Assche, F., Clijsters, H., 1990a. A biological test system for the evaluation of the phytotoxicity of metal-contaminated soils. Environ. Pollut. 66: 157-172.

Van Assche, F., Clijsters, H., 1990b. Effects of metals on enzyme activity in plants. Plant Cell Environ. 13: 195-206.

van der Lelie, D., Schwitzguébel, J.P., Glass, D.J., Vangronsveld, J., Baker, A., 2001. Assessing phytoremediation's progress in the United States and Europe. Env. Sci. Technol. 35: 447A-452A.

Vangronsveld, J., Clijsters, H., 1992. A biological test system for the evaluation of metal phytotoxicity and immobilization by additives in metal contaminated soils. *In*: Metal compounds in environment and life, 4. Interrelation between chemistry and biology. Merian, E., Haerdi, W. (eds.). Science Reviews Inc., Wilmington, USA.

Vangronsveld, J., Colpaert, J., Van Tichelen, K., 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environ. Pollut. 94: 131-140.

Vangronsveld, J., Cunningham, S., 1998. Introduction to the concepts. *In:* Metal contaminated soils: *in situ* inactivation and phytorestoration of. Vangronsveld, J., Cunningham, S. (eds.). Springer-Verlag, New York, USA.

Vangronsveld, J., Ruttens, A., Clijsters, H., 1999. The use of cyclonic ashes of fluidized bed burning of coal mine refuse for long-term immobilization of metals in soils. *In*: Biogeochemistry of trace metals in coal and coal combustion byproducts. Sajwan, K.S. et al. (eds.). Kluwer Academic/Plenum Publishers, New York, USA.

Vangronsveld, J., Sterckx, J., Van Assche, F., Clijsters, H., 1995b. Rehabilitation studies on an old non-ferrous waste dumping ground: effects of revegetation and metal immobilization by beringite. J. Geochem. Expl. 52: 221-229.

Vangronsveld, J., Van Assche, F., Clijsters, H., 1995a. Reclamation of a bare industrial area contaminated by non-ferrous metals: *in situ* metal immobilization and revegetation. Environ. Pollut. 87: 51-59.

Vassilev, A., Schwitzguébel, J.-P., Thewys, T., van der Lelie, D., Vangronsveld, J., 2004. The use of Plants for remediation of metal-contaminated soils. The Scientific WorldJournal 4: 9-34.

Wenzel W.W., Blum W.E.H., 1997. Effect of sampling, sample preparation and extraction techniques on mobile metal fractions. *In*: Biogeochemistry of trace metals. Adriano D.C., Chen Z., Yang S., Iskandar I.K. (eds.). Science Reviews, Northwood, USA.

Yang, J., Mosby, D., Casteel, S.W., Blanchar, R.W., 2001. Lead immobilization using phosphoric acid in a smelter contaminated urban soil. Environ. Sci. Technol. 35: 3553-3559.

PART III

Optimization of a phytotoxicity test (PlantTox)

CHAPTER 4

Influence of physico-chemical soil variables in uncontaminated field soils on the endpoints of a phytotoxicity test (PlantTox)

A. Ruttens, A. De Vocht, W. Geebelen, E. Meers, K. Wouters, M. Mench, R. Carleer, and J. Vangronsveld.

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Abstract

A phytotoxicity test with bean plants (PlantTox), designed to assess soil metal toxicity, was performed on 25 soil samples collected in Flanders (Belgium), in areas with expected low levels of trace element deposition (background). The influence of physico-chemical soil characteristics on morphological and biochemical plant responses was investigated. Biochemical endpoints included the activity of enzymes that are directly or indirectly involved in the anti-oxidative defence systems of plants. Soil pH, cation exchange capacity (CEC), conductivity (EC), organic matter content (OM) and exchangeable contents of Al, Cd, Cu, Fe, Mn, Ni, Pb and Zn were determined. The study aimed to gain insight in the frequent ranges of the test endpoints in non-toxic conditions and to contribute to the determination of the most suited reference level or reference soil for the test when used as a bioassay in risk assessment of contaminated soils. An artificial soil (OECD 207 standard soil for earthworm toxicity) was also included in the soil series.

Multiple regression analysis relating plant responses to physicochemical soil parameters, delivered robust models for all enzymes measured, with R^2 ranging from 0.83 to 0.99. No significant models could be fitted for any of the growth responses measured. OECD standard soil is proposed as a potential candidate general reference soil in the test, at least for soils with pH>4. A soil toxicity-

classification system is presented based on the use of this standard soil as a negative control in the test, and taking the 90th or 10th percentile, respectively, of enzymatic and growth responses in the reference dataset as upper/lower limit of the first phytotoxicity class (i.e. non toxic). An alternative option is also described, based on the calculation of specific reference levels for each contaminated soil, using our regression models and working with their 95% upper prediction limits.

4.1 Introduction

In the area of chemical regulations, biological toxicity tests are required for effect evaluation of some new or existing chemicals. Several toxicity tests are recognized and standardized in this context e.g. by the Organization for Economic Cooperation and Development (OECD) in Europe, by the Environmental Protection Agency (US-EPA) and the US Food and Drug Administration (US FDA) in the United States and by the International Organization for Standardization (ISO). Currently three terrestrial phytotoxicity tests are recognized by these agencies being a germination test, root elongation and an early seedling growth test. A wide variety of plant species can be used for the tests, belonging to well-defined families, which vary among agencies (Fletcher, 1991; Wang and Freemark, 1995).

A tendency exists to use the recognized toxicity tests also as bioassays to assess the ecotoxicity of contaminated field soils (Frische, 2003; Marwood et al., 1998; Schultz et al., 2004; Simini et al., 1995). In a bioassay a comparison is made between the biological responses of a test organism exposed to a potentially contaminated soil sample and to an uncontaminated reference soil (negative control). Sometimes a positive control sample, with a high (known) toxicity response is also included (Norton, 1996). Bioassays are indispensable tools to complement soil chemical analysis, in site-specific risk assessments and in the evaluation of remediation programs (Debus and Hund, 1997; Meers et al, 2006; Thomas et al., 1986). When phytotoxicity tests are applied as bioassays, to evaluate the toxicity of potentially contaminated field samples, the biological responses measured in the tests, may not only be affected by the presence of potentially toxic substances in the soil, but also by some physico-chemical characteristics (e.g. soil texture, nutrient status, pH,...) inherently connected to soil type or to land use and cropping history. A reliable and sensitive interpretation of results therefore depends to a large extent on the choice of an appropriate reference soil (Frische, 2003). This choice should be based on knowledge of the main soil properties that influence test results on reference soils and for example an additional check of the soil sample to be assessed and the reference soil for these properties before the test start.

The influence of the soil nutrient status on plant growth responses, which are used as endpoints in the recognized toxicity tests mentioned above, is generally recognized. Even if it is theoretically possible to adjust nutrient levels of the contaminated soil to the level of the reference, this would be complex and even not desirable, because changed nutrient levels may directly or indirectly affect soil toxicity (Gong et al., 2001).

Alternative phytotoxicity endpoints have been described in literature, including e.g chlorophyll fluorescence (Hulsen, 2002; Krugh and Miles, 1996; Marwood et al., 2001), enzyme capacities (Byl and Cline, 1991; Van Assche and Clijsters, 1988, 1990a) and fatty acid compositions (Verdoni et al., 2001). As far as we know, data on the influence of edaphic factors on these biochemical responses have not yet been published.

Gong et al. (2001) pointed the need to evaluate and refine existing phytotoxicity tests to increase their applicability and suitability for use as bioassays. The present study aims to contribute to the refinement of a phytotoxicity test with bean plants (*Phaseolus vulgaris*) (Van Assche and Clijsters, 1990a; Vangronsveld and Clijsters, 1992), in order to improve its applicability as a bioassay. The test is based on the analysis of growth parameters and biochemical plant responses, in particular enzyme activities that are directly or indirectly involved in the anti-oxidative defence systems of plants, including guaiacol peroxidase (GPOD; E.C. 1.11.1.7), malic enzyme (ME; E.C. 1.1.1.40), glutamate dehydrogenase (GlDH; E.C. 1.4.1.2) and iso-citrate dehydrogenase (ICDH; E.C. 1.1.1.42). These antioxidative enzymes are functional in plants even under normal conditions, and control the level of reactive oxygen species (ROS). Their activity, however, was found to increase in plants exposed to metals, and other stress factors, such as drought, salt stress, chilling, heat chock, air pollutants (ozone and SO_2), pathogen attack, etc., which induce an increased production of reactive oxygen species (ROS) (Mittler, 2002). These increases in enzyme capacities play a role in the plant stress metabolism (Foyer and Noctor, 2005). Studies with bean plants (*Phaseolus vulgaris L. cv Limburge vroege)* have shown that the extent of enzyme induction depended on the metal concentration in the plant (Van Assche et al., 1988; Mench et al., 2000). Because of this strong relationship the activity of these enzymes was considered to reflect the degree of metal toxicity the plant is exposed to. These observations in combination with metal specific changes in the isoperoxidase pattern, have been the basis for using enzymatic parameters as responses in a phytotoxicity test. Detailed information on the concept and design of the test can be read in Van Assche and Clijsters (1990b) and Vangronsveld and Clijsters, (1994). Initially the test aimed to detect metal toxicity, but since many different stress factors involve oxidative stress, all these stress factors are likely to affect the test endpoints. When applied as a bioassay on soil samples, under controlled environmental conditions, the test may therefore be able to detect phytotoxicity caused by a wide variety of contaminants.

Over the last decade this bioassay was often applied to assess the reduction of soil phytotoxicity after amendment with metal immobilizing soil additives such as, Fe- and Mn oxides, steel shots (Fe^0, iron grit), cyclonic ashes, hydroxyapatite, zeolite, bentonite and basic slags (Vangronsveld et al., 1995a, 1996; Mench et al., 1994, 2000; Geebelen et al., 2002). According to the proposed toxicity classification system, soils are classified in toxicity class 2 (slightly toxic) when enzyme responses increase by a factor 1.25 or 1.75 compared to the control. Our main concern related to this system is that such differences also seem to exist between different control conditions. In some tests for example, enzyme capacities in plants grown on a reference soil amended with a metal immobilizing soil additive (cyclonic ashes) were significantly lower than those on unamended reference soils (Geebelen et al., 2002; Vangronsveld et al., 1995b). In other cases test plants grown on detoxified contaminated soils (containing a metal immobilizing additive) had lower enzyme capacities than plants grown on the reference soil (a sandy, uncontaminated soil) (Geebelen et al., 2002; Vangronsveld et al., 1995a). The differences were enzyme dependent, but could reach factors ranging from 1.5 to 3. Also morphological parameters were sometimes significantly lower in plants grown on the reference soil than on a detoxified contaminated soil. These observations suggest that plant responses in the test indeed are subject to influences, other than metal toxicity.

In the present study a series of 25 potential reference soils with different texture and with various land use and cropping history, were collected throughout Flanders (Belgium). Physico-chemical soil characteristics (pH, cation exchange capacity (CEC), conductivity (EC), organic matter content (OM), total and exchangeable metal contents of Zn, Cd, Cu, Pb, Ni, Al, Fe and Mn) were determined and the phytotoxicity test with bean plants was applied as described by Van Assche and Clijsters (1990a). The aim of the study was:

- first, to gain insight in the frequent values of the endpoints of the test obtained on reference soils,
- second, to evaluate the influence of the soil characteristics on the endpoints of the test,
- and third, to determine the most suited reference soil/level for the test to allow a more reliable interpretation of test results.

An artificial soil was included in the soil series (OECD 207 standard soil for earthworm toxicity) because this artificial soil, with standardized composition, could also serve as a potential reference.

4.2 Materials and methods

4.2.1 Collection of soil samples

Twenty-five soil samples were collected throughout various geographical regions of Flanders, leading to a soil series including sandy, loamy and clayey soils (Table 4.1). The soils were collected in areas with expected low levels of trace element deposition and are further referred to as 'reference soils'. The Belgian soil map (scale 1:20 000) was used to select the sampling locations. Locations were only sampled when the soil type indicated on the map corresponded to the field situation based on interpretations by a pedologist. Per location 20 sub-samples were taken with a spade from the top-soil layer (0-25 cm) and subsequently homogenized. Attention was paid to collect soils with different land uses (mainly arable land and pastures but also fallow land and a few forest soils) in order to reach a large spectrum in soil properties. Samples were air dried at room temperature prior to analysis.

An artificial soil was synthesized in analogy with the OECD 207 standard soil for earthworm toxicity (OECD, 1984): 70% quartz sand (Bricobi white sand, Brico, Belgium), 20% kaolinite clay (ECC international, UK), 10% *Sphagnum* peat (Agratorf white *Sphagnum* peat, Agrofino® products, Arendonk, Belgium) (grounded and sieved on 1 mm using a sample mill Retch, SM100), and adjusted to pH 6 with $CaCO_3$ (Vel, analytical grade $CaCO_3$). This artificial soil was added to the soil series.

4.2.2 Determination of soil characteristics

pH

Soil pH was determined in 1M KCl in a soil: solution ratio of 1:5 after shaking (60 rpm) for 2h and after filtration over a filter paper (Whatman n°1). A standard pH meter (Radiometer, PHM82) with glass electrode (Hamilton, LiqPlast) was used for the measurements after calibration with pH 4.0 and pH 7.0 buffer solution.

Conductivity(EC)

Soil conductivity was measured (WTW, LF 537 conductivity meter, TetraCon 96 electrode; calibrated with 0.1 M KCl solution) in a soil:Milli-Q water ratio of 1:5 (Millipore, Milli-Q Water Purification System) after shaking for 18h and after filtration over a filter paper (Whatman, n°1).

Cation Exchange capacity (CEC)

CEC was determined, using an NH_4-acetate percolation method. Ten g air-dried soil was mixed with 25 g white quartz sand (Aldrich, -50+70 mesh) and filled into glass columns (Ø 2 cm). The soil adsorption complex was saturated with $NH4^+$ ions (250 ml 1M NH_4Ac), the excess of $NH4^+$ was washed with ethanol (10x40ml), and after quantitative removal by K^+ ions (500 ml 1M KCl), $NH4^+$

was determined colorimetrical with a Flame Injection Analyser (FIA, Lachat Instruments, QuickChem 8000 series) using Quickchem® Method 10-107-06-1-K.

Organic matter (OM)

Soil organic matter was determined using the method described by Walkley & Black (Allison, 1965) estimating the soil humus content based on carbon determination after C oxidation under standardized conditions with potassium dichromate in sulphuric acid medium.

Total and exchangeable metal content ($M_{tot.}$, $M_{exch.}$)

Total metal content (*aqua regia*) of the homogenized soil sample was determined using a microwave digestion (Milestone, 1200 MEGA). Exchangeable soil metal contents were measured in $Ca(NO_3)_2$ -extracts (0.1 M; 2h shaking, filtration through Whatman filters n°40). Metals in the extracts were analyzed by Induced Coupled Plasmaspectrometry (ICP, Perkin Elmer, Optima 3000 DV). Blanks, and a certified soil sample of the Community Bureau of Reference (BCR material n° 143, trace elements in a sewage sludge amended soil) were added for quality control of the data.

For total soil metal concentrations attention was focused on Zn, Cd, Cu, Pb and Ni because of their frequent occurrence as contaminants in Flemish soils. Exchangeable contents were in addition determined for Mn, Al, and Fe, because we assumed these metals could potentially determine plant responses in our soil series, due to their high mobility in acid soils and their possible role in oxidative stress reactions.

4.2.3 Cultivation of test plants and measurement of plant responses

Bean plants (*Phaseolus vulgaris* L. cv Limburgse vroege; AVEVE, Belgium) were grown for two weeks under controlled environmental conditions (Sanyo, plant growth chamber SGC170.CFX.J., 22 °C, 65 % relative humidity, 12 h light, photosynthetic active radiation: 150 µmol m^{-2} s^{-1}) and watered daily with distilled water up to 40% (first week) and 60% (second week) of the soil water holding capacity. Seeds were sown in polyethylene pots of 400 ml (4 pots per treatment, 4 plants/pot) after one day vernalization (cold treatment at 4°C) and 4h imbibition at room temperature. Two weeks after sowing, morphological parameters (total above ground biomass, shoot length, primary leaf area, root weight) were measured for each plant. Leaf and root material was rinced with distilled water prior to sampling. For enzyme analysis, samples (1 g fresh weight) of primary leaves and roots were frozen immediately in liquid nitrogen and stored at –70°C. All leaves were cut in two over the middle vain, and subsequently divided in two parts for element and enzyme analysis respectively, to avoid unequal distribution of old and young tissue in the samples. Root samples were obtained as homogenized sub-samples form the entire root after cutting it in 1 cm pieces.

Within 3 weeks following harvest, frozen plant tissue was homogenized with a Polytron PT 3000 homogenizer in ice-cold Tris-HCl buffer (0.1 M, pH 7.8) containing 1mM EDTA, 1mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (5 ml buffer g FW^{-1}). The homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 20000g (4°C) (centrifuge Jouan, MR22i). Subsequently the capacity (i.e. the potential activity measured *in vitro* under non-limiting conditions of substrate and coenzyme) of the following enzymes was measured spectrophotometrically (Shimadzu, UV-1602) at 25°C in the supernatant as described by Van Assche et al. (1988): guaiacol peroxidase (GPOD; E.C. 1.11.1.7), malic enzyme (ME; E.C. 1.1.1.40), glutamate dehydrogenase (GlDH; E.C. 1.4.1.2) and iso-citrate dehydrogenase (ICDH; E.C. 1.1.1.42). Enzyme capacity was expressed in milli-Units (mU) per gram FW. These enzymes are related to the plants stress responses (Van Assche and Clijsters, 1990b) and will be further referred to as ‘stress enzymes’.

4.2.4 Statistical analysis

Correlation matrices were obtained using the STATISTICA software package (Basic statistics and tables module) (STATISTICA, 1994). Multiple regression models were constructed using the proc REG statement of the SAS software package (SAS, 2004). All analyses were done at the 95% confidence level. We started with a model including all covariates and deleted manually, step by step, the non-significant variables ($p<0.05$) until a satisfactory fit was found. Afterwards quadratic terms and interactions between the linear effects were added when they showed to be significant ($p<0.05$). A full stepwise backward approach would include all covariates, quadratic and interaction terms in the initial model, but due to the large number of variables this was not feasible in our case. For all retained models the normality of residuals as well as their heteroscedasticity was confirmed graphically. Prediction intervals were calculated using Bonferroni type confidence bounds according to the formula 'Prediction Interval' = $Y_h \pm B\ s\{Y_{h(new)}\}$, with $B = t(1-\alpha/2g;\ n-p)$, g the number of interval estimates, n the number of observations, and p the number of parameters used in the model (Neter et al., 1990). Application of this formula implies that the size of the prediction interval increases when an increasing number of predictions are made simultaneously.

4.3 Results

4.3.1 Soil characteristics

Table 4.1 presents general soil characteristics and land use at the different sample locations. Based on land use, samples were classified as arable land, pastures, fallow-land or forest samples. Texture classes represented in the dataset were Z: sand, S: loamy sand, L: sandy loam, P: light sandy loam A: loam and E: clay (for texture classification see Maréchal and Tavernier, 1974). Soil pH

Table 4.1 Soil characteristics and land use at the different sample locations. EC=electric conductivity, CEC=cation exchange capacity, OM=organic matter content (values are means of two measurements).

	Land use	Soil serie*	pH-KCl	EC (µS/cm)	CEC (cmol kg^{-1})	OM (%)
1	garden	Zcg	6.1	388	13.6	9.3
2	field	Zcg	5.1	506	11.8	4.7
3	forest	Zcg	4.0	511	9.7	5.6
4	forest	Zbf	3.1	272	5.4	3.7
5	pasture	Zbf	4.4	319	5.4	3.7
6	field	Zbf	5.0	225	4.7	3.0
7	field	Zaf	5.5	276	10.1	1.8
8	forest	Zap	3.2	222	2.9	1.7
9	fallow	Scf	4.8	319	6.5	1.3
10	field	Scc	5.8	381	7.9	2.1
11	fallow	Scc	7.4	494	9.7	2.4
12	field	Pcc	6.1	443	7.8	2.7
13	field	Pdc	7.2	643	12.6	2.5
14	pasture	Lhc	4.4	455	10.4	3.3
15	forest	Lhc	4.0	739	19.4	6.8
16	field	Lbp	7.4	645	17.1	4.0
17	fallow	Ldc	5.7	631	7.1	2.1
18	fallow	Ldc	5.3	578	10.1	2.7
19	field	Ldx	7.1	862	22.3	3.2
20	field	Aba	7.2	908	17.9	3.3
21	field	Aba	6.3	745	19.1	2.5
22	pasture	Aba	5.3	683	14.8	2.1
23	field	Aba	7.5	593	11.9	2.4
24	pasture	El	7.3	1361	40.0	9.2
25	pasture	Ebb	6.9	1154	40.0	11.5
26	OECD	/	5.9	512	14.2	5.6

*Soil series presented according to the Belgian soil map and classification system. Z=sand, S=loamy sand, L=sandy loam, P= light sandy loam A=loam and E=clay; second and third letter of the soil series refer to soil drainage class and soil profile respectively) (Maréchal and Tavernier, 1974).

(pH-KCl) varied from 3.1 to 7.5 and conductivity (EC) ranged from 222 to 1361 µS/cm. Organic matter (OM) and cation exchange capacity (CEC) respectively fell in the ranges 1.3%-11.5% (OM) and 2.9-40 cmol kg^{-1} (CEC). Total metal contents of most soils (except soils 16, 20, 25) were in the following ranges (mg kg^{-1} DW): Zn: 11-68, Cu: 2-25, Cd: 0.1-0.6, Pb: 13-49. These values are close to or in the range of Flemish background values for metals in soils. According to the

Flemish legislation maximum background values (determined as 90th percentile of background samples) are 62 mg Zn kg^{-1} DW, 17 mg Cu kg^{-1} DW, 0.8 mg Cd kg^{-1} DW and 40 mg Pb kg^{-1} DW, for a standard soil with 2% clay and 10% organic matter (Belgisch Staatsblad, 1996). Samples 16, 20 and 25 contained increased total metals levels in particular for Cd and Zn. Cadmium and Zn concentrations in these samples were respectively 3.4 mg Cd kg^{-1} DW and 458 mg Zn kg^{-1} DW in sample 16; 1.5 mg Cd kg^{-1} DW and 125 mg Zn kg^{-1} DW in sample 20 and 3.2 mg Cd kg^{-1} DW and 288 mg Zn kg DW^{-1} in sample 25. These samples however, were not removed from the dataset because they were unlikely to be phytotoxic, due to their high pH. In Table 4.2 exchangeable levels of Zn, Cd, Cu, Ni, Al, Mn and Fe are listed. These results confirm the absence of high mobile metal fractions in soils 16, 20 and 25. Exchangeable Pb in all soils was below detection limit (<0.25 mg kg^{-1}).

The matrix of linear correlation between soil characteristics (Table 4.3) shows several significant relationships between the soil variables. It is beyond the scope of this study to discuss all different interrelationships observed, however they were calculated because the presence of these correlations may induce multicollinearity when the variables are used together as covariates in multiple regression models. To verify the presence of multicollinearity Variance Inflations Factors (VIFs) were calculated when regression models were built. With exception of the GlDH model in which Al had a very high VIF (VIF= 52), VIFs were found not to be greater than 10 when interacting variables or power terms were not considered. This observation indicates that in general the degree of multicollinearity is limited (Neter et al., 1990). The presence of numerous interactions and power terms in the models, however, makes interpretation of VIFs difficult. Model interpretations, in terms of individual effects of the covariates, therefore need to be approached with care.

Table 4.2 Exchangeable soil metal contents in 0.1 M $(CaNO_3)_2$ extracts (values are means of two measurements).

mg kg^{-1}	$Zn_{exch.}$	$Cu_{exch.}$	$Cd_{exch.}$	$Al_{exch.}$	$Mn_{exch.}$	$Fe_{exch.}$
1	3.34	<0.25	0.04	0.39	4.55	0.36
2	14.80	0.43	0.08	12.10	16.60	0.40
3	23.10	0.39	0.08	76.40	8.70	2.23
4	2.05	0.78	0.04	38.30	1.68	14.50
5	12.10	0.74	0.12	15.30	45.70	1.32
6	18.40	0.68	0.19	9.80	58.40	0.79
7	1.06	1.23	0.05	0.97	2.25	0.31
8	2.38	1.02	0.04	49.10	2.82	14.12
9	3.69	1.15	0.09	6.33	33.20	1.17
10	1.15	0.29	0.03	1.75	5.90	0.45
11	0.25	0.26	0.03	0.11	2.36	0.28
12	4.99	0.37	0.06	0.59	1.40	0.18
13	0.43	0.28	0.03	0.06	0.12	0.09
14	5.48	0.34	0.10	19.70	4.75	0.76
15	8.15	0.35	0.18	29.80	35.80	1.86
16	0.71	0.65	0.06	0.07	2.47	0.14
17	1.03	0.83	0.04	0.22	0.81	0.12
18	1.84	0.92	0.08	0.62	40.50	0.32
19	0.25	0.28	0.03	0.05	0.41	0.10
20	0.25	0.46	0.03	0.05	0.03	0.14
21	0.25	0.41	0.02	0.23	10.70	0.18
22	1.95	0.38	0.06	0.28	4.65	0.11
23	0.27	1.65	0.07	0.23	0.11	0.28
24	0.25	0.32	0.03	<0.05	0.20	0.24
25	1.66	<0.25	0.05	<0.05	0.36	0.22
26	0.25	<0.25	0.03	5.51	1.52	1.32

Remark: exchangeable Pb was < 0.25 mg kg^{-1} in all samples.

4.3.2 Plant responses

Growth parameters

In general, large differences were found between minimum and maximum values of growth parameters (Figure 4.1). Only shoot length was rather uniform in the entire dataset. Total above ground biomass, leaf area, shoot length and root growth varied with a factor of 2.5, 4.8, 1.6 and 3.7 respectively.

Table 4.3 Matrix of linear correlation between soil characteristics in the soil series. Significant correlations (95% level) are indicated in bold. EC=electric conductivity, CEC=cation exchange capacity, OM=organic matter, metals refer to exchangeable metals determined in 0.1M $Ca(NO_3)_2$.

	pH	OM	CEC	EC	$\text{Zn}_{\text{exch.}}$	$\text{Cd}_{\text{exch.}}$	$\text{Cu}_{\text{exch.}}$	$\text{Ni}_{\text{exch.}}$	$\text{Al}_{\text{exch.}}$	$\text{Mn}_{\text{exch.}}$	$\text{Fe}_{\text{exch.}}$
pH	**1.00**	.12	**.50**	**.57**	**-.49**	**-.43**	-.15	**-.37**	**-.73**	**-.40**	**-.64**
OM	.12	**1.00**	**.74**	**.57**	.13	.08	**-.43**	**.47**	.09	-.07	-.11
CEC	**.50**	**.74**	**1.00**	**.93**	-.29	-.23	**-.41**	.22	-.30	-.30	-.33
EC	**.57**	**.57**	**.93**	**1.00**	-.31	-.26	**-.40**	.13	-.35	-.31	**-.42**
$\text{Zn}_{\text{exch.}}$	**-.49**	.13	-.29	-.31	**1.00**	**.67**	-.08	.35	**.62**	**.56**	.01
$\text{Cd}_{\text{exch.}}$	**-.43**	.08	-.23	-.26	**.67**	**1.00**	.14	**.65**	**.27**	**.82**	-.07
$\text{Cu}_{\text{exch.}}$	-.15	**-.43**	**-.41**	**-.40**	-.08	.14	**1.00**	-.22	**.04**	.18	.25
$\text{Ni}_{\text{exch.}}$	-.37	**.47**	.22	.13	.35	**.65**	-.22	**1.00**	**.26**	.38	-.02
$\text{Al}_{\text{exch.}}$	**-.73**	.09	-.30	-.35	**.62**	.27	.04	.26	**1.00**	.10	**.62**
$\text{Mn}_{\text{exch.}}$	**-.40**	-.07	-.30	-.31	**.56**	**.82**	.18	.38	**.10**	**1.00**	-.08
$\text{Fe}_{\text{exch.}}$	**-.64**	-.11	-.33	**-.42**	.01	-.07	.25	-.02	**.62**	-.08	**1.00**

Closer examination of the data revealed that three samples with very poor plant growth (soil samples 3, 4 and 8) enhanced variations to a large extent. These three samples originated from sandy forest soils and had a low pH (between 3.1 and 4) with a concomitant high content of exchangeable Fe and Al (Table 4.2). A fourth forest soil (sample 15) supported a better plant growth. This soil also had a low pH and high exchangeable contents of Al, but was characterized by a lower exchangeable Fe content and higher CEC, conductivity and organic matter content (sandy loam soil) compared to the other forest samples. Exclusion of the extreme responses from the dataset resulted in strong reductions of the variations across the soil series: above ground biomass (1.1-1.5 g plant^{-1} FW), leaf area (19-26 cm^{2}) and shoot length (7.9 – 10.9 cm) only varied with a factor 1.4 and root weight with a factor 1.8 (0.6 - 1.1 g plant^{-1} FW). Responses of the bean plants on OECD soil were well within the range of responses on natural (non forest) reference soils. Within a soil sample relative standard deviations of the responses never exceeded 20%.

Linear correlations between soil characteristics and growth responses are presented in Table 4.4. Most remarkable are the positive relationships between the different growth responses and soil pH, and the negative correlations between growth responses and exchangeable levels of Fe and Al. This mainly reflects the poor plant growth on the forest soils. Leaf area and total aboveground biomass in addition were positively correlated to CEC and EC.

Multiple linear regression analysis including pH, CEC, EC, OM, exchangeable contents of Zn, Cd, Cu, Ni, Al, Fe, and Mn and their 2-way interaction terms as potential covariates did not result in significant models for any of the growth responses measured.

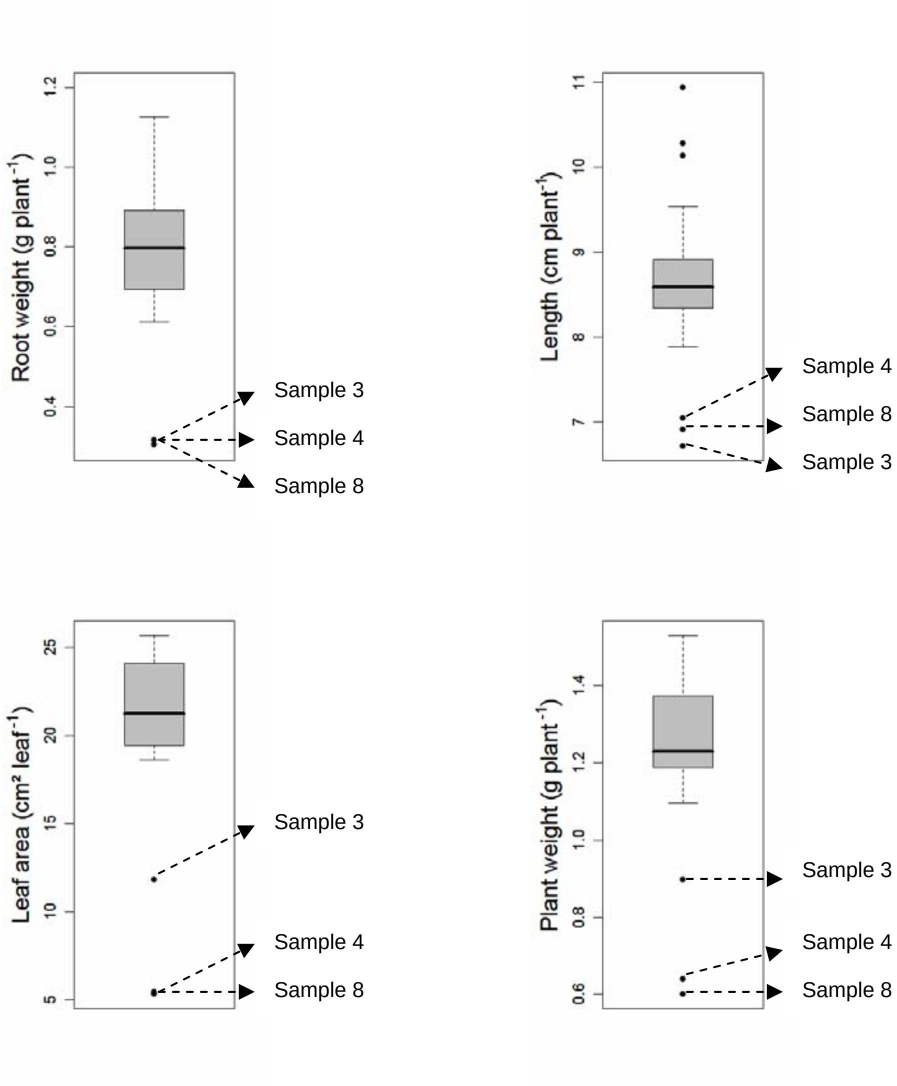

Figure 4.1 Box plots of the growth responses of bean plants (*Phaseolus vulgaris*) grown on a series of reference soils, indicating median, quartiles (boxes), whiskers (bars) (which correspond to data range limits or maximum 1.5 times the inter-quartile distance), and some extreme observations (•) of the dataset (data on fresh weight basis).

Biochemical parameters

On the three forest soils on which plant growth was inhibited (soils 3, 4 and 8), also enzymatic responses in leaves frequently were out of the range of the other soils (Figure 4.2). GPOD capacity in leaves varied with a factor 40 when the forest samples were included, but only varied with a factor 1.7 without the forest samples. The variation factor in ME was reduced from 3.6 to 1.7 after exclusion of the forest samples. Variation factor of ICDH was 4.4 when forest samples were included and 2.2 without forest samples. ICDH capacity was high on one of the forest soils but on the contrary dropped to very low values on the two most acid soils.

Root enzyme responses on forest soils were less extreme than leaf enzymes and growth responses (Figure 4.2). Variation ranges without forest samples were 3.7, 2.0, and 1.8 for GPOD, ME and GlDH respectively, and 6.4, 2.7, 1.9 for the same enzymes when forest samples were included.

All enzyme capacities in bean plants grown on OECD soil were within the response range on natural (non-forest) reference soils.

Similarly to growth responses, the matrix of linear correlation between enzyme capacities and soil characteristics (Table 4.4) showed significant relationships between several enzymes (GPOD and ME in leaves and roots) and the soil factors pH, $Al_{exch.}$ and $Fe_{exch.}$. The direction (sign) of these relationships was opposite to the direction of the relationship observed between these variables and growth responses, reflecting increases in enzyme capacities when growth was reduced. ME (in leaves and roots) in addition showed a negative relationship with EC, suggesting a higher ME response when EC is lower. GPOD in roots correlated significantly with most of the soil variables measured. While this enzyme tended to decrease with increasing pH, EC and CEC (negative correlations), its capacity increased with increasing levels of exchangeable Fe, Al and Cu.

ICDH in leaves and GlDH in roots showed a behavior that differed from the other enzymes, since sometimes reductions in the capacity of these enzymes were observed in response to increasing metal concentrations. ICDH in leaves for example was significantly reduced with increasing $Fe_{exch.}$ levels and GlDH in roots showed the same behavior in response to increasing $Al_{exch.}$ concentrations.

Table 4.4 Matrix of linear correlation between soil characteristics and plant responses. Significant correlations (95% level) are indicated in bold. GPOD=guaiacol peroxidase; ME=malic enzyme; ICDH=isocitrate dehydrogenase; GlDH=glutamate dehydrogenase; EC=electric conductivity, CEC=cation exchange capacity, OM=organic matter, metals refer to exchangeable metals determined in 0.1M $Ca(NO_3)_2$.

	Growth responses				Enzymes in leaves			Enzymes in roots		
	Plant weight	Shoot length	Leaf area	Root weight	GPOD	ME	ICDH	GPOD	ME	GLDH
pH	**.68**	**.49**	**.72**	**.42**	**-.54**	**-.73**	.01	**-.65**	**-.59**	.18
OM	.17	-.19	.24	.13	-.16	-.08	.33	-.22	-.046	-.23
CEC	**.46**	.14	**.55**	.24	-.32	-.37	.25	**-.45**	-.30	-.16
EC	**.47**	.20	**.58**	.24	-.39	**-.43**	.32	**-.53**	**-.40**	-.14
$Zn_{exch.}$	-.24	-.35	-.26	.02	-.06	.24	**.37**	.22	.24	-.20
$Cd_{exch.}$	.05	-.08	.03	.25	-.15	.06	**.41**	.19	.21	.24
$Cu_{exch.}$	-.29	-.27	-.32	-.29	.27	.22	-.39	**.46**	.21	.25
$Ni_{exch.}$	.09	-.17	.14	.15	-.09	.06	**.43**	.05	.12	-.11
Al_{exch}	**-.65**	**-.65**	**-.73**	**-.51**	**.55**	**.80**	.01	**.70**	**.69**	**-.42**
Mn_{exch}	-.09	-.07	-.06	.14	-.14	.01	.26	.17	.18	.39
Fe_{exch}	**-.80**	**-.57**	**-.87**	**-.76**	**.95**	**.92**	**-.61**	**.77**	**.75**	-.26

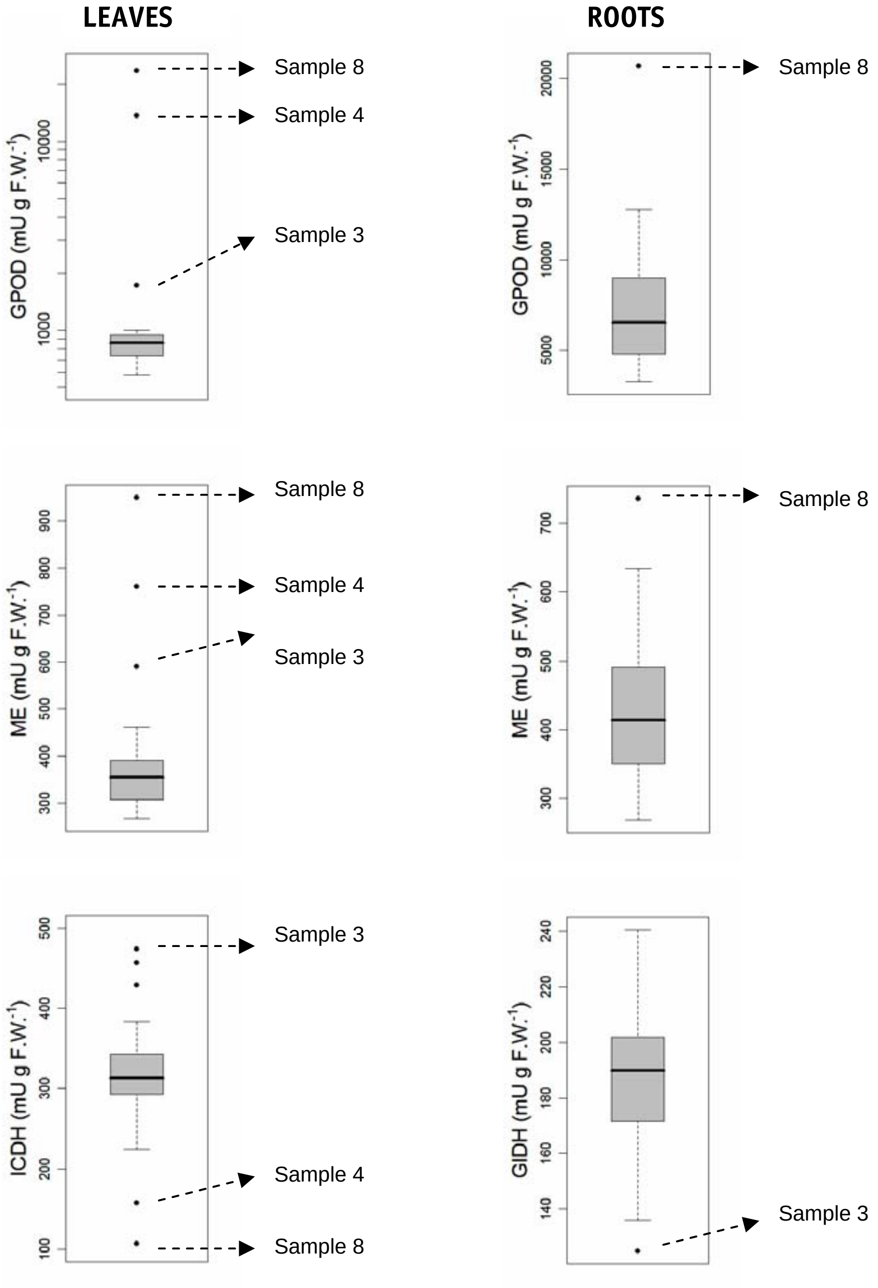

Figure 4.2 Box plots of enzymatic responses (mU g^{-1} FW) of bean plants (*Phaseolus vulgaris)* grown on a series of reference soils, indicating median (black line), quartiles (boxes), whiskers (bars) (which correspond to data range limits or maximum 1.5 times the inter-quartile distance), and some extreme observations (•) of the dataset. GPOD= guaiacol peroxidase, ICDH= isocitrate dehydrogenase, ME= malic enzyme, GlDH= glutamate dehydrogenase.

Multiple linear regression analysis (including pH, conductivity, CEC, %OM, exchangeable concentrations of Zn, Cd, Cu, Pb, Fe, Mn, Al and the necessary interaction terms) resulted for all leaf and root enzymes in significant and strong models describing the relationship between enzyme capacity and soil parameters (Table 4.5, equation 1-7). Linear predictors represented in the enzyme models were soil EC, pH and exchangeable Fe, Al, Mn and Zn for leaves and roots. In roots exchangeable Cu and OM were sometimes present as additional predictor variables. Plots of measured versus predicted enzyme capacities are shown in Figure 4.3. Good fits were obtained for all enzymes.

Table 4.5 Multiple linear regression models describing enzymatic responses of bean seedlings (*Phaseolus vulgaris*) grown on reference soils, as a function of soil characteristics. GPOD=guaiacol peroxidase; ME=malic enzyme; ICDH=isocitrate dehydrogenase; GlDH=glutamate dehydrogenase; EC=electric conductivity, CEC=cation exchange capacity, OM=organic matter, metals refer to exchangeable metals determined in 0.1M $Ca(NO_3)_2$.

Model		R^2	R^2_{adj}
$\mathbf{GPOD}_{leaves} = 997.07 - 0.30\ EC + 4.93\ Zn - - 0.016\ Mn^2 - 21.13\ Fe^2 - 0.81\ Al^2 + 0.028\ Al^2{*}Zn + 0.00036\ Al^2{*}\ Mn^2 + 0.059\ Al^2{*}\ Fe^2$	(equation 1)	0.99	0.99
$\mathbf{ME}_{leaves} = 447.12 - 19.33\ pH + 0.00073\ EC - 206.57\ Fe^2 + 1.51\ Al^2 - 0.0033\ Al^2{*}EC + 0.028\ Al^2{*}\ Fe^2 + 0.60\ Fe^2{*}EC$	(equation 2)	0.97	0.95
$\mathbf{ICDH}_{leaves} = 1531.65 - 381.79\ pH + 27.56\ pH^2 + 0.12\ EC - 34.95\ Fe$	(equation 3)	0.85	0.82
$\mathbf{GPOD}_{roots} = 4379.79 + 9122.11\ Cu - 5083.05\ Fe + 1.11\ EC + 3460.91\ Cu{*}Fe + 10.02\ EC{*}Fe - 14.89\ EC{*}Cu$	(equation 4)	0.86	0.82
$\mathbf{ME}_{roots} = 647.08 - 0.29\ EC - 88.6\ OM + 145.50\ Fe - 6.68\ Fe^2 - 223\ Cu^2 + 0.075\ OM{*}EC + 86.67\ OM{*}Cu^2$	(equation 5)	0.83	0.76
$\mathbf{GlDH}_{roots} = 352.31 + 10.80\ pH - 8.48\ Zn + 18.37\ Mn - 175.95\ Fe + 10.59\ Fe^2 + 5.10\ Al - 0.46\ EC + 0.00024\ EC^2 - 3.12\ pH{*}Mn$	(equation 6)	0.85	0.75

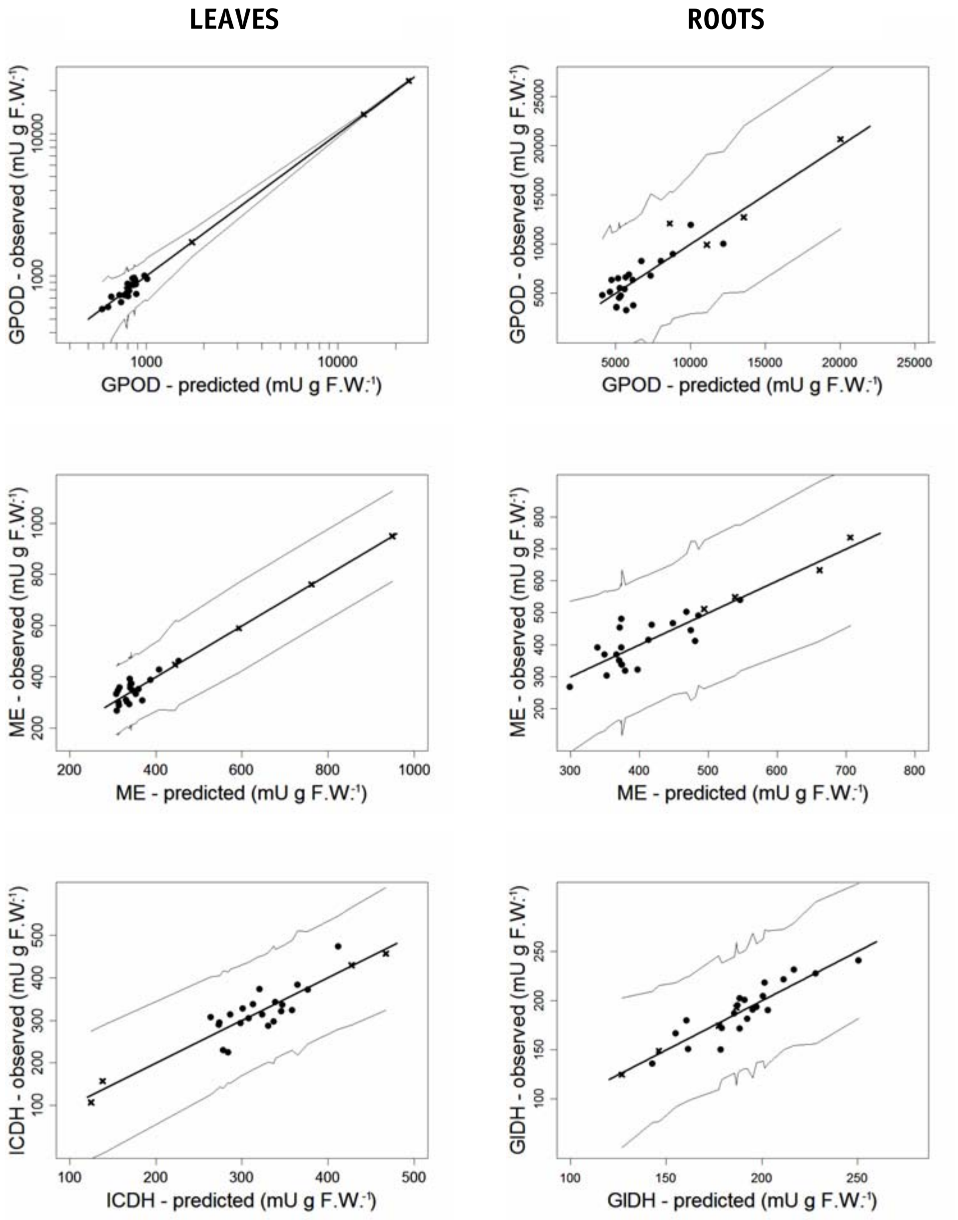

Figure 4.3 Observed versus fitted values of the proposed models (Table 4.4) predicting enzyme responses in function of soil characteristics in uncontaminated reference soils; intervals around the fit present prediction limits (25 predictions). For GPOD in leaves a log scale is used to obtain a better presentation of the data points. GPOD= guaiacol peroxidase, ME= malic enzyme, ICDH= isocitrate dehydrogenase, GlDH= glutamate dehydrogenase. Black symbols '•' are from non-forest samples; 'x' symbols represent forest samples.

4.4 Discussion

4.4.1 Soil characteristics

Relationships between soil characteristics (Table 4.3) are inherently linked to the nature of soil types. Sandy soils, due to their low fraction of small sized reactive soil particles, tend to have low CECs, a low buffering capacity, a low pH and low conductivity. Clayey soils, on the contrary, have a large reactive surface, and by consequence high CEC, pH and EC (Brady, 1984). This is illustrated by the observed positive relationships between pH, CEC and EC (Table 4.3). For most metals, pH is the main factor determining their mobility because of increased sorption and precipitation reactions at higher pH (Adriano, 2001). Correspondingly, significant negative correlations were found between pH and the exchangeable content of Zn, Cd, Al, Mn and Fe (Table 4.3). The behavior of Cu in soils is highly determined by its strong affinity for organic material (Mc Bride and Martinez, 2000; Kabata-Pendias and Pendias, 1984), which may explain the observed negative correlation between exchangeable Cu and OM. Soil CEC and EC were positively correlated with OM and with each other. Since exchange sites on soil organic matter contribute to soil CEC, the relationship between both parameters is evident.

4.4.2 Plant responses

Growth parameters

Despite the presence of some linear relationships between soil parameters and plant responses, no significant multiple regression models were found predicting growth responses based on the soil parameters measured. This poor prediction potential is likely due to the fact that some important covariates determining plant growth have not been analyzed in our study. Available levels of macronutrients (N, P, K) are generally known to determine to a large extent plant growth responses. Gong et al. (2001) for example reported that soil nutrient levels strongly influenced growth responses of *Lepidium sativum* (cress) and *Avena sativa* (oat) in an early seedling growth test. Possibly multiple relations with the variables measured are present but can not be modelized because of the relatively small dataset.

Biochemical parameters

In contrast to growth responses, enzyme capacities were predicted well based on the soil parameters measured (Table 4.5, equation 1-6). This indicates that macronutrient levels in soils do not, or only to a limited extent, influence the capacities of the stress-enzymes in 14 day-old bean plants. Predictor variables present in the models were EC, pH, OM and exchangeable levels of Zn, Cu, Al, Fe or Mn. As explained earlier, the presence of significant correlations between some of the soil parameters may hamper an interpretation of the models, in terms of the individual effect of each variable. The model for GlDH in roots explicitly showed a very high VIF for $Al_{exch.}$ (VIF=52), which indicates the presence of strong multicollinearity and advises against model interpretation. The positive coefficient of $Al_{exch.}$, suggesting an increase in GlDH with increasing $Al_{exch.}$ contents, illustrates the problem since a significant negative correlation was present between $Al_{exch.}$ and GlDH (Table 4.3). For the other models, correlations between variables were weaker and some careful suggestions for interpretation are made below, because some general trends were noticed, and are worth mentioning. All enzyme models (Table 4.5) contained $Fe_{exch.}$ as linear predictor. In the model for GPOD and ME in the roots, $Cu_{exch.}$ was present next to $Fe_{exch.}$. Iron and Cu are redox active elements and are both able to perform one-electron-transfer reactions. They are considered to be direct inducers of reactive oxygen species (ROS) (Schützendübel and Polle, 2002). Especially their involvement in Fenton-like reactions in which very reactive hydroxyl radicals are produced ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + °OH + OH^-$), makes them very strong inducers of oxidative stress. On the other hand, both elements also occur as functional metals in superoxide dismutases (SOD), a group of enzymes responsible for the dismutation of superoxide radicals into hydrogen peroxide and oxygen ($2\ 0_2^{-}$° + $2H^+ \rightarrow H_20_2 + 0_2$) (Alscher et al., 2002). The presence of Fe_{exch} and Cu_{exch} in the models indicates that even in background conditions the level of these elements may influence the ROS level and thus the capacities of enzymes involved in anti-oxidative defense reactions. The appearance of $Cu_{exch.}$ only in root models is likely related to its accumulation in roots (Cuypers et al., 2002). According to the dual role of Cu mentioned above, increasing exchangeable soil Cu concentrations (and thus likely increasing concentrations in roots) induced or reduced GPOD and ME capacity in roots, depending on the situation. ME capacity in roots for example increased with increasing $Cu_{exch.}$ only when $Cu_{exch.} > 0.38*OM(\%)$ (Table 4.5). When $Cu_{exch.}$ concentrations were below this value, which was often the case, increasing $Cu_{exch.}$ tended to reduce ME capacity. For all samples in the soil series increase in $Fe_{exch.}$ seemed to result in an induction of GPOD and ME capacity in roots. In leaves increases of soil $Fe_{exch.}$ concentrations resulted in an induction of GPOD and ME, only if $Al_{exch.}$ was also high ($Al_{exch.} > 18.9$ for GPOD induction; $Al_{exch.} > (206.57 - 0.6EC)/0.028$ for ME induction) (Table 4.5).

In addition to $Fe_{exch.}$ and $Cu_{exch.}$, also $Al_{exch.}$ and $Mn_{exch.}$ were frequently present in the enzyme models, often in interaction with each other, with $Fe_{exch.}$, pH or EC. Aluminum has been reported to cause oxidative stress in plants (Boscolo et al., 2003 and references therein). Its presence in several models is therefore not surprising**.** Despite the role of Mn as a micronutrient (like Cu and Fe, Mn is e.g. a functional metal in some SODs), high levels of available Mn are known to become toxic for plants and to limit plant production (El Jaoual and Cox, 1998; Mench et al., 1994) Mn phytotoxicity is sometimes observed in acidic soils, in soils under reducing conditions, after unequilibrated fertilization (e.g. with high rates of superphosphate) and in soils deficient in Ca, Mg, Fe or P (El Jaoual and Cox, 1998; Adriano, 2001).

Several models show that increasing concentrations of the elements mentioned above resulted in enzyme inductions only when the concentration of one of the other elements was also high. GPOD capacity in leaves for example increased with $Mn_{exch.}$ levels if $Al_{exch.} > 6.67$ ($Al^2_{exch.} > 0.016/0.00036$; Table 4.5). When additional interaction effects were present, the critical level could be dependent on the level of another soil parameter; e.g. if $Al^2_{exch.} > (206.57 - 0.6EC)/0.028$ than ME_{leaves} increased when $Fe_{exch.}$ increased (Table 4.5).

Conductivity was the only soil characteristic present in all models. The EC effect on enzyme capacities varied in function of the enzyme considered, but most often higher conductivities were related to lower enzyme capacities. High conductivities may reduce the toxic effect caused by an element, due to an increased competition for uptake of the toxic element.

A decreasing capacity of ICDH was predicted with increasing $Fe_{exch.}$ levels (Table 4.5, equation 3). The very low ICDH capacities in plants grown on sample 4 and sample 8 (forest samples) were related to extremely high $Fe_{exch.}$ levels. Also $Al_{exch.}$ levels in these samples were among the highest in the dataset. These low ICDH values seem to reflect a loss of the functionality of the enzyme. Even if the effect was less pronounced for GlDH also this enzyme showed, among all reference soils, the lowest capacity in plants grown on two of the forest samples (sample n° 3 and n° 4), and demonstrated a significant negative correlation with $Al_{exch.}$ (Table 4.4). In all other cases the highest enzyme capacities were observed in plants grown on forest samples (Figure 4.3). Inhibitions of antioxidative enzymes in bean plants in conditions of high metal exposure, have been reported also in other studies (Schützendübel and Polle, 2002; Meers et al., 2006). We do not know the exact mechanism of the enzyme inhibitions in plants grown on the forest soils. In case of ICDH competition of Fe for the active site of this metallo-enzyme (Mn required in active centrum) (Engbersen and de Groot, 1992) could be a possible explanation. Glutamate dehydrogenase on the other hand requires Ca for optimal functioning (Scheid et al, 1980), of which the availability may be low in the forest soils. The potentially strong inhibitions make interpretation of the ICDH response in a phytotoxicity test difficult, because, in contrast with the other enzymes, low capacities may represent not only a very low but also a very high degree of toxicity.

The results discussed above generally demonstrate that bean plants grown on different reference soils, may be exposed to different levels of ROS production and therefore exhibit different levels of enzymes that are directly or indirectly involved in the anti-oxidative response. The extreme enzyme capacities and very poor growth response of bean plants on samples n° 3, 4 and 8 (forest soils) suggest a toxic response likely caused by Fe and/or Al. Therefore the PlantTox test may not be suited to detect additional toxicities (Zn, Cu,...) in very acid soils (pH<4) such as the forest samples in this study. Further research is necessary to answer this question.

4.4.3 Selection of the most appropriate reference levels

Based on the results discussed above, different options can be proposed to select suited ‘reference levels’ for the PlantTox test.

1. A first option is to ***use only one reference soil*** in large screening experiments. Because of the outlying observations on samples n°3, 4 and 8 (forest soils), this option seems not possible if acid soils (pH 4) are included in the test series, but it may be justified when testing other soils, that have physico-chemical characteristics (with exception of the metal level, of course) in the range of the values found in our reference soils. When very acid soils are

excluded a rather limited range in response variation was observed, over a wide variety of potential reference soils. This range in response variation, even if generally close to a factor 2, is for most enzymes relatively small compared to the range in a situation where metal-contaminated samples are assessed. In the latter case for example increases in enzyme capacities with factors up to 6 and 7, 4 and 6, 40 and 47 have been found for ME, ICDH and GPOD in the leaves, and factors up to 3 and 6, 7 and 9, 9 and 13 for ME, GlDH and GPOD in roots (Vangronsveld and Clijsters, 1992; Vangronsveld et al., 1995a) on a heavily contaminated soil originating from an old Zn smelter site. Growth responses of surviving plants as low as 5% of the control level for leaf area and shoot weight, and 15 % of the control level for shoot length have been found in very toxic conditions. In extremely toxic conditions, plants even failed to germinate.

Depending on the selection of the unique reference soil and its further use, the 'one-reference-soil-approach' holds the risk of false positive or false negative results. If we assume that false positives are to be avoided as much as possible, the reference level should be sufficiently high to cover the upper limits of the range of reference responses.

Van Assche and Clijsters (1990a) and Vangronsveld and Clijsters (1992) have proposed a classification system to interpret the results of the PlantTox test, based on the % induction in enzyme capacities and the % reduction in growth responses, compared to the response on a reference soil. In their studies an agricultural soil (soil n°1 of this study) was used as a reference. In this study responses of the bean plants on OECD soil were found to be well within the range of responses on natural (non forest) reference soils. Since this OECD soil is artificially made in the laboratory, its composition is supposed to be more or less constant throughout different periods and labs. Field soils are known to be subject to seasonal fluctuations or variations due to e.g fertilizer applications or liming history. These treatments can affect soil parameters such as conductivity, exchangeable Fe content,... which were shown to influence enzymatic responses

(Table 4.5). Such potential variations in reference level reduce the reproducibility of test results (difference between control and polluted response may vary from test to test). Therefore we now propose the use of OECD substrate as a potential general reference soil for the PlantTox test assuming that the composition (and characteristics) of this artificial substrate can be controlled better compared to the characteristics of field soils. However also in case of OECD soil the constancy of responses needs further investigation because differences in peat quality, sand or clay origin may influence the characteristics of this artificial substrate.

After combining the knowledge on the range of plant responses in reference conditions, and on the plant responses on OECD substrate compared to the other non-toxic soils, the original indexation in phytotoxicity classes can be recalibrated as presented in Table 6. The non-toxic upper limit of most enzymatic responses was selected as the 90% percentile of each enzyme response in our soil series (after exclusion of samples 3, 4 and 8); for growth responses the 10th percentile was taken. An exception was made for ME in roots and GPOD in roots. For these enzymes the 75th percentile of our dataset was considered as non-toxic upper limit because of the strong responsiveness of these models to exchangeable Cu, in combination with the large variation range in case of GPOD. The response values corresponding to the respective percentiles were expressed as percentage of the OECD response to obtain the upper/lower limits of toxicity class n°1. The limits of the highest toxicity class are based on the maximum observed inductions/reductions under toxic conditions (cfr. supra), and the range of each class was given a fixed size for each response. When the selected percentiles did not differ statistically significant from the OECD response, the upper limit of the first class was shifted with 10% maximum to obtain a significant difference. Compared to the existing system (Vangronsveld and Clijsters, 1992) this revised system is based on a profound knowledge of responses in reference conditions, and avoids the determination of false positive results. It can however not rule out the occurance of false negatives, in particular for soils resulting in low intrinsic enzyme responses. GPOD in roots, which showed the largest response variation among reference soils, will likely be a less sensitive parameter in such soils. This problem can potentially be overcome when the second option is used (see below).

As discussed earlier the OECD reference and the developed system are not suited for acid soils (pH≤4) with very high levels of exchangeable Al, Fe or Mn. In general the proposed system is only valid for soils with characteristics within the ranges of the values in this dataset.

Table 4.6 Revised system for classification of soils in phytotoxicity classes based on PlantTox results (values are expressed as percentages of values obtained on OECD standard soil).

	Class 1 (not toxic)	Class 2 (slightly toxic)	Class 3 (moderately toxic)	Class 4 (strongly toxic)
Growth responses				
Shoot length	>85	84-65	64-45	<45
Leaf area	>85	84-65	64-45	<45
Root weight	>80	79-60	59-40	<40
Enzymes in leaves				
GPOD	<120	120-260	261-420	>420
ME	<125	125-255	256-375	>375
ICDH	<120	120-200	201-280	>280
Enzymes in Roots				
GPOD	<235	235-315	316-395	>395
ME	<175	175-275	276-375	>375
GlDH	<125	125-185	186-245	>245

2. A second possibility for selection of an appropriate reference level for the PlantTox test could be ***the calculation of a specific reference level for each contaminated soil based on its soil characteristics (='model approach').*** Based on the present study potentially suited models for such calculations are available for the stress enzymes (Table 4.5). As a result of the absence of models for growth responses the 'model approach' can only be applied for the

enzymatic responses. Interpretation of growth responses remains useful, but can only be done using the first approach. Taking into account the lower specificity of growth responses compared to the enzymatic responses, priority must be given to the latter in case both response groups lead to different conclusions. As stated earlier this 'model approach' can potentially increase the accuracy of the test because of the use of a flexible reference level, adapted to each individual situation. The upper limit of the 95% prediction interval calculated for the model (Figure 4.3) could be taken as the upper limit of the first toxicity class. Actually however this upper limit (for 25 predictions) is about a factor 1.3, 1.4 and 1.5 higher/lower than the predicted value for GPOD, ME and ICDH in leaves respectively and, a factor 2.0, 1.5 and 1.3 for GPOD, ME and GlDH in roots (Figure 4.3; calculated values not shown). These factors are relatively large compared to the total variation among non-toxic soils. Moreover exchangeable metal concentrations representative for non-toxic soils should be determined more precisely to enter these values in the regression equations. Before the 'model approach' can become profitable additional data collection and modeling work seem necessary to improve the fits and to reduce the size of prediction intervals. A question, which also needs to be answered, is the one about the constancy of the OECD response (and in fact the response on any given soil) at different cultivation moments or throughout different laboratories. Depending on the answer, it may be necessary to adjust for differences between tests when applying the models. This question should be subject of future research.

3. Theoretically <u>other options</u> are also available to select adequate reference soils, but compared to the first two approaches these options have more disadvantages. The options include:

-the use, for each potentially contaminated soil sample to be assessed, a reference soil with physico-chemical soil characteristics similar to the potentially contaminated sample, based on the knowledge of the response determining parameters in reference conditions, as shown in Table 4.5 (equation 1-6). The search for such soils, however, as well as their inclusion in the test is complicated and time-consuming and may not be realistic, particularly not in large screening experiments.

-the use of a reference soil bank, with a limited number of soil types could overcome the problem of the potential large number of reference soil needed in the previous option. The soil types could be selected based on the information available in the models (Table 4.5, equation 1-6). However, because of the complexity of the models also this option seems difficult to adopt.

4.5 Conclusions

This study demonstrates that morphological and biochemical plant responses, detected in a phytotoxicity test with bean plants (PlantTox), (Van Assche and Clijsters,1990a), vary across a series of reference soils. When acid forest soils (pH 4) are excluded, these variations, even if generally close to a factor 2, are small compared to a test situation where also (metal) contaminated soil samples are included. The test can therefore undoubtedly be used to detect phytotoxicity of metal contaminated soils. Regression models relating plant responses to physicochemical soil parameters, delivered relevant models for all enzyme capacities measured (GPOD, ME and ICDH capacities in leaves, and for GPOD ME and GLDH capacity in roots) explaining respectively 99%, 97%, 85%, 86%, 83%, and 85%, of the response variation. Responses of the stress enzymes in general were determined to a large extent by soil pH, conductivity and exchangeable levels of Zn, Cu, Al, Fe or Mn. Remarkable was the inability to find significant multiple regression models for prediction of growth responses. This observation suggests that growth responses, in contrast to enzyme capacities, are to a large extent determined by other soil factors than those investigated in our study.

Available soil levels of macronutrients (N, P, K) and some physical or biological soil factors are possible candidates.

Based on the results obtained two possible approaches were proposed for future applications of the PlantTox test: a ‘one-reference-soil approach’ and a ‘model approach’ (reference calculation). In analogy with the classification system proposed by Van Assche and Clijsters (1990a) and Vangronsveld and Clijsters (1992), we developed a toxicity classification method (Table 4.6), based on the use of OECD standard soil (OECD, 1984) as a general reference (negative control) soil.

CHAPTER 5

Variations in morphological and enzymatic responses of *Phaseolus vulgaris* grown on OECD 207 standard soils

A. Ruttens, M. Mench., A. De Vocht, W. Geebelen, E. Meers and J. Vangronsveld.

Plant and Soil. Article in preparation.

Abstract

OECD standard soil, prepared according to the prescriptions of guideline N° 207 of the Organization for Economic Cooperation and Development (OECD), has been proposed as negative control soil in a phytotoxicity test with bean plants (*Phaseolus vulgaris*). The so-called PlantTox test is based on the analysis of morphological and enzymatic responses of the test plants and was designed to assess the phytotoxicity of potentially contaminated soils. The present study was designed to gain insight in the constancy/variance of morphological and enzymatic responses of *Phaseolus vulgaris* grown on OECD 207 standard soils with varying origins of their constituents (peat, kaolinite, sand). The study aims to contribute to an optimization of the PlantTox protocol. Results revealed similar growth responses (relative deviations ranging from 5.8% to 11.5%) but differential enzymatic responses (relative deviation ranging from 17.4% to 83.8%) when different origins of the constituents were used. The different enzymatic responses could be related mainly to differences in conductivity (EC) and organic matter content (OM) of the various standard soils. These results raise questions to the general suitability of OECD 207 standard soil as negative control soil in the PlantTox test. We conclude that the term 'OECD standard soil' should be interpreted with care since its composition, by definition, is not completely standardized.

5.1 Introduction

When phytotoxicity tests are performed as bioassays on soil samples, to evaluate the toxicity of potentially contaminated field soils, the choice of an appropriate negative control soil is extremely important (Frische et al, 2003). Selection of an unsuited negative control soil (further referred to as reference soil) will easily lead to misinterpretation of test results and to false positive or false negative conclusions about the toxicity of a substrate. For a phytotoxicity test using *Phaseolus vulgaris* as a test plant (PlantTox test; described first by Van Assche and Clijsters, 1990a), morphologic and enzymatic plant responses on a series of potential reference soils were investigated in Chapter 4. Plant responses on an artificial soil, prepared according to the prescriptions of OECD guideline N° 207 (OECD, 1984), were found to be well in the range of responses on non-toxic field soils. OECD artificial soil has been introduced in ecotoxicity studies as a standardized substrate to overcome soil type dependence of results when testing the toxicity of substances (because of the soil type dependence of bioavailability) (LØkke and Van Gestel, 1998). Based on the results in Chapter 4 we proposed the OECD 207 standard soil as potential reference soil for the PlantTox test, assuming that its composition (and resulting plant responses), in contrast to field soils, could be kept constant between different tests. In analogy with Van Assche and Clijsters (1990a), a classification scheme was proposed to assign a 'Phytotoxicity Index' to tested soils based on a comparison of bean plant responses on contaminated soils with responses obtained on OECD standard soil (Chapter 4). The insight in the range of responses in reference conditions and the resulting well-funded classification scheme, make of the PlantTox test a potentially reliable phytotoxicity test.

In this chapter, a comparison is made between responses of bean plants grown simultaneously on standard soils prepared according to the prescriptions of guideline N° 207 of the Organization for Economic Cooperation and Development (OECD, 1984), but with varying commercial origins of the soil constituents. Large response differences among different standard soils would place constraints to the general applicability of the toxicity classification scheme and would ask for remedial measures. We propose that relative deviations (RD) of plant responses among different standard soils should be on average not higher than 20% to guarantee the general applicability of the proposed classification system (Chapter 4).

Before the start of the main experiment the reproducibility of plant responses at a given moment on one of the standard soils was verified to get insight in the variability between replicates, and to ensure a reliable interpretation of the results of the main experiment.

5.2 Materials and Methods

5.2.1 Preparation of standard soils

OECD standard soils were prepared according to OECD guideline N°207 (OECD, 1984): 70% sand, 20 % kaolinite and 10% *Sphagnum* peat were mixed, and the resulting soil was adjusted to pH 6 with $CaCO_3$.

Peat was grounded and sieved through a 1 mm sieve (sample mill, Retch, SM 100) prior to use. It was mixed with the other compounds in a humid condition, but a correction for its water content was made. Drying of peat in an oven is not recommended because rehydration may become difficult (LØkke and Van Gestel, 1998).

Different commercial origins of the soil constituents were used to obtain three different OECD soils: OECD1 (white sand: 'Gamma, Belgium'; kaolinite: kaolin CH112F, Keramikos, Haarlem, The Netherlands; white *Sphagnum* peat: turba EE3120, A.S. Biomix LTD, Estonia), OECD2 (white sand: 'Brico, Belgium'; kaolinite: China clay, Fluka (Sigma-Aldrich group), Buchs, Switzerland'; white

Sphagnum peat: Isratorf, Duitse blonde turf, NV Van Israel, Geraardsbergen, Belgium) and OECD3 (white sand Bricobi, Brico, Belgium; kaolinite: China clay, ECC international, UK; white *Sphagnum* peat: Agratorf, Agrofino® products, Arendonk, Belgium). The necessary amount of $CaCO_3$ (Vel, analytical grade) for each soil was determined based on a fitted calibration line expressing pH in function of increasing doses of $CaCO_3$ (ranging between 0.1% to 0.6%). Prior to pH measurements soils were allowed to equilibrate at room temperature for 3 days after addition of $CaCO_3$.

5.2.2 Determination of physicochemical characteristics of the standard soils

Soil pH, conductivity (EC), organic matter content and exchangeable levels of Zn, Cu, Al, Fe and Mn were determined in the different standard soils. These parameters were selected based on their presence in model equations predicting enzymatic responses in bean plants grown on a series of field soils with background metal concentrations (Chapter 4). Soil pH was determined in 1M KCl in a soil:solution ratio of 1:5 after shaking (60 rpm) for 2h and after filtration over a filter paper (Whatman n°1). Soil conductivity was measured (WTW, LF 537 conductivity meter and TetraCon 96 electrode, calibrated with 0.1 M KCl solution) in a soil:Milli-Q water ratio of 1:5 (Millipore, Milli-Q Water Purification System) after shaking for 18h and after filtration over a filter paper (Whatman n°1). Organic matter content of the soils was determined using the method described by Walkley & Black (Allison, 1965) estimating the soil humus content based on carbon determination after C oxidation under standardized conditions with potassium dichromate in sulphuric acid medium. Soil organic matter content in this method is assumed to be the double of the determined C content. Extractions with $Ca(NO_3)_2$ (0.1 M, soil:solution ratio 1:5, 2 h shaking at 65 rpm, filtration through Whatman filter paper n°40) were used to estimate exchangeable soil metal contents (Boisson et al., 1999). Metals in the extracts were analyzed by Induced Coupled Plasma Spectrometry (ICP, Perkin Elmer, Optima 3000 DV).

5.2.3 Cultivation of test plants and measurement of plant responses

Bean plants (*Phaseolus vulgaris* cv Limburgse vroege) were grown for two weeks in a growth chamber under controlled conditions (22 °C, 65 % relative humidity, 12 h light, photosynthetic active radiation: 150 µmol m^{-2} s^{-1}) (Sanyo, plant growth chamber SGC170.CFX.J). Seeds were sown in polyethylene pots of 400 ml (4 plants/pot; 4 pots per 'treatment') after 24h vernalization (cold treatment at 4°C) followed by 4h imbibition in tap water at room temperature. Soils were watered daily with distilled water up to 40% of their water holding capacity during the first week and to 60% in the second week. Fourteen days after sowing, morphological parameters (shoot length, primary leaf area, root weight) were measured for each plant. Samples of primary leaves and roots (1 g fresh weight, FW), were frozen in liquid nitrogen and stored at –70°C for enzyme analysis. Half leaves, obtained by cutting the leaves over the middle vain, were pooled to obtain representative leaf samples; root samples were obtained as homogenized sub-samples from the entire root system after cutting in 1-cm pieces. Within 3 weeks following harvest, frozen plant tissue was homogenized with a Polytron PT 3000 homogenizer in ice-cold Tris-HCl buffer (0.1 M, pH 7.8) containing 1mM EDTA, 1mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (5 ml buffer g^{-1} FW). The homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 20000g (4°C) (Jouan, centrifuge MR22i). Subsequently the capacity (i.e. the potential activity measured *in vitro* under non-limiting conditions of substrate and coenzyme) of the following enzymes was measured spectrophotometrically (Shimadzu, UV-1602) at 25°C in the supernatant as described by Van Assche et al. (1988): guaiacol peroxidase (GPOD; E.C. 1.11.1.7), malic enzyme (ME; E.C. 1.1.1.40), glutamate dehydrogenase (GlDH; E.C. 1.4.1.2) and isocitrate dehydrogenase

(ICDH; E.C. 1.1.1.42). Enzyme capacity was expressed in milli-Units (mU) per gram fresh weight.

5.2.4 Experimental set-up

5.2.4.1 Experiment 1: Reproducibility of plant responses

At three different moments throughout the year (February, April and September) three replicates of OECD1 soil (OECD1, OECD1′, OECD1″; each replicate consisted of 4 pots), were analyzed to check the reproducibility of the plant responses at a given moment, on a given soil. The design of the experiment is illustrated in Figure 5.1.

5.2.4.2 Experiment 2: Effect of variations in the origin of OECD constituents

The main aim of this study was to evaluate possible influences of the origin of soil constituents of the standard soil on plant responses. Therefore bean plants were grown at the same moment on three standard soils prepared according to the OECD guidelines (OECD, 1984), but composed of constituents with different commercial origins (OECD1, OECD2 and OECD3) (Figure 5.1).

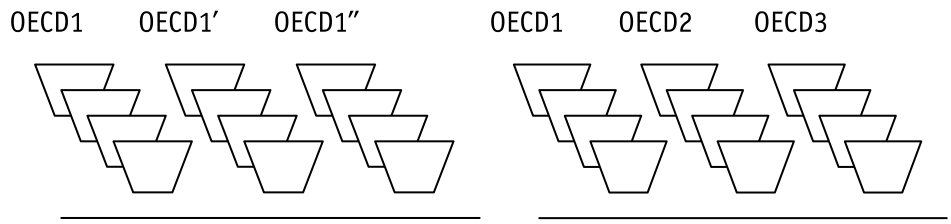

Figure 5.1 Design of experiment 1 (left picture) to compare plant responses between different replicate series of OECD1 soil at a given moment. OECD1, OECD1′ and OECD1″ represent three replicate series of OECD1 soil (4pots/′treatment′), and experiment 2 (right picture) to compare plant responses between different OECD soils at a given moment. OECD1, OECD2 and OECD3 represent three OECD soils with different origins of soil constituents (4pots/′treatment′). In each of the experiments 4 replicate pots are present (each containing 4 plants) ().

5.2.5. Statistical analysis

Because of the small samples sizes (4 pots/treatment), a non-parametric statistical test (Kruskall-Wallis) was used to detect differences between 'treatments'. When significant differences were observed, a multiple comparison procedure was used to separate the means ($p<0.05$) (Conover, 1980).

5.3 Results and discussion

5.3.1 Physico-chemical characteristics of the standard soils

Differences in physico-chemical characteristics of the different standard soils were observed depending on the origin of the compounds (Table 5.1). Despite the same amount of peat present in the different soils, a different percentage soil organic carbon was measured. This demonstrates that peat characteristics were different depending on its origin. The soil data will be used in paragraph 5.3.3.2 to explain differences in enzyme capacities in leaves and roots of bean plants grown on the different soils.

Table 5.1 Physico-chemical characteristics of the different standard soils after pH adjustment. Data are means ± standard error of 2 replicates. EC = electric conductivity, OM = organic matter, $M_{exch.}$ refers to exchangeable metal concentrations in 0.1M $Ca(NO_3)_2$ extracts.

	OECD1	OECD2	OECD3
pH-KCl			
before pH adjustment	3.4 ± 0.2	3.0 ± 0.1	2.7 ± 0.1
after pH adjustment	6.0 ± 0.1	5.8 ± 0.1	5.9 ± 0.1
EC (µS/cm)	376 ± 9	427 ± 2	500 ± 11
OM (%)	10.0 ± 0.8	10.5 ± 0.6	7.9 ± 0.7
$Zn_{exch.}$ (mg kg^{-1} DW)	0.70 ± 0.05	0.51 ± 0.04	0.35 ± 0.04
$Cu_{exch.}$ (mg kg^{-1} DW)	<0.25	<0.25	<0.25
$Al_{exch.}$(mg kg^{-1} DW)	7.6 ± 0.4	3.3 ± 0.2	5.7 ± 0.3
$Fe_{exch.}$ (mg kg^{-1} DW)	1.6 ±0.1	3.2 ± 0.2	1.4 ± 0.2
$Mn_{exch.}$ (mg kg^{-1} DW)	0.81 ±0.09	0.60 ± 0.07	1.01 ± 0.08

The calibration lines for pH-adjustment of the 3 different standard soils is presented in Figure 5.2. While in OECD1 and OECD2 a similar amount of $CaCO_3$ (0.41% in OECD1; 0.40% in OECD2) was needed to adjust the soil pH to 6, a higher $CaCO_3$ dose was needed in OECD3 (0.59%). This corresponds to the lowest initial pH of OECD3 (Table 5.1).

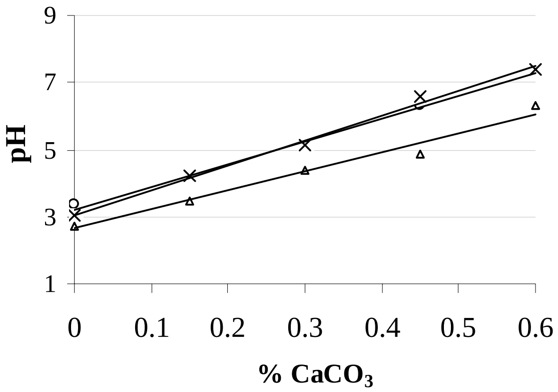

Figure 5.2 Calibration lines for pH adjustment of the three different standard soils. Legend: OECD1 (o), OECD2 (x) , OECD 3(△). Equations: OECD1: $y = 6.7467x + 3.216$, $R^2 = 0.99$; OECD2: $y= 7.4067x + 3.058$, $R^2 = 0.99$; OECD3: $y= 5.6933x + 2.644$, $R^2 = 0.98$.

5.3.2 Experiment 1: reproducibility of plant responses

Plant responses obtained on different replicate series of OECD1 soil (Figure 5.1) are presented in Table 5.2. The experiment was repeated 3 times (February, April, September) for the purpose of validation of test results. At each moment all growth parameters (leaf area, shoot length, root weight), enzymes capacities in leaves (GPOD, ME and ICDH) and enzyme capacities in roots (GPOD, ME and GlDH) were found to be equal (no significant difference at the 95% level). This illustrates that the plant responses on a given soil at a given moment are reproducible. This reproducibility of results is crucial for a reliable interpretation of possible effects observed in the main experiment (paragraph 5.3.3). Relative deviations among moments between the lowest and the highest observations of a particular response are also given in Table 5.2. These relative deviations were calculated as the difference between the lowest and the highest mean obtained for a certain response, expressed as percentage of the lowest mean. Relative deviations ranged from 0.4% to 20.0% for growth responses, from 3.7% to 20.4% for enzyme capacities in leaves, and from 1.9% to 17.4% for enzyme capacities in roots, depending on the parameter and moment considered. Despite the sometimes relatively large variations (up to 20%), the reproducibility of results within one moment was concluded to be satisfying, considering the biological nature of the samples.

In comparison with the variations observed at a certain moment, much larger response variations appeared between different cultivation moments (Table 5.1). Relative deviations in this case (calculations not shown) ranged from 21.0% to 54.6% for growth responses, from 46.0% to 70.5% for enzyme capacities in leaves and from 10.3% to 91.3% for enzyme capacities in roots. The low observed deviation for one of the roots enzymes (10.3% for ME) may just be a coincidence, since larger variations were observed among replicates at a same moment (cfr. above). Potential parameters responsible for variation among cultivation moments are numerous and include plant related factors as well as factors related to experimental conditions.

Among plant related factors, endogenous rhythms may influence plant responses even when grown under controlled environmental conditions (Colepicolo et al., 1992). ‘Endogenous rhythms’ regulate many aspects of plant physiology and development not only in relation to circadian circumstances, but also related to seasonal variations (Johnson, 2001). The genetic identity of some of the components of the circadian oscillator has recently been described (Webb, 2003). Another plant factor of potential importance is the nutrient content in the seed, which may be related not only to seed size but also to carry-over effects from the soil the seeds were produced on. Such factors are not necessarily constant for different seed lots.

Table 5.2 Plant responses on three replicate series of OECD 1 soil, repeated at three different moments. Data indicate mean ± standard error of four replicates. No differences in responses were observed between three replicates series within 1 moment at the 95% level. GPOD = guaiacol peroxidase, ME = malic enzyme, GlDH = glutamate dehydrogenase, ICDH = isocitrate dehydrogenase. Enzyme capacities expressed in mU g^{-1} FW. RD = Relative Deviation = difference between the lowest and the highest mean obtained for a certain response (at a given moment), expressed as percentage of the lowest mean (at that moment).

	Growth responses			Enzyme capacity in leaves			Enzyme capacity in roots		
February	Shoot length (mm)	Leaf area (cm^2 leaf^{-1})	Root weight (g plant^{-1})	GPOD	ME	ICDH	GPOD	ME	GlDH
OECD1	92.5±1.6	22.8±0.5	1.13±0.07	822±89	702±19	794±36	3002±152	384±17	256±26
OECD1′	92.9±0.8	21.7±0.6	0.95±0.11	844±137	767±59	793±29	3084±33	380±12	233±11
OECD1″	92.6±1.2	20.8±0.4	1.14±0.08	867±50	673±13	822±36	2996±132	377±19	242±7
RD (%)	**0.4**	**9.6**	**20.0**	**5.5**	**14.0**	**3.7**	**2.9**	**1.9**	**9.9**
April									
OECD1	74.3±3.2	25.8±0.7	1.69±0.05	1207±23	471±24	456±42	4964±332	330±13	260±16
OECD1′	81.1±2.2	27.6±1.3	1.63±0.07	1108±62	480±48	485±58	4610±180	350±18	223±14
OECD1″	79.9±2.4	25.7±2.1	1.66±0.08	1332±108	516±35	474±44	5010±474	345±31	207±15
RD (%)	**9.2**	**7.4**	**3.7**	**20.2**	**9.6**	**6.4**	**8.7**	**6.1**	**11.6**
September									
OECD1	100.3±4.4	24.5±1.4	1.23±0.07	576±44	500±13	794±13	5539±106	344±19	215±13
OECD1′	94.3±1.1	23.1±1.2	1.23±0.06	601±76	566±18	773±11	5812±266	404±16	229±17
OECD1″	94.3±1.3	22.4±0.2	1.19±0.02	553±61	470±24	690±29	5549±100	386±21	199±16
RD (%)	**6.4**	**9.4**	**3.4**	**8.7**	**20.4**	**15.1**	**4.9**	**17.4**	**15.1**

Even if experimental conditions are controlled as good as possible some sources of variation may be related to these conditions. Uncontrolled variations in the peat compound (which is sold in a humid state) due to aging reactions can not be ruled out and may affect the composition of the standard soil. Also climatic conditions in the growth chamber (e.g light quality and quantity, temperature, humidity) and (in case of enzyme measurements) age of the reagents may be subject to slight (unintentional) variations depending on the test moment and may contribute to the observed differences in plant responses.

The large range of plant response variation among test moments, clearly demonstrates the necessity to include a negative control sample every time the PlantTox test is performed. The observations certainly ask for further investigation and clarification. Furthermore, addition of positive control samples to the PlantTox test, with constant composition and toxicity, would be of great value to further improve its reliability. At each test moment this could possibly allow the adjustment of test interpretations to a calibration curve based on the positive control samples, or at least it would offer the possibility to confirm a constant interpretation of test results.

5.3.3 Experiment 2: Effect of variations in the origin of OECD soil constituents

5.3.3.1 Morphological parameters

Growth parameters of bean plants cultivated on the 3 different OECD soils (OECD1, OECD2, OECD3) are summarized in Table 5.3. No significant influence of the origin of soil constituents was observed on growth responses. Relative deviations between the lowest and the highest value ranged from 5.8% to11.5% for the different growth responses. This variability was well in the range of the variability observed between replicates of a same standard soil at a certain moment (see above).

Table 5.3 Growth responses on OECD soils with different origins of soil constituents (OECD1, OECD2, OECD3). Data indicate mean ± standard error of four replicates. Data followed by the same letter are not statistically different at the 95% level.

	Growth responses		
	Shoot length (mm)	Leaf area (cm^2 leaf $^{-1}$)	Root weight (g plant $^{-1}$ FW)
OECD1	92.5±1.6 (a)	22.8±0.5 (a)	1.13±0.07 (a)
OECD2	94.3±2.5 (a)	23.1±0.4 (a)	1.05±0.04 (a)
OECD3	87.2±1.1 (a)	21.9±0.6 (a)	1.17±0.04 (a)

5.3.3.2 Biochemical parameters

In contrast to growth parameters, enzyme capacities showed differential responses in plants grown on the different OECD soils (Figure 5.3). In leaves as well as roots the capacities of all enzymes were the highest in plants grown on OECD3 soil. Relative deviations between the lowest and the highest enzyme capacities on the different standard soils were 17.4%, 27.6% and 29.8% for ME, ICDH and GPOD capacities in leaves and 30.7%, 48.1% and 63.8% for ME, GlDH and GPOD capacities in roots. Such large deviations were never observed among replicates of a same standard soil (see above). The most aberrant enzyme responses in plants grown on the OECD3 soil correspond to the different calibration line of pH-adjustment (Figure 5.1) of this soil. In Chapter 4 regression relationships were determined between physico-chemical soil properties and enzyme capacities in bean plants grown on a range of non-toxic field soils. For the three different standard soils under study in the present chapter we measured the soil properties that were present as predictor variables in the various models. This allowed us to verify if the observed differences in enzyme capacities between standard soils could be predicted based on the specific characteristics of the soils. The absolute values of the predictions do not necessarily match the observations in the present study because of the previously mentioned differences in enzyme capacities among cultivation times (Table 5.2). Relative differences between the predictions for the 3 OECD soils are our main concern.

The results of the predictions are shown in Table 5.4. Predictions were not made for ICDH in leaves and GlDH in roots because model equations for these variables were reported to be influenced considerably by the presence of some acid forest samples with deviating responses in the dataset of Chapter 4.

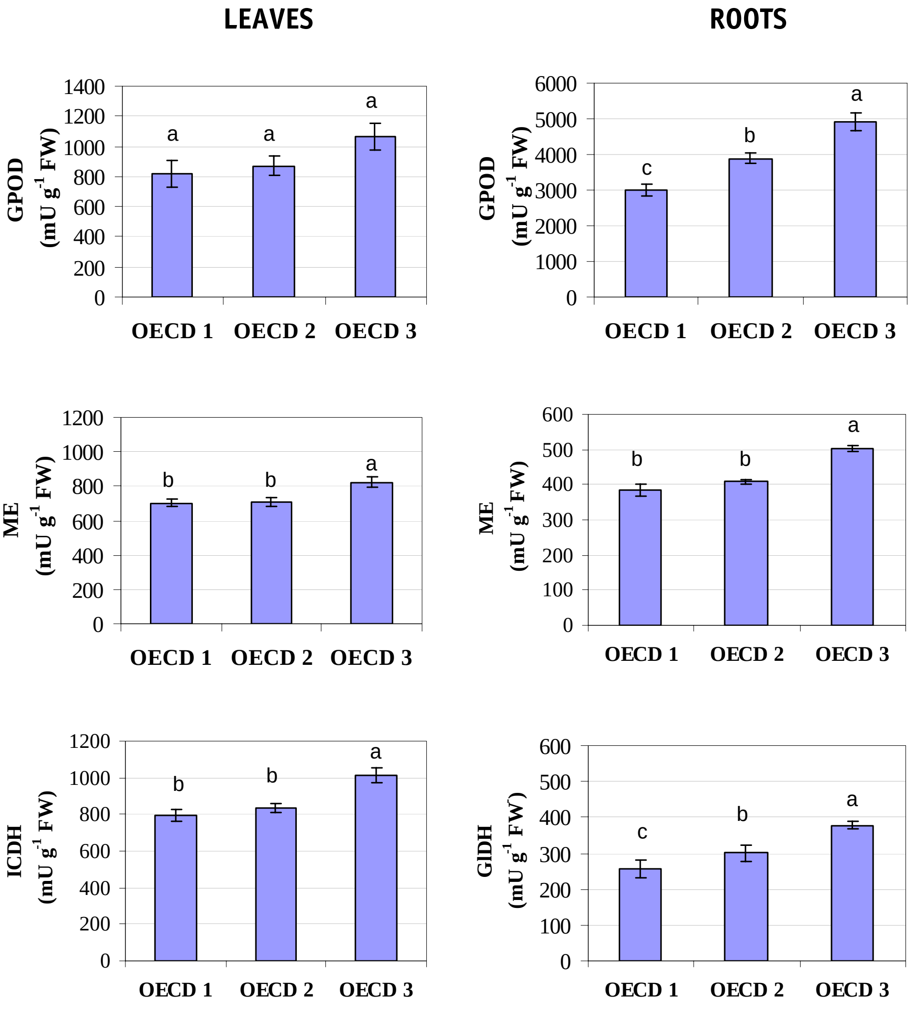

Figure 5.3 Enzyme capacities in leaves and roots of 14-day old *Phaseolus vulgaris* seedlings grown on OECD soils with different origins of soil constituents (OECD1, OECD2, OECD3). Bars indicate mean ± standard error of 4 replicates. Letters above the bars refer to statistical significant differences ($p<0.05$). GPOD = guaiacol peroxidase, ME = malic enzyme, GlDH = glutamate dehydrogenase, ICDH = isocitrate dehydrogenase.

Table 5.4 Prediction of enzyme capacities (mU g^{-1} FW) in bean plants grown on the different OECD soils based on previously described regression equations (Chapter 4). Attention should be focused on relative differences among soils, and not to absolute data. GPOD = guaiacol peroxidase, ME = malic enzyme, GlDH = glutamate dehydrogenase, ICDH = isocitrate dehydrogenase, OM=organic matter, EC= electrical conductivity, CEC=cation exchange capacity, metals refer to exchangeable metal concentrations in 0.1M $Ca(NO_3)_2$ extracts.

Model equations	Soil	Predicted	Observed
$\mathbf{GPOD}_{leaves} = 997.07 - 0.30\,EC + 4.93\,Zn$	OECD1	830	822
$- 0.016\,Mn^2 - 21.13\,Fe^2 - 0.81\,Al^2 + 0.028$	OECD2	856	872
$Al^2{*}Zn + 0.00036\,Al^2{*}\,Mn^2 + 0.059\,Al^2{*}\,Fe^2$	OECD3	803	1067
$\mathbf{ME}_{leaves} = 447.12 - 19.33\,pH + 0.00073\,EC$	OECD1	360	702
$- 206.57\,Fe^2 + 1.51\,Al^2 - 0.0033\,Al^2{*}EC$	OECD2	354	710
$+ 0.028\,Al^2{*}\,Fe^2 + 0.60\,Fe^2{*}EC$	OECD3	428	824
$\mathbf{GPOD}_{roots} = 4379.79 + 9122.11\,Cu$	OECD1	5313	3001
$- 5083.05\,Fe + 1.11\,EC + 3460.91\,Cu{*}Fe$	OECD2	5581	3885
$+ 10.02\,EC{*}Fe - 14.89\,EC{*}Cu$	OECD3	6166	4915
$\mathbf{ME}_{roots} = 647.08 - 0.29\,EC - 88.6\,OM$	OECD1	86	384
$+ 145.5\,Fe - 6.68\,Fe^2 - 223\,Cu^2$	OECD2	56	408
$+ 0.075\,OM{*}EC + 86.67\,OM{*}Cu^2$	OECD3	267	503

The highest enzyme capacities observed in plants grown on OECD3, were also predicted by the regression equations. The higher EC and lower %OM of this soil compared to the other standard soils, were to a large extent responsible for these high predictions. In order to check if indications for a similar phenomenon could also be found in other studies, a literature search on Zn toxicity data obtained on OECD substrate was performed. The results revealed different EC50 values for *Eisenia Fetida* (cocoon production) and *Folsomia candida* (juvenile production) in different studies (see Lock and Janssen, 2001 and references therein). According to Spurgeon and Hopkin (1996) for example EC50 in *Eisenia fetida* was 462 mg Zn kg^{-1}; Spurgeon et al. (1997) reported values between 234 mg Zn kg^{-1} and 382 mg Zn kg^{-1}, while Lock and Janssen (2001) found an EC50 of 704 mg Zn kg^{-1} (95% confidence limits: 658-754).

Similarly Zn EC50 for *Folsomia candida* varied from 487 mg Zn kg^{-1} according to Smit and Van Gestel (1998) to 900 mg Zn kg^{-1} according to Sandifer and Hopkin (1997). In all studies, OECD artificial soil was prepared as prescribed by OECD (1984), adjusted to the same pH (6), and respecting a similar equilibration time and exposure period. Even if differences in the background of the laboratory cultured animals (genetic background, food supply,...) and differences in test temperature (Smit and Van Gestel, 1997) are considered to determine these variations to a large extent (LØkke and Van Gestel, 1998), it can not be excluded that differences in the origin of OECD constituents may have contributed to the effect.

5.3.4 Difficulties arising from the difference in plant responses between different standard soils

As a result of the potential differences in plant responses among different standard soils (OECD1, OECD2, OECD3), questions raise about the general suitability of OECD standard soil as negative control in the PlantTox test. Application of the toxicity classification system proposed in Chapter 4. for interpretation of PlantTox results (which is based on the interpretation of relative differences between the response on an OECD standard soil (negative control) and the potentially contaminated soil sample to be assessed), may lead to a different classification of the soil sample depending on the origin of the constituents used for preparation of OECD standard soil (negative control soil). Interpretation of PlantTox results, may not be completely reproducible when OECD soils with different origins of the constituents are used. This affect the accuracy of the system in a negative way. Additional research is needed to find a negative control of which the composition can be kept completely constant and which at the same delivers plant responses well in the range of non-toxic field soils. Addition of nutrient solution to an inert substrate or use of other standard soils could be a possible option. In the mean time we suggest to prepare the OECD standard soil for application as negative control in the PlantTox test with compounds in origin or characteristics as similar as possible to the ones used in Chapter 4. This soil corresponds to OECD3 in the present chapter.

5.4 Conclusions

When bean seedlings were grown on standards soils prepared according to the prescriptions of guideline N° 207 of the Organization for Economic Cooperation and Development (OECD, 1984), but with varying commercial origins of the compounds, differential enzymatic responses of the test plants were obtained. Relative deviations in enzyme responses among the different standard soils ranged between 17.4% and 63.8%, and were considerably higher than deviations observed among replicates of a same standard soil. The differences in responses among standards soils could be related mainly to differences in EC and OM content of these soils.

As a result of the potential differences in plant responses between different standard soils (OECD1, OECD2, OECD3), questions can be posed to the general suitability of OECD standard soil as negative control in the PlantTox test. Generally we advice to interpret the term 'OECD standard soil' with care since its composition, by definition, is not completely controlled.

PART III
References

Adriano, D.C., 2001. Trace elements in terrestrial environments: biogeochemistry, bioavailability and risks of metals. Springer, New York, USA.

Allison, L.E., 1965. Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. *In*: Agronomy Monograph 9. Black, C.A. *et al.* (eds.). ASA, Madison, Wisconsin, USA.

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53: 1331-1341.

Belgisch Staatsblad, 1996. Decree of the Flemish government of 5 march 1996 concerning the Flemish regulation on soil remediation (Vlarebo). Belgisch Staatsblad - Moniteur Belge, 27.03.1996. (modified on repeated occasions, latest change BS 5.08.2004).

Boisson, J., Mench, M., Vangronsveld, J., Ruttens, A., Kopponen, P., De Koe, T., 1999. Immobilization of trace metals and arsenic by different soil additives: evaluation by means of chemical extractions. Commun. Soil Sci. Plant Anal. 30: 365-387.

Boscolo P.R.S., Menossi, M., Jorge, R.A., 2003. Aluminium-induced oxidative stress in maize. Phytochemistry. 62: 181-189.

Brady, N.C., 1984. The nature and properties of soils. Macmillan, New York, USA.

Byl, T.D., Klaine, S.J., 1991. Peroxidase Activity as an indicator of sublethal stress in the aquatic plant *Hydrilla Verticillata* (Royle). *In*: Gorusch J.W., Lower, W.R., Wang, W., Lewis, M.A. (eds.). Plants for toxicity assessment. second volume, ASTM STP 115. American Society for Testing and Materials, Philadelphia, USA.

Clijsters, H., Cuypers, A., Vangronsveld, J., 1999. Physiological responses to heavy metals in higher plants; defence against oxidative stress. Z. Naturforsch. 54c: 730-734.

Colepicolo, P., Camarero, V.C.P.C., Hastings, J.W., 1992. A circadian-rhythm in the activity of superoxide dismutase in the photosynthetic alga *Gonyaulax-Polyedra*. Chronobiol. Int. 9(4): 266-268.

Conover, W.J., 1980. Practical nonparametric statistics. Wiley, New York, USA.

Cuypers, A., Vangronsveld, J., Clijsters, H., 2002. Peroxidases in roots and primary leaves of *Phaseolus vulgaris*. Copper and Zinc Phytotoxicity: a comparison. J. Plant Physiol. 159: 869-876.

Debus, R., Hund, K. 1997. Development of analytical methods for the assessment of ecotoxicological relevant soil contamination. Part B: ecotoxicological analysis of soil and soil extracts. Chemosphere 35 (1/2): 239-261.

El Jaoual, T., Cox, D.A., 1998. Manganese toxicity in plants. J. Plant Nutr. 21(2): 353-386.

Engbersen, J.F.J., De Groot, A.E., 1992.Inleiding in de bio-organische chemie. Pudoc, Wageningen, The Netherlands.

Fletcher, J., 1991. Keynote speech: A Brief Overview of Plant Toxicity Testing. *In*: Plants for toxicity assessment, second volume. Gorsuch J.W. et al. (eds.). ASTM STP 115, American Society for Testing and Materials, Philadelphia, USA.

Foyer, C., Noctor, G., 2005. Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28: 1056-1071.

Frische, T., 2003. Ecotoxicological evaluation of in situ bioremediation of soils contaminated by the explosive 2,4,6 trinitrotoluene (TNT). Environ. Pollut. 121: 103-113.

Geebelen, W., Vangronsveld, J., Clijsters, H., Adriano, D.C., Carleer, R., 2002. Amendment induced immobilization of lead in a lead-spiked soil: evidence from phytotoxicity studies. Water Air Soil Pollut. 140: 261-278.

Gong, P., Wilke, B.M., Strozzi, E., Fleischmann, S., 2001. Evaluation and refinement of a continuous seed germination and early seedling growth test for the use in the ecotoxicological assessment of soils. Chemosphere. 44(3): 491-500.

Hulsen, K., Top, E.M., Höffe, M., 2002.Chlorophyll fluorescence-to monitor microbial degradation of linuron applied in low concentrations to bean plants. New Phytol. 154: 821-829.

Johnson, C.H., 2001. Endogenous timekeepers in photosynthetic organisms. Annu. Rev. Physiol. 63: 695-728.

Kabata Pendias, A., Pendias, H., 1984. Trace elements in soils and plants. CRC Press, Boca Raton, FL, USA.

Krugh, B.W., Miles, D., 1996. Monitoring the effects of five "nonherbicidal" pesiticide chemicals on terrestrial plants using chlorophyll fluorescence. Environ. Toxicol. Chem, 15/4: 495-500.

Lock K., and Janssen C.R., 2001. Modeling Zn Toxicity for terrestrial invertebrates. Environ. Toxicol. Chem. 20(9): 1901-1908.

LØkke, H., Van Gestel, A.M., 1998. Soil toxicity testing in risk assessment of new and existing chemicals. *In*: Handbook of soil invertebrate toxicity tests. LØkke, H., Van Gestel, A.M. (eds.). John Wiley & Sons Ltd., West Sussex, U.K.

Maréchal, R., Tavernier, R., 1974. Atlas van België. Pedologie. Nationaal Comité voor Geografie, Ghent, Belgium.

Marwood, C.A., Solomon, K.R., Greenberg, B.M., 2001. Chlorophyll fluorescence as a bioindicator of effects on growth in aquatic macrophytes from mixtures of polycyclic aromatic hydrocarbons. Envrion. Toxicol. Chem 20: 890-891.

Marwood, T.M., Knoke, K., Yau, H., Lee, J.T., Trevors, A., Suchorski-Tremblay, A., Flemming, C.A., Hodge, V., Liu, D.L., Seech, A.G., 1998. Comparison of toxicity detected by five bioassays during bioremediation of diesel fuel-spiked soils. Environ. Toxicol. Water Qual. 13(2): 117-126.

McBride, M.B., Martinez, C.E., 2000. Copper phytotoxicity in a contaminated soil: Remediation tests with adsorptive materials. Environ. Sci. Technol. 34: 4386-4391.

Meers, E., Ruttens, A., Geebelen, W., Vangronsveld, J., Samson, R., Vanbroekhoven, K., Vandegehuchte, M., Diels, L., Tack, F.M.G., 2006. Potential use of the plant antioxidant network for environmental exposure assessment of heavy metals in soils. Environ. Mon. Assess. *In Press*.

Mench, M., Manceau, M., Vangronsveld, J., Clijsters, H., Mocquot, B., 2000. Capacity of soil amendments in lowering the phytoavailability of sludge-borne Zn. Agronomie. 20: 383-397.

Mench, M., Vangronsveld, J., Didier, V., and Clijsters, H., 1994. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. Environ. Pollut. 86: 279-286.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410.

Neter, J., Wasserman, W., Kutner, M., 1990. Applied linear statistical models. Richard D. Irwin. Inc., Boston, MA, USA.

Norton, D. (ed.), 1996. Early seedling growth protocol for soil toxicity screening. Department of Ecology, Publication N° 96-324, Washington State, USA.

OECD, 1984. Guideline for testing of chemicals, N° 207. Earthworm, acute toxicity test. OECD publications, Organization for Economic Cooperation and Development, Paris, France.

Sandifer , R.D., Hopkin, S.P., 1997. Effects of temperature on the relative toxicities of Cd, Cu, Pb and Zn to *Folsomia candida* (Collembola). Ecotoxicol. Environ. Saf. 37: 125-130.

SAS, 2004. SAS/ STAT User' s Guide Version 9, SAS Institute Inc., Carey, NC, USA.

Scheid, H.W., Ehmke, A., Hartmann, T., 1980. Plant NAD-dependent glutamate dehydrogenase. Purification, molecular properties and metal ion activation of the enzymes from *Lemna minor* and *Pisum sativum*. Z. Naturforsch. 35(3/4): 213-221.

Schultz, E., Joutti, A., Raisanen, M., Lintinen, P., Martikainen, E., Lehto, O., 2004. Extractability of metals and ecotoxicity of soils from two old wood impregnation sites in Finland. Sci. Tot. Env. 326(1-3): 71-84.

Schützendübel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53: 1351-1365.

Simini, M., Wentsel, R.S., Checkai, R.T., Phillips, C.T., Chester, N.A., Major, M.A., Amos, J.C., 1995. Evaluation of soil toxicity at Joliet Army ammunition plant. Environ. Toxicol. Chem. 14(4): 623-630.

Smit, C.E., Van Gestel, C.M.A., 1997. Influence of temperature on the regulation and toxicity of zinc in *Folsomia candida* (Willem) (Collembola:Isotomidea) in relation to bioavailability in soil. Environ. Toxicol. Chem. 16: 1177-1186.

Spurgeon, D.J., Hopkin, S.P., 1996. Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. Pedobiologia 40: 80-96.

Spurgeon, D.J., Tomlin, M.A., Hopkin, S.P., 1997. Influence of temperature on the toxicity of zinc to the earthworm *Eisenia fetida*. Bull. Environ. Contam. Toxicol. 58: 283-290.

STATISTICA, 1994. Statistica for the Macintosh. User's manual Vol I, II. Statsoft, Tulsa, OK, USA.

Thomas, J.M., Skalski, J.R., Cline, J.F., McShane, M.C., Simpson, J.C., 1986. Characterisation of chemical waste site contamination and determination of its extent using bioassays. Environ. Toxicol. Chem. 5: 487-501.

Van Assche, F., Cardinaels, D., Clijsters, H., 1988. Induction of enzyme capacity in plants as a result of heavy metal toxicity: dose-response relations in *Phaseolus vulgaris* L., treated with zinc and cadmium. Environ. Pollut. 52: 103-115.

Van Assche, F., Clijsters, H., 1990a. A biological test system for the evaluation of the phytotoxicity of metal-contaminated soils. Environ. Pollut. 66: 157-72.

Van Assche, F., Clijsters, H., 1990b. Effects of metals on enzyme activities in plants. Plant Cell Environ. 13: 195-206.

Vangronsveld, J., Clijsters, H., 1992. A biological test system for the evaluation of metal phytotoxicity and immobilization by additives in metal contaminated soils. *In:* Metal compounds in environment and life, 4. Interrelation between chemistry and biology. Merian E, Haerdi W. (eds.). Science Reviews Inc., Wilmington, USA.

Vangronsveld, J., Clijsters, H., 1994. Toxic effects of metals. *In*: Plants and the chemical elements. Farago, M.E. (ed.). VCH Verlagsgesellschaft, Weinheim, Germany.

Vangronsveld, J., Colpaert, J., Van Tichelen, K., 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environ. Pollut. 94: 131-140.

Vangronsveld, J., Sterckx, J., Van Assche, F., Clijsters, H., 1995b. Rehabilitation studies on an old non-ferrous waste dumping ground: effects of revegetation and metal immobilization by beringite. J. Geochem. Expl. 52: 221-229.

Vangronsveld, J., Van Assche, F., Clijsters, H., 1995a. Reclamation of a bare industrial area contaminated by non-ferrous metals: *in situ* metal immobilization and revegetation. Environ. Pollut. 87: 51-59.

Verdoni, N., Mench, M., Bessoule, J.J., Vangronsveld, J., 2001. Fatty acid composition of tomato leaves as biomarker of metal-contaminated soils. Environ. Toxicol. Chem. 20: 382-388.

Wang, W., Freemark, K., 1995. The use of plants for environmental monitoring and assessment. Ecotoxicol. Environ. Saf. 3: 289-301.

Webb, A.A.R., 2003. The physiology of circadian rhythms in plants. New Phytol. 160: 281-288.

Part IV

Conclusions and Perspectives

MAIN RESULTS AND CONCLUSIONS

In the north east of Belgium (Campine region; Dutch: de Kempen) large areas of land are contaminated with metals (mainly Zn, Cd, Pb) due to the historical presence of several zinc smelters. *In situ* immobilization and phytostabilization have been proposed as potential low-cost remediation alternatives for large scale metal contaminations (Vangronsveld and Cunningham, 1998; Vassilev et al., 2004). The objectives of this thesis were (1) to asses the potential of several metal immobilizing soil treatments to reduce the risks associated with the soil contamination in the Kempen on the long term, and (2) to optimize a phytotoxicity test (PlantTox) aiming at an accurate detection of soil phytotoxicity, prior to and after metal immobilization in highly contaminated soils.

In relation to i*n situ* metal immobilization and phytostabilization, the following conclusions were drawn from this work (**PART II**):

Durability and efficiency of metal immobilization by CAH (=cyclonic ashes-type H) is high compared to liming in an acid sandy soil collected at a former agricultural field (Balen)

Liming is the oldest and likely most widely adopted technique for immobilization of metals in soils. Its metal immobilizing capacity results from an increase in pH, but the effect decreases rapidly in time due to dissolution and leaching of the liming agent. As a result liming has to be repeated frequently (every 3 to 5 years) to maintain low metal availabilities. Soil amendments with a more durable and/or stronger metal immobilizing effect are required for sustainable *in situ* immobilization and phytostabilization purposes.

In literature conflicting results are reported about the long-term metal immobilization behavior of cyclonic ashes. Field experiments at an old zinc smelter site, and simulation experiments in soil columns both demonstrate a durable effect of the treatment (Vangronsveld et al., 1995, 1996, 1999; Bouwman and Vangronsveld, 2004). In contrast, studies based on acid extractions and isotopic dilution techniques (Geebelen et al., 2006; Lombi et al., 2003) report an active immobilization mechanism of cyclonic ashes similar to that of lime.

The first chapter of this work aimed to collect additional information about the long-term effects of several soil treatments. A simulation experiment, based on water leaching in soil columns, was used to compare the durability and efficiency of soil treatment with CAH (a type of cyclonic ashes), SS (an Fe-rich material), and CAH + SS on the one hand, with that of liming on the other hand. The results showed a more sustainable immobilization effect by CAH compared to the effect of liming during the 26-years simulation period.

We hypothesize that conflicting results among studies are related to:

- the soil type (texture + initial pH) of the contaminated soil
- the difference in 'stabilization time'
- the use of different types of cyclonic ashes
- a slow and mild acidification in the present study, compared to stronger acidic chemical techniques in former studies (Lombi et al., 2002b; Geebelen et al., 2006).

After a simulation period of 26 years, soil pore water concentrations of Zn, Cd and Pb in the CAH treatment were respectively a factor of 9.4 and 5.8 and >2.4 lower compared to results obtained in the untreated soil. Plant accumulation of the same elements in the CAH treatment at that moment was still reduced with a factor 2.6, 2.0 and 1.4 for Zn, Cd and Pb respectively. The effect of CAH+SS was even stronger, at least for Zn and Cd (cfr. below). In the LIME and SS treatment, on the contrary, reduction in plant metal accumulation reached a maximum factor of only 1.4 at the end of the experiment. Moreover in the lime treatment an increase of Pb concentrations in the soil pore water was observed during the first simulated years, while this increase was not present in the CAH treatment. The results demonstrate that soil treatment with cyclonic ashes, or cyclonic ashes + steel shots, has potentials for durable reductions of plant metal uptake and metal leaching in metal contaminated soils. A concern about potential groundwater pollution in the Kempen caused by metal leaching was expressed recently (Degryse, 2003).

The combined use of cyclonic ashes and steel shots [CA(H)+SS] shows a stronger metal immobilizing effect than the individual treatments

In a simulation experiment in soil columns (Chapter 1) as well as in a lysimeter experiment under natural climatologic conditions (Chapter 2), all measured response parameters (exchangeable soil metal concentrations, metal concentrations in soil pore water or leachate water, plant metal concentrations) indicated a stronger metal immobilization by CA+SS compared to the effect of each of these compounds applied separately. In Chapter 1, CAH was the type of cyclonic ashes used, whereas in Chapter 2 and Chapter 3 cyclonic ashes from Beringen ('beringite') were used. For a few of the response parameters in Chapter 1 the difference between CAH alone, and CAH+SS was not statistically significant, but the same trend was (almost) always present. Only Pb concentrations in the soil pore water of the columns (Chapter 2) were temporarily higher after CAH+SS compared to CAH alone which is likely due to a high mobility of Pb present in SS. The high observed efficiency of the combined treatment confirms the results obtained by Boisson et al. (1999).

The use of a mixture of products generally offers the advantage that metals can be immobilized by different mechanisms and on different binding sites and was shown to be effective on previous occasions (Brown et al., 2004; Hettiarachchi et al., 2000).

Two additional hypotheses may explain the high efficiency of the combination of CA and SS (Chapter 2):

- an increased efficiency of metal binding on Fe or Mn oxides due to the high pH induced by the presence of CA;
- an increased precipitation of Fe-oxides (and thus (co)-precipitation of metals) on clay particles introduced in the soil by CA addition.

Experiments using a combination of lime and SS may reveal the relative importance of the two (hypothetic) mechanisms.

Combination of compost (C) with the inorganic amendments improves plant responses, but attention should be paid to Cu and Pb leaching when organic matter applications are considered in the context of phytostabilization projects

There is much circumstantial evidence for the importance of organic matter in metal solubility control (Mc Bride et al., 1997, Sauvé et al., 1998). Organic matter additions may increase soil pH and add exchange capacity, which both contribute to a decrease of metal availability. Different types of organic matter have been evaluated in the context of phytostabilization studies (Basta et al., 2001; Berti and Cunningham, 1997; Brown et al., 2003a, 2003b; McBride and Martinez, 2000; Shuman et al., 2002). On the other hand, organic matter additions can increase the amount of soluble organic ligands and eventually lead to the opposite effect (Martinez and Mc. Bride, 1999; Shuman et al., 2002). Application of compost is an attractive option in phytostabilization projects.

A low water holding capacity and low fertility at industrial sites often reduce the chance on a successful revegetation, while these characteristics can be improved by compost addition (Brady, 1984). However, because of the reasons mentioned above, a careful evaluation of its effect is necessary.

In the present work a lysimeter set-up under natural environmental conditions (Chapter 2, Chapter 3) was used to evaluate and compare the effect of four inorganic and/or organic treatments [compost (C), C+CA, C+CA+SS, CA+SS] on metal phytotoxicity, plant availability of metals and metal leaching in an industrial contaminated soil (Overpelt). The cyclonic ashes used in this experiment originated from the former coal mine in Beringen and are alternatively known as 'beringite'. Integration of results showed that addition of compost alone slightly reduced exchangeable soil metal contents, plant metal accumulation and phytotoxicity compared to the untreated soil. Zn and Cd leaching were also reduced, but Cu and Pb leaching on the contrary strongly increased. The differential leaching behavior among elements in the C treatment can be explained by differences in stability constants of metallo-organic complexes, which are generally higher for Cu and Pb than those for Zn and Cd (Mc Bride and Martinez, 2000; Kabata-Pendias and Pendias, 1984).

When compost was combined with inorganic metal immobilizing amendments (CA+SS) it had a beneficial effect on plant responses additional to the inorganic amendment alone. In the combined C+CA+SS treatment, the inorganic amendments were able to compensate for the increases in Cu and Pb leaching caused by the addition of compost. In the combined treatment of C+CA, on the contrary, increases were still observed, even if small compared to the compost treatment alone. Our results demonstrate that attention should be paid to Cu and Pb leaching when organic matter additions are considered for *in situ* immobilization or phytostabilization of metal contaminated soils.

C+CA+SS showed to be an effective treatment for amendment assisted phytostabilization of an industrially contaminated soil (Overpelt)

Treatment of the contaminated Overpelt soil with C+CA+SS (Chapter 2, Chapter 3), resulted after a one-year evaluation period in:

- a decrease of the soil 'Phytotoxicity Index' from 3.5 to 1.7 (on a scale from 1 to 4),
- a reduction of Zn, Cd, Cu and Pb concentrations in *Agrostis capillaris L.* with a factor of respectively 12.3, 7.8, 2.2 and >1.8 compared to plants grown on the untreated soil,
- a high diversity of spontaneously colonizing plant species on the lysimeters in comparison with the untreated lysimeter,
- an overall reduction in Zn, Cd and Pb leaching with a factor of respectively 120, 63 and 1.6 compared to metal amounts leached from the untreated soil; the effect on Pb leaching was not completely clear because Pb concentration in leachate of the untreated soil were below the detection limit of our measurements (<0.05 mg/l).

The **second experimental part** of this thesis **(PART III)** focused on the optimization of a phytotoxicity test with bean plants (*Phaseolus vulgaris* L.) (Van Assche and Clijsters, 1990; Vangronsveld and Clijsters, 1992). The so-called PlantTox test is based on the analysis of growth parameters and biochemical plant responses, in particular enzyme activities that are directly or indirectly involved in the anti-oxidative defence systems of plants, including guaiacol peroxidase (GPOD; E.C.: 1.11.1.7), malic enzyme (ME; E.C.: 1.1.1.40), glutamate dehydrogenase (GlDH; E.C.: 1.4.1.2) and isocitrate dehydrogenase (ICDH; E.C.: 1.1.1.42). When phytotoxicity tests are applied as bioassays, to evaluate the toxicity of potentially contaminated field samples, a reliable and sensitive interpretation of results depends to a large extent on the choice of an appropriate reference soil (Frische, 2003). The present work aimed to gain insight in the frequent ranges of the endpoints of the PlantTox test in non-toxic conditions, and to contribute to the determination of the most suitable reference level or reference soil for the test when used as a bioassay in risk assessment of contaminated soils.

From the second experimental part of this study the following conclusions were drawn:

In screening experiments to detect phytotoxicity in a series of soil samples, the use of only one negative control soil is a potential option for the PlantTox test

A phytotoxicity test with bean plants (PlantTox), designed to assess soil metal toxicity, was performed on 25 soil samples collected in Flanders (Belgium), in areas with expected low levels of trace element deposition (further referred to as reference soils) (Chapter 4). Attention was paid to the collection of soils with different land uses (mainly arable land and pastures but also fallow land and a few forest soils) in order to reach a large spectrum in soil properties. When very acid soils were excluded, endpoints of the PlantTox test showed a rather limited range in response variation, over a wide variety of potential reference soils. Variation factors of enzymatic responses across the series of reference soils, only ranged from 1.7 ($GPOD_{leaves}$) to 3.7 ($GPOD_{roots}$)(after exclusion of acid forest soils). In contrast; when strongly metal contaminated soils were assessed by means of the PlantTox test (Vangronsveld and Clijsters, 1992; Vangronsveld et al., 1995) increases in enzyme capacities with factors up to 7, 6 and 47 have been found for ME, ICDH and GPOD in the leaves, and with factors up to 6, 9 and 13 for ME, GlDH and GPOD in roots) compared to the reference soil. Based on the relatively small variation range under non-toxic conditions, the use of only one reference soil seems acceptable, at least if very acid soils (pH<4) are excluded.

Multiple regressions models, relating enzymatic plant responses to physico-chemical soil parameters, allowed the prediction of enzyme reference levels for each particular soil, within the uncertainties determined by the prediction limits

When the PlantTox test was applied on a series of non-toxic soils, multiple regression analysis relating plant responses to physico-chemical soil parameters, delivered significant models for all enzymes measured with R^2 ranging from 83% to 99% (Chapter 4). Soil pH, conductivity and exchangeable levels of Zn, Cu, Al, Fe or Mn were present as X-variables in the regression equations. No significant models could be fitted for any of the growth responses. This observation suggests that growth responses, in contrast to enzyme capacities, are to a large extent determined by other soil factors than those investigated in our study. Available soil levels of macronutrients (N, P, K) and some physical or biological soil factors are possible candidates. Application of the models potentially allows the use of a flexible reference level in the PlantTox test, adapted to each individual soil. However, due to the relatively large prediction limits of the models compared to the total range of response variation in non-toxic conditions (cfr. supra) practical benefits of this ‘model approach’ could not (yet) be obtained.

OECD artificial soil was proposed as a possible general reference soil for the PlantTox test and a corresponding toxicity classification system was developed

Van Assche and Clijsters (1990) and Vangronsveld and Clijsters (1992) have proposed a classification system to interpret the results of the PlantTox test, based on the % induction in enzyme capacities and the % reduction in growth responses compared to the response on a reference soil. In their studies an agricultural soil was used as a reference. The results obtained in the present study demonstrated that responses of bean plants grown on OECD 207 artificial soil were well within the range of responses on natural (non forest) reference soils. OECD artificial soil was therefore proposed as a potential general reference soil for the PlantTox test (one-reference-soil approach) assuming that the composition (and characteristics) of this artificial substrate can be controlled better compared to the characteristics of field soils. The original indexation in phytotoxicity classes was recalibrated (Chapter 4) by combination of our knowledge about the range of plant responses in reference conditions on the one hand, and the plant responses on OECD substrate relative to the other non-toxic soils on the other hand. The non-toxic upper limit of most enzymatic responses was selected as the 90% percentile of each enzyme response in our soil series (after exclusion of 3 very acid samples); for growth responses the 10th percentile was taken. The system thereby avoids the detection of false positive results; false negatives on the opposite can not be ruled out.

Variations in enzymatic responses among OECD soils, prepared using constituents that had a different (commercial) origin, raise questions about the suitability of this soil as negative control in the PlantTox test

A comparison was made between responses of bean plants grown simultaneously on artificial soils prepared according to the prescriptions of guideline N° 207 of the Organization for Economic Cooperation and Development (OECD, 1984), but with varying commercial origins of the soil constituents (Chapter 5). Results revealed similar growth responses (relative deviations ranging from 5.8% to 11.5%) but differential enzymatic responses (relative deviation ranging from 17.4% to 63.8%) when different origins of the constituents were used. The different enzymatic responses could be related mainly to differences in conductivity (EC) and organic matter content (OM) of the various standard soils. This observation argues against the general suitability of OECD artificial soil in the PlantTox test.

PERSPECTIVES

The exploitation of biological and chemical processes to reduce the inherent risk associated with metal contaminated sites is an emerging branch of science (Kamnev and van der Lelie, 2000). *In situ* immobilization of metals, by means of metal immobilizing soil amendments, whether or not in combination with phytostabilization approaches, has been proposed as a low cost and environmentally sound alternative to current remediation options (Vangronsveld and Cunningham, 1998). A high observed durability of the immobilizing effect of cyclonic ashes (CAH) compared to lime in this study opens perspectives for *in situ* applications of such treatment in the Kempen. Whereas liming, because of the temporary character of the immobilization effect, offers little guarantees for a durably save agricultural production on metal contaminated land, application of cyclonic ashes seems to have a more permanent effect on metal mobility and bioavailability in soils (accumulation in plants). More research into the capability of this treatment to reduce metal concentrations in crops (in particular Cd) below consumption limits, in various contaminated soils, is necessary before practical applications can be considered. The results suggest that the search for cyclonic ashes or other clay-like materials with characteristics even more similar to the original ashes from Beringen is worth to be continued. Combination of cyclonic ashes with SS can increase the extent of reductions in cases where the effect of cyclonic ashes alone is insufficient.

Higher plants are an essential part of a healthy and balanced ecosystem. They produce oxygen and organic substances on which most other life forms depend. Plants provide food and nesting habitat for insects, invertebrates, birds, mammals, etc. They have an essential role in the processes of nutrient cycling and soil stabilization. Toxic effects on plants may have severe consequences for

wildlife, and the whole ecosystem. Phytotoxicity tests are therefore an indispensable part in ecotoxicological risk assessments of contaminated sites (Wang and Freemark, 1995) both prior to and after site remediation.
The influence of the soil nutrient status on plant growth responses, which are used as endpoints in most standardized toxicity tests (OECD, 1984; US-EPA 1996a, 1996b; ISO, 1993, 1995), is generally recognized. Even if it is in theory possible to adjust nutrient levels of the contaminated soil to the level of the reference, this would be complex and even not desirable, because a change in nutrient levels may directly or indirectly affect soil toxicity (Gong et al., 2001). Toxicity tests, making use of alternative test endpoints, such as the PlantTox test in this study, potentially less dependent on soil nutrient levels, could be of great value to improve the accuracy of toxicity assessments of contaminated soils.

More research is needed to further improve the PlantTox protocol. Related to the 'model approach' improved regression equations, able to predict reference responses within smaller prediction limits, may lead to an improved accuracy of test interpretations. When a flexible reference level can be used, adapted to each individual situation, both false positive and false negative conclusions about the toxicity of a substrate can be avoided (better). Application of the 'one–reference-soil' approach, as developed in this study, on a dataset including contaminated samples should reveal the possibilities and limitations of the proposed system. For future applications of this approach, however, an alternative to the OECD 207 artificial soil as negative control should be found of which the composition can be held completely constant throughout different test moments and laboratories. Addition of nutrient solution to an inert substrate or the use of other standard soils may be an option. Furthermore inclusion of positive control samples in the PlantTox test, with constant composition and toxicity, would be of great value to further improve its reliability. At each test moment, this potentially allows the adjustment of test interpretations to a calibration curve, based on the positive control samples. At least it would offer the possibility to confirm a constant interpretation of test results. When using the 'model approach', a negative or positive control sample could also serve to adjust for differences in test moments caused by a variety of factors that are hard to control such as endogenous rhythms or unintended variations in experimental conditions (Chapter 5).

Like other plants species on the lists of regulatory agencies (OECD, US-EPA), *Phaseolus vulgaris*, used in the PlantTox test, is an agronomic species. It is largely unknown and subject of considerable debate, whether or not the sensitivity of agronomic crops generally correspond to the sensitivity of native species (of the same family) in ecosystems. Paschke et al. (2000) showed that phytotoxicity thresholds of Zn and Cu were higher in 7 grass species of Western North America than those of some agronomic crops that are currently used in risk assessments. They concluded that the use of agronomic species in phytotoxicity test, might lead to false positive conclusions about the necessity for remedial actions at contaminated sites. The use of agronomic species, including the use of *Phaseolus vulgaris* as a test species in phytotoxicity tests may therefore be questionable. However, as long as information about the relative sensitivities of different species remains limited, both wildtype and agronomic species can considered equally suited for use in phytotoxicity test. Moreover, to protect also the more sensitive species in ecosystems, it may be advisable to include tests with a sufficiently high degree of sensitivity in toxicity evaluations.

Recent evolutions in the area of oxidative stress and oxidative signaling in plants, whether or not in combination with molecular approaches, open doors for the selection of new stress-related plant responses possibly suited for use in phytotoxicity tests. A question, which remains open, is whether or not enzymatic endpoints measured on seedlings are indicative for ecosystem effects.

This question is subject to debate and no study has ever been performed to resolve it. Further research is necessary to relate effects measured in the PlantTox test to ecological field observations.

PART IV
References

Basta, N.T., Gradwohl, R., Snethen, K.L., Schroder, J.L., 2001. Chemical immobilization of lead, zinc and cadmium in smelter-contaminated soils using biosolids and phosphate rock. J. Environ. Qual. 30: 1222-1230.

Berti, W.R., Cunningham, S.D., 1997. In-place inactivation of Pb in Pb–contaminated soils. Environ. Sci. Technol. 31: 1359-1364.

Boisson, J., Mench, M., Vangronsveld, J. *et al.*, 1999. Immobilization of trace metals and arsenic by different soil additives: evaluation by means of chemical extractions. Commun. Soil Sci. Plant Anal. 30: 365-387.

Bouwman, L.A., Vangronsveld, J., 2004. Rehabilitation of the nematode fauna in a phytostabilized heavily zinc-contaminated, sandy soil. J. Soils Sediments. 4: 17-23.

Brady, N.C., 1984. The nature and properties of soils. Macmillan Publishing Company, New York, USA.

Brown, S.L., Chaney, R.L., Hallfrish, J., Ryan, J.A., Berti, W.R., 2004. In situ soil treatments to reduce the phyto- and bioavailability of lead, zinc and cadmium. J. Environ. Qual. 33: 522-531.

Brown, S.L., Chaney, R.L., Hallfrish, J.G., Xue, Q., 2003a. Effect of biosolid processing on the availability of lead in an urban soil. J. Environ. Qual. 32: 100-108.

Brown, S.L., Henry, C.L., Chaney, R.L., Compton, H., De Volder, P.S. 2003b. Using municipal biosolids in combination with other residuals to restore metal-contaminated mining area's. Plant Soil 249: 203-215.

Degryse, J., 2003. Leaching of cadmium and zinc from spodosols: from laboratory to field scale. Doctoraatsproefschrift n° 581 aan de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen van de K.U.Leuven.

Fletcher, J., 1991. Keynote speech: A Brief Overview of Plant Toxicity Testing. *In*: Plants for toxicity assessment, second volume. Gorsuch J.W. et al. (eds.). ASTM STP 115, American Society for Testing and Materials, Philadelphia, USA.

Frische, T., 2003. Ecotoxicological evaluation of in situ bioremediation of soils contaminated by the explosive 2,4,6 trinitrotoluene (TNT). Environ. Pollut. 121: 103-113.

Geebelen, W., Sappin-Didier, V., Ruttens, A., Yperman, J., Carleer, R., Bongué-Boma, K., Mench, M., Vangronsveld, J., 2006. Evaluation of cyclonic ash and commercial Na-silicates for heavy metal immobilisation purposes in contaminated soils in Flanders. Environ. Pollut. *In Press*.

Gong, P., Wilke, B.M., Strozzi, E., Fleischmann, S., 2001. Evaluation and refinement of a continuous seed germination and early seedling growth test for the use in the ecotoxicological assessment of soils. Chemosphere. 44(3): 491-500.

Hettiarachchi, G.M., Pierzynski, G.M., Ransom, M.D., 2000. In situ stabilization of soil lead using phosphorus and manganese oxide. Environ. Sci. Technol. 34: 4614-4619.

ISO, 1993. International Organization for Standardization. Soil Quality. Determination of the effect of Pollutants on soil Flora. Part 1: Method for the Measurement of Inhibition of Root Growth. ISO 11269-1.

ISO, 1995. International Organization for Standardization. Soil Quality. Determination of the effect of Pollutants on soil Flora. Part 2: Effects of chemicals on the Emergence and Growth of Higher Plants. ISO 11269-2.

Kabata-Pendias, A., Pendias, H., 1984. Trace elements in soils and plants. CRC Press, Boca Raton, FL, USA.

Kamnev, A.A., van der Lelie, D., 2000. Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation. Biosci. Rep. 20: 239-258.

Lombi, E., Zhao, F., Wieshammer, H., Zhang, G., McGrath, S.P., 2002a. *In situ* fixation of metals in soils using bauxite residue: Biological effects. Environ. Pollut. 118: 435-444.

Lombi, E., Zhao, F., Wieshammer, H., Zhang, G., McGrath, S.P., 2002b. *In situ* fixation of metals in soils using bauxite residue: Chemical assessment. Environ. Pollut. 118: 445-452.

Lombi, E., Hamon, R.E., McGrath, S.P., McLaughlin, M.J., 2003. Lability of Cd, Cu and Zn in polluted soils treated with lime, beringite and red mud and identification of a non-labile colloidal fraction using isotopic dilution techniques. Environ. Sci. Technol. 28: 1219-1228.

Martinez, C.E., Mc Bride, M.B., 1999. Dissolved and labile concentrations of Cd, Cu, Pb and Zn in aged ferrihydrite-organic matter systems. Environ. Sci. Technol. 32: 743-748.

Mc Bride, M., Sauvé, S., Henderschot, W., 1997. Solubility control of Cu, Zn, Cd and Pb in contaminated soils. European Journal of Soil Sciences 48: 337-346.

McBride, M.B., Martinez, C.E., 2000. Copper phytotoxicity in a contaminated soil: Remediation tests with adsorptive materials. Environ. Sci. Technol. 34: 4386-4391.

OECD, 1984. Guideline for testing of chemicals, N° 207. Earthworm, acute toxicity test. OECD publications, Organization for Economic Cooperation and Development, Paris, France.

Paschke, M.W., Redente, E.F., Levy, D.B., 2000. Zinc toxicity thresholds for important reclamation grass species of the western Unites States. Environ. Toxicol. Chem. 19(11): 2751-2756

Sauvé, S., McBride, M., Hendershot, W., 1998. Soil solution speciation of lead (II): effect of organic matter and pH. Soil Sci. Soc. Am. J. 62: 618-621.

Shuman, L.M., Dudka, S., Das, K., 2002. Cadmium forms and plant availability in compost-amended soil. Commun. Soil Sci. Plant Anal. 33: 737-748.

U.S. EPA, 1996a. Ecological Effects Test Guidelines. Terrestrial Plant Toxicity, Seedling Emergence. U.S. Environmental Protection Agency. EPA 712-C-96-153. OPPTS 850.4100.

U.S. EPA, 1996b. Ecological Effects Test Guidelines. Terrestrial Plant Toxicity, Seed Germination/Root Elongation Toxicity Test. U.S. Environmental Protection Agency. EPA 712-C-96-154. OPPTS 850.4200.

Van Assche, F., Clijsters, H., 1990. A biological test system for the evaluation of the phytotoxicity of metal-contaminated soils. Environ. Pollut. 66: 157-72.

Vangronsveld, J., Clijsters, H., 1992. A biological test system for the evaluation of metal phytotoxicity and immobilization by additives in metal contaminated soils. p. 117-125. *In*: Metal compounds in environment and life, 4 (Interrelation between chemistry and biology). Merian, E., Haerdi, W. (eds.). Science Reviews Inc., Wilmington, USA.

Vangronsveld, J., Colpaert, J., Van Tichelen, K., 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environ. Pollut., 94: 131-140.

Vangronsveld, J., Cunningham, S., 1998. Introduction to the concepts. *In*: Metal contaminated soils: i*n situ* inactivation and phytorestoration. Vangronsveld, J., Cunningham, S. (eds.). Springer-Verlag, New York, USA.

Vangronsveld, J., Ruttens, A., Clijsters, H., 1999. The use of cyclonic ashes of fluidized bed burning of coal mine refuse for long-term immobilization of metals in soils. *In*: Biogeochemistry of coal and coal combustion byproducts. Sajwan et al. (eds.). Plenum Academic/Plenum Publishers, New York, USA.

Vangronsveld, J., Van Assche, F., Clijsters, H., 1995. Reclamation of a bare industrial area contaminated by non-ferrous metals: *in situ* metal immobilization and revegetation. Environ. Pollut. 87: 51-59.

Vassilev, A., Schwitzguébel, J.-P., Thewys, T., van der Lelie, D., Vangronsveld, J., 2004. The use of plants for remediation of metal-contaminated soils. Scientific WorldJournal. 4: 9-34.

Wang, W., Freemark, K., 1995. The use of plants for environmental monitoring and assessment. Ecotoxicol. Environ. Saf. 3: 289-301.

BIBLIOGRAFIE

International journals

Boisson, J., Mench, M., Vangronsveld, J., **Ruttens, A.**, Kopponen, P., De Koe, T., 1999. Immobilization of trace metals and arsenic by different soil additives: evaluation by means of chemical extractions. Commun. Soil Sci. Plant Anal. 30:365-387.

Boisson, J., **Ruttens, A.**, Mench, M., Vangronsveld, J., 1999. Evaluation of hydroxyapatite as a metal immobilizing soil additive for the remediation of polluted soils. Part 1. Influence of hydroxyapatite on metal exchangeability in soil, plant growth and plant element concentrations. Environ. Pollut., 104: 225-233.

Mench, M, Bussière, S., Boisson, J., Castaing, E., Vangronsveld, J., **Ruttens, A.,** De Koe, T., Bleeker, P., Assuncao, A., Manceau, A., 2003. Progress in remediation and revegetation of the barren Jales gold mine spoil after in situ treatments. Plant Soil 249: 187-202.

Meers, E., Unamuno, V.R., Vandegehuchte, M., Vanbroekhoven, K., Geebelen, W., Roeland, S., Vangronsveld, J., Diels, L., **Ruttens, A.**, Du Laing, G., Tack, F.M.G., 2005. Soil solution speciation of Cd as affected by soil characteristics in unpolluted and polluted soils. Environmental Toxicol. Chem. 24(3): 499-509.

Meers, E., **Ruttens, A.**, Samson, D., Hopgood, M., Tack, F.M.G., 2005. Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. Chemosphere 58(8): 1011-1022.

Meers, E., **Ruttens, A.**, Hopgood, M., Lesage, E., Tack, F.M.G., 2005. Potential of *Brassica rapa*, *Cannabis sativa*, *Helianthus annuus* and *Zea mays* for phytoextraction of heavy metals from calcareous dredged sediment derived soils. Chemosphere 61(4): 561-572.

Geebelen W., Bijnens, O., Claeys, N., Adriaenssens, E., Makkonen, S, **Ruttens, A.**, Carleer, R., Yperman, J., Clijsters, H., Holopainen, T., Vangronsveld, J., 2005. Transplanted lichens as biomonitors for atmospheric fluoride pollution near two fluoride point sources in Flanders (Belgium). Belg. J. Bot. 138: 141-151.

Ruttens, A., Colpaert, J., Mench, M., Boisson, J., Carleer, R., Vangronsveld, J., 2006. Phytostabilization of a metal contaminated sandy soil. II: Influence of compost and/or inorganic metal immobilizing soil additives on metal leaching. Environ. Pollut. *In press.*

Ruttens, A., Mench, M., Colpaert, J., Boisson, J., Carleer, R., Vangronsveld, J., 2006. Phytostabilization of a metal contaminated sandy soil. I: Influence of compost and/or inorganic metal immobilizing soil additives on phytotoxicity and plant availability of metals. Environ. Pollut. *In press.*

Ruttens A., De Vocht A., Geebelen W., Meers E., Wouters K., Mench M., Carleer R. and Vangronsveld J., 2006. Influence of physico-chemical soil variables in uncontaminated field soils on the endpoints of a phytotoxicity test (PlantTox). Environ. Toxicol. Chem. Submitted for publication.

Ruttens A., Geebelen W., Mench M., Carleer R., Clijsters H. and Vangronsveld J., 2006. Long-term sustainability of metal immobilization by soil additives: cyclonic ashes vs. lime. Environ. Pollut. MS in preparation.

Ruttens A., Mench M., De Vocht A., Geebelen W., Meers E., Vangronsveld J., 2006. Variations in morphological and enzymatic responses of *Phaseolus vulgaris* grown on OECD 207 standard soils. Plant Soil. MS in preparation.

Geebelen, W., Sappin-Didier, V., **Ruttens, A**., Yperman, J., Carleer, R., Bongué-Boma Kwèlè, Mench, M., Vangronsveld, J., 2006. Evaluation of cyclonic ash and commercial Na-silicates for heavy metal immobilization purposes in contaminated soils in Flanders. Environ. Pollut. *In press*.

Meers, E., **Ruttens, A.**, Geebelen, W., Vangronsveld, J., Samson, R., Vanbroekhoven, K., Diels, L., Tack, F.M.G., 2006. Using the Plant Antioxidant Network for Environmental Exposure Assessment of Heavy Metals in Soils. Environ. Monit. Assess. *In press*.

Meers, E., Vandecasteele, B., **Ruttens, A.**, Vangronsveld, J., Tack, F.M.G., 2006. Comparison of the potential of five willow species (*Salix* sp.) for phytoextraction of heavy metals. Environ. Exp. Bot. *In press*.

Mench, M., Vangronsveld, J., Beckx, C., **Ruttens, A**., 2006. Progress in assisted natural remediation of an arsenic contaminated agricultural soil. Environ. Pollut. *In press*.

Meers, E., Du Laning, G., Vanbroekhoven K., Geebelen W., **Ruttens, A.**, Vandegehuchte, M., Samson, R., Diels, L., Vangronsveld, J., Tack, F.M.G., Verloo, M., 2006. Comparison of Cd extractability from soils and sediments by commonly used single extraction protocols. Geoderma. *In Press*.

Chapters in books

Vangronsveld, J., **Ruttens, A**., Clijsters, H., 1999. The use of cyclonic ashes of fluidized bed burning of coal mine refuse for long-term immobilization of metals in soils. *In*: Biogeochemistry of coal and coal combustion byproducts. Sajwan et al. (eds.). Plenum Academic/Plenum Publishers, New York, USA. pp. 223-233.

Vangronsveld, J., **Ruttens, A**., Mench, M., Boisson, J., Lepp, N.W., Edwards, R., Penny, C., van der Lelie, D., 1999. *In situ* inactivation and phytoremediation of metal and metalloid-contaminated soils: field experiments. *In*: Wise et al. (eds.). Bioremediation of contaminated soils. Marcel Dekker Inc., New York, USA. pp.859-884.

Vangronsveld J. and **Ruttens A.**, 1999. *In situ* reclamation techniques for heavy metal contaminated soils. *In*: Soil fertility, soil biology and plant nutrition interrelationships. Siqueira, J.O. et al., (eds.). Brazilian Soil Science Society / Federal University of Lavras / Soil Science Department (SBCS/UFLA/DCS). pp. 389-404.

Vangronsveld, J., Diels, L., van der Lelie, D., Bierkens, J., Corbisier, P., Spelmans, N., Adriaensens, E., Carleer, R., Van Poucke, L., **Ruttens, A.,** Colpaert, J., Clijsters, H., 2002. Réhabilitation par immobilisation des métaux. Site de Lommel Maatheide (Belgique). *In:* Les elements traces métalliques dans les sols. Approches fonctionneles et spatiales. Baize, D., Tercé, M. (eds.). INRA, Paris, France. pp. 393-408.

Mench M., Vangronsveld J., Lepp N., Bleeker P., **Ruttens A.,** Geebelen W., 2006. Phytostabilisation of metal-contaminated sites. Phytoremediation of metal-contaminated soils. Morel J.-L. et al. (eds.). NATO Science Series. Earth and environmental sciences, Volume 68. Springer, Dordrecht, The Netherlands. pp. 109-190.

Some afterthoughts

When I started this work ,

I hoped to find answers to some intriguing questions.

Never I realized that each answer

would provoke so many new questions.

But like in a puzzle, in which every tiny little piece,

makes the picture a bit more complete,

I hope this work could contribute a piece, no matter how small,

to the fascinating world of sciences.