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Remediation of Pb Contaminated Soils by Phytoextraction and Amendment induced Immobilization: Biological Aspects

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ABBREVIATIONS

AAS	Atomic absorption spectroscopy
APOD	Ascorbate peroxidase
BCR	Community bureau of reference
CA	Cyclonic ash
CAT	Catalase
CEC	Cation exchange capacity
CDC	Critical deficiency concentration
CTC	Critical toxicity concentration
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DOM	Dissolved organic matter
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EXAFS	Extended X-ray absorption fine structure
FW	Fresh weight
GDH	Glutamate dehydrogenase
GPOD	Guaiacol peroxidase
GI	Gastro-intestinal
GR	Glutathione
GSSG	Glutathione disolphide
HFO	Hydrous ferrous oxides
HMO	Hydrous manganese oxides
ICDH	Isocitrate dehydrogenase
ICP	Induced couples plasma spectroscopy
MDHA	Monodehydroascorbate reductase
ME	Malic enzyme
OECD	Organization for economic cooperation and development
OM	Organic matter
PBET	Physiologically based extraction test
PC	Phytochelatins
PI	Phytotoxicity index
PR	Phosphate rock
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SPOD	Syringaldazine peroxidase
ST	Steelshot
TEL	Tetraethyllead
TML	Tetramethyllead
XRD	X-ray diffraction

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SUMMARY

This study examines the biological aspects related to alternative remediation strategies for Pb contaminated soils: EDTA induced Pb phytoextraction and amendment induced immobilization of soil Pb by means of inorganic soil amendments.

The physiological effects of Pb-EDTA and EDTA were studied on bean plants (*Phaseolus vulgaris* L. Limburgse vroege), grown under strictly controlled conditions on a Hoagland nutrient solution. Addition of Pb-EDTA to the growth medium increased the capacity of enzymes belonging to three different compartments of defence mechanisms against oxidative stress in roots and primary leaves, before effects on plant morphology or chlorophyll content were observed. Pb-EDTA further drastically reduced Ca, Fe, Mn and Zn content in primary leaves. EXAFS analysis revealed that only 60-70% of all Pb in leaves was complexed to EDTA. Our data did not permit to conclude whether the observed phytotoxic response is due to the presence of Pb-EDTA, unchelated Pb or their combination. SPOD appeared to be an early biomarker for Pb-EDTA induced stress, possibly due to its role in cell wall lignification. Addition of EDTA alone decreased Fe, Mn, and Zn content in primary leaves of bean plants but did not induce any stress reaction.

Several soil amendments known to immobilize Pb were compared on a soil spiked with increasing concentrations of Pb: bentonite (1% w/w), zeolite (0.5%), cyclonic ash (5%), compost (5%), lime (1%), steelshot (1%) and hydroxyapatite (1%). Bentonite (-24%) addition resulted in the lowest reductions in $Ca(NO_3)_2$ extractable soil Pb and therefore only slightly reduced phytotoxicity. Steelshot (-58%) and zeolite (-74%) were more efficient in reducing $Ca(NO_3)_2$ extractable Pb: both amendments however appeared to have negative effects on plant growth probably due to release of toxic amounts of Fe and Mn (steelshot) and destruction of soil texture (zeolite). Substantial reductions in $Ca(NO_3)_2$ extractable Pb were observed when cyclonic ash (-90%), compost (-90%), hydroxyapatite (-94%) and lime (-98%) were applied. In general, these amendments reduced Pb phytotoxicity concomitant with reduced Pb concentration in lettuce tissue (*Lactuca sativa* Hilde). Cyclonic ash, lime and compost further improved plant growth and reduced oxidative stress at low Pb concentrations due to soil pH increase mitigating Al or Mn toxicity (occurring at the low pH of the original uncontaminated soil).

A following experiment examined Pb immobilization on 10 soils contaminated with Pb (and in some cases Cu, Zn, Cd, As) from different origin using lime (1% w/w), a mix of cyclonic ash and steelshot (5% CA + 1% ST), and North Carolina phosphate rock (1 or 2% PR). In general, $CaCl_2$ extractable Pb (PR= -30%; CA+ST= -77%; lime= -82%), Cu (PR= -48%; CA+ST= -61%; lime= -88%) and Zn (PR= -37%; lime= -75%; CA+ST= -79%), were reduced more efficiently when lime and cyclonic ash and steelshot were used than when phosphate rock was

applied. The amendments appeared less efficient in immobilizing Cd (lime= -16%; PR= -21%; CA+ST= -33%). Also, lime and cyclonic ash and steelshot were more effective in reducing plant metal uptake and in mitigating soil phytotoxicity than phosphate rock did. CA+ST did not increase $CaCl_2$ extractable Fe but did significantly increase $CaCl_2$ extractable Mn on most soils.

Phosphate rock was the only amendment which significantly decreased Pb bioavailability in the gastro-intestinal tract, probably due to pyromorphite formation ($(Pb_5(PO_4)_3X$ where X= OH, F or Cl). Only on 3 soils however PR significantly decreased PBET Pb. On the other hand, phosphate rock significantly increased PBET As in a few cases, probably due to competition between PO_4^{3-} and AsO_4^{3-} for the same sorption sites in soil. The combination CA+ST decreased PBET Cd and As on 3 soils, probably due to binding on Fe-and Mn-oxides. Our data indicate that metal/metalloïd immobilizing efficacy depends to a substantial degree on soil characteristics and present metal species. Additional experiments indicated that only low amounts of PO_4^{3-} were released by the PR used in our study: possibly PR more efficiently immobilized metals in case higher amounts were used (e.g. 5% w/w).

SAMENVATTING

Deze thesis heeft als doel de biologische aspecten te onderzoeken die verbonden zijn aan alternatieve bodemsaneringstechnieken voor Pb gecontamineerde substraten. Meer in het bijzonder wordt aandacht besteed aan fytoextractie van Pb met behulp van EDTA en Pb immobilizatie door toevoeging van metaalimmobilizerende bodemadditieven.

De fysiologische effecten van Pb-EDTA en EDTA op planten werden onderzocht aan de hand van bonenplanten (*Phaseolus vulgaris* L. Limburgse vroege), gekweekt op Hoagland voedingsoplossing onder strikt gecontroleerde klimatologische omstandigheden. De capaciteit van enzymen, die deel uitmaken van 3 verschillende compartimenten van de verdedigingsmechanismen van de plant tegen oxidatieve stress, was verhoogd na toevoeging van Pb-EDTA aan de voedingsoplossing. Deze responsen traden op voordat effecten op groei en chlorofylgehalten waargenomen werden. Voorts werden sterk verlaagde gehalten aan Ca, Fe, Mn en Zn gemeten in primaire bladeren. EXAFS analyse toonde aan dat slechts 60-70% van het Pb aanwezig in de bladeren gecomplexeerd was met EDTA; het is niet duidelijk of de gevonden fytotoxische respons te wijten is aan de aanwezigheid van Pb-EDTA, niet-gechelateerd Pb of een combinatie van beide. SPOD bleek een vroege biomarker te zijn voor Pb-EDTA geïnduceerde stress, mogelijks door zijn rol in celwandlignificatie. Toediening van enkel EDTA resulteerde in een verlaging vna de Fe, Mn, en Zn gehalten in de plant maar induceerde geen stress respons.

Pb immobilizatie werd in eerste instantie getest aan de hand van een bodem waaraan 6 Pb concentraties werden toegevoegd. Volgende bodemadditieven werden getest: bentoniet (1%), zeoliet (0.5%), cycloonassen (5%), compost (5%), kalk (1%), staalgrit (1%) en hydroxyapatiet (1%). De kleinste reductie in $Ca(NO_3)_2$ extraheerbaar Pb werd gevonden na bentoniet toevoeging (-24%); de daling in plantbeschikbaar Pb bleek onvoldoende om alle fytotoxische effecten te elimineren. Staalgrit (-58%) en zeoliet (-74%) reduceerden het plantbeschikbaar Pb in hogere mate dan bentoniet: beide bodemadditieven bleken echter nefaste gevolgen te hebben voor de planten, waarschijnlijk door de vrijstelling van toxische hoeveelheden Fe en Mn (staalgrit) en door aantasting van de structuur van de bodem (zeoliet). Plantbeschikbaar Pb werd sterk verlaagd wanneer cycloonassen (-90%), compost (-90%), hydroxyapatiet (-94%) en kalk (-98%) werden toegevoegd. Toevoeging van deze bodemadditieven reduceerde de fytotoxiciteit van Pb en verlaagde het Pb gehalte in sla (*Lactuca sativa* Hilde), gekweekt op deze bodems. Voorts bevorderden cycloonassen, kalk en compost de groei van de testplanten en werd oxidatieve stress, waarschijnlijk veroorzaakt door Al of Mn toxiciteit ten gevolge van de zure pH van de originele niet gecontamineerde bodem, teniet gedaan door pH verhoging.

In een volgend experiment werd de immobilizatie onderzocht van metalen in 10 bodems gecontamineerd met verschillende vormen van Pb, in een aantal gevallen gecombineerd met andere metalen zoals Cu, Zn, Cd, As. Als additieven werden kalk (1%), een mengsel van cycloonassen en staalgrit (5% CA+ 1% ST), en een fosfaaterts afkomstig uit Noord-Carolina (1 of 2% PR), gebruikt. Over het algemeen bleken $CaCl_2$ extraheerbare concentraties aan Pb (PR= -30%; CA+ST= -77%; kalk= -82%), Cu (PR= -48%; CA+ST= -61%; kalk= -88%) en Zn (PR= -37%; kalk= -75%; CA+ST= -79%) sterker gereduceerd na toevoeging van kalk en de combinatie cycloonassen en staalgrit, dan wanneer fosfaaterts gebruikt werd. De additieven bleken minder efficiënt bij het reduceren van $CaCl_2$ extraheerbaar Cd (kalk= -16%; PR= -21%; Ca+ST= -33%). Kalk en CA+ST reduceerden fytotoxiciteit en metaalopname door planten in sterkere mate dan fosfaaterts. Toevoeging van CA+ST verhoogde de hoeveelheid $CaCl_2$ extraheerbaar Mn maar niet Fe.

Biobeschikbaar Pb in het maag-darm kanaal (PBET) daalde na toediening van fosfaaterts, waarschijnlijk door vorming van pyromorfiet ($Pb_5(PO_4)_3X$ waar X= OH, F of Cl). Dit was echter slechts het geval op 3 bodems. Toevoeging van fosfaaterts resulteerde in een aantal gevallen in een mobilizatie van As, waarschijnlijk als gevolg van competitie tussen PO_4^{3-} en AsO_4^{3-} voor dezelfde adsorptieplaatsen in de bodem. Op 3 bodems werden reducties in PBET extraheerbaar Cd en As gevonden na CA+ST toevoeging, waarschijnlijk door binding aan Fe en Mn-oxiden. Onze resultaten wijzen erop dat metaalimmobilizatie onder meer afhangt van de aanwezige metaal species en van bodemkarakteristieken. Bijkomende experimenten toonden voorts aan dat het gebruikte fosfaaterts slechts kleine hoeveelheden PO_4^{3-} vrijstelde: naar alle waarschijnlijkheid kunnen betere resultaten met PR bekomen worden wanneer grotere hoeveelheden worden toegevoegd (bv. 5%).

CHAPTER I

INTRODUCTION

1. LEAD: OCCURRENCE AND HEALTH RISKS

Lead (Pb) is a bluish-grey heavy metal that is found in small quantities in the earth's crust. Natural Pb occurrence in top horizons of different soils from various countries ranges from 3 to 189 mg kg^{-1}, and averages 32 mg kg^{-1} (Kabata-Pendias & Pendias, 1992). Although there are more than 200 minerals of Pb, only a few are common, with galena (PbS), cerussite ($PbCO_3$), and anglesite ($PbSO_4$) as the most important (Adriano, 2001).

In the last fifty years huge amounts of Pb have been extracted for use in rechargeable batteries, pigments and other compounds, rolled and extruded products, cable sheathing, alloys, shot and ammunition, and gasoline additives (Ewers & Schlipköter, 1991, Adriano, 2001). Its extensive use worldwide and widespread dissemination in the environment have resulted in numerous contamination cases, elevating Pb concentration in soils up to 1000 - 10 000 mg kg^{-1} and even higher (Berti et al., 1998). Soil contamination with Pb results from various activities, such as mining and smelting, combustion of gasoline containing the anti-knock additives tetraethyl- and tetramethyl-Pb, use of Pb-based paints and explosives, land application of municipal biosolids, use of certain fertilizers and pesticides, battery recycling, and use of Pb bullets (Adriano, 2001).

In contrast to Cu or Zn which are essential heavy metals in many organisms, Pb is neither an essential nor a beneficial element for plants or animals. Increased Pb concentrations are known to drastically decrease microbiota in soil (U.S. EPA, 1986), while toxic effects are observed in plants grown on soils near smelters or mines, or in soils treated with Pb containing pesticides (Koeppe, 1981; Dudka & Adriano, 1997). Furthermore, Pb has been singled out as the most frequent source of accidental poisoning in domestic animals (NAS, 1972). For example, one of the soils tested within this thesis was collected at a contaminated site in Belin-Beliet (France) after horses had died. Close examination revealed increased Pb concentrations in soil up to 1859 mg kg^{-1}, due to atmospheric deposition near a battery breaking facility.

Humans may be exposed to Pb via inhalation (automobile exhaust, dust), ingestion (Pb in vegetables, childrens pica behaviour for soil, paint) and dermal contact (organo-Pb-compounds). Once absorbed, Pb is concentrated in blood where the major Pb fraction is associated with hemoglobin. Pb interferes with several enzymatic steps in the haem pathway inhibiting hemoglobin production by the body. Pb has a half-life in blood of approximately 25 days, in soft tissue about 40 days, while the majority is stocked in bone and teeth for more than 25 years (Adriano, 2001). Elevated blood Pb concentrations result in high blood pressure, kidney diseases, brain damage, reproductive abnormalities, abnormal vitamin D metabolism, coma and even death under certain circumstances (ATSDR, 1993; Lowny et al., 1998). Children are more sensitive because they absorb and retain more Pb (30-50%) in proportion to their body weight than adults do (Mushak et al., 1989. US EPA, 1990).

Major acute exposures to Pb need not occur for Pb poisoning to develop. The body accumulates Pb over a lifetime and releases it slowly, so chronic exposures to even small doses over time can cause Pb poisoning. The most sensitive target of Pb poisoning in humans is the nervous system (ATSDR, 1992, 1993). Studies found that primary school children with high tooth Pb levels but with no known history of Pb poisoning had larger deficits in psychometric intelligence scores, speech and language processing attention, and classroom performance than children with lower levels of Pb (Needleman, 1979). After executing over 25 years of epidemiological research concerning Pb health effects, Needleman (2000) recently attributed Pb to be responsible for the behaviour of 11 to 38% of the criminals in the US. The U.S.

National Academy of Sciences states that Pb blood concentrations of around 10 µg dL^{-1} in children are associated with disturbances in early physical and mental growth and in later intellectual functioning and academic achievement (NAS, 1993), while in 1984, 17% of all US children had a blood Pb concentration over 15 µg dL^{-1}. This percentage increased when only children from inner cities (30%) were sampled, while in Mexico city, blood Pb exceeded this level in 70% of all children (D. Adriano, oral communication). The U.S. EPA has classified Pb as a probable human carcinogen; no reference dose or concentration is yet established (U.S. EPA, 1993).

2. Pb BIOAVAILABILITY IN SOIL

2.1. Pb cleanup guidelines

In the United States no national soil heavy metal cleanup standards are currently available, although many state agencies have developed their own standards that vary from those of other states (Bryda & Sellman, 1994). Such guidelines are normally based on total metal analysis of soils (Vangronsveld & Cunningham, 1998). In Europe, there is only one set of critical levels that apply to all the countries of the EU, which establishes limit values for concentrations of metals in soils that should not be exceeded when sewage sludge is applied in agriculture (Reiniger, 1997). The directive has been implemented in the form of national laws. In many cases, these critical values have been extended to soils in general and are not only limited to the application of sewage sludge (Adriano, 2001).

In Flanders (Belgium), following cleanup values for Pb are adopted, as a function of the intended use of the remediated site: 200 mg Pb kg^{-1} for natural parks and agricultural soils, 400 mg Pb kg^{-1} for residential areas, 1500 mg Pb kg^{-1} for recreational areas and 2500 mg Pb kg^{-1} for industrial areas. These five classes are extensively described in the legislation ‘Besluit van de Vlaamse Regering houdende achtergrondwaarden, bodemsaneringsnormen en toepassingen van gereinigde grond' and are based on a standard soil sample containing 10% clay and 2% OM (Van Gehuchte et al., 1997).

Soils act as a sink for pollutants mainly due to adsorption processes which bind inorganic and organic pollutants with varying strengths (Alloway & Ayres, 1993). Pb in soil may be present (1) as simple or complexed ions in the soil solution; (2) as easily exchangeable ions; (3) organically bound; (4) occluded by or co-precipitated with metal (Fe, Mn) oxides, carbonates or phosphates; or (5) as ions in crystal lattices of primary minerals (Cunningham et al., 1995; McLaren & Crawford, 1973; Soon & Bates, 1982). There is a large consensus among the scientific community to believe that the risk for living organisms associated to the presence of heavy metals in the environment is determined for a major part by the solubility of the various heavy metal-bearing phases present, rather than by the total elemental concentration (Manceau et al., 1996).

2.2. Bioavailability

Bioavailability can be defined as the potential for a living organism to take up metal from food (i.e., oral) or from the abiotic environment (i.e., external) to the extent that the metal may become involved in the metabolism of the organism. More specifically, it refers to biologically available metal that can be taken up by an organism and can react with its metabolic machinery (Campbell, 1995), or it refers to the fraction of the total metal that can interact with a biological target (Vangronsveld & Cunningham, 1998).

In ecotoxicology, bioavailability can be broadly defined as the portion of a chemical in the environment that is available for biological action, such as uptake by an organism (Rand, 1995). The conventional approach involves feeding groups of individuals known amounts of the chemical in question over a period of time. The portion of the total dose retained in the organism at the end of the feeding period is measured after withholding of food for sufficient time to allow complete clearance of the food from the gut (Newman, 1995). Bioavailability is then estimated by mass balance under the assumption that all chemicals measured in the organism are assimilated.

2.3. Factors influencing Pb bioavailability

Metal bioavailability in soils depends on the physical, biological, and chemical characteristics of the medium affected, the metal species, and the biological species (Adriano, 2001).

2.3.1. Soil characteristics

Studies of Pb sorption by soils indicate that pH, redox status, OM, clay content, Al, Fe and Mn oxides, phosphate, sulphate and carbonate content are important parameters determining Pb mobility and bioavailability in soils (Bataillard et al., 2001; Koeppe, 1981; Van Stichelen, 1994; Xian & Shokohifard, 1989; Zimdahl & Skogerboe, 1977).

It was recognized early on that Pb toxicity was inversely proportional to soil pH (Griffith, 1919). An increase in soil pH ionises pH-dependent exchange sites, resulting in greater Pb sorption to soil particles (Berti & Cunnigham, 1998). Reddy & Patrick (1977) found that Pb uptake by both roots and tops decreased with increasing pH and suspension redox potential. After analysing 84 Pb contaminated soils, Sauvé et al. (1997) proposed an equation which allows calculation of the activity of Pb^{2+} ions in soil based on pH and total Pb content only, underlining the importance of soil pH on Pb solubility [$p\text{Pb}^{2+} = 0.62 \text{ x pH} - 0.84 \text{ x } \log_{10}(\text{Total Pb}) + 6.78$]. Reactions involving organic matter (OM) play a predominant role in the adsorption of Pb by soils. A common mechanism of complexation by OM for Pb^{2+} is the adsorption of Pb^{2+} ions onto humic materials through an ion exchange process between the ion adsorbed and the H^+ ions from the OM. While OM may serve as an immobile complexer for Pb, it can also facilitate the transport of this element in soils via the formation of soluble complexes with DOM (Pinheiro et al., 2000). In general, the ability of OM to mobilize or immobilize metals depends on the nature of the OM, pH, redox, and competing ions and ligands. Sauvé et al.

(1998b) for example found that 30 to 50% of dissolved Pb in a contaminated orchard soil treated with leaf compost was present as soluble OM complexes at low pH and up to 80 to 99% at pH ~7. Soil type, in particular the soil's clay content, has been shown to influence plant uptake of Pb as well as sorption of Pb by soil materials. The affinity of Pb for mineral soil components is typically as follows: clay > silt > sand (Adriano, 2001). Al, Fe and Mn oxides may exert an important role on Pb adsorption by soils (Ross & Negra, 2001). In measuring the adsorption of several trace elements by synthetic Mn and Fe oxides, McKenzie (1980) found that Pb adsorption by Mn oxides was up to 40 times greater than that by the Fe oxides. Martinez & McBride (1998) found that Pb was adsorbed more strongly by iron oxides than Co, Cu, Mn, Ni or Zn. Phosphates in soil can bind Pb resulting in the formation of pyromorphite ($Pb_5(PO_4)_3X$ where X= OH, F or Cl). Pb phosphates are less soluble than oxides, hydroxides, carbonates, and sulfates under soil-surface conditions (Cotter-Howells, 1996; Ruby et al., 1994; Sauvé et al., 1998b). Lee et al. (1976) and Koeppe & Miller (1970) attributed observed phytotoxicity on Pb contaminated substrates to phosphate deficiency, as a result of pyromorphite formation, rather than Pb phytotoxicity. Finally, carbonates and sulphates present in soil can complex Pb rendering it less mobile.

2.3.2. Source term

Different Pb species exhibit different rates of Pb dissolution, depending on their chemistry and particle size distribution, the mechanism by which they dissolve, and the geochemistry of the soils in which they are present (Ruby et al., 1999).

Automotive exhausts contain Pb in more readily soluble form (PbClBr and $PbSO_4$) compared to Pb oxide species (e.g. PbO_x, $PbO \cdot PbSO_4$), present after smelter operations (Davis et al., 1992). Pb oxide species in turn are more soluble than galena (PbS) and anglesite ($PbSO_4$) associated with ore and mining sites. At mining and smelting sites, Pb minerals may be encapsulated within other soil mineral grains, such as quartz or slag, which further limits their solubility (Ruby et al., 1999). The most common forms of Pb in paint are red Pb (Pb_3O_4), white Pb

[$2PbCO_3 \cdot Pb(OH)_2$], basic Pb sulphate ($PbO_2 \cdot PbSO_4$), and Pb chromate ($PbCrO_4$) (Davis & Wixson, 1988; Heaton, 1940; Ruby et al., 1994). Due to the relatively high solubility of Pb oxide and carbonate species, and the small particle size, small particles of Pb released from paint weathering are considered to be highly mobile (Ruby et al., 1999). Elemental Pb particles (Pb present in acid batteries, cable coverings, and ammunition) that are deposited in soils quickly form coatings of highly bioavailable Pb oxide (Ruby et al., 1999).

2.3.3. Biological species

In pharmacology studies, Pb bioavailability is defined as the fraction of an administered dose that reaches the blood from the gastro-intestinal tract. In the soil-plant environment, bioavailability is generally interpreted as the fraction of the total metal that interacts with plant roots (also referred to as phytoavailable fraction). It is clear that in both cases different conditions will determine Pb solubility and bioavailability.

In case of the soil-plant environment, it should be noted that essential heavy metals (Cu, Zn) are more readily taken up than non essential elements (Pb, Cd). Metal uptake further depends on plant species, cultivar, nutritional status and stage of development (Jolly & Brown, 1989; Marschner, 1995; Römheld & Marschner, 1990). A focal point of soil plant interactions is the microecosystem surrounding the plant roots, the rhizosphere. This microecosystem 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). Both plant roots and microbes exude inorganic and organic compounds, e.g. protons, CO_2/HCO_3^-, and various functional groups possessing acidifying, chelating and/or reductive power. These compounds can trigger a range of chemical reactions and biological transformations resulting in altered state (phase), speciation, and solubility, of chemical elements and compounds relative to the bulk soil. Dicotyledons and non-grass monocotyledons for example mediate Fe uptake by means of a plasmamembrane bound ferric reductase that transfers electrons from intracellular

NADH to Fe (III) chelates in the rhizosphere (Buckhout et al., 1989; Chaney et al., 1972). The ferrous ions (Fe^{2+}) released from the chelates by this process are subsequently transported into the cytoplasm via a separate transport protein (Kochian, 1991). On the other hand, grasses are known to release low-molecular weight, high affinity Fe(III)-chelating compounds (phytosiderophores), which solubilize ferric Fe in the rhizosphere and are recognised for uptake by specific membrane receptors (Chaney, 1987; Bienfait, 1988). Rhizosphere acidification was also observed in case of phosphate deficiency (Moorby et al. 1988). However, experiments with *Alyssum murale* showed that reduction of the rhizosphere pH by increased proton release is probably not the mechanism by which this plant species solubilises heavy metals from soils (Bernal & McGrath, 1994). Acidification was not the mechanism either by which the Zn hyperaccumulator *Thlaspi caerulescens* mobilises Zn in the soil (McGrath, et al., 1997). It has been shown that grasses release phytosiderophores (e.g. mugineic acid, avenic acid) upon Zn and Fe deficiency which can mobilize Zn, Fe, Cu and Mn from soil (Kinnersely, 1993; Zhang et al., 1989, Walter et al., 1994). Wiren et al. (1996) proposed two pathways for the uptake of Zn from Zn-phytosiderophores in grasses: (1) via the transport of the free Zn cation, and (2) the uptake of non-dissociated Zn-phytosiderophore complexes. Mench & Martin (1991) collected dissolved root exudates from tobacco and corn and observed increased extractability of Mn and Cu, while Ni and Zn were not affected. The chemical nature of phytosiderophores can differ among plant species and even cultivars, but typically one dominant phytosiderophore is characteristic for a certain genotype (Römheld & Marschner, 1990).

On the other hand, the importance of root colonizing bacteria and the presence of mycorrhizal fungi should not be underestimated. It is known that plant uptake of certain mineral nutrients such as Fe and Mn may be facilitated by rhizospheric microorganisms (Crowly et al., 1991; Barber & Lee, 1974). Salt et al. (1995) found that several strains of *Pseudomonas* and *Bacillus* were capable of increasing the total amount of Cd accumulated from hydroponic solution by 2 weeks old *Brassica juncea* seedlings. Fungi are known to exudate organic compounds such as citric and oxalic acid which assist both (essential) metal and anionic (e.g. phosphate) nutrition of fungi (Gadd, 1999). Sayer et al. (1999) recently demonstrated that chloropyromorphite, one of the most thermodynamically stable Pb minerals in the surface environment (Cotter-Howells, 1996), can be solubilised by organic-acid-producing fungi (*Aspergillus niger*). On the other hand, Jentschke et al. (1998) described that mycorrhizal infection reduced Pb content in *Picea abies* roots.

2.2.4. Time

Because soils are open, dynamic systems subject to short-term fluctuation and (partly irreversible) long-term changes (soil formation), the bioavailability of trace elements also varies with time (Wenzel et al., 2002). For instance, trace elements bound in the crystal lattices of clay minerals are typically not accessible to plant roots in the short term. However, such elements may become partially available when these minerals are transformed in the course of soil formation, weathering or – even in relatively short periods – as a consequence of root activities (Gobran et al., 1999).

2.4. Estimating Pb phytoavailability

An empirical approach commonly used for studying the mobility and bioavailability of metals in soils is the use of sequential extraction techniques (Chlopecka, 1997). Sequential fractionation attempts to extract successively each metal pool from the soil, starting with the most easily solubilized metals down to the most recalcitrant. The successive extraction reagents are intended to dissolve selectively various chemically defined pools usually identified as the following: soluble, exchangeable, carbonate bound, oxide bound, organic matter bound, and residual. Several sequential extraction schemes have been described for soil and sediments (Ahnstrom & Parker, 1999; Rauret et al., 1999; Tessier et al., 1979). Sequential extraction schemes however have limitations, because they do not extract chemically well-defined forms or species of metals, but rather only operationally defined forms (Rauret et al., 1999). There is a general agreement that only a limited fraction of Pb in soil is available for uptake by plants (Davies, 1995). The content of a metal in soil solution (i.e., soluble) plus the 'weakly adsorbed' content (i.e., exchangeable) provide a good measure of the plant-available amount (Adriano, 2001). Single extractants such as $CaCl_2$, $Ca(NO_3)_2$, etc. have been used to extract this fraction.

3. PHYSIOLOGICAL EFFECTS OF Pb ON PLANTS AND DEFENCE MECHANISMS

3.1. Pb uptake and translocation

3.1.1. Uptake by roots

The movement of Pb from the external solution into the cell walls is a non-metabolic, passive process, driven by diffusion (Marschner, 1995). Apoplastic transport is only possible as far as the endodermis and is limited or retarded by the high cation exchange capacity of cell walls (Tung & Temple, 1996; Wu et al., 1999). This cation exchange capacity is largely determined by the presence of pectin compounds such as galacturonic and glucuronic acids. Pb ions have been found to compete for the negatively charged sites of these acids, which are normally saturated with Ca^{2+} (Wierzbicka, 1998). Ca and heavy metals are bound with decreasing affinity in the order Pb > Cr > Cu > Ca > Zn (Ernst, 1998b). The metal binding capacity of cell walls is not only species specific (in general, high for dicotyledonous species, lower for monocotyledonous species), but also genotype specific, as shown for the difference between British and German populations of the perennial grass *Agrostis capillaris* (Ernst, 1976). In addition, cell walls of roots exposed to Pb accumulate large amounts of $PbCO_3$, formed from the respiratory CO_2 (Jarvis & Leung, 2001; Salt et al., 1995). Several workers have reported the occurrence of Pb precipitates on root cell walls (Malone et al., 1974; Przymusinski & Woźny, 1985; Qureshi et al., 1986; Theiss, 1990; Antosiewicz & Wierzbicka, 1999). Dushenkov et al. (1995) observed Pb precipitation on roots and at the bottom of a hydroponic container of *Brassica juncea*, grown on distilled water with only Pb (added as $Pb(NO_3)_2$) in the growth medium. Infrared spectroscopic analysis of the precipitate identified Pb phosphate as its major component. Cotter-Howells et al. (1999) identified pyromorphite type structures on the outer cell walls of root cells of the metal tolerant cultivar of *Agrostis capillaris* using X-ray absorption spectroscopy. The physiological significance of such $Pb\text{-}PO_4^{3-}$ deposits however is unclear; the precipitation of $Pb\text{-}PO_4^{3-}$ compounds in plants could be a detoxification mechanism or simply a passive, antagonistic reaction between Pb and PO_4^{3-} (Cotter-Howells et al., 1999).

As a result, apoplastic Pb movement is relatively low and only a minor fraction of Pb is taken up into cells (Seregin & Ivanov, 1998). Using X-ray probe analysis, Qureshi et al. (1986) reported that Pb occurred in the cytoplasm of root tips excised from a Pb-sensitive clone of *Anthoxanthum odoratum* while Huang et al. (1997a) localized Pb in cortical cells of sunflower roots using scanning electron microscopy. Within cells, Pb has been localized in vacuoles, the Golgi apparatus, the endoplasmic reticulum, chloroplasts, mitochondria and nuclei (for a review see Seregin & Ivanov, 2001). Theiss (1990) observed Pb in plasmodesmata of *Lepidium sativum* seedlings suggesting symplastic Pb transport.

There is little information in the literature concerning mechanisms of Pb transport into plant cells. In case of heavy metals, competition for the same transmembrane carrier between essential heavy metals and non-essential heavy metals has been observed. The relative lack of selectivity in transmembrane ion transport may partially explain why non-essential heavy metals can enter cells even against a concentration gradient (Marschner, 1995). For example, kinetic data demonstrate that essential Cu and Zn and non-essential Ni and Cd compete for the same transmembrane carrier (Clarkson & Luttge, 1989). A possible transport pathway for Pb entering the cell could be through plasmamembrane Ca^{2+}-channels. Using isolated root-cell plasma membrane vesicles, Huang et al. (1997a) found that Pb can significantly inhibit such channels. The inhibition of the Ca^{2+} channel activity could result from Pb blockage of the channel, or Pb competing with Ca^{2+} for the transport pathway. Comparable results are found in animal systems where there is experimental evidence showing that Pb^{2+} is transported into cells via Ca^{2+} channels (Tomsig & Suszkiw, 1991; Audesirk, 1993). The role of such transmembrane metal transporters in metal uptake was only recently highlighted in the study of hyperaccumulation mechanisms. Lasat et al. (1996) found a higher density of Zn transporters per unit of membrane area in *Thlaspi caerulescens* roots compared to *Thlaspi arvense.* Additionally, physiological data suggest the presence of a high-affinity and highly expressed Cd transporter in the root cell plasma membrane of a specific ecotype (Ganges) of *T. caerulescens* while another tested ecotype (Prayon) possibly seemed to mediate its Cd uptake by Zn transporters (Lombi et al., 2000, 2001).

3.1.2. Pb translocation from roots to shoots

Once ions have entered the root, they can either be stored or exported to the shoot. To enter the xylem vessels, the ions have to cross the Casparian strip, which separates the cortex from the central cylinder. Because of this barrier, the transport of solutes from the cortex must in principle occur through the endodermal cells (symplastically). The endodermis however is not a perfect barrier against the apoplastic pathway of ions from the cortex into the stele: at at least two sites along the root axis this barrier may be ‘leaky’ (Marschner, 1995). At the root apex, the Casparian band is not yet fully developed allowing apoplastic transport into the stele (Huang & Van Steveninck, 1989). On the other hand, apoplastically occurring elements can penetrate the stele in basal root zones where lateral roots emerge from the pericycle in the stele and where structural continuity of the endodermis is ruptured transiently during penetration of the lateral roots of the cortex (Marschner, 1995). Wierzbicka (1987) demonstrated that in onion meristems, Pb^{2+} was transported along both symplast and apoplast. The relative importance of the two pathways within the cortex depends on factors such as external ion concentration, root hair formation, and suberization of the rhizodermal cells (Marschner, 1995).

In general Pb translocation in plants is low, mainly due to Pb accumulation in epidermis and cortex cells in roots (Fodor et al., 1998). Huang & Cunningham (1996) found that plant species differ significantly in Pb uptake and translocation. For 50 species/cultivars tested, shoot Pb concentrations differed among plant species by more than 10 fold when grown in nutrient solution, and by more than 20 fold when grown in Pb contaminated soil. Kumar et al. (1995) found that some cultivars of *Brassica juncea* showed a strong ability to accumulate Pb in shoots. When the tissue distribution of the Pb in the shoots of selected cultivars of *B. juncea* was investigated, almost 90% of the Pb was found in stems, and only a small proportion (5-10%) was detected in leaves. Xylem cell walls have a high cation exchange capacity which probably retards the movement to the shoots and further to the leaves.

It should be mentioned that in order to guarantee the availability and solubility of trace elements for their use in mineral nutrition, mechanisms should be present which prevent sorption, hydrolysis, and non-specific chemical reactions that could occur in the uptake and translocation processes. Similar processes must affect pollutant elements as well. For cations, this problem is most likely avoided by organic complexation. A substantial body of qualitative information indicates that the nutrient ions Ca, Co, Fe, Mn, and Zn exist in organically complexed forms in xylem exudates (Tiffin, 1977) but also non-essential trace elements have been shown to be complexed. Most research has focused on identifying the intracellular metal ligands involved in hyperaccumulator plants, because it is clear that most of the metal taken up by hyperaccumulator plants must be chelated if acute metal toxicity is to be avoided (Baker et al., 2000). Plants are known to accumulate Ni readily and unlike most non-essential elements Ni is mobile in plants and accumulates in seeds (Mishra, 1974). In the Ni hyperaccumulator *Alyssum lesbiacum*, free histidine was produced as a specific and proportional response to Ni exposure (Krämer et al., 1996). It was hypothesised that histidine could be important both as a high-affinity ligand for nickel detoxification and in facilitating nickel transport out of the root in the xylem. Not only in Ni transport, but also in

Zn transport histidine seems to play a role. Using X-ray absorption, Salt et al. (1999) demonstrated that the majority of intracellular Zn in roots of the hyperaccumulator *Thlaspi caerulescens* was found to be coordinated with histidine. Contrary to this observation, an amino acid analysis of the root exudates of *T. caerulescens* revealed that the concentration of histidine was found to be not dependent on the Zn concentration in the root (Lodewijckx, 2001). The exact role of free histidine actually still is speculative and will remain so until more mechanistic data are available. Sarret et al. (2001) recently showed that Zn was predominantly present as Zn phosphate dihydrate in roots and leaves of *Phaseolus vulgaris* when added as Zn sulphate in solution, while Pb was predominantly found in leaves as cerussite (Pb carbonate) when the plant was grown in Pb nitrate solution.

3.1.3. Foliar deposition and uptake of Pb

It is a general consensus that atmospheric Pb increases the Pb content of vegetation, primarily by particulate deposition (Adriano, 2001). There are two primary sources of environmental Pb particulates: emissions from mining and smelting operations, and exhaust emission from automobiles. Particle Pb is quite insoluble and therefore is not expected to enter the leaf surface in substantial amounts. Generally high percentages of the Pb deposited on vegetation surface can be removed by water or mild detergents indicating that Pb is externally located. For example, in vegetables cultivated near a contaminated non ferro site in G.D. Luxemburg, Pb content in leaves was reduced by 70% after washing with tap water (Administration de l’Environment, 1990, 1992). Bassuk (1986) observed significant increases in Pb concentrations in lettuce plants exposed to automobile exhaust. Washing with vinegar (acetic acid) or liquid detergent reduced the Pb content to similar levels as those in unexposed plants. Carlson et al. (1976) measured Pb movement from leaf surfaces (fumigated with 1-3 µm diameter $PbCl_2$ aerosol), to non-fumigated plant tissues; they observed negligible movement of Pb into and/or within the plant. Large differences in aerosol deposition may occur between plant species because of differences in their surface structure (Wedding et al., 1975). The main barrier of foliar uptake are epicuticular waxes and the cuticular membrane (Arvik & Zimdahl, 1974). The chemical and physical nature of the waxes may act as a barrier to the entry of Pb into the leaf, i.e. particulates may be trapped within the matrix. Consequently, only small amounts of Pb can penetrate the cuticle even after extended exposure and exceptional conditions of Pb solubility (Zimdahl & Arvik, 1974).

3.2. Physiological effect of Pb on plants

At high concentrations, Pb uptake in plants has been reported to result in stunted growth, leaf epinasty and chlorosis (Johnson et al, 1977). However, at lower degrees of pollution, these visible symptoms are less pronounced or can even be absent, whereas at the cellular level several processes are affected, due to increased local metal concentrations.

One of the main effects of metals, responsible for the multidirectional effects on diverse aspects of cell metabolism, is the decline in enzyme activities. Decreased enzyme activities are mainly due to metal binding on SH-groups of enzymes and substitution of essential elements in enzymes (Van Assche & Clijsters, 1990b; Vangronsveld & Clijsters, 1994). Metal binding to SH-groups, essential for the stabilization of the enzyme tertiary structure, affect the enzyme conformation and thus its activity. Blocking SH-groups inhibits the activity of over 100 currently known enzymes (Seregin & Ivanov, 2001). Metals such as Pb, Ni and Co belong to the 'borderline' class according to the classification based on ligand affinity. Consequently, they react with O-containing ligands (characteristic for 'Class A' elements), and with N- or S- ligands (typical for 'Class B' elements) as well. They can be arranged in the following series of increasing 'class B' character: Zn < Ni < Co < Cd < Cu < Pb (Nieboer & Richardson, 1980).

3.2.1. Mineral nutrition

There are several ways metals can affect macro- and micronutrient uptake. For example, Pb can compete with ions of comparable valency and ionic radii, such as Ca (Ca^{2+} = 114 ρm *vs* Pb^{2+}= 133 ρm) (Shannon 1976). Huang et al. (1997a) found that Pb can significantly inhibit Ca^{2+} transport channels. Kennedy & Gonsalves (1987, 1989) showed that Pb caused an inhibition of Mg^{2+}-ATP-ase activity in maize roots and also affected ATP-ase related processes such as H^{+}- ion efflux and transmembrane potentials in maize. Stefanov et al. (1992, 1993) found that Pb alters the plasma membrane permeability, resulting in leakage of ions such as K and other solutes. Such membrane alterations can occur due to complexation with sulphydryl and carboxyl groups, or lipid peroxidation. The latter can be initiated by the formation of free radicals which interfere with membrane structure and can induce lipoxygenase activity (Gora & Clijsters, 1989). Xiong (1997) postulated that high Pb concentrations bound to the cell walls can change the elasticity and plasticity of the cell walls leading to large depositions on membranes, in turn resulting in interfered physiological membrane processes.

3.2.2. Water regime

A decline in transpiration rate and water content in plants treated with Pb^{2+} has been reported (Barcelo & Poschenrieder, 1990; Wóźny et al., 1995). Various mechanisms underlie these effects. Growth retardation for example results in a reduced leaf area, the major transpiring organ. Pb has also been described to increase ABA content, which induces stomatal closure (Seregin & Ivanov, 2001). Pb has further been described to lower the contents of compounds maintaining cell turgor and cell wall plasticity, which can result in a lower water potential (Barcelo & Poschenrieder, 1990; Burzynski & Jacob, 1983).

3.2.3. Inhibition of photosynthesis

In *in vivo* experiments, reduced rates of photosynthesis were reported in several plant species when soil Pb was elevated (Bazzaz et al., 1974a,b, 1975; Carlson &

Bazzaz, 1977; Carlson et al., 1975; Keller & Zuber, 1970; Rolfe & Bazzaz, 1975). Several workers reported decreased chlorophyll contents in leaves after Pb exposure (Fiusello & Molinari, 1973; Wozny et al., 1995; Xiong, 1997).

In vitro studies on isolated chloroplasts showed that Pb affects the photosynthetic activity on the level of both photosystem I and II: PS I was reported to be less sensitive than PS II (Miles et al., 1972; Bazzaz & Govindjee, 1974; Wong & Gonvindjee, 1976; Wrisher & Meglaj, 1980; Becerril et al., 1988). Hampp et al. (1973a) found that photosynthetic CO_2 fixation in isolated spinach chloroplasts was inhibited by Pb while studies using roots and leaves of spinach further demonstrated inhibition of RubisCo and non cyclic photophosphorylation (Hampp et al., 1973b). *In vitro* experiments further showed that Pb significantly inhibited the activity of δ-aminolaevulinic acid (ALA) dehydratase, an enzyme which converts δ-ALA into porphobilinogen in the synthesis of chlorophyll, by binding to SH-groups of the enzyme (Prassad & Prassad, 1987).

3.2.4. Growth and morphogenesis

As most Pb accumulates in roots, root growth is more sensitive to Pb than shoot growth. Pb can directly affect root growth by binding to cell wall polysaccharides, decreasing cell wall plasticity. On the other hand, it is clear that other affected processes, such as decreased photosynthesis, will negatively influence plant growth. Several investigations have found that Pb exposure results in mitotic irregularities (Ahlberg et al., 1972; Bahlsberg Pahlson, 1989; Liu et al., 1994; Przymusinski & Wóźny, 1985; Przymusinski et al., 1991; Sekerka & Bobak, 1974). The numerous nucleophilic centres in nucleic acids are favourite binding sites for Pb ions. Indirectly, oxygen free radicals can cause DNA damage. Effects of Pb on the function of various enzymes involved in the nucleic acid metabolism can also indirectly contribute to alterations in the genetic information by affecting replication fidelity (Seregin & Ivanov, 2001), affecting cell division.

3.2.5. Pb increases the activity of enzymes of the anti-oxidative stress metabolism

After application of heavy metals, an increased activity of a number of enzymes is frequently observed in several plant organs. Several workers observed an enhanced peroxidase and catalase activity in plants after Pb exposure (Hoxha et al., 1985; Lee et al., 1976; Maier, 1978; Xiong, 1997). Van Assche et al. (1988) described different threshold values for Zn and Cd in relation to root and shoot growth and increase of enzyme activities. For several metals it was demonstrated that the increase in activity can be the result of modification of gene expression (Kurepa et al., 1997; Thomas et al., 1998; Xiang & Oliver, 1998). Therefore metals can induce enzyme activity as a result of *de novo* protein synthesis (Xiang & Oliver, 1998). The question rises what the physiological meaning of this enzyme induction should be. Actually, metal phytotoxicity is considered to result in oxidative stress (De Vos & Schat, 1991; Cuypers et al., 1999). Elements such as Cu produce various reactive oxygen species (Koppenol, 1994). Lipid peroxidation products and hydrogen peroxide can accumulate in the plant tissue after metal treatment (Girotto, 1985; Weckx & Clijsters, 1997). The increased activity of enzymes caused by toxic amounts of metals is often a defence against metal imposed oxidative stress.

It is generally accepted that the production of active oxygen species is an unavoidable consequence of the operation of the photosynthetic electron transport chain in an oxygen containing atmosphere (Foyer et al., 1994a). Molecular oxygen in its ground state ($^3O_2$) has a low reactivity (Asada, 1996). The reduced and excited species of oxygen on the other hand are highly reactive and can interact with cellular components. In plant systems singlet oxygen ($^1O_2$) derives from chlorophyll systems linked with electron transport; excitation of O_2 to its singlet state can be achieved when several biological pigments (e.g. chlorophyll) are illuminated in the presence of O_2 (Halliwell & Gutteridge, 1984). It also arises as a by-product in lipoxygenase activity. $^1O_2$ is highly destructive and can react with almost all biological molecules (Canedas, 1989).

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

In a one electron oxidation chain molecular oxygen is converted to water with the formation of active oxygen intermediates. The formation of superoxide radical ($O_2^{\circ -}$) *in vivo* is mainly caused by the electron transfer from ferredoxin to oxygen (Asada, 1996). Hydrogen peroxide is formed through dismutation of the superoxide radical. Transition metal ions (Fe, Cu) can easily perform one electron oxidoreduction reactions resulting in the formation of these harmfull oxygen species. Examples of such reactions are the Fenton and Haber-Weiss reaction:

$$\begin{array}{l}
\mathbf{M^{(n+1)+} + O_2^{\circ -} \longrightarrow M^{n+} + O_2} \\
\mathbf{M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + OH^{\circ} + OH^{-}} \quad \textbf{(Fenton reaction)} \\
\hline
\mathbf{O_2^{\circ -} + H_2O_2 \longrightarrow O_2 + OH^{\circ} + OH^{-}} \ \textbf{(HaberWeiss reaction)}
\end{array}$$

The Fenton and Haber-Weiss reaction result in the production of the very reactive hydroxyl radicals (OH°), which react immediately with every biological molecule in their vicinity, producing secondary radicals of variable reactivity. Since Pb only has oxidation states +II and +IV it can not perform one electron oxidoreduction reactions and thus cannot directly induce oxygen radicals. Binding of Pb to functional groups of (membrane) proteins or substitution of essential metals in enzymes however can cause electron leakages (e.g. in the photosynthetic or mitochondrial electron transport) causing reactive oxygen species which in turn will increase lipid peroxidation, eventually resulting in cell death, tissue death, and growth reduction (Gwóźdź et al., 1997). Pb has been described to induce oxidative stress in other species, such as rats (Daggett et al., 1997; Patra et al., 2001), hamsters (Ercal et al., 1996), and humans (Costa et al., 1997). Ye et al. (1999) recently found a positive correlation between the presence of Pb in human blood and SOD activity.

3.3. Defence mechanisms against metal toxicity

3.3.1. Defence at the plant level

Root exudates can reduce metal toxicity in plants (Mench et al., 1988). Common root exudates with metal-complexing properties are citric and oxalic acid and many phenolic acids. However, such exudates can also increase metal solubility in the soil (Walter et al., 1994; Rengel et al., 1998). In addition, release of respiratory CO_2 by roots is known to precipitate Pb as $PbCO_3$, while Pb-phosphate precipitation on root cell walls also has been reported (Dushenkov et al., 1995; Jarvis & Leung, 2001; Salt et al., 1995).

Changes in the plant metal uptake can also be the consequence of a modification of metal fluxes through the plasma membrane after infection with mycorrhizal fungi. This symbiosis might be of special importance in several plant species since it can modify the host's nutrient and trace element uptake. It is known that mycorrhizal infection with metal tolerant fungi reduces metal uptake by the host plant (Jentschke et al., 1998; Sayer et al., 1999; Van Tichelen et al., 1999, 2001).

Reduction of root to shoot metal translocation can protect sensitive and essential metabolic processes in the aerial plant organs (e.g. photosynthesis) (Vangronsveld & Clijsters, 1994). There is a general consensus that Pb primarily accumulates in the roots and is poorly translocated to other plant parts (Fargasova, 1994; Lane & Martin, 1980; Liu et al., 1994; Qureshi et al., 1986: Theiss, 1990).

3.3.2. Plant defence at the cellular level

3.3.2.1. Mechanisms which prevent interaction between metals and their sites of action

Metal toxicity depends on the plant species, the nature of the metal and its concentration. In Figure 1.1 possible cellular defence mechanisms against metal toxicity are depicted (adapted from Marschner, 1995).

Cell walls (1) demonstrate some binding capacity towards metals. The cell wall of root cells is directly exposed to metals available in the soil water phase. As mentioned earlier, a major part of the total root Pb content was found to be associated with the root cell walls in various plants (Ernst et al., 1990; Bringezu et al., 1999; Chang et al., 1999). Once the metal has crossed the cell wall various mechanisms are available to protect the symplast. At the plasma membrane level 3 mechanisms can be distinguished: (2) restricted influx, (3) active efflux and (4) chelation at the cell wall-plasmamembrane interface. Malone et al. (1974) found that Pb taken up by corn roots was concentrated in dictyosome vesicles. Dictyosome vesicles containing cell wall material fused with one another to encase the Pb deposit. This encased deposit which was surrounded by a membrane migrated towards the outside of the cell where the membrane surrounding the deposit fused with the plasmalemma. The material surrounding the deposit then fused with the cell wall.

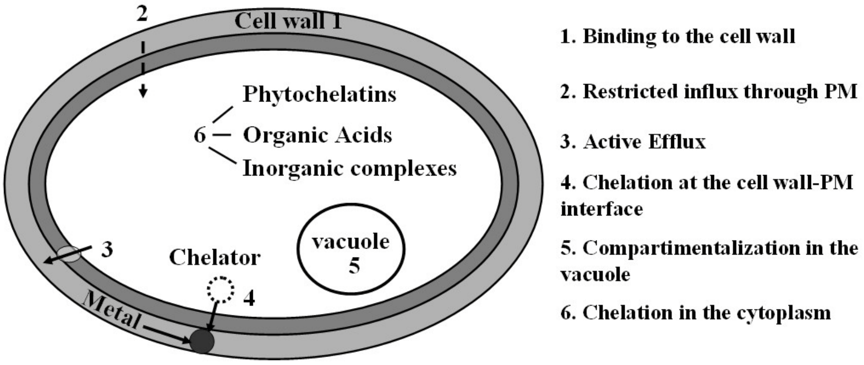

Fig. 1.1: Possible defence mechanisms against metal toxicity.

In the cell the metal can be (5) translocated into the vacuole which makes interference with metabolic processes impossible (Coleman et al., 1997a,b). It has been shown that Zn binds to malate and this complex is transported into the vacuole where Zn is released and binds to oxalate, and malate is transported back to the plasma (Harmens et al., 1994). Electron microscope analysis of Pb treated plants has shown Pb deposition in vacuoles of *Lupinus luteus* (Przymusinski & Wozny, 1985) and *Allium cepa* (Antosiewicz & Wierzbicka, 1999).

Finally, in the cytoplasm (6) several binding agents can be distinguished which form stable complexes with the metal and avoid disturbance of physiological and biochemical processes. For example, Pb can form stable complexes with glutathione (Christie & Costa, 1984). A major role however has been attributed to phytochelatins which demonstrate a unique affinity towards metal binding (Rauser, 1990). Although most evidence for their working is gathered using Cd, several workers report phytochelatin induction upon Pb exposure (Grill et al., 1987, 1988, 1989; Gwóźdź et al., 1997; Salt et al., 1995). Various heavy metals have quite specific impacts on the responsiveness of the phytochelatin syntheses. At the same exposure metal concentration, induction phytochelatin synthesis decreases in following order: Hg > Cd > As > Ag > Cu > Ni > Sb > Au > Sn > Se > Bi > Pb > Zn (Grill et al., 1987).

3.3.2.2. Enzyme induction

As mentioned earlier, Pb is known to increase the activity of several enzymes of the anti-oxidative stress metabolism, e.g. guaiacol and ascorbate peroxidases in plants (Gwźódź et al., 1997; Lee et al., 1976; Maier, 1978; Xiong, 1997). Both enzymes detoxify peroxides. Guaiacol peroxidases are mainly located in the vacuole, cytosol and cell wall, and deliver physiologically active reaction products (e.g. lignification process) in the cell wall. Ascorbate peroxidases are principally found in the chloroplast and cytosol.

In plants 3 types of superoxide dismutase isoenzymes are distinguished: Fe-SOD (in chloroplast), Mn-SOD (in mitochondria) and the Cu-Zn-SOD (chloroplast, cytosol). Since they are metalloenzymes, metal deficiency affects their capacity. Metal excess however also modifies these enzymes (Weckx & Clijsters, 1996). Przymusinski et al. (1995) observed SOD induction upon Pb exposure in plants. Hoxha et al. (1985) and Gwźódź et al.(1997) further found a stimulation of peroxisomal catalase in *Zea mays* and *Lupinus luteus* after application of Pb to the growth medium. All these enzymes are characterized for their reaction with the reactive oxygen species peroxide and superoxide.

Recently, a second group of enzymes – involved in the ascorbate-glutathione pathway - was found to be increased under heavy metal stress (Cu, Zn; Cuypers et al., 2000; Gupta et al., 1999; Foyer, 1993). In this pathway peroxides are detoxified by ascorbate peroxidase; the oxidized substrate is recycled by monodehydroascorbate reductase using NADP as reducing coenzyme (Fig. 1.2, after Cuypers, 2000). Monodehydroascorbate is readily oxidized to dehydroascorbate which in turn is converted to ascorbate by dehydroascorbate reductase. The necessary electrons for this reaction are supplied by glutathione which in turn is reduced by glutathione reductase with NADPH as the final electron donor.

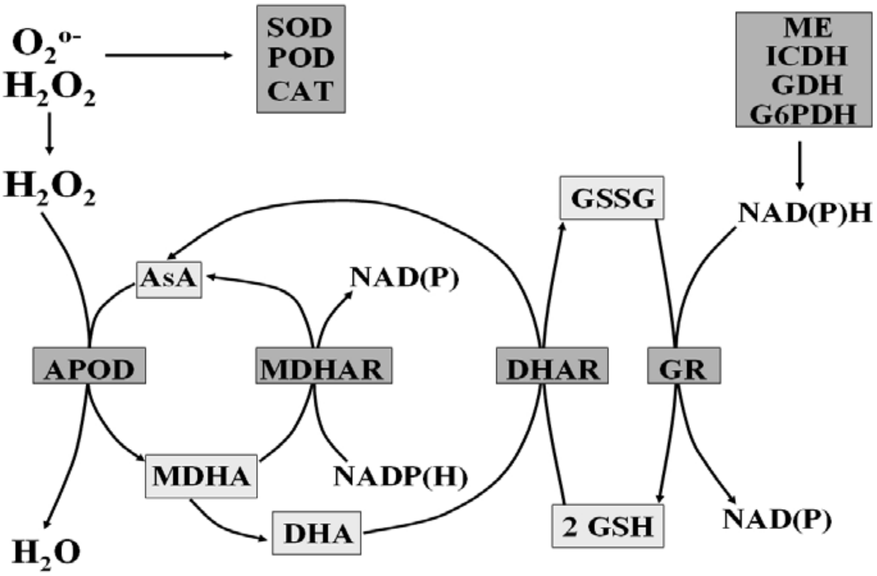

Fig. 1.2: The ascorbate-glutathione pathway and its relation with other enzymes known to be induced under oxidative stress.

A third group of heavy metal induced enzymes concerns enzymes involved in or closely related to the Krebs cycle (isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), malic enzyme (ME)) (Van Assche et al., 1988). It is suggested that the capacity of these enzymes is increased to provide the plant cell with sufficient reducing power to compensate for the decrease in ATP and NADPH normally provided by the metal-sensitive photosynthetic reactions and the NADPH used in the ascorbate glutathione pathway (Clijsters et al., 1999).

3.3.3. Enzymatic changes in plants as a diagnostic criteria of a biological test for the evaluation of phytotoxicity of metal-polluted soils

Since Pb and other heavy metals increase the activity of enzymes belonging to the anti-oxidative stress metabolism, the evaluation of phytotoxicity of metal-polluted soils and other substrates can be realised using enzymatic parameters. Mench (oral communication) observed a high correlation between oxidative stress measured in plants under controlled environmental conditions and plant yield *in situ*. Van Assche & Clijsters (1990) and Vangronsveld & Clijsters (1992) developed a biological test system for phytotoxicity assessment of metal contaminated substrates integrating enzyme induction (POD, ME, GDH, ICDH) with metal specific changes in the isoperoxide pattern and data of morphological parameters (shoot length, primary leaf area, root weight) of *Phaseolus vulgaris* (cv. Limburgse vroege). In short, bean plants are cultivated on soils under controlled environmental conditions for 2 weeks. After 2 weeks plants are harvested and morphological plant parameters are measured. Root and leaf material is stored (-70°C) for (iso)enzyme analysis. The enzyme capacities are measured spectrophotometrically (Van Assche et al., 1988). Anionic isoperoxidases are separated by polyacrilamide gel electrophoresis on a gradient slab gel and stained with benzidine and H_2O_2 (Van Assche & Clijsters, 1990). The values obtained for each parameter examined can be transposed into phytotoxicity classes: each parameter is used to classify the substrate in a given phytotoxicity class (calculated in comparison with values generated on plants cultivated on non polluted substrate). The classification system developed for *Phaseolus vulgaris* cv. Limburgse vroege is presented in Table 1.1. The mean value of the toxicity class numbers obtained for each biological parameter is the ‘phytotoxicity index’ for a given substrate. It ranges from non toxic (1), over slightly (2) or moderately (3) toxic to strongly toxic (4).

Table 1.1: Classification of biological data into phytotoxicity classes, depending on the magnitude of the effect. Ranges apply to the relative (percentage) values of the results as compared to the control values except for D-band isoperoxidases, whose relative capacity is presented as % of the total isoperoxidase capacity of the sample (according to Van Assche & Clijsters, 1990; Vangronsveld & Clijsters, 1992).

		Degree of phytotoxicity (class number)			
Parameters		Not toxic (1)	Slightly toxic (2)	Moderately toxic (3)	Strongly toxic (4)
Shoot length		>85	85-70[a]	70-50	<50
Primary leaf area		>85	85-70	70-50	<50
Root weight		>85	85-70	70-50	<50
Enzyme capacity					
Leaf	POD	<150	150-325	325-500	>500
	ME, ICDH	<125	125-175	175-250	>250
Root	POD,ME, GLDH	<125	125-175	175-250	>250
D-isoperoxides (x100)					
	Leaf	0	0-25	35-50	>50
	Root	0	0-15	15-30	>30

In comparison with chemical analysis of metal polluted soils, biological test systems consider the total phytotoxic effect, since it is the result of the mutual interactions of the metals present in the soil with other soil factors and the plant. Since toxic effects occur first on a cellular level, the application of enzymatic parameters results in a very sensitive phytotoxicity test, in comparison to tests only measuring morphological parameters.

4. RECLAMATION OF METAL CONTAMINATED SOILS

4.1. Engineering approaches

4.1.1. Metal removal

Metals can be removed from soils by (1) excavation, (2) soil washing, (3) electroreclamation and (4) volatilization.

(1) Soil excavation and landfilling requires removal of the contaminated soil to a special landfill and subsequent site restoration which may include backfilling with clean soil and establishment of vegetation (Smith et al., 1995; Soesilo & Wilson, 1997).

(2) Two types of soil washing exist: (a) particle separation techniques and (b) leaching techniques. (a) Particle separation techniques remove the finer fractions of the soil where metals tend to accumulate. This technique reduces the volume of material going into the landfill by separating out the relatively clean fraction of sand and silt. (b) Leaching techniques remove solubilized metals using chemical extractants (H_2SO_4, HCl, CH_3COOH,..) and can be conducted *in situ* or on excavated soil.

(3) Electroreclamation is based on an electrokinetic process which occurs when a direct current is applied between a cathode and anode placed in the soil: cations migrate to the cathode and anions to the anode (Lageman, 1993).

(4) thermal treatment heats the soil until contaminants are volatilized after which they are collected and treated. Only mercury can be removed from soils by this technique.

Excavation is not feasible on larger scales due to high costs involved, the limited number of appropriate landfill sites and the limited availability of good replacement soil. Soil washing as a particle separation technique may effectively remediate soils in which the Pb is associated with small soil particles (e.g. the clay fraction in soils) (Cunningham & Berti, 1999) but is not suited for large scale application as stated above. Leaching techniques have several limitations; they are only effective on very permeable soils, reactors should be constructed with anticorrosive material and extractants may have a detrimental impact on the environment. In *in situ* applications the risk exists that solubilized contaminants leach into the underlying water table (Rulkens, 1993). Electroreclamation is expensive to install, requires substantial amounts of electricity to run and must frequently be maintained.

4.1.2. Metal stabilization

(1) A common method of remediating soils contaminated with heavy metals is to place a physical barrier (cap) on the soil surface that prevents water infiltration and soil erosion. Caps may consist of clean soil, a layer of clay, a layer of asphalt, or a

combination of these materials. In some cases, vertical barriers may be installed to minimize groundwater flow through the contaminated soil.

(2) contaminated soil may be stabilized by mixing with solidifying materials such as cement or other pozzolanic materials, or other suitable agents. Addition of cement increases pH of the soil matrix up to 10 at which many metals form insoluble complexes. Such pH however cannot support plant growth.

(3) Vitrification involves converting the contaminated matrix to a solid block of glass-like material using a heat source. The soil is heated to 1600-2000°C until the matrix melts; after cooling, the molten soil forms a very durable and impermeable glass-like material containing the contaminants.

Cement application may require long-term monitoring as most weathering processes may result in lower pH which may cause cement to break down and release the contaminants (Vangronsveld & Cunningham, 1998). Vitrification on the other hand is effective but extremely expensive and disruptive to the site.

Cunningham & Berti (1999) calculated financial costs for remediation of a one hectare area, contaminated primarily with Pb to a depth of 30 cm. Metal removal techniques proved to be the most expensive: excavation and landfilling was estimated to cost US$1.5 million, followed by soil washing (particle separation technique); US$790.000. Site stabilization was cheaper: asphalt capping for site use as a parking lot costs US$140.000-160.000.

4.2. Phytoremediation

A low cost remediation technique that is environmentally sound and equally protective of human health and the environment would be a valuable and viable alternative to current remediation methods. Research has been directed towards applying biological and chemical processes to exploit or alter the different forms of Pb that exist in soil to reduce the health and safety hazard associated with contaminated soils (Cunningham & Berti, 1999). Examples of such remediation strategies are the so-called phytoremediation techniques (for a review, see Schwitzguébel et al., 2002). For phytoremediation of metal-polluted soils, two possible techniques, both aiming at a reduction of the bioavailable fraction of metals in the soil, can be distinguished: (1) phytoextraction (metal bioextraction by means of hyperaccumulating plants), and (2) in place inactivation (*in situ* metal inactivation by means of soil amendments, mostly combined with the establishment of a vegetation cover, i.e. phytostabilization) (Vangronsveld & Cunningham, 1998; Brown et al., 2001). Both of these techniques are relatively simple and low cost[1], however each technique resolves the risk to the environment and human health through contrasting approaches (Cunningham & Berti, 1999).

4.1.1. Phytoextraction

Phytoextraction is the process where metal accumulating plants are used to transport and concentrate heavy metals from the soil into the shoots in order to reduce soil concentrations of heavy metals to environmentally acceptable levels (Kumar et al., 1995; Salt et al., 1995). Dried, ashed, or composted plant residues, highly enriched in heavy metals may be isolated as hazardous waste or recycled as biometal ore (phytomining). Soils with light to moderate toxic metal contamination might be suitable for growing metal accumulating plants for toxic metal cleanup. Most heavily contaminated soils however do not support plant growth. And even if plants tolerating huge concentrations were obtained, their remedial potential for such soils would be limited due to the long time needed to completely cleanup these substrates.

It is evident that plants used in these processes need some characteristics in order to efficiently fulfil phytoextraction. The ideal plant should be able to tolerate and accumulate high levels of heavy metals preferably in the aboveground parts, should have a rapid growth rate and the potential to produce a high biomass in the field and should be easy to harvest (Vangronsveld et al., 2000).

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[1] Estimation of costs involved in immobilizing Pb on the same polluted substrate as mentioned under 4.1 (using Fe oxides or a PO_4^{3-} source) are estimated to result in a cost of US$53.000 (Cunningham & Berti, 1999).

4.1.1.1. Heavy metal hyperaccumulator plant species

Inventarisation of plants from areas where the soil is enriched with heavy metals due to geological factors or pollution, resulted in a wide range of different species (Baker & Brooks, 1989; Reeves & Baker, 2000). Three basic strategies are used by plants growing on metalliferous soils (Fig. 1.3; Baker, 1981). Metal excluders effectively prevent heavy metals from entering their aerial parts over a broad range of metal concentrations in the soil; however they can still contain large amounts of metals in their roots. Excluders include members of the grass family (e.g. sudangrass, bromegrass, fescue, etc.). Metal non-excluders actively accumulate metals in their shoots and can be divided into two groups: indicators and hyperaccumulators. Heavy metal levels in the tissues of indicator species generally reflect metal levels in the soil while hyperaccumulators concentrate metals in their shoots to levels far exceeding those present in the soil or in the non-accumulating species growing nearby. Indicators include the grain and cereal crops (e.g. corn, soybean, wheat, oats, etc.). Hyperaccumulators are found in several plant families (for a review see Baker et al., 2000).

Among these plant types, hyperaccumulators seem to be the most interesting candidates for phytoextraction processes because of the high accumulation rates of heavy metals in the harvestable parts. Hyperaccumulation implies the existence of mechanisms for metal detoxification within the plant, most likely involving chelation of the metal cation by ligands and/or sequestration of metals away from sites of metabolism in the cytoplasm, notably into the vacuole or cell wall (Baker et al., 2000). Generally a plant is considered a hyperaccumulator if per kilogram dry weight more then 1000 mg (= 0.1%) Co, Cu, Cr, Pb, Ni or 10 000 mg Mn or Zn is accumulated (Baker & Brooks, 1989). The list of metal hyperaccumulating plants is expanding rapidly and the largest numbers collected in the temperate zone belong to the Brassicaceae while in the tropic regions the Euphorbiaceae are the best represented (Baker & Brooks, 1989). There are 400 known hyperaccumulators in the world. The majority of described species concern Ni hyperaccumulation (317 taxa); 14 taxa (from 6 families) have been described as Pb hyperaccumulators

(Baker et al., 2000); among those *Polycarpae synandra* (1040 mg kg^{-1} DW; Cole et al., 1968), *Minuartia verna* (1400 mg kg^{-1} DW; Baker, 1981); *Armeria maritima* (1600 mg kg^{-1} DW; Baker & Brooks, 1989), *Thlaspi alpestre* (2740 mg kg^{-1} DW; Baker, 1981) and *Thlaspi rotundifolium* (8200 mg kg^{-1} DW; Reeves & Brooks, 1983).

However, all these reported hyperaccumulators have a very slow growth rate and low limited biomass production, which limits their use for Pb phytoextraction (Huang et al., 1997).

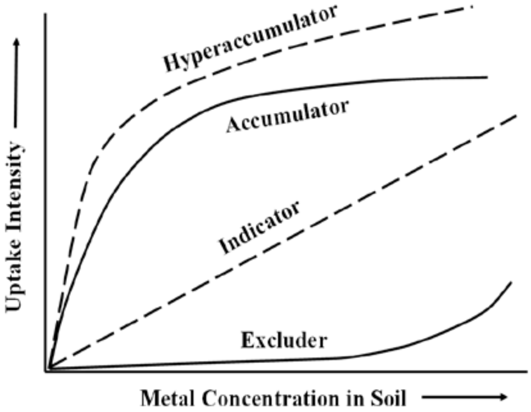

Fig. 1.3: Relative uptake and bioaccumulation potential among plant species.

Recently, it was found that high whole-plant concentrations could be achieved by exposing non-tolerant species to high levels of soluble Pb for short periods (even though the plants did not survive the treatment). Addition of synthetic chelates to the root medium was found to dramatically enhance Pb concentrations found in aboveground plant parts. For example, Huang et al. (1997), found that 24 h after applying 1 g EDTA kg^{-1} contaminated soil, Pb concentration in corn xylem sap increased 140-fold, and net Pb translocation from roots to shoots increased 120-fold as compared to the control (no EDTA). EDTA addition resulted in shoot concentrations in excess of 1% DW in *Zea mays*, *Pisum sativum* and *Brassica juncea* (Blaylock et al., 1997; Huang & Cunningham, 1996; Huang et al., 1997b).

Pb normally has limited solubility and bioavailability in soils due to formation of stable complexes with organic matter, sorption to oxides and clays, and precipitation as carbonated, hydroxides and phosphates (McBride, 1994; Adriano, 2001). EDTA showed to drastically increase Pb mobility in soil (Huang and Cunningham 1996, Huang et al. 1997b, Tejowulan and Hendershot 1998, Epstein et al. 1999, Papassiopi et al. 1999, Wu et al. 1999). Chemically, Pb chelation by EDTA in soil is perfectly explainable, due to the high affinity of the chelating agent for Pb (Skoog et al., 1996). The formation of Pb-EDTA is expected to be the dominant metal-EDTA complex in most soils between pH 5.2 and pH 7.7 (Blaylock et al., 1997).

EDTA addition however also showed to drastically improve Pb uptake and translocation to upper plant parts (Blaylock et al. 1997; Epstein et al. 1999; Huang and Cunningham 1996; Huang et al. 1997b; Jarvis & Leung, 2001). Vassil et al. (1998) observed EDTA in the shoots and xylem sap of *B. juncea*, and found a strong correlation between Pb and EDTA accumulated in shoots. Their findings suggested plant uptake of the chelator, in contrast to Fe(III)-EDTA which is known to dissociate before plant uptake. A plasma membrane-bound reductase is responsible for the reduction of Fe^{3+} to Fe^{2+} rendering the complex less stable while increased acidification of the rhizosphere due to H^+-ATPase results in increased solubilization of the complex (Chaney et al., 1972; Marschner et al., 1986, Wallace and Wallace, 1992; Marschner, 1995). Metal-chelate uptake however is known to occur; root-exuded phytosiderophores (e.g. mugineic acid) complexed with Fe are reported to be absorbed by graminaceous plant species (Marschner, 1995).

Vassil et al. (1998) further found that Pb content in *B. juncea* shoots drastically increased when the EDTA concentration in the growth medium was enhanced. The authors suggested that a threshold concentration of EDTA was required to ‘induce’ accumulation of high concentrations of Pb-EDTA. The authors speculated that at these threshold concentrations, synthetic chelates destroy the physical barrier(s) in roots that normally function to control uptake and translocation of solutes, allowing leakage of soil solution with Pb-EDTA into the plants, ultimately resulting in plant death. Both Zn^{2+} and Ca^{2+} ions are involved in stabilizing plasma membranes (Pasternak, 1988). Vassil et al. (1998) suggested that EDTA would remove stabilizing plasmamembrane ions such as Zn^{2+} and $Ca^{2+,}$ resulting in membrane damage.·The specific pathway of Pb-EDTA to enter the plant and eventually the plant xylem in the presence of low EDTA not causing membrane damage, is not known (Wu et al., 1999).

4.1.2. In place inactivation - Phytostabilization

4.1.2.1. In-place inactivation

In-place inactivation (immobilization) stabilizes soil Pb both chemically and physically through the use of soil amendments and a vegetative cover. Soil amendments alter the existing Pb chemistry in the soil and reduce the biological availability of Pb by inducing the formation of very insoluble Pb species. Amendments that have been examined for their effectiveness in reducing Pb availability include phosphates, liming agents, Fe and Mn oxides, organic materials, aluminosilicates**.** In general, preference is given to cheap materials such as industrial waste products or minerals.

Phosphates

Last decade, several workers reported that addition of PO_4^{3-} (hydroxyapatite, phosphate rock, phosphoric acid, tri super phosphate, Thomas Basic Slags) may substantially reduce bioavailable Pb (Berti and Cunningham, 1997; Boisson et al., 1999; Chen et al., 1997; Chlopecka and Adriano, 1996; Hodson et al., 2000, 2001; Laperche et al., 1996, 1997; Ma, 1996; Ma and Rao, 1997; Ma et al., 1993, 1994a,b; 1995; 1997; Mench et al., 1994b, 1997; Pierzynski & Schwab, 1993; Rabinowitz, 1993, 1997; Ruby et al., 1994, 1996; Yang et al., 2001; Zhang et al., 1997; Zhang & Ryan, 1999a,b).

Rabinowitz (1993) found that phosphate treatment decreased soil Pb extracted with 0.1 M HCl and 10 % citric acid by as much as 43-76%. Berti & Cunningham (1997) and Ma & Rao (1997) observed a dramatic shift in sequential chemical extraction of Pb towards more recalcitrant fractions after adding 0.5% PO_4^{3-} to a Pb contaminated soil while Mench et al. (1994a) found that an alkaline phosphate containing slag reduced Pb concentrations in the aerial tissues of ryegrass and tobacco by 40% and 55% respectively. Other workers also observed reductions in plant Pb uptake (Boisson et al., 1999; Chlopecka & Adriano, 1997a,b). Phosphate treatments also reduced soil bioavailable Pb extracted during the stomach phase of the PBET (Berti & Cunningham, 1997; Brown & Chaney, 1997; Ruby et al., 1994). Ma et al. (1993) found that hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] dissolves and reacts with Pb as is described in following reactions:

$$Ca_{10}(PO_4)_6(OH)_{2(s)} + 14H+_{(aq)} \rightleftarrows 10Ca^{2+}_{(aq)} + 6H_2PO_4^-{}_{(aq)} + 2H_2O$$

$$10Pb^{2+}_{(aq)} + 6H_2PO_4^-{}_{(aq)} + 2H_2O \rightleftarrows Pb_{10}(PO_4)_6(OH)_{2(s)} + 14H^+_{(aq)}$$

The reactions occur to completion, forming a fairly stable precipitate of hydroxypyromorphite ([$Pb_{10}(PO_4)_6(OH)_2$] in the absence of competing metals (Al, Cu, Fe, Cd) and ligands (EDTA, CO_3^{2-}) (Cotter-Howells, 1996; Laperche et al., 1996; Ma, 1996; Ma et al., 1994a,b). Solubility data indicate that Pb pyromorphites are substantially less soluble than the Pb forms generally found in ore minerals (PbS, $PbSO_4$, $PbCO_3$), paint [e.g. $PbCO_3 \cdot Pb(OH)_2$], and automobile emission-affected particulates (Ruby et al., 1999). Jones et al. (1997) and Hettiarachchi & Pierzynski (1999) found strong reductions in PBET extractable Pb when an acetic acid treatment was followed by TSP addition or when phosphoric acid was applied. Treatment of soils with acids indeed may increase Pb available in soil solution to react with added soluble phosphates. Some research however found that treatment with acid enhances Pb inactivation when more soluble phosphate materials are used, but not when less soluble materials are used (e.g. hydroxyapatite) (Berti et al., 1998). Furthermore, acids may increase the solubility of other metals in soil which are not as easily complexed with phosphates. This approach also may produce excess PO_4^{3-} that may reach water systems causing nutrient enrichment and eutrophication. Boisson et al. (1999) found that phosphate applied to soils contaminated with Pb and As may increase As bioavailability and leachability, possibly because of phosphate and arsenate competition for soil sorption sites.

Only few data are available on immobilization of other metals and metalloids in soil after hydroxyapatite/phosphate rock application. Chen et al. (1997) observed reductions in Zn (-29%) and Cd (-49%) concentrations in soil solution while Chlopecka & Adriano (1997b) found decreased Zn and Cd uptake by maize after apatite addition. Several mechanisms have been proposed explaining hydroxyapatite induced immobilization of other metals: (1) ion exchange processes at the surface (Chen et al., 1997; Middelburg & Comans, 1991; Xu et al., 1994), (2) surface complexation (Chen et al., 1997; Xu et al., 1994), (3) precipitation of some amorphous to poorly crystalline, mixed metal phosphate (Ma et al., 1994b; Wright et al., 1995) and (4) substitution of Ca in hydroxyapatite by other metals during recrystallization (coprecipitation).

Liming agents

Addition of lime [e.g. $CaCO_3$, $(Ca,Mg)CO_3$, $Ca(OH)_2$, CaO] can be considered as the oldest and possibly most widely adopted immobilization technique for cationic metals in the soil solution (Mench et al., 1998). Generally, an increase in soil pH ionises pH-dependent exchange sites (lime will 'bind' H^+ ions sorbed to binding sites on the surface of soil particles), raising cation exchange capacity (CEC). This may result in greater Pb sorption to soil particles. Addition of lime also favours the formation of $PbCO_3$ (Berti & Cunnigham, 1998).

Liming agents have been demonstrated to reduce Pb solubility and soil phytotoxicity while in general plant Pb uptake is reduced (Albesel & Cottenie, 1985; Mench et al., 1994a; Pierzynski & Schwab, 1993; Zimdahl & Foster, 1976). Elkhatib et al. (1991) compared Pb sorption in three soils with different $CaCO_3$ content and found the highest adsorption capacity in the soil with the highest $CaCO_3$ content. Chlopecka & Adriano (1997) observed strong increases in ryegrass and corn biomass and decreases in Pb uptake in ryegrass after lime treatment on a Pb spiked soil. Mench et al. (1997) observed significant reductions in water and HOAc-exhangeable Pb in a Pb contaminated soil amended with lime. Ryegrass yield increased and Pb content in shoots in general decreased.

Whereas the mobility of cations generally decreases at a higher pH, the mobility of anions, such as molybdenate and arsenate will increase if the pH is increased. Also, substantial amounts of trace elements (e.g. Pb, Cu) can be bound to OM; an increase in soil pH may change the solid-solution partitioning of the OM, resulting in an increased dissolved organic matter (DOM) concentration in the soil solution, resulting in increased leaching to deeper soil layers or ground water (e.g. Tipping & Woof, 1991). Chlopecka & Adriano (1996) noted that liming is only effective in the short-term; repeated applications are required to maintain metal immobilization.

Fe- and Mn oxides[2]

Materials containing Fe and Mn hydrous oxides often have a great affinity for Pb and other heavy metals. The high reactivity of the hydrous oxides results from the presence of hydroxyl groups; these form an ideal template for bridging trace metals because the OH-OH distance matches well with the coordination polyhedra of trace metals (Mench et al., 1998).

Mn oxides

The reactivity of the different Mn oxides varies widely, due to differences in structure and surface groups. The most reactive form is synthetic birnessite (Osté, 2001). Extended X-ray Absorption Fine Structure (EXAFS) showed that Pb forms

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[2] Strictly speaking, oxidation of Fe in soil can result in formation of iron oxides, iron oxyhydroxides and iron hydroxides (Hargé, 1997). In the following, all these compounds will be referred to as ‘oxides’. HFO and HMO will be used to as specific terms: HFO (hydrous ferric oxide) as a specific 2 line ferrihydrate. HMO (hydrous manganese oxide) as the Mn oxide that is obtained by titration of a potassium permanganate solution with an excess of HCl (its structure is not well known) (Manceau et al., 1992b).

inner-sphere surface complexes[3] on birnessite (Manceau et al., 1997). Mench et al. (1994a,b; 1997) observed strong reductions in soil extractable Pb [$Ca(NO_3)_2$, HOAc] after application of hydrous Mn oxides (HMO) to a limed-silty soil (1112 mg Pb kg^{-1}) and an arable soil (250 mg kg^{-1}), contaminated by aerial deposition near a Pb/Zn smelter and tetraethyllead respectively. Pb concentration in ryegrass shoots was decreased, while concentrations in tobacco shoots or bean leaves were not altered. Mench et al. (1994a) also observed significant reductions in water and $Ca(NO_3)_2$ extractable Cd and Zn upon HMO application while metal content in shoots of ryegrass (Cd, Zn) and tobacco (Cd) and in bean leaves (Cd) decreased. These Mn oxides (birnessite, HMO) however are not readily available (Mench et al., 1998).

Fe oxides

Laboratory and field studies confirm that metals accumulate in Fe oxides (Kabata-Pendias & Pendias, 1992; Hiller & Brümmer, 1995; Martinez & McBride, 1998). Chlopecka & Adriano (1996) observed strong decreases in available Zn after IRM (Iron Rich Material, also Fe-rich) application, a byproduct of TiO_2 production, containing 50% to 60% hydrous Fe oxides. IRM however has a high chloride content (Salinger et al., 1994); in cited experiments the authors washed the additive up to 10 times before application. Large scale application appears problematic due to the high cost involved in IRM washing and the fact that IRM is not available in large quantities (A. Knox, oral communication). Another method for application of Fe or Mn oxides to contaminated soils is by materials which release Fe or Mn in soils (Mench et al., 1998). A gradual oxidation of Fe allows the metal ions to coprecipitate within the newly formed ferric (hydr)oxide. Steelshot, an industrial material containing mainly Fe (97%) and impurities such as Mn (0.6-1%), Si (0.8 to 1.2%), C (0.8-1.2%), and Cr (0.2-0.5%), corrode readily and oxidise into several

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[3] Inner sphere complexes are defined as covalent linkages between the adsorbed ion and the reactive surface with no water of hydration between the adsorbed ion and the surface functional group (Sposito, 1984).

Fe and Mn oxides in soils (Sappin-Didier, 1995). It is suggested that the Fe and Mn oxides released into the soil may coat soil particles, developing a large surface for reaction with trace elements in solution (Mench et al., 1998). Sappin-Didier (1995) found that steelshot with larger particle size (e.g. 1.7 mm and 2.4 mm) were less effective in reducing shoot Cd and Zn uptake compared to soils amended with finer steelshot. Mench et al. (1997) observed reductions in water extractable Pb after steelshot application on a soil contaminated with tetraethyellead while water or $Ca(NO_3)_2$ extractable Pb was not significantly altered on a substrate contaminated from smelter activities (Mench et al., 1994a). Steelshot application further reduced Pb uptake in shoots of ryegrass, while Pb uptake was not altered in tobacco or bean (Mench et al., 1994a). The same workers also observed decreases in Cd and Zn extracted with water or $Ca(NO_3)_2$ upon steelshot addition while Vangronsveld et al. (1994) described efficient As immobilization.

Reducing conditions may decrease sorption capacity of these materials and dissolve Fe and Mn oxides, possibly releasing adsorbed Pb (Berti et al., 1998). Mench et al. (1994b) noticed that plant species (ryegrass, tobacco) were able to influence the Pb inactivating capacity of both HFO and HMO. HFO was less effective in reducing plant Pb concentrations in ryegrass than in tobacco. This effect may have been a result of Fe solubilisation by phytosiderophores exuded from the ryegrass roots.

Organic materials

Organic materials such as composts, biosolids and peat are also prospects for use in *in situ* inactivation of Pb in contaminated soils. Organic matter often has high CEC and may adsorb Pb on pH-dependent exchange sites, such as negatively charged phenolic and carboxylic sites (Berti et al., 1998; Osté, 2001). Organic matter has a high affinity for Pb and forms strong complexes with soil Pb. Organic amendments may also benefit plant growth by increasing soil moisture holding capacity, improving soil texture, and providing plant nutrients (e.g. N, P).

Berti & Cunningham (1997) found reductions in exchangeable and leachable Pb after adding composted leaves to a Pb contaminated soil while Scialdone et al. (1980) observed reduced plant Pb uptake after application of animal wastes or peat. Bassuk (1986) evaluated Pb uptake by lettuce on a Pb spiked soil ($PbCl_2$) using different types of organic matter. According to Pb immobilizing effectiveness, amendments could be arranged as follows: muck soil > manure > ground up leaves > sphagnum peat.

However, there remain some uncertainties regarding the use of organic amendments. It is possible that degradation of organic matter will release organically complexed Pb over time, causing it to be more available (possibly resulting in increased plant uptake) until it is redistributed to other soil fractions (Martinez & McBride, 1999). Some by-products and wastes (e.g. some sludges and manures) may contain heavy metals that could contribute to the contaminant levels already present at the site. High rates of nutrient-rich material such as manures may contribute excess N and P to the environment, which may spur eutrophication.

Aluminosilicates

Clays

Montmorrilonite type clays have been applied for their high cation exchange capacity (Osté, 2001). Krebs-Hartmann (1997) found that montmorillonite clays reduced extractable Zn and Cd while Sims & Boswell (1978) observed a decrease in metal content in wheat leaves (Cd, Zn) after addition of bentonite (a commercial montmorillonite clay) to a contaminated sludge treated soil.

Zeolites

Zeolites are crystalline, hydrated aluminosilicates that possess an infinite three-dimensional crystal structure. Their immobilizing potential depends on their ion-exchange capabilities which result from substitution of Al^{3+} for Si^{4+} in the silicate tetrahedral, creating fixed negatively charged sites throughout the structure (Mench et al., 1998). Zeolites possess ion sieve properties. The internal structure consists of a series of interconnecting channels and cages with specific dimensions. These either trap or exclude ions depending on size. Synthetic and natural zeolites have been investigated with respect to metal uptake. Chlopecka & Adriano (1996, 1997c) showed that clinoptilolite substantially reduced phytoavailable ($CaCl_2$) Pb, Cd and Zn concentrations (-21% to –31% for Pb) and metal uptake by maize (-16% to –56% for Pb) and barley (-18% to –46% for Pb) on a silt loam soil spiked with flue dust. Shanableh & Kharabsheh (1996) observed reductions in leachable Pb (-40% to –97%), Cd and Ni after application of faujasite and phillipsite. Gworek (1992) found reductions in Pb uptake by lettuce up to 73% and 77% after application of synthetic zeolites (4A-type, 13X-type) to a Pb contaminated soil.

Cyclonic ash

Cyclonic ash (formerly called beringite) is a modified aluminosilicate that originates from the fluidised bed burning of coal refuse (from the former coal mine of Beringen, North East of Belgium). The burnt material contains about 30% coal; the remaining fraction is inorganic and mainly consists of schists. 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: (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' (due to the 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 (Mench et al., 1998).

When added to a soil containing 3240 mg Pb kg^{-1}, beringite reduced Pb extractability (ammonium acetate-EDTA) by as much as 32% (Berti et al., 1998). Beringite addition also has been reported to decrease extractable Zn, Cu, Cd and Ni, metal leaching and plant metal uptake (Vangronsveld et al., 1996, Vangronsveld, 1998a,b, Boisson & Mench, 1998). Results from simulation and field experiments indicate that beringite has a long-lasting effect on bio-availability and leaching of metals from both heavily contaminated industrial soils and garden soils (Mench et al., 1994a, Vangronsveld et al., 1990, 1995a,b, 1996; Vangronsveld and Clijsters, 1992; Wessolek & Fahrenhorst, 1994).

Coal Fly ashes

Fly ashes are waste products from coal fired power plants (Carlson & Adriano, 1993). The composition can vary depending on the parent coal and the operating conditions, but it is frequently composed of a ferro-aluminosilicate mineral in which Al, Si, Fe, Ca, K and Na are the predominant elements (Bache & Lisk, 1990; Mench et al., 1998). Shende et al. (1994) observed a decrease in heavy metal uptake by maize in a calcareous soil after coal fly ash addition. Several studies conclude that the effect of fly ash is comparable to the effect of lime (Bache & Lisk, 1990; Marschner, 1995).

4.1.2.2. Phytostabilization

Effective and durable immobilization of metals reduces leaching and bioavailability. Subsequently, a vegetation can develop to physically stabilize the soil, ultimately resulting in the development of a new ecosystem (Vangronsveld et al., 2000). Not only does this render the site aesthetically pleasing, the vegetative cover also provides pollution control and stability to the soil. A vegetation cover drastically reduces lateral wind erosion. In addition, metal percolation to the groundwater may be reduced due to higher evapotranspiration rates relative to bare soils (Vangronsveld et al., 1995; Wenzel et al., 1999). Plants may also help to stabilize metals by accumulating and precipitating metals in the roots or by adsorption on root surfaces. Less commonly, plants may assist in altering the chemical form of contaminants by changing the soil environment (e.g. pH, redox potential) around the plant roots. The microorganisms (bacteria and fungi) living in the rhizosphere of these plants may also have an important role in these processes.

For example, hyphae of mycorrhizal fungi can immobilize metals and constrain their translocation to shoots and mechanically bind together root particles making the soil more resistant to erosion (Leyval et al., 1997). Ideally, plants should not accumulate metals in aboveground tissues that could pass to higher trophic levels. Phytostabilization of metal contaminated soils requires metal-tolerant plants and/or plants tolerant of the growing conditions for a given site. Often, chosen plants include grasses or other fast growing plants to provide complete surface coverage, produce many shallow roots to stabilize the soil and take up soil water, and require no further maintenance once established.

4.1.2.3. Studies comparing soil amendments

Only few studies compared Pb immobilizing soil amendments (Chlopecka & Adriano, 1997a,b,c; Mench et al., 1994a,b, 1997). Different amendments however were tested on different soils making comparisons of amendments difficult. Most studies focussed mainly on chemically evaluating Pb immobilization in soil.

CHAPTER II

AIM AND OUTLINE OF THE THESIS

An unfortunate by-product of industrialization has been the contamination of soil, sediment, and water resources with toxic metals and metalloids (Vangronsveld et al., 2000). Activities such as mining and smelting, the widespread use of Pb-based paints, the manufacturing and testing of explosives and the use of anti-knock agents in gasoline drastically increased Pb concentrations in soil. Elevated Pb levels in the soil cause concern to human health and the environment. Current remediation methods to Pb contaminated soils are expensive, environmentally invasive, and labor intensive (Vangronsveld & Cunningham, 1998). The last decade, the scientific community showed increased interest in the application of low-cost remediation techniques, such as phytoremediation where plants are used to remove (phytoextraction) or render harmless contaminants (phytostabilization; often in combination with soil amendments) (Cunningham & Berti, 1993).

In general, soil Pb solubility, plant uptake and translocation to aboveground plant parts is low, limiting phytoextraction possibilities. Recent research suggests that certain organic chelates such as EDTA may directly address all these factors, and mobilize Pb into aerial plant tissues (Blaylock et al., 1997; Huang et al., 1997b). Several questions however remain unanswered, like if, and how Pb-EDTA enters plant cells, and how it is translocated to aboveground plant parts. Or, if Pb-EDTA induces oxidative stress, as is known in case of Pb, or if chelation renders Pb less phytotoxic in analogy with metal complexation with organic acids and peptides (Rauser 1990) ? And if so, at which concentration phytotoxicity is observed. On the other hand it is important to know what effect EDTA has on the uptake of other essential plant elements.

Several inorganic soil amendments were found to effectively immobilize (inactivate) soil Pb. Most research however focused on chemically assessing Pb inactivation, using selective or sequential soil extractions. Studies comparing different amendments under similar circumstances are rare, while on the other hand almost no information is available on the immobilizing efficacy of the same amendment in soils with different Pb source term. As it is the intention to establish a vegetation cover on amended soils *in situ*, it is important to examine what possible phytotoxic or deficiency effects might occur after amendment application. For example, Boisson et al. (1999) recently indicated that phosphate application can mobilize other harmfull elements such as arsenic, or can immobilize essential elements in such way that nutrient deficiency occurs in plants (Boisson et al., 1999). Also, as soil ingestion by children is one of the main transfer pathways to humans, it is important to examine to what degree these amendments can alter Pb bioavailability in the gastro-intestinal tract.

Chapter III describes the effects of Pb-EDTA and EDTA on plants grown on hydroponics. Phytotoxicity is assessed in bean plants (*Phaseolus vulgaris* L. cv. Limburgse vroege), using measurements of capacities of three groups of enzymes involved in the defence against oxidative stress; this in order to determine whether Pb-EDTA and EDTA alone induce oxidative stress. The effect of Pb-EDTA and EDTA on element chelation in solution and on nutrient uptake is examined.

In **Chapter IV** the immobilizing capacity of 7 soil amendments with different working mechanism is compared on a soil spiked with 6 lead concentrations. Soil amendment application was evaluated using a biological test combining morphological and biochemical plant parameters. In addition, phytotoxicity data are linked with metal mobility and plant Pb uptake.

Chapter V examines the effect of mined phosphate rock, a mix of cyclonic ash and steelshot, and lime on the phytoavailability of Pb, Cu, Zn, Cd, As, Ni, Fe, Mn in ten Pb-contaminated soils with different properties, total metal/metalloid content, and source term. The metal/metalloid immobilizing efficacy of the amendments is evaluated using selective soil extractions ($CaCl_2$), a phytotoxicity test (OECD 208) and metal/metallood uptake by plants.

In **Chapter VI** the bioavailability of Pb, Cd and As in the gastro-intestinal tract after oral intake is described in ten Pb-contaminated soils with different properties, total metal/metalloid content, and source term using a modified *in vitro* Physiological Based Extraction Test (PBET). The effect of mined phosphate rock, a mix of cyclonic ash and steelshot, and lime on PBET extractable Pb, Cd and As bioavailability is examined.

In **Chapter VII**, a summarising discussion is provided, as well as conclusions from this work.

CHAPTER III

EFFECTS OF Pb-EDTA AND EDTA ON OXIDATIVE STRESS REACTIONS AND MINERAL UPTAKE IN *PHASEOLUS VULGARIS*

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ABSTRACT

Sequestration of Pb by synthetic chelates has been reported to increase bioavailability, uptake, and translocation of this metal in plants. In this work the potential phytotoxic effects of Pb-EDTA were investigated in *Phaseolus vulgaris* L. cv. Limburgse vroege plants grown on hydroponics. Addition of 50 µ*M* Pb-EDTA to the nutrient solution caused a significant induction of syringaldazine peroxidase (SPOD; EC 1.11.1.7) in roots and primary leaves and guaiacol peroxidase (GPOD; EC 1.11.1.7) in leaves. Addition of 100 µ*M* Pb-EDTA further exacerbated ascorbate peroxidase (APOD; EC 1.11.1.11), GPOD, dehydroascorbate reductase (DHAR; EC 1.8.5.1), gluthathione reductase (GR; EC 1.6.4.2) and malic enzyme (ME; EC 1.1.1.40) in roots and APOD and ME in primary leaves. Addition of 200 µ*M* Pb-EDTA also induced DHAR in leaves. This induction of peroxidases (SPOD, GPOD, APOD), enzymes of the ascorbate-glutathione cycle (DHAR, GR in roots) and of an $NADP^+$ reducing enzyme in roots and primary leaves indicates that oxidative stress has been initiated. At 200 µ*M* Pb-EDTA, chlorophyll *a* and *b* content in leaves was significantly reduced while visible effects on root morphology and shoot length were observed, while no significant morphological effects were found in the leaves confirming the sensitive character of the measured enzymes as plant stress indicators. Elevation of the Pb-EDTA concentration in the growth medium significantly reduced the content of Ca, Fe, Mn and Zn taken up by plants, probably due to ion leakage as a result of observed toxicity. Addition of up to 200 µ*M* EDTA increased chelation of divalent cations in nutrient solution resulting in reduced plant uptake of Zn, Fe, Mn. This did not result in phytotoxicity.

1. INTRODUCTION

Phytoextraction has recently received increased attention as a remediation technology for Pb polluted soils (Cunningham & Ow, 1996; Berti et al., 1998). Phytoextraction uses metal-accumulating plants which transport and concentrate metals from the soil into the harvestable parts of roots and shoots (Kumar et al., 1995). Lead, however, has limited solubility and bioavailability in soils due to formation of stable complexes with organic matter, sorption to oxides and clays, and precipitation as carbonates, hydroxides, and phosphates (McBride, 1994; Adriano, 2001). Pb is further known to accumulate primarily on cell walls of roots with only limited amounts translocated to the shoots (Huang et al., 1997a; Xiong, 1997). This suggests limited application of phytoextraction to Pb cleanup. However, recent studies have shown that addition of chelates to the soil, e.g. EDTA, can considerably enhance Pb solubility (Huang & Cunningham, 1996; Huang et al., 1997b; Epstein et al., 1999; Wu et al., 1999). EDTA has been reported to drastically improve uptake and translocation of Pb from the roots to the shoots of plants (Huang & Cunningham, 1996; Blaylock et al., 1997; Huang et al., 1997b; Epstein et al., 1999). Vassil et al. (1998) and Epstein et al. (1999) found that Pb is taken up in its complexed form and translocated to the above-ground plant parts as Pb-EDTA. Vassil et al. (1998) further suggested that a treshold concentration of EDTA is required to ‘induce’ accumulation of high Pb-EDTA concentrations in shoots; the authors speculated that the underlying mechanism for such accumulation would be H-EDTA induced destruction of the physiological barrier(s) in roots that control mineral uptake and translocation to the shoots, allowing leakage of soil solution with Pb-EDTA into the plants, ultimately resulting in plant death.

Low intracellular Pb concentrations however are known to substantially increase the formation of active oxygen species, giving rise to oxidation of thiol groups of enzymes and peroxidation of polyunsaturated fatty acids in membranes, eventually causing cell death in plants (Lee et al., 1976; Pukacka & Kuiper, 1988; Hoxha et al., 1985). Active oxygen species are generated in various reactions and metabolic processes where oxygen is involved, for example photosynthesis, where excitation of O_2 to its singlet state is achieved when biological pigments (e.g. chlorophyll) are illuminated in the presence of O_2 (Halliwell & Gutteridge, 1984; Foyer & Noctor, 2000). In an electron oxidation chain, molecular oxygen is converted to water with the formation of active oxygen intermediates ($O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$). Above a certain threshold concentration the physiological status of the cell changes irreversibly resulting in an induction in the capacity of enzymes which quench active oxygen species (Van Assche et al., 1988). Measurement of enzymes quenching oxidative stress was proven to be a sensitive plant stress indicator (Van Assche et al., 1988; Foyer et al., 1994; Vangronsveld & Clijsters, 1994).

Besides Pb, other possibly present elements show a high affinity for EDTA in soil (Fe^{3+}, Cu^{2+}, Al^{3+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}; Skoog et al., 1996) which might substantially reduce the concentration of free EDTA. The question rises which effects Pb-EDTA has on plants if present EDTA concentrations are too low to induce membrane injury and the metal-chelate complex can not ‘leak’ into the plants. If taken up the metal-chelate complex is expected to be less phytotoxic in analogy with metal complexation with organic acids and peptides, such as phytochelatins (Wang et al., 1992; Keltjens & Van Beusichem, 1998).

We studied the effects of Pb-EDTA and EDTA on plants grown on hydroponics. Hydroponic studies have the advantage of a precisely controlled plant-growth medium, allowing detailed calculation of the behaviour of the added metal-chelator complex and its interaction with other essential plant nutrients in solution. Phytotoxicity was assessed in bean plants (*Phaseolus vulgaris* L. cv. Limburgse vroege), using measurements of the enzyme capacity of three types of antioxidative defence systems, in order to determine whether Pb-EDTA and EDTA alone induced oxidative stress.

2. MATERIALS AND METHODS

2.1. Plant material and Pb treatment

Bean seeds received a cold treatment (+4°C) for 3 days to break dormancy and to synchronize germination. They were transferred to a growth chamber to germinate between two layers of water-soaked rock wool for 4 days. Subsequently seed coats were removed and seedlings with a root length of approximately 1.5 cm were grown in 3-mm thick polystyrene squares by fixing the roots through 5-mm holes (9 plants/treatment). The polystyrene was floated on 3 L of aerated full strengths Hoagland's solution (pH 5.0) in 3.5 L polyethylene beakers; Fe was added as $FeSO_4.7H_2O$. To keep Fe in solution, it was complexed with EDTA (Fe/EDTA ratio 2/1) (Wallace, 1983). Nutrient solution was replaced every 2 days; pH was monitored daily. Plants were grown under controlled conditions in a 12-h photoperiod at 65 % relative humidity and day/night temperatures of 22°C. Light was supplied by cool white fluorescent lamps (L= 140W/20SA; Osram, Augsburg, Germany) at a photosynthetic photon flux density of 165 μmol m^{-2} s^{-1} at the leaf level. Final concentrations of 0 (=untreated), 10, 50, 100 and 200 μM Pb, added as $Pb(NO_3)_2$, were complexed with ethylenediaminetetraacetate (EDTA) in a 1/1 ratio (Pb/EDTA). In addition, a control series was used containing 50 and 200 μM EDTA in Hoagland's medium. No data were collected when Pb alone was added as the metal was immediately precipitated with phospates and sulfates present in nutrient solution.

Plants were harvested 14 days after the start of treatment, and morphological parameters (root weight, shoot length, leaf weight) were measured; samples of roots and primary leaves were collected for enzyme analysis and determination of metal concentration. For enzyme measurements, samples of 1 g fresh weight were frozen in liquid nitrogen and stored at -70°C; samples for metal analysis were rinsed with distilled water and dried immediately at 80°C.

2.2. EDTA speciation in nutrient solution

Speciation of nutrients possibly complexing EDTA was calculated using the computer program Solgaswater (Eriksson, 1979). Acidity and stability constants were obtained from A.E. Martell and R.M. Smith, “Critically Selected Stability Constants of Metal Complexes” NIST database version 2.0; the data were chosen for pH 5.0, 25° C and an ionic strength of 0.1 *M*.

2.3. (Iso-)enzymatic analysis

Frozen tissue was homogenized with a Polytron PT 3000 homogenizer in ice cold 0.1 *M* Tris-HCl buffer (pH 7.8), containing 1 m*M* EDTA, 1 m*M* 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 20 000 g (4°C). The enzyme activity was measured spectrophotometrically in the supernatant at 25°C. These measurements were performed under non-limiting conditions of substrate and coenzyme, therefore, it is further named enzyme capacity (expressed in U g^{-1} FW).

The capacities of enzymes belonging to 3 groups of antioxidative defence systems was measured. A first group contained peroxidases occurring in the apoplast (SPOD), the symplast (APOD) or in both (GPOD) eliminating peroxides. Capacity of APOD (EC 1.11.1.11), GPOD (EC 1.11.1.7) and SPOD (EC 1.11.1.7) were measured according to Gerbling et al. (1984), Bergmeier et al. (1974) and Imberty et al. (1984) respectively. A second group is involved in the ascorbate-glutathione cycle which participates in the cellular protection against reactive oxygen species (Foyer, 1993; Foyer et al., 1994). In this pathway peroxides are detoxified by ascorbate peroxidase; the oxidized substrate is recycled by dehydroascorbate reductase (DHAR), which receives the necessary electrons from glutathione. The reduction of the latter substrate is catalyzed by glutathione reductase (GR) with NADPH as a final electron donor. Analysis of DHAR (EC 1.8.5.1) and GR (EC 1.6.4.2) was determined according to Foyer et al. (1989) and Bergmeier et al. (1974), respectively. From the third group, malic enzyme (ME) was chosen as an enzyme of the intermediary metabolism (Vangronsveld & Clijsters, 1994), enhancing the reducing the capacity of the cell by reducing $NADP^+$ to NADPH which serves as an electron donor in the ascorbate-glutathione pathway. ME (EC 1.1.1.40) capacity was determined at 340 nm (Hulme et al., 1963).

2.4. Chlorophyll and carotenoid content

Leaf samples (0.5 g) were selected randomly from the plants, and homogenized in a porcelain mortar in acetone (10 ml) with some $CaCO_3$. The extract was centrifuged at 2000 g for 5 min. A portion of the extract (500 µl) was diluted with acetone up to 5 ml. Absorbance of the supernatant was recorded at 663 nm, 646 nm and 470 nm spectrophotometrically. Chlorophyll and carotenoid content was determined following Lichtenthaler & Wellburn (1983).

2.5. Metal analysis

Leaf material was microwave digested in purified HNO_3 (70-71%). Metal concentration was determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES).

2.6. Statistical analysis

Enzyme capacities and pigment concentrations are the means of 9 samples taken from 3 independent experiments; 3 replicates were considered for nutrient analysis. A Kruskal-Wallis test was performed to detect difference between treatments for each measured parameter ($p < 0.05$). When difference was detected, multiple comparisons were performed to separate the means.

3. RESULTS

3.1. Metal complexation by EDTA in the nutrient solution

Table 3.1 shows that, as a result of dissociation of the Fe-EDTA complex, Cu^{2+} (100%) and Zn^{2+} (98.6%) were almost completely complexed by EDTA in the Hoagland solution, while Fe^{2+} (31.4%) and Mn^{2+} (14.8%) were bound to a lesser degree; Ca^{2+} and Mg^{2+} occurred as free ions. However, when 50 µ*M* EDTA alone was added Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} were almost completely complexed and at the 200 µ*M* EDTA level, 60% of Ca^{2+} was chelated. No free EDTA was detected at the 50 µ*M* level except when 200 µ*M* EDTA was applied that yielded a 10 µ*M* free EDTA concentration in solution. Addition of Pb-EDTA did not significantly change the complexation status of other nutrients since the chelated metal remained present in the solution as a complex (99.97%) irrespective of the applied concentration (only 0.03% of the complex dissociated resulting in a free Pb^{2+} concentration of 0.09 µ*M*).

Table 3.1: Complexation degree of Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} in Hoagland's solution as MEDTA^{2-} (expressed in %) calculated using the Solgaswater software (Eriksson, 1979).

Metalion	**Ca^{2+}**	**Mg^{2+}**	**Fe^{2+}**	**Mn^{2+}**	**Zn^{2+}**	**Cu^{2+}**	**Pb^{2+}**
Hoagland	0	0	31.45	14.85	98.64	100	---
+ 50 µ*M* EDTA	16.67	0	99.82	99.54	100.00	100	---
+ 200 µ*M* EDTA	60.00	0	99.98	99.95	100.00	100	---
+ 10 µ*M* Pb-EDTA	0	0	31.59	14.94	98.65	100	99.97
+ 50 µ*M* Pb-EDTA	0	0	32.15	15.27	98.69	100	99.97
+ 100 µ*M* Pb-EDTA	0	0	32.80	15.65	98.72	100	99.97
+ 200 µ*M* Pb-EDTA	0	0	34.00	16.38	98.79	100	99.97

3.2. Growth response

Fig. 3.1 shows that application of EDTA alone had no effect on root weight, stem length or leaf weight of the bean plants. Application of Pb-EDTA to the growth medium resulted in a gradual decrease in root weight. Root biomass was significantly reduced by 72% when 200 µ*M* Pb-EDTA was applied. Taproot length was not affected while lateral roots were substantially reduced in size (max. 20 mm in length) and number (results not shown). Addition of 200 µ*M* Pb-EDTA to the growth medium further significantly reduced shoot length (-46%); no significant difference was detected in leaf weight.

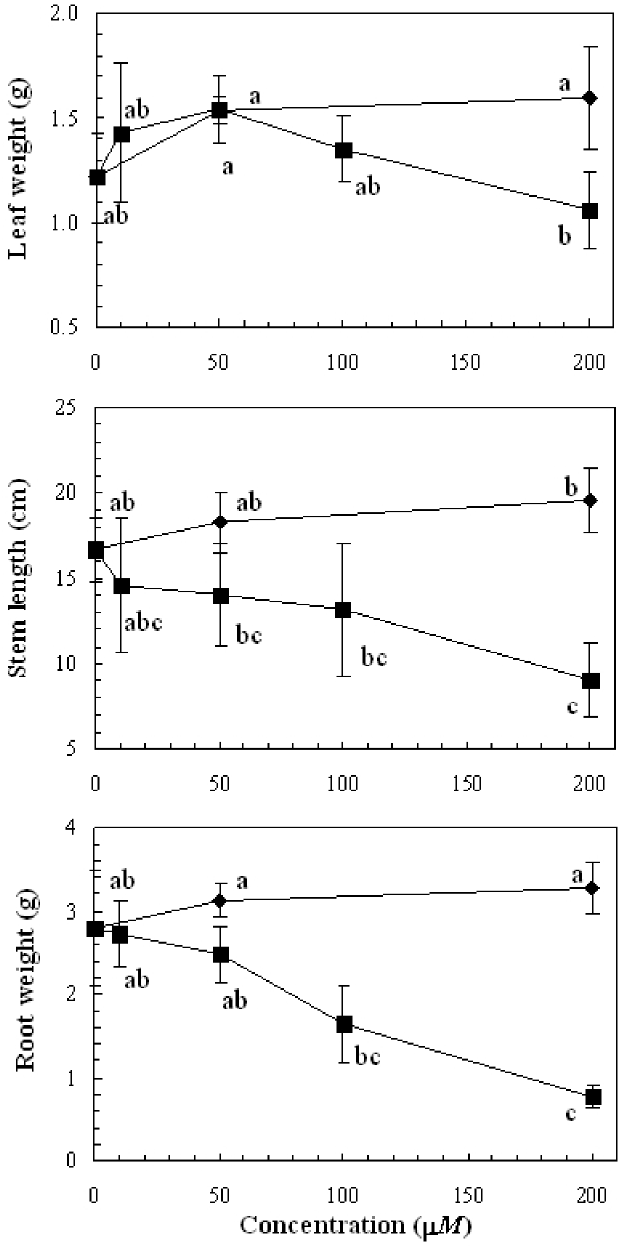

Fig. 3.1: Root weight (g), shoot length (cm) and leaf weight (g) of 14 days old *Phaseolus vulgaris* seedlings treated with Pb-EDTA (■) and EDTA (♦). Values are means and SD (n=27).

3.3. Antioxidative defence in roots

Addition of EDTA alone did not affect the capacity of the enzymes measured (Table 3.2). However, when the Pb-EDTA complex was added, enzyme capacities were affected. The presence of 50 µ*M* Pb-EDTA significantly induced SPOD (+289% when 200 µ*M* Pb-EDTA was applied) in roots. The SPOD pool is primarily located in the apoplast (Imberty et al., 1984; Asada, 1992; Cuypers, 2000) which has more direct contact with the metal concerned. This could explain the high induction of the enzyme.

Table 3.2: Capacity of APOD, GPOD, SPOD, DHAR, GR and ME (U g^{-1} FW) in roots of 14 days old *Phaseolus vulgaris* seedlings treated with several concentrations of EDTA and Pb-EDTA. Values are means, SD (in parentheses); n = 9.

	APOD	**GPOD**	**SPOD**	**DHAR**	**GR**	**ME**
Hoagland	13.7 (4.6)^{ab}	13.9 (5.2)^{a}	26.5 (9.6)^{a}	0.20 (0.03)^{a}	0.36 (0.08)^{ab}	0.71 (0.07)^{a}
+ 50 µ*M* EDTA	12.6 (2.5)^{a}	11.3 (3.3)^{a}	25.6 (4.8)^{a}	0.24 (0.04)^{a}	0.34 (0.09)^{a}	0.71 (0.07)^{a}
+ 200 µ*M* EDTA	14.2 (2.2)^{ab}	13.2 (2.1)^{a}	29.2 (5.0)^{ab}	0.24 (0.05)^{ab}	0.38 (0.07)^{ab}	0.75 (0.08)^{a}
+ 10 µ*M* Pb-EDTA	13.8 (2.4)^{ab}	13.1 (1.9)^{a}	33.2 (15.7)^{ab}	0.22 (0.05)^{a}	0.37 (0.04)^{ab}	0.73 (0.06)^{a}
+ 50 µ*M* Pb-EDTA	15.1 (1.6)^{b}	13.5 (4.8)^{a}	37.0 (9.7)^{b}	0.23 (0.05)^{a}	0.39 (0.06)^{b}	0.70 (0.06)^{a}
+ 100 µ*M* Pb-EDTA	20.7 (4.2)^{c}	19.7 (7.5)^{b}	59.9 (9.8)^{c}	0.33 (0.09)^{b}	0.50 (0.14)^{c}	0.90 (0.12)^{b}
+ 200 µ*M* Pb-EDTA	32.2 (5.9)^{c}	34.9 (9.1)^{c}	103.1 (4.4)^{c}	0.52 (0.11)^{c}	0.85 (0.19)^{c}	1.09 (0.05)^{b}

Addition of 100 µ*M* Pb-EDTA to the growth medium resulted in a significant increase of all other enzymes measured. The increased DHAR (+153%) and GR (+135%) capacities suggest an active involvement of the ascorbate-gluthathione cycle in cellular defence against oxidative stress caused by Pb. DHAR reduces dehydroascorbate to ascorbate which in turn oxidizes as a substrate for APOD. DHAR therefore oxidizes glutathione while GR reduces it. Recently, Gupta et al. (1999) described the induction of the cycle (including metabolites) in a case where toxic Cu concentrations were applied. Induction of the peroxide quenching enzymes GPOD (+152%; partly intracellular) and APOD (+135%; mainly intracellular) indicates an increased intracellular peroxide level while the induction in ME (+53%) capacity is assumed to provide extra reducing power for the ascorbate-gluthatione cycle (Clijsters et al., 1999). SOD quenching superoxide was also found to be induced when Pb-EDTA was added under similar circumstances

(Geebelen et al., unpublished data). The induction of these enzymes strongly suggests the presence of reactive oxygen species (superoxide, peroxide) and therefore it can be concluded that Pb-EDTA in the growth medium induces oxidative stress in roots.

3.4. Antioxidative defence in primary leaves

No significant increase in enzyme capacity was observed when EDTA alone was added to the growth medium (Table 3.3). As in roots, addition of complexed Pb induced oxidative stress in leaves. For example, application of 50 µ*M* Pb-EDTA led to an induction of SPOD (+291% when 200 µ*M* Pb-EDTA was applied) and GPOD (+229%). A correlation between SPOD and increased lignification again occured since good indications for a metal (*in casu* Cd) induced stimulation of lignin biosynthesis in bean leaves have been demonstrated (Ciscato, 2000). Addition of 100 µ*M* Pb-EDTA led to significant increases in APOD (+204%) and ME (+117%) while DHAR (+149%) was significantly increased when 200 µ*M* Pb-EDTA was applied. No increase in GR was observed. The strong enzyme induction confirms that increased amounts of Pb-EDTA in the growth medium also induce oxidative stress in leaves.

Table 3.3: Capacity of APOD, GPOD, SPOD, DHAR, GR and ME (U g^{-1} FW) in primary leaves of 14 days old *Phaseolus vulgaris* seedlings treated with several concentrations of EDTA and Pb-EDTA. Values are means, SD (in parentheses); n = 9.

	APOD	**GPOD**	**SPOD**	**DHAR**	**GR**	**ME**
Hoagland	6.6 (0.9)^{a}	0.72 (0.31)^{a}	0.64 (0.17)^{a}	0.19 (0.13)^{ab}	0.41 (0.12)	0.21 (0.05)^{a}
+ 50 µ*M* EDTA	7.3 (1.6)^{ab}	0.89 (0.31)^{ab}	0.76 (0.18)^{ab}	0.21 (0.05)^{ab}	0.47 (0.18)	0.23 (0.03)^{a}
+ 200 µ*M* EDTA	7.8 (1.4)^{ab}	0.79 (0.12)^{ab}	0.74 (0.15)^{ab}	0.23 (0.05)^{ab}	0.39 (0.12)	0.26 (0.07)^{ab}
+ 10 µ*M* Pb-EDTA	7.1 (2.2)^{ab}	0.84 (0.36)^{ab}	0.67 (0.35)^{a}	0.18 (0.13)^{a}	0.40 (0.12)	0.25 (0.03)^{a}
+ 50 µ*M* Pb-EDTA	6.9 (1.6)^{ab}	0.98 (0.25)^{bc}	0.93 (0.23)^{b}	0.22 (0.08)^{ab}	0.49 (0.12)	0.27 (0.06)^{ab}
+ 100 µ*M* Pb-EDTA	8.3 (1.9)^{b}	1.19 (0.36)^{c}	2.22 (0.77)^{c}	0.25 (0.08)^{b}	0.50 (0.18)	0.29 (0.04)^{b}
+ 200 µ*M* Pb-EDTA	20.2 (2.8)^{c}	2.35 (0.64)^{d}	2.48 (0.70)^{c}	0.47 (0.18)^{c}	0.51 (0.91)	0.46 (0.05)^{c}

3.5. Chlorophyll and carotenoid content in primary leaves

Addition of EDTA alone to the growth medium did not significantly alter the content of chlorophyll *a*, *b* or carotenoids in bean leaves (Table 3.4). Addition of 200 *µ*M Pb-EDTA however significantly reduced the content of chlorophyll *a* and *b*. Chlorophyll *a* and *b* content were reduced with respectively 26 and 33%. Pb-EDTA did not significantly alter carotenoid content in leaves.

Table 3.4: Chlorophyll *a*, chlorophyll *b* and carotenoid content (mg g^{-1} FW) in primary leaves of 14 days old *Phaseolus vulgaris* seedlings treated with several concentrations of EDTA and Pb-EDTA. Values are means, SD (in parentheses); n = 9.

	chlorophyll *a*	**chlorophyll *b***	**carotenoid**
Hoagland	0.97 (0.09)^{a}	0.24 (0.02)^{a}	0.16 (0.02)
+ 50 µ*M* EDTA	0.98 (0.05)^{a}	0.24 (0.02)^{a}	0.16 (0.01)
+ 200 µ*M* EDTA	0.88 (0.04)^{ab}	0.22 (0.01)^{ab}	0.16 (0.01)
+ 10 µ*M* Pb-EDTA	0.96 (0.12)^{a}	0.21 (0.03)^{ab}	0.16 (0.02)
+ 50 µ*M* Pb-EDTA	0.90 (0.08)^{ab}	0.21 (0.02)^{ab}	0.16 (0.01)
+ 100 µ*M* Pb-EDTA	0.87 (0.06)^{ab}	0.20 (0.02)^{ab}	0.14 (0.02)
+ 200 µ*M* Pb-EDTA	0.72 (0.09)^{b}	0.16 (0.03)^{b}	0.13 (0.02)

3.6. Pb and nutrient uptake

Application of EDTA alone led to a significant decrease in Fe (-50% when 200 µ*M* EDTA was applied), Mn (-40%) and Zn (-47%) content in primary leaves (Table 3.5). Concentrations however did not pass critical deficiency levels in primary leaves as defined by Marschner (1995). From Table 3.1 it is clear that EDTA in the nutrient solution resulted in increased chelation of Fe^{2+} (99.98%), Mn^{2+}, (99.95%), Zn^{2+} (100%), Cu^{2+} (100%) and Ca^{2+} (60%). EDTA chelation of these elements results in an enlarged diameter which might limit uptake of the molecules due to decreased permeation through cell wall pores and transport proteins in membranes. Also chelated cations virtually have no ability to bind to carboxylic groups in the apoplasm as increased element concentration in the vicinity of active uptake sites at the plasma membrane increases their uptake (Marschner 1995).

Table 3.5: Content of Mg, Ca, K, (g kg^{-1} DW) and Fe, Mn, Zn and Cu (mg kg^{-1} DW) in primary leaves of 14 days old *Phaseolus vulgaris* seedlings treated with several concentrations of EDTA and Pb-EDTA and concentrations required for optimal growth (Mg, Ca, K) and critical deficiency levels (Fe, Mn, Zn, Cu) according to Marschner (1995). Values are means. SD (in parentheses); n = 3.

	K	**Ca**	**Mg**	**Fe**	**Mn**	**Zn**	**Cu**
Hoagland	34.8 (4.3)	33.0 (0.6)^{ab}	9.3 (0.7)	305 (25)^{a}	273 (12)^{a}	50.2 (4.0)^{a}	11.2 (4.0)
+ 50 µ*M* EDTA	34.2 (2.2)	32.9 (1.4)^{a}	8.9 (0.7)	178 (3)^{bc}	149 (6)^{c}	28.5 (3.7)^{b}	7.7 (1.5)
+ 200 µ*M* EDTA	38.0 (1.5)	36.9 (1.5)^{a}	10.2 (0.6)	151 (6)^{c}	164 (15)^{c}	26.8 (3.0)^{b}	8.4 (1.4)
+ 10 µ*M* Pb-EDTA	35.1 (1.3)	30.9 (0.5)^{bc}	9.1 (1.0)	242 (15)^{ab}	259 (15)^{a}	45.6 (2.8)^{a}	10.8 (3.8)
+ 50 µ*M* Pb-EDTA	35.5 (1.5)	27.9 (0.3)^{cd}	8.3 (0.2)	130 (6)^{d}	196 (7)^{bc}	29.6 (2.5)^{b}	7.0 (2.5)
+ 100 µ*M* Pb-EDTA	39.8 (3.6)	26.3 (0.5)^{d}	8.0 (0.3)	134 (7)^{d}	190 (10)^{bc}	31.8 (1.7)^{b}	7.8 (2.0)
+ 200 µ*M* Pb-EDTA	35.7 (2.7)	19.0 (0.8)^{e}	7.3 (0.4)	116 (8)^{d}	164 (28)^{c}	19.3 (12.1)^{c}	12.5 (3.3)
Opt. / Def. conc.	20-50	1-50	5	50-150	10-20	15-20	3-5

Pb content in primary leaves increased up to 928 mg kg^{-1} DW when Pb-EDTA concentration in the nutrient solution was elevated (Fig. 3.2). Application of 50 µ*M* Pb-EDTA caused a significant decrease in Ca (-42% when 200 µ*M* was applied), Fe (-62%), Mn (-40%) and Zn (-62%) content in leaves. Application of 50 µ*M* Pb-EDTA and higher decreased Fe concentration in primary leaves within the range of 50-150 mg kg^{-1}, the critical deficiency level (Marschner 1995). In case of Ca and Mn a gradual decrease is observed when Pb-EDTA concentration in the growth medium is elevated (Table 3.5).

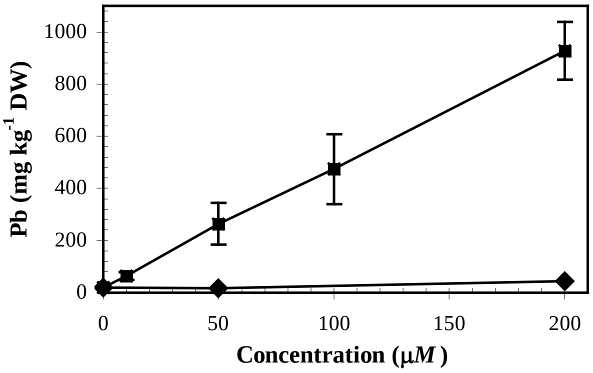

Fig. 3.2: Pb content in primary leaves (mg kg^{-1} DW) of 14 days old *Phaseolus vulgaris* seedlings treated with Pb-EDTA (■) and EDTA (♦). Values are means and SD (n=3).

4. DISCUSSION

Table 3.1 shows that almost all Pb present in the nutrient solution was complexed to EDTA (99.97 %). EXAFS analysis however indicated that in primary leaves of bean plants, grown under the same conditions as mentioned in this paper, only 60-70% of Pb is present as Pb-EDTA (Sarret et al., 2001). This implies that as much as 30-40% of the complex has split. Fe-EDTA is known to dissociate before plant uptake. A plasma membrane-bound reductase is responsible for the reduction of Fe^{3+} to Fe^{2+} rendering the complex less stable while increased acidification of the rhizosphere due to H^+-ATPase results in increased solubilization of the complex (Chaney et al., 1972; Marschner et al., 1986). Recently, Zn was found to be present in roots and leaves of *Phaseolus vulgaris* predominantly as Zn phosphate dihydrate, regardless of its form in nutrient solution ($ZnSO_4$ or Zn-EDTA), suggesting split-uptake of the Zn-EDTA complex at the root level as well (Sarret et al., 2001). In case of Pb-EDTA, split through reduction is not possible; however, rhizosphere acidification could possibly result in dissociation of the Pb-EDTA complex.

On the other hand, several workers found indications that Pb is taken up as Pb-EDTA by plants. Jarvis and Leung (2001) grew *Chamaecytisus palmensis* in hydroponic culture exposed to $Pb(NO_3)_2$ with and without the addition of EDTA. Using transmission electron microscopy, the authors observed heavy depositions of unchelated Pb in intracellular spaces, material adhering to the cell wall and the middle lamellae of roots, while no Pb was observed in upper plant parts. In case EDTA was added, no or only small quantities of Pb were observed in roots while Pb deposits were detected in chloroplasts and plasmodesmata in shoot tissues, suggesting that most or all of the chelated Pb had moved from the root to the shoot. Epstein et al. (1999) further found a positive correlation between Pb and EDTA accumulation in both plant tissue and xylem sap of *Brassica juncea* grown on Pb contaminated soils after EDTA addition. It is however not clear if part of the Pb-EDTA complex is split before uptake in analogy to Fe-EDTA and Zn-EDTA, or if all Pb is taken up as Pb-EDTA and that part of the complex is split in the plant, after uptake. Addition of 200 µ*M* Pb-EDTA to the nutrient solution increased Pb content in primary leaves up to 928 mg kg^{-1} DW. The fact that 30-40% of the Pb-EDTA complex is split results in a concentration of 278-371 mg unchelated[1] Pb kg^{-1} DW. The main part of this concentration range exceeds the toxicity threshold for Pb in plant leaves mentioned by Kabata-Pendias and Pendias (1992) (30-300 mg Pb kg^{-1} DW), suggesting that unchelated Pb should be responsible for the phytotoxic responses observed.

Addition of 50 µ*M* Pb-EDTA to the nutrient solution significantly increased SPOD in roots and SPOD and GPOD in primary leaves. The capacity of all other enzymes (APOD, GPOD, DHAR, GR, ME) significantly increased in roots and leaves (except GR in leaves) when 100 µ*M* Pb-EDTA was added while at 200 µ*M* Pb-EDTA the content of chlorophyll *a* and *b* was reduced as well as root weight and shoot length of the bean plants. The early enzyme induction, relative to changes in chlorophyll content and plant morphology underline the sensitive character of the measured enzymes. Especially the early induction of the apoplastic occurring SPOD in roots and leaves is interesting. Unchelated Pb is known to mainly accumulate in cell walls (Xiong, 1997). SPOD catalyzes the final enzymatic step in lignin biosynthesis and recent research already postulated that lignification is an important process for the plant to cope with metal stress as this leads to more extracellular binding sites for the metal (Olson & Varner, 1993; Padu, 1999; Degenhardt & Gimmler, 2000). From this point of view, SPOD might be an early biomarker for Pb and Pb-EDTA.

Unchelated Pb is further known to inhibit enzyme activities due to interaction with sulphydryl groups or due to Pb induced deficiency of elements essential to the enzyme (e.g. Zn, Seregin & Ivanov, 2001). For example, Pb is known to inhibit δ-ALA-dehydratase, an enzyme catalyzing the conversion of δ-ALA into porphobilinogen in the synthesis of chlorophyll (Prassad & Prassad, 1987). The

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[1] 'Unchelated' here means that Pb is not chelated to EDTA, but this does not exclude Pb chelation to other naturally occurring ligands (e.g. citrate).

observed reductions in chlorophyll *a* and *b* after Pb-EDTA addition to the nutrient solution (Table 3.4) are probably due to Pb induced inhibition of δ-ALA-dehydratase.

Pb-EDTA further significantly reduced Ca, Fe, Mn and Zn content in primary leaves (Table 3.5). Reductions in essential nutrients will decrease the plants vitality and its ability to cope with (metal) stress. Both Ca and Zn ions are involved in stabilizing plasma membranes; Ca and Zn deficiency has been described to result in increased membrane leakage of cell solutes (Pasternak, 1988; Marschner, 1995). Fe content in our bean plants even reached the critical deficiency level defined by Marschner (1995). Several enzymes of the anti-oxidative stress metabolism (catalase, peroxidase, FeSOD) contain Fe (Marschner, 1995). Therefore, Fe deficiency eventually can affect the plants defence system against oxidative stress.

As mentioned above, the elevated concentrations of unchelated Pb found in leaves very probably contribute to the observed phytotoxicity. To what degree Pb-EDTA is phytotoxic is not clear. Pb is, in contrast to transition metals such as Cu and Fe, a non-redox active metal, which can not perform one electron oxidoreduction reactions and thus can not directly induce oxygen radicals. Several other workers however observed anti-oxidative stress reactions in plants when Pb or other non-redox metals such as Cd or Zn were present in the growth medium (Hoxha et al., 1985; Hendry et al., 1992; Weckx et al., 1997; Cuypers et al., 1999). Weckx et al. (1997) found that Zn addition to the growth medium increased lipoxygenase activity in bean leaves, resulting in increased lipid peroxidation. On the other hand, binding of Pb to functional groups of (membrane) proteins or substitution of essential metals in enzymes can cause electron leakages, resulting in the generation of reactive oxygen species (Gwóźdź et al., 1997; Seregin & Ivanov, 2001). Xiong (1997) even postulated that high Pb concentrations bound to the cell walls can change the elasticity and plasticity of the cell walls leading to large depositions on membranes, in turn resulting in interfered physiological membrane processes. The observed reductions in Ca and Zn can also cause electron leakage due to plasma membrane destabilization.

Addition of up to 200 µ*M* EDTA increased EDTA chelation of Ca (up to 60%), Fe (up to 99.9%), Mn (99.9%) and Zn (100%) in Hoagland's solution. In primary leaves of bean plants, Ca content was not significantly altered while Fe, Mn, and Zn content in leaves significantly decreased due to increased EDTA chelation. In none of the cases however critical deficiency concentrations were exceeded, and EDTA addition had no effect on enzymes, pigment content or plant morphology. Vassil et al. (1998) however observed severe toxicity when EDTA concentrations up to 2.5 m*M* were added to *Brassica juncea* plants grown on hydroponics. The authors suggested that free EDTA was taken up by *B. juncea* and that various essential divalent cations (Fe^{2+}, Zn^{2+}, Cu^{2+}) would be bound in the leaf cells, disrupting leaf cell biochemistry and ultimately causing cell death. In our study however applied EDTA concentrations were much lower: addition of 200 µ*M* EDTA resulted in a free EDTA concentration of 10 µ*M* in solution. Obviously this was too low to induce any phytotoxic effects.

Resuming, the induction in the capacity of enzymes belonging to three different "compartments" of mechanisms involved in anti-oxidative defence after Pb-EDTA addition strongly suggests the presence of active oxygen species in both plant organs, inferring that the plant is allocating an increased amount of its energy into anti-oxidative defence. Due to its early and pronounced reaction, SPOD might be a dependable indicator of Pb-EDTA induced stress in plants. Our data do not permit to distinguish between phytotoxicity associated with Pb alone or the Pb-EDTA complex, as EXAFS analysis indicated that part of the Pb-EDTA complex is split. At elevated Pb-EDTA concentrations in the growth medium, chlorophyll *a* and *b* content decreased suggesting Pb induced inhibition of δ-ALA-dehydratase. Ion leakage probably decreased Ca, Fe, Mn and Zn concentrations in leaves which could render the plant less vital in its defence against metal stress at higher Pb-EDTA concentrations.

Addition of EDTA alone to the growth medium increased EDTA chelation of most divalent cations, resulting in decreased plant uptake of Fe, Mn and Zn. EDTA application however did not induce any anti-oxidative stress response. Addition of

200 µ*M* EDTA resulted in a free EDTA concentration of 10 µ*M* in solution. Obviously this was too low to induce any phytotoxic effects.

CHAPTER IV

AMENDMENT-INDUCED IMMOBILIZATION OF LEADIN A LEAD-SPIKED SOIL: EVIDENCE FROM PHYTOTOXICITY STUDIES

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ABSTRACT

Lead immobilization was evaluated on soils spiked with increasing concentrations of Pb (as Pb-acetate) using the following soil amendments: bentonite, zeolite, cyclonic ash, compost, lime, steelshot, and hydroxyapatite. The immobilization efficacy of the amendments was evaluated according to the following criteria: $Ca(NO_3)_2$-extractable Pb as an indicator of Pb phytoavailability, morphological and enzymatic parameters of bean plants (*Phaseolus vulgaris*) as indicator of phytotoxicity**,** and Pb concentration in edible tissue of lettuce (*Lactuca sativa*). The lowest reductions in $Ca(NO_3)_2$-extractable soil Pb occurred when bentonite and steelshot were applied. Phytotoxicity from application of steelshot was confounded by toxic amounts of Fe and Mn released from the by-product which killed the lettuce seedlings. Addition of zeolite induced poor plant growth independent of Pb concentration due to its adverse effect on soil structure. Substantial reductions in $Ca(NO_3)_2$-extractable Pb were observed when cyclonic ash, lime, compost and hydroxyapatite were applied. In general, these amendments reduced Pb phytotoxicity concomitant with reduced Pb concentration in lettuce tissue. Cyclonic ash, lime and compost further improved plant growth and reduced oxidative stress at low soil Pb concentrations due to soil pH increase mitigating Al or Mn toxicity.

1. INTRODUCTION

Lead (Pb) is generally recognised as an environmental toxin to wildlife, domestic animals, and humans (Page & Chang, 1993). Despite efforts by many industrialized countries to reduce Pb emission (e.g. Pb alkyls in gasoline), worldwide Pb emissions are still increasing (Adriano, 2001). Most soil Pb contamination is a result of anthropogenic activities such as mining and smelting, combustion of gasoline containing anti-knock additives tetraethyl and tetramethyl Pb, use of Pb-based paints and explosives, land application of sewage sludge, use of some fertilizers and pesticides, battery recycling, and use of Pb bullets (Adriano, 2001). Immobilization of Pb has been touted as a promising alternative for the generally expensive and disruptive conventional remediation techniques to reduce environmental health risks posed by Pb-contaminated sites (Berti & Cunningham, 1997; Berti et al., 1998, Vangronsveld & Cunningham, 1998).

Several types of soil amendment to immobilize Pb have been evaluated. The use of phosphate in the form of hydroxyapatite (Laperche et al., 1996, 1997; Ma, 1996; Ma et al., 1993, 1994a,b; Zhang et al., 1997) and phosphate rock (Chlopecka & Adriano, 1997a,b,c; Ma & Rao, 1997; Ma et al., 1997; Ma et al., 1995) has received much attention. Phosphates induce the formation of Pb phosphate (pyromorphite) which is stable and highly insoluble even at low pH (Ruby et al., 1994; Zhang et al., 1997). Liming agents are applied for their alkalinity which elevates the surface charge and Pb sorption onto soil particles (Mench et al., 1994b); the formation of insoluble Pb carbonates is favoured as well (Berti et al., 1998). Hydrous Fe- and Mn oxides, and similar by-products (iron-rich material) have been shown to immobilize Pb by surface adsorption and to a lesser degree, by occlusion of the metal into the oxide structure (Berti & Cunningham, 1997; Manceau et al., 1992a; Mench et al., 1994b). Addition of organic matter has shown to immobilize Pb by forming strong complexes with soil Pb (Zimdahl & Foster, 1976; Scialdone et al., 1980; Bassuk, 1986). Some synthetic zeolites (Gworek, 1992; Rebedea & Lepp, 1995) and natural aluminosilicates (Chlopecka & Adriano, 1997a; Shanableh & Kharabsheh, 1996) have been proved to reduce Pb leaching, while Vangronsveld and Clijsters (1992) measured reduced Pb availability using cyclonic ash, a by-product from the fluidized-bed burning of coal refuse.

In situ immobilization of metals in contaminated soils can be combined with phytostabilization (Vangronsveld et al., 1995); a vegetation cover which reduces wind erosion and water percolation (Vangronsveld et al., 1993). The soil additives mentioned above have shown their potential to immobilize Pb. Comparison of effective agents under the same conditions is rare and has mainly focused on measuring reductions in phytoavailable soil Pb using chemical extractions (Berti & Cunningham, 1997). When plants are used as a biological indicator, only metal concentration in the tissue is typically determined (Chlopecka & Adriano, 1997b).

In this paper, the immobilization potential of hydroxyapatite, lime, steelshot, compost, zeolite, cyclonic ash, and bentonite was evaluated using a soil artificially contaminated with increasing amounts of Pb. Their relative efficiency to immobilize Pb was evaluated by measuring (1) $Ca(NO_3)_2$-extractable soil Pb, (2) phytotoxicity, and (3) Pb concentration in edible tissue of lettuce.

2. MATERIALS & METHODS

2.1. Soil treatment

Increasing amounts of Pb were added as Pb-acetate [$(CH_3CO_2)_4Pb)$] (0, 250, 500, 1000, 1500, and 2000 mg Pb kg^{-1}) to an acid sandy soil (pH = 4.27; pH-KCl = 3.55; electrical conductivity = 375 µS cm^{-1}; CEC = 5.35 cmol kg^{-1}; 4.05 % OM). For each concentration the necessary amount of Pb-acetate was brought in solution and added to the soil which was mixed (Kenwood Major) during Pb addition to make sure the metal was evenly distributed in the soil. The following soil amendments were used (w/w): 1% bentonite (commercial grade**,** consisting mainly of montmorillonite; Osmo, Gonthier, Belgium), 0.5% zeolite (13-X type; Molecular Sieves, Union Carbide, USA), 5% cyclonic ash [modified aluminosilicate from the burning of coal refuse from an old coal mine in Beringen consisting of coal (30 %) and mainly schists; N.V. Mijnen, Belgium], 5% compost (commercial grade, consisting of recycled plant material; Intercompost, Belgium), 1% lime [commercial grade (($(Ca,Mg)CO_3$)$_,$ AVEVE, Belgium], 1% steelshot [industrial waste by-product containing mainly iron (97% Fe^0), and Mn (0.6-1%), Si (0.8-1.2%), C (0.8 to 1.2%), and Cr (0.2 to 0.5%); Grenaille**,** Wheelabrator Allevard, Belgium] and 1% hydroxyapatite [$3Ca_3(PO_4)_2 \cdot Ca(OH)_{2;}$ Aldrich, USA]. The rates of amendment addition were based on previous studies (Boisson et al., 1999; Mench et al., 1994a; Vangronsveld et al., 1995, 1996; Rebedea & Lepp, 1995) as well as preliminary greenhouse experiments.

The amendments were added 30 days after Pb equilibration in soil. Then the potted spiked soils were wetted and re-equilibrated over a period of 100 days at room temperature before measurements were performed.

2.2. $Ca(NO_3)_2$- extractable trace elements

Soil samples were air dried (60 °C; 48 h) and sieved through a 2 mm sieve preceeding analysis: electrical conductivity and pH were determined after shaking 10 g soil for 18 h with 25 ml distilled water; pH-KCl was determined after shaking 10 g soil for 1 h with 25 ml 1 *M* KCl. Trace element phytoavailability was assessed using a modified $Ca(NO_3)_2$-extraction (Sappin-Didier, 1995). Samples of 5 g soil were shaken in plastic flasks with 50 ml 0.1 *M* $Ca(NO_3)_2$ for 24 h. Extracts were filtered through ash-free paper (Whatman 42) into acid-washed (10% HCl) glass labware; analysis was performed in triplicate. Solution trace element concentrations were determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES). Three blanks (only extractant) were run to determine background concentrations.

2.3. Plant response

The plant response was assessed by a biological test system using beans (*Phaseolus vulgaris* cv. Limburgse vroege) as a bio-indicator (Van Assche & Clijsters, 1990a; Vangronsveld & Clijsters, 1992). After 1 day vernalisation and overnight imbibition, seeds were sown in polyethylene pots of 400 ml (3 pots per treatment, 5 plants/pot). The plants were cultured in a growth chamber under controlled conditions (22 °C, 65% relative humidity, 12 h light, photosynthetic active radiation: 150 µmol m^{-2} s^{-1}) and watered daily with deionized water. Fourteen days after sowing, morphological parameters (shoot length, primary leaf area, root weight) were measured for each plant and samples (1 g fresh weight) of primary leaves and roots were stored at –70°C for (iso-)enzyme analysis. Activity of the following enzymes were determined spectrophotometrically (Shimadzu UV-1602) according to Van Assche et al. (1988): guaiacol peroxidase (GPOD), malic enzyme (ME) and glutamate dehydrogenase (GDH) in roots and GPOD and ME in primary leaves. These measurements were performed under non-limiting conditions of substrate and coenzyme, therefore, it is further named enzyme capacity (expressed in mU g^{-1} FW). In addition to peroxidase measurement by reaction with guaiacol (GPOD), anionic iso-enzymes of POD were stained after separation by polyacrylamide gel electrophoresis on a 7.5-16% gradient slab gel (Van Assche and Clijsters, 1990a). Lead concentration in plant tissue was not measured since all plant material was used in plant response assessment.

2.4. Lead concentration in lettuce

After harvesting the bean seedlings for biological assays, lettuce (*Lactuca sativa* cv. Hilde) seedlings grown for 3 weeks on a non-contaminated substrate, were transplanted to the treated Pb-spiked soils (3 pots per treatment; 3 plants per pot) and grown in a greenhouse for 4 more weeks. The above-ground plant parts were rinsed with distilled water, dried at 80°C and microwave digested (Milestone MLS-1200 MEGA) in purified HNO_3 (70-71%). Lead concentrations were determined by flame or graphite furnace AAS, depending on the concentration of the element. Blanks (only HNO_3) and standard samples (ICHTJ Virginia Tobacco Leaves (CTA-VTL-2); recovery within 90-110%) were run to determine Pb concentration in the extractant and to check the accuracy of microwave digestion and AAS analysis.

2.5. Statistical analysis

A TWO WAY ANOVA was performed to evaluate the effect of Pb concentration, amendment addition, and/or their interaction. Significant interactions were observed between Pb concentration and amendment addition (data not shown). Consequently, data were analysed separately for each Pb concentration using a non-parametric test. A Kruskal-Wallis test was performed to detect difference between amendments. When difference was detected ($p < 0.05$), multiple comparisons were performed to separate the means (indicated as letters in columns). Since data were analysed per Pb concentration, letters are row specific and cannot be compared between rows. To examine effects of Pb concentration, another Kruskal-Wallis test was performed to detect difference within the unamended soil only. When difference was detected ($p < 0.05$), multiple comparisons were performed to separate the means (indicated in roman numbers, between brackets). Root weight and shoot length of bean plants were based on 15 measurements, primary leaf area on 30 and above-ground weight of lettuce on 9 while 3 replicates were considered for pH, extractable Pb, Pb content in lettuce, and enzyme capacities.

3. RESULTS

3.1. Soil quality

Increasing Pb concentratons did not significantly increase the pH of the unamended soil (Table 4.1). Bentonite and steelshot slightly increased pH, although this increase is not always significant relative to the unamended. Zeolite (6.65 at 2000 mg Pb kg^{-1}), cyclonic ash (6.25) and compost (6.43) significantly increased pH. Application of lime increased pH even higher (7.52). No significant increase in pH was observed after addition of hydroxyapatite.

Table 4.1: pH of Pb-spiked soils (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) amended with several soil additives. Values are means (n=3). Means having the same letter within rows or the same number within the unamended column are not significantly different ($p < 0.05$) (letters are row-specific and can not be compared between rows).

	unamended	1% bentonite	0.5% zeolite	5% cyclonic ash	5% compost	1% lime	1% steelshot	1% hydroxyapatite
0	$4.91^{(I)a}$	5.53^{bc}	6.80^{e}	5.92^{cd}	6.27^{d}	7.62^{f}	5.85^{cd}	5.06^{ab}
250	$4.73^{(I)a}$	5.49^{b}	6.21^{c}	6.20^{c}	6.31^{c}	7.65^{d}	5.36^{ab}	5.09^{ab}
500	$4.55^{(I)a}$	5.12^{ab}	6.18^{c}	6.06^{c}	6.26^{c}	7.58^{d}	5.35^{b}	5.06^{ab}
1000	$4.56^{(I)a}$	5.39^{b}	6.05^{c}	6.48^{c}	6.31^{c}	7.61^{d}	5.36^{b}	5.05^{ab}
1500	$4.75^{(I)a}$	5.36^{b}	6.51^{c}	6.01^{c}	6.27^{c}	7.52^{d}	5.11^{ab}	5.02^{ab}
2000	$5.18^{(I)a}$	5.40^{a}	6.65^{b}	6.25^{b}	6.43^{b}	7.52^{c}	5.58^{a}	5.26^{a}

The $Ca(NO_3)_2$-extractable Pb fraction in the unamended soil significantly increased up to 492 mg kg^{-1}, when total soil Pb was elevated up to 2000 mg kg^{-1} (Table 4.2). $Ca(NO_3)_2$-extractable Pb was significantly reduced when soil amendments were applied. At the highest soil Pb concentration, bentonite reduced $Ca(NO_3)_2$-extractable Pb to 372 mg kg^{-1} (i.e. 24% decrease relative to the unamended); further reductions were obtained with steelshot (207 mg kg^{-1}; 58% decrease) and zeolite (142 mg kg^{-1}; 71% decrease). However, stronger immobilization occurred when cyclonic ash (50 mg kg^{-1}; 89.9% decrease), compost (48 mg kg^{-1}; 90.2% decrease), hydroxyapatite (31 mg kg^{-1}; 94% decrease), or lime (11 mg kg^{-1}; 98% decrease) were applied.

Table 4.2: $Ca(NO_3)_2$-extractable Pb (mg kg^{-1} dry soil) of lead enriched soil (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) treated with several soil additives. Values are means (n=3). Means having the same letter within rows or the same number within the unamended column are not significantly different ($p < 0.05$) (letters are row-specific and can not be compared between rows).

	unamended	1% bentonite	0.5% zeolite	5% cyclonic ash	5% compost	1% lime	1% steelshot	1% hydroxyapatite
0	$9^{(I)a}$	7^{a}	6^{a}	7^{a}	7^{a}	6^{a}	7^{a}	6^{a}
250	$57^{(I,II)d}$	35^{c}	26^{bc}	9^{a}	9^{a}	7^{a}	17^{ab}	7^{a}
500	$108^{(II)e}$	80^{d}	39^{c}	13^{ab}	14^{ab}	7^{a}	31^{bc}	8^{a}
1000	$261^{(III)d}$	181^{c}	90^{b}	24^{a}	26^{a}	8^{a}	70^{b}	16^{a}
1500	$413^{(IV)e}$	267^{d}	93^{b}	37^{a}	40^{a}	9^{a}	175^{c}	22^{a}
2000	$492^{(V)d}$	372^{c}	142^{b}	50^{a}	48^{a}	11^{a}	207^{b}	31^{a}

Electrical conductivity substantially increased upon amendment addition (lime= 420 µS cm^{-1}, hydroxyapatite= 440 µS cm^{-1}, zeolite= 476 µS cm^{-1}, bentonite= 614 µS cm^{-1}, cyclonic ash= 878 µS cm^{-1}, compost= 889 µS cm^{-1}) in comparison to the unamended soil (201 µS cm^{-1} at 2000 mg Pb kg^{-1}) with the exception of steelshot (195 µS cm^{-1}). To what degree amendment addition alters conductivity depends on the amount of salts present in the amendment as well as their solubility. Several amendments are known to release substantial amounts of Ca (lime, hydroxyapatite, cyclonic ash) (Mench et al., 1998). Amendment addition further substantially increased $Ca(NO_3)_2$ extractable Mg (cyclonic ash= 209 mg kg^{-1}; lime= 196 mg kg^{-1}; compost= 136 mg kg^{-1}; unamended= 73 mg kg^{-1}), K (compost= 483 mg kg^{-1}; unamended= 47 mg kg^{-1}), P (hydroxyapatite= 52 mg kg^{-1}; compost= 11 mg kg^{-1}; unamended= 5.5 mg kg^{-1}), Fe (steelshot= 58 mg kg^{-1}; unamended= 16 mg kg^{-1}) and Mn (steelshot= 50 mg kg^{-1}; unamended= 3.3 mg kg^{-1}).

3.2. Plant response

3.2.1. Morphological parameters

Plant response to Pb spiking and soil amendment addition was evaluated by collecting selected plant parameters. In unamended soil, root weight significantly decreased when soil Pb concentration rose above 1000 mg kg^{-1} (Fig. 4.1). Application of bentonite and to a lesser degree, steelshot and hydroxyapatite, resulted as well in a decrease in root weight with increasing Pb concentration, while addition of zeolite strongly reduced root growth independent of soil Pb. Cyclonic ash, compost, and lime increased root weight relative to plants grown on unamended soil, even at low Pb concentrations.

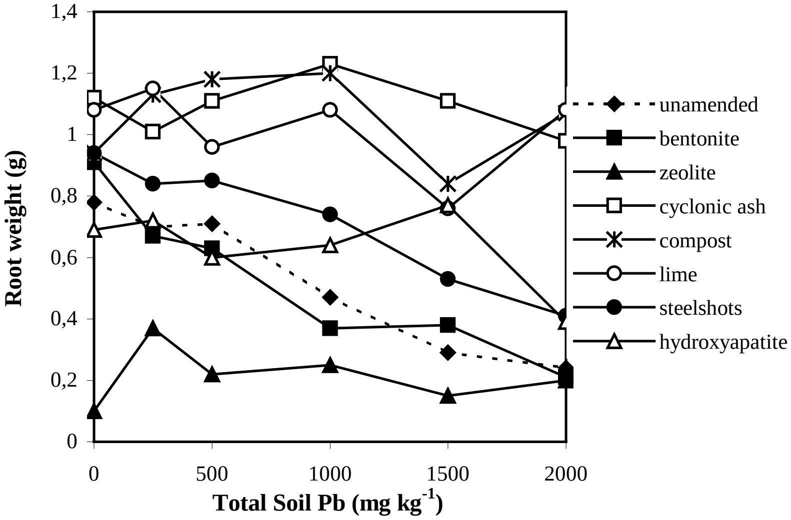

Fig. 4.1: Average individual root weight (g) of 14 day-old *Phaseolus vulgaris* seedlings grown on Pb-spiked soils (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) amended with several soil additives. Values are means (n=15).

No significant inhibitory effect of Pb addition was observed for shoot length (data not shown). Leaf area of the test plants significantly decreased when Pb concentrations exceeded 1500 mg Pb kg^{-1} (Fig. 4.2). Addition of zeolite affected leaf area at all Pb concentrations compared to the unamended. In contrast, cyclonic ash and compost and to a lesser degree, lime and hydroxyapatite, substantially increased leaf area at all Pb concentrations.

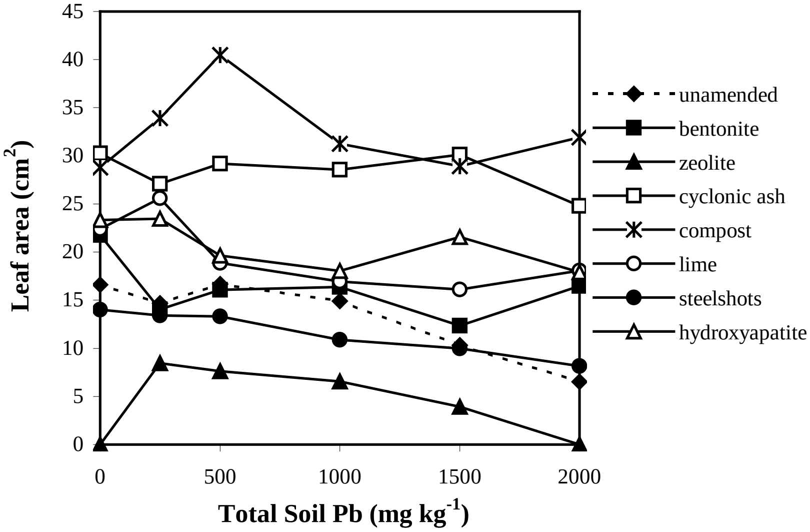

Fig. 4.2: Average individual primary leaf area (cm^2) of 14 day-old *Phaseolus vulgaris* seedlings grown on Pb-spiked soils (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) amended with several soil additives. Values are means (n=30).

3.2.2. Biochemical parameters

In roots of plants grown on unamended soil, GPOD capacity was significantly increased at 1000 mg Pb kg^{-1}, while ME (1500 mg Pb kg^{-1}) and GDH (2000 mg Pb kg^{-1}) were induced at higher concentrations (Table 4.3), as a direct or indirect defense against induced oxidative stress (Van Assche et al., 1988; Van Assche & Clijsters, 1990b; Vangronsveld & Clijsters, 1994). At elevated soil Pb concentrations, the capacity of all enzymes significantly decreased when cyclonic ash, compost and lime were applied; steelshot and hydroxyapatite significantly decreased GDH and ME while bentonite significantly decreased GDH. Cyclonic ash and lime significantly decreased GPOD at low soil Pb concentrations. In contrast, hydroxyapatite increased GDH at low soil Pb concentration (0 mg Pb kg^{-1}). Addition of zeolite significantly increased GPOD and ME, irrespective of soil Pb concentration. Bentonite application did not significantly alter enzyme capacities.

Table 4.3: Capacity of guaiacol peroxidase (GPOD), glutamate dehydrogenase (GDH) and malic enzyme (ME) (mU g^{-1} fresh weight) in roots of 14 day-old *Phaseolus vulgaris* seedlings grown on Pb-spiked soils (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) amended with several soil additives (NA: data not available). Values are means (n=3). Means having the same letter within rows or the same number within the unamended column are not significantly different ($p < 0.05$) (letters are row-specific and can not be compared between rows).

	unamended	1% bentonite	0.5% zeolite	5% cyclonic ash	5% compost	1% lime	1% steelshot	1% hydroxyapatite
GPOD								
0	$13703^{(I)c}$	9753^{bc}	20518^{d}	6870^{ab}	8796^{bc}	5293^{a}	11074^{bc}	9584^{bc}
250	$13205^{(I)cd}$	11707^{bc}	26492^{e}	4482^{a}	9865^{b}	4306^{a}	17993^{d}	8966^{ab}
500	$14092^{(I)b}$	15554^{bc}	24002^{c}	5794^{a}	8299^{ab}	5315^{a}	13736^{b}	11218^{ab}
1000	$18551^{(II)c}$	14890^{bc}	22059^{d}	5749^{a}	10270^{ab}	4326^{a}	15299^{bc}	14604^{bc}
1500	$18470^{(II)c}$	16428^{c}	23762^{d}	6160^{ab}	10696^{bc}	4491^{a}	15922^{c}	12691^{bc}
2000	$17233^{(II)b}$	14753^{b}	NA	7147^{a}	10198^{a}	6508^{a}	17134^{b}	NA
GDH								
0	$157^{(I)\ a}$	146^{a}	156^{a}	145^{a}	125^{a}	195^{ab}	89^{a}	341^{b}
250	$210^{(I,II)a}$	145^{a}	103^{a}	84^{a}	130^{a}	183^{a}	172^{a}	269^{a}
500	$212^{(I,II)ab}$	302^{b}	179^{ab}	121^{ab}	91^{a}	188^{ab}	140^{ab}	237^{ab}
1000	$325^{(I,II)b}$	148^{ab}	223^{ab}	83^{a}	137^{ab}	213^{ab}	129^{ab}	235^{ab}
1500	$360^{(I,II)b}$	169^{ab}	164^{ab}	124^{a}	84^{a}	196^{ab}	129^{a}	275^{ab}
2000	$469^{(II)d}$	61^{a}	NA	128^{bc}	97^{ab}	193^{c}	140^{bc}	175^{bc}
ME								
0	$536^{(I)ab}$	580^{ab}	878^{b}	382^{a}	325^{a}	360^{a}	392^{a}	442^{ab}
250	$478^{(I)ab}$	613^{bc}	787^{c}	311^{a}	448^{ab}	287^{a}	448^{ab}	337^{a}
500	$602^{(I,II)c}$	694^{c}	1098^{d}	319^{ab}	454^{bc}	296^{a}	430^{ab}	408^{ab}
1000	$701^{(I,II)b}$	711^{b}	944^{c}	279^{a}	528^{b}	303^{a}	554^{b}	524^{b}
1500	$825^{(II)d}$	684^{c}	974^{d}	335^{a}	470^{ab}	380^{a}	566^{bc}	479^{ab}
2000	$806^{(II)c}$	680^{bc}	NA	316^{a}	517^{b}	288^{a}	507^{b}	828^{c}

In primary leaves of plants grown on unamended soil, GPOD and ME capacity were significantly increased when soil Pb was elevated (Table 4.4). As in roots, addition of bentonite did not significantly alter this enzyme capacity while zeolite significantly increased GPOD independent of soil Pb. Cyclonic ash, compost, lime and hydroxyapatite significantly decreased enzyme capacities at elevated soil Pb. Cyclonic ash (GPOD, ME), compost (GPOD, ME) and lime (ME) further significantly decreased the enzyme capacities at low Pb soil concentration. In contrast, steelshot and hydroxyapatite significantly increased GPOD at low Pb concentrations. New isoenzymes of POD were detected in primary leaves at low Pb soil concentrations when steelshot and hydroxyapatite were used (data not shown). New isoenzymes were also found in leaves of bean plants cultivated on unamended soil, at 2000 mg Pb kg^{-1}.

Table 4.4: Capacity of guaiacol peroxidase (GPOD) and malic enzyme (ME) (mU g^{-1} fresh weight) in primary leaves of 14 day-old *Phaseolus vulgaris* seedlings grown on Pb-spiked soils (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) amended with several soil additives (NA: data not available). Values are means (n=3). Means having the same letter within rows or the same number within the unamended column are not significantly different ($p < 0.05$) (letters are row-specific and can not be compared between rows).

	unamended	1% bentonite	0.5% zeolite	5% cyclonic ash	5% compost	1% lime	1% steelshot	1% hydroxyapatite
GPOD								
0	$1147^{(I)b}$	924^{ab}	NA	540^{ab}	381^{a}	1083^{b}	1956^{c}	1805^{c}
250	$1204^{(I)bc}$	736^{a}	3553^{e}	647^{a}	452^{a}	746^{ab}	2646^{d}	1518^{c}
500	$1154^{(I)a}$	672^{a}	5643^{c}	584^{a}	199^{a}	1038^{a}	2616^{b}	2414^{b}
1000	$1024^{(I)ab}$	775^{ab}	3075^{d}	790^{ab}	207^{a}	1740^{b}	2459^{c}	2607^{cd}
1500	$2110^{(II)ab}$	1279^{ab}	9227^{c}	579^{a}	193^{a}	1000^{ab}	5436^{bc}	2514^{ab}
2000	$5112^{(III)b}$	NA	7164^{b}	612^{a}	201^{a}	1219^{a}	7777^{b}	1120^{a}
ME								
0	$768^{(I)ab}$	602^{ab}	NA	501^{ab}	449^{a}	628^{ab}	782^{ab}	803^{b}
250	$833^{(I,II)bc}$	619^{ab}	1081^{c}	370^{a}	385^{a}	396^{a}	822^{bc}	618^{ab}
500	$757^{(I)bc}$	646^{bc}	1040^{c}	435^{ab}	361^{a}	471^{ab}	744^{bc}	719^{bc}
1000	$677^{(I)bc}$	688^{bc}	859^{cd}	489^{ab}	382^{a}	494^{ab}	864^{cd}	903^{d}
1500	$923^{(I,II)c}$	622^{bc}	1069^{c}	414^{ab}	294^{a}	316^{ab}	1005^{c}	745^{bc}
2000	$1067^{(II)c}$	NA	1080^{c}	414^{ab}	375^{a}	412^{ab}	1055^{c}	662^{b}

3.2.3. Phytotoxicity index

Morphological as well as biochemical data were used to calculate phytotoxicity indices (PI) according to Vangronsveld & Clijsters (1992). According to this scheme soils can be classified into 4 phytotoxicity classes: not toxic (PI=1), slightly toxic (PI=2), moderately toxic (PI=3), strongly toxic (PI=4).

From Table 4.5 it is evident that in the unamended soil, phytotoxicity increased from not toxic (class 1) to moderately toxic (class 3) with increasing soil Pb concentration. The majority of the amendments reduced Pb phytotoxicity. With bentonite, Pb was not toxic at 500 and with steelshot at 1000 mg kg^{-1}; at higher concentrations the substrate became slightly toxic. Treatment with cyclonic ash, compost, lime, and hydroxyapatite resulted in complete mitigation of phytotoxicity across soil Pb concentrations.

However, application of zeolite induced abnormal plant growth irrespective of soil Pb concentration. Zeolite apparently created an unfavourable soil structure (i.e. dispersion from abundant Na^+ ions) as indicated by a dry, compacted surface layer (0-5 cm) upon water addition.

Table 4.5: Phytotoxicity indices for Pb-spiked soils (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) amended with several soil additives.

	unamended	1% bentonite	0.5% zeolite	5% cyclonic ash	5% compost	1% lime	1% steelshot	1% hydroxyapatite
0	1	1	2	1	1	1	1	1
250	1	1	2	1	1	1	1	1
500	1	1	3	1	1	1	1	1
1000	2	2	3	1	1	1	1	1
1500	2	2	3	1	1	1	2	1
2000	3	2	3	1	1	1	2	1

3.3. Lead concentration in lettuce

Pb concentration in lettuce tissue significantly increased when soil Pb concentration was enhanced (Table 4.6). At the highest Pb concentration (2000 mg Pb kg^{-1}), up to 24.5 mg Pb kg^{-1} fresh weight were found in the edible parts of lettuce. This amount exceeds the guideline value for Pb of 0.8 mg kg^{-1} in green vegetables as recommended by the German Federal Health Agency (Ewers, 1991). The low pH (5.18) of the sandy soil used and its low organic matter concentration (4.05%) were conducive to high Pb availability, resulting in a high $Ca(NO_3)_2$-extractable Pb concentration of 492 mg kg^{-1} (at 2000 mg Pb kg^{-1}). All amendments significantly reduced Pb concentration in lettuce shoots (Table 4.6). At 2000 mg Pb kg^{-1}, addition of compost reduced Pb tissue concentration by 46% (13.2 mg kg^{-1}; relative to the unamended) while 65% reduction was measured when bentonite (8.7 mg kg^{-1}) and cyclonic ash (8.5 mg kg^{-1}) were applied. Application of zeolite was problematic at 2000 mg kg^{-1} (bad soil structure due to dispersion from Na^+ saturation killed the test plants) but tissue analysis from the 1500 mg kg^{-1} rate (9.6 mg kg^{-1}) suggests it's effect was similar to that found with bentonite, cyclonic ash or compost. The lowest concentration however was found with hydroxyapatite (6.8 mg kg^{-1}; 72% reduction) and lime (4.9 mg kg^{-1}; 80% reduction). No data were obtained with steelshot as all lettuce seedlings died, probably due to Mn ($Ca(NO_3)_2$-extractable Mn: +1415% relative to the unamended) or Fe (+262%) toxicity**.**

Table 4.6. Pb content in edible parts of *Lactuca sativa* (mg Pb kg^{-1} fresh weight) grown on lead enriched soil (0, 250, 500, 1000, 1500, 2000 mg Pb kg^{-1}) treated with several soil additives, average individual above-ground weight of lettuce (g) and total Pb extracted from the soil by the same plant (µg Pb plant^{-1}) (NA: data not available). Values are means (n=3 (Pb content), 9 (weight), 3 (Pb uptake)) Means having the same letter within rows or the same number within the unamended column are not significantly different ($p < 0.05$) (letters are row-specific and can not be compared between rows).

	unamended	1% bentonite	0.5% zeolite	5% cyclonic ash	5% compost	1% lime	1% hydroxyapatite
Pb tissue content (mg Pb kg^{-1} FW)							
0	$0.4^{(I)a}$	0.5^{a}	NA	0.3^{a}	0.3^{a}	0.6^{a}	0.3^{a}
250	$10.4^{(II)d}$	5.8^{c}	2.5^{b}	2.3^{b}	2.2^{b}	2.9^{bc}	0.3^{a}
500	$7.8^{(II)c}$	6.5^{c}	5.5^{bc}	3.1^{b}	3.6^{b}	4.2^{b}	0.8^{a}
1000	$12.2^{(II)c}$	6.3^{b}	8.9^{bc}	6.5^{b}	7.1^{b}	4.2^{ab}	2.0^{a}
1500	$15.2^{(III)d}$	8.0^{b}	9.6^{bc}	10.5^{c}	10.2^{c}	4.2^{a}	3.8^{a}
2000	$24.5^{(IV)c}$	8.7^{ab}	NA	8.5^{ab}	13.2^{b}	4.9^{a}	6.8^{ab}
Above-ground weight (g)							
0	$0.29^{(I)a}$	3.10^{c}	NA	1.47^{b}	1.99^{b}	4.18^{c}	3.28^{c}
250	$0.39^{(I)a}$	3.60^{c}	0.62^{a}	1.68^{b}	1.41^{b}	3.43^{c}	4.32^{c}
500	$0.36^{(I)a}$	2.04^{b}	0.33^{a}	2.10^{b}	1.85^{b}	2.50^{b}	4.14^{c}
1000	$0.33^{(I)a}$	0.89^{ab}	0.24^{a}	1.42^{ab}	1.26^{ab}	1.87^{b}	4.57^{c}
1500	$0.32^{(I)a}$	0.48^{a}	0.44^{a}	1.31^{b}	1.50^{b}	1.43^{b}	3.28^{c}
2000	$0.22^{(I)a}$	0.26^{a}	NA	1.15^{b}	1.33^{b}	1.15^{b}	2.48^{c}
Total Pb extracted (µg Pb plant^{-1} FW)							
0	$0.1^{(I)a}$	1.6^{bc}	NA	0.5^{a}	0.6^{a}	2.4^{c}	0.8^{ab}
250	$4.1^{(III)a}$	18.0^{c}	1.5^{a}	3.9^{a}	3.1^{a}	9.9^{b}	1.2^{a}
500	$2.8^{(II)a}$	13.3^{c}	1.8^{a}	6.6^{ab}	6.6^{ab}	10.4^{bc}	3.3^{a}
1000	$4.0^{(III)ab}$	5.6^{ab}	2.1^{a}	9.1^{b}	9.0^{b}	7.8^{b}	9.1^{b}
1500	$4.9^{(III)a}$	3.8^{a}	4.2^{a}	13.8^{b}	15.2^{b}	6.1^{a}	12.4^{b}
2000	$5.4^{(III)ab}$	2.2^{a}	NA	9.9^{b}	17.5^{c}	5.7^{ab}	16.9^{c}

Total Pb uptake from the soil (µg Pb plant^{-1}) was calculated as Pb tissue concentration might be diluted by a much higher plant biomass production after application of amendments. Uptake of Pb increased up to 5.4 µg Pb plant^{-1} on unamended soil (Table 4.6). Amendment addition did not significantly decrease total Pb uptake from the soil by plants (Table 4.6). In contrast, except zeolite, all amendments increased Pb uptake.

This is not surprising since treatment with most additives significantly reduced soil phytotoxicity which resulted in a strongly increased biomass production: e.g. at 2000 mg Pb kg^{-1}, above-ground weight of the seedlings increased by 422% (cyclonic ash), 424% (lime), 505% (compost) and 1029% (hydroxyapatite), relative to the unamended (Table 4.7).

4. DISCUSSION

Based on plant response criteria, significant immobilization of soil Pb occurred when cyclonic ash, compost, lime, or hydroxyapatite were applied. $Ca(NO_3)_2$-extractable Pb concentrations were reduced and phytotoxicity mitigated by these amendments. In general, development of roots and above-ground parts of the plants were enhanced by these amendments irrespective of the soil Pb concentration. At 0 mg Pb kg^{-1}, cyclonic ash (leaves + roots), compost (leaves) and lime (roots) even significantly reduced enzyme capacities. The weak plant growth on the unamended soil might be caused by Al or Mn toxicity due to the low pH of the soil (pH-H_2O= 4.92; pH-KCl= 3.55). Increases of pH observed after amendment addition would have mitigated Al or Mn toxicity (Marschner, 1995) and may explain the observed increase in growth and decrease of oxidative stress. These soil amendments further released nutrients (Mg, K, Ca (cyclonic ash), Ca (lime), N, P, Mg, K (compost)) facilitating plant development on the acid, sandy soil.

Application of bentonite, zeolite, and steelshot decreased $Ca(NO_3)_2$-extractable Pb. However these amendments were unable to reduce phytotoxicity and oxidative stress. Bentonite caused a gradual increase of phytotoxicity from not toxic (class 1) to slightly toxic (class 2) with increasing Pb concentration, suggesting poor immobilization of Pb by this additive (the lowest reduction in $Ca(NO_3)_2$-extractable Pb was found with this additive, i.e. 24% reduction at 2000 mg kg^{-1}; Table 4.2). Although $Ca(NO_3)_2$-

extractable Pb was reduced from 492 to 142 mg kg^{-1} (at 2000 mg Pb kg^{-1}), application of zeolite was detrimental irrespective of soil Pb concentration due to destruction of soil structure. Bentonite and zeolite resulted in the lowest plant Pb uptake from the soil (µg Pb lettuce plant^{-1}). This however is mainly due to a substantial reduction in plant biomass, rather than a significant reduction in Pb concentration (mg Pb kg^{-1} FW) in comparison to other amendments. For example in case of bentonite, Pb uptake was significantly higher at low Pb soil concentrations (250 and 500 mg kg^{-1}; table 4.7) relative to the other amendments. At these concentrations Pb was not phytotoxic for lettuce resulting in a high plant biomass and consequently a high Pb uptake. The addition of steelshot caused a phytotoxic effect due to Fe or Mn phytotoxicity, resulting in the induction of new D-band peroxidases in beans (Van Assche & Clijsters, 1990a) and the death of lettuce seedlings.

The effect of lime confirms the relevance of high alkalinity in mitigating Pb availability (Sauvé et al., 1997). Addition of lime decreased $Ca(NO_3)_2$-extractable Pb by 98% (at 2000 mg kg^{-1}), reduced phytotoxicity and decreased Pb tissue concentration in lettuce. Our data further indicate cyclonic ash as a valuable Pb immobilizing agent, as has been observed earlier in the laboratory (Vangronsveld et al., 1990, Vangronsveld & Clijsters, 1992) and in the field (Vangronsveld et al., 1995, 1996). A field experiment (Maatheide, Belgium) shows that – 12 years after addition of cyclonic ash – pH of the soil (7.3-7.4) is still the same as was observed 2 and 5 years after addition (Vangronsveld et al., 1996) while extrapolated data from a laboratory study indicate stable immobilization of Zn and Cd for 80 years (Wessolek & Fahrenhorst, 1994). The increased pH can however mobilize As; for this reason addition of cyclonic ash is recently combined with steelshot on soils where soil metal as well as As concentrations are elevated. On the other hand application of cyclonic ash can result in Mn deficiency on soils low in Mn. Addition of hydroxyapatite significantly reduced $Ca(NO_3)_2$-extractable soil Pb and Pb tissue concentration in lettuce. The additive probably has the highest capacities for long term Pb immobilization as Pb pyromorphite is known to be one of the most thermodynamically stable lead minerals in the surface environment (Cotter-Howells, 1996). Root weight of bean plants however declined upon the addition of hydroxyapatite, while GDH (roots) and GPOD (leaves) were induced at low soil Pb concentrations (Table 4.3, 4.4), causing the phytotoxicity index to rise to an average of 1.33, independent of the soil Pb concentration (this value was not shown in Table 4.5 where means were rounded to whole numbers). The slight induction of oxidative stress might be explained due to a mineral deficiency as a result of strong immobilization of essential elements ($Ca(NO_3)_2$-extractable Zn (-59%), Mn (-39%) and Fe (-36%) in comparison to the unamended soil) as was earlier found by Boisson et al. (1999). Furthermore, after hydroxyapatite addition no increase in soil pH was measured although this can be very helpful in mitigating soil phytotoxicity (e.g. in case of Al and Mn toxicity). Boisson et al. (1999) further found increased mobilization of As after hydroxyapatite treatment.

In conclusion, all tested soil amendments significantly reduced $Ca(NO_3)_2$- extractable Pb while cyclonic ash, compost, lime and hydroxyapatite neutralised phytotoxicity at elevated soil Pb concentrations. Cyclonic ash, compost and lime further neutralised Al or Mn toxicity on the acid soil by increase of pH resulting in increased plant biomass and reduced enzyme induction (at low soil Pb concentrations). The other amendments may be questionable due to their inefficient Pb immobilization (bentonite), their potential adverse effect on soil structure (zeolite), or plant nutrition (steelshot).

The OECD-208 plant phytotoxicity test: *Brassica napus*, *Trifolium pratense* and *Lolium perenne* grown on 10 Pb contaminated soils treated with a combination of cyclonic ash and steelshot (CA+ST), North Carolina phoshate rock (PR) and lime. The plants are grown under standardised environmental conditions (20°C, 65% RH, 12 h photoperiod); after 5 weeks plants are harvested and above-ground biomass is measured.

CHAPTER V

EFFECT OF AMENDMENTS ON Pb, Cu, Zn, As, Cd, Ni, Fe AND Mn PHYTOAVAILABILITY AND PHYTOTOXICITY IN Pb CONTAMINATED SOILS

1. INTRODUCTION

Lead is a heavy metal that is non-essential to microorganisms, plants, animals and humans. As other metals, its exposure can result in toxicity depending on the level and organism. Its extensive use and widespread disposal in the environment has resulted in numerous Pb-contaminated soils (Adriano, 2001). *In situ* immobilization of soil Pb by the addition of inorganic soil amendments has been considered a cost-effective and environmentally acceptable alternative for the remediation of Pb contaminated soils (Zhang & Ryan, 1999a,b; Ryan et al., 2001). Amendment-induced metal immobilization will, in most cases, be combined with phytostabilization, giving rise to the development of a vegetation cover which reduces wind erosion, surface runoff and water percolation, eventually resulting in the creation of a new landscape (Vangronsveld et al. 1993, 1996).

Last decade a lot of papers examined the effect of phosphate sources such as hydroxyapatite and phosphate rock on Pb immobilization (Laperche et al., 1996, 1997; Ma, 1996; Ma et al., 1993, 1994; 1995; 1997; Ma and Rao, 1997; Zhang et al., 1997). In most soils, Pb however is not the only metal present in elevated concentrations. Processes such as pyrometallurgical production resulted in the emission of huge amounts of Cd, Cu, Zn, As,... as well. In order to evaluate soil phytotoxicity and sustainability of the treatment, it is important to examine the effects of amendments on these elements as well, because side effects can arise.

In contrast to the high amount of papers devoted to PO_4^{3-} induced Pb immobilization, literature discussing the effects of PO_4^{3-} on (im)mobilization of metals such as Cu, Zn or Cd is rare. In aqueous solutions, hydroxyapatite led to immobilization of Cd, Cu, Fe(II), Ni and Zn (Chen et al., 1997; Ma et al., 1994; Xu et al., 1994). Several mechanisms have been proposed explaining hydroxyapatite induced immobilization of other metals: (1) ion exchange processes at the surface (Chen et al., 1997; Middelburg & Comans, 1991; Xu et al., 1994), (2) surface complexation (Chen et al., 1997; Xu et al., 1994), (3) precipitation of some amorphous to poorly crystalline, mixed metal phosphate (Ma et al., 1994b; Wright et al., 1995) and (4) substitution of Ca in hydroxyapatite by other metals during recrystallization (coprecipitation) (Suzuki et al., 1981; Jeanjean et al., 1994, Xu et al., 1994). The last mechanism appears more significant for Cd^{2+} sorption than for Zn^{2+} sorption (Xu et al., 1994). This might be due to the fact that the ionic radius of Cd^{2+} (0.097 nm) is closer to the one of Ca^{2+} (0.094 nm) than the radius of Zn^{2+} (0.074 nm) so that isomorphic substitution of Ca^{2+} by Cd^{2+} is more favourable. The isomorphic substitution by Cu^{2+} (ionic radius 0.069 nm) would be less favourable than by both Cd^{2+} and Zn^{2+}. Sery et al. (1996) demonstrated that Cd effectively occupied Ca crystallographic sites using EXAFS. It seems difficult to quantitatively estimate the proportions of the removal due to any specific mechanism and it appears that the different mechanism all work more or less together (Wright et al., 1995, Xu et al., 1994).

Boisson et al. (1999) however observed increased As uptake by bean plants after hydroxyapatite addition to soil collected at the site of a former Zn smelter. The authors attribuated increased mobility of As in soil to be directly related to the presence of increased PO_4^{3-} in the soil since PO_4^{3-} and AsO_4^{3} are known to compete for the same sorption sites due to chemical and physical similarities. Boisson et al. (1999) also noticed that application of 5% hydroxyapatite resulted in decreased uptake of essential trace elements and probably led to Mn-deficiency in maize.

Cyclonic ash and steelshot both have been reported to immobilize Pb, Zn, Cu, Cd and Ni (Boisson & Mench, 1998, Geebelen et al., 2002, Mench et al., 1994a,b, 1997, 1998, Sappin-Didier, 1995, Vangronsveld et al., 1994, Vangronsveld, 1998b). The high metal immobilizing capacity of cyclonic ash is supposed to be based on a multiple effect: adsorption onto highly reactive sites due to a pH effect, coprecipitation associated with Al, Fe and Mn oxides and crystal growth and metal diffusion into the mineral surface (Mench et al., 1998). Steelshot releases Fe and Mn which corrode into Fe and Mn oxides. The OH-OH distance in Fe and Mn oxides matches well with the coordination polyhedra of many trace metals. Therefore, such hydroxyl groups form an ideal template for bridging trace metals (Manceau et al., 1992a). The increase in pH after cyclonic ash addition can result in As mobilization, while on the other hand too strong immobilization of Mn has been reported after cyclonic ash addition (Mench et al., 2002). Steelshot has been reported to immobilize As[1], and release Mn (Mench et al., 1998, 2002, Sappin-Didier, 1995, Vangronsveld et al., 1994). For this reason, addition of cyclonic ash is recently combined with steelshot on soils where soil metal as well as As concentrations are elevated. The mobility of metals such as Pb, Zn, Cu, Cd, and Ni is highly dependent on soil pH: with increasing pH, the solubility of most trace cations will decrease (Geebelen et al., 2002; Knox et al., 2000; Mench et al., 1994a, 1997, 1998). On the other hand, pH increase is known to increase the solubility of dissolved organic matter (DOM), while substantial amounts of trace cations (Cu, Pb) may be bound to DOM (Sauvé et al., 1998a,b).

The present study examined the immobilization of Pb, Cu, Zn, As and Cd on 10 soil contaminated with different Pb concentrations from different sources. Three different treatments were compared: (1) a combination of cyclonic ash and steelshot, (2) North Carolina phosphate rock, and (3) dolomitic lime. The goal is (1) to evaluate the immobilizing efficacy of potentially toxic elements such as Pb, Cd, As, Cu and Zn, (2) to see if soil amendments theirselves possibly contribute to

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[1] As solubility is particularly high between pH 5-5 and 7.

the release of such non essential elements, and (3) to check if soil treatment affects the availability of essential plant elements (Cu, Zn, Fe, Mn) due to too strong immobilization.

2. MATERIALS & METHODS

2.1. Soil material and amendments

Ten Pb-contaminated soils were collected from various locations in the U.S. and Europe. Soils passed a 9.5-mm sieve and were homogenized. Soil tests (% organic C, CEC, soil texture, $CaCl_2$ extraction) were performed on air-dry material passing a 2-mm sieve. The soil samples reflect eight different sources of Pb enrichment: tetraethyl lead, paint, car exhaust, smelting, sewage sludge biosolid, ore, spike [$PbSO_4$ (40%), $PbCO_3$ (25%), PbO (20%), $PbCl_2$ (15%)], and battery breaking. Soil properties are shown in Table 5.1.

Table 5.1: Location, contamination source, pH, % organic C, CEC (in cmol kg^{-1}), and soil texture of the ten Pb-contaminated soils.

Name/location	Contamination source	pH	% C	CEC	Soil texture
Paimboeuf (France)	[+]	5.0	1.9	9.09	Loamy sand
New Orleans, LA (USA)	Paint, car exhaust	7.5	7.0	26.5	Sand
Joplin, MO (USA)	Smelting	6.6	0.1	2.2	Loamy sand
Pribram (Czech Republic)	Smelting	6.1	1.8	6.9	Sand
Hoboken (Belgium)	Smelting	3.7	1.3	23.2	Loam
Moreno Valley, CA (USA)	Sewage sludge	7.4	2.0	14.5	Loamy sand
Leadville, CO (USA)	Pb ore	4.1	3.0	20.0	Sandy loam
Aiken, SC (USA)	Pb (reagent grade) spike	6.6	1.1	21.1	Loamy sand
Bazoches (France)	Battery breaking	8.1	2.1	28.0	Silt loam
Belin-Beliet (France)	*	7.0	2.9	4.1	Sand

[+] Aerial deposition near a TEL production facility

* Aerial deposition near a battery breaking facility

Soil pH measured in 1:2 soil/water suspension, % C determined by combustion method, CEC determined in double acid (0.5N HCl +0.25N H_2SO_4; Adams and Evans 1962), soil texture determined according to Bouyoucos (1936).

Total metal concentrations in the 10 soils, as well as concentrations from non contaminated soils given by Kabata-Pendias & Pendias (1992) and background values described in VLAREBO (VLAams REglement betreffende de BOdemsanering), the Flemish environmental legislation concerning soil cleanup

(Van Gehuchte et al., 1997), are shown in Table 5.4 (results section). These VLAREBO values are based on several surveys of soils (mean clay 10%, OM 2%) collected throughout Flanders from sites remote from sources of metal pollution (Tack et al., 1995). The table further shows cleanup values per metal as described in VLAREBO. Cleanup values vary according to the intended land use for the remediated site. These threshold values were defined using models estimating the transfer of contaminants from soil to man by different pathways (Stringer, 1990). In total, 5 classes are distinguished: the lowest values are set for natural parks (class I) and agricultural terrains (class II). The highest values are described for industrial terrains (class V). Cleanup values increase as follows: residential area (class III) < recreational area (class IV) < industrial area (class V) (see also Appendix 1). When cleanup values are exceeded, soils should be remediated up to the level of background concentration using the BATNEEC principle (Best Available Technique Not Exceeding Extensive Costs). Background values do not differ according to intended use of the substrate. Background and cleanup values are based on a standard soil sample containing 10 % clay and 2 % OM; in case clay and OM content vary, correction factors have to be taken into account (see Appendix 1). In this chapter, total metal concentrations in the 10 Pb contaminated soils will be compared with VLAREBO background and cleanup values. In order to avoid too complex data presentation, no correction for clay and OM content is calculated. Since no VLAREBO values are set for total Fe and Mn concentrations, soil data will be compared with values from non contaminated soils mentioned by Kabata-Pendias and Pendias (1992). Furthermore, only cleanup values from classes I, II and V will be used.

Soil amendments were applied in the following ratio: cyclonic ash (5%; from another origin than the formerly called beringite) + steelshot (1%) (further referred to as CA+ST), North Carolina phosphate rock [1%, except Joplin and Leadville soil (2%)] (further referred to as PR), and lime (1%; dolomitic lime, commercial grade). The applied amendment ratios were based on experience from former experiments (CA+ST, lime) (e.g. Adriaenssens et al., 1999; Geebelen et al., 2002) or were based on batch studies in case of PR addition (these batch data are discussed in the results section 3.1 from this chapter). After amendment application, the soils were wetted and re-equilibrated over a period of 4 months before experimentation.

The soil amendments were analysed for total metal content after HNO_3/HCl microwave digestion. Water exchangeable metal content was determined after shaking 5 g amendment with 50 ml distilled water for 24 h.

2.2. Evaluation methodology

Exchangeable metal content was determined by shaking 1.5 g of soil (< 2 mm) for 1 h (25°C) with 12 ml of 1 *M* $CaCl_2$ (pH 7.0). Concentrations are compared with exchangeable metal concentrations from a non contaminated reference soil sample (Zutendaal, Belgium), obtained by shaking 2 g soil with 20 ml 0.1 *M* $Ca(NO_3)_2$ for 2 h(25°C). Following exchangeable concentrations were found on Zutendaal soil: 1.33 mg Pb kg^{-1}, 0.72 mg Cd kg^{-1}, 0.00 mg Cu kg^{-1}, 5.00 mg Zn kg^{-1}, 36 mg Fe kg^{-1}, 3.65 mg Mn kg^{-1}. The $Ca(NO_3)_2$ extractable Cd content appeared to be elevated, probably due to antropogenic Cd enrichment and was replaced by 0.14 mg Cd kg^{-1} (M. Mench, oral communication).

Phytotoxicity was assessed using the OECD-208 test (1984). The test included three plant species (*Brassica napus*, *Trifolium pratense* and *Lolium perenne*). From each species, 20 seeds were sown in 100 g soil; three replicates per species were used. Plants were grown under standardised environmental conditions (20°C, 65% RH, 12 h photoperiod). After 5 weeks, plants were harvested and the above-ground biomass was measured.

Metal uptake by plants was assessed using *Zea mays*; three corn seeds were sown per 300 g soil (6 replicates) and grown in a growth chamber under the same conditions as the OECD test for 4 weeks. Corn leaves were rinsed with distilled water, dried at 80°C and microwave digested in purified HNO_3 (70-71 %). Leaves of 3 replicates (from 9 plants) were confined in order to obtain enough material for the analysis. Table 5.2 summarizes minimum concentrations in maize leaves below which deficiency symptoms (decreased biomass production) are described (later referred to as CDC: Critical Deficiency Concentration). Further, treshold concentrations (above which above-ground biomass is reduced) are shown for each element in maize leaves (L3) (later referred to as CTC: Critical Toxicity Concentration). For Pb no specific treshold concentration for maize was found in literature; here the general toxicity range defined for higher plants is used as defined by Kabata-Pendias & Pendias (1992).

In order to evaluate metal deficiency/toxicity, the maximum values described in literature are used as a minimum border while the minimum values described for toxicity are used, in order to very sensitively evaluate possible growth reductions. It should be noted that most of these treshold values for maize have been determined using mono-metallic and hydroponic cultures. In this study the presence of increased concentrations of other metals in the soil might influence phytotoxicity. The Critical Deficiency Concentrations (CDC) are 5 mg Cu kg^{-1}, 25 mg Zn kg^{-1}, 100 mg Fe kg^{-1}, 15 mg Mn kg^{-1}. On the other hand, the lowest treshold values were chosen as Critical Toxicity Concentrations (CTC): 30 mg Pb kg^{-1}, 79 mg Cd kg^{-1}, 30 mg As kg^{-1}, 5 mg Ni kg^{-1}, 27 mg Cu kg^{-1}, 1750 mg Zn kg^{-1}, 750 mg Fe kg^{-1}, 200 mg Mn kg^{-1}.

Table 5.2: Min. concentrations (deficiency) and treshold concentrations (toxicity) in maize plants (L3).

	Pb	**Cd**	**As**	**Ni**	**Cu**	**Zn**	**Fe**	**Mn**
Min. conc. Maize (defic.)	--	--	--	--	2 (3)-5[a,b]	15-251[c]	24-100[d]	15[e]
Treshold maize (L3)	30-300*[a]	79[f]	30[g]	5-10[h]	27-52[i]	1750[j]	750[d]	200[b]

[a] Kabata-Pendias & Pendias (1992); [b] Marschner (1995); [c] Loué (1994); [d] Mortvedt et al. (1991); [e] Halliday & Trenkel (1992); [f] Lagriffoul et al. (1998); [g] Briers (1998); [h] Juste & Mench (1992); [i] Mocquot et al. (1996); [j] Boisson et al. (1999)

All chemicals used in this study were of analytical grade or better. All analysis were done using disposable plastic labware. Soil and plant metal analysis was done by ICP-MS; amendment analysis was done by ICP-AES. Blanks were run for background correction and other sources of error. Multi-element standards were run every 20 samples and recovery was found to be within 100 ± 10 %. For quality control, standard reference plant material (National Institute of Standards and Technology, 1515) was used to ensure that recovery was within the certified range values.

2.3. Statistical analysis

All analysis was done in triplicate, except corn tissue assay ($n = 2$). A Kruskal-Wallis test was performed to detect differences between treatments for each measured parameter ($p < 0.05$). When differences were detected, multiple comparisons were performed to separate the means.

3. RESULTS

3.1. Evaluation of the amount of PR to be applied to Pb contaminated soils

Chlopecka & Adriano (1996, 1997c) applied 0.4% North Carolina PR to a soil spiked with different amounts of metal contaminated flue dust and observed drastic reductions upon Pb phytoavailability and plant metal uptake after addition. Wright et al. (1995) studied the metal immobilizing capacities of North Carolina PR and pointed out that, based on the amount of Pb present in soil solution, the precise amount of PO_4^{3-} to be added can be calculated, using the molar ratio's present in the formed pyromorphites (Appendix 2). In Table 5.3, the calculated percentages of PR to be added to the different soils are given, based on $Ca(NO_3)_2$ extractable Pb and total Pb.

Besides Pb, several other cations (Al, Cu, Fe(II), Cd, Zn, Ni, Ca) are known to inhibit Pb immobilization probably due to precipitation of metal phosphates, decreasing the amount of dissolved PO_4^{3-} available for precipitation with Pb ions (Ma et al., 1994). Also, PO_4^{3-} can be sorbed by or react with other soil components such as iron and aluminium oxides and clays (Ma & Rao, 1999; Menon et al., 1995). Taking into account these factors, Wright et al. (1995) concluded that the addition of 1% PR should be enough to remediate Pb contaminated soils.

Table 5.3: Calculated amounts of North Carolina PR (in %) to be added to the Pb contaminated soils (based on Pb/PO_4^{3-} molar ratio in pyromorphite). Calculations were based on 1 *M* $CaCl_2$ extractable Pb (mg kg^{-1}) and total Pb (mg kg^{-1}) concentrations in the soils.

	$CaCl_2$ Pb	**Total Pb**
Paimboeuf	0.0016	0.01
New Orleans	0.0012	0.16
Joplin	0.0648	0.15
Pribram	0.0062	0.07
Hoboken	0.0026	0.02
Moreno Valley	0.0008	0.01
Leadville	0.0307	0.10
Aiken	0.0045	0.02
Bazoches	0.0009	0.02

We performed batch studies where soluble Pb was determined after soil was shaken for 48 hours with 0, 1, 2 and 5% PR (Appendix 2). In general, addition of higher amounts of PR resulted in higher Pb immobilization. On most soils, the highest relative reductions (i.e. over 80% of the reduction observed after 5% addition) were observed after addition of 1% PR (Paimboeuf, Pribram, Hoboken, Moreno Valley, Aiken, Bazoches). On Joplin and Leadville soil a more gradual reduction was observed when increasing amounts of PR were added. Ma et al. (1995) found that Pb immobilization increased with reaction time; it is expected that metal immobilization will increase in pot experiments which will be equilibrated for 4 months upon amendment addition. Several workers recently reported that high amounts of PO_4^{3-} can immobilize essential elements to a degree that causes plant deficiency (Boisson et al., 1999; Knox, oral communication). PO_4^{3-} addition has been further reported to mobilize As (Boisson et al., 1999). It was decided to use 1% PR except on Joplin and Leadville soil where 2% PR was added (also because much higher extractable Pb concentrations were observed here, in the mg L^{-1} range).

3.2. Total and $CaCl_2$ extractable trace element content in soils and amendments

From Table 5.4 it is clear that total soil Pb exceeds VLAREBO background values (40 mg Pb kg^{-1}) in all soils. Total Pb exceeds VLAREBO class I and II cleanup values (200 mg Pb kg^{-1}) in 9 soils. Total Cd exceeds VLAREBO background value (0.8 mg Cd kg^{-1}) on 9 soils while on 7 soils VLAREBO class I and II cleanup values (2 mg Cd kg^{-1}) are exceeded (New Orleans, Joplin, Pribram, Hoboken, Moreno Valley, Leadville, Aiken). Total As passes the VLAREBO background values (19 mg As kg^{-1}) on 5 soils; on 3 soils As exceeds VLAREBO class I and II cleanup values (45 mg As kg^{-1}) (Pribram, Hoboken, Leadville). Total Ni exceeds the VLAREBO background values (9 mg kg^{-1}) on 6 soils (New Orleans, Hoboken, Moreno Valley, Bazoches); VLAREBO class I and II cleanup values (100 mg Ni kg^{-1}) are never exceeded. Total Cu is above VLAREBO background (17 mg Cu kg^{-1}) on 9 soils; only on 2 soils, VLAREBO class I and II cleanup values (200 mg Cu kg^{-1}) are exceeded (New Orleans, Leadville). Total Zn exceeds VLAREBO background values (62 mg Zn kg^{-1}) on all soils; VLAREBO class I and II cleanup values (600 mg Zn kg^{-1}) are exceeded on 5 soils (New Orleans, Joplin, Pribram, Hoboken, Leadville). On 2 soils total Fe (New Orleans, Leadville) and Mn (Leadville, Pribram) passed the average concentration for non contaminated soils (Fe=38000 mg kg^{-1}, Mn=1000 mg kg^{-1}) mentioned by Kabata-Pendias & Pendias (1992). Total Mn is low on Joplin and Belin-Beliet soil.

Table 5.5 shows total metal concentrations (in mg kg^{-1}) and $CaCl_2$ exchangeable metal concentrations (expressed as % of the total concentration) in the 10 Pb contaminated soils. $CaCl_2$ extractable metal concentrations substantially differed among the soils, ranging from 0 to 51% of total Pb, 20-91% of total Cd, 0-12% of total Cu and 0-54% of total Zn. $CaCl_2$ extractable Pb, Cu and Zn were very low (0%) on New Orleans soil despite the high total Pb (6065 mg kg^{-1}), Cu (938 mg kg^{-1}) and Zn (3842 mg kg^{-1}) concentrations present. Also, Aiken (648 mg kg^{-1}) and Bazoches (598 mg kg^{-1}) soil contained comparable total Pb concentrations, while $CaCl_2$ extractable Pb concentrations substantially differed among both soils [Aiken= 166 mg kg^{-1} (=26%) *vs* Bazoches= 1.3 mg kg^{-1} (=0%)]. On the other hand, comparable Cd concentrations were extracted with $CaCl_2$ on Joplin (2.8 mg kg^{-1}) and Hoboken (2.0 mg kg^{-1}) soil, while total Cd concentrations substantially differed between both soils (89.1 mg kg^{-1} resp. 2.2 mg kg^{-1}). Metal bioavailability appears to substantially differ among soils, due to difference in soil characteristics and present metal species. From this point of view, a directive based on total metal content might not adequately reflect metal bioavailability and related health risks.

Table 5.4: Total trace element concentrations (in mg kg^{-1}) of 10 contaminated soils (determined in HF-HNO_3 acid microwave digestion) and average concentrations for non contaminated soils worldwide (according to Kabata-Pendias, 1992). VLAREBO background and cleanup values (Flanders, Belgium) for each element are added as described in the 'Besluit van de Vlaamse Regering houdende achtergrondwaarden, bodemsaneringsnormen en toepassingen van gereinigde grond' Class I and II cleanup values are used for natural parks and agricultural terrains, class V values for industrial areas (Van Gehuchte et al., 1997).

Name/location	Pb	Cd	As	Ni	Cu	Zn	Fe	Mn
Paimboeuf (France)	188	0.0	19	10	32	69	16346	277
New Orleans, LA	6065	4.8	42	42	938	3842	114475	1776
Joplin, MO	5444	89.1	8	6	32	18204	8059	8
Pribram (Czech Republic)	2663	17.8	368	14	74	3173	26227	3333
Hoboken (Belgium)	656	2.2	75	36	155	766	34000	106
Moreno Valley, CA	236	7.7	10	65	147	438	28022	460
Leadville, CO	3857	18.0	164	6	491	3060	51047	928
Aiken, SC	678	2.7	7	4	11	482	6329	162
Bazoches (France)	598	0.8	18	24	18	67	19896	703
Belin-Beliet (France)	1859	1.0	9	3	122	160	4864	80
Non contaminated (Kabata-Pendias, 1992)	2-44	0.53	4.4-9.3	22	20	64	38000[a]	1000[b]
Background (VLAREBO)	40	0.8	19	9	17	62	--	--
Cleanup I,II (VLAREBO)	200	2	45	100	200	600	--	--
Cleanup V (VLAREBO)	2500	30	300	700	800	3000	--	--

[a] according to Lepp (1981); [b] according to Bowen (1979)

Exceedings of VLAREBO background values are marked in light grey, exceedings of VLAREBO Class I and II cleanup values are indicated in dark grey.

Table 5.5: Total Pb, Cd, Ni, Cu and Zn concentrations (in mg kg^{-1}) of 10 contaminated soils (determined in HF-HNO_3 acid microwave digestion) and $CaCl_2$ extractable metal concentrations (expressed as a % of the total metal content).

Name	Pb		Cd		Ni		Cu		Zn	
	Total	$CaCl_2$	Total	$CaCl_2$	Total	$CaCl_2$	Total	$CaCl_2$	Total	$CaCl_2$
Paimboeuf	188	23%			10	93%	32	3%	69	13%
New Orleans	6065	0%	4.8	33%	42	98%	938	0%	3842	0%
Joplin	5444	36%	89.1	3%			32	2%	18204	3%
Pribram	2663	5%	17.8	69%	14	90%	74	0%	3173	19%
Hoboken	656	4%	2.2	91%	36	79%	155	12%	766	40%
Moreno Valley	236	1%	7.7	42%	65	93%	147	1%	438	7%
Leadville	3857	26%	18.0	87%			491	5%	3060	54%
Aiken	648	26%	2.7	22%			11		482	8%
Bazoches	598	0%	0.8	38%	24	98%	18	0%	67	1%
Belin-Beliet	1859	51%	1.0	80%			122	1%	160	34%

Only metal concentrations that exceeded the VLAREBO background values are shown.

Water exchangeable and total metal concentrations in amendments are shown in Table 5.6. Steelshot and cyclonic ash contained 75 mg Pb kg^{-1} resp. 38 mg Pb kg^{-1}, while 8 resp. 6 mg Pb kg^{-1} was found in phosphate rock and lime. Steelshot and phosphate rock contained up to 60 mg Cd kg^{-1}. Steelshot contained high amounts of Ni (591 mg kg^{-1}), Cu (834 mg kg^{-1}), Fe (922332 mg kg^{-1}= 92 %), Mn (7715 mg kg^{-1}) and Cr (1289 mg kg^{-1}).

Water extractable metal concentrations were low; cyclonic ash released low amounts of Pb (0.7 mg kg^{-1}) while 0.2 mg Mn kg^{-1} were found with steelshot. Cyclonic ash (0.3 mg kg^{-1}), steelshot (3.9 mg kg^{-1}) and phosphate rock (1.6 mg kg^{-1}) released small amounts of Fe. Steelshot also released low concentrations of Mn (0.2 mg kg^{-1})[2].

Table 5.6: Total and water extractable metal concentrations (mg kg^{-1}) in cyclonic ash, steelshot, NC phosphate rock and lime.

	Pb	Cd	As	Ni	Cu	Zn	Fe	Mn	Cr
Total content (mg kg^{-1})									
cyclonic ash	38	< 1	< 2	58	41	152	20015	698	99
steelshot	75	60	< 2	591	834	79	922332	7715	1289
phosphate rock	8	60	< 2	19	15	441	4758	57	148
lime	6	< 1	< 2	4	3	11	3748	155	6
Water extractable (mg kg^{-1})									
cyclonic ash	0.7	< 0.1	< 0.2	< 0.1	< 0.1	< 0.1	0.3	< 0.1	< 0.1
steelshot	< 0.4	< 0.1	< 0.2	< 0.1	< 0.1	< 0.1	3.9	0.2	< 0.1
phosphate rock	< 0.4	< 0.1	< 0.2	< 0.1	< 0.1	< 0.1	1.6	< 0.1	< 0.1
lime	< 0.4	< 0.1	< 0.2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

3.3. Amendment induced metal immobilization evaluated per soil

Paimboeuf

Soil: The contaminated site is located near the Loire estuary in West France (Arrouays et al., 1996; Mench et al., 1997). Lead tetraethyl and methyl have been produced since 1938, at a rate of 20 000 to 40 000 tons per year, in a nearby industry. The soil sample was collected in an old abandoned garden close to the

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[2] The amount of Mn released is determined by the oxidation rate (time that ST are kept in water) (M. Mench, oral communication).

TEL and TML production facility (300 m distance). EXAFS analysis revealed that Pb was present as Pb^{2+} and that it was complexed to functional groups of humic substances (Manceau et al., 1996). Total Pb (188 mg kg^{-1}) and Cu (32 mg kg^{-1}) exceeded VLAREBO background values (As, Ni, and Zn equal background values) (Table 5.4). Total metal concentrations did not exceed VLAREBO class I and II cleanup values. Mench et al. (1997) found that steelshot (1%), cyclonic ash (1%), hydrous manganese oxide (HMO; 1%), and lime (1%) all reduced water extractable Pb by 70%. All amendments reduced Pb content in ryegrass: steelshot (-35%), CA (-41%), lime (-46%) and HMO (-95%). $CaCl_2$ extractable Pb (44 mg kg^{-1}) and Mn (41 mg kg^{-1}) were elevated on unamended Paimboef soil relative to the Zutendaal reference soil (1.33 mg Pb kg^{-1}, 3.65 mg Mn kg^{-1}); soil pH is 5.0.

Fig. 5.1: Abandoned garden where the soil sample was taken, 300 m from the TEL and TML production facility, Paimboeuf, West France.

Amendment addition: All amendments significantly increased soil pH in the order PR (5.4) < CA+ST (5.8) < lime (7.2) (Table 5.7). Addition of CA+ST and lime further significantly increased electrical conductivity. $CaCl_2$ extractable Pb significantly decreased upon amendment addition in the following order: PR (-41%) < CA+ST (-89%) < lime (-93%). $CaCl_2$ extractable As was below detection of the ICP-MS in all cases (< 0.004 mg kg^{-1}). All amendments further significantly decreased $CaCl_2$ extractable Cu: CA+ST = PR (-37%) < lime (-68%). CA+ST (-46%) and lime (-69%) significantly decreased $CaCl_2$ extractable Zn while PR (-33%) and lime (-76%) significantly decreased $CaCl_2$ extractable Mn. PR significantly increased $CaCl_2$ extractable Cd (+80%), while CA+ST significantly increased $CaCl_2$ extractable Ni (+133%) and Mn (+58%).

Plant response: Fe (3142 mg kg^{-1}) and Mn (740 mg kg^{-1}) content in leaves of maize grown on unamended Paimboeuf soil exceeded the Critical Toxicity Concentration (CTC[3]) (Fe= 750 mg kg^{-1}; Mn= 200 mg kg^{-1}) (Table 5.7). All 3 amendments drastically reduced maize Fe content; in case of CA+ST (-79%) and lime (-81%) concentrations dropped below the CTC. Mn content in maize was only reduced after lime application (-77%). In contrast to the increased $CaCl_2$ extractable Mn concentrations after CA+ST application, its concentration is not increased in maize leaves, but is still above the CTC. Lime reduced Cu (-36%), while CA+ST increased Cu (35.9 mg kg^{-1}; +73%) above the CTC (27 mg kg^{-1}). All amendments further decreased Pb (PR= -47%; CA+ST= –50%; lime= -76%) and Zn (PR= -76%; CA+ST= -82%; lime= -84%) content in maize leaves. According to the number of concentrations in excess of the CTC, the amendments can be classified as followed: unamended (2) < CA+ST (2) = PR (2) < lime (0).

The average above-ground maize biomass (harvested on 1 kg soil) drastically increased from 0.5 g (unamended) to 2.7 g (PR= +391%), 4.1 g (lime= +645%) and 4.7 g (CA+ST= +775%) after amendment addition. In other words, despite the fact that Cu and Mn content in maize leaves grown on CA+ST amended soil exceeded the CTC, maize biomass is similar to biomass obtained on lime amended soil (where no CTC was exceeded). Fe content appeared to be more important in determining above-ground maize biomass. Amendment addition did not

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[3] The meaning of the CTC (Critical Toxicity Concentration) and CDC (Critical Deficiency Concentration) is explained in the material & method section of this chapter.

significantly increase above-ground biomass of *B. napus*, *T. pratense*, and *L. perenne*. Ameliorative effects are observed for *B. napus* in case CA+ST and lime were used; due to the high variability between replicates this increase however was not significant.

Table 5.7: Soil and plant data on unamended, CA+ST, PR and lime amended Paimboeuf soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in Paimboeuf and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	5.0 (0.2)^{a}	5.8 (0.0)^{c}	5.4 (0.0)^{b}	7.2 (0.0)^{d}		
conductivity	159 (33)^{a}	1550 (82)^{c}	265 (17)^{ab}	346 (21)^{b}		
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	44 (6)^{d}	5 (0)^{b}	26 (1)^{c}	3 (0)^{a}	1.33	
Cd	0.1 (0.0)^{a}	0.1 (0.0)^{a}	0.3 (0.0)^{b}	0.1 (0.0)^{a}	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	0.7 (0.1)^{a}	1.6 (0.5)^{b}	0.7 (0.1)^{a}	0.4^{a}		
Cu	1.0 (0.0)^{c}	0.6 (0.1)^{b}	0.6 (0.0)^{b}	0.3 (0.1)^{a}	0.00	
Zn	9 (0)^{b}	5 (2)^{a}	7 (2)^{ab}	3 (2)^{a}	5.00	
Fe	15 (8)	18 (9)	18 (9)	10 (1)	36	
Mn	41 (0)^{c}	65 (3)^{d}	27 (1)^{b}	10 (0)^{a}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	9.0	4.5	4.8	2.2	> 30	
Cd	< DL	< DL	< DL	< DL	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	< DL	< DL	< DL	< DL	> 5	
Cu	20.7	**35.9**	17.8	13.3	> 27	< 5
Zn	257	47	61	42	> 1750	< 25
Fe	**3142**	646	**1548**	604	> 750	< 100
Mn	**740**	**632**	**740**	173	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	0.35 (0.17)	0.96 (0.63)	0.13 (0.11)	1.18 (0.86)		
T. pratense	0.054 (0.14)	0.038 (0.028)	0.033 (0.021)	0.047 (0.052)		
L. perenne	0.12 (0.12)	0.08 (0.07)	0.04 (0.02)	0.12 (0.04)		
Z. mays	0.5	4.7	2.7	4.1		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

New Orleans

Soil: New Orleans soil is contaminated by paint and car exhaust; total contents of Pb (6065 mg kg^{-1}), Cd (4.8 mg kg^{-1}), Cu (938 mg kg^{-1}) and Zn (3842 mg kg^{-1}) exceed VLAREBO class I and II cleanup values while total As (42 mg kg^{-1}) and Ni

(42 mg kg^{-1}) exceed VLAREBO background concentrations (Table 5.4). In contrast to the relatively high amounts of total Pb, Cd, Cu and Zn present in Leadville soil, only limited amounts appeared $CaCl_2$ extractable. $CaCl_2$ extractable Pb (13 mg kg^{-1} *vs* 1.3 mg kg^{-1}), Cd (1.6 mg kg^{-1} *vs* 0.14 mg kg^{-1}) and Zn (19 mg kg^{-1} *vs* 5 mg kg^{-1}) only slightly passed the concentrations found on Zutendaal reference soil (Table 5.8). The soil has a high CEC (26.5 cmol kg^{-1}) and a pH of 7.5.

Amendment addition did not significantly alter soil pH; CA+ST significantly increased conductivity (Table 5.8). CA+ST (-31%) and PR (-23%) significantly decreased $CaCl_2$ extractable Pb, while CA+ST (-11%) and PR (-14%) significantly decreased $CaCl_2$ extractable Cd. CA+ST significantly decreased $CaCl_2$ extractable Zn (-30%). Addition of CA+ST significantly increased $CaCl_2$ extractable Mn (+762%).

Plant response: Cu content (36.6 mg kg^{-1}) in maize leaves grown on unamended New Orleans soil rose above the CTC (27 mg kg^{-1}). CA+ST and lime decreased Cu content down to 28.6 (-22%), and 28.2 mg kg^{-1} (-23%), still slightly above the CTC. Zn content in maize leaves decreased upon CA+ST addition (-23%). The concentration of other elements did not substantially change after amendment addition. All amendments exceeded the CTC once (Cu).

Maize plants grown on unamended New Orleans soil produced the highest biomass from all soils, even exceeding maize biomass on all other amendment treated soils. Similarly, *B. napus*, *T. pratense* and *L. perenne* developed substantial above-ground plant biomass. Consequently, New Orleans soil did not appear phytotoxic. Amendment addition did not substantially increase biomass of any of the 4 plant species used.

Joplin

Soil: Since mid 1800s, Pb and Zn mining and smelting activities have taken place in Jasper County, MO (USA). These operations elevated metal concentrations across much of the area (about 6500 ha), including Joplin. The soil sample is taken at Center Creek, north of Web City in Jasper. The ore body in Joplin consisted primarily of PbS; the sample has been milled (not smelted) and an unknown part of the PbS is assumed to be oxidized (S. Brown, oral communication).

Table 5.8: Soil and plant data on unamended, CA+ST, PR and lime amended New Orleans soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in New Orleans and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	7.5 (0.1)^{ab}	7.5 (0.0)^{ab}	7.4 (0.0)^{a}	7.6 (0.0)^{b}		
conductivity	1617 (154)^{a}	2537 (572)^{b}	1740 (92)^{ab}	1807 (234)^{ab}		
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	13 (0)^{b}	9 (1)^{a}	10 (1)^{a}	13 (1)^{b}	1.33	
Cd	1.6 (0.0)^{b}	1.4 (0.0)^{a}	1.4 (0.0)^{a}	1.7 (0.0)^{b}	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	0.7 (0.2)	1.0 (0.3)	0.6 (0.1)	0.6 (0.1)		
Cu	0.8 (0.0)	1.3 (0.5)	0.8 (0.0)	1.1 (0.0)	0.00	
Zn	19 (1)^{b}	13 (2)^{a}	17 (2)^{ab}	20 (2)^{b}	5.00	
Fe	12 (5)	11 (3)	9 (2)	10 (1)	36	
Mn	3 (0)^{a}	29 (2)^{b}	2 (0)^{a}	5 (0)^{a}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	5.9	4.4	4.8	4.0	> 30	
Cd	< DL	0.2	0.3	0.3	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	< DL	< DL	< DL	2.2	> 5	
Cu	**36.6**	**28.6**	**37.1**	**28.2**	> 27	< 5
Zn	188	144	212	189	> 1750	< 25
Fe	456	393	298	336	> 750	< 100
Mn	76	102	82	74	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	3.92 (1.32)	3.61 (0.83)	5.51 (0.79)	5.24 (0.51)		
T. pratense	0.295 (0.090)	0.155 (0.046)	0.132 (0.052)	0.251 (0.056)		
L. perenne	0.46 (0.06)	0.35 (0.24)	0.49 (0.18)	0.50 (0.09)		
Z. mays	8.8	10.2	9.1	10.6		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Total Pb (5444 mg kg^{-1}), Cd (89 mg kg^{-1}) and Zn (18204 mg kg^{-1}) are in excess of VLAREBO class I and II cleanup values (Table 5.4). Total Cu slightly passes VLAREBO background values; total Mn is low (8 mg kg^{-1}). $CaCl_2$ extractable Pb (1949 mg kg^{-1} vs 1.3 mg kg^{-1}), Cd (2.8 mg kg^{-1} vs 0.14 mg kg^{-1}) and Zn (472 mg kg^{-1} vs 5.0 mg kg^{-1}) on unamended Joplin soil drastically exceeded concentrations found in Zutendaal reference soil (Table 5.9). Soil pH is 6.6.

<u>Amendment addition</u>: Addition of CA+ST and lime significantly increased soil pH (up to 7.2 resp. 7.7) and conductivity (Table 5.9). All amendments significantly decreased $CaCl_2$ extractable Pb in the following order: PR (-33%) < CA+ST (-85%) < lime (-90%). $CaCl_2$ extractable Cd also significantly decreased with lime (-48%), CA+ST (-59%) and PR (-64%). Amendment addition further significantly decreased $CaCl_2$ extractable Zn, in the order PR (-42%) < CA+ST (-86 %) = lime (-87 %). Lime addition significantly decreased $CaCl_2$ extractable Mn (-81%), while CA+ST significantly increased $CaCl_2$ extractable Mn (+885%).

<u>Plant response</u>: Pb (171 mg kg^{-1}) and Zn (3869 mg kg^{-1}) content in maize leaves harvested on unamended Joplin soil drastically exceeded their respective CTCs (Pb= 30 mg kg^{-1}; Zn= 1750 mg kg^{-1}) (Table 5.9). All amendments substantially decreased Pb content in maize leaves (CA+ST= -65%; PR= -70%; lime= -81%). Lime decreased Pb back to just above the CTC. All amendments further drastically reduced Cd content in maize leaves (PR= -87%; lime= -93%; CA+ST= -97%). In agreement with data from $CaCl_2$ extractable Zn, all amendments substantially decreased Zn content in maize leaves (PR= -55%; lime= -86%; CA+ST= -90%). Addition of CA+ST and lime decreased Zn content in maize below the CTC. Addition of lime substantially decreased Mn content in maize leaves (25 mg kg^{-1}) to a level nearing the CDC (15 mg kg^{-1}). According to the number of concentrations in excess of the CTC, the amendments can be classified as followed: unamended (2) = PR (2) < CA+ST (1) = lime (1).

Amendment addition substantially increased above-ground biomass of maize (PR= +229%; CA+ST= +382%; lime= +465%). The unamended soil further appeared too phytotoxic for *B. napus* and *T. pratense* seedlings, while *L. perenne* plants grew only sporadically. PR significantly increased above-ground biomass of *T. pratense*, while lime significantly increased above-ground biomass of *B. napus* and *T. pratense*. CA+ST significantly increased above-ground biomass of all 3 test plants.

Table 5.9: Soil and plant data on unamended, CA+ST, PR and lime amended Joplin soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in Joplin and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	6.6 (0.0)^a	7.2 (0.0)^b	6.6 (0.1)^a	7.7 (0.0)^c		
conductivity	526 (221)^a	2270 (66)^b	593 (55)^a	1720 (575)^b		
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	1949 (75)^d	296 (17)^b	1299 (123)^c	198 (5)^a	1.33	
Cd	2.8 (0.1)^b	1.2 (0.0)^a	1.0 (0.0)^a	1.5 (0.0)^a	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	0.9 (0.1)^{ab}	1.1 (0.3)^b	0.7 (0.0)^{ab}	0.5^a		
Cu	0.5 (0.0)	0.6 (0.1)	0.4 (0.0)	0.3	0.00	
Zn	472 (16)^c	68 (12)^a	273 (23)^b	63 (0)^a	5.00	
Fe	27 (16)	17 (9)	14 (4)	25 (11)	36	
Mn	1 (0)^b	13 (1)^c	1 (0)^a	0 (0)^a	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	**171.1**	**60.5**	**52.0**	**32.2**	> 30	
Cd	19.4	0.5	2.5	1.3	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	< DL	1.8	< DL	< DL	> 5	
Cu	<DL	14.3	10.2	11.4	> 27	< 5
Zn	**3869**	404	**1756**	529	> 1750	< 25
Fe	NA	402	730	291	> 750	< 100
Mn	95	139	75	25	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	0.00 (0.00)^a	0.96 (0.22)^{bc}	0.34 (0.16)^{ab}	1.10 (0.45)^c		
T. pratense	0.000 (0.000)^a	0.130 (0.056)^c	0.016 (0.009)^b	0.105 (0.045)^{bc}		
L. perenne	0.02 (0.01)^a	0.13 (0.04)^b	0.07 (0.04)^{ab}	0.11 (0.04)^{ab}		
Z. mays	0.9	4.1	2.8	4.8		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Pribram

Soil: A Pb/Zn smelter is located in Pribram, 50 km SW of Prague (Czech Republic) (Chlopecka & Adriano, 1997b, Knox, 2001). Total Pb (2633 mg kg^{-1}), Cd (17.8 mg kg^{-1}), As (368 mg kg^{-1}) and Zn (3173 mg kg^{-1}) in Pribram soil exceed VLAREBO class I and II cleanup values (Table 5.4). Total Ni and Cu exceed VLAREBO background values. From all tested soils, Pribram contains the highest total Mn content (3333 mg kg^{-1}). Knox (2001) recently found that PR (5%) substantially reduced $CaCl_2$ extractable Pb (-60%), Cd (-55%) and Zn (-54%). The authors further observed substantial reductions of Pb in maize roots (-50%) and leaves (-44%) (Chlopecka & Adriano, 1997b). $CaCl_2$ extractable Pb (139 mg kg^{-1} *vs* 1.3 mg kg^{-1}), Cd (12.3 mg kg^{-1} *vs* 0.14 mg kg^{-1}), Zn (618 mg kg^{-1} *vs* 5.0 mg kg^{-1}), and Mn (199 mg kg^{-1} *vs* 3.6 mg kg^{-1}) were substantially increased on unamended Pribram soil relative to Zutendaal reference soil (Table 5.10). Soil pH is 6.1.

<u>Amendment addition</u>: CA+ST and lime significantly increased soil pH (up to 7.0 resp. 7.6); CA+ST significantly increased conductivity (Table 5.10). All amendments significantly decreased $CaCl_2$ extractable Pb, in the order PR (-18%) < lime (-83%) < CA+ST (-91%). All amendments significantly decreased $CaCl_2$ extractable Cd: PR (-12%) < lime (-29%) < CA+ST (-38%). $CaCl_2$ extractable As was below detection of the ICP-MS (< 0.004 mg kg^{-1}). $CaCl_2$ extractable Zn is significantly decreased as follows: PR (-28%) < lime (-78%) < CA+ST (-86%).

Addition of lime significantly decreased $CaCl_2$ extractable Ni (-59%), while CA+ST and lime significantly decreased $CaCl_2$ extractable Mn (by 75% resp. 57%). Addition of PR significantly increased $CaCl_2$ extractable Mn (+34%).

<u>Plant response</u>: Mn content in maize leaves grown on unamended Pribram soil exceeded the CTC (1003 mg kg^{-1} *vs* 200 mg kg^{-1}) (Table 5.10). Addition of CA+ST (-86%) and lime (-84%) drastically reduced Mn content below the CTC; PR further increased Mn in leaves up to 1567 mg kg^{-1}. Both CA+ST and lime also substantially reduced Zn content in maize leaves (CA+ST= -80%; lime= -69%). Amendment addition did not substantially alter Pb, Cd or Fe content, while CA+ST increased Cu content above the CTC (28.5 mg kg^{-1} *vs* 27 mg kg^{-1}). According to the number of concentrations in excess of the CTC, the amendments can be classified as followed: unamended (1) = CA+ST (1) = PR (1) < lime (0).

Above-ground biomass of maize substantially increased in case PR was added (+69%). PR addition did not significantly increase above-ground biomass of any of the OECD test plants. CA+ST significantly increased above-ground biomass of *B. napus* (+259%) and *T. pratense* (+517%). Addition of lime significantly increased above-ground biomass of *B. napus* (+786%), *T. pratense* (+1014%) and *L. perenne* (+103%).

Table 5.10: Soil and plant data on unamended, CA+ST, PR and lime amended Pribram soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in Pribram and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	6.1 (0.0)^a	7.0 (0.0)^b	6.2 (0.0)^a	7.6 (0.0)^c		
conductivity	597 (94)^{ab}	1733 (49)^c	665 (51)^b	479 (25)^a		
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	139 (4)^d	13 (1)^a	114 (1)^c	24 (2)^b	1.33	
Cd	12.3 (0.2)^d	7.6 (0.5)^a	10.9 (0.3)^c	8.8 (0.4)^b	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	1.4 (0.1)^b	1.4 (0.4)^b	1.3 (0.0)^b	0.6 (0.1)^a		
Cu	0.3 (0.0)	0.3 (0.0)	0.4 (0.0)	0.4 (0.1)	0.00	
Zn	618 (9)^d	85 (13)^a	445 (21)^c	135 (2)^b	5.00	
Fe	12 (5)	8 (6)	10 (2)	12 (3)	36	
Mn	199 (2)^c	50 (8)^a	269 (5)^d	86 (3)^b	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	4.5	4.7	4.2	6.4	> 30	
Cd	7.3	6.3	5.8	3.9	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	1.5	NA	NA	1.0	> 5	
Cu	15.7	**28.5**	17.4	20.4	> 27	< 5
Zn	1410	287	1067	443	> 1750	< 25
Fe	339	572	254	337	> 750	< 100
Mn	**1003**	141	**1567**	162	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	0.37 (0.12)^a	1.33 (0.17)^b	0.76 (0.30)^a	3.28 (0.13)^c		
T. pratense	0.036 (0.009)^a	0.222 (0.094)^b	0.074 (0.033)^{ab}	0.401 (0.088)^c		
L. perenne	0.31 (0.07)^a	0.34 (0.17)^{ab}	0.30 (0.10)^a	0.63 (0.08)^b		
Z. mays	4.5	5.0	7.5	4.9		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Hoboken

<u>Soil</u>: The Hoboken soil sample is collected in Hoboken (Belgium), a region where a lead smelter contaminated a lot of terrains in the past. Total Pb (656 mg kg^{-1}), Cd (2.2 mg kg^{-1}), As (75 mg kg^{-1}) and Zn (766 mg kg^{-1}) exceed VLAREBO class I and II cleanup values while Ni (36 mg kg^{-1}) and Cu (155 mg kg^{-1}) contents exceed VLAREBO background values (Table 5.4). $CaCl_2$ extractable Pb (27 mg kg^{-1} *vs* 1.3 mg kg^{-1}), Cd (2.0 mg kg^{-1} *vs* 0.14 mg kg^{-1}), Cu (18.9 mg kg^{-1} *vs* 0.0 mg kg^{-1}), Zn (310 mg kg^{-1} *vs* 5.0 mg kg^{-1}) and Mn (36 mg kg^{-1} *vs* 3.65 mg kg^{-1}) on unamended

Hoboken soil were substantially higher than in the Zutendaal reference soil (Table 5.11). Soil pH at the location where the sample was collected is low (3.7).

Amendment addition: All amendments significantly increased soil pH, in following order: PR (4.2) < CA+ST (4.8) < lime (6.7) (Table 5.11). Amendments significantly decreased $CaCl_2$ extractable Pb with 59% (PR), 74% (CA+ST) and 81% (lime). All amendments further significantly decreased $CaCl_2$ extractable Cd in the following order: PR (-13%) < lime (-27%) < CA+ST (-60%). $CaCl_2$ extractable As was below ICP-MS detection in all cases (< 0.004 mg kg^{-1}). CA+ST (-36%) and lime (-77%) significantly decreased $CaCl_2$ extractable Ni. All amendments significantly decreased $CaCl_2$ extractable Cu: PR (-49%) < CA+ST (-85%) < lime (-97%). All amendments further significantly decreased $CaCl_2$ extractable Zn; PR reduced $CaCl_2$ extractable Zn by 22 %, while CA+ST and lime addition decreased $CaCl_2$ extractable Zn by 88 and 87 % respectively. Lime significantly reduced $CaCl_2$ extractable Mn (-56%), PR had no effect while CA+ST significantly increased $CaCl_2$ extractable Mn (+61%).

Plant response: Ni (15.5 mg kg^{-1}) and Mn (300 mg kg^{-1}) content in maize leaves harvested on unamended Hoboken soil exceeded the CTC (Ni= 5 mg kg^{-1}; Mn= 200 mg kg^{-1}) (Table 5.11). All amendments substantially reduced Ni content in maize leaves (PR= -52%; lime= -69%; CA+ST= -74%). CA+ST and lime reduced Ni below the CTC. CA+ST further increased Mn content in maize leaves (+94%), PR had no effect while lime addition decreased Mn content below the CTC (-48%). Lime further substantially increased Cu content above the CTC (34.9 mg kg^{-1} *vs* 27 mg kg^{-1}). Amendment addition also decreased Cd content: PR (-37%) < CA+ST (-45%) < lime (-65%). Zn content in maize leaves was reduced in the same order: PR (-32%) < CA+ST (-83%) < lime (-87%). Pb content was not reduced after amendment addition; in contrast, amendment addition resulted in increased Pb content (unamended= 2 mg kg^{-1}; CA+ST= 3.4 mg kg^{-1}; PR= 9.4 mg kg^{-1}; lime= 5.0 mg kg^{-1}). Amendment addition further slightly decreased Fe content in maize leaves (lime= -23%; CA+ST= -31%; PR= -45%). According to the number of concentrations in excess of the CTC, the amendments can be classified as followed: unamended (2) = PR (2) < CA+ST (1) = lime (1).

Table 5.11: Soil and plant data on unamended, CA+ST, PR and lime amended Hoboken soil. Soil pH and conductivity, CaCl$_2$ extractable metal concentrations found in Hoboken and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	3.7 (0.0)^{a}	4.8 (0.0)^{c}	4.2 (0.0)^{b}	6.7 (0.0)^{d}		
conductivity	2483 (46)	2570 (147)	2627 (220)	2603 (240)		
CaCl$_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	27 (10)^{c}	7 (1)^{ab}	11 (2)^{b}	5 (3)^{a}	1.33	
Cd	2.0 (0.0)^{d}	0.8 (0.0)^{a}	1.7 (0.0)^{c}	1.5 (0.0)^{b}	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	7.5 (0.2)^{c}	4.8 (1.0)^{b}	7.5 (0.1)^{c}	1.7 (0.0)^{a}		
Cu	18.9 (0.4)^{d}	2.8 (0.3)^{b}	9.7 (0.3)^{c}	0.5 (0.1)^{a}	0.00	
Zn	310 (4)^{c}	36 (6)^{a}	242 (7)^{b}	39 (2)^{a}	5.00	
Fe	15 (5)	29 (11)	9 (2)	11 (2)	36	
Mn	36 (0)^{b}	58 (6)^{c}	33 (0)^{b}	16 (0)^{a}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	2.0	3.4	9.4	5.0	> 30	
Cd	8.1	3.7	5.1	2.8	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	**15.5**	4.0	**7.5**	4.8	> 5	
Cu	20.3	15.9	24.3	**34.9**	> 27	< 5
Zn	1074	179	725	140	> 1750	< 25
Fe	485	333	267	373	> 750	< 100
Mn	**300**	**582**	**312**	155	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	0.08 (0.03)^{a}	0.70 (0.15)^{b}	0.01 (0.01)^{a}	0.98 (0.44)^{b}		
T. pratense	0.000 (0.000)^{a}	0.038 (0.014)^{b}	0.007 (0.002)^{a}	0.045 (0.011)^{b}		
L. perenne	0.05 (0.02)	0.07 (0.04)	0.00 (0.00)	0.08 (0.06)		
Z. mays	4.9	8.9	7.1	6.4		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Amendment addition increased above-ground weight of maize plants in the order: lime (+29%) < PR (+43%) < CA+ST (+81%). PR addition did not significantly alter above-ground biomass of the OECD test plants. Addition of CA+ST and lime significantly increased above-ground biomass of *B. napus* (+775% resp. +1125%) and *T. pratense* (no plants grew at the unamended Hoboken soil).

Moreno Valley

Soil: The soil sample is taken from Moreno Field station, Moreno Valley, California (University of California); the soil has been extensively treated with sewage sludge (approx. 1080 tons ha^{-1}). Total Pb (656 mg kg^{-1}) and Cd (7.7 mg kg^{-1}) in Moreno Valley soil exceed VLAREBO class I and II cleanup values while total Ni (65 mg kg^{-1}), Cu (147 mg kg^{-1}), and Zn (438 mg kg^{-1}) are above the VLAREBO background level (Table 5.4). $CaCl_2$ extractable metal concentrations were low (extractable Pb and Cu were not substantially increased): only $CaCl_2$ extractable Cd (3.2 mg kg^{-1} *vs* 0.14 mg kg^{-1}) and Cu (30 mg kg^{-1} *vs* 5.0 mg kg^{-1}) were slightly elevated relative to the Zutendaal reference sample (Table 5.12). Soil pH is 7.4.

Amendment addition: Addition of lime significantly increased soil pH (7.9), while PR significantly decreased pH (7.2) (Table 5.12). CA+ST had no effect on pH but significantly increased soil conductivity. All amendments significantly decreased $CaCl_2$ extractable Pb in the order PR (-13%) < lime (-40%) < CA+ST (-67%). CA+ST (-24%) and lime (-9%) significantly decreased $CaCl_2$ extractable Cd. $CaCl_2$ extractable As was below detection of the ICP-MS in all cases (< 0.004 mg kg^{-1}). CA+ST (-33%) and lime (-35%) significantly decreased $CaCl_2$ extractable Ni. Amendment addition did not significantly alter $CaCl_2$ extractable Cu. All amendments however significantly decreased $CaCl_2$ extractable Zn, in the order PR (-20%) < lime (-49%) < CA+ST (-71%). Addition of CA+ST significantly increased $CaCl_2$ extractable Mn (+630%).

Plant response: Cu content in leaves of maize harvested on unamended Moreno Valley soil slightly exceeded the CTC (27.9 mg kg^{-1} *vs* 27 mg kg^{-1}) (Table 5.12). PR slightly reduced Cu content (-8%) while CA+ST (-36%) and lime (-39%) substantially decreased Cu in maize leaves (below the CTC). All amendments further increased Fe content in maize (CA+ST= +79%; PR= +146%; lime= +209%), exceeding the CTC. PR further increased Pb content (+229%) in maize while CA+ST increased Ni content (+68%). Cd, Zn and Mn content were not substantially altered after amendment addition. According to the number of concentrations in excess of the CTC, the amendments can be classified as followed: unamended (1) = PR (1) = CA+ST (1) = lime (1).

Amendment addition did not significantly alter above-ground biomass of maize or any of the test plants used in the OECD test.

Table 5.12: Soil and plant data on unamended, CA+ST, PR and lime amended Moreno Valley soil. Soil pH and conductivity, CaCl$_2$ extractable metal concentrations found in Moreno Valley and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	7.4 (0.0)^{b}	7.4 (0.0)^{b}	7.2 (0.0)^{a}	7.9 (0.0)^{c}		
conductivity	418 (46)^{a}	1570 (200)^{b}	705 (95)^{a}	824 (222)^{a}		
CaCl$_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	1.5 (0.1)^{d}	0.5 (0.1)^{a}	1.3 (0.0)^{c}	0.9 (0.2)^{b}	1.33	
Cd	3.2 (0.1)^{c}	2.4 (0.1)^{a}	3.2 (0.0)^{c}	2.9 (0.1)^{b}	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	4.7 (0.1)^{b}	3.1 (0.6)^{a}	4.6 (0.1)^{b}	3.0 (0.2)^{a}		
Cu	0.9 (0.0)	0.6 (0.0)	1.0 (0.0)	1.3 (0.7)	0.00	
Zn	30 (1)^{d}	9 (1)^{a}	24 (2)^{c}	15 (1)^{b}	5.00	
Fe	13 (5)	13 (0)	11 (2)	24 (12)	36	
Mn	4 (0)^{a}	27 (2)^{b}	2 (0)^{a}	3 (0)^{a}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	1.4	2.4	4.6	1.4	> 30	
Cd	1.5	1.1	1.4	1.1	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	2.8	4.7	< DL	< DL	> 5	
Cu	**27.9**	17.9	25.7	17.0	> 27	< 5
Zn	86	73	125	105	> 1750	< 25
Fe	422	**757**	**1037**	**1303**	> 750	< 100
Mn	86	109	72	72	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	2.00 (1.37)	1.37 (0.77)	1.27 (0.59)	1.86 (0.39)		
T. pratense	0.146 (0.091)	0.182 (0.089)	0.049 (0.019)	0.263 (0.120)		
L. perenne	0.24 (0.13)	0.26 (0.20)	0.05 (0.02)	0.21 (0.09)		
Z. mays	3.7	4.5	4.8	3.5		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Leadville

<u>Soil</u>: the town Leadville (Colorado, USA) got its name in 1875, when 2 prospectors found that the heavy sands left by former gold miners contained high Pb contents imbued with a pyritic ore base. Since then, over 2000 mines were established to

extract Pb but also Cu and Zn (Dunn, 1998; Eusden et al., 2002). Leadville and surroundings got highly contaminated with Pb and other metals which at present still forms a reason for health concern. The soil sample used in our study is taken at a farmers field in Lake County, south of Leadville (S. Brown, oral communication). This area received large quantities of trace metals through direct deposition of mine tailings during active hydraulic mining and through application of contaminated irrigation water (Levy et al., 1992).

Total concentrations of Pb (3857 mg kg^{-1}), Cd (18.0 mg kg^{-1}), As (164 mg kg^{-1}), Cu (491 mg kg^{-1}) and Zn (3060 mg kg^{-1}) in the soil exceed VLAREBO class I and II cleanup values (Table 5.4). Total Fe is elevated (51047 mg kg^{-1} *vs* 38000 mg kg^{-1} on average for non contaminated soils). $CaCl_2$ extractable Pb (1002 mg kg^{-1} *vs* 1.3 mg kg^{-1}), Cd (15.6 mg kg^{-1} *vs* 0.14 mg kg^{-1}), Cu (24 mg kg^{-1} *vs* 0.0 mg kg^{-1}), Zn (1657 mg kg^{-1} *vs* 5.0 mg kg^{-1}), and Mn (634 mg kg^{-1} *vs* 3.65 mg kg^{-1}) were substantially increased on unamended Leadville soil relative to the values obtained on the Zutendaal reference soil (Table 5.13). Soil pH is low (4.1).

<u>Amendment addition</u>: All amendments significantly increased soil pH: PR (4.7) < CA+ST (5.0) < lime (6.1) (Table 5.13). All amendments significantly reduced $CaCl_2$ extractable Pb in the following order: PR (-43%) < CA+ST (-64%) < lime (-89%). CA+ST (-25%) and PR (-28%) also significantly decreased $CaCl_2$ extractable Cd. As was below detection of the ICP-MS (< 0.004 mg kg^{-1}). $CaCl_2$ extractable Cu was significantly reduced in the order: PR (-67%) = CA+ST (-76%) < lime (-97%). $CaCl_2$ extractable Zn was significantly reduced in the order PR (-43%) < lime (-68%) = CA+ST (-71%).

CA+ST did not significantly alter $CaCl_2$ extractable Mn, while PR (-40%) and lime (-18%) significantly decreased $CaCl_2$ extractable Mn. CA+ST significantly increased $CaCl_2$ extractable Ni (+121%).

Fig. 5.2: Farmers field in Lake County, south of Leadville, Colorado (USA), contaminated through direct deposition of mine tailings during active hydraulic mining and through application of contaminated irrigation water.

<u>Plant response</u>: Zn (7491 mg kg^{-1}) and Mn (4624 mg kg^{-1}) content in maize leaves harvested on unamended Leadville soil drastically exceeded their respective CTCs (Zn= 1750 mg kg^{-1}; Mn= 200 mg kg^{-1}) (Table 5.13). Due to severe toxicity only limited plant biomass was available for ICP-MS analysis on unamended soil (Table 5.13): Pb, Cu and Fe concentrations were below detection limit probably due to the fact that not enough biomass could be digested. All amendments substantially reduced Zn content in the order PR (-61%) < CA+ST (-77%) < lime (-89%). In case of CA+ST, Zn content equalled the CTC (1757 mg kg^{-1}) while with lime Zn content was reduced below the CTC (795 mg kg^{-1}). All amendments further substantially reduced Mn in the order CA+ST (-22%) < PR (-36%) < lime (-62%). Even lime application however could not reduce Mn (1768 mg kg^{-1}) below the CTC (200 mg kg^{-1}). CA+ST (30.7 mg kg^{-1}) and lime (36.3 mg kg^{-1}) addition resulted in Cu contents in maize leaves exceeding the CTC (27 mg kg^{-1}), while PR increased Fe (838 mg kg^{-1}) above the CTC (750 mg kg^{-1}). Maize leaves harvested on unamended Leadville soil also contained high levels of Cd (61.4 mg kg^{-1}). All amendments substantially reduced this concentration down to 21.7 mg kg^{-1}

(CA+ST= -65%), 16.9 mg kg^{-1} (PR= -72%) and 8.7 mg kg^{-1} (lime= -86%). Lime addition resulted in the lowest Pb content in maize leaves. According to the number of concentrations in excess of the CTC, the amendments can be classified as follows: unamended (too phytotoxic) < CA+ST (3) = PR (3) < lime (2).

Amendment addition drastically increased above-ground maize biomass from 0.3 g (unamended) up to 3.2 g (PR) and 5.2 g (lime) and 5.7 g (CA+ST). None of the 3 OECD species survived on unamended Leadville soil. PR addition slightly improved growth of *L. perenne* while CA+ST slightly increased above-ground biomass of *B. napus* and *L. perenne*. Lime however was the only amendment which significantly increased above-ground biomass of all OECD species.

Table 5.13: Soil and plant data on unamended, CA+ST, PR and lime amended Leadville soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in Leadville and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	4.1 (0.1)^{a}	5.0 (0.0)^{c}	4.7 (0.0)^{b}	6.1 (0.0)^{d}		
conductivity	3847 (959)	3583 (108)	3163 (90)	3717 (64)		
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	1002 (28)^{d}	364 (21)^{b}	569 (13)^{c}	108 (2)^{a}	1.33	
Cd	15.6 (0.4)^{b}	11.7 (0.2)^{a}	11.2 (0.2)^{a}	15.2 (0.3)^{b}	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	2.2 (0.1)^{a}	4.8 (1.1)^{b}	1.7 (0.1)^{a}	0.9 (0.1)^{a}		
Cu	24 (1)^{c}	6 (0)^{b}	8 (0)^{b}	1 (0)^{a}	0.00	
Zn	1657 (45)^{c}	480 (73)^{a}	948 (32)^{b}	537 (15)^{a}	5.00	
Fe	19 (12)	20 (11)	13 (4)	14 (2)	36	
Mn	634 (21)^{c}	583 (49)^{bc}	382 (11)^{a}	519 (17)^{b}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	NA	14.1	11.3	6.8	> 30	
Cd	61.4	21.7	16.9	8.7	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	< DL	6.8	< DL	2.8	> 5	
Cu	NA	**30.7**	19.0	**36.3**	> 27	< 5
Zn	**7491**	**1757**	**2920**	795	> 1750	< 25
Fe	NA	281	**838**	322	> 750	< 100
Mn	**4624**	**3593**	**2981**	**1768**	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	0.00 (0.00)^{a}	0.12 (0.07)^{a}	0.00 (0.00)^{a}	0.75 (0.11)^{b}		
T. pratense	0.000 (0.000)^{a}	0.000 (0.000)^{a}	0.000 (0.000)^{a}	0.055 (0.027)^{b}		
L. perenne	0.00 (0.00)^{a}	0.04 (0.03)^{a}	0.07 (0.06)^{a}	0.12 (0.04)^{b}		
Z. mays	0.3	5.7	3.2	5.2		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Aiken

Soil: an uncontaminated soil from Aiken, SC (USA) was spiked with different Pb forms [$PbSO_4$ (40%), $PbCO_3$ (25%), PbO (20%), $PbCl_2$ (15%)]. Total Pb (678 mg kg^{-1}) and Cd (2.7 mg kg^{-1}) exceed VLAREBO class I and II cleanup value; total Zn (482 mg kg^{-1}) exceeds the VLAREBO background value (Table 5.4). Chlopecka & Adriano (1997a) found that addition of lime (1%) and PR (2.5%) increased maize yields by 59 % resp. 73 %, while both amendments reduced Pb content in rye tissue by resp. 40 % and 57 %. $CaCl_2$ extractable Pb (166 mg kg^{-1} *vs* 1.3 mg kg^{-1}), Cd (0.6 mg kg^{-1} *vs* 0.14 mg kg^{-1}) and Zn (40 mg kg^{-1} *vs* 5.0 mg kg^{-1}) were elevated on unamended Aiken soil relative to the Zutendaal reference soil (Table 5.14). Soil pH is 6.6.

Amendment addition: Soil pH significantly increased upon CA+ST (7.0) and lime (7.8) addition (Table 5.14). Soil conductivity significantly increased after CA+ST addition. CA+ST (-83%) and lime (-52%) significantly decreased $CaCl_2$ extractable Pb while PR significantly increased $CaCl_2$ extractable Pb (+25%). CA+ST (-86%) and lime (-64%) also significantly reduced $CaCl_2$ extractable Zn. CA+ST significantly enhanced $CaCl_2$ extractable Mn (+387%).

Plant response: Fe content (1134 mg kg^{-1}) in maize leaves grown on unamended Aiken soil exceeded the CTC (750 mg kg^{-1}). PR substantially increased Fe in maize leaves (up to 1949 mg kg^{-1},= + 72%), while CA+ST (349 mg kg^{-1}) substantially reduced Fe below the CTC. In agreement with $CaCl_2$ data, strong decreases in Pb content were observed after CA+ST (-55%) and lime (-64%) addition. All amendments also decreased Mn content in maize leaves by 57-61%. According to the number of concentrations in excess of the CTC, the amendments can be classified as follows: unamended (1) = PR (1) = lime (1) < CA+ST (0).

CA+ST substantially increased above-ground maize biomass (+94%), while PR decreased above-ground biomass (-65%). Amendment addition did not significantly alter above-ground biomass of any of the OECD test plants.

Table 5.14: Soil and plant data on unamended, CA+ST, PR and lime amended Aiken soil. Soil pH and conductivity, CaCl$_2$ extractable metal concentrations found in Aiken and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	6.6 (0.0)^{b}	7.0 (0.0)^{c}	6.3 (0.0)^{a}	7.8 (0.0)^{d}		
conductivity	277 (18)^{a}	1417 (70)^{b}	369 (31)^{a}	339 (13)^{a}		
CaCl$_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	166 (1)^{c}	29 (3)^{a}	208 (17)^{d}	80 (3)^{b}	1.33	
Cd	0.6 (0.0)	0.5 (0.0)	0.6 (0.0)	0.6 (0.0)	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	0.7 (0.0)	1.0 (0.3)	0.8 (0.3)	0.5		
Cu	0.2	0.2 (0.0)	0.2 (0.0)	0.6 (0.4)	0.00	
Zn	40 (2)^{c}	5 (0)^{a}	34 (3)^{c}	14 (2)^{b}	5.00	
Fe	16 (8)	11	11 (2)	15 (3)	36	
Mn	4 (0)^{a}	19 (2)^{b}	6 (1)^{a}	3 (0)^{a}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	14.5	6.5	12.8	5.2	> 30	
Cd	< DL	< DL	< DL	< DL	> 79	
As	< DL	< DL	< DL	< DL	> 30	
Ni	< DL	< DL	< DL	< DL	> 5	
Cu	10.6	9.9	<DL	10.1	> 27	< 5
Zn	129	86	175	154	> 1750	< 25
Fe	**1134**	349	**1949**	**1232**	> 750	< 100
Mn	174	74	70	68	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	0.69 (0.15)	0.63 (0.04)	0.74 (0.28)	0.86 (0.31)		
T. pratense	0.069 (0.029)	0.083 (0.031)	0.049 (0.032)	0.087 (0.033)		
L. perenne	0.16 (0.08)	0.07 (0.03)	0.06 (0.05)	0.14 (0.06)		
Z. mays	3.1	6.0	1.1	2.3		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

Bazoches

Soil: The soil sample is taken 100 m from a former battery breaking site. Total Pb content (598 mg kg^{-1}) exceeds VLAREBO class I and II cleanup values while total Ni (24 mg kg^{-1}) slightly exceeds VLAREBO background values (Table 5.4). CaCl$_2$ extractable Pb (1.3 mg kg^{-1} *vs* 1.3 mg kg^{-1}), as well as other metal concentrations on unamended Bazoches soil, were not elevated relative to the Zutendaal reference soil (Table 5.15). Soil pH is 8.1.

Amendment addition: Addition of lime significantly increased soil pH (8.3); CA+ST (7.7) and PR (7.9) slightly reduced pH (Table 5.15). CA+ST significantly increased soil conductivity. Amendment addition did not significantly reduce $CaCl_2$ extractable Pb. $CaCl_2$ extractable As was below detection in all cases (< 0.004 mg kg^{-1}). CA+ST significantly increased $CaCl_2$ extractable Mn (+3200%).

Table 5.15: Soil and plant data on unamended, CA+ST, PR and lime amended Bazoches soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in Bazoches and Zutendaal (reference) soil (in mg kg^{-1}), metal concentrations in maize leaves (in mg kg^{-1} DW), average above-ground biomass of *Brassica napus*, *Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil), average above-ground biomass of maize (g FW per kg soil).

	unamended	**CA+ST**	**PR**	**lime**		
pH	8.1 (0.0)^{c}	7.7 (0.0)^{a}	7.9 (0.0)^{b}	8.3 (0.0)^{d}		
conductivity	196 (83)^{a}	1597 (81)^{b}	374 (29)^{a}	257 (77)^{a}		
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**	
Pb	1.3 (0.3)	2.2 (0.7)	1.5 (0.0)	1.4 (0.1)	1.33	
Cd	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.14	
As	< 0.004	< 0.004	< 0.004	< 0.004		
Ni	0.5 (0.0)	1.0 (0.4)	0.5	0.4		
Cu	< 0.002	0.2 (0.0)	0.2 (0.0)	< 0.002	0.00	
Zn	1 (0)	1 (0)	2 (1)	1 (0)	5.00	
Fe	19 (9)	15 (7)	10 (2)	12 (3)	36	
Mn	1 (0)^{a}	16 (3)^{b}	1 (0.0)^{a}	1 (0)^{a}	3.65	
Element content maize leaves (mg kg^{-1} DW)					**CTC***	**CDC***
Pb	5.1	3.6	16.0	NA	> 30	
Cd	< DL	< DL	< DL	NA	> 79	
As	< DL	< DL	< DL	NA	> 30	
Ni	1.9	1.3	< DL	NA	> 5	
Cu	10.9	12.1	12.8	NA	> 27	< 5
Zn	36	50	47	NA	> 1750	< 25
Fe	329	270	**1500**		> 750	< 100
Mn	122	142	135	NA	> 200	< 15
Average above-ground plant biomass (g) per pot (100 g soil)						
B. napus	1.57 (0.24)^{ab}	1.73 (0.36)^{b}	0.55 (0.34)^{a}	0.61^{ab**}		
T. pratense	0.234 (0.206)	0.329 (0.102)	0.000 (0.000)	0.125		
L. perenne	0.33 (0.14)^{ab}	0.42 (0.03)^{b}	0.06 (0.07)^{a}	0.25^{ab}		
Z. mays	8.0	7.5	3.1	NA		

* Concentrations in maize leaves exceeding CTC of CDC are marked.

** Due to the limited amount of soil sample, only one replicate was used for the OECD test on lime amended Bazoches soil. For the same reason, maize plants were not sewn. Consequently, no data on metal analysis are available for lime amended Bazoches soil.

Plant response: None of the measured elements exceeded any CTC in leaves of maize grown on unamended Bazoches soil (Table 5.15). Amendment addition did not substantially alter metal uptake, unless in case of PR application where Pb (+214%) and Fe (+356%) content in maize leaves increased. PR addition elevated

Fe content in maize above the CTC (1500 mg kg^{-1} *vs* 750 mg kg^{-1}). According to the number of concentrations in excess of the CTC, the amendments can be classified as followed: PR (1) < unamended (0) = CA+ST (0) = lime (0).

CA+ST addition did not substantially alter above-ground maize biomass, while PR drastically decreased above-ground weight of maize plants (-61%). None of the amendments significantly altered biomass of the OECD test plants relative to plants grown on unamended Bazoches soil.

Fig. 5.3: Sample location Belin-Beliet (France), Pb contaminated from battery breaking.

Belin-Beliet

Soil: The soil sample is taken on a grassland located 100 m from a former lead battery reclamation facility, which closed operation several years ago. Pb sulphate and silica-bound Pb were found to be the predominant Pb forms present (Manceau et al., 1996). Total soil Pb (1859 mg kg^{-1}) exceeds VLAREBO class I and II cleanup value; total Cu (160 mg kg^{-1}) and Zn (160 mg kg^{-1}) exceed VLAREBO background values (Table 5.4). $CaCl_2$ extractable Pb (940 mg kg^{-1} *vs* 1.3 mg kg^{-1}), Zn (55 mg kg^{-1} *vs* 5.0 mg kg^{-1}), Cd (0.8 mg kg^{-1} *vs* 0.14 mg kg^{-1}) and Mn (16 mg kg^{-1} *vs* 3.65 mg kg^{-1}) were elevated in comparison to the Zutendaal reference soil (Table 5.16). Soil pH is 7.0.

Table 5.16: Soil and plant data on unamended, CA+ST, PR and lime amended Belin-Beliet soil. Soil pH and conductivity, $CaCl_2$ extractable metal concentrations found in Paimboeuf and Zutendaal (reference) soil (in mg kg^{-1}), above-ground biomass of *Brassica napus, Trifolium pratense* and *Lolium perenne* (in g FW per 100 g soil).

	unamended	**CA+ST**	**PR**	**lime**	
pH	7.0 (0.0)^{a}	7.3 (0.0)^{b}	7.1 (0.1)^{a}	8.4 (0.0)^{c}	
ccnductivity	109 (54)^{a}	1210 (221)^{b}	155 (8)^{a}	193 (26)^{a}	
$CaCl_2$-extractable elements (mg kg^{-1})					**Zutendaal (Ref.)**
Pb	940 (113)^{d}	241 (30)^{a}	757 (22)^{c}	344 (6)^{b}	1.33
Cd	0.8 (0.1)^{c}	0.5 (0.0)^{a}	0.7 (0.0)^{bc}	0.6 (0.0)^{ab}	0.14
As	< 0.004	< 0.004	< 0.004	< 0.004	
Ni	0.7 (0.1)	1.1 (0.4)	0.6 (0.1)	0.5 (0.0)	
Cu	1.7 (0.1)^{b}	3.6 (0.3)^{c}	1.5 (0.1)^{b}	0.6 (0.1)^{a}	0.00
Zn	55 (6)^{c}	5 (1)^{a}	31 (2)^{b}	5 (0)^{a}	5.00
Fe	18 (2)	10 (9)	12 (2)	19 (2)	36
Mn	16 (2)^{b}	22 (3)^{c}	9 (0)^{a}	6 (0)^{a}	3.65
Average above-ground plant biomass (g) per pot (100 g soil)					
B. napus	1.26	1.62	0.95	1.52	
T. pratense	0.100	0.237	0.14	0.279	
L. perenne	0.20	0.24	0.22	0.30	

<u>Amendment addition</u>: Addition of CA+ST (7.3) and lime (8.4) significantly increased soil pH; CA+ST significantly increased conductivity (Table 5.16). All amendments significantly decreased $CaCl_2$ extractable Pb in the order PR (-19%) < lime (-63%) < CA+ST (-74%). $CaCl_2$ extractable Zn was significantly decreased in the order PR (-44%) < CA+ST = lime (-91%). Lime significantly reduced $CaCl_2$ extractable Cu (-64%). CA+ST (-37%) and lime (-24%) significantly decrease $CaCl_2$ extractable Cd. PR (-46%) and lime (-65%) significantly decreased $CaCl_2$ extractable Mn. CA+ST significantly increased $CaCl_2$ extractable Cu (+120%) and Mn (+35%).

Plant response: No maize material was grown due to the limited amount of the soil sample. For the same reason only one replicate could be used for the OECD test. From these data it appeared that CA+ST and lime has a positive effect on above-ground biomass of *T. pratense*.

3.4. Amendment induced metal immobilization evaluated per metal

In Table A3.1 (Appendix 3) significant changes in $CaCl_2$ extractable elemental concentrations are given for the examined elements and amendments (relative to the unamended). The right column summarizes the average alteration in $CaCl_2$ extractability caused by the amendment (expressed in %). Table A3.2 (Appendix 3) summarizes alterations in metal content in maize leaves, harvested after 4 weeks on 9 soils (all except Belin-Beliet).

Pb

Amendment addition significantly decreased $CaCl_2$ extractable Pb on most soils. PR and lime significantly reduced $CaCl_2$ extractable Pb on 8 soils; CA+ST on 9. None of the applied amendments significantly altered $CaCl_2$ extractable Pb on Bazoches soil. $CaCl_2$ extractable Pb concentration on unamended Bazoches soil however was extremely low (only 0.2% of the total Pb), resulting in concentrations comparable to uncontaminated Zutendaal soil [1 mg Pb kg^{-1} (Bazoches) *vs* the 1.3 mg kg^{-1} (Zutendaal)]. This might be due to the presence of a relatively insoluble Pb species, in combination with a high soil pH (8.1, CEC=28.0 cmol kg^{-1}). In contrast to PR and CA+ST, lime addition did not significantly alter $CaCl_2$ extractable Pb on New Orleans soil, a soil already high in pH (7.5). PR addition significantly increased $CaCl_2$ extractable Pb on Aiken soil (+25%).

On all other unamended soils, following $CaCl_2$ extractable Pb concentrations were measured: 2 mg kg^{-1} (Moreno Valley), 13 mg kg^{-1} (New Orleans), 27 mg kg^{-1} (Hoboken), 44 mg kg^{-1} (Paimboeuf), 139 mg kg^{-1} (Pribram), 166 mg kg^{-1} (Aiken), 940 mg kg^{-1} (Belin-Beliet), 1002 mg kg^{-1} (Leadville), and 1949 mg kg^{-1} (Joplin). Addition of PR resulted in the lowest decreases in $CaCl_2$ extractable Pb. Only on

Hoboken soil $CaCl_2$ extractable Pb was reduced with more than 60 % (-61%). Lime and CA+ST significantly reduced $CaCl_2$ extractable Pb by more than 60% on 6 (all soils except New Orleans, Moreno Valley, Aiken, Bazoches) resp. 8 (all soils except New Orleans and Bazoches) soils. Water extractions showed that CA released small amounts of Pb (Table 5.6). On none of the tested soils, CA+ST significantly increased $CaCl_2$ extractable Pb. On average, $CaCl_2$ extractable Pb was reduced in following order: **PR (-30%) < CA+ST (-77%) ≤ lime (-82%)**. Comparable results were found when a specific bacterial Pb biosensor was applied (Appendix 4).

Pb content in maize leaves harvested on unamended Joplin soil (171 mg kg^{-1}) passed the CTC (30 mg kg^{-1}); amendment addition drastically reduced this concentration down to 60 mg kg^{-1} (CA+ST), 52 mg kg^{-1} (PR) and 32 mg kg^{-1} (lime). On other soils, amendment addition in general decreased Pb content as well (Paimboeuf, New Orleans, Aiken). In case Pb was low (Hoboken=2 mg kg^{-1}, Moreno Valley=1.4 mg kg^{-1}) reductions were less pronounced; in some cases even slight increases were observed. On average, amendments could be classified according to Pb reducing content in maize as follows: **PR (-56%) = CA+ST (-59%) < lime (-73%).**

Cd

On 3 soils (Paimboeuf=0.1 mg kg^{-1}; Aiken= 0.6 mg kg^{-1}; Bazoches= 0.3 mg kg^{-1}), $CaCl_2$ extractable Cd was below or slightly exceeded background concentrations for uncontaminated soils (0.14 mg Cd kg^{-1}). Here, amendment addition did not significantly alter $CaCl_2$ extractable Cd, except on Paimboeuf soil (PR= +84 %; from 0.1 mg kg^{-1} to 0.3 mg kg^{-1}).

Following $CaCl_2$ extractable Cd concentrations were found on other soils: 0.8 mg kg^{-1} (Belin-Beliet), 1.6 mg kg^{-1} (New Orleans), 2.0 mg kg^{-1} (Hoboken), 2.8 mg kg^{-1} (Joplin), 3.2 mg kg^{-1} (Moreno Valley), 12.3 mg kg^{-1} (Pribram) and 15.6 mg kg^{-1} (Leadville). Amendment addition significantly reduced $CaCl_2$ extractable Cd on 5 (PR, lime), and all 7 (CA+ST) of these soils. PR was ineffective on Moreno Valley and Belin-Beliet soil; lime did not significantly alter $CaCl_2$ extractable Cd on New Orleans soil and Leadville soil, where the highest $CaCl_2$ extractable Cd was measured on unamended soil (15.6 mg kg^{-1}).

On average relatively low reductions in Cd extractability were observed upon amendment addition: **lime (-16%) < PR (-21%) < CA+ST (-33%)**. On several soils higher reductions were observed e.g. Joplin ($CaCl_2$ extractable Cd unamended= 2.8 mg kg^{-1}; CA+ST= -59%; PR= -64%; lime= -47%) or Hoboken ($CaCl_2$ extractable Cd unamended= 2.0 mg kg^{-1}; CA+ST=-60%).On other soils, where $CaCl_2$ extractable Cd was particularly high [e.g. Pribram (12.3 mg Cd kg^{-1}), Leadville (15.6 mg kg^{-1})], only lower reductions were observed [Pribram: -38% (CA+ST), -12% (PR), -29% (lime); Leadville: -25% (CA+ST); -28%(PR), no significant reduction with lime]. The success of Cd immobilization appears to substantially depend on factors such as metal speciation and soil characteristics.

Cd content in maize leaves never exceeded the CTC (79 mg kg^{-1}). Amendment addition decreased Cd content in maize with more than 15 % in all cases, except on Pribram (CA+ST= -14%) and Moreno Valley (PR= -7%) soil. On average, Cd content in maize leaves was reduced by **66% (CA+ST) = 68% (PR) < 82% (lime)**.

As

$CaCl_2$ extractable As was below detection of the ICP-MS on all soils (< 0.004 mg kg^{-1}).

Ni

On 6 soils, $CaCl_2$ extractable Ni was below 1 mg kg^{-1} (Paimboeuf=0.7 mg kg^{-1}, New Orleans=0.7 mg kg^{-1}, Joplin=0.9 mg kg^{-1}, Aiken=0.7 mg kg^{-1}, Bazoches=0.5 mg kg^{-1}, Belin-Beliet=0.7 mg kg^{-1}). On these soils, amendment addition did not significantly alter Ni extractability, except on Paimboeuf soil where CA+ST increased $CaCl_2$ extractable Ni (from 0.7 mg kg^{-1} up to 1.6 mg kg^{-1}).

$CaCl_2$ extractable Ni was above 1 mg kg^{-1} on 4 soils (Pribram=1.4 mg kg^{-1}, Hoboken=7.5 mg kg^{-1}, Moreno Valley=4.7 mg kg^{-1}, Leadville=2.2 mg kg^{-1}). PR did not significantly alter $CaCl_2$ extractable Ni on any of the tested soils. Lime significantly reduced $CaCl_2$ extractable Ni on Pribram (from 1.4 mg kg^{-1} to 0.6 mg kg^{-1}), Hoboken (7.5 mg kg^{-1} to 1.7 mg kg^{-1}) and Moreno Valley (4.7 mg kg^{-1} to 3.0 mg kg^{-1}) but not on Leadville soil. CA+ST significantly reduced $CaCl_2$ extractable Ni on Hoboken (from 7.5 mg kg^{-1} to 4.8 mg kg^{-1}) and Moreno Valley soil (from 4.7 mg kg^{-1} to 3.1 mg kg^{-1}). CA+ST however significantly increased $CaCl_2$ extractable Ni on Paimboeuf (from 0.7 mg kg^{-1} up to 1.6 mg kg^{-1}) and Leadville soil (from 2.2 mg kg^{-1} to 4.8 mg kg^{-1}) soil; probably Ni is released by ST during oxidation as was also observed by Mench et al. (2002). According to Ni reducing effectiveness, the amendments can be arranged as follows: **CA+ST (+6%) = PR (-4%) < lime (-54%)**.

Only from one soil, Ni uptake data were available for all amendments (Hoboken). Here, Ni content in maize leaves was **reduced by 52% (PR)**, **69% (lime)** and **74% (CA+ST)**.

Cu

On 5 soils, $CaCl_2$ extractable Cu was below 1 mg kg^{-1} (Joplin=0.5 mg kg^{-1}, Pribram=0.3 mg kg^{-1}, Moreno Valley=0.9 mg kg^{-1}, Aiken=0.2 mg kg^{-1}, Bazoches < 0.002 mg kg^{-1}). Amendment addition did not significantly alter $CaCl_2$ extractable Cu on these soils.

$CaCl_2$ extractable Cu equalled or exceeded 1 mg kg^{-1} on following soils: Paimboeuf (1.0 mg kg^{-1}), New Orleans (0.8 mg kg^{-1}), Belin-Beliet (1.7 mg kg^{-1}), Hoboken (18.9 mg kg^{-1}) and Leadville (24.2 mg kg^{-1}). All amendments significantly decreased $CaCl_2$ extractable Cu on Paimboeuf (CA+ST= -37%; PR= -37%; lime= -68%), Hoboken (PR= -49%; CA+ST= -85%; lime= -98%) and Leadville (PR= -67%; CA+ST= -76%; lime= -98%) soil. None of the amendments significantly altered Cu extractability on New Orleans soil. On Belin-Beliet soil, PR did not significantly alter $CaCl_2$ extractable Cu; lime significantly decreased Cu extractability (from 1.7 mg kg^{-1} to 0.6 mg kg^{-1}) while CA+ST significantly increased $CaCl_2$ extractable Cu (from 1.7 to 3.6 mg kg^{-1}). In order of Cu immobilizing effectiveness, the amendments can be classified as follows: **PR (-48%) ≤ CA+ST (-61%) < lime (-88%).**

Cu content in maize showed huge variation depending on amendment and soil. In most cases, PR addition did not influence Cu uptake while increases as well as decreases in Cu uptake were observed after CA+ST and lime addition. The CTC (27 mg kg^{-1}) was exceeded 3 times with CA+ST, 2 times with PR and one time with lime, although concentrations never were drastically elevated. In 3 cases Cu content stayed below 30 mg kg^{-1} (New Orleans with CA+ST= 28.6 mg kg^{-1} and lime 28.2 mg kg^{-1}; Pribram with CA+ST= 28.5 mg kg^{-1}) while slightly higher values were observed on Paimboeuf (CA+ST=35.9 mg kg^{-1}) and New Orleans (PR=37.1 mg kg^{-1}) soil. On average, Cu content in maize was not altered (CA+ST= +5%; PR= +1%; lime= -6%).

Zn

$CaCl_2$ extractable Zn is elevated on all soils except Bazoches (1 mg kg^{-1}), relative to the uncontaminated reference soil (5 mg kg^{-1}). On Bazoches soil, amendment addition did not significantly alter $CaCl_2$ extractable Zn.

Following Zn concentrations were extracted on unamended soils (using $CaCl_2$): 9 mg kg^{-1} (Paimboeuf), 19 mg kg^{-1} (New Orleans), 30 mg kg^{-1} (Moreno Valley), 40 mg kg^{-1} (Aiken), 55 mg kg^{-1} (Belin-Beliet), 310 mg kg^{-1} (Hoboken), 472 mg kg^{-1} (Joplin), 618 mg kg^{-1} (Pribram) and 1657 mg kg^{-1} (Leadville). CA+ST significantly reduced $CaCl_2$ extractable Zn on all (9) these soils; lime significantly reduced $CaCl_2$ extractable Zn on 8 soils (all except New Orleans) while PR significantly reduced $CaCl_2$ extractable Zn on 6 soils (all except Paimboeuf, New Orleans, Aiken).

The highest reduction in $CaCl_2$ extractable Zn after PR addition was –44% (on Belin-Beliet soil). CA+ST and lime significantly reduced $CaCl_2$ extractable Zn by more than 60 % on 7 soils. The amendments can be arranged in following order relative to their Zn immobilizing potential: **PR (-37%) < lime (-75%) ≤ CA+ST (-79%)**. Both CA+ST and lime showed a high Zn immobilizing efficiency, of equal order as the reductions found for Pb (CA+ST= -77%, lime= -82%).

In agreement with the strong reductions observed in $CaCl_2$ extractions, Zn content in maize was drastically reduced after amendment addition. Addition of CA+ST and lime reduced Zn by more than 76 % on 5 resp. 4 soils. PR and lime addition only slightly increased Zn uptake on Moreno Valley and Aiken soil. In case of CA+ST, the CTC (1750 mg kg^{-1}) was once slightly exceeded on one soil (1757 mg kg^{-1}; Leadville). PR addition exceeded the CTC 2 times (1756 mg kg^{-1} on Joplin; 2920 mg kg^{-1} on Leadville), while Zn content did not exceed the CTC after lime addition. According to average reductions observed in maize uptake, amendments can be ordered as follows: **PR (-51%) < CA+ST (-79%) = lime (-83%).**

Fe

$CaCl_2$ extractable Fe concentrations found on the 10 unamended soils did not pass background concentrations found on an uncontaminated reference soil (36 mg kg^{-1}). None of the applied amendments significantly altered $CaCl_2$ extractable Fe in any of the tested soils, indicating that the Fe released by ST precipitated. On average **CA+ST and lime reduced $CaCl_2$ extractable Fe by 9%**, while **PR reduced $CaCl_2$ extractable Fe by 29%**[4].

On most soils, amendment addition reduced Fe uptake. Fe content exceeded the CTC (750 mg kg^{-1}) 3 times with PR (Paimboeuf=1548 mg kg^{-1}, Moreno Valley= 1037 mg kg^{-1}; Aiken=1949 mg kg^{-1}), 2 times with lime (Moreno Valley= 1303 mg kg^{-1}, Aiken= 1232 mg kg^{-1}) and 1 time with CA+ST (Moreno Valley= 757 mg kg^{-1}). Fe uptake was reduced in following order: **PR (-10%) < lime (-30%) < CA+ST (-49%)**.

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[4] It might be surprising that, on average, PR reduces $CaCl_2$ extractable Fe by 29%, and that in none of the soils this decrease was significant. In general however, Fe analysis resulted in higher differences between replicates (higher standard deviations).

Mn

On 5 soils, $CaCl_2$ extractable Mn was substantially higher than concentrations observed on an uncontaminated reference soil (Paimboeuf=41 mg kg^{-1}, Pribram=200 mg kg^{-1}, Hoboken=36 mg kg^{-1}, Leadville=635 mg kg^{-1}, Belin-Beliet=16 mg kg^{-1}) (Zutendaal reference soil=3.65 mg kg^{-1}). Lime significantly reduced $CaCl_2$ extractable Mn on all these soils; PR significantly reduced $CaCl_2$ extractable Mn on all soils except Pribram (significant increase: +35%) and Hoboken (no alteration). The highest $CaCl_2$ extractable Mn was found on Pribram and Leadville soil. Addition of CA+ST significantly reduced $CaCl_2$ extractable Mn on Pribram soil while no alteration was observed on Leadville soil. On all other 8 soils, CA+ST addition significantly increased $CaCl_2$ extractable Mn: Paimboeuf (from 41 mg kg^{-1} to 65 mg kg^{-1}), New Orleans (from 3 mg kg^{-1} to 29 mg kg^{-1}), Joplin (from 1 mg kg^{-1} to 13 mg kg^{-1}), Hoboken (from 36 mg kg^{-1} to 58 mg kg^{-1}), Moreno Valley (from 4 mg kg^{-1} to 27 mg kg^{-1}), Aiken (from 4 mg kg^{-1} to 19 mg kg^{-1}), Bazoches (from 1 mg kg^{-1} to 16 mg kg^{-1}), Belin-Beliet (from 16 mg kg^{-1} to 22 mg kg^{-1}). The fact that $CaCl_2$ extractable Mn increased upon ST addition, while no alteration in Fe extractability was observed is due to the fact that Fe precipitates at low pH (3.5) than Mn does (5.5) (M. Mench, oral communication).

On average, CA+ST reduced $CaCl_2$ extractable Mn by 6%; this means that the reduced amount of Mn (in mol) observed on Pribram soil (from 199 mg kg^{-1} to 50 mg kg^{-1}) is almost equal to the increased amounts of Mn on the 9 other soils.

Mn content in maize leaves exceeded the CTC (200 mg kg^{-1}) on 4 unamended soils (Paimboeuf, Pribram, Hoboken, Leadville). Addition of CA+ST dropped Mn content in maize grown on Paimboeuf (-15%), Pribram (-86%), and Leadville (-22%) soil; on 3 soils Mn in maize however still passed the CTC (Paimboeuf, Hoboken, Leadville). Concerning the other soils, CA+ST increased Mn uptake on 3 soils (New Orleans, Joplin, Moreno Valley), while on Aiken soil Mn uptake was reduced. Addition of PR in general reduced Mn uptake, but on 4 soils the CTC was still exceeded (Paimboeuf, Pribram, Hoboken, Leadville). Mn in maize was most efficiently reduced by lime; only on Leadville soil Mn content in maize (1768 mg kg^{-1}) exceeded the CTC. On average, amendments can be arranged as follows according to reduction in Mn uptake: **PR (-17%) < CA+ST (-24%) < lime (-65%)**.

3.5. Plant response

In general, amendment addition improved above-ground plant biomass of the OECD plant species grown on the Pb contaminated soils (Table 5.17). PR addition significantly increased above-ground biomass of *B. napus* and *T. pratense* on Joplin soil. CA+ST addition significantly increased plant growth on Joplin (*B. napus, T. pratense, L. perenne*), Pribram (*B. napus, T. pratense*) and Hoboken (*B. napus, T. pratense*) soil. PR (*L. perenne*) and CA+ST (*B. napus, L. perenne*) also allowed plant growth on Leadville soil, in contrast to the unamended soil which appeared severely phytotoxic (Table 5.12) Lime addition significantly increased above-ground plant biomass on Joplin (*B. napus, T. pratense*), Pribram (*B. napus, T. pratense, L. perenne*), Hoboken (*B. napus, T. pratense, L. perenne*) and Leadville (*B. napus, T. pratense, L. perenne*) soil.

Table 5.17: Number of soils where plant biomass was not significantly altered and soils where significant increases in above-ground plant biomass were observed for *B. napus*, *T. pratense*, *L. perenne* (g). Above-ground biomass of *Zea mays* could not be statistically analysed due to the limited sample size (n=2); changes are mentioned in case above-ground biomass increased with more than 50 % relative to the unamended soil. No data could be obtained for Bazoches (*Z. mays*) and Belin-Beliet (*Z. mays*, 3 OECD species) soil, due to limited sample size. In case no plant growth occurred on unamended soils, increase could not be expressed in %.

		No significant change	**increase in above-ground biomass**
B. napus	CA + ST	6	Joplin, Pribram(+260%), Hoboken(+697%)
	PR	8	Joplin
	lime	5	Joplin, Pribram(+787%), Hoboken(+1081%), Leadville
T. pratense	CA + ST	6	Joplin, Pribram(+514%), Hoboken
	PR	8	Joplin
	lime	5	Joplin, Pribram(+1012%), Hoboken, Leadville
L. perenne	CA + ST	8	Joplin
	PR	9	
	lime	6	Pribram(+100%), Hoboken, Leadville
Z. mays	CA + ST	3	Paimboeuf(+780%), Joplin(+381%), Hoboken(+81%), Leadville(+1734%), Aiken(+92%)
	PR	4	Paimboeuf(+403%), Joplin(+226%), Pribram(+68%), Leadville(+950%)
	lime	5	Paimboeuf(+664%), Joplin(+464%), Leadville(+1565%)

On some soils, it is clear that this increased plant growth can be attributed to amendment induced immobilization of toxic metal concentrations. Unamended Joplin and Leadville soil clearly were phytotoxic; none of the OECD test plants survived here (except a few individuals of *L. perenne* on Joplin) while clear phytotoxicity symptoms were observed on *Z. mays* (dwarf growth, chlorosis) (Table 5.9, 5.13). Pb (171 mg kg^{-1} *vs* CTC=30 mg kg^{-1}) and Zn (3869 mg kg^{-1} *vs* CTC=1750 mg kg^{-1}) content in maize leaves harvested on unamended Joplin soil and Zn (7491 mg kg^{-1} *vs* CTC=1750 mg kg^{-1}) and Mn (4624 mg kg^{-1} *vs* CTC=200 mg kg^{-1}) content in maize leaves grown on unamended Leadville soil drastically exceeded their respective CTC.

On the other hand, it should be noted that cyclonic ash, phosphate rock and lime contain and release substantial amounts of Ca, Mg, and other essential elements (K, Na, P) (Table 5.18). Release of essential elements by amendments will in general increase plant growth; it also increases the plants' vitality and consequently its defence against metal induced stress.

Table 5.18: Total and water extractable concentrations (mg kg^{-1}) of Ca, Mg, K, Na, P and S in cyclonic ash, steelshot, North Carolina phosphate rock and lime.

	Ca	Mg	K	Na	P	S
Total content (mg kg^{-1})						
cyclonic ash	46376	8280	11018	2362	91	13344
steelshot	205	< 1	62	< 1	107	403
phosphate rock	358029	3593	1271	7160	126703	19444
lime	302886	50646	2081	214	80	5295
Water extractable (mg kg^{-1})						
cyclonic ash	13286	0,2	127	26	< 0,5	5056
steelshot	7	< 0,1	2	< 0.1	0,7	1
phosphate rock	614	69	34	102	4	217
lime	43	278	35	16	< 0,5	7

4. DISCUSSION

Table 5.19 resumes the observed alterations in $CaCl_2$ extractability (average over 10 soils, in %), 4 months after amendment addition. In a first column, the alterations are given as observed under the studied conditions, i.e. with addition of

5% CA + 1% ST, 1% PR (except Joplin and Leadville, 2%) and 1% lime. On average, both lime and the combination of CA+ST drastically reduced $CaCl_2$ extractable Pb (-82% resp. -77%), Zn (-75% resp. -79%) and Cu (-88% resp. -61%). The observed reductions in metal mobility are in agreement with published data on metal immobilization upon CA, ST or lime addition (Adriaenssens et al., 1999; Chlopecka & Adriano, 1996; Geebelen et al., 2002; Mench et al. 1994ab, 1997, 2000, Vangronsveld et al., 1995a,b, 1996, 1999, 2000). Our data showed that in case $CaCl_2$ extractable Pb, Zn and Cu concentrations were elevated, addition of CA+ST and lime significantly reduced metal extractability, indicating that these amendments can successfully be applied to a wide range of metal contaminated soils. The good results obtained with lime ($CaCO_3$) underline the key role soil pH plays in determining metal bioavailability. It should be noted that in case original soil pH was relatively high [e.g. New Orleans (7.5)] lime appeared less effective in decreasing metal mobility, as $CaCO_3$ dissolution is limited in calcareous soils (Osté, 2001). Here, amendments with other working mechanisms (like CA+ST) can be used. CA is known to substantially increase soil pH as well in a first phase (due to the presence of CaO and MgO), while coprecipitation associated with Al, Fe and Mn oxides (CA, ST) and crystal growth and metal diffusion into the mineral surface (CA) have been suggested as well (Mench et al., 1998).

In general, the applied amendments were less effective in reducing $CaCl_2$ extractable Cd (lime= -16%; PR= -21%; CA+ST= -33%). On some soils substantial amounts of Cd were immobilized [e.g. Joplin: -59% (CA+ST); -64% (PR); -48% (lime)] while on others (e.g. Leadville where $CaCl_2$ extractable Cd= 15.6 mg kg^{-1}), only low reductions were observed [-25% (CA+ST); -28% (PR); no significant reduction with lime]. The Cd immobilizing efficacy of the treatments appeared to substantially differ between soils and probably depends to a substantial degree on Cd speciation and soil characteristics.

Table 5.19: Average alteration in $CaCl_2$ extractable metal concentration (calculated using data from 10 soils) in % after amendment addition. Averages are expressed per kg soil (with application of 5 % CA+1%ST, 1% PR (2% on Joplin + Leadville), 1 % lime) and per 10 g (1%) amendment used. % alteration in metal content observed in maize leaves upon amendment addition (Ni uptake data are only based on 1 soil (Hoboken)) and number of times the CTC and TC[*] are exceeded.

		% Δ kg^{-1} soil	**% Δ 10 g^{-1} amendm.**	**% Δ uptake**	**> CTC**	**> TC**
Pb	CA+ST	-77	-13	-59	1	
	PR	-30	-17	-56	1	
	lime	-82	-82	-73	1	
Cd	CA+ST	-33	-5	-66		
	PR	-21	-13	-68		
	lime	-16	-16	-82		
Ni	CA+ST	+6	1	(-74)		
	PR	-4	-2	(-52)	1	
	lime	-54	-34	(-69)		
Cu	CA+ST	-61	-10	5	3	
	PR	-48	-32	1	1	
	lime	-88	-80	-6	2	
Zn	CA+ST	-79	-13	-79	1	1
	PR	-37	-23	-51	2	2
	lime	-75	-75	-83		
Fe	CA+ST	-9	-2	-49	1	1
	PR	-29	-24	-10	3	3
	lime	-9	-9	-30	2	2
Mn	CA+ST	-6	-1	-24	3	3
	PR	-22	-9	-17	4	4
	lime	-31	-31	-65	1	1

* CT (Critical values) are less sensitive than CTC (further explained in the text)

$CaCl_2$ extractable As was below DL of the ICP-MS in all cases (0.004 mg kg^{-1}). CH_3COOH (first step of BCR sequential extraction schedule as described by Rauret et al., 1999) extractable As was low on most soils. Addition of PR however substantially increased CH_3COOH extractable As on several soils (Joplin: 0.17 mg kg^{-1} *vs* 0.01 mg kg^{-1} on unamended soil; Pribram: 23.4 mg kg^{-1} *vs* 2.2 mg kg^{-1}; Hoboken: 6.33 mg kg^{-1} *vs* 0.34 mg kg^{-1}; Leadville: 1.10 mg kg^{-1} *vs* 0.14 mg kg^{-1}; Belin-Beliet: 1.35 mg kg^{-1} *vs* 0.35 mg kg^{-1}), probably due to PO_4^{3-} - AsO_4^{3-} competition for the same sorption sites (Woolson, 1983; Peryea, 1991). Addition of CA+ST significantly decreased CH_3COOH extractable As on several soils (New Orleans: 0.15 mg kg^{-1} *vs* 0.42 mg kg^{-1} on unamended soil; Pribram: 1.34 mg kg^{-1} *vs*

2.16 mg kg^{-1}; Bazoches: 0.19 mg kg^{-1} *vs* 0.86 mg kg^{-1}), probably due to As binding on Fe oxides (Fendorf et al., 1997; Manning et al., 1998).

PR addition (1% on all soils except 2 % on Joplin and Leadville soil) resulted in the lowest reductions in $CaCl_2$ extractable Pb (-30%), Zn (-37%), Cu (-48%). It was expected that higher amounts of Pb would be immobilized 4 months after PR addition due to higher PO_4^{3-} release, relative to batch studies where PR was only equilibrated with soil for 48 h. In comparison with batch studies however, lower $CaCl_2$ extractable Pb amounts were found in pot experiments (Table 5.20). Moreover, PR addition did not significantly alter $CaCl_2$ extractable Pb on 2 soils (Aiken, Bazoches) while reductions in Pb mobility of 80% resp. 58% were observed using batch studies. In the batch studies a soil sample of 2 g was mixed with 30 ml of water; the relatively high volume of water might have influenced PR dissolution, in comparison to the pot experiments. Essential nutrient uptake by maize did not reach deficiency levels after PR addition. Water extractions further revealed that only low amounts of PO_4^{3-} are released by PR (Table 5.17). Possibly a substantial part of the metal load might have been bound by sorption processes, explaining the high reductions in metal mobility found with water extractions (in batch studies), and the lower reductions observed with $CaCl_2$. From this point of view, water extractions might not be a good indicator for evaluating PR induced Pb immobilization.

When alterations in $CaCl_2$ extractable metal mobility were recalculated and expressed as if all amendments were applied using the same % (1%), differences between CA+ST and PR declined. Moreover, PR appeared slightly more efficient in immobilizing $CaCl_2$ extractable Pb (PR= -17% *vs* CA+ST= -13%), Cd (PR= -13 % *vs* CA+ST= -5%), Cu (PR= -23% *vs* CA+ST= -10%) and Zn (PR= -23% *vs* CA+ST= -13%) per 10 g amendment added (Table 5.19). Knox et al. (2001) recently reported substantially higher reductions in $CaCl_2$ extractability for Pb (-60%), Cd (-55%), and Zn (-54%) on Pribram soil after addition of 5% PR as were found in our study using 1 % PR (Pb= -18%; Cd= -12%; Zn= -28%) (Table 5.10), indicating that addition of higher PR amounts increases the amount of immobilized metals.

Table 5.20: Alterations in extractable Pb (in %) upon PR addition (1 % for all soils except Joplin and Leadville where 2 % is used) in batch studies (extracted with water) and pot experiments (extracted with 1 *M* $CaCl_2$). NS = difference is not significant ($p < 0.05$).

	Batch (H_2O)	**Pot ($CaCl_2$)**
Paimboeuf	-82	-41
New Orleans	--	-23
Joplin	-61	-33
Pribram	-48	-18
Hoboken	-99	-59
Moreno Valley,	-66	-13
Leadville	-44	-43
Aiken	-80	NS
Bazoches	-58	NS
Belin-Beliet	NA	-19

Table 5.19 further summarizes the % alteration observed in metal content found in maize leaves. All amendments drastically reduced Pb, Cd and Zn concentrations in maize leaves. Amendment addition also decreased Ni uptake (CA+ST= -74%, PR= -52%, lime= -69%) although these data are only based on one soil (Hoboken). No reduction in Cu uptake was observed (CA+ST= +5%, PR= -1%, lime= -6%), despite the substantial reductions in $CaCl_2$ extractability (CA+ST= -61%, PR= -48%, lime= -88%).

The relation between $CaCl_2$ extractable metal concentrations and metal concentrations observed in maize plants was examined using linear regression. Data analysis revealed that maize uptake data were not normally distributed, even after performing a log transformation, limiting the use of regression analysis. Fig. 5.4 plots both parameters for Pb, Cd, Zn and Cu. In general, elevated $CaCl_2$ extractable soil metal concentrations result in elevated metal concentrations in maize (e.g. Pb). It is however clear that several other factors influence plant metal uptake, such as plant nutritional status, plant biomass, substrate toxicity (possibly caused by other metal ions),… This is illustrated for Cu, where substantial differences in maize leaf content (10-37 mg Cu kg^{-1} DW) were observed at comparable $CaCl_2$ extractable Cu concentrations in soil. It should further be noted that Pb and Cd are non-essential plant elements and are of no use to the plant, while Zn and Cu are essential for the normal functioning of plants.

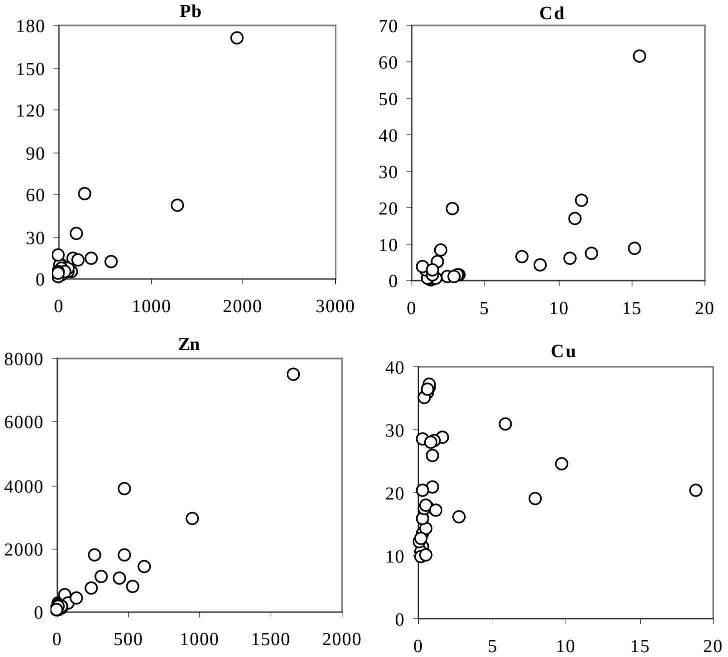

Fig. 5.4: $CaCl_2$ extractable metal concentrations in soil (mg kg^{-1}) (X-axis) *vs* metal concentrations in leaves of 5 weeks old maize plants (mg kg^{-1} DW) (Y-axis); n=36 (Zn), 34 (Pb), 32 (Cu), 23 (Cu).

Plant metal uptake increased with increasing $CaCl_2$ extractable Zn concentration; no such relationship was found for Cu. Probably, $CaCl_2$ extractable Cu were too low to substantially increase plant uptake. In a few cases, Cu concentrations exceeded the CTC (27 mg kg^{-1} DW) (6 times). In our study however the most sensitive treshold concentration was used (Mocquot et al., 1996 mentioned a treshold range varying between 27-52 mg kg^{-1}). None of the observed Cu concentrations passed the upperlevel of this range (52 mg kg^{-1})[5].

Table 5.19 shows the number of times metal concentrations exceeded their respective CTC concentrations in maize leaves. Amendment addition reduced the

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[5] In contrast, Zn content in maize leaves drastically exceeded its CTC (1750 mg kg^{-1} DW) on several soils (e.g. 3869 mg kg^{-1} DW in leaves harvested on unamended Joplin soil, 7491 mg kg^{-1} DW in leaves harvested on Leadville soil).

number of CTC exceedings in following order: **unamended (12) = PR (12) < CA+ST (9) < lime (6)**. As already mentioned, we used the most sensitive critical concentrations mentioned in literature, i.e. 5 mg Ni kg^{-1} [Juste & Mench (1992) mention 5-10 mg Ni kg^{-1}], 27 mg Cu kg^{-1} [Moucquot et al. (1996) mention 27-52 mg kg^{-1}], 30 mg Pb kg^{-1} [Kabata-Pendias & Pendias (1992) mention 30-300 mg kg^{-1})] (Table 5.3). In case the average values from these ranges (i.e. 7.5 mg Ni kg^{-1}, 40 mg Cu kg^{-1}, 165 mg Pb kg^{-1}) were used as CTC [referred to in Table 5.19 as Toxicity Concentration (TC) in stead of CTC], following reductions in TC were observed upon amendment addition: **unamended (10) < PR (9) < CA+ST (5) < lime (3)** (Table 5.19). Following elements exceeded the TC: **unamended [Pb, Ni, Zn (2 times), Fe (2), Mn (4)] < PR [Zn (2), Fe (3), Mn (4)] < CA+ST [Zn, Fe, Mn (3)] < lime [Fe(2), Mn].**

Our experiments were performed in pots, 4 months after amendment addition. In order to apply these amendments on a large scale, it is necessary to examine the effects of amendment addition in a longer time span, and in a more realistic situation, using lysimeter and field experiments. Laboratory simulation studies indicate stable immobilization of Zn and Cd for 80 years upon CA addition (Wessolek and Fahrenhorst, 1994), while a field experiment on a highly metal contaminated acid sandy soil (Maatheide, Belgium; 20 000 mg Zn kg^{-1}) shows that – 12 years after addition of cyclonic ash – pH of the soil (7.3-7.4) is still the same as was observed 2 and 5 years after addition (Vangronsveld et al., 1996). Addition of cyclonic ash to a terrain contaminated with Cd and Ni due to farmyard manure application, showed that plant Cd availability decreased with elapsed time, –23 % (first year), -32 % (second year), -66 % (third year) (Vangronsveld et al., 2000). Cyclonic ash application has also been suggested to induce the formation of metal silicates in sandy soils (Hargé, 1997). Few long term field data are available on ST immobilization but the available studies (up to 5 years) suggest stable metal immobilization (Vangronsveld et al., 2000). On the other hand, PO_4^{3-} induced pyromorphite [$Pb_5(PO_4)_3X$ where X= OH, F or Cl] is known to be the most insoluble form of Pb in soil under a wide range of environmental conditions

(Nriagu, 1974). However, Sayer et al. (1999) recently showed that fungi (*Aspergillus niger)* as well as plants (*Lolium perenne*) were able to mobilize PO_4^{3-} from pyromorphite when pyromorphite was the only PO_4^{3-} source available in the growth medium, resulting in the formation of less stable Pb compounds (Pb oxalate). In case of lime, amendment addition should be repeated in time to maintain metal immobilization (Knox et al., 2000).

5. CONCLUSIONS

In this chapter the effects of amendment addition [5% CA + 1% ST, 1% PR (except Joplin, Leadville; 2%), 1% lime] on metal mobility in 10 Pb contaminated soils was examined. Three research goals were set: (1) evaluate the immobilizing efficacy of the amendments for potentially toxic elements such as Pb, Cu, Zn, As and Cd, (2) examine if soil amendments contribute to the release of toxic elements, (3) examine if soil treatment affects availability of essential plant elements due to too strong immobilization.

(1) In general, amendment addition reduced $CaCl_2$ extractable Pb, Cd, Cu and Zn concentrations in soil. Pb, Cu and Zn were more efficiently immobilized with lime (Pb= -82%, Cu= -88%, Zn= -75%) and CA+ST (Pb= -77%, Cu= -61%, Zn= -79%) than in case PR was used (Pb= -30%, Cu= -48%, Zn= -37%). Amendments were less effective in reducing $CaCl_2$ extractable Cd (lime= -16%; PR= -21%; CA+ST= -33%); Cd immobilization substantially differed between soils and is probably to a large degree determined by the present metal species and soil characteristics. Lime was the only amendment that efficiently reduced $CaCl_2$ extractable Ni (-54%). $CaCl_2$ extractable Ni was below detection of the ICP-MS but CH_3COOH extractions indicated that PR addition can mobilize substantial amounts of As. When immobilization was expressed as mol reduction per 10 gram (1%) of amendment added, PR equalled and in some cases even was more effective than CA+ST. The applied North Carolina PR only released low amounts of PO_4^{3-}; the

addition of higher amounts of PR (5-10%) probably will substantially increase metal immobilization. Metal uptake by maize drastically reduced upon amendment addition: on average, all amendments decreased Pb, Cd, Zn and Ni uptake by more than 50% (Table 5.19). The number of times the CTC values were exceed was drastically reduced in the order PR < CA+ST < lime (Table 5.19); plant growth substantially increased on amended soils.

(2) Amendment analysis revealed that total metal concentrations were elevated in some amendments, e.g. Pb in ST (75 mg kg^{-1}) and CA (38 mg kg^{-1}), Cd in ST and PR (both 60 mg kg^{-1} in PR) and Ni (591 mg kg^{-1}), Cu (834 mg kg^{-1}), Fe (92%), Mn (7715 mg kg^{-1}) and Cr (1289 mg kg^{-1}) in ST (Table 5.6). Metal concentrations were below ICP-MS detection in almost all cases when water was used as extractant: only small amounts of Pb (CA), Fe (CA, ST, PR) and Mn (ST) were detected. On a limited amount of soils, CA+ST application significantly increased $CaCl_2$ extractable Ni (Paimboeuf, Leadville) and Cu (Belin-Beliet), while $CaCl_2$ extractable Mn significantly increased on 8 soils. These increases occurred on soils which in general were low in $CaCl_2$ extractable Ni, Cu or Mn and did not induce accumulation of phytotoxic amounts of these metals in maize (i.e. concentrations exceeding the CTC). When elevated $CaCl_2$ extractable concentrations of the metal in question were present in unamended soils, CA+ST significantly reduced phytoavailable Ni (Hoboken, Moreno Valley), Cu (Paimboeuf, Hoboken, Leadville) and Mn (Pribram). In former experiments, Mn concentrations released by ST were efficiently bound by CA (beringite). The applied CA in this study appeared to be less efficient in binding Mn. In minor cases, PR addition significantly increased $CaCl_2$ extractable Pb (Aiken), Cd (Paimboeuf) and Mn (Pribram); here also, this increase was too low to substantially influence plant uptake. Addition of lime did not increase metal phytoavailability on any of the examined soils.

(3) Amendment addition did not significantly alter $CaCl_2$ extractable Fe while $CaCl_2$ extractable Cu (CA+ST, PR, lime), Zn (CA+ST, PR, lime) and Mn (PR, lime) were reduced in case concentrations were elevated in unamended soils (Table

5.15). In case normal concentrations (not elevated) were present, amendment addition in general did not reduce these levels. In none of the examined soils, amendment addition reduced essential metal concentrations in maize leaves below the Critical Deficiency Concentrations (CDC). In contrast, amendments released essential elements (e.g. Ca, Mg) which probably beneficially influenced plant growth.

Our study examined metal immobilization 4 months after amendment addition using pot experiments. Future experiments should focus on assessing long term effects on one hand, while lysimeter and field studies should gain a more realistic insight in order to apply these amendments *in situ* on a large scale.

Since mid 1800s, Pb and Zn mining and smelting activities have taken place in Jasper County, MO (USA). These operations elevated metal concentrations across much of the area (about 6500 ha). Approximately 2600 residential properties have been contaminated with > 800 mg Pb kg^{-1}. Recent surveys (1994, 1995) showed that 14% of the children under 7 years of age living in the historic smelting area had blood Pb levels > 10 µg dL^{-1} (Yang et al., 2001). Such concentrations in general are associated with disturbances in early physical and mental growth and in later intellectual functioning and academic achievement (NAS, 1993). A soil sample was collected in Center Creek, north of Web City in Jasper, County, MO. The sample (in the text referred to as Joplin) contained 5444 mg Pb kg^{-1}, 89 mg Cd kg^{-1} and 18 204 mg Zn kg^{-1}.

CHAPTER VI

Pb, Cd AND As BIOAVAILABILITY IN THE GASTRO-INTESTINAL TRACT AFTER AMENDMENT ADDITION

1. INTRODUCTION

The primary human health risk posed by Pb-contaminated soils is through the direct ingestion of soil by children (Brown & Chaney, 1999). Standard procedures recommended by the U.S. Environmental Protection Agency (EPA) for estimating soil Pb exposure assume that a typical child will ingest 100 mg soil per day and that 30 % of the ingested Pb will be absorbed into the systemic circulation (US EPA, 1990). Elevated blood Pb concentrations can result in high blood pressure, kidney diseases, brain damage, impaired function in peripheral nerve system, reproductive abnormalities, abnormal vitamin D metabolism, coma and even death under certain circumstances (ATSDR, 1993; Lowny et al., 1998). The U.S. National Academy of Sciences states that Pb blood concentrations of around 10 µg dL^{-1} in children are associated with disturbances in early physical and mental growth and in later intellectual functioning and academic achievement (NAS, 1993).

In vivo animal feeding studies are typically used to determine to what degree ingested soil is solubilized in the gastro-intestinal (GI) tract and taken up in the blood (Ruby et al., 1993, 1996). Such studies however have the disadvantage of being expensive and time consuming (Lowney et al., 1998). Ruby et al. (1996) developed an *in vitro* physiologically based extraction test (PBET) for the prediction of metal availability incorporating GI tract parameters representative of a human (including stomach and small intestine pH and chemistry, soil-to-solution ratio, stomach mixing, stomach emptying rates). Results of the PBET correlated linearly with data obtained in *in vivo* studies using rats. The developed procedure however was labor intensive and not suited for the evaluation of large numbers of samples. Brown & Chaney (1999) substantially simplified this *in vitro* method allowing the analysis of large numbers of samples. Results from this modified procedure correlated well with results from animal feeding studies as well as with results obtained using the original *in vitro* procedure (Brown & Chaney, 1999).

This chapter compares the metal immobilizing efficacy of phosphate rock, a mix of cyclonic ash and steelshot, and lime on ten Pb-contaminated soils with different properties, total metal content, and source term of metals. Besides Pb, extractability of other toxic metals (Cd, As) is examined using the simplified PBET *in vitro* procedure proposed by Brown & Chaney (1999). Following definitions will be used:

Metal bioavailability = the fraction of ingested metal that is solubilized in the gastro-intestinal tract and that can be adsorbed into systematic circulation (in mg kg^{-1})

Metal bioaccessibility = the fraction of total ingested metal concentration that is solublized in the GI tract and that can be adsorbed into systematic circulation (bioavailable metal fraction / total metal fraction in soil; expressed in %).

2. MATERIALS & METHODS

Ten Pb-contaminated soils were collected from various locations in the U.S. and Europe. The soil samples reflect eight different sources of Pb enrichment: tetraethyl lead, paint, car exhaust, smelting, sewage sludge biosolid, ore, spike [$PbSO_4$ (40%), $PbCO_3$ (25%), PbO (20%), $PbCl_2$ (15%)], and battery breaking**.** Soil properties are shown in Table 5.1 and 5.4 (former chapter). Soil amendments were applied in the following ratio: cyclonic ash (5%; from different origin than the formerly called beringite) + steelshot (1%), North Carolina phosphate rock [1%; except Joplin and Leadville soil (2%)], and lime (1%). After amendment addition, the soils were wetted and re-equilibrated over a period of 4 months before experimentation.

Metal bioaccessibility was measured using the modified PBET (physiologically based extraction test) procedure described by Brown and Chaney (1999): 0.35 g soil (< 2 mm) was shaken (30 rpm) with 35 ml of synthetic gastric solution (0.4 M glycine; pH 2.2) for 1 h at 37°C in a water bath. Samples were filtered (Whatman 40) and separated in 2 fractions: one for pH measurement, the other for metal analysis. Samples were generated in disposable plastic labware; all chemicals used in this study were of analytical grade or better. Metal analysis was done by ICP-MS; blanks were run for background correction and other sources of error. Multi-element standards were run every 20 samples and recovery was found to be within 100 ± 10 %.

All analysis were done in triplicate. A Kruskal-Wallis test was performed to detect differences between treatments for each measured parameter ($p < 0.05$). When differences were detected, multiple comparisons were performed to separate the means.

3. RESULTS

After soil reaction, soil pH of the extractant remained 2.2 on all soils, and with all treatments (data not shown).

Pb

Proportionally, the highest Pb bioaccessibility was observed on Belin-Beliet (76%), Joplin (61%), and Aiken (56%) soil (Table 6.1). Pb bioaccessibility on other soils varied between 1% (Hoboken) and 32% (Pribram). The highest biovailable Pb concentrations on unamended soils were observed on Joplin (3307 mg kg^{-1}), Belin-Beliet (1415 mg kg^{-1}), New Orleans (1323 mg kg^{-1}), Pribram (848 mg kg^{-1}) and Leadville (464 mg kg^{-1}) soil (Table 6.1).

Phosphate rock was the only additive able to reduce bioavailable Pb. On three soils PBET Pb was significantly reduced (Joplin = -21%, Pribram = -26%, Hoboken= -50%). On other soils, including those with the highest Pb bioaccessibility (Belin-Beliet, Aiken), PR application did not significantly alter Pb bioavailability. CA+ST and lime did not significantly decrease PBET Pb. Lime addition significantly increased PBET Pb on Joplin (+26%) and Leadville (+43%) soil.

Table 6.1: PBET Pb (mg kg^{-1}) on unamended soils and on soils amended with cyclonic ash and steelshot (CA+ST), phosphate rock (PR), and lime (DL=0.02 mg Pb kg^{-1}). Total Pb in soil (mg kg^{-1}) and Pb bioaccessibility (BA= PBET extracted Pb/total Pb; in %) in unamended soil. Values are means, SD (in parentheses); n = 3.

	unamended	**CA + ST**	**PR**	**lime**	**Total Pb**	**% BA**
Paimboeuf	39.1 (2.6)^{ab}	39.4 (5.9)^{ab}	35.9 (3.8)^{a}	47.9 (4.8)^{b}	188	21
New Orleans	1323 (234)	1267 (165)	1185 (131)	1230 (281)	6065	22
Joplin	3307 (204)^{b}	3345 (260)^{b}	2596 (76)^{a}	4158 (366)^{c}	5444	61
Pribram	848 (65)^{b}	800 (118)^{ab}	626 (30)^{a}	801 (94)^{ab}	2663	32
Hoboken	4.64 (0.73)^{b}	3.88 (0.28)^{ab}	2.32 (0.72)^{a}	6.57 (0.94)^{b}	656	1
Moreno Valley	54.1 (5.4)	64.1 (7.5)	62.5 (11.2)	59.9 (17.6)	236	23
Leadville	464 (43)^{a}	536 (40)^{ab}	449 (72)^{a}	662 (32)^{b}	3857	13
Aiken	381 (28)	420 (40)	388 (14)	421 (36)	678	56
Bazoches	164 (10)	164 (14)	135 (10)	152 (9)	598	27
Belin-Beliet	1415 (207)	1427 (225)	1571 (259)	1556 (111)	1859	76

Cd

Bioaccessible Cd was high on most soils; on 5 soils values over 70 % were measured (New Orleans= 93%, Pribram= 72%, Moreno Valley= 70%, Leadville= 70%, Belin-Beliet= 86%) (Table 6.2). On the soil with the highest total Cd content (Joplin= 89 mg Cd kg^{-1}), bioaccessibility was low (4%).

Addition of CA+ST significantly decreased bioaccessible Cd on 3 soils (Joplin= -29%, Hoboken= -57%, Leadville= -18%). PR addition decreased PBET Cd only on Leadville soil (-25%) while slight increases were observed on Paimboeuf (from 0.04 mg kg^{-1} up to 0.24 mg kg^{-1}) and Belin-Beliet (from 0.86 mg kg^{-1} up to 1.27 mg kg^{-1}) soil. Lime addition increased PBET Cd on Leadville soil (from 12.6 mg kg^{-1} up to 14.5 mg kg^{-1}).

Table 6.2: PBET Cd (mg kg^{-1}) on unamended soils and on soils amended with cyclonic ash and steelshot (CA+ST), phosphate rock (PR), and lime (DL= 0.01 mg Cd kg^{-1}). Total Cd in soil (mg kg^{-1}) and Cd bioaccessibility (BA= PBET extracted Cd/total Cd; in %) in unamended soil. Values are means, SD (in parentheses); n = 3.

	unamended	CA + ST	PR	lime	Total Cd	% BA
Paimboeuf	0.04 (0.01)^{a}	0.04 (0.01)^{a}	0.24 (0.01)^{b}	0.05 (0.00)^{a}	0	--
New Orleans	4.66 (0.62)	4.08 (0.34)	4.35 (0.20)	4.27 (0.70)	5	93
Joplin	3.40 (0.21)^{b}	2.42 (0.20)^{a}	3.03 (0.03)^{b}	3.17 (0.28)^{b}	89	4
Pribram	13.0 (1.1)	11.9 (1.7)	10.5 (0.7)	12.8 (1.5)	18	72
Hoboken	1.32 (0.09)^{bc}	0.57 (0.01)^{a}	1.50 (0.06)^{c}	1.07 (0.18)^{b}	2	66
Moreno Valley	5.63 (0.77)	6.12 (0.26)	6.37 (0.53)	5.66 (1.24)	8	70
Leadville	12.6 (0.4)^{b}	10.3 (0.5)^{a}	9.4 (1.0)^{a}	14.5 (0.4)^{c}	18	70
Aiken	1.28 (0.03)^{ab}	1.03 (0.10)^{a}	1.17 (0.09)^{ab}	1.35 (0.13)^{b}	3	43
Bazoches	0.47 (0.03)	0.45 (0.04)	0.45 (0.03)	0.44 (0.02)	1	47
Belin-Beliet	0.86 (0.11)^{ab}	0.67 (0.09)^{a}	1.27 (0.15)^{c}	0.97 (0.04)^{b}	1	86

As

As bioaccessibility was low on all soils; the highest values were found on unamended Bazoches and Belin-Beliet soil (both 9%) (Table 6.3). The highest bioavailable As concentration is found on unamended Pribram soil (12.11 mg kg^{-1}); lower concentrations were found on Bazoches (1.63 mg kg^{-1}), Hoboken (1.61 mg kg^{-1}), and New Orleans (1.55 mg kg^{-1}) soil.

CA+ST application was the only treatment that significantly decreased PBET As. On Pribram (-44%), Hoboken (-41%) and Bazoches (-39%) soil bioavailable As was significantly reduced while on Aiken soil CA+ST addition reduced PBET As below ICP-MS detection limit (0.4 mg kg^{-1}).

Addition of PR significantly increased PBET As on Pribram (+31%), Hoboken (+155%) and Belin-Beliet (+65%) soil while lime significantly increased PBET As on Hoboken soil (+98%).

Table 6.3: PBET As (mg kg^{-1}) on unamended soils and on soils amended with cyclonic ash and steelshot (CA+ST), phosphate rock (PR), and lime (DL=0.4 mg As kg^{-1}). Total As in soil (mg kg^{-1}) and As bioaccessibility (BA= PBET extracted As/total As; in %) in unamended soil. Values are means, SD (in parentheses); n = 3.

	unamended	**CA + ST**	**PR**	**lime**	**Total As**	**% BA**
Paimboeuf	0.49	0.53 (0.06)	0.47	0.60 (0.03)	18	3
New Orleans	1.55 (0.27)	1.20 (0.14)	1.57 (0.17)	1.59 (0.25)	42	4
Joplin	< 0.4	< 0.4	< 0.4	< 0.4	8	--
Pribram	12.11 (0.59)^{b}	6.79 (1.26)^{a}	15.83 (1.41)^{c}	11.71 (1.90)^{b}	368	3
Hoboken	1.61 (0.06)^{b}	0.95 (0.07)^{a}	4.11 (0.11)^{d}	3.19 (0.35)^{c}	75	2
Moreno Valley	0.68 (0.12)	0.69 (0.12)	0.82 (0.04)	0.73 (0.13)	10	7
Leadville	< 0.4	0.51 (0.08)	0.83 (0.05)	0.61 (0.08)	164	--
Aiken	0.47 (0.00)	< 0.4	0.49 (0.04)	0.45	7	7
Bazoches	1.63 (0.10)^{b}	1.00 (0.12)^{a}	1.41 (0.05)^{b}	1.49 (0.06)^{b}	18	9
Belin-Beliet	0.84 (0.14)^{a}	0.62 (0.12)^{a}	1.39 (0.21)^{b}	0.96 (0.11)^{a}	9	9

4. DISCUSSION

Pb bioaccessibility on the 10 Pb contaminated soils varied between 1% (Hoboken) and 76% (Belin-Beliet) and averaged 33%. Berti & Cunningham (1997) found values from 6 to 42% using 3 Pb contaminated soils (tetra(m)ethyllead, leadazide ($Pb(N_3)_2$). The default value recommended by the U.S. Environmental Protection Agency (EPA) for estimating soil Pb exposures in children assumes that 30% of ingested soil Pb will be absorbed into the systemic circulation (US EPA, 1990). Our data indicate that Pb bioaccessibility can substantially vary between soils as was also observed by Ruby et al. (1996). The authors suggested that Pb bioaccessibility depends on the Pb species present and its solubility, as well as site specific soil chemistry. Therefore, application of a default 30% bioaccessibility value may not be appropriate for all types of Pb contamination.

Phosphate rock was the only additive able to reduce bioavailable Pb. Phosphate rock application is known to induce lead pyromorphite [$Pb_5(PO_4)_3X$ where X= OH, F or Cl] formation in soil (Ma et al., 1993), which in general is fairly insoluble even at low pH, explaining the observed reductions in bioavailable Pb (Berti & Cunningham, 1997; Brown & Chaney, 1999; Cotter-Howells, 1996; Laperche et al., 1997; Ma & Rao, 1997; Ma et al., 1997; Ruby et al., 1994). Our results however showed that only on 3 soils PBET Pb significantly decreased upon PR addition (Joplin= -21%, Pribram= -26%, Hoboken= -50%), indicating that only on these soils a more stable Pb compound (like pyromorphite) should be formed. Apparently, the success of PR in inducing pyromorphite formation depends on soil characteristics and present Pb species. For example, Ma & Rao (1999) reported that a higher soil pH reduced the effectiveness of Pb immobilization by PR due to lower PR solubility and consequently lower PO_4^{3-} release. The authors further found that more Pb was immobilized in soils containing greater amounts of total Pb. Fig. 6.1 plots soil pH against $CaCl_2$ extractable Pb: soils where PBET Pb was significantly reduced are presented as black squares.

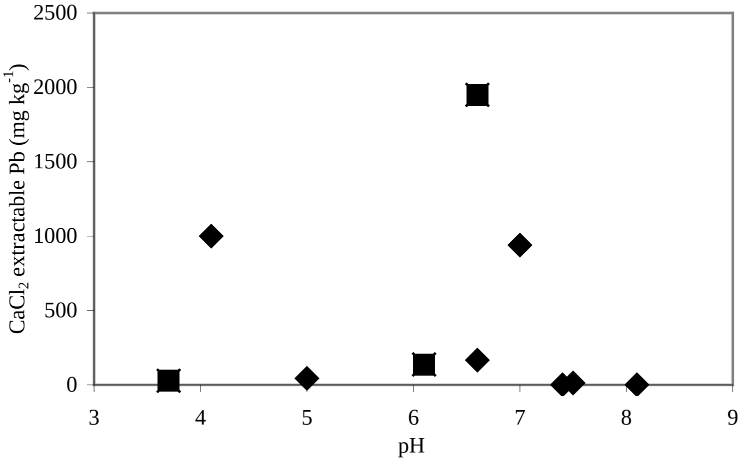

Fig 6.1: $CaCl_2$ extractable Pb (mg kg^{-1}) *vs* soil pH; soils where PBET Pb is significantly reduced upon PR addition are presented by squares.

PBET reductions occurred on the soil with the highest $CaCl_2$ extractable Pb (Joplin, 1950 mg Pb kg^{-1}), and with the lowest pH (Hoboken, pH 3.7). From this point of view however it is surprising that no reductions in PBET Pb were observed on Leadville (pH 4.1; 1000 mg Pb kg^{-1}) or Belin-Beliet soil (pH 7.0; 940 mg Pb kg^{-1}). Other factors however can influence pyromorphite formation. Several cations (Al, Cu, Fe(II), Cd, Zn, Ni, Ca) are known to inhibit Pb immobilization probably due to precipitation of metal phosphates, decreasing the amount of dissolved PO_4^{3-} available for precipitation with Pb ions (Ma et al., 1994b). Also, PO_4^{3-} can be sorbed by or react with other soil components such as iron and aluminium oxides and clays (Ma & Rao, 1999; Menon et al., 1995). Pb can further form hydrocerrusite [$Pb_3(CO_3)_2(OH)_2$] (Ma et al., 1994a).

Water extractions indicated that the applied PR only released low amounts of PO_4^{3-} (Table 5.18). Apparently, substantial differences in solubility and PO_4^{3-} release exist between PR samples coming from different mines, even between different lots from the same mine (L. Ma, oral communication). Ma et al. (1995) compared 14 PRs and all samples were found to substantially remove aqueous Pb. XRD and SEM micrographs however indicated substantial differences among different PR’s. Some resulted in fluoropyromorphite and/or hydrocerrusite formation while results from other samples indicated that sorption mechanisms were responsible for the reduced Pb extractability. The reductions in Pb solubility observed in our batch studies and in $CaCl_2$ extractions are probably the result of sorption processes (ion exchange at the surface, surface complexation, the formation of poorly crystalline or non-crystalline solids) or $Pb_3(CO_3)_2(OH)_2$ formation [PR releases CO_3^{2-} (Ma et al., 1995)], rather than formation of Pb-phosphates. Our data indicate that, due to the low solubility of the PR, much higher amounts (5%-10%, depending on factors such as soil pH, presence of other metal ions, available Pb concentration) should be applied in order to immobilize substantial amounts of Pb through pyromorphite formation.

The low solubility of PR however raises questions about its application in general. Ma & Rao (1999) applied 7 different Florida PRs in batch experiments to a soil heavily contaminated with Pb. All PR’s reduced aqueous Pb concentrations and their effectiveness increased with increasing PR addition. None of the PRs reduced dissolved Pb concentrations below the EPA action level (72.4 n mole L^{-1}), even when concentrations up to 25% (w/w) were used. The authors concluded that although PR has potential in immobilizing Pb, the quantity of PR needed could be uneconomically high for highly contaminated soils. Also, it should be noted that PR contains up to 60 mg Cd kg^{-1} (Table 5.4), and that at a rate of 10% PR addition, this would mean that 6 mg Cd is added per kg treated soil. Laperche et al. (1997) found better results in case PR was ground (< 250 µm), exposing more reactive material to the metal-containing solution (a higher surface increases dissolution kinetics, Wright et al., 1995). Brown & Chaney (1999) found that Pb immobilization substantially increased when liquid PO_4^{3-} sources such as triple superphosphate [TSP = $Ca(H_2PO_4)_2$] fertilizer were used, in comparison with PR. Recently, several workers reported successful Pb immobilization after addition of soluble PO_4^{3-} to the soil (e.g. KH_2PO_4, H_3PO_4, TSP) (Eusden et al., 2002; Hettiarachchi et al., 2000; Yang et al., 2001). Ma & Rao (1999) suggested to mix soluble PO_4^{3-} sources with PR to increase Pb immobilization efficiency. The strong reductions in soil pH as well as the high values of soluble PO_4^{3-} however indicate that this treatment may not be environmentally acceptable (Brown & Chaney, 1999). Also, addition of such high amounts of soluble PO_4^{3-} can drastically increase bioavailable As in soil (Jones et al., 1997).

Addition of CA+ST and lime did not significantly alter PBET Pb; lime even increased PBET Pb on 2 soils. The first months of incubation Pb is mainly adsorbed onto binding sites of the original soil components (CA, lime) and onto highly accessible sites on the surface of the modified clay (CA), which are freed due to a liming effect (Mench et al., 1998). The low pH of the GI tract however easily extracted Pb from these sites. Steelshot corrosion has been described to result in the formation of Fe and Mn oxides which can bind Pb (Mench et al., 1997, 1998). Berti & Cunningham (1997) observed different results when Iron Rich Material[1] was added to 3 Pb contaminated soils: PBET Pb was reduced one soil contaminated with TEL (from 40 to 11%), on a second soil [contaminated with $Pb(N_3)_2$] Pb bioaccessibility increased (from 30 to 45%) while no changes were observed on a third soil [also contaminated with $Pb(NO_3)_2$]. The authors however added 10% IRM (in our study only 1% ST was added), and the PBET test was performed at pH 2.5 (in our experiments PBET was run at pH 2.2). Ainsworth et al. (1994) observed complete Pb desorption (100%) from HFO at pH 2.5, while 20% of Cd and 53% of Co remained sorbed. The authors suggested that Co and Cd were

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[1] IRM is a byproduct of TiO_2 production, and contains 50 to 60% HFO by weight (Berti & Cunningham, 1997).

incorporated into the metal oxide structure due to their smaller atomic radii (resp. 0.072 resp. 0.097 nm), while the larger Pb atoms (0.120 nm) remained associated with the surface. On the other hand, McKenzie (1980) observed that some synthetic Mn oxides were able to sorb Pb at low pH.

As mentioned above, lime increased PBET Pb on Joplin (+25%) and Leadville (+43%) soil. Lime addition also resulted in significantly increased Pb concentrations extracted with acetic acid (BCR1, data not shown) on these soils, while $CaCl_2$ extractable Pb decreased. Possibly this is due to the formation of colloids.

$CaCl_2$ extractable Cd was elevated on 5 soils [2.0 mg kg^{-1} (Hoboken), 2.8 mg kg^{-1} (Joplin), 3.2 mg kg^{-1} (Moreno Valley), 12.3 mg kg^{-1} (Pribram) and 15.6 mg kg^{-1} (Leadville)] (chapter 5). CA+ST significantly decreased $CaCl_2$ extractable Cd on these soils (Hoboken= -60%, Joplin= -59%, Moreno Valley= -24%, Pribram= -38%, Leadville= -25%). PBET Cd was significantly decreased on 3 soils (Joplin= -29%, Hoboken= -57%, Leadville= -18%), while an insignificant reduction was observed on Pribram soil. With exception of Moreno Valley soil, it appears that addition of CA+ST effectively reduced $CaCl_2$ exchangeable as well as PBET extractable Cd. Both cyclonic ash and steelshot have been reported to immobilize Cd, in pot experiments and field studies (Boisson & Mench, 1998; Vangronsveld, 1998b). Addition of cyclonic ash sorbs Cd to the surface of the clay and of the original soil due to pH increase but this effect is probably neutralized at low pH (as is the case in the GI tract). The observed reduction in Cd bioavailability is probably due to Cd binding to Fe oxides and Mn oxides. EXAFS analysis revealed that Mn released by steelshot can transform to a birnessite-like phyllomanganate compound, and birnessite addition has been reported to immobilize Cd (Charlet & Manceau, 1993, Mench et al., 1998). On the other hand, Ainsworth et al. (1994) showed that Cd was less easily desorbed from HFO than Pb at pH 2.5 and suggested that this was due to Cd incorporation into the restructuring Fe oxide (presumably goethite), in contrast to Pb. Incorporation of Cd in goethite is also suggested by Gerth & Brümmer (1983).

PR addition only decreased PBET Cd on Leadville (-25%) soil. Several Cd binding mechanisms have been described for PR (surface complexation, ion exchange, formation of amorphous solids), while also octavite ($CdCO_3$) and cadmium hydroxide [$Cd(OH)_2$] formation have been reported (Wright et al., 1995). Apparently, most of these Cd species are easily solubilized at low pH. On soils low in $CaCl_2$ extractable Cd, PR addition slightly increased $CaCl_2$ extractable Cd (from 0.04 mg kg^{-1} up to 0.24 mg kg^{-1} on Paimboeuf; from 0.86 mg kg^{-1} up to 1.27 mg kg^{-1} on Belin-Beliet soil), possibly due to Cd release (PR contains up to up to 60 mg Cd kg^{-1}; Table 5.6 former chapter)[2]. Lime addition slightly increased PBET Cd on Leadville soil (from 12.6 mg kg^{-1} up to 14.5 mg kg^{-1}).

As bioaccessibility varied between 0% (Joplin, Leadville) and 9% (Bazoches, Belin-Beliet). Rodriguez et al. (1999) found values values between 2.7 and 42.8% on 15 mining/smelter sites using an *in vivo* test (swine). CA+ST was the only treatment that significantly decreased PBET As (Pribram= -44%; Hoboken= -41%; Bazoches= -39%, Aiken= <DL). Several workers have reported As adsorption on Fe-oxides (Lombi et al., 1999b; Mench et al., 1998; Vangronsveld et al., 1994). Both As (III) and As (V) have been described to form inner sphere complexes with hydrous ferric oxide (Fendorf et al., 1997; Manning et al., 1998).

Addition of PR significantly increased PBET As on Pribram (+31%), Hoboken (+155%) and Belin-Beliet (+65%) soil while lime significantly increased PBET As on Hoboken soil (+98%). None of the applied amendments contains significant amounts of As (Table 5.6, chapter 5). In case of PR addition, PO_4^{3-} release could be responsible for the observed increase in As bioavailability. Due to its chemical and physical similarities, it is known that PO_4^{3-} competes for the same sorption sites in soil as arsenate (AsO_4^{3-}) (Woolson, 1983; Peryea, 1991). The fact that PBET As was increased on soils where the highest bioavailable As concentrations were

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[2] Addition of 1% (Paimboeuf) and 2% (Leadville) resulted in an addition of 0.6 resp. 1.2 mg Cd kg^{-1}, from which a part could have been solubilized after PR dissolution.

observed appears to confirm this hypothesis (Pribram=12.11 mg kg^{-1}; Hoboken=1.61 mg kg^{-1}).

The increased PBET As found on Hoboken soil after lime addition might be due to pH increase; lime increased pH of the soil from 3.7 up to 6.7 (As mobility in general increases from pH 5.0 to reach its maximum between pH 6.5-6.9; from here on, As mobility decreases up to pH 8.5-9.0) (M. Mench, oral communication). Lime addition however increased soil pH to a comparable degree on Paimboeuf (5.0 up to 7.2) and Leadville (4.1 up to 6.1) soil while on these soils PBET As was not altered. Paimboeuf soil contains less total As (19 mg kg^{-1} *vs* 75 mg kg^{-1} on Hoboken soil). Leadville contains high total As concentrations (164 mg kg^{-1}). It is clear that factors such soil characteristics and metal speciation substantially influence metal mobility.

4. CONCLUSIONS

Phosphate rock was the only amendment which significantly decreased PBET Pb. Only on 3 soils however significant reductions were observed. In general, reductions occurred on soils with high $CaCl_2$ extractable Pb concentrations or with low pH, facilitating PR dissolution and Pb phosphate formation. We found that Pb immobilization strongly depends on soil characteristics (e.g. pH, presence of other metals) and metal species present in soil. Our data further indicate that the limiting step in pyromorphite formation is the low PO_4^{3-} release by PR. For this reason, higher amounts of PR (e.g. 5 or 10%), possibly combined with acidification or the application of liquid P-forms (e.g. H_3PO_4) are likely to result in higher reductions in PBET Pb.

CA+ST significantly decreased PBET Cd on almost all soils where $CaCl_2$ extractable Cd was elevated, probably as a result of Cd binding to Fe and Mn oxides. The CA+ST treatment further resulted in reduced PBET As concentrations, probably due to As complexation to Fe oxides.

Our data further indicated that PR might release Cd, and that phosphate addition can increase PBET As. On the other hand, pH increase upon addition of lime might mobilize As as well.

CHAPTER VII

GENERAL CONCLUSIONS

This thesis examined biological aspects of recently developed alternative remediation strategies for Pb contaminated soils. These remediation techniques use plants to either remove (phytoextraction) or render harmless contaminants (phytostabilization; often in combination with soil amendments) (Schwitzguébel et al., 2002; van der Lelie et al., 2001).

Under normal conditions, Pb phytoextraction has only limited potential as a remediation technology due to the limited mobility of Pb in soil, the low Pb uptake by plants, and the low translocation of the metal to upper plant parts (McBride, 1994; Xiong, 1997). Last decade however, EDTA was found to drastically increase Pb phytoavailability, Pb plant uptake and Pb translocation (Blaylock et al. 1997; Epstein et al., 1999; Huang et al., 1997b; Huang & Cunningham, 1996; Vassil et al., 1998). Several field experiments have been performed and are still in progress applying EDTA to the contaminated substrate in combination with non hyperaccumulator plants (M. Blaylock, oral communication). In order to optimize phytoremediation potential of such technique, information on metal uptake and plant reaction to increased metal concentrations is essential.

Induction of oxidative stress as an early plant response to metal stress is generally well documented. In this thesis the effects of Pb-EDTA and EDTA on oxidative stress metabolism and mineral uptake in plants are examined. EXAFS analysis revealed that 60-70% of Pb in primary leaves of bean plants is present as Pb-EDTA, while all Pb (99.97%) present in the nutrient solution was complexed to EDTA (Sarret et al., 2001). This suggests that Pb-EDTA is at least partly taken up by plants. It is not clear from our data if the partial split of the complex occurs at the root surface level (as is the case in Fe-EDTA and Zn-EDTA) or if all Pb-EDTA is assimilated as such and split in the plant, after uptake (Chaney et al., 1972, Sarret et al., 2001). The first symptoms of toxicity (induction of SPOD in roots and leaves and GPOD in leaves) occurred when 50 µ*M* Pb-EDTA was applied to the nutrient solution, and when 264 mg Pb kg^{-1} DW was found in primary leaves (Fig. 3.2). The fact that 30-40% of Pb-EDTA is split could suggest that 79-106 mg unchelated[1] Pb kg^{-1} DW is present in leaves, exceeding the minimum toxicity treshold defined by Kabata-Pendias & Pendias (1992) (30-300 mg Pb kg^{-1}).

The early induction of the apoplastic occurring SPOD is interesting. SPOD catalyzes the final enzymatic step in lignin biosynthesis and other workers already postulated that lignification is an important process for the plant to cope with metal stress as this leads to more extracellular binding sites for the metal (Olson & Varner, 1993; Padu, 1999; Degenhardt & Gimmler, 2000). This also demonstrates SPOD might be a valuable biomarker for Pb and Pb-EDTA toxicity. Addition of 50 µ*M* Pb-EDTA further resulted in significant reductions in Ca, Fe, Mn and Zn content in primary leaves.

All other measured enzymes were induced in roots and leaves (except GR in leaves) when higher amounts of Pb-EDTA were added to the nutrients solution (100 µ*M*), but before reductions in chlorophyll content or plant morphology were observed. The induction in the capacity of enzymes representative for three different mechanisms involved in the cellular anti-oxidative defence strategy strongly suggests the presence of active oxygen species in roots and leaves. Several ways have been described in literature for non redox active metals to induce oxidative stress, including increased lipoxygenase activity (Weckx et al., 1997), metal binding to functional groups of (membrane)proteins (Seregin & Ivanov, 2001), membrane destabilization due to mineral deficiency (Pasternak, 1988). An elaboration of other antioxidative mechanisms (e.g. determination of ascorbate,

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[1] 'Unchelated' here means Pb is not chelated to EDTA, but this does not exclude Pb chelation to other naturally occurring ligands (e.g. citrate).

glutathione, determination of free radicals, TBA, lipoxygenase), possibly measured in a time course, as well as the subcellular localization of the different responses could contribute to a better understanding of the underlying mechanisms. Future research should further focus on the behaviour and uptake of Pb-EDTA and on the relation between different Pb species and plant stress.

Addition of EDTA alone decreased plant uptake of Fe, Mn and Zn due to EDTA chelation in the hydroponic solution. At none of the applied EDTA concentrations phytotoxic effects were observed. Only low amounts of ‘free’ EDTA were present in solution (10 µ*M*), which appearently were not phytotoxic.

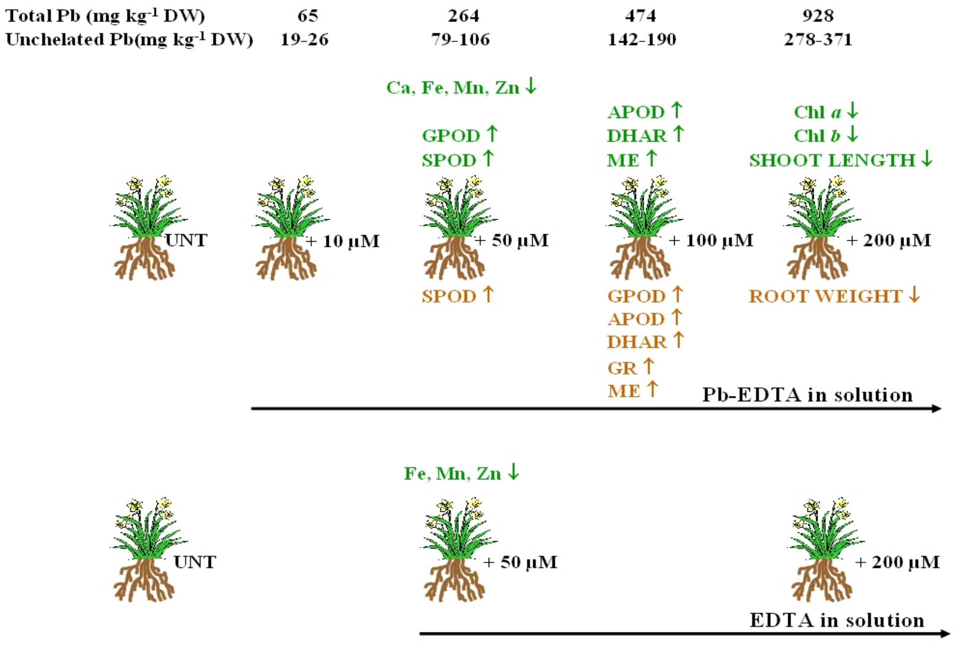

Fig. 7.1: Effects of Pb-EDTA and EDTA on oxidative stress reactions and mineral uptake in *Phaseolus vulgaris.*

Two aspects of Pb immobilization were examined: in a first study several soil amendments were compared on a soil spiked with various Pb concentrations. A second experiment was set up to compare the efficacy of the most efficient amendments from the first study in 10 soils contaminated with different Pb species. Following soil amendments were applied to a soil spiked with Pb concentrations up to 2000 mg Pb kg^{-1}: bentonite (1%), zeolite (0.5%), cyclonic ash (5%), compost (5%), lime (1%), steelshot (1%) and hydroxyapatite (1%). At the highest Pb soil concentration, $Ca(NO_3)_2$ extractable Pb was reduced in following order: bentonite (-24%), steelshot (-58%), zeolite (-71%), cyclonic ash (-90%), compost (-90%), hydroxyapatite (-94%) and lime (-98%). On unamended soil, phytotoxicity increased with increasing soil Pb. Most amendments reduced phytotoxicity, except bentonite, steelshot and zeolite. Bentonite apparently immobilized too low amounts of Pb in order to avoid complete phytotoxicity. In case of steelshot and zeolite addition, negative effects on plant growth were observed independent of soil Pb concentration, probably due to release of toxic amounts of Fe and/or Mn (steelshot) and destruction of soil texture (zeolite). Cyclonic ash, compost, hydroxyapatite and lime decreased Pb phytotoxicity concomitant with reduced Pb concentration in lettuce tissue. A slight induction of oxidative stress was observed upon hydroxyapatite addition, possibly as a result of strong immobilization of essential elements [$Ca(NO_3)_2$ extractable Zn (-59%), Mn (-39%), Fe (-36%)]. Cyclonic ash, lime and compost further improved plant growth and reduced oxidative stress at low Pb concentrations, due to soil pH increase mitigating Al or Mn toxicity.

It was decided to continue experimentation with lime, phosphate rock and a combination of cyclonic ash and steelshot. Phosphate rock (PR) was used in stead of hydroxyapatite, mainly for economic reasons[2]. Cyclonic ash (CA) was combined with steelshot (ST) in order to avoid CA induced Mn deficiency, to neutralize ST induced Fe and/or Mn toxicity and to immobilize As. Amendments were applied in following ratios: 5% CA + 1% ST, 1% lime, 1% PR (except 2 soils where 2% was used); ratios were optimised in former experiments (CA, ST, lime),

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[2] 1 kg North Carolina PR costs approx. 0.2 €, while the applied hydroxyapatite (purified form, Aldrich) costs 1500 €. 1 kg of applied lime costs 0.25-0.37 €, compost 0.01 €, steelshot 1.75 €. The price of cyclonic ash is neglectibly low.

while in case of PR application batch studies were used which indicated efficient Pb immobilization on the 10 soils upon 1 and 2 % PR addition. The treatments were compared on 10 Pb contaminated soils collected worldwide, with different soil characteristics, different Pb concentration and different Pb species. Total metal content did not appear to be a good indicator for metal bioavailability; on some soils comparable $CaCl_2$ extractable metal concentrations were found as in uncontaminated soils, despite the substantially elevated total metal content. In case $CaCl_2$ extractable metal concentrations were elevated relative to an uncontaminated reference soil, amendment addition substantially decreased these concentrations. On average, CA+ST (-77%) and lime (-82%) were more efficient in reducing $CaCl_2$ extractable Pb than PR (-30%). A similar pattern was found when a specific microbial Pb biosensor was used. Several soils further contained elevated Cu, Zn, As and Cd concentrations: Cu (PR= -48%; CA+ST= -61%; lime= -88%) and Zn (PR= -37%; lime= -75%; CA+ST= -79%) were reduced more efficiently when lime and CA+ST were used than in case PR was applied. The amendments appeared less efficient in immobilizing Cd (lime= -16%; PR= -21%; CA+ST= -33%), while $CaCl_2$ extractable As was below DL of the ICP-MS on all soils; CH_3COOH extractable As decreased upon CA+ST application, while addition of PR substantially increased extractable As on several soils.

CA+ST addition increased $CaCl_2$ extractable Mn on most soils; $CaCl_2$ extractable Ni sporadically increased while $CaCl_2$ extractable Fe was not altered. The increased Mn and Ni concentrations did however not affect plant growth. On several soils addition of CA+ST and lime increased plant growth due to mitigation of soil phytotoxicity and due to the release of nutrients while PR addition only sporadically showed this beneficial effect. A comparable pattern was found in plant metal uptake (*Zea mays*): the number of times metal concentrations in maize leaves exceeded critical toxicity concentrations as defined in literature decreased in following order: unamended (12) < PR (12) < CA+ST (9) < lime (6).

The combination CA+ST decreased PBET Cd and As on 3 soils, probably due to binding on Fe-and Mn-oxides. Phosphate rock was the only amendment able to reduce Pb bioavailability in the gastro-intestinal tract, probably due to pyromorphite formation [($Pb_5(PO_4)_3X$ where X= OH, F or Cl)]. On the other hand, PR significantly increased PBET As in a few cases, probably due to competition between PO_4^{3-} and AsO_4^- for the same sorption sites in soil. PR only significantly decreased PBET Pb on 3 soils. Water extractions showed that the applied North Carolina PR released only low amounts of PO_4^{3-}. Apparently PO_4^{3-} release can substantially differ among different PRs. The fact that only on a limited amount of soils reductions in PBET extractable Pb were observed further showed that besides pyromorphite formation, other sorption processes (ion exchange at the surface, surface complexation, formation of poorly crystalline or non-crystalline solids) or $Pb_3(CO_3)_2(OH)_2$ formation were involved. Pyromorphite formation depends on factors such as soil pH (a low pH increases PR dissolution), bioavailable Pb, the presence of CO_3^{2-}, iron and aluminium oxides and clays and the presence of other metals [Al, Cu, Fe(II),Cd, Zn, Ni] which can bind PO_4^{3-}.

The addition of higher amounts of PR (5-10%) or liquid PO_4^{3-} forms might increase Pb immobilization (Eusden et al., 2002; Hettiarachchi et al., 2000; Ma & Rao, 1999; Yang et al., 2001). It is however questionable if such technique is environmentally acceptable, due to the high amount of Cd present in most PR (addition of 10% North Carolina PR adds 6 mg Cd kg^{-1}), the strong reductions in pH (which can mobilize other metals present) and the high values of soluble PO_4^{3-} which can mobilize As.

Resuming, our data indicate that metal immobilization by means of CA+ST and lime is an efficient and environmentally acceptable alternative for the treatment of heavily contaminated substrates (e.g. smelting, battery breaking, TEL production) as well as light contaminated farmlands (e.g. due to sewage sludge). Our findings however are based on short term experiments (4 months); lysimeter and *in situ* experiments are needed to confirm the results obtained in our study. It is expected that lime addition should be repeated after a few years, while field data from the heavily contaminated Lommel-Maatheide site (Belgium) indicate stable metal immobilization upon CA addition for up to 12 years now.

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APPENDIX 1

Vlarebo background and cleanup values

Table A1.1 summarizes background values for standard soil samples (10% clay, 2% OM); Table A1.2 summarizes cleanup values for standard soils. Cleanup values differ according to the intended land use for the remediated site (Class I-V).

Table A1.1: Target values (mg kg^{-1} soil DW) of standard soil samples and correction factors (A, B, C) for the equation, legally adopted in Flanders (Van Gehuchte et al., 1997).

	Target value	A	B	C
As	19	14	0.5	0
Cd	0.8	0.4	0.03	0.05
Cu	17	14	0.3	0
Ni	9	6.5	0.2	0.3
Pb	40	33	0.3	2.3
Zn	62	46	1.1	2.3

Table A1.2: Cleanup values (in mg kg^{-1} standard soil, DW) adopted in Flanders, as a function of the intended use of remediated site.

	Natural park (class I)	Agriculture (Class II)	Residential area (Class III)	Recreational area (Class IV)	Industrial area (Class V)
As	45	45	110	200	300
Cd	2	2	6	15	30
Cu	200	200	400	500	800
Ni	100	100	470	550	700
Pb	200	200	400	1500	2500
Zn	600	600	1000	1000	3000

In case soil sample factors differ from the standard sample (i.e. when clay content ≠ 10% and OM ≠ 2 %), background and cleanup values can be calculated as follows:

$$N(x,y) = N(10,2) \text{ x } \frac{A + xB + yC}{A + 10B + 2C}$$

Where N = background or cleanup value, when the clay content is *x* or 10%, and the OM is *y* or 2 %, x and y are the respective clay and OM contents of the soil sample, *A*, *B*, and *C* are factors depending on the element in question presented in Table A1.1.

APPENDIX 2

A. Calculation of PR to be added based on Pb content

By knowing the concentration of Pb and the mineral it will form when PO_4 is added, the precise amount of PO_4 to be added can be calculated, based on the molar ratio Pb/PO_4, present in the formed pyromorphite (Wright et al., 1995). Chloropyromorphite and hydroxypyromorphite are the two most likely $Pb\text{-}PO_4$ minerals to form under the given conditions. These two minerals exhibit the fastest kinetic rate of formation (Nriagu, 1974). Although the two minerals are different by chemical formula, they both have the same Pb/PO_4 ratios. The following is the calculation for the molar ratio:

Chemical formula for pyromorphite $Pb_5(PO_4)_3(OH, Cl)$

where Pb= 5 x 207.2 g/ mol = 1036 g/mol
and PO_4= 3 x 94.93 g/mol = 284.79 g/mol

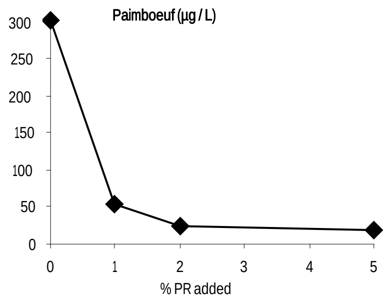
The molar ratio of Pb/PO_4 in pyromorphites = 3.64

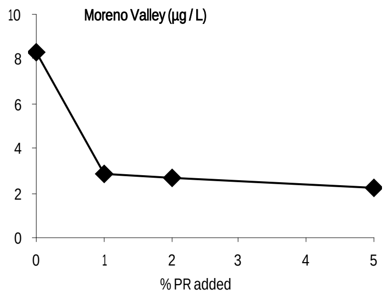
For example, 1.4 mg Pb kg^{-1} is present in the soil solution. This is 6.75 10^{-6} mol Pb kg^{-1}. Knowing that Pb/PO_4 is present in pyromorphite in the ratio 3.64, this means that 1.86 10^{-6} mol PO_4 kg^{-1} should be added, which is 0.0001762 g PO_4 kg^{-1}. Using the fact that 46.84 % of North Carolina phosphate rock is PO_4, one can calculate that 0.00038 g PR is needed, or 0.00004 %.

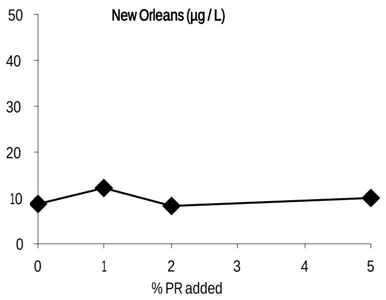
B. Batch studies to determine the amount of PR to be added

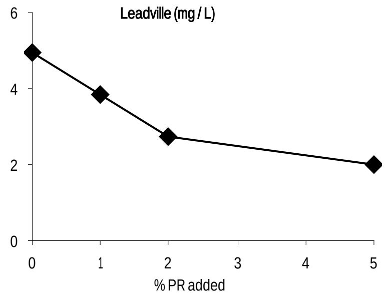
0, 1, 2 and 5% of North Carolina PR was mixed with 2 g of soil in plastic centrifuge tubes; 30 ml distilled water was added. Samples were shaken for 48 hours, tubes were centrifuged and 5 ml samples were taken with a pipet (no difference was found between pipet sampling and filtering the samples) and analysed for Pb by flame AAS or ICP-MS, depending on the concentration (procedure based on Chen et al., 1997). n=2.

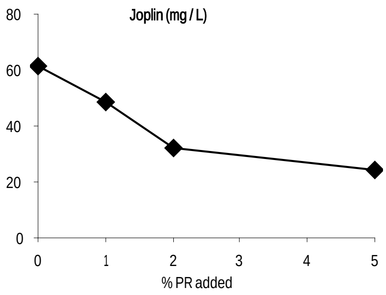
Fig. A2.1: Pb in solution (µg L^{-1}, except Joplin, Leadville and Aiken which are in mg L^{-1}) after shaking 2 g of Paimboeuf, New Orleans, Joplin, Pribram, Hoboken, Moreno Valley, Leadville, Aiken and Bazoches soil with 0, 1, 2 and 5% PR for 48h (next page).

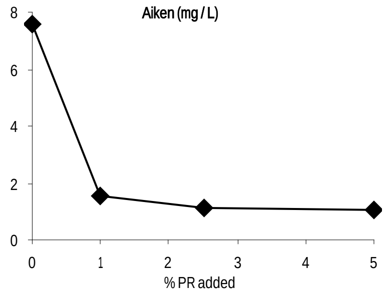

Paimboeuf (µg / L)
300
250
200
150
100
50
0
0
1
2
3
4
5
% PR added

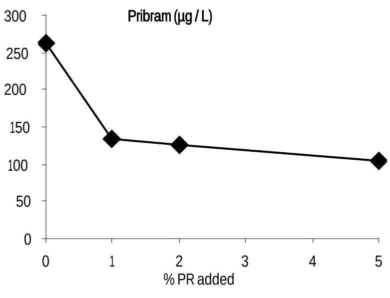

Moreno Valley (µg / L)
10
8
6
4
2
0
0
1
2
3
4
5
% PR added

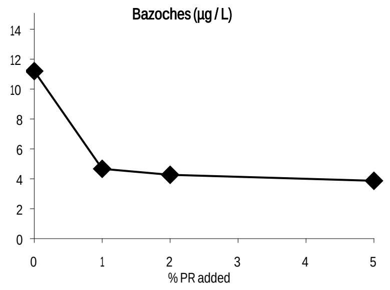

New Orleans (µg / L)
50
40
30
20
10
0
0
1
2
3
4
5
% PR added

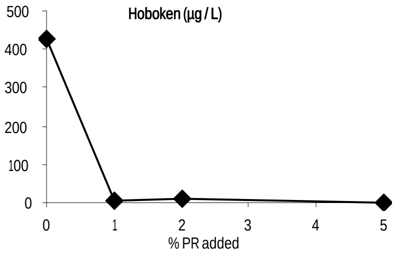

Leadville (mg / L)
6
4
2
0
0
1
2
3
4
5
% PR added


Joplin (mg / L)
80
60
40
20
0
0
1
2
3
4
5
% PR added


Aiken (mg / L)
8
6
4
2
0
0
1
2
3
4
5
% PR added


Pribram (µg / L)
300
250
200
150
100
50
0
0
1
2
3
4
5
% PR added


Bazoches (µg / L)
14
12
10
8
6
4
2
0
0
1
2
3
4
5
% PR added


Hoboken (µg / L)
500
400
300
200
100
0
0
1
2
3
4
5
% PR added

APPENDIX 3

Alterations in $CaCl_2$ extractable metal content and metal uptake by maize upon addition of CA+ST, PR and lime.

Table A3.1: Significant alterations in $CaCl_2$ extractable metal content on each soil after amendment addition (CA+ST, PR, lime) and average alteration in $CaCl_2$ extractability, relative to the unamended soil, calculated with data from 10 soils (expressed in %).

		increase	No change	Significant decrease					Average
		+ > 6 %	+5% to –5%	-6 % to –20 %	-21% to -40%	-41% to -60%	-61% to -80%	-81 to –100%	% Δ*
Pb	CA+ST		**BA**		**NE**		**HO,MO,LE,BE**	**PA,JO,PR,AI**	-77
	PR	**AI**	**BA**	**NE,PR,MO,BE**	**JO**	**PA,LE**	**HO**		-30
	lime		**NE,BA**		**MO**	**AI**	**BE**	**PA,JO,PR,HO,LE**	-82
Cd	CA+ST		**PA,AI,BA**	**NE**	**PR,MO,LE,BE**	**JO,HO**			-33
	PR	**PA**	**MO,AI,BA,BE**	**NE,PR,HO**	**LE**		**JO**		-21
	lime		**PA,NE,LE,AI,BA**	**MO**	**PR,HO,BE**	**JO**			-16
As	CA+ST								
	PR								
	lime								
Ni	CA+ST	**PA,LE**	**NE,JO,PR,AI,BA,BE**		**HO,MO**				+6
	PR		**All soils**						-4
	lime		**PA,NE,JO,LE,AI,BA,BE**		**MO**	**PR**	**HO**		-54
Cu	CA+ST	**BE**	**NE,JO,PR,MO,AI,BA**		**PA**		**LE**	**HO**	-61
	PR		**NE,JO,PR,MO,AI,BA,BE**		**PA**	**HO**	**LE**		-48
	lime		**NE,JO,PR,MO,AI,BA**				**PA,BE**	**HO,LE**	-88
Zn	CA+ST		**BA**		**NE**	**PA**	**MO,LE**	**JO,PR,HO,AI,BE**	-79
	PR		**PA,AI,BA**	**NE**	**PR,HO,MO**	**JO,LE,BE**			-37
	lime		**JO,BA**			**MO**	**PA,PR,LE,AI**	**JO,HO,BE**	-75
Fe	CA+ST		**All soils**						-9
	PR		**All soils**						-29
	lime		**All soils**						-9
Mn	CA+ST	**All but LE,PR**	**LE**				**PR**		-6
	PR	**PR**	**NE,JO,HO,ME,AI,BA**		**PA,LE**	**BE**			-22
	lime		**NE,MO,AI,BA**	**LE**		**PR,HO**	**PA,BE**	**JO**	-31

* The average % alteration is calculated as the average molar alteration in $CaCl_2$ extractable elemental concentration divided by the average total $CaCl_2$ extractable elemental concentration in mmolar. An average reduction of 77 % (in case of CA+ST, Pb) thus means that on average (over the 10 soils), $CaCl_2$ extractable mol Pb is reduced by 77 % when CA+ST is used.

Table A3.2: Alterations on metal content in maize leaves after amendment addition (CA+ST, PR, lime) on soils, relative to the unamended. In the last colums, the number of soils where the CTC* is exceeded are shown, as well as the average alteration in metal content, relative to the unamended.

		increase				No change	decrease				> CTC	Average % Δ
		+ > 76%	+51– 75%	+31- 50%	+16- 30%	+15 to –15%	-16 to – 30%	-31 to 50 %	-51 to 75%	-76 to –100%		
Pb	CA+ST		**HO,MO**			**PR**	**NE**	**PA**	**JO,AI**		**JO**	**-59**
	PR	**HO,MO**				**PR,AI**	**NE**	**PA**	**JO**		**JO**	**-56**
	lime	**HO**		**PR**		**MO**		**NE**	**AI**	**PA**	**JO**	**-73**
Cd	CA+ST					**PR**	**MO**		**HO,LE**	**JO**		**-66**
	PR					**MO**	**PR**	**HO**	**LE**	**JO**		**-68**
	lime						**MO**	**PR**	**HO**	**JO,LE**		**-82**
Ni	CA+ST								**HO**			**-74**
	PR								**HO**		**HO**	**-52**
	lime								**HO**			**-69**
Cu	CA+ST	**PR**	**PA**				**NE,HO**	**MO**			**PA,NE,PR**	**5**
	PR				**HO**	**PA,PR,HO,MO**					**NE**	**1**
	lime		**HO**		**PR**		**NE**	**PA,MO**			**NE,HO**	**-6**
Zn	CA+ST					**MO**	**NE**	**AI**		**PA,JO,PR,HO**	**LE**	**-79**
	PR			**MO,AI**		**NE**	**PR**	**HO**	**JO,LE**	**PR**	**JO,LE**	**-51**
	lime				**MO,AI**	**NE**			**PR**	**PA,JO,HO,LE**		**-83**
Fe	CA+ST	**MO**	**PR**			**NE**		**HO**	**AI**	**PA**	**AI**	**-49**
	PR	**MO**	**AI**				**PR**	**NE,HO**	**PA**		**PA,MO,AI**	**-10**
	lime	**MO**				**PR,AI**	**NE,HO**			**PA**	**MO,AI**	**-30**
Mn	CA+ST	**HO**		**NE,JO**	**MO**	**PA**	**LE**		**AI**	**PR**	**PA,HO,LE**	**-24**
	PR		**PR**			**PA,NE,HO**	**JO,MO**	**LE**	**AI**		**PA,PR,HO,LE**	**-17**
	lime					**NE**	**MO**	**HO**	**JO,LE,AI**	**PA,PR**	**LE**	**-65**

* one none of the soils the CDC was exceeded, for any element

APPENDIX 4

Application of a specific bacterial Pb biosensor

Luminometry assay was carried out using an ANTHOS LUCY1 luminometer (Anthos Labtech b.v., Heerhugowaard, The Netherlands) at 23°C, as previously described (Corbisier *et al.*, 1999). For metal standards, duplicate samples were set up per microtitre assay. As negative controls, eight reaction samples containing redistilled water were included in the test. The bioluminescence emitted (ALU) and the optical density (OD_{620} nm) of the culture was measured over 16 h at 30 min intervals, and processed using the MIKROWIN software, as previously described (Corbisier *et al.*, 1999). Further data processing was carried out using EXCEL 7.0 (Microsoft) or Origin 5.0 (Microcal™Origin™, Microcal Software, Inc., Northampton, MA, USA).

BIOMET detectable Pb was below detection in three soils (Paimboeuf, Pribram, Moreno Valley; Table A4.1). In soils from New Orleans and Hoboken, Pb was detectable in the untreated soil but addition of soil additives reduced the Pb concentration below detection limit (42 µg L^{-1} Pb equivalents; van der Lelie et al., 2000). On the other soils, PR application resulted in a slight or even no decrease in detectable Pb (Joplin, Aiken, Bazoches, Belin-Beliet). Detectable Pb further decreased with lime while the strongest decreases were found after application of CA+ST. Only low levels of Pb-bioavailability was observed in the Leadville samples, independent of the treatment.

Table A4.1: Pb concentration (mg Pb eq. kg^{-1} DW) determined by the BIOMET test in the untreated soils and in soils amended with cyclonic ash and steelshot (CA+ST), PR and lime. Values are means, n = 2, <DL = concentration below BIOMET detection limit.

	unamended	CA+ST	PR	lime
Paimboeuf	< DL	< DL	< DL	< DL
New Orleans	20	< DL	< DL	< DL
Joplin	404	182	404	279
Pribram	< DL	< DL	< DL	< DL
Hoboken	8	< DL	< DL	< DL
Moreno Valley	< DL	< DL	< DL	< DL
Leadville	16	16	16	16
Aiken	17	7	17	8
Bazoches	18	7	18	18
Belin-Beliet	221	53	155	126

BIOMET data clearly showed a high degree of similarity with $CaCl_2$ extracted Pb, i.e. high reductions in extractable Pb with CA+ST and lime, and lower reductions with PR. Vangronsveld et al. (2000) and Tibazarwa et al. (2001) found strong correlations between exchangeable Zn, Cd, or Ni in soils and BIOMET detected metal concentration. The high number of samples beneath BIOMET detection limit however, did not permit to statistically show the same relationship with our data. Also, on Leadville samples, BIOMET only detected low levels of Pb while no differences between amendments were observed (in contrast to $CaCl_2$ extractions, which clearly showed amendment induced reductions in Pb extractability). Analysis of the microbial ecology of the Leadville soil showed that a Pb-resistant *Ralstonia metallidurans* strain, referred to as LV1, was present (10^{+6} cfu g^{-1} soil) as the only culturable microorganism (S. Brown and D. van der Lelie, unpublished results). Further analysis of strain LV1 revealed that it has the same Pb-resistance mechanism as *R. metallidurans* CH34, the parental strain of the Pb-biosensor strain. Pb resistence in strain CH34 involves post-efflux Pb detoxification (Borremans et al., 2001), avoiding re-entry of the ion in the cell. The presence of strain LV1 with a similar Pb-resistance system as the sensor species might have negated the Pb bioavailability in the Leadville soil.