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DOCTORAL DISSERTATION

Metal-phytoextraction: the potential of high biomass crops and improving efficiency using short rotation coppice (SRC) of willow

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Voorwoord

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Summary

In the northeast of Belgium (the Campine region), an area of at least 280 km^2 is historically contaminated with mainly cadmium (Cd), zinc (Zn) and lead (Pb). The negative impacts on inhabitants and the environment in general as well as economic losses in the farming industry urged regional policy makers to strongly recommend the remediation of the metal-contaminated soil. Given the vastness of the area and the diffuseness, moderation and shallowness of the contamination, phytoextraction, *i.e.* the use of plants to extract metals out of the soil and accumulate them in harvestable biomass, is proposed as a good remediation strategy. More specifically, cultivating non-food high biomass crops with moderate metal accumulation capacity reveals promising for this area.

In this thesis, different high biomass species were cultivated and evaluated for several years in field trials on a Cd-Zn-Pb-contaminated soil in the northeast of Belgium to investigate which high biomass crop exposed the highest, but also the most stable (in time) phytoextraction potential on these soils. Biomass production and metal accumulation of pre-selected tobacco clones (*Nicotiana tabacum* L.), pre-selected sunflower mutants (*Helianthus annuus* L.) and a commercial hemp (*Cannabis sativa* L.) were determined for 2-4 subsequent years while the phytoextraction potentials of more than 200 different commercially available and experimental (designed by the Institute of Nature and Forest; INBO) poplar (*Populus*) and willow (*Salix*) clones in short rotation coppice (SRC) were assessed at the end of the first cutting cycle (after 4 growing seasons). The tobacco clones and the sunflower mutants revealed to be efficient extractors of respectively Cd and Zn, while the highest, simultaneous extraction of Cd and Zn was observed using the woody species in SRC. Phytoextraction of Pb appeared to be utopia using the evaluated crops but this is not considered a major problem given the low bioavailability and activity of Pb. The estimated long remediation times (> 60 years) furthermore indicated that crosscuts with economic and (other) environmental advantages are crucial for large-scale implementation of metal phytoextraction. When for the evaluated crops these external benefits, assessed by reviewing literature, were combined with the observed phytoextraction potentials, SRC revealed to be the most

suitable crop for the implementation of metal phytoextraction in the area under investigation.

In parallel with the field evaluations of the high biomass crops, metal phytoextraction using SRC of willow was studied into more detail in this research. SRC of willow is a high biomass crop abundantly investigated in the framework of metal phytoextraction. However, the longer-term effectiveness of SRC phytoextraction applications is rather unknown due to the complex interactions between, and evolutions of the main entities of a phytoextraction system. Therefore, determining effective reduction of metal levels in soil and changes in soil toxicity, and also soil fertility and functionality, in longer-term field experiments with SRC phytoextraction applications is of high importance. The SRC experimental field in the metal-contaminated area in northeast Belgium offers a unique opportunity to this concern. Pseudo-total soil metal concentrations and soil toxicity, estimated using standardized chemical extractions and plant- and invertebrate-based ecotoxicity assays, were assessed in soil managed by 8 years of metal phytoextraction using SRC of willow (Tora; *Salix schwerinnii x Salix viminalis*) and in soil without phytoextraction management. The observed decontamination might indicate a much more effective phytoextraction (48 times in case of Cd removal, 79 times for Zn removal) by 8 years of willow-management than predicted by extrapolating metal removal of Tora after 4 growing seasons. Furthermore, the chemical extractions and all ecotoxicity tests unanimously indicated the willow-managed soil to be less toxic, to different extents, compared to the unmanaged soil. The results all emphasize the environmental benefits of a SRC-phytoextraction-managed soil compared to no management.

To reduce remediation times of metal phytoextraction using SRC of willow and/or increase benefits of phytoextraction synergies, a last part of this research focused on improving willow biomass production and/or metal accumulation. A selection of 3 strategies was proposed to meet this concern: *in situ* clone selection, bioaugmentation of willow with beneficial plant-associated bacteria and fertilization of willow. The *in situ* evaluation of clones, performed on the SRC experimental field in the metal-contaminated area, lead to the selection of 2

experimental (INBO) willow clones, *Salix viminalis* and *Salix alba x alba*. In comparison with the best performing commercial and other experimental clones, these clones exposed by nature higher stem metal concentrations (up to 7% for Cd and 21% for Zn) (*S. viminalis*) or a higher biomass production (up to 4%) (*S. alba x alba*). Bioaugmentation of the previously selected willow clones, evaluated in pot experiments with in total 17 promising bacterial strains, did not result in improved biomass yields nor enhanced metal accumulation or translocation. The fertilizer applications on the contrary, also applied to the former selected willow clones growing in pots, raised productivity levels significantly and in case of *S. alba x alba*, *in planta* metal concentrations also increased. As a result, the tested fertilizers doubled (*S. viminalis*) or even tripled (*S. alba x alba*) phytoextraction efficiency of the selected clones.

In conclusion, of all evaluated high biomass crops for metal phytoextraction in the Campine area, a prominent role is reserved for SRC. Focusing on metal phytoextraction in this region using SRC of willow, the decontamination rate observed on the longer term revealed to be much higher than previously predicted. Moreover, *in situ* clone selection and adjusted fertilization applications seem very promising strategies to further reduce remediation time using this crop.
Finally, our results demonstrate that it is highly recommendable to perform future research on phytoextraction topics as much as possible *in situ*.

Samenvatting

In het noordoosten van België (de Kempen) is een gebied van minstens 280 km^2 vervuild met metalen, voornamelijk cadmium (Cd), zink (Zn) en lood (Pb), als gevolg van voormalige activiteiten van zinksmelters in de buurt. De negatieve gevolgen voor inwoners en het milieu in het algemeen, alsook economische verliezen in de landbouw, hebben regionale beleidsmakers aangezet opdracht te geven deze metaalverontreinigde gronden te saneren. Rekening houdend met de grootte van het gebied en de diffuusheid, matigheid en oppervlakkigheid van de vervuiling, wordt fytoextractie als gepaste saneringsstrategie naar voor geschoven. Fytoextractie is het gebruik van planten om metalen uit de bodem te extraheren en vervolgens te accumuleren in bovengrondse plantendelen die geoogst kunnen worden. Meer specifiek blijkt het telen van niet-eetbare biomassagewassen met een matige metaalaccumulatie veelbelovend voor de sanering van dit gebied.

Aangezien nog niet bekend is welk biomassagewas het hoogst, maar ook meest stabiel (in tijd) fytoextractiepotentieel heeft op deze verontreinigde gronden, worden in deze thesis verschillende biomassagewassen geteeld en geëvalueerd in veldexperimenten van meerdere jaren op een Cd-Zn-Pb verontreinigd terrein in het noordoosten van België. De biomassaproductie en de accumulatie van metalen in geselecteerde tabaksklonen (*Nicotiana tabacum* L.), geselecteerde zonnebloemmutanten (*Helianthus annuus* L.) en commercieel verkrijgbare hennep (*Cannabis sativa* L.) werden bepaald in veldexperimenten van 2 tot 4 opeenvolgende jaren. Daarnaast werd het fytoextractiepotentieel van meer dan 200 verschillende, commercieel beschikbare en niet-commercieel beschikbare (experimentele), populieren- (*Populus*) en wilgen- (*Salix*) klonen in korte omloop rotatie geschat na de eerste rotatiecyclus (na 4 groeiseizoenen). De tabaksklonen en zonnebloemmutanten bleken efficiënte opnemers van respectievelijk Cd en Zn, terwijl de hoogste simultane opname van Cd en Zn werd gerealiseerd met de houtige klonen in korte omloop. Fytoextractie van Pb bewerkstelligen met de geëvalueerde gewassen bleek een utopie. Dit hoeft echter geen beperking te zijn gezien de biobeschikbaarheid en daarmee ook het risico van Pb voor mens en omgeving zeer laag is. De berekende

saneringsperiodes zijn lang (> 60 jaar) en benadrukken dat een synergie met economische en (andere) ecologische voordelen cruciaal is om fytoextractie op grote schaal realistisch te maken. Deze externe opportuniteiten werden voor de geëvalueerde biomassagewassen gedefinieerd aan de hand van een literatuurstudie. Wanneer fytoextractiepotentiëlen en bijkomende economische en ecologische kansen werden gecombineerd, bleek korte omloop hout (KOH) van wilg en populier het meest geschikte gewas voor het toepassen van fytoextractie in het onderzochte gebied.

Naast de veldstudies met de verschillende biomassagewassen werd fytoextractie met KOH van wilg meer in detail bestudeerd. Wilg in korte omloop is een veel onderzocht gewas in het kader van extractie van metalen. Echter, de doeltreffendheid van fytoextractie met KOH op de langere termijn is ongekend als gevolg van de complexe interacties tussen, en veranderingen in de hoofdelementen van een fytoextractiesysteem. Het is daarom van groot belang om de effectieve daling van metaalconcentraties in de bodem en veranderingen in toxiciteitslevel, alsook bodemvruchtbaarheid en –functioneringsvermogen, te onderzoeken in langere termijn KOH fytoextractie-experimenten. Het KOH proefveld in het metaalverontreinigde gebied in het noordoosten van België biedt in dit opzicht een unieke kans. Metaalconcentraties in de bodem en bodemtoxiciteit, onderzocht gebruik makend van gestandaardiseerde chemische extracties en ecotoxiciteitstesten met planten en ongewervelden, werden bepaald in bodem na 8 jaar behandeling met KOH van wilg (Tora; *Salix schwerinnii x Salix viminalis*) en in bodem zonder fytoextractiebehandeling. De geobserveerde sanering na 8 jaar was veel hoger (48 keer hoger in het geval van Cd en 79 keer in geval van Zn) dan voorspeld door het extrapoleren van data verkregen na 4 jaar groei van deze wilg op het proefveld. Daarenboven wezen de chemische bodemextracties en alle ecotoxiciteitstesten unaniem uit dat de met wilg behandelde bodem minder toxisch was dan de bodem zonder fytoextractiebehandeling.

Met het oog op het inkorten van de saneringsduur van fytoextractie met KOH van wilg en/of het verhogen van externe voordelen die gepaard gaan met deze saneringsmethode werd in een laatste deel van deze thesis getracht de biomassaproductie en/of de opname van metalen van wilg te verbeteren. Om

bovenstaand doel te bereiken werden 3 strategieën onderzocht: een *in situ* selectie van klonen, de aanrijking van wilg met plantgeassocieerde bacteriën met voordelige eigenschappen en bemesting van wilg. De evaluatie van klonen *in situ*, uitgevoerd op het KOH proefveld in het metaalverontreinigde gebied, leidde tot een selectie van 2 experimentele wilgenklonen, *Salix viminalis* and *Salix alba x alba*. In vergelijking met de best presterende commerciële en andere experimentele klonen vertonen deze klonen een van nature hogere metaalconcentratie in de stam (tot 7% voor Cd en 21% voor Zn) (*S. viminalis*) of een hogere biomassaproductie (tot 4%) (*S. alba x alba*). De aanrijking van deze geselecteerde wilgenklonen met bacteriën met veelbelovende eigenschappen werd geëvalueerd in potexperimenten. In totaal werden 17 verschillende bacteriën getest maar een verhoogde biomassaproductie of hogere metaalaccumulaties of translocaties konden niet worden vastgesteld. Behandelingen van de wilgen met meststof (in potexperimenten) verhoogde de productie van biomassa daarentegen substantieel en de metaalconcentraties in de plant stegen ook in het geval van de *S. alba x alba* kloon. Bemesting had bijgevolg een verdubbeling (*S. viminalis*) en zelfs een verdriedubbeling (*S. alba x alba*) van de fytoextractie-efficiëntie tot gevolg.

Tot besluit, van alle biomassagewassen die geëvalueerd werden voor fytoextractie van metalen in de Kempen is een vooraanstaande rol weggelegd voor KOH van wilg en populier. Specifiek voor fytoextractie in dit gebied met KOH van wilg, bleek de geobserveerde saneringssnelheid op langere termijn veel hoger te zijn dan eerder voorspeld. Bovendien zijn een *in situ* selectie van klonen en aangepaste bemesting veelbelovende strategieën om de saneringsduur met KOH van wilg verder te reduceren.
Tot slot benadrukken de resultaten in deze thesis dat het sterk aanbevolen is toekomstig fytoextractie-onderzoek zoveel als mogelijk op veldschaal uit te voeren.

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Chapter 1

Introduction

1.1 Metal-contaminated soils

Metals occur in the soil all over the Earth and a lot of them are indispensible for live in general (essential metals). However, non-essential metals but essential metals too, are or become toxic to living organisms at certain concentrations. In this thesis, concentrations of metals (being essential or not) in soil that are potentially toxic for living organisms are referred to as 'toxic metal concentrations', while the soil itself is labeled as 'metal-contaminated'.

The presence of toxic metal concentrations in soils is one of the most serious environmental problems worldwide (Vassilev *et al.* 2004). In Europe, contamination by metals accounts for more than 34% of cases of soil pollution, followed by mineral oil (23.8%) and polycyclic aromatic hydrocarbons (10.9%) (EAA 2014). Metal contamination has been identified by the European Commission as one of the 8 major threats to European soils (Kidd *et al.* 2015). Anthropogenic sources of metal contamination are pyrometallurgical ('smelting') industries, residues from metalliferous mining, combustion of fossil fuels and waste incineration as well as some pesticides and fertilizers used in agriculture (Vassilev *et al.* 2004). This in addition to natural sources of metals like weathering and erosion of rocks, volcanic activities, marine aerosols and forest fires (Nagajyoti *et al.* 2010) and soils that naturally contain high metal concentrations (Vassilev *et al.* 2004).

The Campine region ('Kempen' in Dutch), stretching over the northeast of Belgium and the southeast of the Netherlands (Figure 1.1), is contaminated by cadmium (Cd), zinc (Zn) and lead (Pb). The source of the contamination is

anthropogenic and historical. The contamination imposes a major risk to human health and the environment in general and the remediation of the soil is by no means easy.

1.1.1 Anthropogenic, historical contamination in the Campine region

In Belgium, pyrometallurgical industries initially built up around Liège to extract metals such as Zn and Pb on an industrial scale (Morgan 1985). Flue gasses exited the plants unfiltered and had a devastating impact on the health of many people in densely populated areas such as Liège. Therefore, new Zn-smelters were established in low-populated areas such as in the Campine region in Belgium and the Netherlands in the 19th century. The Campine region was moreover selected because of high quality transport infrastructure (*e.g.* the canal Bocholt-Herentals, a railway ('ijzeren Rijn')) and high unemployment rates. Zinc-smelters were built in Belgium in Overpelt (1880), Balen (1885), Lommel (1904) and Rotem (Dilsen) (1913) (and close to the Belgian border in Budel, the Netherlands (1892)) (Colpaert *et al.* 2004) (Figure 1.1). Unfortunately, the early pyrometallurgical process adopted, the so-called Belgian-type horizontal retort process, was very inefficient (Morgan 1985). Ores containing ZnS, PbS, ZnO and PbO were heated together with charcoal to 1400°C end gaseous Zn and Pb were subsequently collected in water-cooled condensers (while CO and SO_2 gasses excited the plants as flue gas). However, large quantities of Zn and Pb exited the plants as flue gasses as well, and since the ores typically also contained high concentrations of Cd (volatization temperature < 800°C), also substantial amounts of Cd were volatized during the process. As a consequence, large quantities of Cd, Pb, S and Zn were emitted from the pyrometallurgical industries in the northeast of Belgium during the period 1880-1973. Through atmospheric deposition, an area of about 700 km^2 in both Belgium and the Netherlands became historically, moderately contaminated by Cd, Zn and Pb (Staessen *et al.* 1994; Vangronsveld *et al.* 1995a; Hogervorst *et al.* 2007). Furthermore, the discharging of water used for cooling and the application of highly contaminated industrial waste products (retorts, condensers and ores) for railway and road construction, on school and farm yards, *etc.* spread the pollution even further (Verlaek and Weynants 2006). Since 1973, electrochemical processes replaced pyrometallurgical processes resulting in a

drop in atmospheric metal emissions (*e.g.* Cd-emissions in Overpelt decreased from 125 000 kg per year in 1950 to 130 kg year in 1990). However, since metal ions are not degradable, they are still present in elevated concentrations in the Campine region today. Decades after emissions from Zn-smelters have been cut, the so-called metal deserts, of which some span several square kilometers, still witness the toxicity and unsuitability of the soil.

The 7 most suffering municipalities in Flanders, Belgium (Balen and Mol in the province of Antwerp, Hamont-Achel, Hechtel-Eksel, Lommel, Neerpelt and Overpelt in the province of Limburg; Figure 1.1) cover an area of 494 km^2 and count more than 147 000 inhabitants (Schreurs *et al.* 2011). Large areas of this contaminated region are in agricultural use. Since the soil in the Campine region is characterized by a sandy texture, relative low pH values and organic matter content, the uptake of metals in crops and leaching of metals to the groundwater is relatively high (see further) (De Temmerman *et al.* 2003; Kirkham 2006). As a consequence, food and fodder crops produced in this area frequently exceed European and Belgian threshold values for Cd (Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002: Commission Regulation no. 1881/2006) (Witters *et al.* 2009; Ruttens *et al.* 2011). This imposes a serious concern regarding food safety and health of humans and other organisms and additionally threatens crop growth and marketability of the farming industry.

In this research, we focus on the remediation of the metal-contaminated area in the Campine region in Belgium. Metals of concern are Cd, Zn and Pb and, by consequence, further information is focused on these metals.

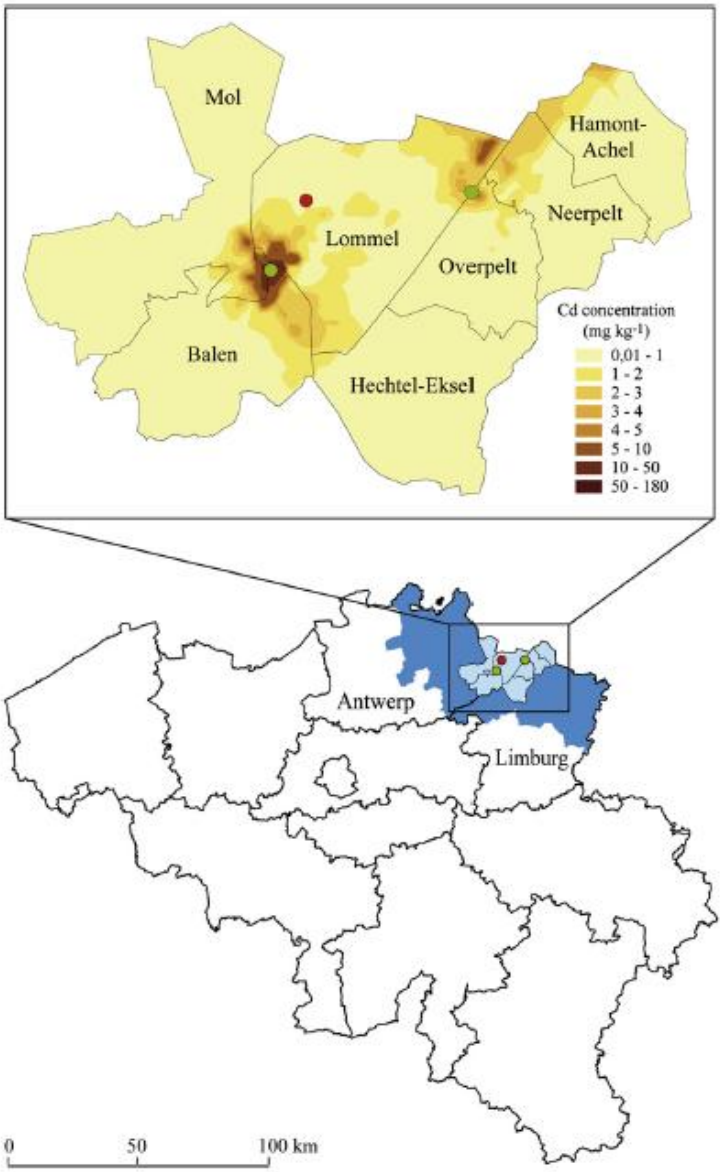

Figure 1.1 Below: location of the Belgian Campine region (shaded in dark blue). Above: Location of the Zn-smelters in Balen, Lommel and Overpelt and the 7 most suffering municipalities. Excavation significantly lowered the contamination level around the dismantled (1974) Zn-smelter in Lommel (Maatheide) (red dot). Source: Schreurs, Voets and Thewys (2011). Target (background) and cleanup values for Cd are respectively 0.7 and 2 mg kg^{-1} dry soil.

1.1.2 Risks related to metal-contaminated soil

A soil containing metals such as Cd, Zn and Pb in toxic concentrations imposes risks for the environment since these metals might be spread through water erosion, dispersed by the wind or leached to ground- or surface waters. In addition, the uptake/intake of the metals by plants and animals might cause serious toxicity symptoms. Before uptake/intake and toxicity of Cd, Zn and Pb in plants and humans are described, 3 important remarks regarding the toxicity of a metal-contaminated soil should be made.

Firstly, in general, only a part of the total metal content in the soil is available to interact with a biological target (Geebelen *et al.* 2003). This part is mainly composed of metals present as free ions, in soluble forms and absorbed to inorganic constituents at ion exchange sites (McGrath *et al.* 2001; Vassilev *et al.* 2004; Nolan *et al.* 2005) and is often lower than 1% of the total metal content in soil (Whiting *et al.* 2001; Braud *et al.* 2006). The risk of a metal-contaminated soil is therefore strongly related to the 'bioavailable' concentration of metals rather than to the total amount present in soil (Vangronsveld *et al.* 2009). However, when the 'bioavailable' pool of metals diminishes through leaching or uptake by plants, it may be replenished from the total metal pool both by the soil buffering capacity and by diffusion processes (Kashem and Singh 2002; Whiting *et al.* 2001). Bioavailability of metals depends on several factors such as metal species, form and concentration, soil structure and soil characteristics (pH, organic matter content, redox potential, concentration of other elements, temperature, humidity,…) (Benavides *et al.* 2005; Vamerali *et al.* 2010). For example, metal cations Cd and Zn occur in exchangeable forms while Pb is mainly being adsorbed to soil particles or precipitated and thus less bioavailable (Clemens 2001; Puschenreiter *et al.* 2001). Furthermore, soil with low levels of ion exchange sites (like nutrient poor, sandy soils) expose by definition a higher metal bioavailability. Also a negative relation between soil pH and metal bioavailability was numerously reported in literature (Zaccheo *et al.* 2006). Since the soil in the Campine region is characterized by a sandy to sandy-loam soil structure, low nutrient levels and slight acidity (pH 4-6), the bioavailability of Cd and Zn is relatively high (Van Slycken *et al.* 2013).

Secondly, the simultaneous presence of various metals can cause synergic or antagonist interactions, thus increasing or lowering the toxicity of 1 metal (Vamerali *et al.* 2010). Experiments with mycorrhizal fungi revealed that the application of a higher Zn concentration reduced Cd toxicity in the fungus (Brunnert and Zadrazil 1985; Colpaert and Van Assche 1992; Hartley *et al.* 1997). Hartley, Cairney and Meharg (1997) furthermore reported that the combined toxicity of Cd, Zn, Pb and Sb was equal to that of Cd alone.

Thirdly, although Cd, Zn and Pb can be toxic to living organisms at certain concentrations, a distinction has to be made between Zn on the one hand and Cd and Pb on the other. Zinc is an essential trace element (micronutrient) for all living organisms (Frassinetti *et al.* 2006; Rout and Das 2009; Nagajyoti *et al.* 2010). It is crucial for cell division in all higher organisms to realize growth and reproduction, for the functionality of more than 300 enzymes, for the stabilization of DNA, for gene expression and for the immune system. Human beings are rarely exposed to an excess of Zn, they are more likely to suffer Zn deficiencies. On the contrary, ecosystems (like the Campine area) can be subjected to an excess of Zn and Zn can easily become toxic to plants when present in excess (Hall 2002; Clemens 2006; Rout and Das 2009; Nagajyoti *et al.* 2010). Cadmium and Pb are non-essential trace elements and can be highly toxic for living organisms already at low concentrations.

Plants and metals

The **uptake** of metals by plants occurs via the roots, generally in association with the uptake of water and other minerals. Besides bioavailability of metals, root characteristics (volume, kinetics, age, *etc.*) additionally play an important role during metal uptake (Lasat 2002).

The entrance of Zn^{2+} into a root cell (crossing the plasma membrane) is mainly mediated by a group of transporters belonging to the Zn- and Fe-regulated transporter protein (ZIP) family (Clemens 2001). These transporters passively move Zn^{2+} using its electrical and/or concentration gradient (Kawashi *et al.* 2011). For Cd^{2+} and $Pb^{2+,}$ being non-essential metal ions, no specific transport mechanisms exist. It is suggested that both metals enter root cells via uptake mechanisms for essential cations (e.g. Fe^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}) exposing a low substrate specificity (Clemens 2001; Benavides *et al.* 2005; Hart *et al.* 2006;

Krämer *et al.* 2007; He *et al.* 2009). The ZIP family of metal transporters and Ca^{2+}-channels are demonstrated to be involved in the opportunistic hitchhiking of Cd^{2+}, while the natural resistance associated macrophage protein (NRAMP) family might also play a role. The mechanisms by which Pb enters the root are less known. Although several authors have reported that Ca^{2+}-channels might be the main pathway (Sharma and Dubey 2005; Wang *et al.* 2007a; Pourrut *et al.* 2011), alternative non-selective pathways, such as cyclic nucleotide-gated ion channels or other low-affinity cation transporters cannot be excluded (Pourrut *et al.* 2011). Once inside a root cell, (the excess of) Zn, Cd and Pb will preferentially bind to nitrogen (N), sulfur (S) and oxygen (O) donors (Nieboer and Richardson 1980; Clemens 2001, 2006). These can be functional groups in enzymes as well as low molecular weight (LMW) ligands that are constitutively present (gluthathione; GSH) or synthesized in response to the presence of metal ions (GSH-derived peptides called phytochelatines; PC) (Clemens 2001; Cobbett and Goldsbrough 2002; Hart *et al.* 2006; Vázquez *et al.* 2006). The potential role of metallothioneins and organic acids as ligands is not discussed here. Metal-LMW-ligand complexes can be transported into the vacuole, probably mainly through ATP-binding cassette (ABC)-transporters (Clemens 2001; Cobbett and Goldsbrough 2002). In the vacuole, high molecular weight (HMW) complexes are formed of which the exact nature is not really understood. The sequestration of excess metal ions in vacuoles is a common detoxification process since it protects the cell contents from toxic effects (Clemens 2001; Cobbett and Goldsbrough 2002; Benavides *et al.* 2005; Sharma and Dubey 2005). In case of Pb, immobilization by negatively charged pectins within the cell wall, precipitation of insoluble Pb salts in intercellular spaces and accumulation in plasma membranes also occur (Pourrut *et al.* 2011).

Besides compartmentalization in the vacuole (and immobilization/precipitation in case of Pb), the metal (complexes) can also be transported upwards to other plant parts via the xylem (Clemens 2001; Pourrut *et al.* 2011). To reach the xylem vessels, radial movement in an apoplastic and/or symplastic way are assumed to be possible in case of Cd and Zn (Di Toppi and Gabbrielli 1999; Clemens 2001; Benavides *et al.* 2005), while Pb mainly moves in the apoplast (Sharma 2005; Pourrut 2011) (Figure 1.2). The symplastic route is considered

more selective since cell membranes have to be crossed. The main barrier metals encounter before reaching the xylem, is the root endodermis which consists of cells equipped with Casparian strips blocking the apoplastic route (Figure 1.2). Cadmium and Zn might pass in a symplastic way, be actively loaded into the xylem, be whether or not bound to ligands and transported upwards with the xylem fluid. The apoplastic transport of Pb however, is highly restricted by the Casparian strips (Sharma and Dubey 2005; Pourrut *et al.* 2011). In this way, Pb is accumulated near the endodermis where a major part is sequestered or excreted. However, a part of the Pb moves up through the vascular tissues and diffuses out in the surrounding tissues (Seregin and Ivanov 2001). It is suggested this can be realized in 2 ways: (1) Pb might pass the Casparian barrier by means of symplastic passage (Sharma and Dubey 2005); (2) near the tip of roots and close to the lateral branches, the Casparian strip is not fully formed and Pb (as well as other metals) can enter the vascular tissues without passing through a cell membrane (Vamerali *et al.* 2010). After penetrating into the vascular cylinder, Pb can again be transported via the apoplastic pathway (Sharma and Dubey 2005; Pourrut *et al.* 2011). It might eventually be loaded into xylem vessels and be transported to other plant parts.

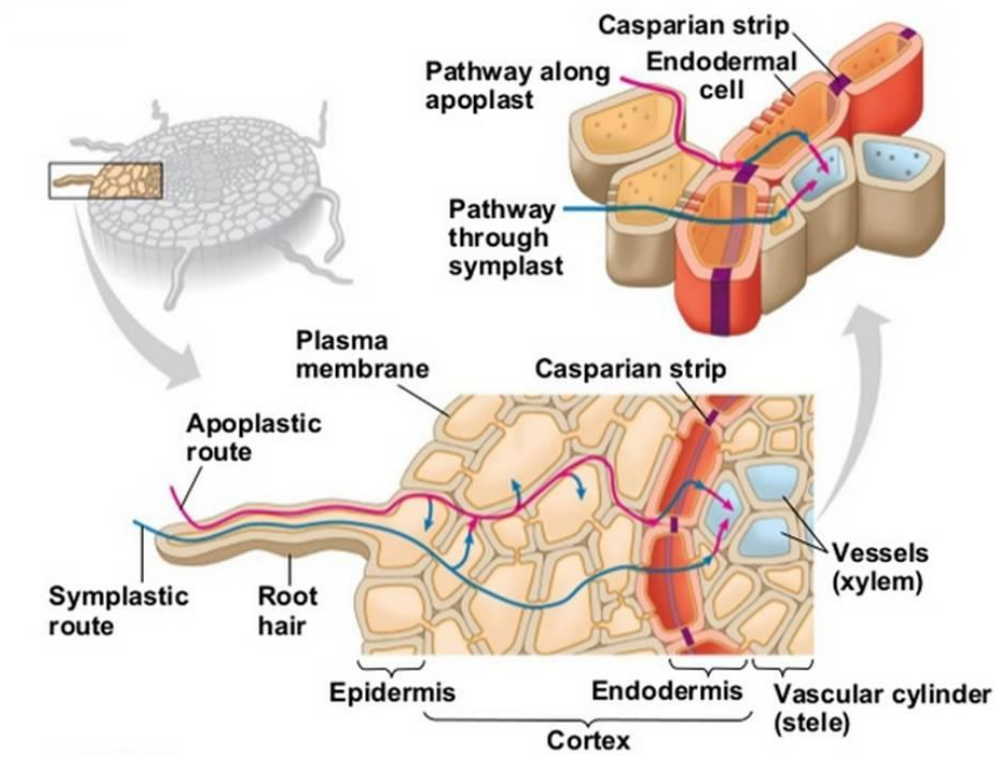

Figure 1.2 Cross section of a root structure and display of the apoplastic and symplastic route by which metal ions are transported from soil to xylem vessels. The passage of the endodermis layer equipped with Casparian strips is highlighted above. The symplastic route is considered more selective since cell membranes have to be crossed. Source: Campbell and Reece (2005).

Although plants have developed a complex network of homeostatic mechanisms (controlling uptake, accumulation, transport and detoxification of metals) in order to maintain the concentration of essential metals within physiological limits and to minimize detrimental effects of non-essential metals (Clemens 2001, 2006; Cobbett and Goldsbrough 2002), at certain levels, the effect of metals on plants becomes toxic. In general, the various **toxic effects** of metals on diverse aspects of the plant metabolism can be related to 3 main characteristics of metals.

(1) Their capability to interact with ligand groups of biomolecules (*e.g.* enzymes or nucleic acids). Cadmium, Zn and Pb are known to have a relatively high affinity for sulphydryl (-SH) and carboxyl (-COOH) groups (Nieboer and Richardson 1980). Since -SH groups are needed for enzyme activity and/or stability, blocking of these groups seriously inhibits functionality of enzymes or even leads to their denaturation (Van Assche and Clijsters 1990; Seregin and Ivanov 2001; Sharma and Dubey 2005). Several toxic effects of Cd, Zn and Pb can be attributed to this 'blocking' mechanism, *e.g.* inhibition of chlorophyll biosynthesis reducing photosynthetic capacities and alteration of the plasma membrane permeability by affecting the ATP-ases in it (Vangronsveld and Clijsters 1994; Küpper *et al.* 1998; Di Toppi and Gabbrielli 1999; Clijsters *et al.* 1999; Clemens 2001; Benavides *et al.* 2005; He *et al.* 2009; Pourrut *et al.* 2011). As the nucleophilic centres in nucleic acids are also favourite binding sites of metal ions, crosslinks between DNA strands, single-strand DNA breaks and chelation or formation of complexes between DNA and metals have been reported (Smeets *et al.* 2005).

(2) Their chemical similarity to essential metals which makes substitutions in *e.g.* metalloenzymes possible. As a result, lower efficiency levels, malfunctioning or loss of functionality of enzymes were reported numerously. A lot of inhibitions in the photosynthetic system (*i.e.* disruption of the electron transport chain) are well-documented (Van Assche and Clijsters 1983, 1986; Krupa and Moniak 1998; Clijsters *et al.* 1999; Smeets *et al.* 2005). Furthermore, the replacement of essential metals by Cd, Zn or Pb might cause deficiency effects. Disturbances and deficiencies of mineral nutrition in general (Nagajyoti *et al.* 2010; Pourrut *et al.* 2011) as well as specific interferences in uptake between above-mentioned

metal ions and essential macronutrients (Ca, Mg, P and K) (Di Toppi and Gabbrielli 1999; Benavides *et al.* 2005; He *et al.* 2009; Nagajyoti *et al.* 2010) have been reported.

(3) Their capability to induce oxidative stress, indirectly, and to activate a sequence of signals in response (Cuypers *et al.* 1999; Foyer and Noctor 2005). Oxidative stress (OS) reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. ROS radicals occur transiently in aerobic organisms because they are also generated in plant cells during normal metabolic processes such as respiration and photosynthesis (Halliwell *et al.* 1999). Although some of them may function as important signalling molecules, all ROS can be extremely harmful to organisms at high concentrations since they can oxidize proteins, lipids and nucleic acids, often leading to alterations in cell structure, mutagenesis and cell death. A variety of proteins (antioxidative enzymes) and a host of non-protein scavengers including GSH, function as scavengers of ROS (Noctor and Foyer 1998). Cd^{2+}, Zn^{2+} and Pb^{2+} are redox-inert metals, not able to induce ROS production through a Fenton-like reaction (Smeets *et al.* 2005). They however indirectly induce OS (increase ROS concentrations in cells) by reducing the pool of antioxidant GSH (see paragraph metal uptake by plants), by disruption of the electron transport chain or by activating Ca-dependent systems affecting Fe-mediated processes (Clemens 2001; Pinto *et al.* 2003; Benavides *et al.* 2005; Sharma and Dubey 2005; Smeets *et al.* 2005; Pourrut *et al.* 2011). Alterations in membrane permeability due to changes in lipid composition and/or lipid peroxidation are reported in relation to Cd, Zn and Pb toxicity by the same authors. Besides the effects explained above, changes in hormonal status and a disturbed water balance are often reported as a result of the presence of toxic amounts of Cd, Zn and/or Pb (Clemens 2001; Benavides *et al.* 2005; He *et al.* 2009; Nagajyoti *et al.* 2010; Pourrut *et al.* 2011).

Cadmium, Zn and Pb are known to induce a very broad range of toxic effects on plants, reflected at the biochemical and physiological level as described above, but eventually also expressed at the morphological level. Without being complete, frequently observed morphological symptoms of Cd, Zn and Pb

toxicity are: inhibited germination/development/growth, dwarf growth, senescence and chlorosis of leaves, leaf epinasty and leaf roll, browning of root tips, root blunt and root thickening, death root tips and leaf tips and possibly overall death (Van Assche and Clijsters 1990; Vangronsveld and Clijsters 1992; Clijsters *et al.* 1999; Cuypers *et al.* 1999; Di Toppi and Gabbrielli 1999; Clemens 2001; Benavides *et al.* 2005; Sharma and Dubey 2005; He *et al.* 2009; Rout and Das 2009).

Humans and metals

When considering Cd, Zn and Pb, metal toxicity to humans is mostly caused by the presence of Cd and Pb since an excess of Zn in humans is seldom occurring. The consumption of contaminated dietary products and ingestion of soil particles are well-recognized routes for **intake** of Cd and Pb of non-smoking individuals in the contaminated area (Nawrot *et al.* 2010) (acute poisoning is not discussed here). However, a growing body of evidence suggests that exposure to soil brought indoors, present as house dust, might also stand for a critical route of exposure (Hogervorst *et al.* 2007). In addition, intake routes resulting from contaminated groundwater applications also exists (Flemish Agency for Care and Health, Cadmiumwebtool). Most of the Cd will end up in the gastrointestinal tract (also the inhaled particles) and will subsequently mainly be stored in kidneys, liver and also testes (Nawrot *et al.* 2010). Since the half-life of Cd in the body is 10-30 years, an age-related cumulative increase in the body of this metal occurs. On the longer term, **toxic effects** are reported to cause renal dysfunction, osteoporosis, lung cancer, anomalies in the arterial system functioning and even an increased risk for cancer overall and total mortality (Järup 2003; Schoeters *et al.* 2006; Hogervorst *et al.* 2007; Nawrot *et al.* 2008, 2010; Schutte *et al.* 2008). Lead may be absorbed in the lungs or in the gastrointestinal tract (Järup 2003). Lead in the blood is bound to erythrocytes and can slowly be eliminated via urine. The accumulation of Pb in the skeleton will however also occur and, while the half-life of Pb in blood is about 1 month, in the skeleton it is 20-30 years. In less serious cases, the most obvious sign of Pb poisoning is disturbance of haemoglobin synthesis, which may eventually lead to anaemia. Long-term continuous exposure might cause Pb encephalopathy (syndrome of global brain dysfunction), characterized by

sleeplessness and restlessness. However, in more severe cases, the affected individual may suffer from acute psychosis, confusion, reduced consciousness, memory deterioration, prolonged reaction time and a reduced ability to understand. Children are especially susceptible since a higher gastrointestinal uptake occurs and Pb can penetrate the still permeable blood-brain barrier. Behavioural disturbances, learning and concentration difficulties are reported as a consequence of incurred brain damage. The overall evidence for Pb as a carcinogen is only weak, the most likely candidates are lung cancer, stomach cancer and gliomas (Steenland and Boffetta 2000). On the basis of sufficient (Cd) or rather insufficient (Pb) evidence in both humans and experimental animals, the International Agency for Research on Cancer (IARC) has classified Cd as a 'human carcinogen' (group 1) and Pb as a 'possible human carcinogen' (group 2) (IARC 2015, http://monographs.iarc.fr/ENG/Classification/).

Cadmium vs. zinc vs. lead

In general, it is observed that the biological availability of Pb in soil is low (Clemens 2001, 2006; Puschenreiter *et al.* 2001; Pulford and Watson 2003) limiting dispersion or leaching risks as well as plant uptake. In addition, Pb taken up by the roots of plants is not easily translocated to aboveground plant parts for reasons explained earlier (Sharma and Dubey 2005; Pourrut *et al.* 2011). Although in the Campine region several hundreds of mg Pb are present per kg of dry soil, this metal is not directly indicated to be the most toxic for humans. Zinc and Cd are more available in the soil and rather easily leached, dispersed or taken up and transported in plants. However, Zn is an essential microelement and an excess of Zn in humans seldom occurs. By consequence, Cd has always been considered the most toxic and dangerous contaminant in the Campine region. Although the detrimental effects resulting from Cd contamination already worried researchers decades ago (Buchet *et al.* 1980; Chang *et al.* 1980; Lauwerys *et al.* 1980; Roels *et al.* 1980, 1981), only in 2006 the Flemish government proposed an action plan (Actieplan Cadmium voor de Noorderkempen). In the Campine region, 18 actions were undertaken, including control, monitoring and contaminant removal actions as well as several awareness and sensitization actions (information in Dutch can be found at http://www.lne.be/themas/milieu-en-gezondheid/acties/cadmiumpro-

blematiek/actieplan-cadmium).

1.2 Remediation options in the Campine region

Since the metal-contaminated area in the Campine region imposes a serious risk for living organisms, the environment and the agricultural sector, remediation of the soil is strongly promoted by the Public Waste Agency of Flanders (OVAM) (Witters *et al.* 2009). The target (background) values and cleanup values for Cd, Zn and Pb in Flanders depend on site-specific characteristics as they are a function of soil destination type, clay content, organic matter content and pH (Vlarebo 2008) (Table 1.1). Since the Campine soil generally is considered to be moderately contaminated with Cd (0-5 mg Cd kg^{-1} dry soil) (Schreurs *et al.* 2011) (although areas with a higher Cd concentration can be found; Figure 1.1) and several hundreds of mg Zn and Pb kg^{-1} dry soil occur (Ruttens *et al.* 2011), the legally determined cleanup values for Cd, Zn and Pb are mostly exceeded (Table 1.1).

Table 1.1 Target (background), cleanup and the in this research measured Cd, Zn and Pb concentrations (mg kg^{-1} dry soil) in the Campine area. The site-specific characteristics that are assumed in the calculations are: destination type: agricultural, pH-KCl: 5, clay: 3% and organic matter: 4%. Formulas can be found at: https://navigator.emis.vito.be/mijn-navigator?woId=23022.

Contaminant (mg kg^{-1} dry soil)	Target values	Cleanup values	Campine values
Cd	0.7	2	1.8-13
Zn	50	282	90-778
Pb	38	200	97-342

The selection of the most appropriate soil remediation method depends on site characteristics, types of contaminants (and speciation), contamination level and the end use of the contaminated area (Mulligan *et al.* 2001). Given the site characteristics, the contaminants and contamination level of the Campine region and the fact that the contamination is diffuse and shallow (mainly located in the agriculturally active soil layer), conventional as well as plant-based remediation technologies (phytoremediation) are possible cleanup options. Both technologies are further discussed below.

1.2.1 Conventional remediation technologies

A number of conventional, civil-engineering-based remediation technologies can be applied in case of a Cd-Zn-Pb-contaminated soil (Mulligan *et al.* 2001). In extreme cases, isolation and containment strategies like landfill covers, encapsulation by physical barriers (*e.g.* of steel or cement) and solidification/stabilization (*e.g.* by vitrification or injecting chemicals) could be applied as well as excavation and dumping the soil elsewhere. Effective treatments of the soil can be categorized *ex situ* or *in situ*. Physical separation (*e.g.* gravity separation, fluidized bed separation, flotation…), soil washing (addition of surfactants and other additives to leach metals) and pyrometallurgical processes (elevated temperature extraction of metals) are commonly used *ex situ* technologies. For *in situ* treatment, soil flushing (with or without additives to leach contaminants) and electrokinetic applications (which induce an electric current in the soil) can be implemented.

However, because of the vastness of the target contaminated area in the Campine region (>280 km^2 in Belgium; Ruttens *et al.* (2008)), conventional remediation technologies render economically and practically inapplicable (Vangronsveld *et al.* 1995a; McGrath *et al.* 2001; Di Baccio *et al.* 2003; Meers *et al.* 2003; Van Ginneken *et al.* 2007; Ruttens *et al.* 2011). Moreover, all conventional techniques either remove the top soil or change its properties substantially which retards or hampers the usage of the soil for agriculture afterwards. Furthermore, until recently, almost no attention was paid to the secondary environmental effects (for instance release of greenhouse gasses) during remediation and the consumption of natural resources. These should be explicitly included and quantified in the evaluation/comparison of different remediation options.

As a result of major practical, economic and environmental drawbacks, traditional civil-engineering-based technologies are considered inapplicable and undesired in the area of interest.

1.2.2 Phytoremediation

An alternative strategy to contain, inactivate, remove or degrade harmful environmental contaminants is the use of plants. This strategy is termed phytoremediation and is considered a relatively cheap, solar-driven, *in situ* remediation technology (Salt *et al.* 1998). Phytoremediation is commonly divided in 5 main subgroups of which phytostabilization and phytoextraction are applicable in case of metal contamination (Pulford and Watson 2003; Vangronsveld *et al.* 2009; Vamerali *et al.* 2010).

Phytostabilization

The stabilization of toxic metal concentrations in the soil is not a real cleanup technology but rather a management strategy to decrease the risks of contaminants (Vangronsveld *et al.* 1995a, 1996; Vangronsveld and Cunningham 1998). Indeed, establishing a vegetative cover on metal-contaminated soil prevents the dispersal of contaminants through water and wind erosion and water percolation compared to bare or sparsely vegetated sites (Vangronsveld *et al.* 2009; Vamerali *et al.* 2010). Moreover, plants may also help to effectively stabilize the contaminants by accumulating and precipitating toxic elements in the roots or adsorb them on root surfaces whereby decreasing their leaching. Microorganisms living in the rhizosphere of these plants likely also play an important role to this concern. Plants should be metal-tolerant and suitable for the site and, ideally, should not accumulate contaminants in aboveground plant tissues, which could end up in the food chain (Vassilev *et al.* 2004; Vamerali *et al.* 2010). Phytostabilization is often combined with the use of soil amendments to immobilize toxic compounds in soil resulting in a reduced biological availability which decreases leaching and dispersion risks as well as plant uptake and eventual toxicity (Vangronsveld *et al.* 2009; Vamerali *et al.* 2010). Possible soil amendments for stabilization are liming agents, beringite, zeolites, iron oxides, phosphates, organic material… (for reviews see Mench *et al.* 1998, Vangronsveld *et al.* 2000; Adriano *et al.* 2004) and the approach can be termed 'aided phytostabilization'. (Aided-)Phytostabilization eventually leads to an attenuation of the impact on site and to adjacent ecosystems, preventing health risks, and might be a temporary or a definitive action (Vangronsveld *et al.* 2009).

Phytoextraction

Phytoextraction is the use of metal-accumulating plants to extract metals out of the soil and concentrate them in harvestable plant parts (Kumar *et al.* 1995; Salt *et al.* 1998). As the plant biomass is harvested and processed, contaminants are permanently removed from the soil. The idea of using plants to clean up contaminated environments is already old and cannot be traced to any particular source (Blaylock and Huang 2000) but Chaney (1983) was the first to re-introduce it as a remediation technology on metal-contaminated soils. The phytoextraction efficiency of a plant (determining the remediation time needed) is determined by the concentration of metals in the harvestable biomass and the amount of harvestable biomass produced. Initially, much interest focused on **hyperaccumulator plants** that are able to take up and tolerate extraordinary high levels of metals (*e.g.* Cd-hyperaccumulators contain by definition > 100 mg Cd kg^{-1} aboveground dry weight while this is > 1000 mg kg^{-1} for Pb-hyperaccumulators and > 10 000 mg kg^{-1} Zn for Zn-hyperaccumulators) (Reeves and Baker 2000). Most of hyperaccumulator plants are endemic to areas of natural mineralization and mine spoils. About 450 angiosperm species have been identified as metal (As, Cd, Co, Cu, Mn, Ni, Pb, Sb, Se, Tl, Zn) hyperaccumulators (Rascio and Navari-Izzo 2011). About 25% of these metal hyperaccumulators belong to the family of *Brassicaceae* and, in particular, to genera *Thlaspi* and *Alyssum*. Zinc hyperaccumulators include *Arabidopsis halleri* and different species of *Thlaspi* among *Brassicaceae* as well as *Sedum alfredii* belonging to the family of the *Crassulaceae*. *A. halleri* and *S. alfredii*, together with *Thlaspi caerulescens* and *T. praecox* are species that, besides Zn, also hyperaccumulate Cd. Recently *Solanum nigrum* (*Solanaceae*) has been noticed as a fifth Cd hyperaccumulator.

An alternative to the use of hyperaccumulators is the use of **non-food high biomass crop species** accumulating only moderate amounts of toxic elements but producing higher biomass yields than hyperaccumulators (Vangronsveld *et al.* 2009; Vamerali *et al.* 2010). Ideally, such crop species should possess as many as possible of the following characteristics: fast growth and high biomass production, extended root system for exploring large soil volumes, good tolerance to high concentrations of metals in soil as well as in plant tissues, high

efficiency in metal uptake and translocation to aboveground parts, adaptability to a wide range of environments/sites, easy to propagate, be non-invasive and without side effects, resistant to pests and pathogens, low water requirements and demanding easy and low-input agricultural management including harvest (Robinson *et al.* 2000; Jensen *et al.* 2009; Vangronsveld *et al.* 2009; Vamerali *et al.* 2010). Common evaluated crops are Indian mustard (*Brassica juncea*), rapeseed (*Brassica napus*), tobacco (*Nicotiana tabacum*), sunflower (*Helianthus annuus*), maize (*Zea mays*), alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris*), switchgrass (*Panicum virgatum*) miscanthus (*Miscanthus sp.*) and giant reed (*Arundo donax*) (Hammer *et al.* 2003; Vangronsveld *et al.* 2009; Vamerali *et al.* 2010; Kidd *et al.* 2015). Tree species often examined in relation to metal-phytoextraction are short rotation coppice (SRC) of willow (*Salix*) and poplar (*Populus*). To be clear, the cultivation of these high biomass crops is by no means to serve industries for production of animal feed or human food.

1.3 Phytoextraction in the Campine region

Since regional policy makers strongly promote the decontamination of the soil in the Campine region (Witters *et al.* 2009), phytoextraction is selected as remediation technology above phytostabilization.

1.3.1 Hyperaccumulator *vs.* high biomass species

The choice of the type of phytoextractor (hyperaccumulator *vs.* high biomass species) depends on the site characteristics (Vangronsveld *et al.* 2009). Two arguments favoring the choice for hyperaccumulators are (i) very high contamination levels (hyperaccumulators will not easily suffer from metal toxicity) and (ii) possible economic profit of the re-cycling of metals from the produced biomass (termed phytomining). However, in the Campine region, metal contamination levels are in general diffuse and moderate and reclaiming of Cd, Zn or Pb from produced biomass is up to date not economically profitable (actually only for nickel (Ni) phytomining is reported to be economically feasible (Chaney *et al.* 2000, 2005, 2007). Furthermore, most of the hyperaccumulator plant species are able to accumulate just 1 metal (Reeves and Baker 2000) and there are only a few species found to hyperaccumulate Cd (McGrath *et al.* 2001; Bert *et al.* 2003; Schwartz *et al.* 2003; Fischerová *et al.* 2006; Rascio and

Navari-Izzo 2011). In addition, most hyperaccumulators are small, herbaceous plants without information on the agricultural management as well as a lack of commercial seed supply (Vamerali *et al.* 2010). Finally, the biomass production of hyperaccumulators is reported to be (very) low limiting their phytoextraction efficiency (Robinson *et al.* 2003; Vassilev *et al.* 2004; Fischerová *et al.* 2006; Dickinson *et al.* 2009; Kidd *et al.* 2015). Therefore, in the Campine region, the potential of high biomass crops with moderate metal accumulation levels is further investigated.

1.3.2 Phytoextraction using high biomass crops in the Campine area

The cultivation of non-food high biomass crops in the Campine region started in 2004 on a metal-contaminated field 500 m northeast of the Zn-smelter in Balen (Figure 1.1). The first experiments were performed in the frame of the EU-FP5 project PHYTAC. The research was continued and the field was extended in 2006 as part of a collaborative project between the Netherlands (Active Soil Management Campine region; ABdK) and Flanders, Belgium (Public Waste Agency of Flanders; OVAM), called 'BeNeKempen', in order to work out a joined strategy to optimize solutions and management for the toxic metal concentrations in the Campine region (http://www.ovam.be/benekempen). The phytoextraction efficiency of SRC of willow and poplar (see further), energy maize and rapeseed were evaluated (Ruttens *et al.* 2008). However, these first studies revealed the estimated remediation time for cleanup of the experimental field to be (unacceptably) long (from 55 up to 2235 year for Cd remediation; depending on species/clone) (Ruttens *et al.* 2008, 2011; Van Slycken *et al.* 2013). This was also reported by almost all other authors investigating high biomass crops on metal-contaminated soils. The information presented so far has led to the conclusion that (for Cd) there is remediation potential, but it is clear that using high biomass crops, even in the most optimistic scenario, at least 60 to 70 years are needed to reduce the total Cd content from 5 to 2 mg kg^{-1} (Vangronsveld *et al.* 2009). Moreover, it is postulated that phytoextraction should preferably not exceed a period of around 10 years to become economically feasible when implemented solely as a remediation technology (Blaylock and Huang 2000). The long remediation time needed is considered the major drawback, the Achilles heel, of metal phytoextraction, seriously limiting its

large-scale application (Robinson *et al.* 2003; Vassilev *et al.* 2004; Dickinson *et al.* 2009). The rather low extraction efficiency is due to a low biomass production and/or low metal accumulation in aboveground biomass. While the first one is related to the suitability of the crop for the contaminated area and the implementation of proper agronomic practices, the most important factors limiting aboveground metal accumulation are a low metal availability in the soil and low metal uptake, translocation, accumulation and tolerance by plants (Kumar *et al.* 1995; Burd *et al.* 2000; Kayser *et al.* 2000; Artursson and Jansson 2003; Quartacci *et al.* 2006; Vangronsveld *et al.* 2009; Weyens *et al.* 2009a,c). To overcome these limitations and improve biomass productivity levels of high biomass crops on metal-contaminated soil, a few strategies are proposed and discussed (without being complete).

1.3.3 Strategies to improve metal phytoextraction using high biomass crops

Species/cultivar/clone selection

Natural variation occurs in the uptake and distribution of essential and non-essential trace elements among crop species and among cultivars and clones within the species level (Grant *et al.* 2008; Kidd *et al.* 2015). Furthermore, productivity levels of a crop and even cultivar/clone vary considerably according to the site specific characteristics like climate and soil properties. Therefore, selection of the most suitable crop/cultivar/clone for a given area, taking into account the present contaminants and contamination level, is considered a first strategy to enhance metal phytoextraction. It might furthermore be profitable to develop crossings and clones with an improved site suitability and/or metal extraction capacity by conventional plant breeding, *in vitro* breeding or chemical mutagenesis (Herzig *et al.* 1997, 2014; Guadagnini 2000; Nehnevajova *et al.* 2007; Schröder *et al.* 2008; Kidd *et al.* 2015).

Genetic engineering

The capacity of a plant to tolerate, accumulate and metabolize toxic amount of metals can be manipulated by means of genetic engineering (Salt *et al.* 1998). Many genes involved in the acquisition, allocation and detoxification of metals have been identified and characterized from a variety of organisms, especially

bacteria and yeasts (Ehrlich 1997). Using this genetic information, transgenic plants overproducing proteins playing a role in chelation, assimilation and membrane transport of metals might be engineered (Vamerali *et al.* 2010). For example, enhanced tolerance and accumulation might be achieved through overproduction of metal chelating molecules such as citrate, phytochelatins (PC), phytosiderophores or overexpression of metal transporter proteins (*e.g.* ZIP proteins). There are several promising examples of successfully transformed plants that exhibite better phytoextraction capacity tested at laboratory scale (Vangronsveld *et al.* 2009). However, the implementation of genetically manipulated organisms (GMOs) is still an open question as its answer strongly depends on public (and governmental) perception.

Optimizing agronomic practices

Another strategy, originally applied to improve productivity levels, is the optimization of agronomic practices. Appropriate planting methods and planting densities, irrigation and fertilization management, pest and weed control, harvest methods, crop rotation and intercropping as well as soil management practices (pre- and post-cultivation) for a number of phytoextraction crops have recently be reviewed by Kidd *et al.* (2015). Although effects of suitable agronomic measures on crop productivity can be obvious, the effects on metal availability, uptake and translocation are not yet extensively studied/known. It is however reported that *e.g.* optimizing rotation length and the time of harvest (especially in case of SRC cultivations) and harvesting the root bole might contribute to improve metal phytoextraction using high biomass crops (Puschenreiter *et al.* 2001; Hammer *et al.* 2003; Dickinson and Pulford 2005; Mertens *et al.* 2006; Kidd *et al.* 2015).

Chelator-assisted phytoextraction

A number of natural and synthetic chelators have been extensively studied in phytoextraction applications in order to increase bioavailability, uptake and translocation of metals (Meers *et al.* 2005, 2008; Parra *et al.* 2008; Vangronsveld *et al.* 2009; Vamerali *et al.* 2010). In general, the chemical additives form stable complexes with metals which are soluble in the soil pore water, thereby available for uptake. It is postulated that metal-chelator

complexes are taken up along an apoplastic pathway and pass through (disrupted) Casparian strips (Vamerali *et al.* 2010). The aminopolycarboxylic acid ethylenediamine-tetraacetic acid (EDTA) has been tested intensively as soil amendment. It has shown (at laboratory scale) to be very effective in enhancing metal mobility and uptake of metals, particularly Pb (Puschenreiter *et al.* 2001; Meers *et al.* 2005; Wang *et al.* 2007b). However, it was also reported that EDTA and/or EDTA-formed metal complexes are toxic for some plants and that high doses inhibited the development of arbuscular mycorrhiza and cause zootoxicity (Geebelen *et al.* 2002; Vassilev *et al.* 2004; Maxted *et al.* 2007). Furthermore, EDTA is poorly photo-, chemo- or biodegradable (Vassilev *et al.* 2004). *In situ* application can cause groundwater pollution by uncontrolled metal dissolution and leaching. To overcome these problems, the use of other, naturally occurring and/or more rapidly biodegradable aminopolycarboxylic acids (*e.g.* nitrilotriacetate (NTA) or ethylene diamine disuccinate (EDDS)), inorganic amendments (*e.g.* elemental sulfur (S) or physiologically acid fertilizers (such as NH_4SO_4)), organic acids and amino acids have been proposed (Kayser *et al.* 2000; Gramss *et al.* 2004; Vassilev *et al.* 2004; Li *et al.* 2005; Evangelou *et al.* 2007; Meers *et al.* 2008; Dirilgen *et al.* 2009). However, chelator-assisted phytoextraction is unlikely to lead to any sort of promising solution. Since the soil zone involved in metal uptake, *i.e.* the volume occupied by roots, usually only represents 1% of soil volume (Marschner and Godbold 1995), the majority of the applied amendments is likely to be far from the uptake site with a high risk of leaching and groundwater or surface water contamination (McGrath *et al.* 2001; Meers *et al.* 2008). In addition, undesirable side effects of the alternative chelators as well as negative effects of soil acidification on soil fertility and structure have been reported as well (Kayser *et al.* 2000; Gramss *et al.* 2004).

Exploiting beneficial bacteria and mycorrhiza

A general soil holds typically approximately 10^9 bacteria per gram of soil (Torsvik and Øvreås 2002; Roesch *et al.* 2007) and these soil bacteria can colonize plant roots in significant numbers (10^5-10^7 colony forming units per gram of fresh weight) (Benizri *et al.* 2001; Hallmann 2001; Compant *et al.* 2010; Croes *et al.* 2013; Weyens *et al.* 2013b). Following root colonization, some of the bacteria can also penetrate plant roots and may establish inside

roots and aerial plant parts as endophytes. Mycorrhization, a fungus in symbiotic relation with plant roots, occurs naturally in a very large number of species (> 90%) with the exception of the *Brassicaceae* family (Javaid 2007).

Both, plant-associated bacteria and mycorrhizal fungi, may improve phytoextraction by one or several of the following mechanisms. (i) They can promote plant health and growth by producing plant growth hormones such as cytokinins, gibberellins or auxins, by enhancing the uptake of essential minerals such as phosphorus, nitrogen and iron, and/or by outcompeting or inactivating pathogens (Lodewyckx *et al.* 2002; Vessey 2003; Baum *et al.* 2015). (ii) They can increase plant metal tolerance by intra- or extracellular sequestration of metals and/or by precipitatation, chelation or binding metals to exopolymers (Bruins *et al.* 2000; Lodewyckx *et al.* 2001; Sessitsch and Puschenreiter 2008; Haferburg and Kothe 2010; Cicatelli *et al.* 2014). (iii) They can increase bioavailability, uptake and eventual translocation of metals as a result of producting chelating compounds such as siderophores and organic acids (Lombnaes *et al.* 2008; Braud *et al.* 2009; Rajkumar *et al.* 2010; Leung *et al.* 2013; Cicatelli *et al.* 2014; Sheikh-Assadi *et al.* 2015).

The exploitation of bacteria and mycorrhizal fungi for application in phytoremediation has been discussed in several reviews (Gadd 2004; Lebeau *et al.* 2008; Weyens *et al.* 2009a,c; Haferburg and Kothe 2010; Leung *et al.* 2013; Sessitsch *et al.* 2013; Haslmayr *et al.* 2014; Phieler *et al.* 2014). In addition, beneficial interactions between bacteria and mycorrhizal fungi in relation to phytoextraction have also been found (Artursson and Jansson 2003; Zimmer *et al.* 2009).

A big environmental advantage of improving metal phytoextraction using plant-associated bacteria or mycorrhizal fungi, is the location and timing of the activities. Since mobilization of metals will merely occur in the root zone and the activity of both is in tight equilibrium with the plant's activity, leaching of solubilized metals will be limited.

1.4 Valorization of metal phytoextraction using high biomass crops

It is very likely that metal phytoextraction using non-food high biomass crops in the Campine region, even taken into account improvements that still can be made, will take longer than the period considered a threshold for metal phytoextraction as a stand-alone technology. However, the production of biomass on degraded land offers cross-cuttings with economic as well as (other) environmental agendas (Dickinson *et al.* 2009). Both synergies seem to be indispensible for the justification, advancement and eventual implementation of metal phytoextraction. Economic revenues and environmental benefits of cultivating high biomass crops on metal-contaminated soil are shortly clarified below, a more elaborate discussion can be found in Chapter 3.

1.4.1 Economic valorization through biomass conversion

Biomass can be used to generate an income in many ways. However, since every product of phytoextraction is potentially hazardous biomass with increased (and potentially toxic) contents of metals, biomass processing is only environmentally sound if the re-entry of metals in the environment is minimized. In this way, for woody biomass produced (like SRC of willow and poplar) direct combustion, gasification and pyrolysis are possibilities. During direct combustion, heat and/or power are generated while gasification converts biomass to a low to medium calorific value gaseous fuel (Vassilev *et al.* 2004). Pyrolysis, the rapid heating of biomass to moderate temperatures (350-650°C) in the absence of oxygen, yields a char, liquid and gas fraction (Lievens *et al.* 2008). For most herbaceous high biomass crops, physical-chemical conversion routes (pressing and extracting vegetable oil from biomass; Vassilev *et al.* 2004)) and pyrolysis seem more appropriate.

In case of woody biomass, the fate of metals and viability of the process was thoroughly investigated for combustion (Šyc *et al.* 2012; Delplanque *et al.* 2013), gasification (Vervaeke *et al.* 2006) and pyrolysis (Lievens *et al.* 2008; Stals *et al.* 2010, 2013; Fletcher *et al.* 2014). It revealed that pyrolysis is the process most controllable regarding fate of hazardous metals (combustion and gasification typically happen at higher temperatures (> 850°C) at which metals

(especially Cd) are more easily volatized). The economic profit from contaminated biomass conversion is also mostly investigated for woody biomass (Voets *et al.* 2011; Kuppens *et al.* 2014, 2015). The outcomes depend on a lot of variables, which are moreover changing over time, leading to highly uncertain results. However, the need for governmental compensations was repeatedly mentioned as well as including CO_2 abatement (and other environmental benefits) in the price of biomass.

It should furthermore be mentioned that all the biomass conversion studies were performed at lab-scale and either denote the environmental and economic constrictions when converting metal-contaminated biomass to energy or do not mention them at all. More research is urgently required to not turn this very important argument into a bottleneck for the implementation of metal phytoextraction.

1.4.2 Environmental benefits of metal phytoextraction using high biomass crops

Environmental benefits of high biomass crops on a metal-contaminated soil, besides a gradual decontamination, are generated by the presence of a vegetation cover (in comparison with no or scarce vegetation) (Vangronsveld *et al.* 1995a, 1995b, 1996; Vangronsveld and Cunningham 1998; Pulford and Watson 2003; Dickinson *et al.* 2009; Zegada-Lizarazu *et al.* 2010; Van Slycken *et al.* 2015). A first benefit of a vegetation cover is a risk reduction regarding spreading of the contaminants. A crop cover prevents dispersion by wind and erosion by water. In addition, uptake of water and transpiration through leaves limits the amount of water percolation and by consequence the leaching of metals to ground and surface waters. Secondly, a vegetation cover increases biodiversity. The presence of a plantation might improve life and quality of life in soil, in waters nearby, on land and in the air. Thirdly, vegetation potentially improves the quality of the soil in many ways. Leaf fall adds significant amounts of organic matter to the surface layers of the soil, promoting nutrient cycling, soil aggregation and water holding ability. Dead tree roots and root exudates also contribute to this. Finally, the growing crops will sequester CO_2 in soil, roots and aboveground biomass, (dependent on the CO_2 input of cultivation) contributing to CO_2 abatement.

Although the environmental benefits are undeniable, until now these are not rewarded for what they are worth. However, it are these positive externalities that tremendously strengthen the label of phytoextraction as a sustainable, risk reducing technology and finding a way to compensate them, which is only the right thing to do, reveals to be crucial for the application of metal phytoextraction.

1.5 Short rotation coppice (SRC)

In general, short rotation coppice (SRC) plantations consist of fast growing trees or shrubs and are characterized by higher wood productivity in time and space than conventional cultivated forests, due to high juvenile growth rates of the trees (Baum *et al.* 2009a). SRC plantations are mainly grown for producing wood fuel for heat and power production (Baum *et al.* 2009a; Dimitriou and Aronsson 2011; Van Slycken *et al.* 2013). The most important tree species grown in SRC in Europe are willow (*Salix sp.*) and poplar (*Populus sp.*), which are characterized by fast juvenile growth, with the capacity for asexual reproduction and an ability to resprout from rootstocks or stools. SRC of willow and poplar is generally harvested in 2-5 year cycles. Agronomic practices from propagation to harvest are well established and straightforward using either labor or machinery. The willow and poplar clones are usually planted as cuttings (of *e.g.* 20 cm) in twin row design, with a row distance of 0.75 m between twins and 1.5 m between twin rows, to facilitate mechanized management and high biomass production. The planting density can vary from 10 000 up to 30 000 (Ruttens *et al.* 2008). Aboveground biomass is typically harvested during the winter and new shoots re-sprout spontaneously from the stools in the following spring (Dimitriou *et al.* 2006; Baum *et al.* 2009a; Van Slycken *et al.* 2013). The estimated lifespan of a SRC plantation is 25-30 years (Defra 2004; Dimitriou *et al.* 2006).

SRC of willow is currently grown commercially on ca. 14 000 ha in Sweden for energy-biomass production (Langeveld *et al.* 2012). Smaller areas of SRC are cultivated in Italy (ca. 6000 ha; mostly poplars), Poland (ca. 3000; mostly willows), UK (ca. 7500 ha; mostly willows), Germany (ca. 5000 ha; poplars and willows) and other European countries. Although these areas cannot be

considered as extensive in comparison to other agricultural crops, a rapid increase of SRC in several European countries has been projected already in the short-term (Dimitriou *et al.* 2009; Evans *et al.* 2010).

A SRC plantation of hundreds of different willow and poplar clones was established on the experimental field in Lommel in 2006 (in the framework of the BeNeKempen project) and is the main non-food high biomass crop investigated on this site.

1.6 European project GREENLAND

The research on the metal-contaminated experimental field that is reported in this thesis was part of the European (EU-FP7) project GREENLAND (Gentle Remediation of Trace Element Contaminated Land) (http://www.greenland-project.eu/). The project (2011-2014) focused on the use of gentle remediation options (GRO) as practical tool for land remediation and risk management. GRO include various (mostly plant-based) approaches to remediate trace element contaminated soils at low cost and without significant negative effects for the environment (*i.e. in-situ* immobilisation/phytoexclusion, phytovolatilisation, phytostabilisation, rhizofiltration, rhizodegradation, phytodegradation/phyto-transformation and phytoextraction). The main aim of GREENLAND was to optimize the applicability of GRO options in the field and to make them fit for practical application. This includes technical questions on one hand (use of biomass, soil tests, biotechnological improvements,…) but also the close involvement of stakeholders on the other hand. At the end of GREENLAND, the efficiency of GRO under various conditions (contamination level, climate,…) was demonstrated. With similar importance, also the beneficial socio-economic impacts (profit from biomass valorization, improvement of land value,…) was shown. Both technological and socio-economic benefits will be important prerequisites for practical application. A decision support tool (DST), developed by GREENLAND, allows stakeholders to take a decision for GRO and a practical handbook facilitates the implementation. Overall, there will be a substantial improvement of soil quality and socio-economic conditions at the local level but also on the European level.

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Chapter 2

Objectives

In the Campine region, northeast of Belgium, the soil is historically contaminated with mainly cadmium (Cd), zinc (Zn) and lead (Pb). In the introduction (**Chapter 1** of the thesis) it became clear that Cd-Zn-Pb-contaminated soils impose serious risks for human health and the environment in general, emphasizing the importance of remediation. Phytoextraction using non-food high biomass crops with moderate metal-accumulating capacities revealed to be a good option for the restoration of this area.

Since it was unknown which high biomass crop exposed the highest, but also the most stable (in time), phytoextraction potential on these soils, different species (tobacco, sunflower, hemp and short rotation coppice (SRC) of willow and poplar) were cultivated and evaluated in field trials on a Cd-Zn-Pb-contaminated soil for several years. The biomass production and metal accumulation in harvestable plant parts are presented, compared and discussed in **Chapter 3**. Besides the phytoextraction potential, crosscuts with economic revenues and (other) environmental benefits of the cultivation of high biomass crops was also addressed.

In parallel with the field studies described in Chapter 3, soil remediation using SRC of willow, a crop abundantly investigated in the context of metal phytoextraction, was investigated in this research. Although frequently evaluated, the longer-term effectiveness of SRC phytoextraction applications is rather unknown due to the complexity of a phytoextraction system. Therefore, determining effective decontamination and investigating changes in soil toxicity, and also soil fertility and functionality, in longer-term field experiments with SRC is of high importance. In **Chapter 4**, analyses of soil managed by 8 years of

metal phytoextraction using SRC of willow (Tora; *Salix schwerinnii x Salix viminalis*) and soil without phytoextraction management were performed to assess decontamination rate and changes in soil toxicity.

Since reducing remediation times of metal phytoextraction using SRC of willow and/or increasing benefits of phytoextraction synergies is in any case progressive, improving biomass production and/or metal accumulation of SRC of willow was aimed. In **Chapter 5** three strategies are explored to meet this concern. The importance of *in situ* selection of best performing clones, bioaugmentation of these selected clones with beneficial plant-associated bacteria and fertilization of these clones are discussed in respectively part I, II and III of this chapter.

The most important findings of this research were summarized and concluded in **Chapter 6**.

Chapter 3

Phytoextraction of Cd-Zn-Pb-contaminated soil using high biomass crops: potential of tobacco, sunflower, hemp and SRC of willow and poplar

Abstract

Phytoextraction was proposed as remediation strategy for agricultural soils in a diffusely Cd-Zn-Pb-contaminated region in northeast Belgium. The use of high biomass crops with sufficient metal accumulation is preferred since these are expected to not only gradually decontaminate the soil but also generate an income through biomass valorization. Since it was unknown which high biomass crop exposed the highest, but also the most stable (in time), phytoextraction potential on these soils, different species were cultivated and evaluated on a Cd-Zn-Pb-contaminated field for several years. Biomass production and metal accumulation of pre-selected tobacco somaclonal variants (*Nicotiana tabacum* L.), pre-selected sunflower mutants (*Helianthus annuus* L.) and commercial hemp (*Cannabis sativa* L.) were evaluated for 2-4 subsequent years while the phytoextraction potentials of poplar (*Populus*) and willow (*Salix*) in SRC were assessed at the end of the first cutting cycle (after 4 growing seasons). The tobacco clones and the sunflower mutants revealed to be efficient extractors of respectively Cd and Zn, while the highest, simultaneous extraction of Cd and Zn was realized with the commercial willow clone Zwarte Driebast followed by the experimental poplar clone (D x (T x M)). However, the time needed to remediate

the moderate contaminated soil to the remediation threshold values is estimated to be at least 60 years. Therefore, economic revenues and environmental advantages from phytoextraction using high biomass crops are crucial and were assessed as well. When combining phytoextraction potential and possible economic and environmental benefits, SRC revealed to be the most suitable crop for the implementation of metal phytoextraction in the area under investigation.

3.1 Introduction

In the northeast of Belgium, at least 280 km^2 are moderately contaminated with metals like cadmium (Cd), zinc (Zn) and lead (Pb) (Hogervorst *et al.* 2007). Until the 1970ies, these metals were emitted by the pyrometallurgical smelters located nearby and contaminated the surrounding region through atmospheric deposition. Phytoextraction, *i.e.* the use of plants to extract metals out of the soil, was proposed as remediation strategy for this area (Ruttens *et al.* 2011). In particular high biomass crops with high to moderate metal accumulating capacity are of interest in this area since these crops not only result in a gradual decontamination of the soil by extraction of metals but can also provide an alternative income for the farmers.

Willow (*Salix*) and poplar (*Populus*) are identified as genera that tend to accumulate high concentrations of more mobile elements (Dickinson *et al.* 2009; Ruttens *et al.* 2011). SRC of willow and poplar for remediation of metal-contaminated soils was already investigated in many European countries like Sweden (Perttu and Kowalik 1997; Klang-Westin and Eriksson 2003), Poland (Landberg and Greger 1996; Perttu and Kowalik 1997), France (Robinson *et al.* 2000), Denmark (Jensen *et al.* 2009), Switzerland (Hammer *et al.* 2003; Rosselli *et al.* 2003), the Czech Republic (Fischerová *et al.* 2006) and the United Kingdom (Dickinson and Pulford 2005; French *et al.* 2006; Maxted *et al.* 2007). The capability of tobacco (*Nicotiana tabacum* L.) to extract Cd out of the soil was already reported by Mench *et al.* in 1989. Also Guadagnini (2000) mentioned excellent Cd accumulation properties and a high biomass productivity of tobacco. Its metal extraction potential was investigated several times at field scale (Vangronsveld *et al.* 2009; Fässler *et al.* 2010; Herzig *et al.* 2014). Sunflower (*Helianthus annuus* L.) is a known bioenergy plant able to accumulate

large amounts of several metals in its aerial tissues, gaining growing interest for phytoremediation purposes (De Maria and Rivelli 2013; Kötschau *et al.* 2014). Decontamination of metal-contaminated soils using sunflower was investigated earlier in pot trials (De Maria and Rivelli 2013; Rivelli *et al.* 2014; Zalewska and Nogalska 2014) and on field scale (Nehnevajova *et al.* 2007, 2009; Fässler *et al.* 2010; Herzig *et al.* 2014; Kötschau *et al.* 2014). Furthermore, Madejón *et al.* (2003) reported very low concentrations of potentially toxic elements in sunflower seeds which limits the risk for food chain contamination. The application of multiple-use plant hemp (*Cannabis sativa* L.) for soil remediation arose in Chernobyl in 1998 and its metal phytoextraction potential was assessed in different pot experiments and field trials (Linger *et al.* 2002; Citterio *et al.* 2003; Shi *et al.* 2012; Fumagalli *et al.* 2014).

Unfortunately, since extrapolations of phytoremediation efficiency based on hydroponical and pot experiments are often unrealistic (Vangronsveld *et al.* 2009) and long lasting experiments on field scale are scarce, there still exist many uncertainties concerning the longer-term effectiveness of phytoextraction (Dickinson *et al.* 2009). An existing large-scale field experiment in the contaminated region in Belgium however, offered a unique opportunity to investigate some aspects to these concerns.

The main goal of this research was to evaluate and compare extraction efficiencies of willow and poplar SRC, tobacco, sunflower and hemp based on longer-term field results. While tobacco, sunflower and hemp were cultivated and analyzed in the framework of the present study, results of SRC crops have been reported already in previous studies (Ruttens *et al.* 2008, 2011; Van Slycken *et al.* 2013, 2015) but data were included for comparison. In this manuscript:

- differences in biomass production and metal accumulation between evaluated poplar and willow clones, tobacco clones and sunflower mutants as well as variations throughout the tested years were reported and interpreted
- economic revenues from cultivating SRC woody crops, tobacco, sunflower and hemp are described and compared
- environmental benefits of the evaluated crops were discussed.

Evaluation of high biomass crops on the metal-contaminated field in Belgium was part of the Greenland EU project (FP7-KBBE-266124) (http://www.greenland-project.eu/). This project addressed several issues according to gentle remediation options (GRO), in general plant-based, to remediate trace element contaminated soils (TECS) at low cost and without significant negative effects for the environment.

3.2 Material and methods

3.2.1 Site description

The metal-contaminated experimental field is located in Lommel (51°12'41'' N; 5°14'32'' E), northeast of Belgium. Belgium has a temperate climate with a mean annual temperature of 10.5°C and an average annual rainfall of 852 mm. The field is a former maize field taken out of production since 1999 and is situated 500 m NE of a Zn smelter. To study the possibilities of cultivating energy crops on metal-contaminated soils, an about 10 ha field experiment was set up in 2006 as a collaboration between Hasselt University, Ghent University and the Research Institute for Nature and Forest (INBO). The soil of the experimental field has a 'sand' texture according to the USDA triangle consisting of 88% sand, 8% silt and 4% clay (Meers *et al.* 2007b). The original pH-H_2O varied between 4.8 and 6.6 while the pH-KCl ranged from 4.4 to 6.0 (Meers *et al.* 2007b; Ruttens *et al.* 2008). Organic matter contents measured were between 1.58 and 8.00%, the EC fluctuated from 50.4 to 175.0 µS cm-1 and measured CEC values were between 5.5 and 8.8 cmol kg^{-1} (Meers *et al.* 2007a,b; analyses by Soil Service of Belgium). Pseudo-total metal concentrations were estimated by *aqua regia* digestion and were in the range of 4-13 mg Cd kg^{-1} DW soil, 210-778 mg Zn kg^{-1} DW soil and 133-342 mg Pb kg^{-1} DW soil (Ruttens *et al.* 2008; Greenland EC project, unpublished results). In Flanders (Belgium) remediation criteria are site-specific as they are a function of destination type, clay, organic matter content and pH (Vlarebo 2008; explained in Witters 2011). For the studied area, calculated remediation thresholds for the soil are 2 mg Cd, 282 mg Zn and 200 mg Pb kg^{-1} DW soil. Plant available metal fractions were estimated by exchangeable metal concentrations determined in

0.01 M $CaCl_2$ and are in the range of 0.07-0.78 mg Cd kg^{-1} DW soil, 2.68-42.21 mg Zn kg^{-1} DW soil and 0.07-0.34 mg Pb kg^{-1} DW soil (Ruttens *et al.* 2008; Van Slycken, unpublished results).

Liming of the field

Because of the low pH of the field, the whole experimental field was limed in 2006 (Ruttens *et al.* 2008). After deep ploughing of the soil, powdered lime (80% $CaCO_3$, 5% $MgCO_3$) was added to the soil at a dose of 6000 kg ha^{-1} and incorporated in the upper 25 cm soil layer using a rotary tiller. After liming, pH-H_2O increased to 5.6-6.7 while the pH-KCl raised to 5.5-6.3 (Ruttens *et al.* 2008, 2011; analyses by Soil Service of Belgium).

3.2.2 Short rotation coppice (SRC) of willow and poplar

Physico-chemical soil characteristics (including soil metal concentrations)

Since the SRC plantation occupied the major part of the experimental field (Figure 3.1), soil characteristics and soil metal concentrations were equalized to data described above in 'Site description'. Additional data were furthermore collected using the same material and methods.

Field preparation

One month before the establishment of the SRC plantation, a glyphosate-based herbicide (Roundup MAX™ 3%) was used (12 L per ha) to eliminate existing weeds (Ruttens *et al.* 2008). Ploughing and carefully harrowing provided a smooth plant bed. A fence, put 70 cm deep into the soil, was set up around the field site to reduce damage by rabbits to the young trees.

Plant material and planting

Eight commercially available willow clones (Belders, Belgisch Rood, Christina, Inger, Jorr, Loden, Tora and Zwarte Driebast) were selected from a Swedish and Dutch breeding program (Ruttens *et al.* 2008) while 5 commercial poplar clones (Grimminge, Koster, Muur, Oudenberg and Vesten) were chosen from a Flemish and Dutch breeding program. Besides commercially available clones (further referred to as 'commercial' clones), also many experimental crossing types

(further referred to as 'experimental' clones), designed by INBO with the aim to remediate Cd-contaminated soils, were tested. A total of 100 poplar clones from 42 different families and 160 willow clones from 11 families were selected. The experimental poplar clones can be divided in 3 groups: *Populus trichocarpa* {T} clones, intraspecific crossings of *P. trichocarpa* x *P. trichocarpa* {T x T} and crossings of *P. trichocarpa* x *P. maximowiczii* {T x M} including 2 backcrossings to *P. deltoides* {D (T x M)}. Also the experimental willow clones can be summarized in 3 groups: a *Salix alba* {A} group with purebred *S. alba* and intraspecific crossings of *S. alba* with *S. alba/S. rubens/S. fragilis,* a *S. viminalis* {V} group and a third group comprising crossings of *S. viminalis* x *S. viminalis* {V x V} derived from the second group.

In April 2006, cuttings (20 cm) of all clones were planted on the experimental field in a twin row design using an adapted leek planting machine. The twin row design, with a row distance of 0.75 m between twins and 1.5 m between twin rows, was opted to allow harvest machines to cut twin rows in one track without damaging the stubs of neighbouring rows. Planting distances in the row are 30 and 60 cm for commercial willow clones (respectively 30 000 and 15 000 plants ha^{-1}) and 60 and 90 cm for commercial poplar clones (respectively 15 000 and 10 000 plants ha^{-1}). Each combination of commercial clone and planting distance was planted in blocks of about 300 m^2 and each block was repeated in fourfold to account for field heterogeneity (Figure 3.1 and Supplementary figure 3.1 p.117). For each tested experimental poplar and -willow clone, 25 to 50 trees were planted in blocks in duplex repetition with planting distances of 90 (poplar) and 60 (willow) cm (see Supplementary figure 3.2 p.118).

Maintenance and harvests

In May and June of the first growing season, weed control actions were performed using a full automatic lawnmower (between twin rows) or by manually spraying with a glyphosate-based herbicide (Roundup MAX™ 3%; 8 L ha^{-1}) (within twin rows) (Ruttens *et al.* 2008). To deal with the rabbits still present on the field, weekly hunting was performed during the first months of the experiment. Because of a very dry period in July 2006, irrigation was applied during 2 days by the use of a sprinkler irrigation system at a rate of 60 m^3 h^{-1}.

In November 2009, after 4 growing seasons, a first harvest of all aboveground biomass was performed. The harvester used was a Belgian corn chipper (brand New Holland) with an adopted head equipped with 2 circular saws able to cut woody stems close to the ground. A forward stabbing tang was used to assemble all stems from twin rows, bending them forward allowing to cut the stems of both rows in one track. The harvester chipped the cut stem immediately. In February 2014, after another 4 growing seasons, a second harvest was performed, this time by the harvester 'Stemster' from the Danish firm Nordic Biomass. The Stemster, specially designed to harvest short rotation coppice, also assembled and cut stems of twin rows close to the ground using 2 circular saws. Cut stems were collected on the field allowing them to dry.

Biomass production, *in planta* metal concentrations and extraction potential

Data concerning biomass production of commercial poplar clones and of most promising (based on phenotypic appearance) experimental poplar and willow clones were abstracted from Van Slycken *et al.* (2015). Data used are based on biomass production after the first 4 growing seasons of poplar and willow clones at highest planting distance (respectively 90 cm and 60 cm). Adjustments were made as productivity was calculated as the mean of yields per ha based on tree harvest and on harvested surface. Furthermore, for the experimental clones, the resulting yields of both blocks were combined in respect to the number of promising clones measured in every of the 2 blocks. Metal concentrations of experimental poplar and willow clones were calculated as the mean of concentrations measured in both blocks taken into account the number of clones measured in every of them. Biomass yields and metal concentrations of commercial willows were derived from Van Slycken *et al.* (2013) and also based on the first 4 growing seasons. The extraction potential is defined as the amount of metals potentially removed from a soil and calculated based on the amount of metals accumulated in harvestable plant parts per unit of surface and time. For every (collection of) poplar and willow clone(s), the extraction potential was calculated by multiplying yearly stem production with the concentration of metals in the woody biomass and expressed in g ha^{-1} yr^{-1}.

3.2.3 Tobacco, sunflower and hemp

Physico-chemical soil characteristics (including soil metal concentrations)

Tobacco, sunflower and hemp were cultivated on ± 1.5 a plots that were adjacent in subsequent years (without overlap) (Figure 3.1). After the 2014 experiment, topsoil samples (0-30 cm) were taken N, S, E and W of the grouped plots under natural grass vegetation (mainly *Agrostis capillaris* and *Holcus lanatus*) that was never part of a cultivation plot. Litter and vegetation cover were removed before sampling. Soil samples were oven-dried and sieved (< 2 mm). Pseudo-total metal (Cd, Zn and Pb) concentrations of the soil samples were estimated by *aqua regia* digestion (Van Ranst *et al.* 1999). Briefly, 0.5 g of oven-dried soil was microwave digested in a HNO_3-HCl solution (1:3 v:v) at 160°C (25 min ramp time, 10 min ventilation). Plant-available fractions were determined using 0.01 M $CaCl_2$ in a 1:5 (w:v) extraction ratio (Van Ranst *et al.* 1999). After 2 h of shaking (120 rpm), the mixture was filtered. Analysis of samples from digestion and $CaCl_2$-extraction were subsequently performed using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies 700 Series). The pH-H_2O and pH-KCl were measured after 1 h of equilibration (120 rpm) with respectively deionised H_2O and 1 M KCl in a 1:5 (w:v) solution. Electrical conductivity (EC) of the soil was determined using a conductivity meter (WTW LF340) and measured after 1 h of equilibration (120 rpm) with deionised H_2O in a 1:5 (w:v) ratio. The effective cation exchange capacity (CEC_e) was calculated as the sum of cations (Ca/20+Mg/12+K/39+Al/9, cations in mg L^{-1}) extracted by 1 M NH_4Cl (Gillman and Sumpter 1986). A 1:10 (w:v) extraction solution was shaken for 2 h (120 rpm) and cations present in the extract were measured using ICP-OES. For all analyses, blanks were included for quality control of the data. Besides, for pseudo-total metal concentrations, a reference soil (CRM 143 R Sewage Sludge Amended Soil, Community Bureau of Reference - BCR N° 230) was included for confirmation of the analysis.

Field preparation

One month before planting of tobacco, sunflower and hemp, the plot was rototilled at least once superficially and once deeply. In 2014, the plot was sprayed before milling with a glyphosate-based herbicide to destroy present weeds and grasses. In 2012, 2013 and 2014, after milling, mushroom manure was spread over the plots at a dose of 5 m^3 a^{-1}. The fertilizer was incorporated into the soil by milling again. The plant bed was left a few weeks to allow leaching of toxic fertilizer compounds. In 2013 and 2014, a small fence (25 cm deep into the soil) was provided around the plot for sunflower cultivation to prevent eating of young sunflower plantlets by rabbits.

Plant material and planting

Seeds of *in vitro* bred tobacco clones (*Nicotiana tabacum* L. *sp.*) and mutant lines of sunflower (*Helianthus annuus* L. *sp.*) were provided by Phytotech Foundation (PT-F) in Bern, Switzerland. Clones and mutants tested were the result of years of selection for enhanced metal tolerance and shoot metal removal as described in Herzig *et al.* (2014). From 2011 to 2014, 2 tobacco somaclonal variant lines were tested: mother clone BAG (*Badischer Geudertheimer*) and derivatives NBCu-10-4 and NBCu-10-8 and mother line FOP (*Forchheim Pereg*) with derivatives NFCu-7-15 and NFCu-7-19. Selected mother cultivars BAG and FOP were considered controls for their respective derivatives. Second generation (F2) descendants of the selected clones were tested in 2011 and 2012 while 3rd generation (F3) offsprings were evaluated in 2013 and 2014. Sunflower mutants tested in the period 2011-2014, belonged to 3 mutant line families (15-35-190-04, 86-35-190-04 and 14-185-04), all resulting of chemical mutagenesis of selected inbred line IBL 04. The latter mother line IBL 04 (without mutagenesis) was planted as control. From the 5th (M5) up to the 8th (M8) generation of different sunflower mutants were evaluated. In 2013 and 2014, also the phytoextraction potential of hemp was tested. Common commercial bird seed was used as source of hemp seed.

Two months before plant date of the tobacco clones on the field, seeds were sown in seedling soil in the greenhouse (day temperature 22°C, night temperature 18°C, air humidity 60%, photoperiod 15 h). A glass cover protected

germinating seeds during the first 2 weeks. Watering was performed to keep the soil moist. After 3 weeks, every tobacco seedling was transferred to a pot made of pressed propagation soil. The plantlets were kept in the greenhouse and watered regularly. When the frost period had gone and at least 2 weeks before planting on the field, plants were put outside to acclimatize. The first week of June, the tobacco plantlets were planted on the field. In 2011 and 2012 planting distance was 80 cm (15 625 plants ha^{-1}) while this was 60 cm in 2013 and 2014 (27 778 plants ha^{-1}). Twenty up to 45 replicates per clone were planted every year.

Seeds of the sunflower mutants were sown in propagation soil 3 weeks prior to planting on the field. The seeds were pushed 2 cm in the soil with a spacing of 5 cm in between. Germinating sunflower seeds were placed outside, however in a sheltered environment. In the first week of June, sunflower seedlings were planted on the field with a planting distance of 15 cm (2011) or 25 cm (2012, 2013, 2014) in the row and 40 cm between the rows (respectively 166 667 plants ha^{-1} or 100 000 plants ha^{-1}). Every year, 20 up to 100 replicates per clone were planted.

The hemp seeds were sown directly on the field at the beginning of June. Seeds were manually pushed in the soil for 3 cm using a planting distance of 5 cm in the row and 12 cm between rows (167 plants m^{-2}). In 2013 and 2014, 6 m^2 of hemp was sown.

As an illustration, a detailed structure of the tobacco-sunflower-hemp plot in 2013 is given in Supplementary information (Supplementary figure 3.3 p.119).

Maintenance and harvest

All plants were watered after planting and were additionally irrigated during the month of June in 2012 and 2013, due to long dry periods. Mowing in between rows of tobacco to control weed growth was done twice in 2011 and 2012. No weed control after planting was done for sunflower and hemp (in all years) or for tobacco in 2013 and 2014. Aboveground biomass of all plants was harvested every year at about 15th of September.

Biomass production, in planta metal concentrations and extraction potential

The survival rate of tobacco and sunflower was not explicitly assessed since basically all planted seedlings survived the whole cultivation period (without visual deficiency symptoms). Aboveground fresh weight (FW) production and height (H) of all tobacco, sunflower and hemp plants was determined on the field directly after harvest (except for the tobacco clones of 2011). For tobacco and sunflower, every year, a group of 10 plants per clone/mutant with a representative FW compared to the overall clone/mutant FW was selected for further investigation in the lab. All selected sunflower and tobacco plants were chipped individually using a garden chipper and chips were air-dried until constant weight (about 2 months). Thereafter, aboveground dry weight (DW) production was determined. DW production of the other, non-selected plants was estimated based on the regression equation expressing the FW-DW relationship of selected plants. In 2011 and 2013, mass of produced sunflower seeds was also measured. Total FW production of hemp was measured for the 6-m^2 plot. Four bales with known FW were selected for further analysis in the lab. After chipping and drying of the bales, DW was determined and based on these results, total DW production for the 6-m^2 hemp plot was estimated.

All chipped and dried plant material was individually hammer-milled (Retsch SM100) to obtain a fine powder. To determine total Cd, Zn and Pb concentrations in the biomass, this powder was wet-digested in Pyrex tubes in a heating block. The digestion consisted of 3 cycles in 1 mL HNO_3 (70%) and 1 cycle in 1 mL HCl (37%) at 120°C for 4 h. Samples were thereafter dissolved in HCl (37%) and diluted to a final volume of 5 mL (2% HCl) with Millipore water. Cadmium, Zn and Pb concentrations in the extracts were measured using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies 700 Series). Milled sunflower seeds (1 mixed sample per mutant) were also analyzed for their metal content. All samples were tested at least in triplicate. Blanks and certified reference material (trace elements in spinach, Standard Reference Material® 1570a, National Institute of Standards and Technology, USA Department of Commerce) were included for quality control of the data.

The potentially extracted amount of metals per hectare and year was calculated for each of the evaluated tobacco and sunflower plants by multiplying the mean aboveground DW production of the clone/mutant (kg ha^{-1} yr^{-1}) with the mean Cd, Zn and Pb concentrations in the selected plants (mg kg^{-1}). The extraction potential of hemp was calculated by multiplying mean DW data and observed metal concentrations.

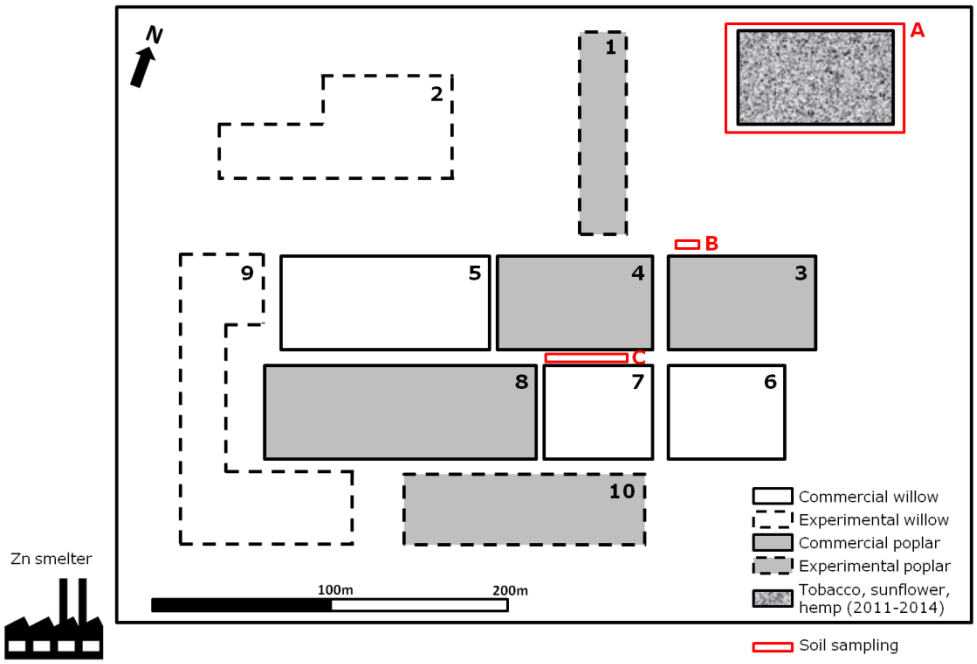


Figure 3.1 Scheme of the experimental field in Lommel with the location of commercial (full lines) and experimental (dotted lines) willow and poplar blocks and the location where plots of tobacco, sunflower and hemp were established in the period 2011-2014. Red boxes depict sampling places for calculations of remediation times (A: moderate, B: high, C: very high contamination level).

3.2.4 Phytoextraction efficiencies of SRC, tobacco, sunflower and hemp

In order to compare phytoextraction efficiencies of different crops, a comparison of estimated extraction potentials and bioconcentration factors (BCF) was adopted. The BCF, defined here as the ratio of metal concentration in

aboveground biomass or wood to total soil metal content, directly allows to compare extraction efficiencies of different crops even on different levels of contamination. For tobacco and sunflower, aboveground metal contents and extraction potentials of identical clones/mutants (disregarding the generation) were averaged over tested years to obtain a more realistic mean. Extraction potentials were assumed to be independent of soil metal concentrations.

For the best performing clones/mutants of all tested species, hypothetical remediation times needed to reduce pseudo-total Cd, Zn and Pb concentrations measured at (A) at the location of the tobacco, sunflower and hemp plots 2011-2014, (B) in the middle of the field, and (C) closer to the zinc smelter (Figure 3.1), respectively referred to as moderate, high and very high contamination level, to remediation thresholds were calculated. Following assumptions were made: (i) species' extraction potentials are independent of soil metal concentrations, (ii) total soil metal content decreases linearly due to a constant yearly extraction, (iii) contamination and rooting depth are 0.5 m, and (iv) the soil density is 1250 kg m^{-3}.

3.2.5 Statistical analyses

Statistical analyses were performed in R 3.1.3 (R Development Core Team, 2013). The effect of tobacco clone/sunflower mutant and year on the dry weight (DW) biomass production per plant and Cd, Zn, and Pb concentrations in the biomass was analyzed using ANOVA. The QQ-plots were used to examine normality of the residuals. In the case of non-normality, transformations of the outcome (logarithmic, inverse, square root, exponential) were performed. When an indication of non-normality was present for all these transformations, a Box-Cox was used. All decisions about the transformations of the outcomes were taken *a priori*. Model-robust standard errors were used in all analyses due to potential differences in the variance of the outcome for different clones/mutants and years. Since interaction between clone/mutant and year was present in all analyses, the differences between clones/mutants for each year and the differences between years for each clone/mutant were analyzed separately. Two-by-two comparisons were conducted using Tukey correction for multiple testing.

3.3 Results

3.3.1 Short rotation coppice (SRC) of willow and poplar

Physico-chemical soil characteristics (including soil metal concentrations)

Physico-chemical soil characteristics (and soil metal concentrations) of the SRC field are described in 'Material and methods' section under 'Site description' and summarized in Table 3.2. Pseudo-total and $CaCl_2$-exchangeable soil metal concentrations reveal to be very heterogeneous throughout the field. Ruttens *et al.* (2008) reported that a certain contamination gradient could be distinguished which corresponds to the main wind direction (SW). However, hot spots of Cd contamination were discovered as well.

Biomass production, metal accumulation and extraction potential of SRC

Concerning stem biomass production, no clear distinction was observed between poplar and willow in general, nor existed a stringent difference between commercial clones and measured (most promising) experimental clones for poplar or willow (Table 3.1). Poplar clones produced between 2.0 (commercial clone Muur) and 10.6 (experimental clone D x (T x M)) tons of stem biomass per hectare and year while willow stem production varied between 1.4 (commercial clone Inger) and 12.3 (commercial clone Zwarte Driebast) t ha^{-1} yr^{-1}. Cadmium and Zn concentrations in the stem ranged between respectively 9-14 and 271-421 mg kg^{-1} DW for experimental poplars and between 7-28 and 272-844 mg kg^{-1} DW for willow with no clear distinction between commercial and experimental clones. The highest Cd and Zn extraction potential within the collection of experimental poplars was observed for the evaluated clone of crossing type D x (T x M) reaching 93 g Cd and 2873 g Zn ha^{-1} yr^{-1}. For willow, the commercial clone Zwarte Driebast showed highest extraction potentials for Cd and Zn, respectively 208 and 5072 g ha^{-1} yr^{-1}.

Table 3.1 Stem biomass production (kg DW ha^{-1} yr^{-1}), Cd and Zn concentrations in the stem (mg kg^{-1} DW) and Cd and Zn extraction potential (g ha^{-1} yr^{-1}) after the first 4 growing seasons of commercial clones and most promising experimental clones planted on the field in Lommel in 2006.

Species (plants ha^{-1})	**Clone type**	**Clone (n° of most promising)**	**Crossing type**	**Stem biomass (kg DW ha^{-1} yr^{-1})**	**$[Cd]_{stem}$ (mg kg^{-1} DW)**	**$[Zn]_{stem}$ (mg kg^{-1} DW)**	**Cd extraction potential (g ha^{-1} yr^{-1})**	**Zn extraction potential (g ha^{-1} yr^{-1})**
Poplar (10 000 plants ha^{-1})	Commercial	Grimminge	D (T x D)	4750	n.d.	n.d.	n.d.	n.d.
		Koster	D x N	3750	n.d.	n.d.	n.d.	n.d.
		Muur	D x N	2000	n.d.	n.d.	n.d.	n.d.
		Oudenberg	D x N	3800	n.d.	n.d.	n.d.	n.d.
		Vesten	D x N	5750	n.d.	n.d.	n.d.	n.d.
	Experimental	(3-5)	{T}	4496-8543 (5920)	10-16 (14)	365-507 (421)	52-131 (82)	1772-3118 (2494)
		(15-23)	{T x T}	2557-13873 (5648)	6-20 (13)	220-627 (358)	30-119 (71)	778-4204 (2023)
		(4-5)	{T x M}	3650-5873 (4830)	7-20 (13)	227-501 (345)	24-96 (65)	828-1993 (1664)
		(1)	{D x (T x M)}	10600	9	271	93	2873
Willow (15 000 plants ha^{-1})	Commercial	Belders	A	1775 ± 1150	8 ± 3	291 ± 128	14 ± 11	517 ± 404
		Belgisch Rood	F	n.d.	n.d.	n.d.	n.d.	n.d.
		Christina	V x V	1675 ± 1500	21 ± 6	640 ± 60	35 ± 33	1072 ± 965
		Inger	Tr x V	1400 ± 875	22 ± 1	844 ± 65	31 ± 19	1182 ± 744
		Jorr	V	1950 ± 1200	18 ± 4	642 ± 153	35 ± 23	1252 ± 826
		Loden	Da	4250 ± 2250	28 ± 13	682 ± 160	119 ± 84	2899 ± 1678
		Tora	S x V	2500 ± 2000	21 ± 1	631 ± 72	53 ± 42	1578 ± 1275
		Zwarte Driebast	Tr	12250 ± 7000	17 ± 3	414 ± 59	208 ± 125	5072 ± 2987
	Experimental	(14-21)	{A}	3118-12765 (5733)	3-12 (7)	138-407 (272)	14-158 (41)	602-4391 (1559)
		(9)	{V}	3626-7663 (5850)	14-26 (21)	380-769 (549)	72-148 (121)	1617-4085 (3212)
		(5-6)	{V x V}	2489-4765 (3858)	18-29 (22)	501-724 (626)	55-107 (84)	1563-3214 (2416)

D: *Populus deltoides*; M: *Populus maximowiczii*; N: *Populus nigra*; T : *Populus trichocarpa*, A: *Salix alba;* Da: *Salix dasyclados* ; F: *Salix fragilis;* S: *Salix schwerinnii;* Tr: *Salix triandra* ; V: *Salix viminalis*; {}: collection of all experimental clones with this crossing type (see 'Material and methods' section for details). n.d. = not detected.

Values are mean, mean ± standard deviation or min-max (mean). n.d.: not detected. Stem biomass production and Cd and Zn concentrations in the stems of commercial poplar clones and all experimental clones were derived from Van Slycken *et al.* (2015), those of commercial willow clones from Van Slycken *et al.* (2013). Extraction potentials were calculated based on the abstracted data.

3.3.2 Tobacco, sunflower and hemp

Physico-chemical soil characteristics (including soil metal concentrations)

The location of the plots for cultivation of tobacco, sunflower and hemp (from 2011 till 2014) is depicted in Figure 3.1 ('Material and methods' section) and corresponding physico-chemical soil characteristics and metal concentrations are presented in Table 3.2. All measured soil characteristics are considered normal compared to field values observed earlier (summarized in first row in Table 3.2). Pseudo-total Zn and Pb concentrations in the soil for tobacco, sunflower and hemp cultivation revealed to be lower than remediation thresholds determined for the studied area (respectively 282 and 200 mg kg^{-1} DW soil).

Climatological data

Climatological data for the cultivation period of tobacco, sunflower and hemp (June-July-August) for the years 2011 until 2014 are given in Table 3.3. Compared to the normal values, *i.e.* mean climatological values for the 30-year period 1981-2010, some deviations were found for the year 2013. Relative air humidity, total rainfall and total days of rain were lower than normal (respectively 7%, 25% and 36% lower) while total hours of sunshine was higher (13%). Furthermore, the mean wind direction (NNE) is different from normal (SW) and less common in general in Belgium. For the other years, differences compared to normal values were observed for total rainfall and total days of rain, which were higher than normal. Finally, in 2011, observed total hours of sunshine were almost 20% lower than normal.

Table 3.2 Physico-chemical characteristics of the topsoil (0-30 cm) on the experimental field in general (SRC field) and specified on the location of tobacco, sunflower and hemp cultivation in the period 2011-2014. A plantation scheme illustrating SRC blocks and tobacco, sunflower and hemp plots can be found in 'Materials and methods' section (Figure 3.1).

Soil sample	**Soil texture**	**pH-H_2O**	**pH-KCl**	**CEC_e (meq 100 g^{-1})**	**EC (µS cm^{-1})**	**Pseudo-total metal concentrations (mg kg^{-1} dry soil)**			**$CaCl_2$-exchangeable metal concentrations (mg kg^{-1} dry soil)**		
						Cd	**Zn**	**Pb**	**Cd**	**Zn**	**Pb**
SRC field	4% clay 8% silt 88% sand	5.6-6.7	5.5-6.3	5.5-8.8	50.4-175.0	4-13	210-778	133-342	0.07-0.78	2.68-42.21	0.07-0.34
Tobacco, sunflower, hemp plots 2011-2014	(Meers *et al.* 2007a)	6.78 ± 0.13	5.58 ± 0.15	7.10 ± 0.70	54.24 ± 5.86	4.05 ± 0.83	234.38 ± 37.99	141.75 ± 16.29	0.21 ± 0.06	11.15 ± 5.12	0.15 ± 0.04

Values for the SRC field are ranges based on data observed in previous research (Meers *et al.* 2007a,b; Ruttens *et al.* 2008; Van Slycken *et al.*, unpublished results). Values for the tobacco, sunflower and hemp plots are mean ± standard deviation of 8 independent replicates.

Table 3.3 Normal[2] and year specific climatological data[1] averaged for the field cultivation period of tobacco, sunflower and hemp (June-August) in the years 2011-2014.

Climatological data (June-August)	**Normal[2]**	**2011[1]**	**2012[1]**	**2013[1]**	**2014[1]**
Mean temp. (°C)	**17.5**	16.7	17.3	18.2	17.3
Mean max. temp. (°C)	**22.1**	21.2	21.9	22.7	21.5
Mean min. temp. (°C)	**13.2**	12.6	13.0	13.7	13.4
Mean rel. air humidity (%)	**74.7**	74.3	76.7	69.3	72.7
Total rainfall (mm)	**224.6**	317.2	271.3	169.2	348.2
Total days of rain (d)	**44**	61	51	28	49
Total hours of sun (h)	**579**	466	534	654	551
Mean wind velocity (m s^{-1})	**2.9**	3.1	3.2	3.2	3.0
Mean wind direction (% of occurrence)	**SW (12.5%)**	WSW (11%)	WSW (11%)	NNE (4.2%)	NNE-NNW-SW (4.2-3.7-12.5%)

[1]Climatological data were measured by the Royal Meteorological Institute of Belgium (KMI) (50°48'17'' N; 4°21'27'' E). [2]Mean climatological values for the 30-year period 1981-2010.

Tobacco

Height of the tested *in vitro* bred tobacco clones varied between 94 (2013: BAG F3) and 182 (2014: NBCu-10-8 F3) cm with an average of 160 cm in 2012, 94 cm in 2013 and 162 cm in 2014 for a mean BAG plant (height not measured in 2011). Aboveground DW production per plant differed significantly throughout the years (Figure 3.2). When comparing the same clonal variants tested in 2011 and 2013, similar productions of aboveground biomass were observed, with values significantly lower than in 2012 and 2014. Between 2012 and 2014, in turn, there was a significant difference for the BAG mother clone and its derivative NBCu-10-8, with double as much DW produced in 2012, while there was no difference observed for the FOP mother clone. Aboveground DW production of a mean BAG plant varied from 43.33 ± 22.63 g in 2011 over 54.27 ± 26.86 g in 2013 and 150.68 ± 47.87 g in 2014 to 336.04 ± 75.13 g in 2012.

In 2011, NBCu-10-8 showed significantly more aboveground biomass than NBCu-10-4 and their mother clone BAG. This observation was however not clearly reproduced in any of the following years. Considering mother clone FOP and its derivatives (NFCu-7-15 and NFCu-7-19), no significant differences were observed. In 2012, DW production of FOP and its derivative NFCu-7-15 was significantly lower than that of BAG and its derivative. However, this was not the case in subsequent years.

Produced aboveground biomass per ha and per year varied between 677 ± 354 kg DW ha^{-1} yr^{-1} (BAG in 2011) and 6031 ± 2381 kg DW ha^{-1} yr^{-1} (FOP in 2014) (Table 3.4). Different planting distances caused some minor changes compared to the rankings and general observations described above.

Considering metal concentrations in aboveground biomass, lowest concentrations for Cd, Zn as well as Pb were found in 2013 (Table 3.4). Concerning the other 3 years, there were no significant differences when comparing Cd concentrations in the same clonal variants, while Zn and Pb concentrations varied significantly. When comparing the different clones within a year, for Cd and Zn no consistent trends were observed in 2011 and 2012, however, in 2013 and 2014, NBCu-10-8 performed (significantly) better than its mother clone BAG. For Pb concentrations, no consistency regarding better performing clones was observed throughout subsequent years.

Calculated extraction potentials for Cd, Zn and Pb seemed to vary widely, from 12 up to 123 g Cd ha^{-1} yr^{-1}, 244 up to 2139 g Zn ha^{-1} yr^{-1} and 9 up to 124 g Pb ha^{-1} yr^{-1} (Table 3.4). Obviously lower amounts of metals were extracted in 2011 and 2013 compared to 2012 and 2014. In general, no clear differences were observed between the group of BAG and its derivatives and the group of FOP and its derivatives. Considering BAG and derivatives, NBCu-10-8 seemed to have the highest extraction potential for Cd, Zn and Pb in 3 out of the 4 years (2011, 2013 and 2014). The extraction potential of FOP seemed to be higher than that of derivative NFCu-7-15 (however only tested in 2012) while performance in comparison with NFCu-7-19 (tested in 2013 and 2014) is ambiguous.

Sunflower

Since rabbits consumed all sunflowers planted in 2012 shortly after planting, no results of 2012 sunflower cultivation could be presented. Of all tested sunflower mutants in the other years, mean height varied between 106 (2014: 15-35-190-04 M8) and 147 (2013: 14-185-04 M5) cm with averages of 139 cm in 2013 and 107 cm in 2014 for an IBL 04 control plant (height not measured in 2011). Aboveground DW production per plant is similar in all years except for mutant line family 15-35-190-04 where significant differences were found with a (significantly) lower production of the 15-35-190-04 M8 mutant in both 2013 and 2014 compared to other 15-35-190-04 mutants (Figure 3.3). A mean IBL 04 control plant produced an aboveground DW of 73 ± 27 g in 2013 and 66 ± 23 g in 2014. In 2013, mutant 14-185-04 M5 performed significantly better than its mother line (IBL 04) and both other derivatives. In 2014, 15-35-190-04 M7 and 14-185-04 M5 tended to produce more biomass than the mother line but this was not statistically confirmed.

Produced aboveground biomass per ha and per year reflected the same rankings and general observations as described above (Table 3.5). However, the high (but rather unrealistic) planting distance applied in 2011, increased biomass production considerably compared to 2013 and 2014. Yields of 7.3 and 6.5 t ha^{-1} yr^{-1} were found for the IBL 04 control in respectively 2013 and 2014.

Considering Cd and Zn concentrations in aboveground biomass, significantly higher values were found in 2014 compared to 2013 for the mother line IBL 04

and for 14-185-04 M5 and 86-35-190-04 M8 (Table 3.5). On the other hand, for the mutant line family 15-35-190-04, very similar concentrations of Cd and Zn were measured throughout the years. Lead concentrations did not differ much between 2013 and 2014 for most of the mutants. Mutant 15-35-190-04 M6 accumulated significantly more Pb in 2011 than mutants of the same line in 2013 and 2014. When comparing metal concentrations of different mutants within a year, 2 things are noticeable: (1) only in 2013 clear differences were observed between mutants with mutant 15-35-190-04 M8 revealing a significantly higher accumulation of Cd and Zn compared to IBL 04 and other derivatives; (2) in 2013 as well as 2014, mutant 86-35-190-04 M8 showed higher (but not significantly higher) Cd, Zn and Pb accumulation in comparison with IBL 04.

Calculated extraction potentials varied between 21 and 87 g Cd ha^{-1} yr^{-1}, 2141 and 8424 g Zn ha^{-1} yr^{-1} and 17 and 114 g Pb ha^{-1} yr^{-1}. Extraction of Cd and Zn decreased from 2011 over 2014 to 2013. Concerning extraction of all 3 metals, better performance than the IBL 04 mother line was observed in 2013 for mutants 86-35-190-04 M8 and 14-185-04 M5 while in 2014 mutants 15-35-190-04 M7 and 14-85-04 M5 took this role. Focusing only on Cd extraction in these years, all but 1 mutant performed better than IBL 04 control.

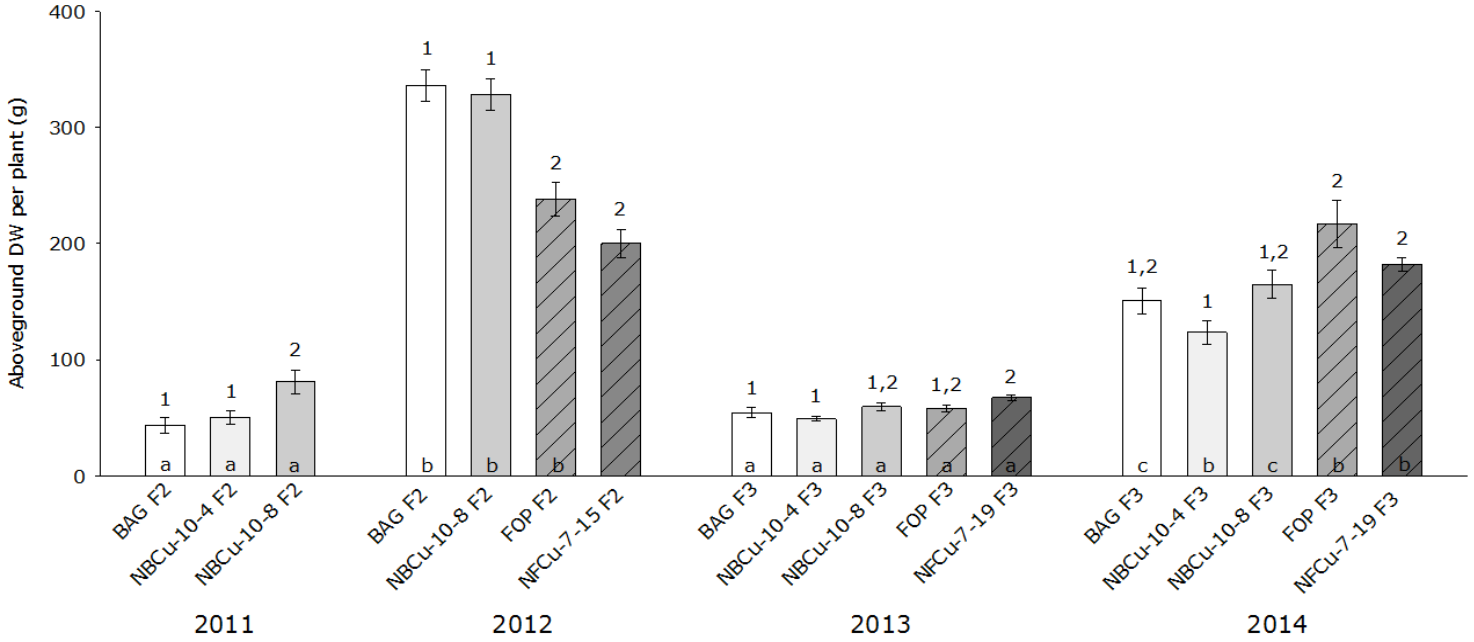


Figure 3.2 Mean aboveground DW production per plant (g) of *in vitro* bred tobacco clones tested in the period 2011-2014. Error bars are standard errors (n=20-45). Different grey shades represent different clones (disregarding the generation). BAG is the mother clone of NBCu clones (bars without pattern) while FOP is the mother clone of NFCu clones (bars with pattern). Numbers 1 and 2 represent clones with significantly different ($p < 0.01$) biomass production within a certain year while letters a-c represent significantly different ($p < 0.001$) biomass production of a certain clone between subsequent years.

Table 3.4 Aboveground biomass production (kg DW ha^{-1} yr^{-1}), Cd, Zn and Pb concentrations (mg kg^{-1} DW) in the aboveground biomass and Cd, Zn and Pb extraction potentials (g ha^{-1} yr^{-1}) of all *in vitro* bred tobacco clones tested in the period 2011-2014.

Year (plants ha^{-1})	***In vitro* bred tobacco clone**	**Gene-ration**	**Biomass production (kg DW ha^{-1} yr^{-1})**	**$[Cd]_{plant}$ (mg kg^{-1} DW)**	**$[Zn]_{plant}$ (mg kg^{-1} DW)**	**$[Pb]_{plant}$ (mg kg^{-1} DW)**	**Cd extraction potential (g ha^{-1} yr^{-1})**	**Zn extraction potential (g ha^{-1} yr^{-1})**	**Pb extraction potential (g ha^{-1} yr^{-1})**
2011	**BAG**	**F2**	677 ± 354	18 ± 4 $^{1\,a}$	495 ± 114 $^{1\,a}$	19 ± 6 $^{1\,a}$	12 ± 7	335 ± 191	13 ± 8
(15 625)	**NBCu-10-4**	**F2**	788 ± 419	18 ± 4 $^{1\,a}$	463 ± 69 $^{1\,a}$	17 ± 5 $^{1\,a}$	14 ± 8	365 ± 202	14 ± 8
	NBCu-10-8	**F2**	1267 ± 688	18 ± 4 $^{1\,a}$	467 ± 90 $^{1\,a}$	17 ± 4 $^{1\,a}$	23 ± 13	591 ± 340	21 ± 13
2012	**BAG**	**F2**	5251 ± 1174	20 ± 4 $^{1,2\,a}$	407 ± 67 $^{1,2\,b}$	24 ± 5 $^{1\,a}$	102 ± 30	2139 ± 594	124 ± 39
(15 625)	**NBCu-10-8**	**F2**	5128 ± 1196	19 ± 3 $^{1\,a}$	392 ± 40 $^{1\,b}$	22 ± 3 $^{1\,b}$	100 ± 27	2012 ± 512	112 ± 30
	FOP	**F2**	3722 ± 1237	18 ± 3 $^{1,2\,a}$	431 ± 46 $^{1,2\,a}$	25 ± 3 $^{1\,a}$	67 ± 25	1605 ± 560	92 ± 32
	NFCu-7-15	**F2**	3129 ± 995	17 ± 1 ^{2}	463 ± 31 ^{2}	29 ± 2 ^{2}	52 ± 17	1447 ± 470	91 ± 30
2013	**BAG**	**F3**	1508 ± 746	9 ± 1 $^{1\,b}$	162 ± 9 $^{1\,c}$	7 ± 0 $^{1\,b}$	14 ± 7	244 ± 121	10 ± 5
(27 778)	**NBCu-10-4**	**F3**	1363 ± 408	10 ± 3 $^{1,2\,b}$	187 ± 33 $^{1,2\,b}$	6 ± 2 $^{1,2\,b}$	14 ± 6	255 ± 89	9 ± 3
	NBCu-10-8	**F3**	1650 ± 623	12 ± 2 $^{2\,b}$	195 ± 19 $^{2\,c}$	7 ± 1 $^{1\,c}$	20 ± 9	321 ± 125	12 ± 5
	FOP	**F3**	1614 ± 501	10 ± 2 $^{1,2\,b}$	166 ± 22 $^{1,2\,b}$	6 ± 1 $^{1,2\,b}$	17 ± 6	268 ± 91	10 ± 4
	NFCu-7-19	**F3**	1868 ± 369	9 ± 1 $^{1\,a}$	171 ± 16 $^{1,2\,a}$	5 ± 1 $^{2\,a}$	18 ± 4	320 ± 70	10 ± 2
2014	**BAG**	**F3**	4186 ± 1330	18 ± 2 $^{1,2\,a}$	281 ± 29 $^{1\,d}$	9 ± 1 $^{1\,c}$	76 ± 25	1177 ± 393	37 ± 12
(27 778)	**NBCu-10-4**	**F3**	3432 ± 1168	16 ± 2 $^{2\,a}$	284 ± 38 $^{1\,c}$	11 ± 3 $^{1,2\,c}$	56 ± 21	973 ± 357	36 ± 15
	NBCu-10-8	**F3**	4577 ± 1483	25 ± 5 $^{1\,a}$	406 ± 67 $^{2\,a,b}$	10 ± 1 $^{1,2\,d}$	113 ± 42	1859 ± 676	46 ± 16
	FOP	**F3**	6031 ± 2381	20 ± 2 $^{1,2\,a}$	316 ± 26 $^{1,2\,c}$	12 ± 2 $^{2\,c}$	123 ± 49	1907 ± 769	72 ± 31
	NFCu-7-19	**F3**	5055 ± 696	18 ± 3 $^{1,2\,b}$	297 ± 43 $^{1,2\,b}$	10 ± 3 $^{1,2\,b}$	93 ± 20	1501 ± 301	51 ± 16

Values are mean ± standard deviation of at least 20 (biomass production) or at least 5 (metal concentrations) biological replicates. Different grey shades represent different *in vitro* bred clones (disregarding the generation). BAG is the mother clone of NBCu clones while FOP is the mother clone of NFCu clones. Superscript numbers 1 and 2 represent clones with significantly different Cd ($p < 0.05$), Zn and Pb ($p < 0.01$) concentrations within a certain year while superscript letters a-d represent significantly different Cd, Zn and Pb ($p < 0.001$) concentrations of a certain clone between subsequent years.

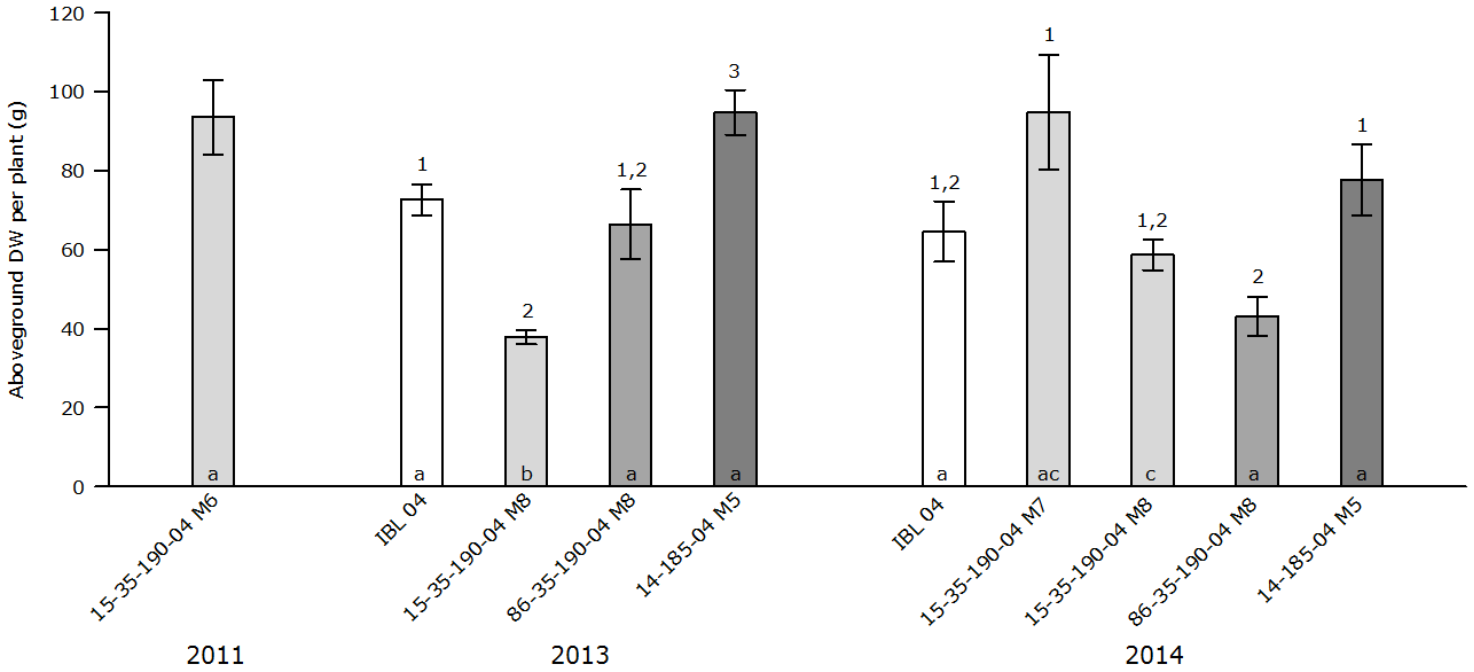


Figure 3.3 Mean aboveground DW production per plant (g) of sunflower mutants tested in the period 2011-2014. Error bars are standard errors (n=10-90). Different grey shades represent different mutant line families (disregarding the generation). IBL 04 is the mother line of all mutants. Numbers 1-3 represent mutants with significantly different ($p < 0.01$) biomass production within a certain year while letters a-c represent significantly different ($p < 0.001$) biomass production of a certain mutant between subsequent years.

Table 3.5 Aboveground biomass production (kg DW ha^{-1} yr^{-1}), Cd, Zn and Pb concentrations (mg kg^{-1} DW) in the aboveground biomass and Cd, Zn and Pb extraction potentials (g ha^{-1} yr^{-1}) of all sunflower mutants tested in the period 2011-2014.

Year (plants ha^{-1})	**Mutant**	**Gene-ration**	**Biomass production (kg DW ha^{-1} yr^{-1})**	**$[Cd]_{plant}$ (mg kg^{-1} DW)**	**$[Zn]_{plant}$ (mg kg^{-1} DW)**	**$[Pb]_{plant}$ (mg kg^{-1} DW)**	**Cd extraction potential (g ha^{-1} yr^{-1})**	**Zn extraction potential (g ha^{-1} yr^{-1})**	**Pb extraction potential (g ha^{-1} yr^{-1})**
2011 (166 667)	**15-35-190-04**	**M6**	15596 ± 7675	6 ± 1 ^{a}	540 ± 82 ^{a}	7 ± 1 ^{a}	87 ± 45	8424 ± 4340	114 ± 59
2013 (100 000)	**IBL 04**		7263 ± 2673	3 ± 1 $^{1\,a}$	351 ± 56 $^{1,3\,a}$	3 ± 1 $^{1,2\,a}$	21 ± 9	2550 ± 1023	23 ± 10
	15-35-190-04	**M8**	3787 ± 1730	6 ± 2 $^{2\,a}$	565 ± 58 $^{2\,a}$	4 ± 1 $^{1\,b}$	24 ± 13	2141 ± 1002	17 ± 9
	86-35-190-04	**M8**	6642 ± 6711	4 ± 1 $^{3\,a}$	433 ± 54 $^{3\,a}$	8 ± 3 $^{3\,a}$	27 ± 28	2873 ± 2925	51 ± 54
	14-185-04	**M5**	9477 ± 5430	3 ± 1 $^{1,3\,a}$	320 ± 102 $^{1\,a}$	3 ± 1 $^{2\,a}$	29 ± 18	3037 ± 1993	28 ± 18
2014 (100 000)	**IBL 04**		6457 ± 2280	6 ± 2 $^{1\,b}$	619 ± 183 $^{1\,b}$	4 ± 1 $^{1\,a}$	38 ± 17	3994 ± 1841	23 ± 11
	15-35-190-04	**M8**	5869 ± 777	7 ± 1 $^{1\,a}$	545 ± 84 $^{1\,a}$	4 ± 0 $^{1\,b}$	41 ± 9	3199 ± 650	23 ± 4
	15-35-190-04	**M7**	9482 ± 4829	6 ± 1 $^{1\,a}$	601 ± 144 $^{1\,a}$	4 ± 1 $^{1\,b}$	61 ± 32	5697 ± 3205	35 ± 20
	86-35-190-04	**M8**	4314 ± 1566	8 ± 1 $^{1,2\,b}$	818 ± 368 $^{1\,b}$	5 ± 2 $^{1\,a}$	33 ± 13	3528 ± 2040	26 ± 13
	14-185-04	**M5**	7775 ± 3371	5 ± 1 $^{1,3\,b}$	535 ± 116 $^{1\,b}$	3 ± 1 $^{1\,a}$	42 ± 20	4160 ± 2016	22 ± 11

Values are mean ± standard deviation of at least 10 (biomass production) or at least 5 (metal concentrations) biological replicates. Different grey shades represent different mutant line families (disregarding the generation). IBL 04 is the mother line of all mutants. Superscript numbers 1-3 represent mutants with significantly different Cd, Zn and Pb ($p < 0.05$) concentrations within a certain year while superscript letters a and b represent significantly different Cd, Zn and Pb ($p < 0.001$) concentrations of a certain mutant between subsequent years.

Sunflower seeds

Mean seed yield, measured for mutants in 2011 and 2013, varied between 7.17 and 23.14 g per plant (Table 3.6). Maximum values for Cd and Zn content found were respectively 1.95 and 121.63 mg kg^{-1} seed. Lead values were in all cases below the detection limit.

Table 3.6 Mean seed yield per plant (g) and metal concentrations (mg kg^{-1} seed) in the seeds of sunflower mutants tested in 2011 and 2013.

Year	Mutant	Gene-ration	Yield (g $plant^{-1}$)	$[Cd]_{seed}$ (mg kg^{-1})	$[Zn]_{seed}$ (mg kg^{-1})	$[Pb]_{seed}$ (mg kg^{-1})
2011	**15-35-190-04**	**M6**	18.17 ± 12.22	1.19	121.63	b.d.l.
2013	**IBL 04**		12.30 ± 8.16	1.34	111.16	b.d.l.
2013	**15-35-190-04**	**M8**	7.17 ± 3.51	1.42	66.46	b.d.l.
2013	**86-35-190-04**	**M8**	11.44 ± 6.31	1.95	64.28	b.d.l.
2013	**14-185-04**	**M5**	23.14 ± 8.12	0.96	87.25	b.d.l.

Yield values are mean ± standard deviation of at least 10 biological replicates. Metal concentrations were determined for 1 mixed seed sample per mutant. b.d.l. = below detection limit.

Hemp

Hemp plots of 6 m^2 were established in 2013 and 2014 using the same seed source and planting technique. However, germination of seeds in 2014 was puny and hemp cultivation was considered unsuccessful that year (no data collected). Mean height of plants in 2013 was about 2 m. Aboveground biomass production, Cd, Zn and Pb concentrations in the biomass and metal extraction potentials of hemp is summarized in Table 3.7. After 1 growing season, hemp was estimated to extract 1.22 kg of Zn and about 7 and 44 g of respectively Cd and Pb per ha.

Table 3.7 Aboveground biomass production (kg DW ha^{-1} yr^{-1}), Cd, Zn and Pb concentrations (mg kg^{-1} DW) in the aboveground biomass and Cd, Zn and Pb extraction potentials (g ha^{-1} yr^{-1}) of hemp tested in 2013.

Year (plants m^{-1})		**Biomass production (kg DW ha^{-1} yr^{-1})**	**$[Cd]_{plant}$ (mg kg^{-1} DW)**	**$[Zn]_{plant}$ (mg kg^{-1} DW)**	**$[Pb]_{plant}$ (mg kg^{-1} DW)**	**Cd extraction potential (g ha^{-1} yr^{-1})**	**Zn extraction potential (g ha^{-1} yr^{-1})**	**Pb extraction potential (g ha^{-1} yr^{-1})**
2013 (167)	**Hemp**	17496	0.4 ± 0.1	70 ± 7	3 ± 0	7 ± 2	1221 ± 115	44 ± 9

Values are mean ± standard deviation of 8 biological replicates.

3.3.3 Phytoextraction efficiencies of SRC, tobacco, sunflower and hemp

Figure 3.4 summarizes the Cd and Zn extraction potentials of all evaluated plants on the experimental field. In case of poplar and willow clones, (available) data were abstracted from Table 3.1. In case of tobacco and sunflower, extraction potentials of identical clones/mutants (disregarding the generation) were averaged over all tested years but 2011 (no fertilizer application and too high planting distance in case of sunflower). Tobacco clones had to be tested in at least 2 out of the 3 remaining years to be incorporated. The combined standard deviation was calculated using the total variance (incorporating deviance of each group and deviance of the mean of group means). When comparing the evaluated species, sunflower mutants cluster together at high Zn extraction levels and low Cd extraction levels. The tobacco clones had a rather low Zn extraction and showed moderate amounts of Cd removal. The available poplar and willow clones covered a large range of Cd and Zn extraction and suggest a rather linear trend in combined Cd and Zn removal. Lead extraction efficiency could only be evaluated for tobacco, sunflower and hemp (Figure 3.5) and seemed to increase from the tested sunflower mutants over hemp to tobacco clones BAG, NBCu-10-8 and FOP. For all species tested and concerning all 3 metals, standard deviations were very high. The bioconcentration factor (BCF) could only be calculated if soil pseudo-total metal content was known, which was not the case for commercial poplar clones nor for experimental poplars and willows. BCFs of Cd and Zn, determined for the remaining tested clones/mutants were, with exception of hemp, almost always > 1 (Table 3.8). The extraction of Cd was the highest for tobacco and some willow clones (Loden, Inger, Tora) while Zn extraction efficiency was highest for the sunflowers tested, followed by the commercial willows. The efficiency of Pb extraction, estimated for tobacco and sunflower, was very low (≤ 0.10).

Based on Cd and Zn extraction potentials, Zwarte Driebast revealed to be the best performing willow clone, while an experimental poplar of the group {D x (T x M)} was the most successful poplar clone (Figure 3.4). The sunflower mutant line 15-35-190-04 and tobacco clone NBCu-10-8 demonstrated highest mean Cd

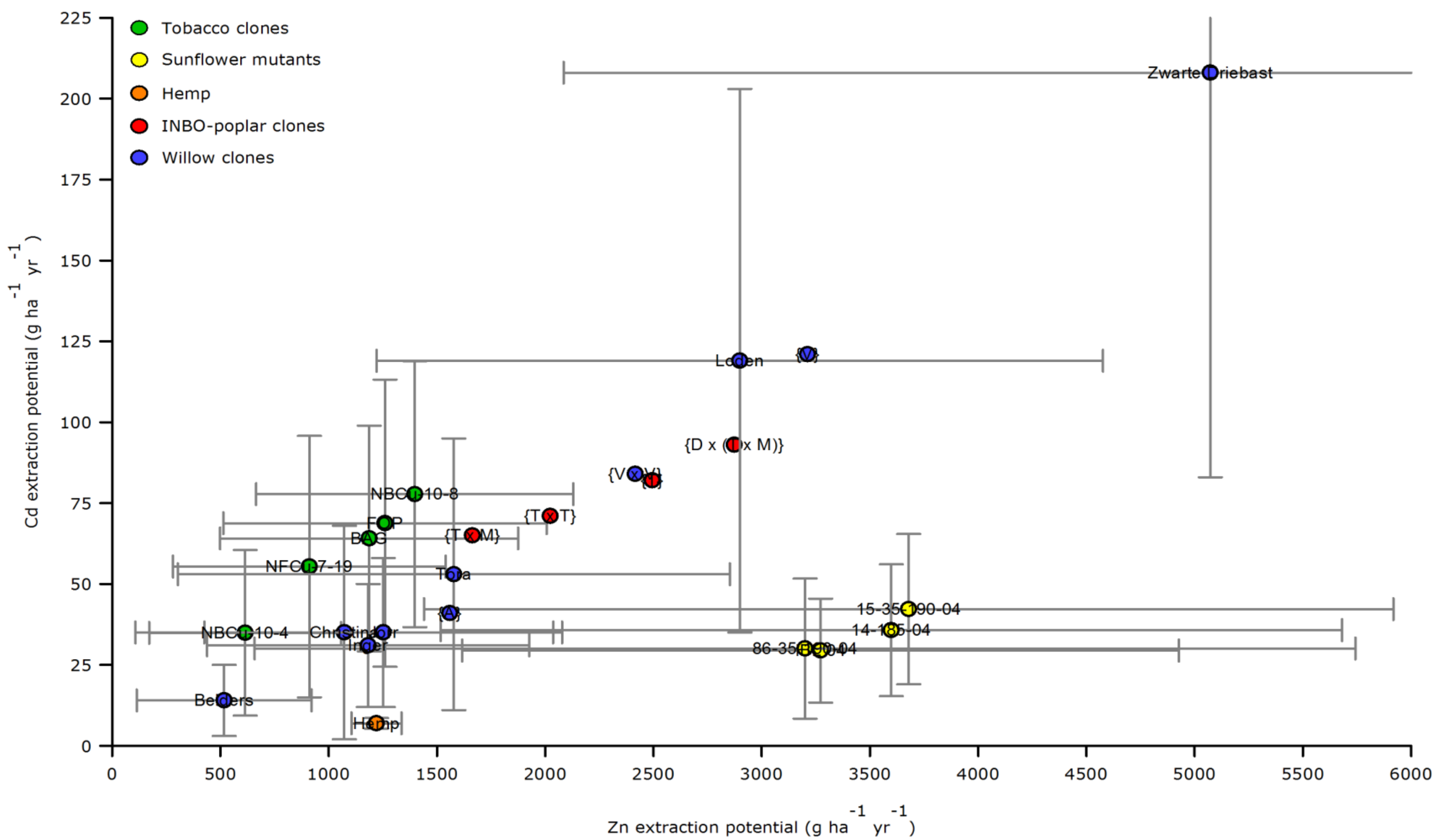
and Zn extraction in their groups. Hypothetical remediation times calculated for these best performing clones/mutant revealed shortest decontamination of Cd as well as Zn concentrations on the experimental field is most likely realized with Zwarte Driebast (Table 3.9). It would take a time span of 60 ± 36 years to clean the moderately contaminated soil to remediation thresholds. Phytoextraction of Pb in the (very) high contaminated part of the field in Lommel by tested plants revealed to be highly unrealistic. Disregarding Pb, the metal determining the remediation time seemed to depend on contamination level and species used.

Table 3.8 Mean bioconcentration factors (BCF) of Cd, Zn and Pb for tobacco clones, sunflower mutants, hemp and commercial willow clones evaluated on the experimental field. The BCF is defined as the ratio of metal concentration in aboveground biomass/stems to total soil metal content. In case of tobacco, sunflower and hemp, BCFs are based on *in planta* metal concentrations averaged for identical clones/mutants over all tested years but 2011 (results not shown) and mean pseudo-total metal concentrations in the soil as determined earlier (Table 3.2). In case of commercial willow clones, BCFs are based on stem metal concentrations (Table 3.1) and mean pseudo-total metal concentrations in the soil of the different clonal blocks copied from Van Slycken *et al.* (2013).

		Pseudo-total soil metal concentrations (mg kg^{-1} dry soil)			**BCF**		
Species	**Clone/mutant**	**Cd**	**Zn**	**Pb**	**Cd**	**Zn**	**Pb**
Tobacco	**BAG**				3.95	1.21	0.09
	NBCu-10-4				3.21	1.00	0.06
	NBCu-10-8				4.69	1.41	0.09
	FOP				3.95	1.30	0.10
	NFCu-7-19				3.46	1.04	0.06
Sunflower	**IBL 04**	4.05	234.38	141.75	0.99	2.07	0.02
	15-35-190-04				1.73	2.43	0.03
	86-35-190-04				1.48	2.67	0.04
	14-185-04				0.99	1.83	0.02
Hemp	**(2013)**				0.10	0.30	0.02
Commercial willow	**Belders**	7.50	450.00	n.d.	1.07	0.65	n.d.
	Christina	6.70	370.00	n.d.	3.13	1.73	n.d.
	Inger	6.70	430.00	n.d.	3.28	1.96	n.d.
	Jorr	6.30	357.00	n.d.	2.86	1.80	n.d.
	Loden	6.30	377.00	n.d.	4.44	1.81	n.d.
	Tora	6.50	359.00	n.d.	3.23	1.76	n.d.
	Zwarte Driebast	5.50	299.00	n.d.	3.09	1.38	n.d.

n.d. = not detected.

Figure 3.4 Cadmium and Zn extraction potentials (g ha^{-1} yr^{-1}) of poplar, willow and tobacco clones, sunflower mutants and hemp evaluated on the experimental field. In case of poplar and willow clones, results reflect data from Table 3.1. In case of tobacco and sunflower, extraction potentials of identical clones/mutants were averaged over all tested years but 2011. Values are mean ± standard deviation.
D: *Populus deltoides*; M: *Populus maximowiczii*; N: *Populus nigra*; T : *Populus trichocarpa*, A: *Salix alba;* Da: *Salix dasyclados* ; F: *Salix fragilis;* S: *Salix schwerinnii;* Tr: *Salix triandra* ; V: *Salix viminalis*; {}: collection of all experimental clones with this crossing type (see 'Material and methods' section for details).


Tobacco clones
Sunflower mutants
Hemp
INBO-poplar clones
Willow clones
Cd extraction potential (g ha-1 yr-1)
Zn extraction potential (g ha-1 yr-1)

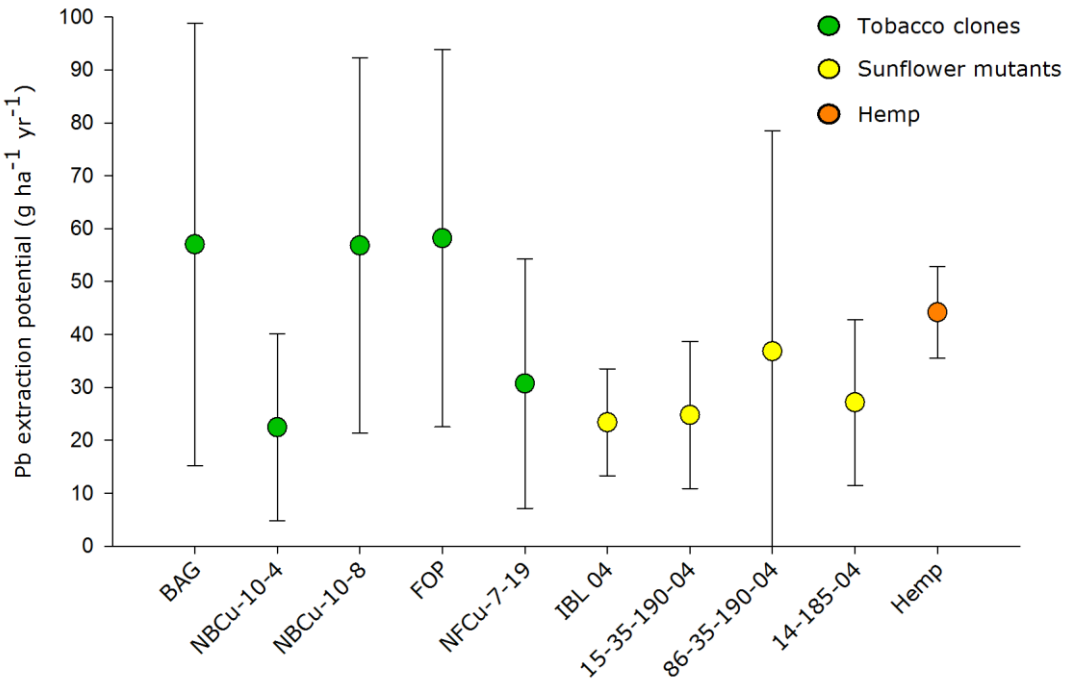


Figure 3.5 Lead extraction potentials of evaluated tobacco clones, sunflower mutants and hemp cultivated on the experimental field. In case of tobacco and sunflower, extraction potentials of identical clones/mutants were averaged over all tested years but 2011. Values are mean ± standard deviation.

Table 3.9 Hypothetical remediation times (years) of best performing clones/mutant of tested species (see Figure 3.4) to reduce 1 mg of Cd, Zn and Pb kg^{-1} DW soil or very high, high or moderate field Cd, Zn and Pb concentrations to remediation thresholds (2 mg Cd, 282 mg Zn and 200 mg Pb kg^{-1} DW soil). Assumptions made were: (i) species' extraction potentials are independent of soil metal concentrations (contamination level), (ii) total soil metal content decreases linearly with time due to a constant yearly extraction, (iii) contamination and rooting depth are 0.5 m, and (iv) the soil density is 1250 kg m^{-3}.

Hypothetical remediation times (years)					**Contamination level**							
						Very high			**High**			**Moderate**
Species	**Clone/mutant**	**1 mg Cd kg^{-1} DW soil**	**1 mg Zn kg^{-1} DW soil**	**1 mg Pb kg^{-1} DW soil**	**13 mg Cd kg^{-1} DW soil**	**708 mg Zn kg^{-1} DW soil**	**316 mg Pb kg^{-1} DW soil**	**7 mg Cd kg^{-1} DW soil**	**429 mg Zn kg^{-1} DW soil**	**217 mg Pb kg^{-1} DW soil**	**4 mg Cd kg^{-1} DW soil**
Poplar	**D x (T x M)**	67	2	n.d.	739	927	n.d.	336	320	n.d.	134
Willow	**Zwarte Driebast**	30 ± 18	1 ± 1	n.d.	331 ± 199	525 ± 309	n.d.	150 ± 90	181 ± 107	n.d.	60 ± 36
Tobacco	**NBCu-10-8**	80 ± 42	4 ± 2	110 ± 67	881 ± 463	1905 ± 999	>10000	401 ± 211	657 ± 345	1864 ± 1145	160 ± 84
Sunflower	**15-35-190-04**	149 ± 81	2 ± 1	250 ± 140	1637 ± 896	724 ± 440	>10000	744 ± 407	250 ± 152	4250 ± 2380	298 ± 163
Hemp	**(2013)**	893 ± 255	5 ± 0	142 ± 29	9821 ± 2806	2181 ± 205	>10000	4464 ± 1276	752 ± 71	2415 ± 494	1786 ± 510

Values are mean ± standard deviation. n.d. = not detected.

3.4 Discussion

3.4.1 Potential of metal phytoextraction using high biomass crops

In this study, metal phytoextraction of short rotation coppice (SRC) of willow and poplar clones, selected tobacco clones, sunflower mutants and hemp was evaluated and compared. On a Cd-Zn-Pb-contaminated field, annual crops (tobacco, sunflower, hemp) were cultivated for 4 subsequent years, while the woody crops in short rotation were examined after 4 continuous growing seasons.

Biomass productivity levels of SRC depend on site-specific conditions, clonal selection, climatic conditions, plant spacing and management. For SRC of willow, expected biomass productivity is between 6 and 10 t ha^{-1} yr^{-1} in Sweden (Dimitriou *et al.* 2006) while higher values (10-20 t ha^{-1} yr^{-1}) were considered common by Maxted *et al.* (2007). Annual yields reported for poplars in SRC are between 10 and 15 t ha^{-1} in less intensive conditions (Laureysens *et al.* 2004). Finally, Zegada-Lizarazu *et al.* (2010) described an average biomass yield between 10 and 12 t ha^{-1} yr^{-1} for poplar and willow in temperate climates. After 4 growing seasons of poplar and willow clones on the experimental field in Lommel, biomass productivity was low (mostly < 5 t ha^{-1} yr^{-1}; Table 3.1) compared to generally expected yields. The lower productivity levels could be attributed to the nutrient poor, sandy characteristics of the soil (Van Slycken *et al.* 2015). Given the lack of fertilization and irrigation, the productivity of willow and poplar clones will be less in comparison with SRC cultures on regular, more fertile soils. Furthermore, yields obtained after the first growing seasons tend to be lower than yields from later cutting cycles (Aronsson *et al.* 2014; Van Slycken *et al.* 2015) since the plant will allocate a considerable amount of its energy to the establishment of the root system during the first years. When biomass production of the commercial willow clone Tora was estimated after another 3 growing seasons (beginning 2013), a yield of 5401 ± 3791 kg ha^{-1} yr^{-1} was assessed which is more than double compared to the first growing cycle (results not shown). The high variability in stem biomass production and Cd and Zn

concentrations observed, can be attributed to the heterogeneity of the field given that the same clone is planted in different plots on different locations of the field. However, for Zwarte Driebast, the high biomass variability could not be explained by total levels of soil contaminants or nutrient conditions (Van Slycken *et al.* 2013). Furthermore, clonal differences in extraction efficiency are occurring (in the supposition that replicates are evenly spread over the field) and for willow, these are described in Chapter 5, part I.

Tobacco plant height and dry weight production per plant differed significantly between the years while this was not the case for sunflowers (Figures 2 and 3). Since both crops were cultivated next to each other on the same plots, this might suggest that the tobacco clones are more susceptible to yearly variations in climatological conditions, field preparation and maintenance actions and/or the quality of the seed might differ substantially between years. For example, the low biomass production in 2011 could partly be the result of no fertilizer application, no irrigation (except just after planting) and clearly less hours of sun compared to normal values and the other years. The low amount of biomass produced in 2013, might be related to (a combination of) 3 features. Firstly, plantlets were quite old (about 11 weeks) when the field was ready for planting. A growth spurt might already have taken place when the plants were still in pots, hampering their biomass production seriously. Secondly, because no weed control was executed before and after planting, a considerable amount of weeds (mainly *Polygonum sp.*) grew in between the tobacco plants competing for nutrients and water. Finally, compared with the other years and normal values, a lower quantitiy of rain but a higher amount of sun were observed in this year which might have induced drought stress to some extent. Aboveground yields of tobacco not only varied considerably over the years on our field but also did not reach yield values as reported by Kayser *et al.* (2000) (10-12.5 t ha^{-1} yr^{-1}), Fässler *et al.* (2010) (8.5-10.5 t ha^{-1} yr^{-1}) or Herzig *et al.* (2014) (24.7-37.5 t ha^{-1} yr^{-1}) obtained in phytoremediation field experiments in a temperate region (Table 3.4). Aboveground production of an IBL 04 sunflower plant on the metal-contaminated soil in Lommel was comparable with biomass productions of this inbred line found in earlier years on the metal-contaminated site in Rafz, Switzerland (93.7 g, 68 ± 17 g and 78 ± 8.6 g) (Nehnevajova *et al.* 2007,

2009) (Figure 3.3). Yield values per hectare and year seem to be rather low compared with yields reported for sunflowers cultivated on metal-contaminated soils in a temperate region, being from 7.5 up to 29 t ha^{-1} yr^{-1} (Kayser *et al.* 2000; Fässler *et al.* 2010; Herzig *et al.* 2014) (Table 3.5). The high yield calculated in 2011, is rather unrealistic. A planting distance of 15 cm in the row caused sunflowers to 'zigzag' in search for light. This is however impossible on a larger scale.

The significant differences in concentrations of Cd, Zn and Pb of a tobacco clone or sunflower mutant throughout the years cannot be explained by differences in soil metal content since all experiments were conducted in a very narrow area of the field with identical soil characteristics (Table 3.2, 3.4 and 3.5). Also Fässler *et al.* (2010) found considerable year-to-year variations in metal uptake of tobacco and sunflower in field trials. It is speculative which (combination of) factors (climatic, seed quality/generation, field preparation and management, planting distance…) are responsible for the abundant significant differences found between years. The lower concentrations found in 2013 could not be explained. Moreover, for sunflower mutants of the mutant line 15-35-190-04 no differences in Cd and Zn concentrations were observed throughout the years. This further complicates formulating a plausible hypothesis.

As a result of the high variations in tobacco biomass production and metal concentrations, the extraction potentials for Cd, Zn and Pb differed widely throughout the years (about a factor of 10 for every metal) (Table 3.4). Concerning extraction potentials of sunflower mutants in 2013 and 2014, variation is more limited (about a factor 3 for Cd and Pb, a factor 2.5 for Zn) (Table 3.5).

The 3-4 years of tobacco and sunflower cultivation on the field in Lommel could reveal some information concerning stable improvements after selection based on somaclonal variation and conventional *in vitro* breeding (tobacco) or chemical mutagenesis (sunflower). For tobacco, there are no consistent indications for derivates producing more biomass than mother variants (Table 3.4). Concerning metal accumulation, only NBCu-10-8, for which (significantly) higher Cd and Zn concentrations compared to BAG were observed in 2 out of the 4 years, might announce a slightly improved metal-accumulating clone. When combining

biomass productivity and metal accumulation and averaging over the years, mean Cd and Zn removal of NBCu-10-8 indeed was higher than mean values of BAG (Figure 3.4). Regarding the sunflowers, in 2013 and 2014 mutant 86-35-190-04 M8 showed an improved metal accumulation compared to control and higher yields than for the mother line were obtained with mutant 14-185-04 M5 (Table 3.5). However, the large biomass increments of these mutants (so called 'giant mutants') compared to control (Nehnevajova *et al.* 2007, 2009) were not observed. Averaging extraction potentials over both years revealed a slight, likely stable extraction improvement for mutant lines 15-35-190-04 and 14-185-04 (Figure 3.4). In case of willow and poplar, the vegetative propagation of selected clones imposes a big advantage to this concern. Vegetative propagation increases the potential to maintain improved characteristics of a certain genotype/cultivar/clone/variant (Zegada-Lizarazu *et al.* 2010). Furthermore, using stem cuttings to establish clonal plantations is expected to reduce variability between plants compared to plants raised from seeds (Dickinson *et al.* 2009).

Metal contents in the tested sunflower seeds are similar to values reported by Lombi *et al.* (1998) who cultivated sunflower on a sandy soil with 3.57 mg Cd and 412 mg Zn kg^{-1} DW soil (Table 3.6). However, seed yield per plant in Lommel was lower in comparison with this pot trial (7-23 g *vs.* 45 g). De Maria and Rivelli (2013) reported that Cd contents in seeds of sunflowers grown in contaminated soil in pots never exceeded the toxicity threshold values considered for livestock fodder imposed by the European Commission (European Commission 2002). Mean Cd contents in seeds of all but 1 sunflower mutant grown in Lommel, however, exceeded threshold values for feed material of vegetable origin applied in Europe (1 mg Cd kg^{-1} at 12% moisture content of the material). The application of the seeds for pet feed production is legally justified (threshold 2 mg Cd kg^{-1} at 12% moisture content of the material) however raises questions of concern.

Biomass production of hemp in Europe is reported to be about 10 tons per hectare and year (Citterio *et al.* 2003). Based on the 6 m^2 plot in Lommel in 2013, biomass production is estimated to reach almost 75% more (Table 3.7). Although results could not be confirmed in additional field trials, this could be an

indication of sufficient growth of hemp on this metal-contaminated site in Belgium, taking into account that fertilization is applied. Cadmium shoot content was however very low compared to values observed in other hemp phytoextraction studies (11.4-33.3 mg kg^{-1} and 66 mg kg^{-1} Cd), even in respect to the higher Cd concentrations in soil used in these pot trials (respectively 25 mg kg^{-1} Cd and 82 mg kg^{-1} Cd) (Citterio *et al.* 2003; Shi *et al.* 2012).

As a result of the heterogeneity of the field (in case of the tree clones) and yearly variations in many factors (in case of tobacco and sunflower), variability is high when considering mean extraction potentials of all evaluated clones/mutants (Figures 4 and 5). In general however, extraction potentials together with BCFs (Table 3.8) tend to indicate sunflower as a highly efficient Zn extractor while tobacco revealed a more pronounced extractor of Cd. This was also found by Kayser *et al.* (2000) and Fässler *et al.* (2010). Most evaluated poplar and willow clones showed a phytoextraction potential in between that of tobacco and sunflower. However, the large range of (combined) Cd and Zn extraction covered by these clones gives considerable cause for optimism that clone selection and/or conventional breeding approaches may provide additional clones with a higher combined extraction of Cd and Zn. Although the poplar and willow clones were in general cultivated on the parts of the field containing higher levels of Cd and Zn, BCFs for commercial willow clones indicate they possess high efficiencies regarding Cd and Zn extraction compared to tobacco and sunflower. BCFs ≥ 1 furthermore confirm very efficient extraction (accumulation of metals in the crops relative to the soil) (Dickinson and Pulford 2005; Kötschau *et al.* 2014) of Cd and Zn by sunflowers, tobacco and commercial willows. Hemp on the contrary, showed a very low extraction efficiency and had only some low potential for Zn extraction due to its high biomass production. Most remediation studies with hemp indeed report a low phytoextraction potential and too slow soil restorations (Linger *et al.* 2002; Citterio *et al.* 2003; Fumagalli *et al.* 2014).

Phytoextraction of Pb using the species tested in Lommel obviously is utopia (Table 3.9). The BCFs indicate a very inefficient uptake of Pb by aboveground biomass. However, its low bioavailability in the soil, and even increased inactivation by a vegetation cover (Chaney *et al.* 1997), makes that

concentrations of Pb in soil, even when exceeding remediation thresholds, rarely cause problems for agriculture (plant Pb uptake).

Care needs to be taken with the calculated remediation times (Table 3.9). Firstly, all tested species are assumed to possess a steady extraction potential, independent of soil metal concentrations. It is however not evaluated to what extent extraction potentials of sunflower, tobacco, hemp, willow or poplar depend on levels of soil contamination. Secondly, the yearly linear decrease in total soil metal concentration due to phytoextraction likely is a simplistic vision compared to the real situation. It assumes that a species' biomass production, its metal accumulation (or at least the product of both) and the bioavailability of metals in the soil does not change over time. Several authors (Robinson *et al.* 2003, 2006; Koopmans *et al.* 2007; Van Nevel *et al.* 2007; Manzoni *et al.* 2011) proposed decay models incorporating, to some extent, soil chemistry (with all kinds of sorption, retention and leaching processes to describe evolutions in the 'bioavailable' metal pool), changes in plant metal accumulation and biomass production over time *etc.*. However, involving more factors increases uncertainty in turn and since no model is acknowledged to be valid in all cases, the most simple approach is used here. Nevertheless, the remediation times of the different crops are differential enough to conclude willow clone Zwarte Driebast will likely need the shortest time to restore the metal contents in Lommel to the legislative threshold values. The experimental poplar clone D x (T x M) is the second best option. Furthermore, a thorough study of the individual clones in the experimental groups of INBO crossing types might unravel other clones suitable for phytoextraction purposes. On the contrary, the tobacco clones and sunflower mutants used were the result of respectively *in vitro* breeding and mutagenesis followed by continuous breeding and selection for improved phytoextraction efficiencies. Further enhancing the remediation potential in these groups is therefore not very likely.

It should be mentioned that remediation times for Cd on the moderately contaminated soil in Lommel are long and become unacceptably long in case of highly contaminated soil. This is considered the most important limitation, the Achilles heel, of phytoextraction limiting large-scale applications (Linger *et al.* 2002; Vassilev *et al.* 2004; Dickinson *et al.* 2009). Therefore, adding other

profits is essential for the implementation of phytoextraction. This added value can be achieved when economic and environmental aspects of metal-phytoextraction join the picture.

3.4.2 Economic aspects of metal phytoextraction using high biomass crops

Economic and environmental aspects are described here with the aim to distinguish between the phytoextracting crops evaluated in this research. In order to make a comparison between economic aspects of the different crops, most common conversion processes for the metal-contaminated biomass are explained in advance.

The economic profit of a phytoextracting crop is determined by the difference between input costs and output revenues. Costs to make are related to the establishment, the maintenance and the harvest of the plantation and eventual storage of the crops or crop residuals. Tobacco, sunflower and hemp need to be germinated, planted, fertilized, maintained (irrigation, weeding), harvested and/or stored yearly (see 'Material and methods' section) while SRC plantations, after the establishment phase, typically have a lifetime of 20 to 30 years with low-labor intensive management (harvest and eventually fertilization every 2-7 years) (Zegada-Lizarazu *et al.* 2010; Dimitriou *et al.* 2011) as well as the possibility of storage on the field. Furthermore, in case of the annual crops, a rotation cycle with other crops is highly recommended (Kidd *et al.* 2015), increasing the intensity of such cultures even more. Therefore, concerning economic input, a plantation of SRC seems much more attractive than the cultivation of annual crops. Also Zegada-Lizarazu *et al.* (2010) reported short rotation woody crops in principle have lower agricultural inputs and lower production costs compared to annual crops. Biomass can be used to generate an income in many ways. However, since every product of phytoextraction is potentially hazardous biomass with higher (to toxic) contents of metals, biomass processing is only environmentally sound if the re-entry of hazardous metals in the environment is minimized. Potential non-justified uses of biomass as lignocellulosic feedstock (*e.g.* SRC wood for pulp or board industries) or as feed or fodder supply (*e.g.* tobacco for tobacco industry, sunflower for vegetable oil

or as fodder crop…) are not reported here (except in case of hemp). Also mixtures of supplying feedstock and possible pre- and post-treatments of contaminated biomass/products are not explained here. Biomass conversion techniques described for the SRC wood crops tested in this research are direct combustion, gasification and pyrolysis while for tobacco and sunflower physical-chemical conversion and pyrolysis are considered. Environmental issues and economics are discussed if information is available.

During direct combustion, heat and/or power are generated. It is a technique commercially used around the world and the main destination of willow wood in Sweden, a country with about 14 000 ha of willow SRC (Dimitriou *et al.* 2011). The fate of metals and viability of the process when combusting metal-contaminated *Salix* biomass was assessed by Šyc *et al.* (2012) and Delplanque *et al.* (2013). In both cases, the highest amount of Cd was recovered in the flue gas (fly ash), which indicated combustion to be problematic and unjustified without installation of highly efficient filters. Moreover, metal content in the ash exceeded the limits of respective policies disallowing its utilization as fertilizer.

Biomass gasification converts biomass to a low to medium calorific value gaseous fuel (Vassilev *et al.* 2004). This fuel can be used to generate heat and electricity by direct firing in engines, turbines, and boilers after suitable clean up. Alternatively, the gas can be reformed to produce fuels such as methanol and hydrogen, which could then be used in *e.g.* fuel cells or micro turbines. Compared to combustion, gasification-based systems may be more beneficial in terms of economies of scale and clean and efficient operation. Fixed bed downdraft gasification of willow wood, cultivated on a metal-contaminated site in Belgium, was evaluated by Vervaeke *et al.* 2006. Again Cd, Zn and Pb were enriched in the fly ashes compared to the bottom ashes. Also, Cd and Zn concentrations in the bottom ashes exceeded Flemish threshold values for use of this fraction as a fertilizer.

A third technique for woody biomass conversion is pyrolysis. Pyrolysis, the rapid heating of biomass to moderate temperatures (350-650°C) in the absence of oxygen, yields a char, liquid and gas fraction (Lievens *et al.* 2008). The pyrolysis gas is mainly used for internal energy provision (Kötschau *et al.* 2014). The liquid fraction, the pyrolysis oil, can be used as a substitute for fossil fuels to

generate heat and electricity or can be upgraded and used as a transport fuel. Besides, the liquid has potential to produce a range of specialty and commodity chemicals (Vassilev *et al.* 2004). The pyrolysis char can be used as an energy carrier (directly or after pelletising), as a soil amendment (biochar) or can be upgraded to active carbon (AC). Biochar might enhance structure and fertility of the soil and increase carbon sequestration while AC has potential as a filter medium for gas and water treatment or in the food industry (Fletcher *et al.* 2014; Kötschau *et al.* 2014). Pyrolysis of metal-contaminated willow leaves and branches, with contamination levels comparable to those in this research, revealed metals to be concentrated in the ash/char fraction at low process temperatures (350°C) (Stals *et al.* 2010). The metal free liquid and gas fractions are reported to be suitable for fuel and chemical stock applications. A preliminary pyrolysis experiment with willow wood from the field trial in Lommel, focusing on the quality of the liquid fraction, revealed a restricted use of the liquid fraction as fuel due to a rather low higher heating value and a rather high water content (Greenland EC project, unpublished results). The recovery of Cd from the native biomass into the pyrolysis liquid was about 7 wt% (1.31 up to 2.22 mg Cd kg^{-1} DW). Biochar derived from pyrolysis of phytoremediation willow wood was characterized by Fletcher *et al.* (2014). Quality of the char as well as concentrations of metals in it can be regulated by changing process parameters. However, the authors mentioned fate of the metals should be thoroughly investigated to prevent possible air pollution during char production. Furthermore, Cd concentrations in the willow wood used as feedstock were much lower (2.36 mg kg^{-1} DW) than those in willow wood in this research (Table 3.1). Characteristics of activated char from fast pyrolysis of short rotation hardwood cultivated for phytoremediation in Flanders showed to be comparable to those of a commercial reference (Stals *et al.* 2013). The fate of metals was however not explained. Flash pyrolysis using Cd-Cu-Pb-Zn contaminated sunflower was performed by Lievens *et al.* (2008) who reported the metals to be concentrated in the ash/char fraction at low process temperatures (350°C). Pyrolysis of tobacco was studied extensively (amongst many others) (Pütün *et al.* 2007; Akalin and Karagöz 2011; Cardoso *et al.* 2011; Yang *et al.* 2011; Wu *et al.* 2015) but never in relation to phytoremediation. A preliminary pyrolysis

experiment with metal-contaminated tobacco and sunflower showed the pyrolysis liquid from the tobacco and sunflower had potential as renewable fuel because of its relative low water content and high calorific value but low yield could compromise the economic viability of this valorization (Greenland EC project, unpublished results). Up to 12.3 wt% of Cd and 10.9 wt% of Cu in native biomass were concentrated in the oil fraction. In a second preliminary pyrolysis experiment, metal-contaminated tobacco was pyrolyzed in order to obtain AC's through physical activation of the char fraction (Greenland EC project, unpublished results). The AC's revealed to expose an efficient removal of Cr(VI) in aqueous solution. If metal leaching during treatment application is prevented and the AC's would be handled as waste afterwards, concentrating metals in the pyrolysis char fraction might be desirable.

The physical-chemical conversion route applies only to biomass from which vegetable oil can be obtained like sunflower, tobacco and hemp. It consists of pressing and extracting oil from the biomass (Vassilev *et al.* 2004). Vegetable oils can be used in special engines or in diesel engines after an esterification step to produce oil methyl esters. However, no results were found in relation to metal-contaminated biomass. Hemp cultivated on the field in Lommel could, because of its very low metal content, also be used for all kinds of industrial applications (insulation, rope, clothes, paper, particle board,…) (Linger *et al.* 2002; Citterio *et al.* 2003; Zegada-Lizarazu *et al.* 2010). It is furthermore very likely that hempseed oil, derived from the produced hemp, has no restrictions for application (lubricants, paints, inks, fuel, plastics, cosmetic and pharmaceutical products).

An advantage of gasification and pyrolysis is the generation of fuels and in case of pyrolysis potentially also products (active coal, biochar, chemicals…) more valuable than electricity/heat. Power from biomass is in many cases not economic because power is generated from a large base of fossil-fueled plants (Vassilev *et al.* 2004). Also Voets *et al.* (2011) reported biomass-integrated gasification and flash pyrolysis are expected to energetically and economically perform better than combustion. Pyrolysis moreover has the advantage that biomass conversion and energy generation can be decoupled since pyrolysis oil can be stored and transported (Vassilev *et al.* 2004; Voets *et al.* 2011). Another

important benefit from pyrolysis is that process parameters like temperature can be regulated. Combustion and gasification typically happen at higher temperatures (> 850°C) at which metals (especially Cd) are more easily volatized. By consequence, (expensive) gas cleaning is required before releasing gas into the atmosphere (combustion) or using it as a fuel (gasification). During pyrolysis, most metals remain in the char fraction as long as the process temperature is below 450°C (Lievens *et al.* 2008; Stals *et al.* 2010).

The possible economic revenues from described valorization opportunities for the evaluated crops were searched for in literature. No relevant information was found for valorization of metal-contaminated tobacco, sunflower or hemp. For metal-phytoextraction using short rotation willow, economic revenues were investigated mostly in Flanders and results are summarized here. Economics of gasification and flash pyrolysis of short rotation willow wood in Flanders, studied by Voets *et al.* (2011), revealed flash pyrolysis to be more profitable at small-scale SRC conversion (electrical capacity 5-10 MW). However, combined heat and power production is required and a substantial amount of produced heat should be sold. Economics of SRC conversion were also studied by (amongst others) Mitchell *et al.* (1995), Dornburg and Faaij (2001) and Bridgwater *et al.* (2002). In general combustion revealed to be the most expensive conversion route and pyrolysis seems to be better suited for small capacities and gasification for higher capacities. A techno-economical assessment of fast pyrolysis of contaminated willow by Kuppens *et al.* (2015) indicated that the profitability stands or falls with operational scale (*i.e.* the available amount of willow) and the heat turnover (*i.e.* the guaranteed demand for heat). Since both factors are highly uncertain in Flanders, chances are high that the possible price range for phytoextracting willow is not able to cover cultivation costs. A compensation for the income loss of the farmers by the government, using complementing feedstocks and/or processing of the char fraction into an apprized product (*i.e.* activated carbon), are possible routes to reduce economic risks. Kuppens *et al.* (2014) indeed noted that as long as the AC from phytoremediating crops can be sold at market prices, the processing costs (of activation and fume gas treatment for removal of volatizing metals) are expected to be more than compensated. Pyrolysis process conditions in favor of

char production might therefore be preferred. When assessing the economics of phytoextraction, ideally, CO_2 abatement should be incorporated as well (Witters *et al.* 2012a,b). Indeed, when produced biomass is used for renewable energy production, the energy generated is considered greenhouse gas neutral. However, the external benefit of CO_2 abatement is not included correctly in the price of biomass and can as such not yet be taken into account in economic valorization. Considering the differences in conversion techniques for evaluated crops and the lack of economic revenues for most of them, it is difficult to distinguish between the tested crops. In any case, the SRC on the experimental field showed a more stable (and probably increasing) biomass production over the years compared to the annuals. A higher biomass production by short rotation woody crops over annuals was also generalized for all kinds of soils by Zegada-Lizarazu *et al.* (2010). In addition, the poplar and willow wood can be sold whenever economic profit is highest or income generation is needed since harvest can be done between 2-7 years and storage (drying) on the field is possible for a variable length of time.

To conclude the economic aspects of metal-phytoextraction, an important remark should be made. The use of biomass from phytoremediation to generate bioenergy is very often claimed to be an economic valorization of the technique, compensating for the long remediation time required. In addition, it is manifested as a sustainable use of marginal land for 'green' energy production, gaining more and more attention in primary energy production worldwide (Schröder *et al.* 2008; Dickinson *et al.* 2009; Witters *et al.* 2012a; Kidd *et al.* 2015). However, the problem of contaminated crop conversion/disposal has not been addressed extensively (Sas-Nowosielska *et al.* 2004; Witters *et al.* 2012a). Moreover, all the studies described above were performed at lab-scale and either denote the environmental and economic constrictions when converting metal-contaminated biomass to energy or do not mention them at all. To our knowledge, there are nowadays no contaminated biomass integrated bioenergy systems operating at a commercial scale. Some authors (Lievens *et al.* 2008; Stals *et al.* 2010) even reported handling and disposal of metal enriched plant wastes is the bottleneck of valorization (breakthrough) of the obtained biomass (phytoremediation). To illustrate this, many tons of poplar and willow wood,

harvested after the second growth cycle on the experimental field in Lommel, could not be given a sustainable, environmentally sound, economically profitable destination.

3.4.3 Environmental benefits of metal phytoextraction using high biomass crops

To compare environmental benefits of phytoextraction using SRC woody species and annual crops (tobacco, sunflower, hemp), an untreated (bare) metal-contaminated soil was taken as reference. The gradual decontamination of the soil by evaluated species is extensively argued before and is not considered in this paragraph. Other environmental benefits are generated by the presence of a vegetation cover on metal-contaminated land compared to no or scarce vegetation. A first benefit of a vegetation cover is a risk reduction regarding spreading of the contaminants. Crop growth prevents dispersion by wind and erosion by water (Vangronsveld *et al.* 1995a,b; Pulford and Watson 2003; Dickinson *et al.* 2009). In addition, uptake of water and transpiration through leaves, might limit the leaching of metals to ground and surface waters. Secondly, a vegetation cover increases biodiversity (Vangronsveld *et al.* 1996; Zegada-Lizarazu *et al.* 2010; Van Slycken *et al.* 2015). The presence of a plantation might improve life and quality of life in soil, in waters nearby, on land and in the air. Thirdly, vegetation potentially improves the quality of the soil in many ways (Pulford and Watson 2003; Dickinson *et al.* 2009). Leaf fall adds significant amounts of organic matter to the surface layers of the soil, promoting nutrient cycling, soil aggregation and water holding ability. Dead tree roots and root exudates also contribute to this. Finally, the growing crops will sequester CO_2 in soil, roots and aboveground biomass, (dependent on the CO_2 input of cultivation) contributing to CO_2 abatement (Van Slycken *et al.* 2015). Given the advantages of a vegetation cover on metal-contaminated soil compared to reference (inaction), it is possible to distinguish between evaluated species regarding environmental benefits. Since the establishment of SRC means the continuous presence of a vegetation cover (or at least of the root system) for several decades, reduction of risks, improvement of soil quality and carbon sequestration will be higher than for a plantation (rotation) with annuals like

tobacco, sunflower or hemp that have a seed-to-seed life cycle of only a few months (Fumagalli *et al.* 2014; Van Slycken *et al.* 2015). Furthermore, compared to annual crops, SRC plantations demand much less frequent mechanical management (tillage, fertilization, harvest). This seriously limits soil disturbance and compaction which in turn enhances environmental benefits (*e.g.* increase C sequestration in soil, prevent nutrient leaching, lower greenhouse gas emissions (Zegada-Lizarazu *et al.* 2010; Dimitriou *et al.* 2012a,b)). Moreover, since harvest of SRC takes place in the dormant season (when the maximum amount of nutrients and carbohydrates are translocated to the roots), depletion of soil nutrients is less intense than in case of annuals (Zegada-Lizarazu *et al.* 2010). Therefore, yearly fertilization, a burden to the environment in many ways, is not needed. Finally, poplar and willow are native species in the cold and temperate regions of the Northern hemisphere while tobacco (tropical and subtropical America), sunflower (North and South America) and hemp (Central and South Asia) are not. This enhances the leading role of SRC plantations for phytoextraction in the Campine area not only because of biodiversity issues but also regarding expected productivity and undesired variations due to climatic factors.

Although economic revenues of metal-phytoextraction are (still) uncertain, the environmental benefits are undeniable. Unfortunately, none of the external benefits (environmental benefits from a vegetation cover as well as CO_2 abatement when production of renewable energy) are rewarded when implementing phytoextraction. One could discuss if government intervention is needed, how this should be undertaken and if it eventually would convince land owners, farmers or investors to apply phytoextraction. However, this is beyond the scope of this research. In any case, further investigating sustainable, economically profitable and environmentally sound conversion of metal-contaminated biomass together with studying positive externalities of phytoextraction and a way to compensate them is crucial. These findings will label phytoextraction as a sustainable technology and hopefully lead to large-scale, commercial implementations.

Conclusion

In a 4-year field experiment in the northeast of Belgium, the phytoextraction potential of some high biomass crops was evaluated and compared. In general, biomass productivity of short rotation coppice (SRC) of willow and poplar, tobacco, sunflower and hemp revealed to be rather low on the metal-contaminated soil and could, in case of tobacco and sunflower, not completely be explained nor controlled. Moreover, productivity of tobacco varied considerably over the tested years as influenced by a lot of factors. Also metal accumulation in aboveground biomass of tobacco and sunflower varied over the years without proper clarification. As a result, the phytoextraction potential of both differed enormously, especially for tobacco, causing large variations when predicting phytoextraction potentials. Furthermore, the selected tobacco clones and sunflower mutants showed no significant, stable improvements in metal extraction compared to their mother clones/lines although years of selection for phytoextracting purposes had preceded. The only successful experiment with hemp on the contaminated soil revealed a very high biomass production but a low phytoextraction potential. The shortest remediation time for simultaneous Cd and Zn clean up was achieved using commercial willow clone Zwarte Driebast, followed by the experimental poplar clone D x (T x M). Moreover, the large range of (combined) Cd and Zn extraction covered by the SRC clones gives considerable cause for optimism that clone selection and/or conventional breeding approaches may provide additional clones with a high combined extraction of Cd and Zn.

A major drawback of metal phytoextraction using the evaluated high biomass crops is the long time needed to clean the soil. As such, it is mostly difficult to make a strong case for phytoextraction as a stand-alone technology. Economic revenues through biomass conversion and a rewarding for environmental benefits of a phytoextracting crop plantation are essential for large-scale, commercial implementation of metal-phytoextraction. Therefore, research regarding sustainable, economically profitable and environmentally sound conversion of metal-contaminated biomass as well as compensation systems for external benefits is of primordial importance.

Given the extraction potential and available information on economics and external benefits, SRC is defined to be the most suitable crop for metal phytoextraction in the area under investigation.

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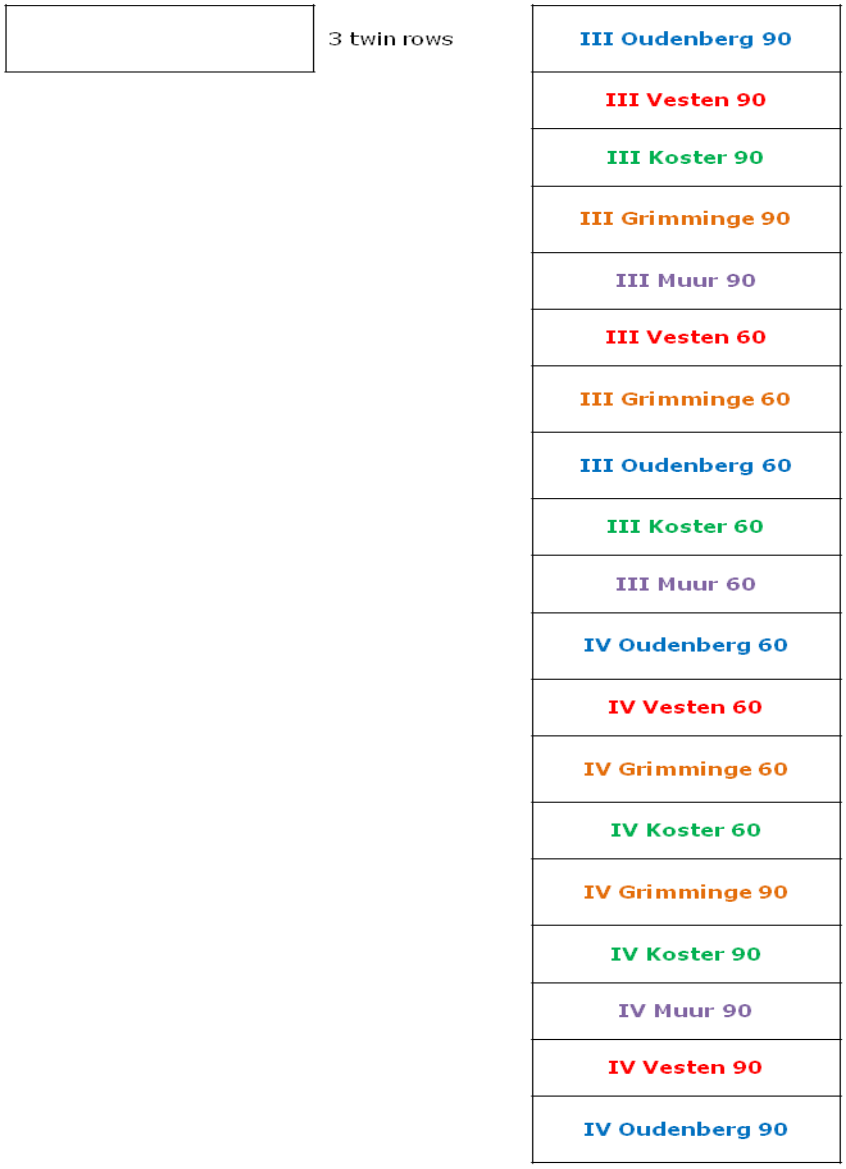
Supplementary information



Supplementary figure 3.1 Detailed structure of commercial poplar block 8 (see Figure 3.1 in 'Material and methods' section). Different colors represent different clones. Numbers 90 and 60 depict plant distance (cm) in the row.

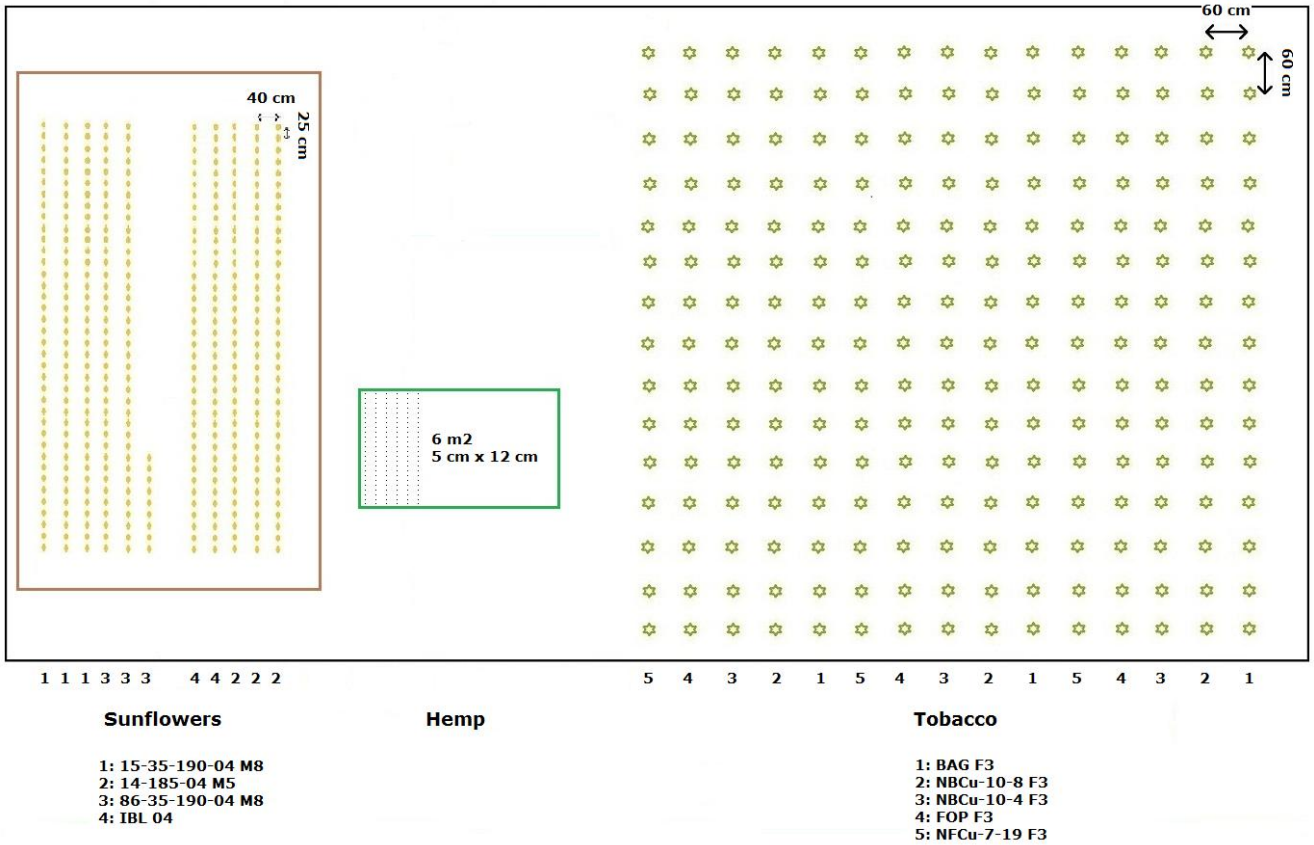
V86	*Salix viminalis*
V86	*Salix alba*
S89	*Salix alba x Salix alba*
S88	*Salix rubens half-sib*
S89	*Salix rubens x Salix alba*
S97	*Salix alba x Salix fragilis*
S97	*Salix viminalis x Salix viminalis*
S97	*Salix viminalis x Salix viminalis*

1 row of 25 trees

2 rows of 25 trees

			V86.136	V86.165	/	S89.005/7
			V86.145	V86.150	S89.005/238	S89.005/226
			V86.124	V86.173	S89.005/210	S89.005/42
			V86.144	V86.162	S89.005/26	S89.005/15
			S89.005/47	V86.156	S89.005/109	V86.188
			V86.161	V86.133	S89.005/177	S97.005/18
			V86.203	V86.159	S89.005/171	S89.005/78
			V86.164	V86.???	S89.005/148	S89.005/4
			V86.169	V86.166	S89.005/59	S89.005/28
			V86.160	V86.196	S89.005/241	S89.005/229
			S97.012/2	S97.011/3	S97.010/3	S97.010/4
			S97.012/10	S97.011/2	S97.011/1	S97.012/6
			S97.012/1	S97.010/6	S97.012/9	S97.012/7
			S97.010/5	S97.012/8	S97.012/11	S97.010/1
			S97.012/4	S97.012/5	S97.012/3	S97.010/2
S97.005/13	S89.016/41	S88.003/160	S89.007/85	S89.002/21	S89.001/53	V86.068
S97.005/15	S89.016/73	S88.003/173	S89.007/42	S89.002/58	S89.001/28	V86.027
S97.005/???	S89.016/2	S88.003/13	S89.007/90	S89.002/5	S89.001/8	V86.053
S97.005/4	S89.016/79	S88.003/21	S89.007/70	S89.002/20	S89.001/2	V86.019
S89.016/97	S97.005/7	S89.005/135	S89.007/28	S97.011/1	S89.001/40	S89.005/187
S97.005/11	S89.016/22	S88.003/204	S89.007/33	S89.002/39	S89.001/25	V86.088
S97.005/2	S89.016/53	S88.010/120	S89.007/79	S89.002/13	S89.001/49	V86.041
S97.005/12	S89.016/36	S88.003/34	S89.007/57	S89.002/47	S89.001/26	V86.043
S97.005/20	S89.016/86	S88.010/68	S89.007/55	S89.002/38	S89.001/42	V86.019
S97.005/27	S89.016/46	S88.003/120	S89.007/108	S89.002/19	S89.001/12	V86.023
			S97.007/12	S97.007/1	S97.007/21	S97.007/24
S97.007/11	S97.007/29	S97.007/2	S97.007/30	S97.007/5	S97.007/13	S97.007/22
S97.007/26	S97.007/3	S97.007/???	S97.007/32	S97.007/14	S97.007/28	S97.007/8
S97.007/10	S97.007/7	S97.007/25	S97.007/6	S97.007/19	S97.007/???	S97.007/16
S97.007/9	S97.007/27	S97.007/23	S97.007/17	S97.007/20	S97.007/4	S97.007/31

Supplementary figure 3.2 Detailed structure of experimental willow block 2 (see Figure 3.1 in 'Material and methods' section). Different colors represent different crossing types. Plant distance in the row is 60 cm.



Supplementary figure 3.3 Detailed structure of the tobacco-sunflower-hemp plot in 2013.

Chapter 4

Eight years of phytoextraction using SRC of willow: effective decontamination and changes in soil toxicity

Abstract

The different entities in a SRC phytoextraction system as well as their complex interactions are changing over time. By consequence, extrapolations of phytoextraction results (decontamination levels, risk reduction, soil quality changes, *etc.*) obtained in hydroponic, pot or short-term/small-scale field experiments are highly unreliable. This implies that the longer-term effectiveness of metal phytoextraction using short rotation coppice (SRC) is rather unknown. Therefore, determining effective reduction of metal levels in soil and changes in soil toxicity, and also soil fertility and functionality, in longer-term field experiments with SRC phytoextraction applications is crucial. The experimental field in the metal-contaminated area in northeast Belgium offers a unique opportunity to this concern. Analyses of soil managed for 8 years by metal phytoextraction using SRC of willow (Tora; *Salix schwerinnii x Salix viminalis*) and soil without phytoextraction management were performed in this research. This report describes the tangible decontamination based on pseudo-total soil metal concentrations as well as changes in soil toxicity observed using standardized chemical extractions and ecotoxicity assays based on plants (dwarf beans (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.) and turnip (*Brassica rapa*)) and invertebrates (tiger worm (*Eisenia andrei*) and nematode (*Caenorhabditis elegans*)). The observed decontamination after 8 years revealed

to be much higher (48 times in case of Cd removal, 79 times for Zn removal) than predicted by extrapolating metal removal of Tora based on biomass analysis after 4 growing seasons. Furthermore, all chemical extractions and all ecotoxicity tests unanimously indicated the willow-managed soil to be less toxic, to different extents, compared to the unmanaged soil. The results all emphasize the environmental benefits (soil remediation, reduced toxicity and leaching risks, improved soil quality) of a SRC-phytoextraction-managed soil compared to no management. Further evaluating the benefits of longer-term metal phytoextraction might eventually strongly contribute to the realization of large-scale applications of this remediation technology.

4.1 Introduction

A phytoextraction system is composed of 4 main entities: the soil, the soil solution, microorganisms and the plant (Lasat 2000; Landberg and Greger 2002; Koopmans *et al.* 2007; Mench *et al.* 2009). The system components together with their complex interactions determine the overall remediation efficiency under prevailing conditions. Although the efficiency of a phytoextraction system can be estimated at a certain moment by direct measurements, future predictions come along with high uncertainties since changes in the components and their interactions are very likely to happen. To illustrate, possible evolutions in the entities related to soil and plant for a phytoextraction system with SRC of willow are shortly described here. SRC of willow is reported to need more than one cutting cycle in order to express its full potential of productivity (Labrecque and Teodorescu 2003; Van Slycken *et al.* 2015), and a doubling or even tripling of yield values in later cutting cycles were repeatedly observed (Hofmann-Schielle *et al.* 1999; Aronsson *et al.* 2014). Moreover, the characteristics of the soil and changes in soil conditions over time (*e.g.* depletion of nutrients, acidification, *etc.*) play a major role in possible future yield increments. Metal accumulation in the stem might decrease with stand age, as demonstrated by Hammer *et al.* (2003) and suggested by Mertens *et al.* (2006). This might again happen due to an interaction between soil and plant. Mertens *et al.* (2006) reported that fast developing root systems in young willow SRC plantations explore more substrate with relatively more available Cd and Zn. On the longer

term however, metal bioavailability might decrease due to uptake of bioavailable metals and insufficient replenishing from the total metal pool. Furthermore, *Salix* roots are known to grow deeper with time (Keller *et al.* 2003). In this way, the extending root system might avoid the shallow (0 - 0.5 m) contaminated layer, leading to less metal uptake. Finally, if stem biomass productivity levels increase, a dilution effect might lower stem metal concentrations. For *Salix*, a dilution due to growth was observed when following up 1 growing season (Dinelli and Lombini 1996) and when investigating fertilization effects over 3 years (Klang-Westin and Perttu 2002).

Given the complexity of a(n) (evolving) SRC phytoextraction system, it is proposed that extrapolations from hydroponics, pot trials or short-term/small-scale field experiments are unreliable (as was also reported for phytoremediation systems in general by Dickinson *et al.* 2009 and Vangronsveld *et al.* 2009). Therefore, it is concluded here that there is no other reliable way of assessing phytoextraction efficiencies on the longer term than to evaluate such systems in practice. A large-scale (about 10 ha), long-term (since 2006) field experiment with hundreds of different poplar and willow clones in SRC in the Cd-Zn-Pb-contaminated region in Belgium (Ruttens *et al.* 2008) offers a unique opportunity to this concern because field trials like these are very scarce (Dickinson *et al.* 2009).

In Chapter 3, metal removal and remediation time were determined based on biomass (stem production and metal accumulation) analysis of clones after 4 growing seasons (end of the first rotation cycle). In this chapter, remediation time and soil toxicity are evaluated after 8 years of growth (at the end of the second rotation cycle). Since the commercially available willow clone Tora (*Salix schwerinnii x Salix viminalis*) is a frequently tested clone for metal phytoextraction (Meers *et al.* 2003; French *et al.* 2006; Delplanque *et al.* 2013), also in this first, exploratory investigation, the Tora clone was selected for evaluating the effect of 8 years of SRC management.

Determining the level of decontamination, by measuring pseudo-total metal contents in willow-managed (SRC of Tora phytoextraction) soil and adjacent unmanaged (no phytoextraction) soil, is essential to assess the phytoextraction efficiency of a longer-term SRC cultivation,. However, the toxicity and main risks

of a metal-contaminated soil are often presumed to be related to 'bioavailable' metal concentrations instead of total concentrations (Herzig *et al.* 2014; Kumpiene *et al.* 2014). Bioavailability is however a complex term, in which *e.g.* dynamic processes, chemical speciation of the metals and species specific interactions are involved (Meers *et al.* 2007b). Multiple single extractions (*e.g.* EDTA-, NH_4NO_3-, $NaNO_3$-, $CaCl_2$- and water-extractions) can be performed to estimate the potential 'external' bioavailability of metals (although each of these extractions targets a different portion of the 'available' metal content). Furthermore, the presence of internationally accredited extraction procedures (for extraction with EDTA: ÖNORM L 1089:2005; with NH_4NO_3: DIN ISO 19730:2008(E), with $NaNO_3$: Osol, 1998, with $CaCl_2$: Van Ranst *et al.* (1999) and with water: ISO/TS 21268-1:2002) allows making comparisons between all kinds of soils. The toxicity of a soil to living organisms (*i.e.* the 'internal' bioavailability of metals) is complex and difficult to predict solely using extractable/exchangeable amounts of metals as described above (Kumpiene *et al.* 2014). To this respect, internationally accredited (ISO standards) or in literature recognized ecotoxicity assays are very useful as a direct tool for estimating soil toxicity. Examples of plant-based ecotoxicity tests are the dwarf bean (*Phaseolus vulgaris* L.) (Vangronsveld and Clijsters 1992), lettuce (*Lactuca sativa* L.) (ISO 17126:2005) and turnip (*Brassica rapa*) (ISO 11269-2:2012) test. The toxicity responses of plants growing on the metal-contaminated soil are estimated based on parameters like biomass production, % of seed emergence, metal content in the tissues, *etc.*. In case of the dwarf bean test, also activities of stress-related enzymes in roots and leaves are used for classifying toxicity (Vangronsveld and Clijsters 1992). Beans growing on metal-contaminated soil suffer from higher than balanced concentrations of reactive oxygen species (ROS; *e.g.* hydrogen peroxide (H_2O_2)) causing so-called oxidative stress (OS). The activity of guaiacol peroxidase (GPOD), a group of H_2O_2-quenching enzymes which are also involved in the lignification of cells as defense mechanism, are reported to increase in dwarf bean roots and leaves suffering from OS (Smeets *et al.* 2005). Also malic enzyme (ME), glutamate dehydrogenase (GIDH) and iso-citrate dehydrogenase (ICDH), $NAD(P)^+$-reducing enzymes delivering reducing power for optimal functioning of anti-oxidative

enzymes (which oxidize NADPH while quenching ROS), are increased in conditions of OS (Vangronsveld and Clijsters 1994). By consequence, measuring the activities of stress-related enzymes in bean roots (ME, GIDH) and leaves (ME, ICDH) of unmanaged and willow-managed soil provides information regarding the level of OS related to metal toxicity of the soil. The tiger worm (*Eisenia andrei*) (ISO/CD 17512-1:2008) and nematode (*Caenorhabditis elegans*) (ISO 10872:2010) test are examples of invertebrate-based ecotoxicity assays. In these cases, the level of soil toxicity is estimated by introducing the invertebrates into the metal-contaminated soil and determining parameters like the number of offspring produced per adult, growth of the worms, physical soil preference/avoidance *etc.*. Besides the effect of longer-term SRC phytoextraction on soil metal contents and soil toxicity, the impact on general physico-chemical soil characteristics (pH, cation exchange capacity (CEC) and electrical conductivity (EC)) might deliver some additional information regarding soil fertility and functionality.

In this study, the longer-term phytoextraction efficiency of 8 years of Tora SRC was assessed by determining the effective decontamination (subtracting pseudo-total metal concentrations of unmanaged (no phytoextraction) and willow-managed (SRC of Tora phytoextraction) soil). The observed decontamination and related remediation times were compared with metal removals and remediation times based on biomass production of, and metal content in Tora stems after 4 growing seasons. Furthermore, differences in soil toxicity between willow-managed and unmanaged soil were assessed using standardized chemical extractions as well as accredited plant and invertebrate ecotoxicity assays.

The study was conducted as part of the Greenland EU project (FP7-KBBE-266124) (http://www.greenland-project.eu/). This project addressed several issues according to gentle remediation options (GRO), in general plant-based, to remediate trace element contaminated soils (TECS) at low cost and without significant negative effects for the environment. Many TE-contaminated sites and corresponding managements were involved in the project and in order to compare efficacy between them, a selection of a minimum risk assessment battery, combining chemical and ecotoxicity assays, was performed (Kumpiene *et al.* 2014).

4.2 Material and methods

4.2.1 Site description and SRC plantation

The metal-contaminated experimental field is located in Lommel, northeast of Belgium. A description of the site as well as an overview of the SRC plantation can be found in Chapter 3 ('Material and methods' section: Site description, Short rotation coppice (SRC) of willow and poplar) (see also Figure 4.1).

4.2.2 Biomass analysis of Tora after 4 years of growth

Production of stem biomass and metal concentrations (Cd, Zn) in the stem of Tora (*Salix schwerinnii x Salix viminalis*) were measured after the first 4 growing seasons. Data were derived from Van Slycken *et al.* (2013). The extraction potential was calculated by multiplying yearly stem production with the concentration of metals in the woody biomass and expressed in g ha^{-1} yr^{-1}. Results are listed in Chapter 3, Table 3.1.

4.2.3 Soil investigation after 8 years of Tora SRC

Soil sampling

End 2013, topsoil (0-30 cm) was sampled on the field under Tora (plant distance in the row 30 cm) (assigned 'willow-managed soil') and under natural grassland vegetation (mixture of *Agrostis capillaris*, *Holcus lanatus*, *Epilobium angustifolium*, *Juncus effusus*, *Poa pratensis* and *Rumex acetosa*) next to the block with Tora (assigned 'unmanaged soil') (Figure 4.1). Three samples per condition were collected with a spacing of 3 m from each other. Litter and vegetation cover were removed before sampling. Soil samples were sieved (4 mm) but not air-dried.

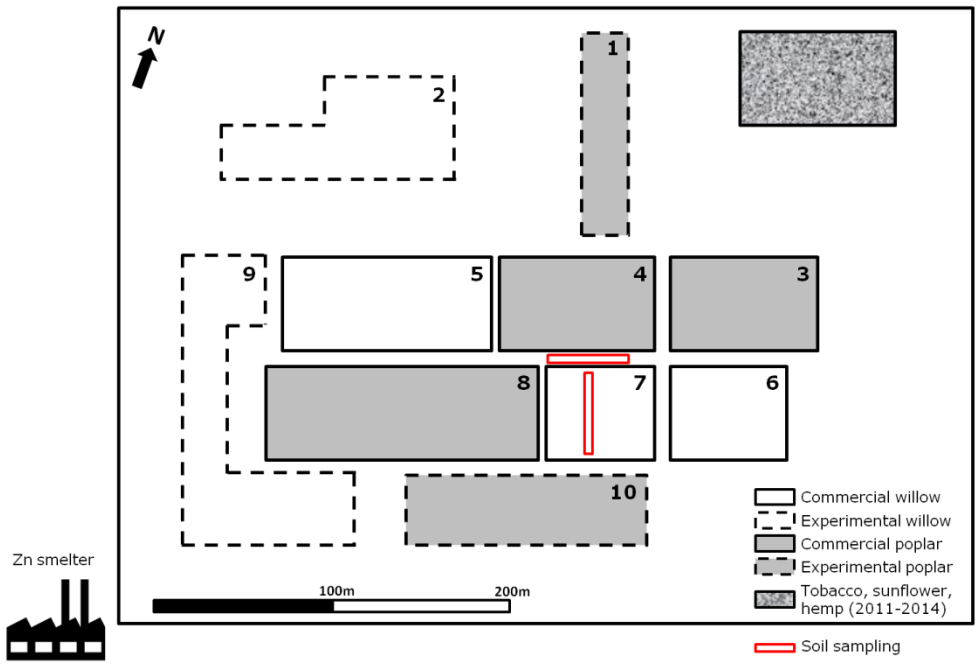


Figure 4.1 Scheme of the experimental field in Lommel with the location of commercial (full lines) and experimental (dotted lines) willow and poplar blocks. Red boxes depict sampling places for estimating the effect of 8 years of SRC of Tora on soil metal content and soil toxicity.

Physico-chemical soil characteristics (including soil metal concentrations)

Soil samples were oven-dried and sieved (2 mm grid) and subsequently analyzed for pH-H_2O, pH-KCl, electrical conductivity (EC) and effective cation exchange capacity (CEC_e). Material and methods are described in Chapter 3 ('Material and methods' section: Tobacco, sunflower and hemp: Physico-chemical soil characteristics).

Pseudo-total Cd, Zn and Pb soil concentrations were determined using *aqua regia* extraction and were performed and analyzed at the Institute of Soil Science and Plant Cultivation State Research Institute (IUNG, Poland). Briefly, 0.5 g air-dried and ground soil was microwave digested at 160 °C, 25 min ramp time and 20 min hold time. Samples were centrifuged and analyzed by ICP-MS. Ethylenediaminetetraacetic acid disodium-dihydrate (**EDTA**)-extractable Cd, Zn and Pb concentrations were evaluated using the Austrian standard procedure

(ÖNORM L 1089:2005). Ten g of air-dried and sieved (< 2 mm) soil was mixed with 100 mL of 0.05 M EDTA solution, shaken for 2 h and filtered. The standard procedure (DIN ISO 19730:2008(E)) was followed for determining ammonium nitrate (**NH_4NO_3**)-extractable metal concentrations. Air dried, sieved (< 2 mm) soil was extracted with 1 M NH_4NO_3-solution in a liquid-to-solid ratio (L:S) of 2.5 L kg^{-1} for 120 min using an end-over-end shaker at room temperature and filtered. Sodium nitrate (**$NaNO_3$**)-extractable metal concentrations were measured according to a Swiss Standard Procedure (Osol, 1998). Fifty mL of 0.1 M $NaNO_3$-solution was added to 20 g of air-dried, sieved (< 2 mm) soil in a 100 mL Erlenmeyer flask. Flasks were closed, shaken for 2 h and filtered. Calcium dichloride (**$CaCl_2$**)-extractable Cd, Zn and Pb concentrations were evaluated as described in Van Ranst *et al.* (1999). Oven-dried and sieved (< 2 mm) soil was extracted with 0.01 M $CaCl_2$ in a 1:5 (w:v) extraction ratio. After 2 h of shaking (120 rpm), the mixture was filtered. **Water** extractable soil metal concentrations were determined by a standard compliance batch leaching test (ISO/TS 21268-1:2002). Soil was air-dried, sieved (< 4 mm) and mixed with 0.001 M $CaCl_2$-solution in a L:S of 2 L kg^{-1}, shaken for 24 h using a rotating device and filtered. Analysis of samples from all single extractions were performed using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies 700 Series).

Remediation time

Based on differences in pseudo-total metal concentrations measured in unmanaged and willow-managed soils, the hypothetical remediation time needed to reduce pseudo-total Cd, Zn and Pb concentrations in the unmanaged soil (in Chapter 3 referred to as 'very high contamination level'), to remediation thresholds (2 mg Cd, 282 mg Zn and 200 mg Pb kg^{-1} DW soil) were calculated. Following assumptions were made: (i) measured decreases in pseudo-total metal concentrations are due to uptake by Tora which is constant over the years, (ii) measured decreases in pseudo-total metal concentrations in the first 0.3 m of soil are valid for the first 0.5 m of soil, (iii) contamination and rooting depth are 0.5 m, and (iv) the soil density is 1250 kg m^{-3}.

Ecotoxicity tests

Ecotoxicity plant tests were performed with dwarf beans (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.) and turnip (*Brassica rapa*). The plantox test using **dwarf beans** was executed as described in Vangronsveld and Clijsters (1992). After 1 day of vernalization and 4 h of imbibitions, the beans (cv. Limburgse vroege) were sown in 400 mL polyethylene pots (4 plants per pot, 3 pots per condition). Plants were grown in a growth chamber (temperature 22 °C, air humidity 65%, photoperiod 12 h, photosynthetically active radiation 150 µmol m^{-2} s^{-1}). After 14 days, morphological parameters (shoot length, fresh weight (FW) of roots, shoots and primary leaves) of each plant were determined. Of every individual, samples (0.5 g FW) of primary leaves and roots were immediately frozen in liquid nitrogen and stored at -70 °C until activity analysis of enzymes related to plant stress responses (referred to as 'stress enzymes'). Within 1 month after harvest, each frozen sample was homogenized with a Polytron PT 3000 homogenizer in ice-cold Tris-HCl buffer (0.1 M, pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 4% insoluble polyvinylpyrrolidone (5 mL buffer g^{-1} FW). The homogenate was passed through a nylon mesh and centrifuged for 10 min at 20 000 g and 4 °C. Subsequently the activity (*i.e.* the potential activity measured *in vitro* under non-limiting conditions of substrate and coenzyme) of the following stress enzymes was measured spectrophotometrically (Shimadzu UV-1602) at 25 °C in the supernatant (Van Assche *et al.* 1988): guaiacol peroxidase (GPOD, EC 1.11.1.7), malic enzyme (ME, EC 1.1.1.40), glutamate dehydrogenase (GIDH, EC 1.4.4.2) and iso-citrate dehydrogenase (ICDH, EC 1.1.1.42). Enzyme activity was expressed in milli-Units (mU) per g FW. Based on biomass production and activity of stress enzymes in relation to beans cultivated on reference soil, phytotoxicity classes (PI) were calculated (1 = not toxic, 4 = highly toxic) (Vangronsveld and Clijsters 1992). The reference soil used was an uncontaminated soil from a kitchen garden (Kolbas *et al.* 2011).

The plantox test using **lettuce** was executed at the Unité Mixte de Recherche BIOdiversité, GÊnes & Communautés (UMR BIOGECO, France) according to the standard procedure (ISO 17126:2005). The same uncontaminated reference soil as for the dwarf bean test was used. Three plastic pots of 0.65 L were filled with

each of the soil samples and placed in a greenhouse. Soils were fertilized with 100 mL of a modified Hoagland n°2 solution to avoid nutrient deficiencies, rehydrated up to their water holding capacity (WHC) by capillarity, and then maintained between 60 and 80% of WHC by daily irrigation with deionized water. One lettuce plantlet (cv. Novappia, 5th-6th leaf stage) was transplanted in each pot. After 48 days of growth, lettuce plant shoots were harvested, thoroughly washed in distilled water and oven-dried at 50ºC until constant weight to obtain dry weight (DW). Subsequently, plant shoots were grinded (<1 mm). Plant material (approx. 0.1 g) was digested in a 2:1 concentrated HNO3:HCl mixture on a hot plate at 130°C, and the concentration of Cd, Pb and Zn was measured by ICP-OES at the Instituto de Investigaciones Agrobiológicas de Galicia-Departamento de Bioquímica del Suelo/Consejo Superior de Investigaciones Científicas (IIAG-CSIC, Spain).

The **turnip** plantox test was performed following the standard procedure (ISO 11269-2:2012) by the Institut National de l'Environnement Industriel et des Risques (INERIS, France). The uncontaminated reference soil used for this test was the LUFA 2.2 soil, commercially provided by the German governmental institution Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer. Plastic pots, 4 replicates per condition, were filled with soil and 20 seeds per replicate were sown. Emergence of seeds was observed where after thinning to 5 shoots between day 4 and day 10 occurred.

After 18 days of growth, shoot production (DW) of turnip was evaluated after oven-drying shoots at 50 °C. The plant growth results were expressed as shoot DW per pot.

Ecotoxicity tests using invertebrates were also performed at INERIS and include the tiger worm (*Eisenia andrei*) avoidance test and nematode (*Caenorhabditis elegans*) growth and reproduction test. Both tests were executed following standard procedures (respectively ISO/CD 17512-1:2008 and ISO 10872:2010) with slight modification for soil testing in case of nematodes (Huguier *et al.* 2013). For the **worm** avoidance test, two-compartment vessels were used filled with either the artificial uncontaminated ISO reference soil and the willow-managed soil or the ISO reference soil and the unmanaged soil or the willow-managed soil and the unmanaged soil. Fifty worms (10 worms in 5 replicates)

were exposed and allowed to make the initial choice on which compartment to enter. After 48 h of exposure, the vessels were separated by inserting a divider and the number of worms were counted in every of the 2 compartments.

For the **nematode** test, individuals as well as the *Escherichia coli* strain OP50 were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, USA). The uncontaminated reference soil is again the LUFA soil. Stock cultures of *C. elegans* were maintained on nematode growth medium (NGM) agar plates spread with *E. coli* as food prior to nematode introduction. The stock culture was starved, resulting in the formation of dauer larvae, a dormant juvenile stage occurring when nutrients/food resources are low. Dauer larvae were transferred to agar plates spread with a fresh lawn of bacteria for obtaining synchronous adults. After 72 h at 20 ± 2 °C, these adults reproduced and the resulting age-synchronous first stage juveniles were used in the test. Of the juveniles, 30 individuals picked randomly, mean initial body length was determined using a binoculair microscope (15-fold magnification). Before the test started, soils were air-dried and then moistened to 80% of their own WHC with growth medium (M9 buffer), subtracting the food volume. The soils were then sealed and stored at 4 °C for 24 h to equilibrate before introducing the invertebrates. Of a 12-well plate, each well (25 mm diameter) was filled with 0.5 g of soil (DW). The food source (*E. coli* strain OP50) was added directly into the wells. Four replicates of 10 juvenile organisms per replicate were placed in the reference and tested soils. After 96 h of exposure, both adults and juveniles were extracted from the tested soils. The number of newly produced juveniles was recorded and presented as the number of offspring per adult. Growth of nematodes was calculated as final body length minus mean initial body length.

Statistical analyses

Statistical analyses were performed in R 3.1.3 (R Development Core Team, 2013). To differentiate between the unmanaged and willow-managed soil samples regarding measured physico-chemical soil characteristics, the Students t-test was applied. Significant differences in variances of the dwarf bean, lettuce, turnip and nematode test results were evaluated with ANOVA. Two-by-two comparisons were conducted using Tukey correction for multiple testing.

4.3 Results

4.3.1 Effect of 8 years of SRC on physico-chemical soil characteristics (including soil metal concentrations)

Willow-managed and unmanaged soil did not differ concerning soil texture and pH (water as well as potential acidity) (Table 4.1). The effective cation exchange capacity (CEC_e) tended to be slightly lower for the willow-managed soil compared to the unmanaged soil while electrical conductivity (EC) was significantly lower for the willow-managed soil. Pseudo-total Cd, Zn and Pb concentrations in soil and all EDTA-, NH_4NO_3-, $NaNO_3$-, $CaCl_2$- and water-extractable (exchangeable) concentrations tended to be lower for the willow-managed than for the unmanaged soil. Some differences were found to be significant ($p < 0.05$).

4.3.2 Effect of 8 years of SRC on estimated remediation time

The hypothetical remediation times needed to reduce pseudo-total soil metal concentrations of 13 mg Cd, 708 mg Zn and 316 mg Pb kg-1 DW soil (*i.e.* the concentrations measured in the unmanaged soil in this research, assigned 'very high contaminated soil' in Chapter 3, Table 3.9) to remediation thresholds (2 mg Cd, 282 mg Zn and 200 mg Pb kg-1 DW soil) were calculated based on Tora biomass analysis after the first 4 years of growth (Chapter 3: Table 3.1) and based on the observed decontamination after 8 years of Tora SRC (Table 4.1). Metal removal and hypothetical remediation time of the latter one was 48 times more efficient/shorter in case of Cd and 79 times in case of Zn compared to calculations based on 4 years of growth.

Table 4.1 Physico-chemical characteristics of the topsoil (0-30 cm) of unmanaged (under natural grassland vegetation on the field) and willow-managed (under SRC of Tora on the field) soil.

Soil sample	Soil texture	pH-H_2O	pH-KCl	CEC_e (meq 100 g^{-1})	EC (µS cm^{-1})	Pseudo-total metal concentrations (mg kg^{-1} dry soil)			$CaCl_2$-exchangeable metal concentrations (mg kg^{-1} dry soil)		
						Cd	Zn	Pb	Cd	Zn	Pb
Unmanaged	4% clay 8% silt 88% sand (Meers *et al.* 2007a)	6.27 ± 0.06 [a]	5.37 ± 0.10 [a]	5.72 ± 0.22 [a]	94.00 ± 10.73 [a]	12.63 ± 0.88 [a]	708.06 ± 79.34 [a]	316.15 ± 36.85 [a]	0.74 ± 0.12 [a]	42.21 ± 9.11 [a]	0.31 ± 0.07 [a]
Willow-managed		6.37 ± 0.03 [a]	5.49 ± 0.02 [a]	5.28 ± 0.53 [a]	69.30 ± 4.06 [b]	9.40 ± 0.53 [b]	548.38 ± 51.37 [a]	226.67 ± 17.53 [b]	0.52 ± 0.03 [a]	26.31 ± 3.24 [a]	0.20 ± 0.01 [a]

Soil sample	EDTA-exchangeable metal concentrations (mg kg^{-1} dry soil)			NH_4NO_3-exchangeable metal concentrations (mg kg^{-1} dry soil)			$NaNO_3$-exchangeable metal concentrations (mg kg^{-1} dry soil)			Water-exchangeable metal concentrations (mg kg^{-1} dry soil)		
	Cd	Zn	Pb	Cd	Zn	Pb	Cd	Zn	Pb	Cd	Zn	Pb
Unmanaged	6.97 ± 0.68 [a]	320.40 ± 51.40 [a]	157.49 ± 23.29 [a]	0.51 ± 0.10 [a]	43.48 ± 11.07 [a]	0.52 ± 0.12 [a]	0.08 ± 0.02 [a]	8.80 ± 3.02 [a]	0.14 ± 0.01 [a]	0.06 ± 0.01 [a]	6.35 ± 1.37 [a]	0.40 ± 0.14 [a]
Willow-managed	5.11 ± 0.31 [b]	217.89 ± 20.66 [a]	106.18 ± 10.45 [b]	0.36 ± 0.01 [a]	27.75 ± 2.85 [a]	0.39 ± 0.04 [a]	0.04 ± 0.00 [a]	4.33 ± 0.32 [a]	0.13 ± 0.01 [b]	0.04 ± 0.00 [b]	3.62 ± 0.52 [a]	0.28 ± 0.05 [a]

Values are mean ± standard deviation of 3 independent replicates. Different superscripts indicate significant differences at the level $p < 0.05$.

Table 4.2 Removal of Cd, Zn and Pb (g ha^{-1} yr^{-1}) and remediation time (years)* based on either analysis of Tora biomass after 4 years of growth (Chapter 3: Table 3.1) or based on observed metal concentrations in the topsoil (0-30 cm) after 8 years of willow (SRC of Tora) management (Table 4.1). *Time needed to reduce pseudo-total soil metal concentrations of 13 mg Cd, 708 mg Zn and 316 mg Pb kg^{-1} DW soil to remediation thresholds (2 mg Cd, 282 mg Zn and 200 mg Pb kg^{-1} DW soil). Assumptions made were: (i) Tora's extraction potential is independent of soil metal concentrations (contamination level), (ii) total soil metal content decreases linearly with time due to a constant yearly extraction or measured decreases in pseudo-total metal concentrations are due to uptake by Tora stems which is constant over the years, (iii) measured decreases in pseudo-total metal concentrations in the first 0.3 m of soil are valid for the first 0.5 m of soil, (iv) contamination and rooting depth are 0.5 m, and (v) the soil density is 1250 kg m^{-3}.

	Metal removal and remediation time based on biomass analysis of Tora after 4 years of growth		**Metal removal and remediation time based on observed decontamination after 8 years of Tora SRC management**	
	g ha^{-1} yr^{-1}	**years**	**g ha^{-1} yr^{-1}**	**years**
Cd	53 ± 42	1297 ± 1028	2523	27
Zn	1578 ± 1275	1687 ± 1363	124750	21
Pb	n.d.	n.d.	69906	10

Values are mean and mean ± standard deviation. n.d. = not detected.

4.3.3 Effect of 8 years of SRC on soil phytotoxicity: Ecotoxicity tests

Fresh weight of aboveground plant parts, primary leaves and roots as well as shoot length of dwarf beans cultivated on unmanaged and willow-managed soil revealed to be similar and (significantly) lower ($p < 0.05$) than results found for the uncontaminated reference soil (Table 4.3). Both unmanaged and willow-managed soils are classified as moderately toxic (PI = 3) in comparison with the reference soil, regarding these bean morphological parameters. Activity of stress enzymes in the leaves was also similar for beans grown on unmanaged and willow-managed soil while the reference soil grown beans revealed (significantly) lower ($p < 0.05$) activities of measured leaf stress enzymes. In the roots of beans cultivated on willow-managed soil, activity of stress enzymes was significantly lower ($p < 0.05$; found for ME) than in roots of unmanaged soil.

Moreover, the activities of root stress enzymes in willow-managed soil were even very similar to the ones found for the plants grown in the reference soil. The calculated PI based on the activities of all measured root enzymes reflected a non-toxic willow-managed soil and a slightly toxic unmanaged soil compared to the reference soil. The total PI (bean biomass production and enzyme activities), classified both unmanaged and willow-managed soil as slightly toxic (PI = 2). Lettuces grown on the uncontaminated reference soil produced more shoot DW and contained significantly less Cd and Zn than plants on contaminated soil (Table 4.4). Regarding willow-managed and unmanaged soil, although no significant differences were found (at the level $p < 0.05$), shoot DW of lettuce tended to be higher and Cd, Zn and Pb concentrations in shoots tended to be lower when grown on willow-managed soil. Turnip DW shoot production decreased in the order LUFA reference soil – willow-managed soil – unmanaged soil with significant differences ($p < 0.05$) between all of them (Table 4.5). Furthermore, emergence of turnip seeds tended to be higher in willow-managed soil compared to the unmanaged soil. The nematodes showed a significantly higher ($p < 0.05$) amount of offspring per adult and seemed to grow better in the willow-managed soil compared to the unmanaged soil (Table 4.5). Remarkably, the uncontaminated LUFA reference soil seemed to be slightly less suitable than the contaminated soils for nematode growth. Worms seemed to prefer the soil from the field (unmanaged and willow-managed) over the uncontaminated ISO reference soil and this preference was more prominent for the willow-managed soil than the unmanaged soil (Figure 4.2, first 2 paired bars on the left). In the worms avoidance experiment conducted with both contaminated soils, worms tended to have a slight preference for the willow-managed soil (Figure 4.2, right pair of bars).

Table 4.4 DW shoot production (g) and metal content (mg kg^{-1} DW) in the shoots of lettuce grown in pots on uncontaminated reference (kitchen garden), unmanaged (under natural grassland vegetation on the field) and willow-managed (under SRC of Tora on the field) soil.

Soil sample	DW shoots (g)	Shoot metal content (mg kg^{-1} DW)		
		$[Cd]_{shoot}$	$[Zn]_{shoot}$	$[Pb]_{shoot}$
Reference (kitchen garden)	4.75 ± 0.21 [a]	0.08 ± 0.00 [a]	20.29 ± 1.93 [a]	0.91 ± 0.68 [a]
Unmanaged	2.68 ± 0.89 [b]	19.76 ± 5.07 [b]	523.04 ± 263.85 [b]	1.43 ± 1.24 [a]
Willow-managed	3.54 ± 0.36 [a,b]	18.05 ± 0.89 [b]	312.43 ± 57.15 [b]	0.75 ± 0.51 [a]

Values are mean ± standard deviation of 3 biological replicates. Different superscripts indicate significant differences at the level $p < 0.05$.

Table 4.5 Results of the turnip and nematodes ecotoxicity tests of uncontaminated reference (LUFA), unmanaged (under natural grassland vegetation on the field) and willow-managed (under SRC of Tora on the field) soil.

Soil sample	Turnip test		Nematode test	
	% of seed emergence	DW shoots (mg)	No. of offspring $adult^{-1}$	Growth of nematodes (µm)
Reference (LUFA)	n.d.	32.59 ± 3.37 [a]	85.48 ± 14.10 [a,b]	828.38 ± 98.56 [a]
Unmanaged	68.75 ± 16.00 [a]	13.76 ± 2.25 [b]	63.73 ± 26.01 [a]	876.04 ± 94.59 [a]
Willow-managed	85.00 ± 4.10 [a]	21.30 ± 2.80 [c]	112.90 ± 16.36 [b]	943.19 ± 50.15 [a]

Values are mean ± standard deviation of 4 technical replicates. n.d. = not detected. Different superscripts indicate significant differences at the level $p < 0.05$.

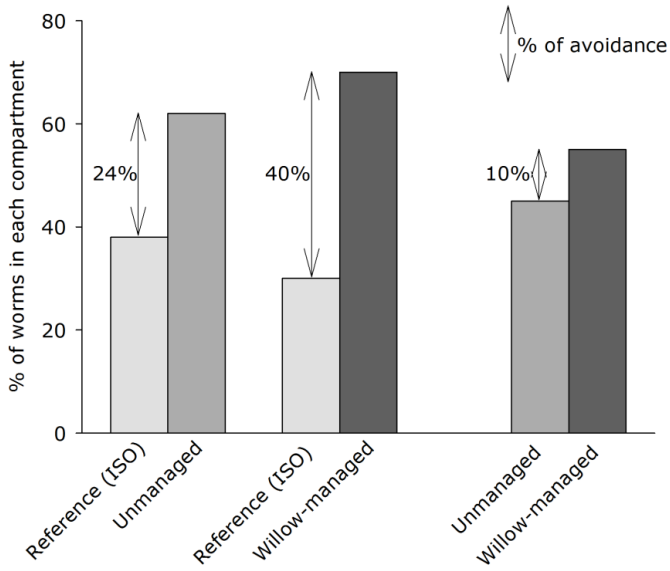


Figure 4.2 Results of the worms avoidance test (sum of 5 technical repetitions). Bars present the percentage of worms in each compartment, arrows define the percentage of avoidance from the soil sample below the arrow compared to the neighbouring soil sample. The first 2 paired bars on the left show the results of the worms avoidance test of uncontaminated reference (ISO) soil versus field soil (unmanaged and willow-managed). The right pair of bars displays the result of the worms avoidance test with both field soils.

Table 4.3 Fresh weight production (FW in g), activities of stress enzymes in leaves and roots (mU g^{-1} FW) and corresponding phytotoxicity indices (PI) of dwarf beans grown in pots on uncontaminated reference (kitchen garden), unmanaged (under natural grassland vegetation on the field) and willow-managed (under SRC of Tora on the field) soil. Total PI reflects the PI of bean morphological parameters together with enzyme activities in leaves and roots.

BIOMASS PRODUCTION	**FW aboveground biomass (g) [PI]**	**FW primary leaves (g) [PI]**	**FW roots (g) [PI]**	**Shoot length (cm) [PI]**	**Morpholo-gical PI**	
Reference (kitchen garden)	1.640 ± 0.064 ^{a} [1]	0.920 ± 0.034 ^{a} [1]	0.866 ± 0.074 ^{a} [1]	11.8 ± 2.5 ^{a} [1]	1	
Unmanaged	1.143 ± 0.114 ^{b} [3]	0.614 ± 0.086 ^{b} [3]	0.614 ± 0.103 ^{b} [2]	6.6 ± 0.1 ^{b} [3]	3	
Willow-managed	0.987 ± 0.067 ^{b} [3]	0.532 ± 0.033 ^{b} [3]	0.716 ± 0.055 ^{a,b} [2]	6.5 ± 0.6 ^{b} [3]	3	
ENZYME ACTIVITIES IN LEAVES	**GPOD (mU g^{-1} FW) [PI]**	**ICDH (mU g^{-1} FW) [PI]**	**ME (mU g^{-1} FW) [PI]**		**Leaf enzyme activities PI**	**Total PI**
Reference (kitchen garden)	410 ± 45 ^{a} [1]	611 ± 79 ^{a} [1]	455 ± 43 ^{a} [1]		1	1
Unmanaged	1347 ± 394 ^{b} [3]	737 ± 112 ^{a,b} [1]	771 ± 116 ^{b} [2]		2	2
Willow-managed	1103 ± 19 ^{b} [2]	801 ± 35 ^{b} [2]	782 ± 63 ^{b} [2]		2	2
ENZYME ACTIVITIES IN ROOTS	**GPOD (mU g^{-1} FW) [PI]**	**GIDH (mU g^{-1} FW) [PI]**	**ME (mU g^{-1} FW) [PI]**		**Root enzyme activities PI**	
Reference (kitchen garden)	7449 ± 1084 ^{a} [1]	281 ± 54 ^{a} [1]	434 ± 47 ^{a} [1]		1	
Unmanaged	11339 ± 1677 ^{a} [2]	335 ± 34 ^{a} [1]	715 ± 50 ^{b} [2]		2	
Willow-managed	7338 ± 2810 ^{a} [1]	269 ± 32 ^{a} [1]	521 ± 86 ^{a} [1]		1	

Values are mean ± standard deviation of 3 biological replicates. PI = phytotoxicity index; 1: not toxic, 2: slightly toxic, 3: moderately toxic. GPOD = guaiacol peroxidase, ICDH = isocitrate dehydrogenase, ME = malic enzyme, GIDH = glutamate dehydrogenase. Different superscripts indicate significant differences at the level $p < 0.05$.

4.4 Discussion

In this research, the longer-term effects of metal phytoextraction using SRC of Tora on soil metal contents and soil toxicity were investigated. Soil was sampled on a Cd-Zn-Pb-contaminated field under an 8-year old SRC culture of Tora (willow-managed soil) and under an adjacent, natural grassland vegetation (unmanaged soil). Eight growing seasons of Tora obviously reduced the amounts of total Cd, Zn and Pb in the soil given the unmanaged soil is the reference (Table 4.1). Based on these reductions, hypothetical remediation times to remediate the soil to legal threshold values shortened tremendously compared with time frames estimated combining stem biomass production and stem metal content of Tora after 4 growing seasons (Chapter 3) (Table 4.2). In both approaches, the remediation times were calculated using a simple linear model. This model has some shortcomings, as described in Chapter 3 ('Discussion' section). In addition, it should be mentioned that there is a growing interest in estimating remediation times based on extractable (*e.g.* $NaNO_3$-extractable (Herzig *et al.* 2014)) instead of pseudo-total soil metal concentrations. The labile metal pool is considered to be more closely related to soil toxicity and associated risks. While in the calculations in this research the labile ('bioavailable') fraction is assumed to be constant over time, there is a lot of uncertainty regarding the replenishment of this labile metal pool by the other soil fractions and more accurate equilibrium models are being developed. Nevertheless, for comparative studies like in this research, the application of a simple, linear model can be justified. However, independent of this model, the remediation times needed based on estimated metal removal of Tora after 4 growing seasons could be considered an overestimation. Two reasons are given to this concern. (1) Since during the first growing seasons a SRC tree will allocate a lot of energy for the establishment of the root system, biomass yields obtained in these first years tend to be lower than yields from later cutting cycles (Hofmann-Schielle *et al.* 1999; Labrecque and Teodorescu 2003; Aronsson *et al.* 2014; Van Slycken *et al.* 2015). Indeed, it was reported earlier (Chapter 3: 'Discussion' section) that the yield of Tora more than doubled during the second growing cycle compared to the first 4 years of growth (5401 ± 3791 *vs.* 2500 ± 2000 kg ha^{-1} yr^{-1} (Table

3.1)). (2) The biomass analyzed in Chapter 3 originated from Tora trees growing in different plots than the Tora in this research and there might be a lower stem metal concentration in the biomass of the former linked to a lower level of soil contamination (6.50 mg Cd and 359.00 mg Zn kg^{-1} DW soil (Table 3.8) *vs.* Table 4.1 for Tora in this research). In turn, the remediation times calculated based on the observed decontamination in the soil after 8 years of Tora cultivation might be underestimations. The difference in pseudo-total metal content between willow-managed and unmanaged soil is assumed to be entirely accumulated in the Tora stems and removed by harvest. However, an unknown amount of the observed metal losses is accumulated in the Tora root system and also present in the leaf litter that was not yet decomposed (sampling was done in early winter). In addition, a part of the metal losses might be explained by possible higher metal leaching in the willow-managed compared to the unmanaged soil. However, the extractable and pseudo-total metal concentrations in unmanaged and willow-managed soil (ratio extractable Cd or Zn to pseudo-total Cd or Zn is smaller for willow-managed compared to unmanaged soil and pH and CEC do not differ between both soils) do not support this event. Nevertheless, even taken into account over- and underestimations are likely to occur, the differences of a factor 48 (Cd removal) and 79 (Zn removal) cannot be explained. Therefore, this exploratory study might indicate a much more effective phytoextraction by 8 years of willow-management than predicted based on the results of biomass analysis after 4 years.

Considering the main risks and toxicity of a metal-contaminated soil to be related to bioavailable metal concentrations, the results found with the chemical extractions (EDTA, NH_4NO_3, $NaNO_3$, $CaCl_2$ and water) indicate the willow-managed soil to be less toxic than the unmanaged soil (Table 4.1). A lower toxicity was also reported earlier by Xue *et al.* (2015) who determined available (EDTA-extractable and NH_4NO_3-exchangeable) Cd, Zn and Pb after 6 years of Tora SRC management on the field in Lommel (the same sampling locations as studied here after 8 years). Dwarf bean morphological parameters as well as activities of stress enzymes in the leaves did not allow to distinguish between unmanaged and willow-managed soil and categorized both as moderately (morphological) and slightly (leaf enzymes) toxic compared to the

uncontaminated reference soil used (Table 4.3). Also the high leaf GPOD activities on willow-managed and unmanaged soil indicate phytotoxicity (Mench *et al.* 2000). However, activities of stress enzymes in the roots reflected a reduced toxicity after 8 years of willow-management versus no management. The unmanaged soil was classified as slightly toxic while the willow-managed soil, with stress enzyme activities in roots comparable to levels found on reference soil, was classified as not toxic. Furthermore, all parameters measured in the lettuce, turnip and nematode tests, as well as all 3 parts of the worm avoidance test, showed slight up to significant indications of reduced toxicity after willow management compared to no management (Tables 4.4 and 4.5, Figure 4.2). The lower number of offspring and growth of nematodes in LUFA reference versus willow-managed and/or unmanaged field soil and the lower attraction of worms to the ISO reference soil compared to the field soils might be the result of other soil characteristics preferred by the invertebrates. In case of the nematodes, this observation was also reported by Kumpiene *et al.* (2014). However, outcomes of the test on unmanaged versus willow-managed soil, exposing similar soil features, are still valid to this concern.

The EC can be used to quantify the concentration of soluble salts (*e.g.* Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-}, SO_4^{2-}, HCO^{3-}, K^{+}, NH_4^{+}, NO_3^{-}, CO_3^{2-}) in soils and varies depending on the amount of moisture held by soil particles, correlating strongly to particle size and texture (Gartley 1995). The EC decreased obviously after Tora management compared to no management and since both samples had the same particle size (texture) and moisture content (at the moment of measurement), the decrease very likely reflects a decrease in soluble salts/nutrients by cultivating and harvesting SRC of willow in comparison with no management (natural grass vegetation). However, Xue *et al.* 2015 concluded that Tora phytoextraction might have long-term beneficial effects on soil fertility and ecosystem services in metal-contaminated soils. Their research on functional gene richness and diversity on the same soils but after only 6 years of phytoextraction management, revealed both parameters were higher in willow-managed soil than in unmanaged soil. The SRC management also increased soil microbial biomass, soil respiration and all measured soil enzyme activities compared to no management. In addition, a study by Touceda-González *et al.*

(in preparation) regarding microbial community structure and activity in (phyto)managed trace element (TE) contaminated soils, including willow-managed and unmanaged soil from the field in Lommel, showed that biological fertility of TE-contaminated soils can be improved by phytomanagement compared to no management.

Conclusion

The effective decontamination and changes in soil toxicity resulting from 8 years of phytoextraction using SRC of Tora on a Cd-Zn-Pb-contaminated soil were determined in this research. The observed decontamination might indicate a much more effective phytoextraction by 8 years of willow-management than predicted based on the results of biomass analysis after 4 years. Resulting remediation times for severely contaminated soil, even if this phytoextraction efficiency is an overestimation, become much more reasonable. Further investigating soil decontamination by SRC clones in the third rotation cycle is highly recommended.

By estimating external and internal bioavailability of metals, using respectively standardized chemical extractions and ecotoxicity assays (plant- and invertebrate-based), a lower toxicity of the willow-managed soil compared to the unmanaged soil was observed. In addition, evidence for improved biological fertility and functionality was provided for willow-managed soil on the metal-contaminated field.

Taken together, the results all emphasize the environmental benefits (soil remediation, reduced toxicity and leaching risks, improved soil quality) of a SRC-phytoextraction-managed soil compared to no management. Further evaluating the benefits of longer-term metal phytoextraction might eventually deliver an important contribution for realizing large-scale applications of this remediation technology.

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Chapter 5

Improving phytoextraction of Cd-Zn-Pb-contaminated soil using SRC of willow: clone selection, bioaugmentation with beneficial plant-associated bacteria and fertilization

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Abstract

Short rotation coppice (SRC) revealed to be suitable for implementation of metal phytoextraction in the metal-contaminated area in the northeast of Belgium. However, improving biomass production and/or metal accumulation of SRC to reduce remediation times and/or increase potential economic revenues, are highly desired. In this research, 3 strategies are proposed to meet this concern: clone selection, bioaugmentation with plant-associated bacteria and fertilization.

Since different species and clones show considerable variations in biomass productivity levels and stem metal concentrations, a first strategy to improve SRC metal phytoextraction is the *in situ* selection of promising clones. *In situ* selection allows examining the suitability of different clones taking into account prevailing environmental/climatic conditions and the specific characteristics of the soil. A second approach to improve biomass production and/or metal accumulation of SRC is the exploitation of plant-associated bacteria. Plant-associated bacteria have the capability to promote plant growth, increase metal uptake and translocation to aboveground plant parts as well as to reduce harmful effects of metal phytotoxicity. Finally, fertilizer applications, implemented all over the world to increase biomass productivity levels of commercial crops, are evaluated as third strategy to improve SRC metal phytoextraction.

The *in situ* evaluation of all SRC clones led to the selection of the 'experimental' willow clones *Salix viminalis* and *Salix alba x alba* which expose respectively second highest Cd and Zn concentrations in the stem and highest stem biomass production. Bioaugmentation of the previously selected willow clones, evaluated in pot trials with up to 17 promising bacterial strains, did not result in improved biomass yields nor enhanced metal accumulation or translocation. The fertilizer applications on the contrary, also applied to the former selected willow clones growing in pots, raised productivity levels significantly and in case of *S. alba x alba*, *in planta* metal concentrations also increased. As a result, the tested fertilizers doubled (*S. viminalis*) or even tripled (*S. alba x alba*) phytoextraction efficiency of the selected clones.

In conclusion, *in situ* selection should be the first step in the process to improve SRC metal phytoextraction. Bioaugmentation to enhance metal phytoextraction was repeatedly reported to be successful but it could not be confirmed in this research for the selected willow clones and selected bacterial strains. Further investigating proper phenotypic selection criteria and mechanisms as well as the extent to which colonization is accomplished can reveal more about bacteria-enhanced phytoextraction of willow cuttings. The effect of fertilizer applications on biomass production as well as metal accumulation of selected clones is highly promising and further research on field scale is strongly recommended.

PART I: Clone selection

5.I.1 Introduction

Salix and *Populus* have been identified as genera that tend to accumulate high concentrations of more mobile elements (Landberg and Greger 1996; Perttu and Kowalik 1997; Robinson *et al.* 2000; Di Baccio *et al.* 2003; Giachetti and Sebastiani 2006; Dickinson *et al.* 2009). However, high levels of variability in metal tolerance and uptake occur within plant families or within a single genus as well as between different populations and cultivars of the same species. Naturally occurring variability in metal accumulation is very likely related to gene expressions although also associations with biomass production, water use and root microflora might exist (Granel *et al.* 2002). In case of willow, obvious differences in Cd uptake were observed between cultivars by Landberg and Greger (1996, 2002), Granel *et al.* (2002), Mleczek *et al.* (2010), Ruttens *et al.* (2011) and Van Slycken *et al.* (2013). Literature on accumulation of metals in poplar is less extensive but Laureysens *et al.* (2004b) reported clonal variations for the uptake of various metals. Besides differences in metal accumulation, also biomass production might vary considerably between clones. Biomass productivity levels of SRC depend on site-specific conditions, climatic conditions, plant spacing and management but also on clonal selection. Significant clonal differences in biomass production were found for willow and poplar on metal-contaminated soil (Laureysens *et al.* 2004a; Ruttens *et al.* 2011; Van Slycken *et al.* 2013). Since clonal variations exist for *Salix* and *Populus* regarding metal accumulation and biomass production, the extraction potential can vary remarkably from one clone to another.

The field trial in the Lommel (Belgium), with more than 100 poplar and willow clones planted (commercially available and experimental crossing types), offers the opportunity to *in situ* select best performing clones in terms of phytoextraction capacity. The identification of clones with naturally occurring high biomass production and/or stem metal concentrations *in situ* is considered a first and indispensible step to improve phytoextraction efficiency in this area.

5.I.2 Material and methods

More than 100 different poplar and willow clones were tested for phytoextraction purposes on a field trial in Lommel, northeast of Belgium. The field site, the establishment of the field and maintenance actions are described in Chapter 3, 'Material and methods' section. Clones evaluated are commercially available poplar and willow cultivars (further referred to as 'commercial' clones) as well as experimental crossing types (further referred to as 'experimental' clones). The latter ones were developed by the Institute for Nature and Forest Research (INBO) in order to evaluate their growth and remediation potential on Cd and Zn contaminated soils (Meiresonne, unpublished results). Evaluated families and crossings of experimental poplar and willow clones can be found in Chapter 3, 'Material and methods' section: Plant material and planting.

Methods for determination of biomass production, metal concentrations in the stem and extraction potentials are also described in 3, 'Material and methods' section: Biomass production, *in planta* metal concentrations and extraction potential.

The bioconcentration factor (BCF), defined here as the ratio of metal concentration in wood to total soil metal content, directly allows to compare extraction efficiencies of different clones even on different levels of contamination. The BCFs of Cd and Zn were calculated for clones with known or determined soil contamination levels. In case soil metal concentrations had to be determined, 3 topsoil samples (0-30 cm) were taken under natural grass vegetation (mainly *Agrostis capillaris* and *Holcus lanatus*) next to the cultivation plot. Litter and vegetation cover were removed before sampling. Soil samples were oven-dried and sieved (< 2 mm). Pseudo-total metal (Cd, Zn and Pb) concentrations of the soil samples were estimated by *aqua regia* digestion (Van Ranst *et al.* 1999) and analysis was subsequently performed using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies 700 Series).

5.I.3 Results

On the field in Lommel, after 4 growing seasons, poplar clones did show higher mortality rates, higher appearance of rust symptoms and chlorosis, more damage by rabbits and a lower uniformity of growth within a clone (Meiresonne, unpublished results). This explains why finally only willows remained in the selection (Table 5.1). Of interest is the performance of the experimental clones (designed by INBO for Cd and Zn remediation) compared to the commercially available and frequently tested clones. Measuring biomass production and metal content of most promising (based on phenotypic appearances) experimental crossing types revealed that interesting clones could be found within these collections (Table 5.1; results for individual experimental clones not shown). Combining stem biomass production and Cd and Zn concentrations in the stem, but also taking into account uniformity of growth and mortality rate, 2 experimental clones were selected. The first clone, a *Salix viminalis* clone, V, member of the experimental *S. viminalis* collection {V}, exhibited the second highest combined Cd and Zn concentration in the stem of all evaluated clones. Commercial willow Loden showed a higher Cd and Zn stem concentration, however, the selected *S. viminalis* clone had potential to improve Cd and Zn extraction with respectively 7% and 21% compared to Loden because of the higher biomass production. The second clone, a *Salix alba x alba*, A x A, member of the experimental *S. alba* collection {A}, revealed the highest stem productivity of all evaluated clones, improving stem biomass production of second best performing clone (commercial willow Zwarte Driebast) with 4%. Furthermore, stem Cd accumulation of the elected *S. alba x alba* was highest of all clones in the {A} collection.

The bioconcentration factor (BCF) of Cd and Zn for the selected *S. viminalis* clone was higher (Cd) or comparable (Zn) to values observed for best performing commercial clones (Table 5.2). In case of *S. alba x alba*, the BCF of both Cd and Zn was rather low compared to BCFs for commercial clones with only Belders exposing even lower BCFs.

Table 5.1 Stem biomass production (kg DW ha^{-1} yr^{-1}), Cd and Zn concentrations in the stem (mg kg^{-1} DW) and Cd and Zn extraction potential (g ha^{-1} yr^{-1}) after 4 growing seasons of commercial, most promising and selected experimental willow clones planted on the field in Lommel in 2006 (planting distance 15 000 plants ha^{-1}).

Clone type	Clone (n° of most promising)	Crossing type	Stem biomass (kg DW ha^{-1} yr^{-1})	$[Cd]_{stem}$ (mg kg^{-1} DW)	$[Zn]_{stem}$ (mg kg^{-1} DW)	Cd extraction potential (g ha^{-1} yr^{-1})	Zn extraction potential (g ha^{-1} yr^{-1})
Commercial	Belders	A	1775 ± 1150	8 ± 3	291 ± 128	14 ± 11	517 ± 404
	Belgisch Rood	F	n.d.	n.d.	n.d.	n.d.	n.d.
	Christina	V x V	1675 ± 1500	21 ± 6	640 ± 60	35 ± 33	1072 ± 965
	Inger	Tr x V	1400 ± 875	22 ± 1	844 ± 65	31 ± 19	1182 ± 744
	Jorr	V	1950 ± 1200	18 ± 4	642 ± 153	35 ± 23	1252 ± 826
	Loden	Da	4250 ± 2250	28 ± 13	682 ± 160	119 ± 84	2899 ± 1678
	Tora	S x V	2500 ± 2000	21 ± 1	631 ± 72	53 ± 42	1578 ± 1275
	Zwarte Driebast	Tr	12250 ± 7000	17 ± 3	414 ± 59	208 ± 125	5072 ± 2987
Experimental	(14-21)	{A}	3118-12765	3-12	138-407	14-158	602-4391
	(9)	{V}	3626-7663	14-26	380-769	72-148	1617-4085
	(5-6)	{V x V}	2489-4765	18-29	501-724	55-107	1563-3214
Selected clones (experimental)	***S. viminalis***	**V ({V})**	**5179**	**25**	**678**	**127**	**3511**
	S. alba x alba	**A x A ({A})**	**12765**	**12**	**344**	**158**	**4391**

A: *Salix alba;* Da: *Salix dasyclados* ; F: *Salix fragilis;* S: *Salix schwerinnii;* Tr: *Salix triandra* ; V: *Salix viminalis*; {}: collection of all experimental clones with this crossing type (see Chapter 3 for details).

Values are mean ± standard deviation or min-max. n.d.: not detected. Stem biomass production and Cd and Zn concentrations in the stems of commercial willow clones were calculated from data published in Van Slycken *et al.* (2013). Data of experimental clones were derived from Van Slycken *et al.* (2015). Extraction potentials were calculated based on the abstracted data.

Table 5.2 Mean bioconcentration factors (BCF) of Cd and Zn for commercial willow clones and selected experimental willow clones evaluated on the experimental field after 4 growing seasons. The BCF is defined as the ratio of metal concentration in stems to total soil metal content. In case of commercial willow clones, BCFs are copied from Table 3.8. For the selected *S. viminalis* and the *S. alba x alba* clone, BCFs are based on stem metal concentrations (Table 5.1) and mean pseudo-total metal concentrations determined in the soil of their plots on the field (in case of *S. viminalis* measured pseudo-total soil concentrations were 5.12 ± 0.07 mg Cd and 372.10 ± 8.41 mg Zn kg^{-1} DW soil, for *S. alba x alba* these were 4.78 ± 0.22 mg Cd and 409.21 ± 7.64 mg Zn kg^{-1} DW soil).

		Pseudo-total soil metal concentrations (mg kg^{-1} dry soil)		**BCF**	
Clone type	**Clone/mutant**	**Cd**	**Zn**	**Cd**	**Zn**
Commercial clones	**Belders**	7.50	450.00	1.07	0.65
	Christina	6.70	370.00	3.13	1.73
	Inger	6.70	430.00	3.28	1.96
	Jorr	6.30	357.00	2.86	1.80
	Loden	6.30	377.00	4.44	1.81
	Tora	6.50	359.00	3.23	1.76
	Zwarte Driebast	5.50	299.00	3.09	1.38
Selected clones	***S. viminalis***	5.12	372.10	4.88	1.82
(experimental)	***S. alba x alba***	4.78	409.21	2.51	0.84

5.I.4 Discussion

To improve metal phytoextraction by SRC, a first strategy is to select species and clones with an *in situ* high biomass productivity level and/or metal accumulation. *In situ* selection allows examining the suitability of different clones taking into account prevailing environmental/climatic conditions and the specific characteristics of the soil. The supremacy of willow over poplar on the field in Lommel, which was also mentioned earlier by Ruttens *et al.* (2011), eventually led to a selection only based on the willows planted (Table 5.1). The selected experimental clone *Salix viminalis* (V) shows second highest combined Cd and Zn concentrations in the stem of all evaluated clones but improves stem metal extractions (up to 7% for Cd and 21% for Zn) compared to the best performing clone (commercial willow Loden) due to a higher biomass production.

Furthermore, the high bioconcentration of Cd and Zn observed for the selected *S. viminalis* clone (highest BCF of all evaluated clones in case of Cd) indicate a very efficient extraction by this clone (Table 5.2). The selected experimental clone *Salix alba x alba* (A x A) exposed highest stem biomass production of all evaluated clones (up to 4% higher than the second best performing clone Zwarte Driebast) (Table 5.1). In addition, both selected clones looked healthy, exhibited a uniform growth and a low mortality rate after 4 growing seasons.

PART II: Bioaugmentation with beneficial plant-associated bacteria

5.II.1 Introduction

A second strategy to improve metal phytoextraction using SRC of willow is the exploitation of their plant-associated bacteria. Plant-associated bacteria include rhizospheric (living in the direct vicinity of the roots), endophytic (colonizing internal plant tissues) and phyllospheric (living in the external regions of the aboveground plant parts) bacteria. Numerous studies (Lebeau *et al.* 2008; Braud *et al.* 2009; Rajkumar *et al.* 2009; Weyens *et al.* 2009a,c; Glick 2010) have already demonstrated that plant-associated bacteria have the capability to promote plant growth, increase metal uptake and translocation to aboveground plant parts as well as reduce harmful effects of metal phytotoxicity.

Bacteria have the ability to increase plant yield and health through a number of different mechanisms, categorized as direct and indirect (Lodewyckx *et al.* 2002). Direct plant growth promotion can be summarized in 3 groups of mechanisms: (1) Bacteria can bio-fertilize the plant by mobilization and provision of nutrients (Vessey 2003). For example, nitrogen (N_2-) fixation is known to occur in rhizosphere and endophyte bacterial species (Dos Santos *et al.* 2012) and these diazothrophs might help the plant to fulfill its nitrogen demand. Bacteria might also facilitate the plant uptake of essential but sparingly available phosphorus (P) by solubilization of inorganic P or mineralization of organic P (Rodríguez *et al.* 2006; Richardson *et al.* 2009). Furthermore, plants can benefit from bacteria that excrete siderophores, low-molecular-weight iron (FeIII)-chelating molecules. The siderophores bind the low soluble Fe-oxides and the resulting ferric-siderphore complexes can be recognized and taken up by the plant (Vessey 2003). (2) Bacteria can produce regulators of plant growth and development (called phytohormones) or modulate plant phytohormone levels (Glick 2010). Many plant-associated bacteria can synthetize one or more phytohormones such as auxins, cytokinins and gibberelines. The most studied member of the auxin family is indole-3-acetic acid (IAA) (Spaepen and Vanderleyden 2011). The stimulatory effect of auxin production has been

associated with enhanced root proliferation resulting in higher total root surface, which leads to better nutrient and water acquisition by the plant and subsequently increased biomass. (3) Bacteria can abate negative effects of stress on plant growth (Glick *et al.* 2007). Under various stresses, the ethylene biosynthesis is induced and higher than normal ethylene levels inhibit root elongation and plant growth. Some bacteria can lower the ethylene levels in the plant by breaking down the ethylene precursor 1-aminocylcopropane-1-carboxylate (ACC) by the production of the enzyme 1-aminocylcopropane-1-carboxylate (ACC) deaminase. Indirectly, plant growth can be promoted by inhibiting growth and activity of plant pathogens or pests. This inhibition can be attributed to a variety of mechanisms: (i) Outcompeting pathogens by competition for space and nutrients. Beneficial bacteria can capitalize on nutrients and spaces limiting the availability to other microbes and pathogens thereby suppressing their growth (Hibbing *et al.* 2010). In some cases also a physical barrier to pathogens is created (*e.g.* by biofilm formation) (Ramey *et al.* 2004). (ii) Direct antagonism towards pathogens by the production of biocontrol agents such as antibiotics and antifungal metabolites (*i.e.* lytic enzymes) (Kobayashi *et al.* 2005). (iii) Activation of induced systemic resistance (ISR) (Heil and Bostock 2002; Compant *et al.* 2005; Kloepper and Ryu 2006). ISR is a state of the plant whereby previous contact with an induction agent makes the plant not only locally but systemically more able to resist subsequent pathogen attack. The capacity of priming the innate immune system of the plant has been described for a number of beneficial bacteria.

Besides promotion of plant growth, plant-associated bacteria can be exploited for increasing metal bioavailability in soil and enhancing metal uptake by plants. Sorbed, precipitated and occluded metals can be solubilized by acidification and redox-changes or through chelation and ligand-induced dissolution (Gadd 2004; Sessitsch *et al.* 2013). The process is primarily assigned to rhizosphere bacteria and root endophytes producing natural chelators such as carboxylic acid anions and siderophores, or organic acids. Siderophores are indeed reported to chelate various other cations than iron improving the uptake of metals by plants (Braud *et al.* 2009; Rajkumar *et al.* 2010). Organic acids in turn may dissociate their protons in the soil solution to form negatively charged ligands capable of

complexing with metallic cations increasing their bioavailability and uptake (Lombnaes *et al.* 2008).

However, increased concentrations of metals in the plant may lead to toxicity. To deal with this phytotoxicity, endophytic bacteria able to sequester metals intra- or extracellular or precipitate, chelate or bind metals to exopolymers are of special interest (Bruins *et al.* 2000; Lodewyckx *et al.* 2001; Sessitsch and Puschenreiter 2008; Haferburg and Kothe 2010). Focusing on the remediation of Cd-contaminated soils, the CZC and CZR efflux mechanisms are of special interest since they allow Cd ions to precipitate onto the bacterial cell wall (Diels *et al.* 1995; Nies 1995; van der Lelie *et al.* 1999). Endophytes equipped with metal resistance/sequestration systems and eventually able to produce natural metal chelating compounds may contribute to metal detoxification in plants, lowering phytotoxicity, but have also potential to increase metal translocation to aerial plant parts (Lodewyckx *et al.* 2001).

Besides bacteria-induced changes in biomass- and metal-related issues as an individual result of excreted compounds or mechanisms described above, a complex interaction between different mechanisms/products might cause other/stronger/weaker alterations. For example, the production of ACC deaminase or IAA might directly or indirectly also be involved in the uptake of metals (López *et al.* 2005; Zaidi *et al.* 2006). Similarly, interference with plant growth hormones resulting in a faster growth whereby escaping pathogens could be considered another mechanism of indirect biocontrol. Furthermore, the activity of plant-associated bacteria is in tight equilibrium with the plant's activity (Kidd *et al.* 2008). This has the advantage that nutrients made available don't get lost but also that the leaching of mobilized metals is prevented. Finally, Sessitsch and Pushenreiter (2008) demonstrated that bacteria with the above-mentioned characteristics/mechanisms are frequently naturally present in plants growing on metal-contaminated sites.

In this part, the bacterial populations associated with the previously selected willow clones (PART I), *S. viminalis* and *S. alba x alba*, growing on the metal-contaminated field in Lommel, are thoroughly characterized in order to select the most promising strains. The genotypic identification is additionally used to gain more insight in the structures of the cultivable plant-associated

communities of both clones while the phenotypic characterization was also used to create a general overview of the traits present in the communities. The effect of inoculating promising strains on biomass production and metal uptake of the selected clones was evaluated in pot experiments using metal-contaminated soil from the field in Lommel as a substrate. The different pot trials were furthermore used to evaluate the influence of certain experimental conditions on twig biomass production and metal uptake and translocation to twigs. Finally, recurring patterns (biomass- and metal-related) observed when growing both selected clones in pots as well as the (dis)similarities between pot results and field data from PART I were discussed.

5.II.2 Material and methods

5.II.2.1 Sampling, isolation and characterization of bacterial strains

Sampling and isolation of bacterial strains

Since the final aim of this study is to use the most promising bacteria for inoculation experiments, only the cultivable bacterial population was investigated. In order to isolate the cultivable bacterial strains associated with the selected clones, rhizosphere soil (in this case the soil directly adhering to the roots), roots and twigs were sampled from willow clones growing on the field in Lommel (block 2, see Chapter 3: Figure 3.1) in October 2010. Three representative, healthy trees were chosen of which samples were collected and combined together to obtain 3 mixed samples. Rhizosphere soil and roots were sampled simultaneously by cutting roots (and the adhering soil) at a depth of 20-30 cm using sterilized equipment and storing roots and soil in 20 mL sterile 10 mM $MgSO_4$ solution. Twig samples of the 3 trees were clustered and transferred in open air.

Rhizosphere soil and root samples were vortexed where after the roots were removed using sterilized equipment. In order to isolate cultivable bacterial strains from the rhizosphere soil, 100 µL of serial dilutions up to 10^{-5} in 10 mM $MgSO_4$ solution were plated on 1/10 strength 869 solid medium (Mergeay *et al.* 1985). To isolate cultivable endophytes, roots and twigs were surface-sterilized

for respectively 1.5 and 2.5 min in a 1% active chloride solution supplemented with one droplet Tween 80 (Merck) per 100 mL solution, and were subsequently rinsed three times in sterile distilled water. The third rinsing solution was plated on 869 medium to check surface sterility (if no growth was observed after 7 days, surface sterilization was considered to be successful). Surface-sterilized root and twig samples were fine-cut using sterilized equipment, transferred to 10 mL sterile 10 mM $MgSO_4$ solution and weighted. Thereafter, both samples were macerated during 1 min (roots) or 2 min (twigs) using a Polytron PR1200 mixer (Kinematica A6). Serial dilutions up to 10^{-4} (roots) and 10^{-3} (twigs) were made and 100 µL of each was plated on 1/10 strength 869 solid media and incubated for 7 days at 30°C. After incubation of the plates, colony forming units were separated into morphologically identical groups, counted and calculated per gram soil or fresh plant weight. Of each morphotype, 1 to 10 replicates (depending on abundance) were purified 3 times and stored in a glycerol solution (15% (w:v) glycerol; 0.85% (w:v) NaCl) at -70°C.

Genotypic identification

From all purified bacterial strains total genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Bacterial DNA concentrations and purity were evaluated with a Nanodrop spectrophotometer (ND-1000, Isogen Life Science). Polymerase chain reaction (PCR) amplification of 16 S rRNA genes was carried out in mixtures containing 50 ng μL^{-1} bacterial DNA, 1.8 mM High Fidelity PCR buffer (Invitrogen, Ghent, Belgium), 1.8 mM MgCl2, 0.2 mM of each of the four deoxynucleoside triphosphates (dNTPs), 0.4 µM of each of the forward (bacteria-specific 16S-26F: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse (universal 16S-1392R: 5'-ACGGGCGGTGTGTRC-3') primers and 1.25 U of High Fidelity Platinum Taq DNA polymerase (Invitrogen, Ghent, Belgium). Cycling conditions consisted of 1 denaturation cycle at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 52°C for 30 s, and 72°C for 3 min, and completed with an elongation step of 10 min at 72°C (Techne TC 5000 PCR Thermal Cycler). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The PCR products were directly used for amplified 16 S rDNA restriction analysis (ARDRA) and sequencing. For ARDRA, 20 µL aliquots of the PCR products were digested

for 2 h at 37°C with 1 U of the four-base specific restriction endonuclease HpyCH4 IV in 1× NEB buffer 1 (New England Biolabs, Beverly, MA, USA). The digestion products obtained were separated by electrophoresis in a 1.5% agarose gel, and visualized by gelred staining and UV illumination. Bacterial strains with the same ARDRA patterns were grouped and the purified PCR product (QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA)) of 1 representative isolate of each group was bi-directionally sequenced by Macrogen Inc. (Amsterdam, the Netherlands) using BigDye^{TM} terminator cycling conditions (Applied Biosystems (ABI) 3730XL). Consensus sequences were obtained with Genious Basic 5.3.6 and sequence matches were searched for on the Ribosomal Database Project II (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and the database of National Center for Biotechnology Information (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Sequences of selected strains were submitted to the EMBL database where accession numbers were assigned (http://www.ebi.ac.uk/ena/).

Phenotypic characterization

All purified bacterial strains were screened a first time (2011 screening) for their potential plant growth promoting characteristics (production of indole-3-acetic acid (IAA), 1-aminocylcopropane-1-carboxylate (ACC) deaminase activity), potential metal uptake enhancing properties (production of siderophores and organic acids) and Cd and Zn tolerance. After 2 years of stock conservation in the freezer (-80 °C), a second screening was performed (2013 screening) on most promising strains from the first screening with additional N_2-fixation and P-solubilization capacity testing. All results were assessed qualitatively. Before screening, strains were grown in 869 medium and subsequently washed 2 times with 10 mM $MgSO_4$. Isolates that were not able to grow in the different test media (incubation for 5 (liquid media) to 7 (solid media) days at 30°C) were considered as not detectable. Media without cell suspension served as control.

Production of IAA was tested according to Gordon and Weber (1951). Bacterial strains were inoculated in 1 mL 1/10 strength 869 medium with 0.5 g L^{-1} L-tryptophan. To 0.5 mL of supernatant, 1 mL Salkowsky reagent ($FeCl_3$-$HClO_4$) was added and positive strains were detected by observing a color change from yellow to pink. **ACC deaminase activity** was determined applying

a protocol adjusted from Belimov *et al.* (2005). Washed bacterial cell pellets were resuspended in 1 mL salts minimal medium with 1 mM ACC as sole nitrogen source. After 3 days at 30°C, cell pellets were resuspended in 0.1 mL Tris-HCl buffer (pH 8.5) and disrupted by the addition of 20 µL toluene. To induce ACC deaminase activity, 10 µL 0.5 M ACC and 100 µL 0.1 M Tris-HCl (pH 8.5) were added. After 30 min the reaction was stopped by adding 0.5 mL 0.56 N HCl and ACC deaminase activity was evaluated visually with an aliquot of the supernatant as described in Belimov *et al.* (2005). Bacterial **nitrogenase activity** was determined in a N-free semi-solid malate-sucrose medium (NFMM) modified from Döbereiner (1989) (Xie *et al.* 2003). For indication of pH changes, 3 mL of bromothymol blue L^{-1} medium was added (Schmid and Hartmann 2007). Anaerobic N_2-fixing capacity was visually rated as a color change from blue to yellowish-green which resulted from the acidification of sugars confirming bacterial growth. For screening **phosphate-solubilizing** bacteria, National Botanical Research Institute's phosphate growth medium (NBRIP) was used (Nautiyal 1999). Ten µL aliquots of washed bacterial strains were inoculated in holes (Ø 0.5 cm) and incubated for 7 days at 30°C. Formation of a clear halo (solubilization zone) around the holes indicated positive strains.

Siderophore production was qualitatively evaluated by the colorimetrical method of Schwyn and Neilands (1987). After incubating strains in 800 µL selective 284 medium with a carbon mix and 0, 0.25 and 3 µM Fe (respectively deficient, optimal and oversupplying Fe conditions), 100 µL of the blue CAS reagent was added and decolorization of CAS was evaluated. **Secretion of organic acids** was assessed using the colorimetric method of Cunningham and Kuiack (1992). Strains were incubated in 800 µL sucrose tryptone medium and, in case organic acids were produced, a color change from red to orange and yellow was observed after addition of 100 µL 0.1% (v:v) alizarine red S.

To test **metal tolerance**, the isolates were plated on selective 284 medium with a carbon mix (Schlegel *et al.* 1961) and 0.0, 0.4, 0.8 and 1.6 mM Cd (added as $CdSO_4$) or 0, 0.6, 1.0 and 2.5 mM Zn (added as $ZnSO_4$). Tolerance for Cd and Zn together was also tested using the same medium and a combination of different Cd and Zn concentrations. After an incubation period of 7 days at 30°C, growth of the isolates was rated visually.

5.II.2.2 Inoculation experiments

Strains selection

In general, the selection of promising strains is based on the results of the phenotypic traits taking into account the original niche of the species, genotype and abundance. Rhizosphere strains are expected to be able to potentially increase metal uptake (produce organic acids and siderophores), promote plant growth by N_2-fixation and P-solubilization as well as to be tolerant to high metal concentrations. Root endophytes are considered promising when exhibiting as much as possible plant growth promoting traits with relatively high metal tolerance and eventually the production of organic acids and siderophores. In turn, the production of IAA and ACC deaminase are indispensable for twig endophytes to be chosen. Selected genotypes should furthermore not be known as plant pathogen and morphotypes of elected strains should be relatively abundant in the isolation.

For the inoculation experiments 2012A and B, choice is based on the 2011 phenotypic screening while the second phenotypic screening in 2013 delivered the strains for inoculation experiment 2013. In inoculation experiment 2014, best isolates of former experiments were evaluated again.

Set up

Biomass production and metal accumulation of the selected clones, whether or not inoculated with selected strains, were evaluated in pot experiments at Hasselt University (30 km from the field). The first experiment (2012A) was conducted in the greenhouse (day temperature 22°C, night temperature 18°C, air humidity 60%, photoperiod 15 h) while later experiments were performed outside in a sheltered environment (under a glass cover) (2012B) or outside in open air (2013 and 2014). The experiments lasted for 60 to 90 days. Details are given in Table 5.3.

Physico-chemical soil characteristics (including soil metal concentrations)

For all experiments, pots (4 L) were filled with 4.8 kg of 4 mm sieved, unsterilized topsoil (0-30 cm) from the contaminated field in Lommel.

Experiments 2012A, 2012B and 2013 were conducted with soil from the same spot on the field while for inoculation experiment 2014, soil from a different spot was taken. Soil was analyzed for pseudo-total and plant available ($CaCl_2$-exchangeable) Cd, Zn and Pb concentrations and for pH-H_2O, pH-KCl, electrical conductivity (EC) and effective cation exchange capacity (CEC_e) before starting the pot experiments. Details on material and methods are described in Chapter 3 ('Material and methods' section: Tobacco, sunflower and hemp: Physico-chemical soil characteristics).

Cultivation and inoculation

Twenty cm cuttings of the selected clones were obtained from a stock plantation of INBO (2012A) or cut on the field (2012B, 2013, 2014) from twigs of 'mother' plants growing in block 2 (see Chapter 3: Figure 3.1). Diameters of cuttings were measured and they were weighed to achieve experimental groups with the same mean cutting diameter and fresh weight. The numbers of replicates (cuttings) used for each examined bacterial strain in each pot trial are listed in Table 5.3. A control group was formed by double as much cuttings and was treated in the same way except for the inoculation that was not performed. To allow root development, cuttings were placed in half strength aerated Hoagland's nutrient solution. After 7 days, inocula of the selected bacteria were added to this Hoagland's nutrient solution. Since a successful inoculation is likely to depend on the growth stage of the bacterial strain to be inoculated (highest success rates were obtained in our laboratory with strains in exponential growth phase at an absorbance value of about 1 (A_{660}=1) (Truyens, personal communication)), growth curves of all selected bacterial strains were drawn to reveal the exact cultivation time needed. In order to do so, bacterial strains were cultivated in 869 liquid medium at 30°C and every hour the absorbance value at 660 nm was measured (visible spectrophotometer Novaspec Plus). To prepare the inocula, bacterial strains were cultivated in 869 liquid medium at 30°C (following the exact same method as when determining the growth curves) until an absorbance value of 1 (A_{660}=1). The culture was centrifuged (20 min at 3220 g) and bacterial pellet was resuspended in 10 mM $MgSO_4$ solution to obtain an inoculum with an absorbance value of 1. The rooted cuttings were placed in the inoculum-Hoagland solution in a 1/10 volume ratio (final bacterial

concentration of 10^8 CFU mL^{-1}) for another 72 h before they were planted in pots. For pot trials 2013 and 2014, plants were additionally inoculated every 2 weeks during the extent of the experiment. The inoculum was prepared as described above and 20 mL of inoculum was poured in every pot.

Maintenance

All plants received the same amount of tap water when watering was needed. On every cutting, all emerging shoots were allowed to develop.

Table 5.3 Practical details and number of replicates for inoculation experiments performed in the period 2012-2014.

Inoculation experiment	Cutting origin/ Growth environment	Duration (days in pot)	Season	Number of bacterial strains tested	Number of replicates (control)	Picture set up
2012A	INBO stock cuttings/ greenhouse	90	February-May	5	10 (20)	
2012B	field cuttings/ outdoor sheltered	60	July-September	6	10 (20)	
2013	field cuttings/ outdoor open	90	June-September	6 + consortium of all	10 (20)	
2014	field cuttings/ outdoor open	70	June-September	2	6 (12)	

Evaluation

Biomass production

At harvest, leaves, twigs, cutting and roots were separated and cutting and roots were washed thoroughly with tap water to remove all traces of soil present on the surface. Fresh weight (FW) biomass production of plant parts was determined for all replicates in all experiments. Dry weight (DW) production was evaluated for half of the replicates (2012A and B) or for all replicates (2013 and 2014) after oven-drying (60°C) plant parts until constant weight. For inoculation experiments 2012A and B, DW production of the other replicates was estimated based on the regression equation expressing the FW-DW relationship of measured plants.

In planta metal concentrations, bioconcentration factor and translocation factor

Cadmium, Zn and Pb concentrations in every plant part (leaves, twigs, cutting and roots) were determined for 3 replicates per inoculated condition and 6 for control (2012A, 2012B, 2014) of for respectively 5 and 10 (2013). Selected plants were representative for the condition regarding DW production.

To determine total Cd, Zn and Pb concentrations in the biomass, air-dried plant material was individually milled obtaining a fine powder, which was wet-digested as described in Chapter 3 ('Material and methods' section: Tobacco, sunflower and hemp: *In planta* metal concentrations). All samples were tested at least in duplicate. Blanks and certified reference material (trace elements in spinach, Standard Reference Material® 1570a, National Institute of Standards and Technology, USA Department of Commerce) were included for quality control of the data.

The bioconcentration factor (BCF) is defined here as the ratio of metal concentration in twigs to total soil metal content. The BCF directly allows to compare extraction efficiencies of different clones even on different levels of contamination (Dickinson and Pulford 2005). The translocation factor (TF) is defined as the metal concentration in twigs to the metal concentration in roots and is an indication of the efficiency with which a metal, that is taken up by the

roots, is transported to the twigs (Lebeau *et al.* 2008). BCF and TF of Cd, Zn and Pb were calculated for every plant evaluated for metal content.

Twig metal extraction

Metal extraction (potential) is defined as the amount of metals that plants can extract out of the soil and accumulate in their harvestable biomass per unit of soil area and time (usually expressed in g ha^{-1} yr^{-1}). In this research, the mean extraction of Cd and Zn was calculated per condition by multiplying mean plant twig production (DW) of the condition (g $plant^{-1}$ 60-90 $days^{-1}$) with mean Cd and Zn concentrations measured in the twigs of the condition (mg kg^{-1}).

Physico-chemical soil characteristics (including soil metal concentrations) at the end of pot experiments

At the end of inoculation experiment 2013, soil (mixture of bulk and rhizosphere) samples of 3 (inoculated conditions) or 6 (control conditions) randomly chosen pots were collected. The soil was analyzed for pseudo-total and plant available ($CaCl_2$-exchangeable) Cd, Zn and Pb concentrations and for pH-H_2O, pH-KCl, electrical conductivity (EC) and effective cation exchange capacity (CEC_e) as described in Chapter 3 ('Material and methods' section: Tobacco, sunflower and hemp: Physico-chemical soil characteristics).

Re-isolation of inoculated bacterial strains

In order to verify for successful colonization of inoculated plants, cultivable bacterial strains were isolated from rhizosphere, roots and twigs of 1 plant per condition in experiments 2012A and B. The procedure is similar to the one described for isolation of strains. Next to 1/10 strength 869 solid medium, colony forming units were also evaluated on selective 284 solid medium with 0.4 mM Cd and 0.8 mM Cd. Strains morphologically similar to the inoculated ones were purified and identified as described in Genotypic identification.

Statistical analyses

Statistical analyses were performed in R 3.1.3 (R Development Core Team, 2013). To determine (dis)similarities between physico-chemical characteristics of the soil used for pot trials 2012A, 2012B and 2013 *vs.* pot trial 2014, a Student

t-test was performed. The effect of inoculation on dry weight (DW) biomass production was analyzed using ANOVA or, in case of possible heteroscedasticity of the residuals (checked by plotting), model-robust standard errors (waldtest). The QQ-plots were used to inspect normality of the residuals. In case of non-normality, transformations of the outcome (logarithmic, inverse, square root, exponential) were tried. When an indication of non-normality was present for all these transformations, a Box-Cox was used. Two-by-two comparisons with the control condition were conducted using Dunnett's test. In case of non-normally distributed errors, the non-parametric Kruskal-Wallis Rank Sum test was applied followed by two-by-two comparisons with the control condition using Gao's test. ANOVA and Dunnett's test to perform two-by-two comparisons with the control were applied to assess the effects of inoculation on *in planta* metal concentrations, bioconcentration and translocation factors and physico-chemical soil characteristics. The same procedure was used to differentiate between soil characteristics of control plants at the start and at the end of inoculation experiment 2013.

To study the effect of plant part on biomass production and on metal concentration for control plants, a mixed model was used. In case of non-normality, the outcome was transformed as described above. A random intercept was used to account for the correlation between measurements for the same plant. DW production of and metal contents in different plant parts were allowed to have a different variance. The Tukey's method was used to deal with multiple testing.

5.II.3 Results

5.II.3.1 Isolation of the plant-associated bacteria of the selected *Salix* clones

The cultivable bacterial populations were isolated from rhizosphere, roots and twigs of the selected *Salix* clones. For both clones, the number of CFU g^{-1} soil or plant tissue decreased from rhizosphere (10^7) to roots (10^7-10^6) and was significantly lower in the twigs (10^3-10^2) (Figure 5.1). A total of 369 bacterial strains were isolated and characterized.

5.II.3.2 Genotypic identification of the plant-associated bacteria

For the *S. viminalis* clone, the number of different genera was highest in the roots (24 different genera), followed by the rhizosphere (17) and the twigs (3) (Figure 5.1). *Pseudomonas* was the only genus present in all compartments. Besides, rhizosphere soil and root tissue had 13 genera in common, whereas *Curtobacterium sp.* and *Sphingomonas sp.* were present in both roots and twigs. *Duganella sp., Nocardioides sp., Ralstonia sp*. and *Streptomyces sp*. were only recovered from rhizosphere soil, whereas species belonging to the genera *Alcaligenes, Chryseobacterium, Microbacterium, Phaeospirillum, Plantibacter, Polaromonas, Rhizobium, Xanthomonas* and some *uncultured bacterium* were only isolated from root tissue.

For the *S. alba x alba* clone, the number of different genera also decreased in the order roots – rhizosphere – twigs, respectively 20, 13 and 10 genera were found. The genera *Arthrobacter, Caulobacter* and *Xanthomonas* were found in all compartments. Furthermore, all 13 genera recovered from the rhizosphere were also found in the roots, whereas *Alcaligenes sp.* and *Rahnella sp.* were present in both roots and twigs. Species belonging to the genera *Duganella, Mesorhizobium, Mycobacterium, Pseudomonas* and *Sphingomonas* were only isolated from the roots, while *Curtobacterium sp., Flavobacterium sp.*, *Frigoribacterium sp., Microbacterium sp.* and some *uncultured bacterium* were only found in the twigs.

The cultivable bacterial populations of the rhizospheres of *S. viminalis* and *S. alba x alba* clones had 9 genera in common comprising respectively 59% and 79% of the population, while the root populations of both clones had 18 genera in common representing 95% and 97% of the root population. *Curtobacterium sp.* were found in the twigs of both clones and comprise 69% of the *S. viminalis* and 18% of the *S. alba x alba* twig population.

5.II.3.3 Phenotypic characteristics of the plant-associated bacteria

Results are based on the 2011 phenotypic screening of all 369 isolates (see Supplementary tables 5.1 and 5.2 pp.255-268). Endophytic strains (root and

twig isolates) producing potentially plant growth promoting traits (IAA or ACC deaminase) were abundant in both clones (from 24% up to 86% of all isolates) with equal percentages found for the twig isolates of both clones (Table 5.4). The rhizosphere community of both clones had an equal percentage of isolates producing organic acids (9%) while production of siderophores was higher for the *S. viminalis* rhizosphere community (47% *vs.* 37% for *S. alba x alba*). Considering the root isolates, the *S. viminalis* clone, with an organic acid production capacity of 17% and a siderophore production of 43%, scored better for potentially metal uptake enhancing traits than the *S. alba x alba* clone.

Metal tolerance in rhizosphere and root strains is in general higher for the *S. viminalis* than for the *S. alba x alba* clone. Remarkably, percentages of metal tolerant strains in the twigs were in general higher for the *S. alba x alba* clone. For both clones, tolerance to Zn revealed to be higher than tolerance to Cd which in turn is higher than tolerance to Cd combined with Zn.

5.II.3.4 Relation between genotypic and phenotypic characterization

When combining Supplementary tables 5.1 and 5.2 (pp.255-268) with Table 5.4, the most important isolated genera for potentially increasing plant growth and metal uptake as well as for metal tolerance could be revealed. In the cultivable root communities of both *S. viminalis* and *S. alba x alba*, *Variovorax sp.*, *Rhizobium sp.* and *Caulobacter sp.* play a prominent role in production of IAA and ACC deaminase. In case of *S. viminalis*, *Spinghobacterium sp.* are additionally very important for ACC deaminase production. The isolated *Curtobacterium sp.* are of high importance for production of plant growth promoting components in the twigs of both clones. Next to this genus, also *Pseudomonas sp.* and *Xanthomonas sp.* reveal to be eminent in case of respectively *S. viminalis* and *S. alba x alba*. *Bacillus* is an important genus for production of organic acids in the cultivable rhizosphere and root communities of both clones while a number of other genera play a substantial role as well. The production of siderophores by isolated rhizosphere strains can mainly be attributed to the genera *Stenotrophomonas*, *Pseudomonas* and *Mesorhizobium* (*S. viminalis*) or *Bacillus*, *Variovorax* and *Polaromonas* (*S. alba x alba*). The

isolated *Sphingobacterium sp.* and *Caulobacter sp.* have the highest percentages of siderophore production in the root communities of respectively *S. viminalis* and *S. alba x alba* while *Variovorax sp.* and *Rhizobium s*p. additionally play an important role in both clones.

Highest percentages of metal tolerant rhizosphere strains were observed for the genera *Arthrobacter*, *Ralstonia* and *Streptomyces* in case of *S. viminalis* while these were *Bacillus*, *Variovorax*, *Chryseobacterium* and *Paenibacillus* in case of *S. alba x alba*. *Sphingobacterium*, *Variovorax*, *Rhizobium* and *Caulobacter* revealed to be prominent genera for metal tolerance in the cultivable root community of *S. viminalis*. In *S. alba x alba*, this role is also played by *Variovorax sp.* supplemented with *Bosea sp.* and a number of others. Metal tolerance of *Curtobacterium sp.* isolated from twig samples of both clones is noteworthy, however, in case of *S. alba x alba* a number of other genera play a substantial role as well.

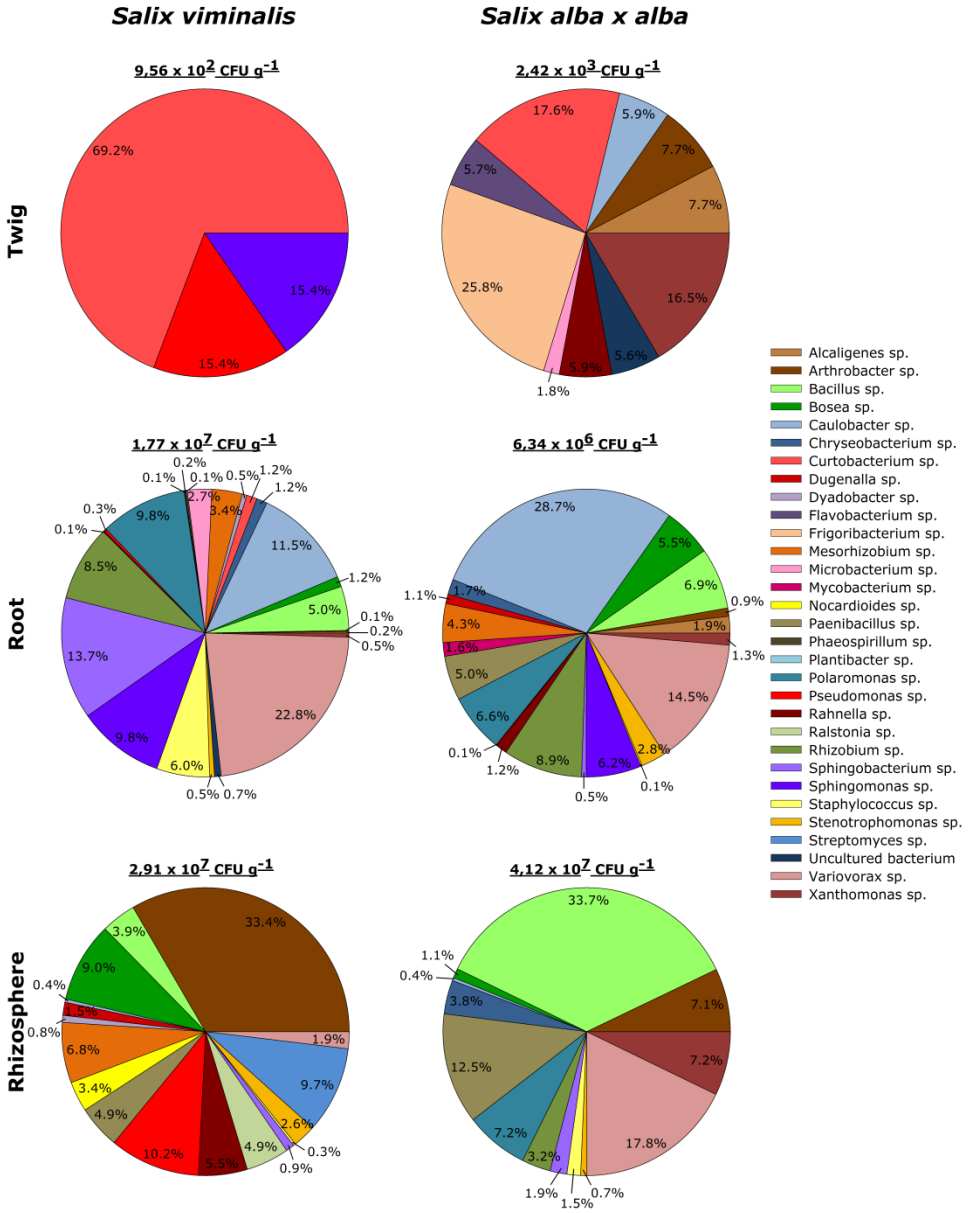

Figure 5.1 Estimated total number of cultivable colony forming units (CFU) per g of twig (top) or root (middle) fresh weight or per g of rhizosphere soil (bottom) for *S. viminalis* (left) and *S. alba x alba* (right). Pie charts: Diversity and relative abundance (%) of cultivable bacterial genera isolated from twigs, roots and rhizosphere of *S. viminalis* and *S. alba x alba*. Each color represents a bacterial genus.

Table 5.4 Total percentages of positive test results per compartment (rhizosphere, roots and twigs) in the 2011 phenotypic screening for the selected *S. viminalis* and *S. alba x alba* clone grown on the metal-contaminated field in Lommel. Results per compartment are calculated based on relative abundances of positively scoring isolates in the compartment. A list of all isolated strains, their relative abundances and results of the *in vitro* phenotypic traits testing can be found in Supplementary information (Supplementary tables 5.1 and 5.2; pp.255-268).

		Plant growth promoting traits		Metal uptake enhancing traits		Phytotoxicity reducing traits (mM)								
Clone	**Compartment (n° of isolates)**	**IAA**	**ACC deam.**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
Salix viminalis	**Rhizosphere (59)**	75	57	9	47	40	61	32	33	56	16	11	36	0
	Roots (120)	41	44	17	43	42	57	44	30	53	17	6	37	4
	Twigs (4)	85	50	0	50	35	35	0	35	35	0	0	0	0
Salix alba x alba	**Rhizosphere (66)**	44	38	9	37	29	63	14	21	51	11	3	19	0
	Roots (91)	54	24	7	30	35	51	22	29	42	14	10	26	4
	Twigs (29)	86	49	28	45	33	61	5	24	37	5	2	9	0

IAA = production of indole-3-acetic acid, ACC deam. = production of 1-aminocyclopropane-1-carboxylate deaminase, OA = production of organic acids, SID = production of siderophores.

5.II.3.5 Inoculation experiments

Strains selection

Table 5.5 lists the selected strains for inoculation experiments 2012A, 2012B and 2013 alongside the willow clone and plant part these strains where originally isolated from and the phenotypic traits these strains exhibit *in vitro*. For the inoculation experiments 2012A and B, choice is based on the 2011 phenotypic screening and respectively 5 and 6 isolates were selected as potentially promoting plant growth and/or metal uptake and/or reducing phytotoxicity. The second phenotypic screening in 2013 delivered the strains for inoculation experiment 2013. In inoculation experiment 2014, isolates resulting in higher Cd and Zn extractions than control in the former 3 experiments were evaluated again. For *S. viminalis* these were Rh1 and Rh2 while for *S. alba x alba* this was Tw1 and Rh4.

It should be mentioned that the second *in vitro* phenotypic screening (in 2013) for the about 100 most promising strains selected based on the first screening (2011 screening) revealed an unsuccessful re-cultivation in 9% of the cases as well as a serious decline in positive test results compared to the first screening (Supplementary table 5.3 p.269). Almost half of the isolates lost the *in vitro* production capacity of IAA and siderophores while 74% and 62% of the isolates no longer scored positively for respectively ACC deaminase production and organic acid production. Regarding metal tolerance *in vitro*, the loss of tolerance to Zn (1 mM and 2.5 mM) is about 50% while tolerance of isolates to Cd (0.8 mM and 1.6 mM) is lost in about 90% of the cases.

Physico-chemical soil characteristics (including soil metal concentrations)

The soil used for pot trials 2012A, 2012B and 2013 was not significantly distinct ($p < 0.05$) from the one used for pot experiment 2014 regarding acidity in water, but potential acidity and EC were significantly higher and the CEC_e significantly lower in the 2014 soil (Table 5.6). Also pseudo-total and $CaCl_2$-exchangeable soil metal concentrations differed significantly ($p < 0.05$) defining the soil for pot trial 2014 as more contaminated.

Table 5.5 Genotypic identification, origin and phenotypic traits of selected bacterial strains for all inoculation experiments with *S. viminalis* and *S. alba x alba* cuttings. Phenotypic test results of selected strains for inoculation experiments 2012A and 2012B are based on the 2011 screening while these for inoculation experiment 2013 resulted from the 2013 screening.

			Plant growth promoting traits				Metal uptake enhancing traits		Phytotoxicity reducing traits (mM)								
Inoculation experiment	**Code**	**Genotypic identification [Accession number] (Origin)**	**IAA**	**ACC deam.**	**N_2-fix.**	**P-sol.**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
2012A	**Rh1**	Rahnella sp. [LN551929] (Rhizosphere strain *S. viminalis*)	+	+	n.d.	n.d.	+	+	+	+	+	+	+	-	+	+	-
	Ro1	Sphingobacterium sp. [LN551928] (Root endophyte *S. viminalis*)	+	+	n.d.	n.d.	+	+	+	+	+	+	+	+	+	+	+
	Ro2	Caulobacter sp. [LN551927] (Root endophyte *S. alba x alba*)	+	+	n.d.	n.d.	+	+	+	+	+	+	+	+	+	+	+
	Tw1	Curtobacterium sp. [LN551926] (Twig endophyte *S. viminalis*)	+	+	n.d.	n.d.	-	+	+	+	-	+	+	-	-	-	-
	Tw2	Pseudomonas sp. [LN551925] (Twig endophyte *S. viminalis*)	+	+	n.d.	n.d.	-	+	-	-	-	-	-	-	-	-	-
2012B	**Rh2**	Bacillus sp.[LN867304] (Rhizosphere strain *S. viminalis*)	-	+	n.d.	n.d.	+	+	+	+	+	+	+	+	-	+	-
	Rh3	Stenotrophomonas sp. [LN867305] (Rhizosphere strain *S. viminalis*)	+	+	n.d.	n.d.	+	+	+	+	-	+	+	-	-	-	-
	Rh4	Bacillus sp. [LN867306] (Rhizosphere strain *S. alba x alba*)	+	-	n.d.	n.d.	+	+	-	+	-	-	+	-	-	-	-
	Ro3	Sphingobacterium sp. [LN867307] (Root endophyte *S. viminalis*)	-	+	n.d.	n.d.	-	+	+	+	+	+	+	-	-	+	-
	Ro4	Microbacterium sp. [LN867308] (Root endophyte *S. viminalis*)	+	+	n.d.	n.d.	+	+	+	+	+	+	+	-	-	+	-
	Ro5	Sphingobacterium sp. [LN867309] (Root endophyte *S. viminalis*)	-	+	n.d.	n.d.	+	+	+	+	+	+	+	+	+	+	-

Inoculation experiment	Code	Genotypic identification [Accession number] (Origin)	Plant growth promoting traits				Metal uptake enhancing traits		Phytotoxicity reducing traits (mM)								
			IAA	ACC deam.	N_2-fix.	P-sol.	OA	SID	0.4 Cd	0.6 Zn	0.4 Cd + 0.6 Zn	0.8 Cd	1.0 Zn	0.8 Cd + 1.0 Zn	1.6 Cd	2.5 Zn	1.6 Cd + 2.5 Zn
2013	**Rh5**	Pseudomonas sp. [LN867310] (Rhizosphere strain *S. viminalis*)	+	+	-	+	+	+	n.d.	n.d.	n.d.	-	-	n.d.	-	-	n.d.
	Rh6	Arthrobacter sp. [LN867311] (Rhizosphere strain *S. viminalis*)	+	+	+	+	+	-	n.d.	n.d.	n.d.	-	-	n.d.	-	-	n.d.
	Ro6	Pseudomonas sp. [LN867312] (Root endophyte *S. viminalis*)	+	+	-	+	+	+	n.d.	n.d.	n.d.	-	-	n.d.	-	-	n.d.
	Ro7	Stenotrophomonas sp. [LN867313] (Root endophyte *S. viminalis*)	+	+	+	+	+	+	n.d.	n.d.	n.d.	-	+	n.d.	-	-	n.d.
	Ro8	Rhizobium sp. [LN867314] (Root endophyte *S. alba x alba*)	+	+	+	+	-	+	n.d.	n.d.	n.d.	-	-	n.d.	-	-	n.d.
	Ro9	Rhizobium sp. [LN867315] (Root endophyte *S. alba x alba*)	+	+	+	+	-	+	n.d.	n.d.	n.d.	-	-	n.d.	-	-	n.d.

Bacterial strains are coded after the plant part originally isolated from: Rh = rhizosphere strain, Ro = root strain and Tw = twig strain. IAA = production of indole-3-acetic acid, ACC deam. = production of 1-aminocyclopropane-1-carboxylate deaminase, N_2-fix. = nitrogenase activity, P-sol. = phosphate solubilizing, OA = production of organic acids, SID = production of siderophores. +, - = positive respectively negative test result for phenotypic trait. n.d. = not detected.

Table 5.6 Physico-chemical characteristics of the topsoil (0-30 cm) used for the inoculation experiments. The soil for experiments 2012A, 2012B and 2013 was sampled on the same spot on the field while the soil used in experiment 2014 was sampled on a different spot.

Inoculation experiment	Soil texture	pH-H_2O	pH-KCl	CEC_e (meq 100 g^{-1})	EC (µS cm^{-1})	Pseudo-total metal concentrations (mg kg^{-1} dry soil)			$CaCl_2$-exchangeable metal concentrations (mg kg^{-1} dry soil)		
						Cd	Zn	Pb	Cd	Zn	Pb
2012A 2012B 2013	4% clay 8% silt 88% sand (Meers *et al.* 2007a)	6.64 ± 0.09 [a]	5.56 ± 0.08 [a]	7.10 ± 0.70 [a]	52.07 ± 4.46 [a]	4.02 ± 0.45 [a]	223.34 ± 20.99 [a]	144.04 ± 11.90 [a]	0.24 ± 0.06 [a]	11.27 ± 3.70 [a]	0.14 ± 0.02 [a]
2014		6.56 ± 0.04 [a]	5.87 ± 0.07 [b]	5.46 ± 0.07 [b]	246.67 ± 79.19 [b]	6.93 ± 0.10 [b]	428.94 ± 6.49 [b]	216.53 ± 7.03 [b]	0.43 ± 0.01 [b]	21.29 ± 0.91 [b]	0.30 ± 0.03 [b]

Values are mean ± standard deviation of 6 (2012A, 2012B and 2013) or 3 (2014) independent replicates. Different superscripts indicate significant differences at the level $p < 0.05$.

Biomass production

For all inoculation experiments, survival rate of the cuttings was nearly 100% and no visible symptoms of phytotoxicity were observed. Mean diameter and DW of cuttings was, for a given experiment and clone, very equal for all conditions (results not shown). Some general observations could be made regarding biomass production in all 4 experiments (Figures 5.2-5.5). *Salix viminalis* control cuttings produced significantly more ($p < 0.001$) twig DW than root DW in all experiments while control *S. alba x alba* plants showed a significantly higher ($p < 0.001$) root than twig production in the longer experiments 2012A and 2013. The *S. viminalis* clone produced in all cases more twig biomass than *S. alba x alba* clones. Leaf DW production could not be compared between clones nor experiments since timing and intensity of autumn leaf fall was different for both clones and experiments.

In experiment **2012A**, the control condition produced a twig DW of 1.81 ± 0.41 g in case of *S. viminalis* and 1.01 ± 0.65 g in case of *S. alba x alba* (Figure 5.2). Inoculation of *S. viminalis* plants with bacterial strain Ro2 resulted in a significantly lower ($p < 0.05$) twig and slightly lower root and leaf DW production compared to control plants. The production of roots, twigs and leaves tended to increase for cuttings inoculated with isolates Rh1 and Tw2 in comparison with control. In case of the *S. alba x alba* clone, inoculation with strains Ro1 and Tw1 tended to increase biomass production compared to the control with a significantly higher ($p < 0.01$) twig production after Tw1 inoculation. The produced twig DW observed for controls in experiment **2012B** was 1.10 ± 0.65 g for *S. viminalis* and 0.47 ± 0.20 g for *S. alba x alba* (Figure 5.3). Inoculation of *S. viminalis* cuttings with isolates Rh2, Rh3 and Ro3 tended to (slightly) increase biomass production compared to control plants. Considering the *S. alba x alba* plants, inoculation with strain Rh4 tended to slightly increase root, twig and leaf production while almost all other inoculated strains tended to decrease DW production compared to control plants. In experiment **2013**, a twig DW production of 2.76 ± 0.84 g and 2.17 ± 0.36 g was measured for the control condition of respectively *S. viminalis* and *S. alba x alba* (Figure 5.4). In case of *S. viminalis*, inoculation with isolate Ro8 seemed to slightly increase biomass production compared to control plants while strains

Rh5, Ro6 and Ro9 seemed to decrease DW production of root, twig and leaf. Biomass production of inoculated *S. alba x alba* cuttings tended to be similar (Ro6, Ro8, CONS) or slightly lower (Rh5, Rh6, Ro7, Ro9) compared to control cuttings. Control *S. viminalis* and *S. alba x alba* cuttings produced a twig DW of respectively 3.20 ± 0.58 g and 2.36 ± 0.60 g in experiment **2014** (Figure 5.5). There were no differences observed between inoculated and control conditions for *S. viminalis* while biomass production of inoculated *S. alba x alba* plants seemed to be slightly lower than biomass production of control cuttings.

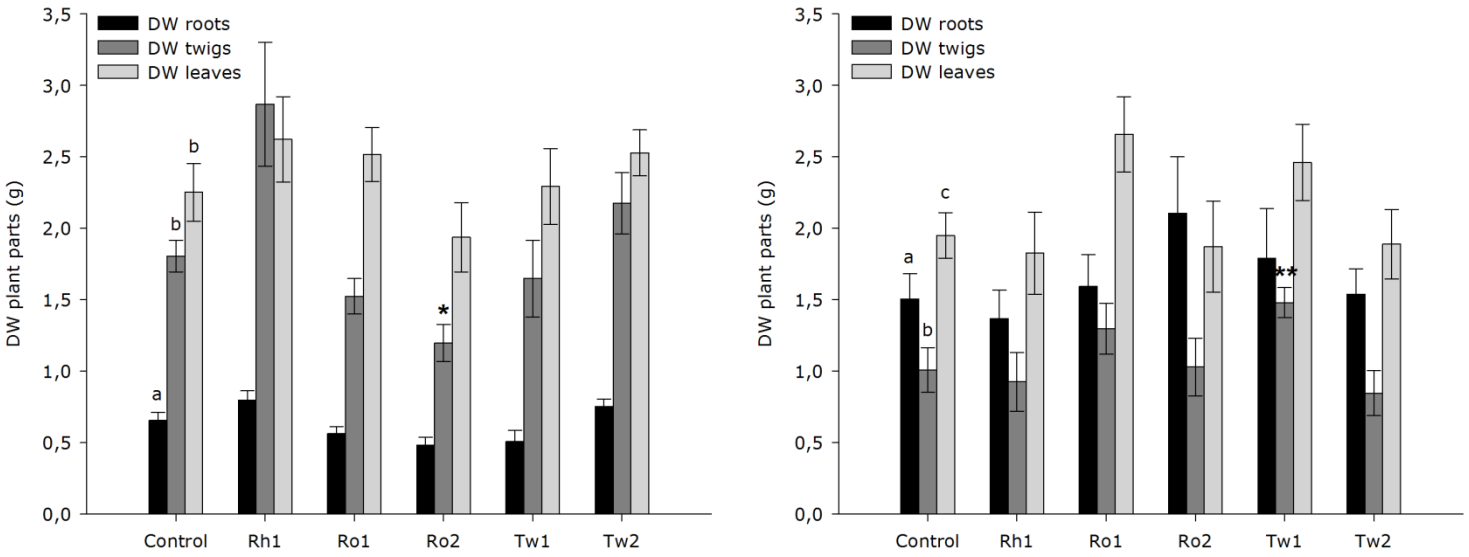

Figure 5.2 Inoculation experiment 2012A: Biomass production. Mean dry weight (DW in g) production of roots, twigs and leaves of control (N = 20) and inoculated (N = 10) cuttings of *S. viminalis* (left) and *S. alba x alba* (right) after 90 days of growth in pots. Error bars are standard errors. Stars indicate a significant different biomass production of a plant part after inoculation compared to the respective control plant part (significance levels: * $p < 0.05$ and ** $p < 0.01$). Letters a-c indicate significantly different ($p < 0.001$) biomass productions between the plant parts of the control condition.

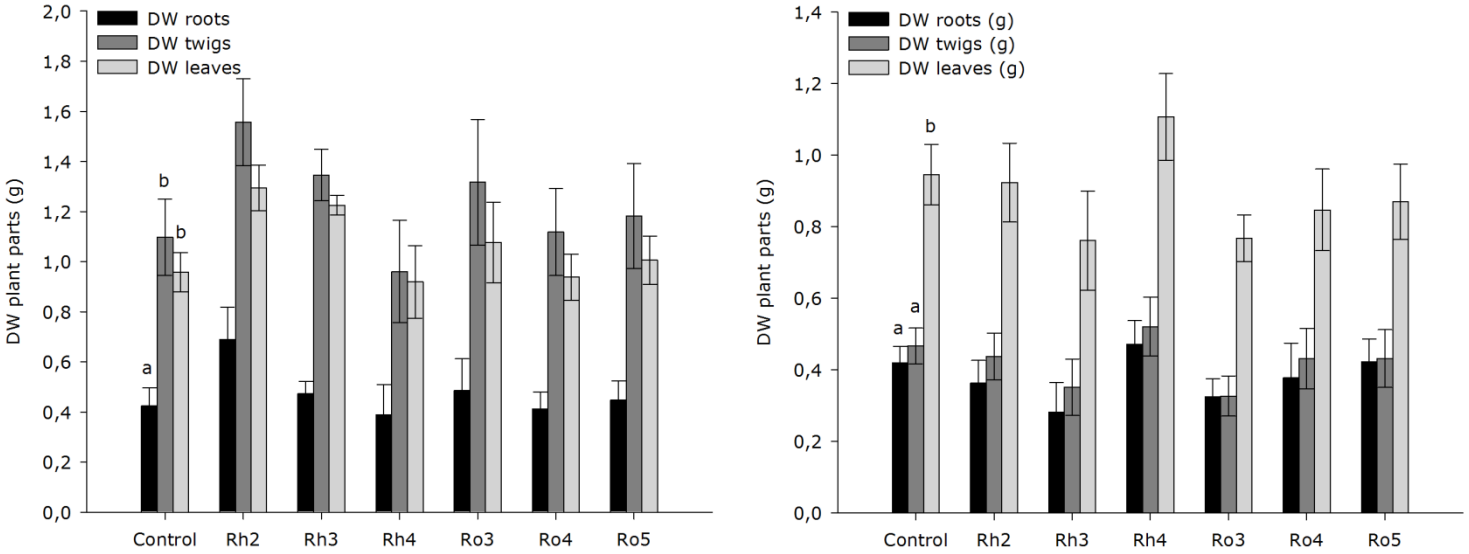

Figure 5.3 Inoculation experiment 2012B: Biomass production. Mean dry weight (DW in g) production of roots, twigs and leaves of control (N = 20) and inoculated (N = 10) cuttings of *S. viminalis* (left) and *S. alba x alba* (right) after 60 days of growth in pots. Error bars are standard errors. Letters a and b indicate significantly different ($p < 0.001$) biomass productions between the plant parts of the control condition.

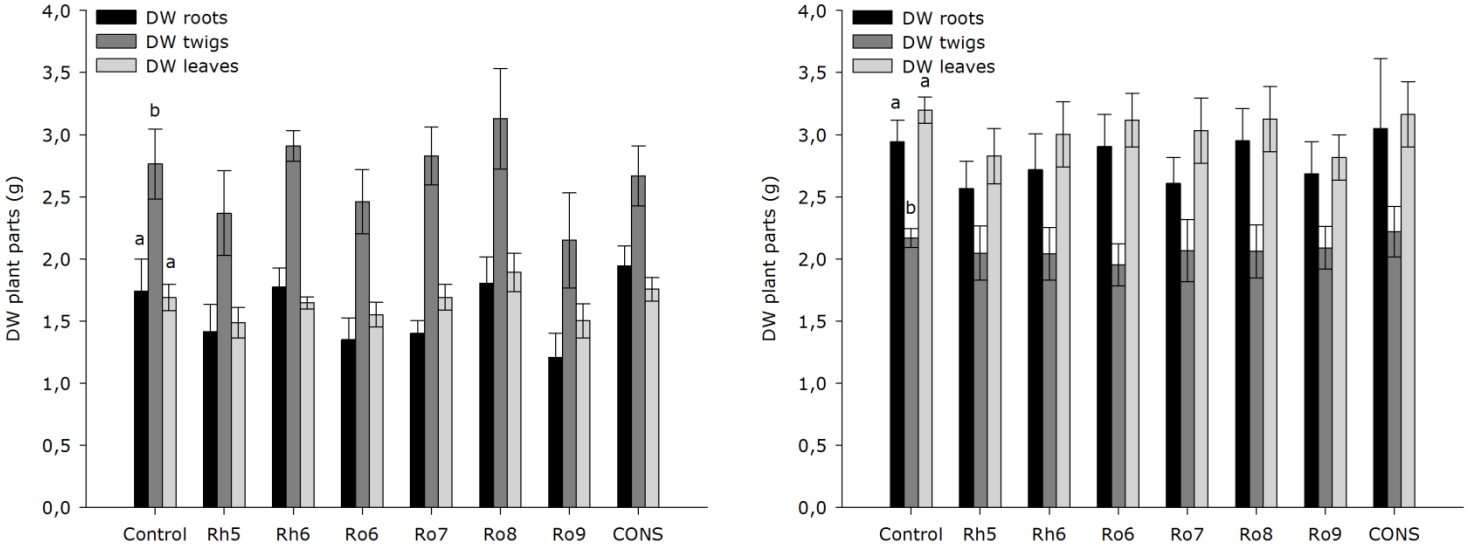

Figure 5.4 Inoculation experiment 2013: Biomass production. Mean dry weight (DW in g) production of roots, twigs and leaves of control (N = 20) and inoculated (N = 10) cuttings of *S. viminalis* (left) and *S. alba x alba* (right) after 90 days of growth in pots. Error bars are standard errors. Letters a and b indicate significantly different ($p < 0.001$) biomass productions between the plant parts of the control condition.

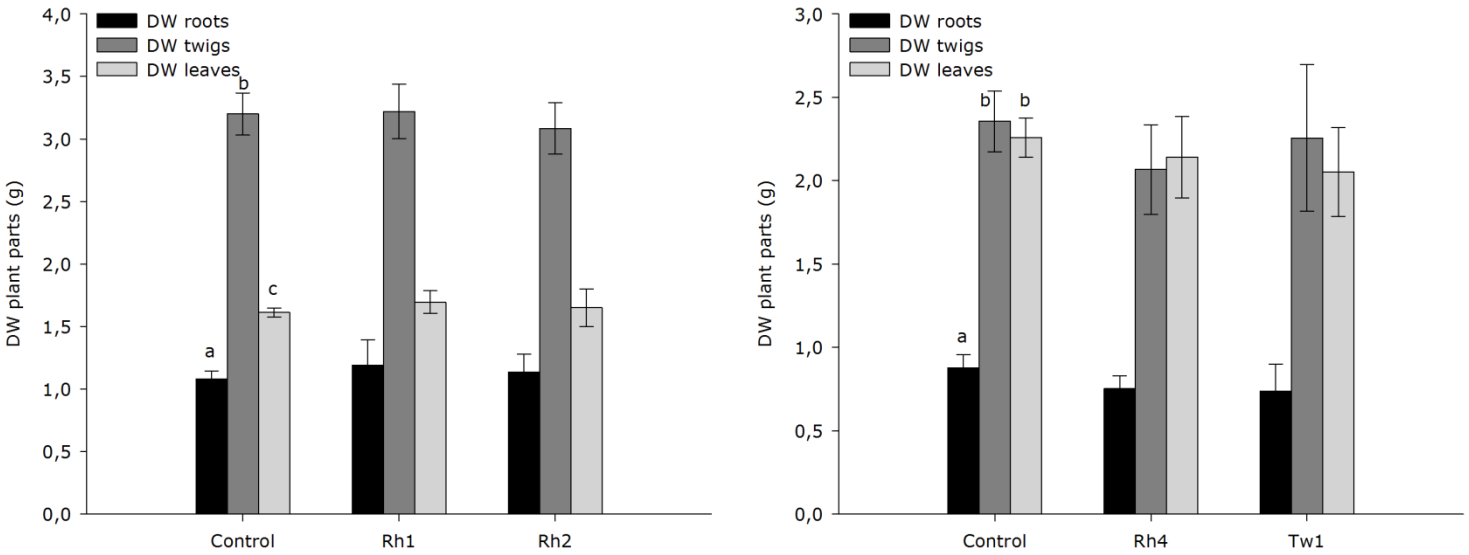

Figure 5.5 Inoculation experiment 2014: Biomass production. Mean dry weight (DW in g) production of roots, twigs and leaves of control (N = 12) and inoculated (N = 6) cuttings of *S. viminalis* (left) and *S. alba x alba* (right) after 70 days of growth in pots. Error bars are standard errors. Letters a-c indicate significantly different ($p < 0.001$) biomass productions between the plant parts of the control condition.

In planta metal concentrations, bioconcentration factor and translocation factor

Cadmium, Zn and Pb concentrations in roots, cutting, twigs and leaves of control plants of all inoculation experiments are listed in Tables 5.7 (*S. viminalis*) and 5.8 (*S. alba x alba*). Lead concentrations in leaves and twigs were frequently (depending on experiment and evaluated clone) below the detection limit of 0.05 mg L^{-1} and were not shown. Concerning Cd and Zn, a few general trends were observed. *Salix viminalis* plants accumulated (significantly) more Cd and Zn in root and leaf tissue than in cutting and twigs while for *S. alba x alba*, accumulation of these metals was, with high significance, highest in the leaves in 3 out of 4 experiments. In all cases, *S. viminalis* had higher Cd and Zn concentrations *in planta* than *S. alba x alba* (roots: on average about 3.5 times more Cd and 2 times more Zn; cutting, twigs and leaves: on average about 2 times more Cd and 1.5 times more Zn). Twig Cd and Zn concentrations decreased in the order of experiments 2012A – 2013 – 2014 – 2012B for *S. viminalis* and in the order 2012A – 2013 – 2012B – 2014 for *S. alba x alba*. Lead was in both clones highly retained in the roots and translocation to cuttings, twigs and leaves was very limited.

For all inoculation experiments and for both clones, inoculated plants followed the same trends of metal partitioning in the different plant parts as their respective controls (results not shown). Significantly different *in planta* metal concentrations were observed after inoculation (compared to control) in all experiments (Table 5.9). However, values after inoculation were not always higher and there were no bacterial strains consistently increasing or decreasing Cd and/or Zn accumulation in more than 1 plant part.

The field cuttings used to set up inoculation experiment 2014 already contained significant amounts of Cd, Zn and Pb (Tables 5.7 and 5.8). In case of Cd and Zn, concentrations in *S. viminalis* cuttings were considerably higher than those in *S. alba x alba* cuttings (respectively 126% and 80% higher). When comparing Cd, Zn and Pb concentrations in cuttings before the experiment with concentrations measured after 70 days of growth in pots, it was observed that for *S. viminalis* the Cd, Zn and Pb concentrations in the cutting increased by performing the pot trial while for *S. alba x alba* the Cd and Zn concentrations in the cuttings decreased.

The bioconcentration factors (BCFs) and translocation factors (TFs) of Cd and Zn for *S. viminalis* and *S. alba x alba* control plants for all inoculation experiments are listed in Table 5.10. BCFs and TFs varied considerably between experiments but some general observations could be made. BCFs were > 1 except in 1 case (*S. alba x alba* in 2014) while TFs > 1 were only found for *S. alba x alba* in 2012A and 2013. The *S. viminalis* clone showed a higher bioconcentration of Cd and Zn than *S. alba x alba*, but the latter one had a clearly more efficient metal translocation from roots to twigs in 3 out of 4 cases (not in 2014). Finally, for both clones, the bioconcentration and translocation of Cd are (slightly) more efficient than the bioconcentration and translocation of Zn. The BCF and TF of Pb could not be calculated due to the unreliability of Pb concentrations measured in the twigs.

For both clones, no significant differences in Cd and Zn bioconcentration or translocation between control and inoculated conditions were observed for experiments 2012A, 2013 and 2014 (BCFs and TFs for inoculated conditions not shown). For experiment 2012B, significantly lower ($p < 0.05$) BCFs of Cd were found after inoculating *S. viminalis* cuttings with strains Rh2, Rh4 and Ro4 (respective values 1.82 ± 0.18, 1.80 ± 0.14 and 1.85 ± 0.09) and a significantly lower ($p < 0.05$) TF of Cd and Zn was observed after inoculating *S. viminalis* cuttings with strain Rh2 (respective values 0.17 ± 0.01 and 0.23 ± 0.03).

Table 5.7 Metal (Cd, Zn, Pb) concentrations (mg kg^{-1} DW) in roots, cutting, twigs and leaves of *S. viminalis* control plants for all inoculation experiments. Metal (Cd, Zn, Pb) concentrations in cuttings used to set up inoculation experiment 2014 (field cuttings, before planting in soil in pots) are given below the dashed line.

***S. viminalis* Control**	**Cd (mg kg^{-1} DW)**				**Zn (mg kg^{-1} DW)**				**Pb (mg kg^{-1} DW)**	
Inoculation experiment	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**
2012A	45.81 ± 7.85 ^{a}	17.77 ± 3.76 ^{b}	19.91 ± 4.71 ^{b}	33.01 ± 9.65 ^{c}	1527.75 ± 121.80 ^{a}	447.16 ± 19.73 ^{b}	685.60 ± 102.25 ^{c}	1690.42 ± 328.47 ^{a}	181.86 ± 22.89 ^{a}	9.78 ± 3.06 ^{b}
2012B	36.98 ± 5.94 ^{a}	21.52 ± 3.94 ^{b}	9.04 ± 0.74 ^{c}	21.80 ± 5.19 ^{b}	947.46 ± 63.02 ^{a}	528.94 ± 45.06 ^{b}	281.64 ± 12.04 ^{c}	1276.96 ± 309.75 ^{d}	123.28 ± 35.25 ^{a}	8.49 ± 1.94 ^{b}
2013	20.70 ± 3.33 ^{a}	14.94 ± 1.64 ^{b}	16.51 ± 2.44 ^{b}	26.81 ± 4.77 ^{c}	958.39 ± 148.84 ^{a}	420.82 ± 55.75 ^{b}	503.05 ± 86.53 ^{b}	1763.74 ± 298.50 ^{c}	68.67 ± 15.41 ^{a}	5.87 ± 1.34 ^{b}
2014	36.88 ± 5.27 ^{a}	18.14 ± 2.21 ^{b}	13.35 ± 1.18 ^{c}	20.16 ± 1.61 ^{b}	1440.30 ± 172.55 ^{a}	527.69 ± 23.15 ^{b}	458.07 ± 41.16 ^{c}	1246.34 ± 125.20 ^{a}	123.22 ± 27.49 ^{a}	6.23 ± 2.07 ^{b}
2014 field cuttings		16.29 ± 0.96				480.80 ± 19.75				3.54 ± 2.93

Values are mean ± standard deviation of 6 (2012A, 2012B, 2014) or 10 (2013) biological replicates. Different superscripts indicate significantly different metal concentrations between the plant parts of a certain control at the level $p < 0.01$ (Cd and Zn) or $p < 0.001$ (Pb).

Table 5.8 Metal (Cd, Zn, Pb) concentrations (mg kg^{-1} DW) in roots, cutting, twigs and leaves of *S. alba x alba* control plants for all inoculation experiments. Metal (Cd, Zn, Pb) concentrations in cuttings used to set up inoculation experiment 2014 (field cuttings, before planting in soil in pots) are given below the dashed line.

***S. alba x alba* Control**	**Cd (mg kg^{-1} DW)**				**Zn (mg kg^{-1} DW)**				**Pb (mg kg^{-1} DW)**	
Inoculation experiment	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**
2012A	12.11 ± 1.13 ^{a}	11.27 ± 3.94 ^{a,b}	15.61 ± 2.19 ^{b}	22.77 ± 4.02 ^{c}	575.29 ± 41.18 ^{a}	253.97 ± 98.70 ^{b}	692.66 ± 202.45 ^{a}	1576.67 ± 245.11 ^{c}	132.31 ± 33.71 ^{a}	5.94 ± 1.33 ^{b}
2012B	11.04 ± 1.17 ^{a}	10.83 ± 1.67 ^{a}	8.19 ± 0.49 ^{b}	16.19 ± 2.06 ^{c}	440.31 ± 48.26 ^{a}	388.73 ± 79.39 ^{a}	240.31 ± 30.56 ^{b}	865.19 ± 89.61 ^{c}	71.01 ± 13.18 ^{a}	9.82 ± 2.77 ^{b}
2013	5.94 ± 0.70 ^{a}	5.68 ± 1.10 ^{a}	8.90 ± 1.11 ^{b}	12.10 ± 1.31 ^{c}	458.00 ± 33.58 ^{a}	257.53 ± 47.21 ^{b}	345.13 ± 32.95 ^{c}	945.75 ± 75.64 ^{d}	67.57 ± 16.65 ^{a}	3.78 ± 1.48 ^{b}
2014	12.21 ± 1.39 ^{a}	4.97 ± 0.71 ^{b}	3.28 ± 0.59 ^{c}	7.68 ± 0.73 ^{d}	865.90 ± 37.60 ^{a}	249.75 ± 26.72 ^{b}	203.14 ± 22.81 ^{c}	671.28 ± 82.19 ^{d}	128.39 ± 9.06 ^{a}	5.53 ± 1.41 ^{b}
2014 field cuttings		7.22 ± 0.83				266.87 ± 15.52				4.41 ± 0.81

Values are mean ± standard deviation of 6 (2012A, 2012B, 2014) or 10 (2013) biological replicates. Different superscripts indicate significantly different metal concentrations between the plant parts of a certain control at the level $p < 0.01$ (Cd) or $p < 0.001$ (Zn and Pb).

Table 5.9 Significantly different *in planta* metal (Cd, Zn, Pb) concentrations between inoculated and control conditions for all inoculation experiments.

Inoculation experiment	Clone	Inoculated strain	Metal in plant part	Value	Significantly higher/ lower; at the level
2012A	*S. viminalis*	Ro2	Zn in roots	1250.49 ± 31.26	lower; $p < 0.05$
2012B	*S. viminalis*	Rh2	Cd in twigs	7.31 ± 0.71	lower; $p < 0.05$
	S. viminalis	Rh4	Cd in twigs	7.23 ± 0.55	lower; $p < 0.01$
	S. viminalis	Ro3	Cd in twigs	7.63 ± 0.42	lower; $p < 0.05$
	S. viminalis	Ro4	Cd in twigs	7.45 ± 0.38	lower; $p < 0.05$
2013	*S. alba x alba*	Ro7	Zn in leaves	1098.06 ± 50.49	higher; $p < 0.01$
	S. alba x alba	Rh5	Pb in cutting	8.80 ± 2.06	higher; $p < 0.01$
	S. alba x alba	Ro6	Pb in cutting	8.21 ± 2.82	higher; $p < 0.05$
	S. alba x alba	Ro7	Pb in cutting	8.21 ± 2.08	higher; $p < 0.05$
2014	*S. viminalis*	Rh2	Cd in leaves	24.10 ± 1.19	higher; $p < 0.05$
	S. viminalis	Rh1	Pb in cutting	11.44 ± 2.88	higher; $p < 0.05$

Values are mean ± standard deviation of 3 (2012A and B, 2014) or 5 (2013) biological replicates.

Table 5.10 Bioconcentration factors (BCFs) and translocation factors (TFs) of Cd and Zn for *S. viminalis* and *S. alba x alba* control plants for all inoculation experiments. The BCF is defined as the ratio of metal concentration in twigs to total soil metal content while the TF is defined as the metal concentration in twigs to the metal concentration in roots.

Control	***S. viminalis***		***S. alba x alba***	
Inoculation experiment	**BCF Cd**	**BCF Zn**	**BCF Cd**	**BCF Zn**
2012A	4.95 ± 1.17	3.07 ± 0.46	3.88 ± 0.54	3.10 ± 0.91
2012B	2.25 ± 0.18	1.26 ± 0.05	2.04 ± 0.12	1.08 ± 0.14
2013	4.11 ± 0.61	2.25 ± 0.39	2.21 ± 0.28	1.55 ± 0.15
2014	1.93 ± 0.17	1.07 ± 0.10	0.47 ± 0.09	0.47 ± 0.05
	TF Cd	**TF Zn**	**TF Cd**	**TF Zn**
2012A	0.43 ± 0.04	0.45 ± 0.06	1.30 ± 0.28	1.20 ± 0.35
2012B	0.25 ± 0.04	0.30 ± 0.03	0.75 ± 0.09	0.55 ± 0.05
2013	0.79 ± 0.11	0.52 ± 0.09	1.52 ± 0.31	0.77 ± 0.14
2014	0.37 ± 0.06	0.32 ± 0.05	0.27 ± 0.04	0.23 ± 0.03

Values are mean ± standard deviation of 3 (2012A, 2012B, 2014) or 5 (2013) biological replicates.

Twig metal extraction

The extracted amounts of Cd and Zn by twigs of *S. viminalis* and *S. alba x alba* control and inoculated plants for every inoculation experiment are listed in Table 5.11. A few general observations could be made. Firstly, for *S. viminalis* control plants the extracted amounts of Cd and Zn were highest in 2013 and 2014, slightly less in 2012A and, by far, lowest in experiment 2012B. For control plants of *S. alba x alba*, extraction of Cd and Zn was highest in experiment 2013, slightly lower in 2012A, obviously lower in experiment 2014 and again, by far, lowest in experiment 2012B. Secondly, *S. viminalis* plants extracted at least 2 times more Cd and Zn than *S. alba x alba* plants in most cases. Thirdly, in all experiments and for all conditions, variation were high with standard deviations of roughly 30% of the mean value.

Some slight (0-10%), medium (11-30%) and strong (> 30%) improvements of Cd and Zn extraction were observed after inoculation in the different experiments. For *S. viminalis* plants, a strong improvement seemed to occur after inoculation with isolate Rh1 in experiment 2012A, a medium increment after inoculating strains Rh2 (experiment 2021B) and Ro8 (experiment 2013) and a slight improvement after inoculation with isolates Tw2 (2012A), Rh3 (2012B), Ro7 (2013) and Rh2 (2014). For the *S. alba x alba* clone, slight increments in Cd and Zn extraction potential tended to appear after inoculation with strains Rh2 (2012B), Tw1 (2014) and with the consortium in experiment 2013. Almost all other clone – bacterial strains combinations seemed to reduce (to different extents) Cd and Zn extraction of twigs.

Physico-chemical soil characteristics (including soil metal concentrations) at the end of inoculation experiment 2013

Soil (mixture of bulk and rhizosphere soil) characteristics after cultivating *S. viminalis* and *S. alba x alba* control plants after 90 days in pots in experiment 2013 are summarized in Table 5.12. In general, actual (pH-H_2O) and potential (pH-KCl) acidity of the soil did not change much in comparison with values measured before the experiment while the CEC_e decreased and the EC increased significantly. Pseudo-total Cd, Zn and Pb concentrations decreased (significantly)

compared to the start of the experiment as was also the case for $CaCl_2$-exchangeable Cd and Zn.

For both clones, no significant differences in pH (H_2O and KCl), CEC_e, EC, pseudo-total and $CaCl_2$-exchangeable metal concentrations between control and inoculated conditions were observed (results of inoculated conditions not shown).

Re-isolation of inoculated bacterial strains

After identifying re-isolated strains that are morphologically similar to the inoculated ones, it revealed that none of the inoculated strains could be re-isolated from the rhizosphere, roots or twigs of inoculated plants in experiments 2012A as well as 2012B. Results are therefore not presented.

Table 5.11 Cadmium and Zn extracted by twigs (mg plant^{-1} 60-90 days^{-1}) of inoculated and control plants of *S. viminalis* (left columns) and *S. alba x alba* (right columns) for all inoculation experiments.

		S. viminalis		***S. alba x alba***	
Inoculation experiment	**Condition**	**Extracted Cd (mg plant^{-1} 90 days^{-1})**	**Extracted Zn (mg plant^{-1} 90 days^{-1})**	**Extracted Cd (mg plant^{-1} 90 days^{-1})**	**Extracted Zn (mg plant^{-1} 90 days^{-1})**
2012A	**Control**	0.036 ± 0.012	1.238 ± 0.339	0.016 ± 0.010	0.698 ± 0.494
	Rh1	0.061 ± 0.023 (+69%)	1.816 ± 0.709 (+47%)	0.012 ± 0.008 (-23%)	0.749 ± 0.561 (+7%)
	Ro1	0.026 ± 0.008 (-26%)	0.806 ± 0.286 (-35%)	0.014 ± 0.007 (-13%)	0.534 ± 0.268 (-23%)
	Ro2	0.018 ± 0.006 (-46%)	0.594 ± 0.146 (-52%)	0.013 ± 0.007 (-15%)	0.528 ± 0.324 (-24%)
	Tw1	0.025 ± 0.012 (-29%)	0.795 ± 0.373 (-36%)	0.015 ± 0.008 (-3%)	0.622 ± 0.331 (-11%)
	Tw2	0.040 ± 0.012 (+11%)	1.290 ± 0.402 (+4%)	0.012 ± 0.006 (-21%)	0.563 ± 0.298 (-19%)
		Extracted Cd (mg plant^{-1} 60 days^{-1})	**Extracted Zn (mg plant^{-1} 60 days^{-1})**	**Extracted Cd (mg plant^{-1} 60 days^{-1})**	**Extracted Zn (mg plant^{-1} 60 days^{-1})**
2012B	**Control**	0.010 ± 0.006	0.309 ± 0.184	0.004 ± 0.002	0.112 ± 0.050
	Rh2	0.011 ± 0.004 (+15%)	0.368 ± 0.125 (+19%)	0.004 ± 0.002 (+8%)	0.117 ± 0.054 (+4%)
	Rh3	0.011 ± 0.003 (+7%)	0.336 ± 0.089 (+9%)	0.002 ± 0.002 (-37%)	0.077 ± 0.050 (-32%)
	Rh4	0.007 ± 0.004 (-30%)	0.296 ± 0.186 (-4%)	0.004 ± 0.002 (+7%)	0.108 ± 0.053 (-4%)
	Ro3	0.010 ± 0.005 (+1%)	0.304 ± 0.167 (-2%)	0.003 ± 0.001 (-29%)	0.077 ± 0.047 (-32%)
	Ro4	0.008 ± 0.003 (-16%)	0.274 ± 0.106 (-11%)	0.004 ± 0.002 (+3%)	0.093 ± 0.054 (-17%)
	Ro5	0.009 ± 0.005 (-6%)	0.354 ± 0.194 (+15%)	0.004 ± 0.002 (-2%)	0.102 ± 0.057 (-9%)

		S. viminalis		***S. alba x alba***	

Inoculation experiment	Condition	Extracted Cd (mg plant^{-1} 90 days^{-1})	Extracted Zn (mg plant^{-1} 90 days^{-1})	Extracted Cd (mg plant^{-1} 90 days^{-1})	Extracted Zn (mg plant^{-1} 90 days^{-1})
2013	**Control**	0.046 ± 0.015	1.390 ± 0.486	0.019 ± 0.004	0.748 ± 0.143
	Rh5	0.041 ± 0.016 (-9%)	1.346 ± 0.518 (-3%)	0.020 ± 0.007 (+3%)	0.698 ± 0.266 (-7%)
	Rh6	0.044 ± 0.004 (-5%)	1.330 ± 0.115 (-4%)	0.019 ± 0.007 (+0%)	0.720 ± 0.254 (-4%)
	Ro6	0.044 ± 0.013 (-4%)	1.362 ± 0.408 (-2%)	0.019 ± 0.006 (+1%)	0.712 ± 0.222 (-5%)
	Ro7	0.048 ± 0.012 (+6%)	1.478 ± 0.410 (+6%)	0.020 ± 0.008 (+2%)	0.743 ± 0.299 (-1%)
	Ro8	0.053 ± 0.018 (+17%)	1.639 ± 0.529 (+18%)	0.019 ± 0.007 (-1%)	0.741 ± 0.262 (-1%)
	Ro9	0.034 ± 0.017 (-25%)	1.117 ± 0.560 (-20%)	0.019 ± 0.005 (-3%)	0.686 ± 0.203 (-8%)
	CONS	0.042 ± 0.010 (-9%)	1.370 ± 0.343 (-1%)	0.020 ± 0.006 (+6%)	0.786 ± 0.234 (+5%)
		Extracted Cd (mg plant^{-1} 70 days^{-1})	**Extracted Zn (mg plant^{-1} 70 days^{-1})**	**Extracted Cd (mg plant^{-1} 70 days^{-1})**	**Extracted Zn (mg plant^{-1} 70 days^{-1})**
2014	**Control**	0.043 ± 0.009	1.466 ± 0.297	0.008 ± 0.002	0.479 ± 0.133
	Rh1	0.042 ± 0.008 (-3%)	1.442 ± 0.290 (-2%)		
	Rh2	0.044 ± 0.008 (+3%)	1.498 ± 0.299 (+2%)		
	Rh4			0.007 ± 0.003 (-5%)	0.427 ± 0.165 (-11%)
	Tw1			0.009 ± 0.004 (+20%)	0.513 ± 0.249 (+7%)

Values are mean ± standard deviation. Grey shades indicate a higher Cd and Zn extraction compared to respective controls (light grey: slight improvement (0-10%), middle grey: medium improvement (11-30%) and dark grey: high improvement (> 30%)).

Table 5.12 Physico-chemical characteristics of the soil (mixture of bulk and rhizosphere soil) in pots of control plants of *S. viminalis* and *S. alba x alba* at the end of inoculation experiment 2013. The values occurring at the start of inoculation experiment 2013 (first row) were copied from Table 5.6.

Soil sampling	Clone	pH-H_2O	pH-KCl	CEC_e (meq 100 g^{-1})	EC (µS cm^{-1})	Pseudo-total metal concentrations (mg kg^{-1} dry soil)			$CaCl_2$-exchangeable metal concentrations (mg kg^{-1} dry soil)		
						Cd	Zn	Pb	Cd	Zn	Pb
At the start of inoculation experiment 2013		6.64 ± 0.09	5.56 ± 0.08	7.10 ± 0.70	52.07 ± 4.46	4.02 ± 0.45	223.34 ± 20.99	144.04 ± 11.90	0.24 ± 0.06	11.27 ± 3.70	0.14 ± 0.02
At the end of inoculation experiment 2013	**S. viminalis**	6.45 ± 0.09*	5.43 ± 0.22	4.97 ± 0.11***	72.52 ± 3.83***	2.67 ± 0.09**	184.80 ± 4.10	131.85 ± 0.14	0.17 ± 0.02**	7.32 ± 1.32*	0.19 ± 0.03**
	S. alba x alba	6.59 ± 0.14	5.75 ± 0.24	5.60 ± 0.12*	65.42 ± 7.88**	2.62 ± 0.16**	181.14 ± 12.84	128.50 ± 10.08	0.17 ± 0.01**	7.05 ± 0.61**	0.14 ± 0.03

Values are mean ± standard deviation of 6 independent replicates. Stars indicate a significantly different mean value at the end of the experiment compared to at the start of the experiment (significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.II.4 Discussion

A second approach for improving metal phytoextraction is inoculation, or bioaugmentation, of the previously selected clones with beneficial plant-associated bacteria. In order to select these advantageous bacterial strains, all cultivable plant-associated bacteria from rhizosphere, root and twig of the selected *Salix* clones were isolated and genotypically and phenotypically characterized. The cultivable community is in general reported to represent 0.3% of the total bacterial community in case of soil (Amann *et al.* 1995) and 0.001-0.1% of the total endophytic bacterial community in plant tissues (Torsvik and Øvreås 2002; Alain and Querellou 2009).

5.II.4.1 Genotypic characterization of the cultivable plant-associated communities of the *S. viminalis* and the *S. alba x alba* clone

The genotypic identification was additionally used to gain more insight in the structures of the cultivable plant-associated communities and the origin and behavior of specific genera (Figure 5.1). The decreasing number of cultivable bacteria recovered from rhizosphere to roots to twigs for both clones is consistent with most reports in literature (Benizri *et al.* 2001; Hallmann 2001; Compant *et al.* 2010) and previous investigations in our laboratory (Croes *et al.* 2013; Weyens *et al.* 2013b). Also the orders of magnitude of cultivable bacteria found in rhizosphere and roots correspond with above-mentioned literature. However, the total amounts of colony forming units isolated from the twigs are rather low for both clones. Many endophytic genera isolated from the roots of both clones were also present in their rhizospheres. Common strains of these genera most likely colonized the roots from the rhizosphere by penetrating the root cortex (Compant *et al.* 2010), especially during side root formation. These strains can be considered as facultative endophytes. Several studies confirm that many endophytic bacteria derive from the rhizosphere (Sessitsch *et al.* 2002; Berg *et al.* 2005; Compant *et al.* 2005; Hardoim *et al.* 2008). Following root colonization, endophytes may colonize various plant organs, which explains the presence of common species in rhizosphere, roots and twigs for both clones.

A number of genera of the *S. viminalis* and the *S. alba x alba* clone were only found as endophytes in roots and/or twigs but not in the rhizosphere. The presence of these species can be due to: (i) they were already present inside the cuttings planted in 2006 and spread over the new emerging roots and twigs from the cutting; (ii) these bacteria originate from the caulosphere or phyllosphere and colonized twigs and/or roots after entering respectively stem or leaf tissue (Berg *et al.* 2005; Compant *et al.* 2010); (iii) the presence of viable but not cultivable bacteria in some tissues while the same species are cultivable from other tissues (Hurek *et al.* 2002; Compant *et al.* 2010). It cannot be excluded that the absence of taxa isolated from roots and/or twigs in the rhizosphere is due to the detection limit of the isolation procedure.

The genotypic identification of isolated strains revealed other interesting information. While the rhizosphere and twig populations of both clones possess respectively several and only a few common genera, their root communities consist of almost all the same genera (Figure 5.1). This probably is the result of a combination of a strong selection by willow regarding bacteria entering from the rhizosphere and/or caulosphere as well as a common population in the cuttings used to establish the field years ago. The genera diversity, that is highest in the roots for both clones, supports this hypothesis. Relatively distinct rhizosphere and twig populations of willow clones growing on the same field as well as willow-associated bacteria comprising high clone or compartment specificity were also reported by Weyens *et al.* (2013b). It is suggested that this results from 2 selection steps: (1) plant rhizodeposits mediate a substrate-driven community shift in the rhizosphere and (ii) the host genotype immune system fine-tunes the microbial profile in the selection of root endophyte assemblages (Bulgarelli *et al.* 2013).

5.II.4.2 Phenotypic characterization of the cultivable plant-associated communities of the *S. viminalis* and the *S. alba x alba* clone

The phenotypic characterization mainly served as a screening procedure aiming to identify potentially advantageous isolates. Furthermore, it provides a general overview of the traits present in the cultivable plant-associated communities of

the selected willow clones on the metal-contaminated field (Table 5.4). It is postulated that IAA and ACC deaminase producing bacteria can promote plant growth and root development (Taghavi *et al.* 2009). IAA indeed can promote root growth and induces proliferation and elongation of root hairs, resulting in a larger root surface and hence the possibility to take up more water and nutrients (Patten and Glick 2002). The high percentages of isolates producing IAA in both clones might to a certain extent contribute to extending the root system and promoting growth of plants on metal-contaminated soil. Bacterial ACC deaminase activity can lower stress ethylene levels in the plant under harmful conditions such as the presence of toxic metal concentrations (Glick *et al.* 2007). In both clones, strains able to produce ACC deaminase were found which could be of importance for plants growing on the metal-contaminated soil reducing metal-induced stress ethylene and thus the adverse effects of increased ethylene levels.

Besides growth promotion, bacterial strains producing compounds that can mobilize metals in the soil near the roots could also improve efficiency of metal phytoextraction. Indeed, siderophores and organic acids producing bacteria, to a certain amount present in the rhizosphere and roots of both clones, can increase the concentrations of plant-available metals resulting in an increased metal uptake (Braud *et al.* 2009; Saravanan *et al.* 2007). However, willows taking up too much Cd and Zn from the contaminated soil may suffer from phytotoxicity since these elements can interfere with the normal cellular processes. Metal-resistant bacteria can sequester and detoxify metals for themselves but also reduce the internal availability of metals in their host plant (Lodewyckx *et al.* 2001). The percentages of strains tolerant for Cd and Zn do presume that tolerant endophytic strains for the inoculation experiment are numerous. Strains belonging to different taxa possessing above-mentioned features were isolated from rhizosphere, roots and twigs of both clones. This natural enrichment of plant-associated bacteria able to cope with a selecting factor is consistent with earlier reports (van der Lelie 1998; Siciliano *et al.* 2001; Sessitsch and Puschenreiter 2008; Barac *et al.* 2009; Weyens *et al.* 2009c).

Results above are derived from the first *in vitro* phenotypic screening in 2011. A strong decline in positive test results was however observed when evaluating

the second phenotypic screening in 2013 for the about 100 most promising strains (Supplementary table 5.3 p.269). It might suggest that bacterial strains happen to lose their beneficial characteristics if conserved in the freezer (in glycerol) in absence of selecting factors. In addition, the number of viable cultivable cells decreased after storage in glycerol. In case of endophytes, this was reported earlier in our laboratory by Eevers *et al.* (2015).

5.II.4.3 Relation between genotypic and phenotypic characterization

When evaluating the relative positive test results of the isolated genera for production of plant growth promoting and metal uptake enhancing traits as well as for metal tolerance (Supplementary tables 5.1 and 5.2; pp.255-268, Table 5.4), some observations could be made. Firstly, although the abundance (CFU g^{-1} of rhizosphere soil of root or twig fresh weight) is a major factor in the calculations of percentages of positive test results, the prominent or negligible role of a genus given a certain phenotypic trait and compartment is still detectable to a certain extent. Secondly, given a certain phenotypic trait and compartment, there is in general no genus or combination of genera specified to play a substantial role in both clones. It could therefore be concluded that the role of a genus in increasing plant growth, metal uptake and/or metal tolerance (ranging from highly important to negligible) is depending on clone, compartment, phenotypic trait and estimated number of CFU g^{-1}. As a result, there is no single genus (or a list of a few genera) assigned as preferable to select beneficial plant-associated bacteria from.

5.II.4.4 Bioaugmenting the *S. viminalis* and *S. alba x alba* clone with beneficial plant-associated bacteria

In order to evaluate the effect of bioaugmenting the previously selected clones with beneficial plant-associated bacteria, inoculation experiments with selected strains and cuttings of *S. viminalis* and *S. alba x alba* clones were performed. The selection of bacterial strains for the inoculation experiments was based on their genotype, abundance and presence of phenotypic characteristics (Table 5.5) as described in 'Material and methods' section. Although all selected strains

were able to produce ACC deaminase and/or IAA, and eventually show nitrogenase and phosphate solubilizing activity, effects of inoculation on biomass production only sometimes seemed to be (slightly) beneficial (Figures 5.2-5.5). Moreover, effects were highly depending on the clone-bacteria combination, the evaluated plant part and the experimental set up. Despite the capability of all selected strains to produce organic acids and/or siderophores, significant increases in *in planta* metal concentrations after inoculation occurred only occasionally (Table 5.9). Since increases were solely observed for either Cd or Zn as well as only for a certain part of the plant, it can be concluded that there were no consistent effects of inoculation on *in planta* Cd and Zn concentrations in any of the experiments. Furthermore, in general, the bioconcentration factors (BCFs) and translocation factors (TFs) of Cd and Zn were similar for control and inoculated plants (Table 5.10). Besides some exceptions in inoculation experiment 2012B, no significant differences were found in this research.

Although there were no frequent, consistent beneficial effects of the inoculated strains on biomass production or metal accumulation, and even though effects seemed to be bacteria, clone and organ specific, increments in twig Cd and Zn extraction were found after inoculation for both clones and in all experiments (Table 5.11). However, there are a few considerations to make. Firstly, variation on extracted amounts of Cd and Zn were high in all cases (standard deviations of about 30% of the mean value), which especially doubts the slight (0-10%) and medium (11-30%) increments found. Secondly, the only strong (> 30%; given the first consideration, this one could be assumed valid) increase in Cd and Zn extraction observed, was the combination of *S. viminalis* cuttings with bacterial strain *Rahnella sp.* (Rh1) in experiment 2012A. However, when this promising strain was evaluated a second time in 2014, it could not at all redeem its promises. When taken into account that the bacterial strains were, besides added during the rooting stage (like in 2012A), additionally poured in the pots every 2 weeks, results are not promising. We might therefore conclude that there is no conclusive evidence for improved metal phytoextraction of the selected willow clones by exploiting the 17 'promising' bacterial strains evaluated here. Possible reasons for failure of bacteria-enhanced phytoextraction in this research are: (i) the inoculated bacterial strains, cultivated in nutrient rich and

wet conditions, might simply have died when inoculated (during rooting stage and in soil in pots) due to a new environment that is potentially experienced as very stressful (personal communication Montesinos). Furthermore, the soil used in the experiments was not sterilized to mimic the *in situ* situation. Therefore, successful bioaugmentation with potential beneficial bacterial strains, even though relatively abundant species were selected, is not evident since competition with trillions of indigenous bacteria occurs (Lugtenberg *et al.* 2001). McLoughlin (1994) described loss of microbial survival after soil inoculation (*i.e.* by competition, grazing and desiccation) is the main drawback of bioaugmentation. (ii) In case of survival of inoculated endophytic strains, the next crucial step required for exhibiting advantageous effects inside the plant, is a sufficient plant colonization (Lugtenberg and Kamilova 2009). The inoculated strains could not be re-isolated from roots, cuttings or twigs with the method used. If colonization was successful or not, could, by consequence, not be confirmed. (iii) If assumed survival and colonization of bioaugmented strains was accomplished successfully, it remains still uncertain to what extent the phenotypic characteristics expressed *in vitro* are exposed *in vivo*. Furthermore, the qualitative assessment of phenotypic traits does not always provide conclusive evidence for the presence of a trait (for example false positives might occur due to multiple underlying mechanisms causing color change). Ambiguous results after inoculation were reported earlier by Weyens *et al.* (2013a). Likewise, Lugtenberg and Kamilova (2009) described lower numbers of success after inoculation from laboratory scale to greenhouse and field conditions.

5.II.4.5 Phytoextraction of Pb

The phytoextraction potential of Pb by twigs could not be calculated due to unreliably low concentrations measured in the twigs. A combination of 2 things might explain this. Firstly, Pb is strongly bound to soil organic matter and soil minerals and therefore mainly precipitated and less bioavailable than for example Cd and Zn (Puschenreiter *et al.* 2001). This was also observed in the Lommel soils which were used. Although a significant amount of Pb was present in the soils (Table 5.6; background value for Pb in Flanders is 38 mg kg^{-1} DW soil (see Chapter 1: Table 1.1)), the availability revealed to be very low (Table

5.6: $CaCl_2$-exchangeablility of Pb is 0.10-0.14% of total Pb in soil *cf.* Cd and Zn 5-6%; Chapter 4: Table 4.1: water availability of Pb is 0.12-0.13% of total Pb in soil *cf.* Cd and Zn 0.43-0.90%). The low 'bioavailability' and the slow diffusion of this element in the soil suggests the Pb uptake by plants to be limited, as confirmed in this study (Tables 5.7 and 5.8). Secondly, most of the Pb taken up by the *Salix* clones was accumulated in the roots with an extremely low translocation to aboveground plant parts. Root systems acting as Pb sink were reported repeatedly in case of willow (Pulford and Watson 2003; Fischerová *et al.* 2006). In conclusion, the phytoextraction of this element by willow is considered insufficient. However, the low bioavailability in the soil, and even increased inactivation by a vegetation cover (Chaney *et al.* 1997), makes that concentrations of Pb in soil even exceeding remediation thresholds rarely cause problems of agricultural or health-related kind.

5.II.4.6 Differences in experimental conditions and possible influences on twig metal extraction efficiency

To take into account possible effects of experimental conditions on twig metal extraction efficiency when juxtaposing different experiments, results of the control plants of all 4 inoculation experiments are compared and discussed here. Biomass production, metal accumulation, BCFs and TFs vary considerably over the experiments. This is related to the different conditions of the experiments, *i.e.* growth environment of the plants, season when performing the experiment as well as climatological conditions in the season, contamination level of the soil used, water supply, duration of the experiment and origin of the cuttings used to establish the pot trials (Tables 2 and 5). Moreover, the 2 clones as well as the different plant parts seem to react in different ways in the different experiments. Regarding twig biomass production, for both clones best results were found in experiments 2013 and 2014 (Figures 5.2-5.5). Experimental conditions like an outdoor environment, summer season and cuttings originating from 'mother' plants growing on the metal-contaminated field might therefore be considered to stimulate twig production, although not known to what extent each of them will. On the contrary, the level of soil metal contamination (2013 *vs.* 2014, Table 5.6) does not seem to influence twig production. A combination of other factors like

the higher EC in the 2014 soil or differences in climatic conditions during summer might correlate with the higher twig biomass in the shorter (2014: 70 days *vs.* 2013: 90 days) experiment. However, the 4 different experiments with their set of experimental conditions do not allow revealing the relation between metal uptake/translocation and experimental conditions. Moreover, BCF and TF are, besides depending on experimental conditions and clone also a function of parameters like chemical form and concentration of the metal (Lebeau *et al.* 2008). However, some things can be said. Compared with outside conditions (2012B, 2013 and 2014), in the greenhouse (2012A) temperature (especially of the soil) and humidity are continuously rather high. These factors might increase the mobility of metals in the pots and subsequent uptake by plants (internal communication Vangronsveld). Also the origin of the cuttings to establish the pot trials has an influence on metal issues. While the cuttings from the INBO stock plantation (2012A) can be considered metal-free at the onset of the experiment, field cuttings (2012B, 2013 and 2014) already contain considerable amounts of Cd, Zn and Pb (Tables 5.7 and 5.8). When using the latter one to carry out a pot trial, metal concentrations in the developing twigs and leaves as well as BCFs and TFs will be different than when starting with metal-free cuttings. Finally, a higher contamination level of the soil (and higher $CaCl_2$-available concentrations of Cd, Zn and Pb) (experiment 2014) does not seem to result in a proportionally higher metal uptake. For *S. alba x alba*, Cd and Zn concentrations in cutting, twigs and leaves were even lower when planted on the higher contaminated soil. Although it is reported in literature that a significant positive relationship between available metal fraction in the soil and metal concentrations in plants exists when growing willow in pots (Vandecasteele *et al.* 2005; Cloutier-Hurteau *et al.* 2014), other differences in experimental conditions between experiments do not allow to confirm this observation. BCFs and TFs are considerably lower for plants on the higher contaminated soil. Decreasing BCFs with increasing contamination levels were also reported in literature (Dickinson and Pulford 2005; Lebeau *et al.* 2008). In this research, for both clones highest twig Cd and Zn concentrations and highest BCFs and TFs were found in the 90-days greenhouse experiment 2012A performed in (early) spring using INBO stock cuttings. Since twig biomass production and twig metal accumulation vary

considerably for control plants over the experiments as a result of different experimental conditions, the extracted amount of metals by twigs also fluctuates highly and will not be further discussed.

5.II.4.7 Clonal differences in pot trials and comparison with field data

The discussion above clearly indicates that comparing results of the different experiments is not reasonable. However, recurring differences between the *S. viminalis* and *S. alba x alba* clone in all experiments, might represent true clonal differences. Discrepancies found between control plants in all experiments are summarized below and compared to characteristics of the clones in the field situation. In all experiments, the *S. viminalis* clone produced more twig biomass than *S. alba x alba* (Figures 5.2-5.5). This is in contrast with observations on the Lommel field where stem biomass production of the *S. alba x alba* clone is significantly higher than of the *S. viminalis* clone after 4 growing seasons (see PART I: Table 5.1). It seems that in the very early stages of development *S. alba x alba* is allocating more energy for developing an extended root system. This was in particular observed in the 90-days experiments (2012A and 2013). Rapid and slow starters and differences in biomass allocation to twigs/leaves or roots among willow clones were also reported by Weih and Nordh (2002).

Regarding Cd and Zn concentrations in twigs (and other plant parts), *S. viminalis* was again performing obviously better than *S. alba x alba* in the pot trials (Tables 5.7 and 5.8). This is in line with results from the field (see PART I: Table 5.1). It should however be mentioned that the concentrations of Cd and Zn in cuttings and twigs are lower than the concentrations in stems on the field. A combination of both factors described underneath might offer an explanation to this concern. (i) The short time for the pot trials not only represents a limited time for metal uptake but very likely also causes the rhizosphere to be not yet well developed. Cloutier-Hurteau *et al.* (2014) reported that willows grown for a few months in pots acquire their nutrients (and metals) from the most available and soluble soil pools and that the rhizosphere will develop more during subsequent growing seasons, leading to increased soil metal solubility and uptake by willows. (ii) The pseudo-total metal concentrations in the soil in pots

are lower than those on the field at the location of *S. viminalis* and *S. alba x alba* cultivation (block 2: 4.95 ± 0.23 mg Cd and 390.65 ± 22.41 mg Zn kg^{-1} DW soil, block 9: 7.26 ± 0.35 mg Cd and 488.12 ± 32.28 mg Zn kg^{-1} DW soil) (measurements of metal concentrations in stems of both clones after 4 growing seasons (PART I: Table 5.1) are based on a wood mixture of individuals from both blocks). Moreover, at the end of experiment 2013, parts of the Cd, Zn and Pb seemed to have leached since the decline in pseudo-total metal concentrations (Table 5.13) cannot be explained by metal uptake in willow plants (a *S. viminalis* control plant extracts 0.33 mg Cd and 11.89 mg Zn in total, a *S. alba x alba* control plant 0.14 mg Cd and 8.01 mg Zn). Thus, over time, metal concentrations in the pots are even lower than originally applied. Both clones clearly prefer to accumulate extracted metals in leaves and/or roots and not in the woody plant parts. Higher Cd and Zn concentrations in the leaves compared to the stems of commercial *Salix* clones on the metal-contaminated field in Lommel were also reported by Van Slycken *et al.* (2013).

When comparing the bioconcentration of Cd and Zn in pot experiments (Table 5.10) with BCFs examined on field scale (Table 5.2), these were not only in the same range but also the same major trends were observed. Firstly, an accumulation of Cd and Zn in twigs relative to the soil (BCF > 1) (Dickinson and Pulford 2005; Kötschau *et al.* 2014) generally happened. Secondly, *S. viminalis* exposed a higher bioconcentration of Cd and Zn than *S. alba x alba*. Thirdly, the bioconcentration of Cd is (slightly) more efficient than the bioconcentration of Zn.

As a result of both, higher twig production and higher Cd and Zn contents in the twigs, *S. viminalis* plants extracted in general about twice as much Cd and Zn than S*. alba x alba* cuttings (Table 5.11). On the metal-contaminated field however, highest extraction potentials were obtained with *S. alba x alba* (see PART I: Table 5.1). The observations above, with the exception of BCF, clearly illustrate that performance of the selected willow clones on field-scale could not be predicted based on results in short-term pot trials.

PART III: Fertilization

5.III.1 Introduction

Plants need several nutrients for proper development and growth. The most important nutrient, accounting for 80% of nutrients taken up by the roots, is nitrogen (N), which is essential for leaf growth and overall development of the plant. The 2 other major macronutrients plants need are phosphorus (P) and potassium (K). Generally, P promotes the development of roots (formation of root hairs), buds, flowers, seeds and fruits, and K stimulates strong stem growth, winter-hardiness, disease resistance, movement of water in plants and promotion of flowering and fruiting. To a lesser extent, plants also need other macronutrients like calcium (Ca), magnesium (Mg) and sulfur (S) as well as small amounts of trace elements such as iron (Fe), manganese (Mn), copper (Cu), zinc (Zn)…

Repeatedly harvesting willow biomass implies the removal of significant quantities of nutrients from a site, resulting in nutrient depletion and decrease of willow productivity (Labrecque and Teodorescu 2003). Fertilizing these agricultural plantations is therefore required to maintain and eventually improve biomass production over the years (Zegada-Lizarazu *et al.* 2010; Dimitriou and Aronsson 2011; Kidd *et al.* 2015). In Sweden, the European leader in using willow SRC for bioenergy production, conventional inorganic fertilizers have been recommended (Dimitriou *et al.* 2011). Inorganic fertilizers are fertilizers containing mined or synthetically produced compounds and do not include carbon-containing compounds (except urea). In a long-term fertilization plan, the amount of fertilizer applied should be a function of nutrient status of the soil, nutrient removal by aboveground harvest and nutrient recycling by foliage litter (Labrecque and Teodorescu 2003). However, in practice, fertilization rates are mostly based on generalized amounts (*e.g.* in Sweden, 70 kg N ha^{-1} yr^{-1} during the first cutting cycle and 60-80 kg N ha^{-1} yr^{-1} in later cutting cycles (Mola-Yudego 2010)). Furthermore, since from a technical and economic point of view annually spreading is difficult, fertilizers are applied after planting and after each harvest (Labrecque and Teodorescu 2003; Dimitriou *et al.* 2011).

Improved nutrient availability is known to enhance the photosynthetic capacity of individual leaves (increased leaf N concentrations) and/or increase the amount and size of leaves, both resulting in a greater photosynthesis and thus a larger general productivity (Bowman and Conant 1994; Merilo *et al.* 2006). According to a survey in Sweden by Dimitriou *et al.* (2011), fertilized willow SRC plantations had on average 38% higher yield than non-fertilized SRC plantations. Labrecque and Theodorescu (2003) reported an 64% increase in biomass production of a *S. viminalis* clone on a sandy site after fertilization with sludge (100 kg N ha^{-1}). In addition, it is suggested (Hytönen 1994) and demonstrated (Labrecque and Teodorescu 2001, 2003) that the increase in productivity of willows after fertilization is more important on sites that are poor in nutrients (like sandy soils) than on nutrient-richer sites. Since N is considered the most important nutrient for plants, the response of SRC of willow to fertilization is mainly studied for N fertilizer applications. Parameters often investigated to this extent are: biomass production (dry weight (DW) production of leaves and stems), stem height and diameter, number of leaves/shoot, leaf DW/shoot, LAI/shoot, leaf N concentration and fertilization response (DW increment (kg) per added amount of N (kg)) (Bowman and Conant 1994; Labrecque and Teodorescu 2001; Lower and Orians 2003; Merilo *et al.* 2006; Aronsson *et al.* 2014). Less frequently examined but also of importance is root development and productivity. Root systems are not only essential to sequester soil nutrients but also to intercept a possible excess of elements and prevent nutrient leaching (Bowman and Conant 1994; Labrecque and Teodorescu 2001).
The effect of fertilizing willow SRC on photosynthetic canopy properties and biomass productivity levels was repeatedly investigated, mostly in the framework of bioenergy production. However, when willow is used for metal phytoextraction, fertilization might not only influence biomass yields but might also affect metal accumulation in the biomass. Wångstrand, Eriksson and Öborn (2007) described that fertilizer applications have an effect on soil conditions and can potentially affect the bioavailability of Cd in soil. Zaccheo, Crippa and Pasta (2006) even stated that the effects of plant N nutrition can be exploited to enhance the efficiency of Cd phytoextraction using sunflower. Other information regarding alterations in Cd concentrations as a result of N-fertilizer applications

focused mainly on the edible crops wheat and barley. However, the reported effects are not unambiguous. Landberg and Greger (2003) found Cd in wheat grains decreased with increasing N fertilization rate and N concentration. The authors explained the decrease by a dilution effect caused by the increase in biomass production. Gavi *et al.* (1997) found no effects of N fertilization on the grain Cd concentration while a positive response (higher Cd concentration with higher N fertilizer rate and higher N concentration in wheat and barley grain) was reported by others (Singh *et al.* 1992; Oliver *et al.* 1993; Grant *et al.* 1995; Mitchell *et al.* 2000; Wångstrand *et al.* 2007). Reported alterations in metal availability are related to ion exchange reactions in the soil solution, acidification of the soil and speciation and complexation of metals (Wångstrand *et al.* 2007). The type of fertilizer (and chemical form) and the amount applied are key factors (Kidd *et al.* 2015). For example, the different effects on external pH when N is applied as ammonium (NH_4^+) or nitrate (NO_3^-) to plants is well known (Bloom *et al.* 2002). NH_4^+-nutrition of higher plants results in rhizosphere acidification due to proton excretion by root cells (Haynes 1990; Hinsinger *et al.* 2003) and may result in a local metal mobilization (Zaccheo *et al.* 2006). The uptake of metals by plants is however not solely explained by availability of metals in the soil. Root kinetics and interactions between metals and added nutrients as well as between metals themselves (*e.g.* Cd-Zn and Cd-Fe interactions) play a role as well (Cloutier-Hurteau *et al.* 2014; Kidd *et al.* 2015). Another factor of prime importance is the availability of water. The uptake and use of water and nutrients (N) is frequently recognized to be strongly interrelated (Weih and Nordh 2002; Lower and Orians 2003; Weih *et al.* 2011).
Fertilizer applications are not only recommended to obtain biomass productivity levels of willow SRC on the longer term but also have potential to significantly improve biomass yields on the nutrient-poor, sandy soil on the metal-contaminated field of interest. Furthermore, although not yet investigated for willow, fertilization treatments might be exploited to increase metal mobility in the rhizosphere and subsequent uptake by plants. Regarding potential improvements in both parameters, fertilization is proposed as a third strategy to improve metal phytoextraction of willow SRC.

In this section, the effects of different commercial NPK-fertilizers and self-composed soil-adjusted fertilizer treatments on the phytoextraction efficiency of the selected *S. viminalis* and *S. alba x alba* clone were determined. Biomass production and metal accumulation of fertilized willow cuttings were evaluated in pot trials using the metal-contaminated soil originating from the field in Lommel as a substrate. Biomass effects were observed by counting number of leaves, measuring twig length and determining DW production of roots, twigs and leaves. Also the fertilization response was defined. Potential influences of fertilizer applications on physico-chemical soil characteristics and *in planta* metal concentrations were outlined as well.

5.III.2 Material and methods

5.III.2.1 Set up

Biomass production and metal accumulation of fertilized and control (not fertilized) *S. viminalis* and *S. alba x alba* cuttings were evaluated in 2 pot experiments (referred to as fertilization experiments 2013 and 2014), performed at Hasselt University (30 km from the field). Both experiments were performed outside (without cover) and lasted for 90 (2013) or 70 (2014) days. Details are given in Table 5.13.

5.III.2.2 Physico-chemical soil characteristics (including soil metal concentrations)

For both fertilization experiments, pots (4 L) were filled with 4.8 kg of 4 mm sieved, unsterilized topsoil (0-30 cm) from the contaminated field in Lommel. The soils used for these fertilization experiments are the same as for inoculation experiments 2013 and 2014. Soil characteristics (pseudo-total and plant available Cd, Zn and Pb concentrations, pH-H_2O, pH-KCl, electrical conductivity (EC) and effective cation exchange capacity (CEC_e) are listed in PART II, Table 5.6.

5.III.2.3 Cultivation and fertilization

Twenty cm cuttings of the selected clones were cut on the field from twigs of 'mother' plants growing in block 2 (see Chapter 3: Figure 3.1). Diameters of cuttings were measured and they were weighed (before rooting) to achieve experimental groups with the same mean cutting diameter and fresh weight. Numbers of replicates (cuttings) used for each examined fertilizer are listed in Table 5.13. A control group was formed by double as much cuttings and was treated in the same way except for the fertilization that was not performed. To allow root development, cuttings were placed in half strength aerated Hoagland's nutrient solution for 10 days. Thereafter they were planted in pots. Fertilizers were added to the soil in pots either by mixing with the soil volume (2013) or by scattering on the surface of the soil after planting of the cuttings (2014).

The selected fertilizers for fertilization experiment 2013 were named 'NKMg13' and 'OSMO'. NKMg13 refers to a self-composed fertilizer containing commercially available grains of nitrogen (N) (ammonium nitrate (NH_4NO_3); 27% N, 4% MgO) and potassium (K) (potassium chloride (KCl); 60% K_2O) and powdery magnesium (Mg) (100% MgO). The amount of elements added was based on nutrient requirements for willow cultivation and amount of (bioavailable) nutrients already present in the soil, resulting in an advice of 160 kg N ha^{-1} yr^{-1}, 150 kg K_2O ha^{-1} yr^{-1} and 110 kg MgO ha^{-1} yr^{-1} (analysis and advice by Soil Service of Belgium). OSMO refers to Substral Osmocote NPK 14-13-13, a commercial slow release (life time about 6 months) granular fertilizer for all kind of garden plants. It contains 14% N (7.2% NO_3-N, 6.8% NH_4-N), 13% P_2O_5, 13% K_2O and 7% SO_3. For fertilization experiment 2014, fertilizer treatments were named 'NKMg14' and 'YARA'. NKMg14 is the same self-composed fertilizer as in experiment 2013 but the amount of elements was calculated per surface instead of volume. YARA refers to Yara Opticrop NPK 17-4-13, a commercial granular fertilizer used in agriculture. It contains 17% N (6.8% NO_3-N, 10.2% NH_4-N), 4% P, 13% K, 1.3% Mg and 3% S. The amounts of elemental fertilizers added per pot are listed in Table 5.14.

Table 5.13 Practical details and number of replicates for fertilization experiments 2013 and 2014.

Fertilization experiment	Cutting origin/ Growth environment	Duration (days in pot)	Season	Fertilizers tested	Number of replicates (control)	Picture set up
2013	field cuttings/ outdoor open	90	June-September	NKMg13, OSMO	10 (20)	
2014	field cuttings/ outdoor open	70	June-September	NKMg14, YARA	6 (12)	

Table 5.14 The amount of elemental fertilizers per pot (g) for every fertilization treatment in fertilization experiments 2013 and 2014. The NKMg-fertilizers were self-composed based on recommended doses of N, K and Mg by the Soil Service of Belgium. OSMO refers to Substral Osmocote NPK 14-13-13 and YARA stands of Yara Opticrop NPK 17-4-13.

		Fertilization experiment 2013		**Fertilization experiment 2014**	
Element		**NKMg13[b]**	**OSMO[c]**	**NKMg14[d]**	**YARA[e]**
N	**Form [g N-fertilizer pot^{-1}][a]**	NH_4NO_3 (27% N) [0.20]	NH_4NO_3 (14% N) [1.68]	NH_4NO_3 (27% N) [0.55]	NH_4NO_3 (17% N) [0.55]
P	**Form [g P-fertilizer pot^{-1}] [a]**	/	P_2O_5 (13% P) [1.56]	/	P (4% P) [0.13]
K	**Form [g K-fertilizer pot^{-1}] [a]**	KCl (60% K_2O) [0.19]	K_2O (13% K) [1.56]	KCl (60% K_2O) [0.52]	K (13% K) [0.42]
Mg	**Form [g Mg-fertilizer pot^{-1}] [a]**	MgO (100% MgO) [0.14]	/	MgO (100% MgO) [0.38]	Mg (1.3% Mg) [0.04]
S	**Form [g S-fertilizer pot^{-1}] [a]**	/	SO_3 (7% S) [1.56]	/	S (3% S) [0.10]

[a] effective amount of elemental fertilizer added, *e.g.* in case of NKMg13, 0.20 g of N-fertilizer added corresponds to 0.74 g of NH_4NO_3 (27% N) grains added

[b] based on a dose of 160 kg N, 150 kg K_2O and 110 kg MgO per year and per ha-volume agriculturally active (surface of 1 ha, depth of 0.3 m and soil density of 1200 kg m^{-3}).

[c] based on a dose of 3 g L^{-1} as recommended by the manufacturer for pot plants.

[d] based on a dose of 160 kg N, 150 kg K_2O and 110 kg MgO per year and per ha-surface.

[e] based on a dose of 160 kg N per year and per ha-surface.

5.III.2.4 Maintenance

All plants received the same amount of tap water when watering was needed. On every cutting, all emerging shoots were allowed to develop.

5.III.2.5 Evaluation

Biomass production

Just before harvesting the plants, the length of all developed twigs was measured and the number of leaves per twig was determined.

At harvest, leaves, twigs, cutting and roots were separated and cutting and roots were washed thoroughly with tap water to remove all traces of soil present on the surface. Dry weight (DW) biomass production was evaluated for all replicates after oven-drying (60°C) plant parts until constant weight. In addition, the weight per leaf and per unit of twig length (respectively DW $leaf^{-1}$ and DW cm^{-1} twig) were calculated for every plant.

To compare the efficiency of the different fertilizer treatments, the fertilization response was determined for every fertilization treatment. The fertilization response is defined here as the mean increment in biomass production (of the treatment compared to control) to the amount of N-fertilizer added (kg DW kg^{-1} N-fertilizer).

In planta metal concentrations, bioconcentration factor and translocation factor

Cadmium, Zn and Pb concentrations in every plant part (leaves, twigs, cutting and roots) were determined for 5 replicates per fertilization treatment and for 10 replicates in case of the control condition in 2013, or for respectively 3 and 6 replicates in 2014. Selected plants were representative for the condition regarding DW production. Material and methods are described in PART II ('Material and methods' section: *In planta* metal concentrations, bioconcentration factor and translocation factor).

The bioconcentration factor (BCF) and translocation factor (TF) of Cd, Zn and Pb were calculated for every selected plant. Methods can be found in the same paragraph in PART II.

Twig metal extraction

Metal extraction (potential) is defined as the amount of metals plants can extract out of the soil and accumulate in their harvestable biomass per unit of soil area and time (usually expressed in g ha^{-1} yr^{-1}). In this research, the mean

extraction of Cd and Zn was calculated per condition by multiplying mean plant twig production (DW) of the condition (g plant^{-1} 70 or 90 days^{-1}) with mean Cd and Zn concentrations measured in the twigs of the condition (mg kg^{-1}).

Physico-chemical soil characteristics (including soil metal concentrations) at the end of fertilization experiments

At the end of fertilization experiment 2013, soil (mixture of bulk and rhizosphere) samples of 3 (NKMg13 and OSMO treatment) or 6 (control conditions) randomly chosen pots were collected. The soil was analyzed for pseudo-total and plant available ($CaCl_2$-exchangeable) Cd, Zn and Pb concentrations and for pH-H_2O, pH-KCl, electrical conductivity (EC) and effective cation exchange capacity (CEC_e). Details on material and methods are described in Chapter 3 ('Material and methods' section: Tobacco, sunflower and hemp: Physico-chemical soil characteristics).

5.III.2.6 Statistical analyses

Statistical analyses were performed in R 3.1.3 (R Development Core Team, 2013). The effect of fertilization on dry weight (DW) biomass production, total twig length, total number of leaves, DW leaf^{-1} and DW cm^{-1} twig was analyzed using ANOVA or, in case of possible heteroscedasticity of the residuals (checked by plotting), model-robust standard errors (waldtest). The QQ-plots were used to inspect normality of the residuals. In the case of non-normality, transformations of the outcome (logarithmic, inverse, square root, exponential) were tried. When an indication of non-normality was present for all these transformations, a Box-Cox was used. Two-by-two comparisons with control were conducted using Dunnett's test. In case of non-normally distributed errors, the non-parametric Kruskal-Wallis Rank Sum test was applied and two-by-two comparisons with control were performed using Gao's test.

ANOVA followed by Dunnett's test to perform two-by-two comparisons with control was applied to assess the effects of fertilization on *in planta* metal concentrations, bioconcentration and translocation factor and physico-chemical soil characteristics.

5.III.3 Results

5.III.3.1 Climatological data

Climatological data for the main cultivation period of the willow cuttings (June-July-August) for the years 2013 and 2014 are given in Table 5.15. Mean (as well as mean min./max.) temperature was slightly higher in 2013 compared to 2014. Furthermore, relative air humidity, total rainfall and total days of rain were lower in 2013 while total hours of sunshine and wind velocity were higher than in 2014.

Table 5.15 Normal[2] and year specific climatological data[1] averaged for the main cultivation period (June-August) of *S. viminalis* and *S. alba x alba* cuttings in pot in 2013 and 2014. [1]Climatological data were measured by the Royal Meteorological Institute of Belgium (KMI) (50°48'17'' N; 4°21'27'' E). [2]Mean climatological values for the 30-year period 1981-2010.

	Normal	2013	2014
Mean temp. (°C)	**17.5**	18.2	17.3
Mean max. temp. (°C)	**22.1**	22.7	21.5
Mean min. temp. (°C)	**13.2**	13.7	13.4
Mean rel. air humidity (%)	**74.7**	69.3	72.7
Total rainfall (mm)	**224.6**	169.2	348.2
Total days of rain (d)	**44**	28	49
Total hours of sun (h)	**579**	654	551
Mean wind velocity (m s^{-1})	**2.9**	3.2	3.0
Mean wind direction	**SW**	NNE	NNE-NNW-SW
(% of occurrence)	**(12.5%)**	(4.2%)	(4.2-3.7-12.5%)

5.III.3.2 Biomass production

In fertilization experiment 2013, about 60% of the *S. viminalis* cuttings fertilized with osmocote (OSMO) was not considered healthy. Leaves started to curl and became chlorotic after 8 weeks in pot and from week 11 on, twig tops started to hang over and wilt. By consequence, no results for the *S. viminalis* clone with OSMO treatment are given. Slightly yellowing of leaves was also observed for *S.*

alba x alba plants fertilized with osmocote (experiment 2013), but most of them exhibiting no wilting twig tops. Almost all other plants in 2013 and 2014 survived showing no visible symptoms of phytotoxicity or other health-related problems. Mean diameter and DW of cuttings was, for a given experiment and clone, very equal for all conditions (results not shown). A general observation could be made regarding biomass production in both fertilization experiments (Figures 5.6 and 5.7). Fertilization of *Salix viminalis* cuttings increased productivity, resulting in bigger plants with the same proportion of plant parts compared to the control plants. In both experiments, the production of twigs was higher than the production of roots and leaves. *S. alba x alba* cuttings also showed a higher (aboveground) productivity after fertilization but the twig biomass increased disproportionally. By consequence (with exception of the OSMO condition in 2013) fertilized plants had clearly more twig DW than leaf and root DW, which was not observed for the control plants.

For the ***S. viminalis*** clone, all fertilization treatments (NKMg13 in experiment 2013, NKMg14 and YARA in experiment 2014) increased production of roots, twigs and leaves significantly compared to the control (Figure 5.6). In 2014, the YARA treatment improved growth of twigs and leaves more than the NKMg14 fertilization. Fertilizing ***S. alba x alba*** plants increased production of twigs and leaves compared to control plants, however, not significantly in case of the NKMg14 treatment in experiment 2014 (Figure 5.7). Root growth was only significantly improved by the YARA treatment (2014) and was even reduced after OSMO treatment (2013). In general, best results in 2013 were reached with fertilizer NKMg13 and in 2014 with the YARA treatment.

In both experiments, compared to control plants, total twig length and number of leaves per plant as well as DW cm^{-1} twig and DW $leaf^{-1}$ increased after fertilizing ***S. viminalis*** plants (highly significant results found for the NKMg14 and YARA treatment in experiment 2014) (Figure 5.8). For the ***S. alba x alba*** clone, however, results were contrasting (Figure 5.9). In both experiments, total twig length was (significantly) higher after fertilizer treatments but, while number of leaves increased significantly in 2013 (after NKMg13 and OSMO treatment), the amount of leaves tended to decrease in 2014 (for the NKMg14 as well as YARA treatment). Furthermore, leaf mass and twig mass per length

unit were (significantly) higher after both fertilizer treatments in 2014 but seemed rather unchanged in 2013 fertilizer treatments.

The fertilization response (biomass increment compared to control in kg DW kg^{-1} N-fertilizer added) for both clones and both experiments is rendered in Table 5.16. In general, the fertilization response of twigs was about half (*S. viminalis*) or more than half (*S. alba x alba*) of the response in total biomass (sum of root, twig and leaf responses). For ***S. viminalis*** in experiment 2014, highest biomass increases per amount of N-fertilizer were obtained using fertilizer Yara Opticrop. In case of the ***S. alba x alba*** clone, the NKMg13 treatment showed the highest response in experiment 2013 while this role was for the YARA treatment in experiment 2014.

Table 5.16 Fertilization responses (kg DW kg^{-1} N fertilizer) of total biomass (sum of roots, twigs and leaves produced) and of twigs alone of fertilized *S. viminalis* and *S. alba x alba* plants in fertilization experiments 2013 (90 days) and 2014 (70 days).

Fertilization response (kg DW kg^{-1} N-fertilizer)	**Condition**	***S. viminalis***		***S. alba alba***	
		Total biomass	**Twigs**	**Total biomass**	**Twigs**
2013	**NKMg13**	30.68 ± 11.22	15.03 ± 5.10	25.88 ± 13.57	15.82 ± 5.48
	OSMO			1.78 ± 2.14	1.00 ± 0.99
2014	**NKMg14**	8.47 ± 3.24	4.58 ± 1.65	4.08 ± 4.36	2.34 ± 2.26
	YARA	16.29 ± 4.87	8.20 ± 2.72	16.07 ± 3.32	9.08 ± 2.04

Values are mean ± standard deviation.

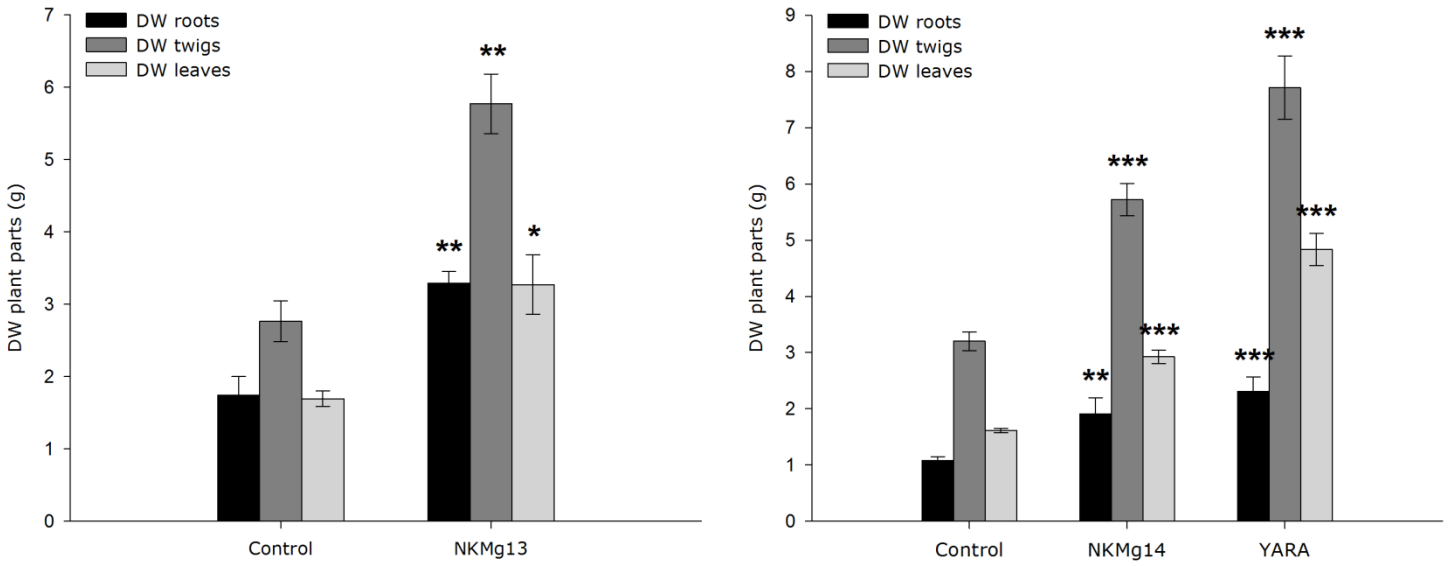

Figure 5.6 *S. viminalis* fertilization experiments: Biomass production. Left graph: Fertilization experiment 2013, right graph: Fertilization experiment 2014. Bars represent mean dry weight (DW in g) production of roots, twigs and leaves of control (2013: N = 20, 2014 N = 12) and fertilized (2013: N = 10, 2014 N = 6) cuttings after 90 (2013) or 70 (2014) days of growth in pots. Error bars are standard errors. Significance levels: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (compared to the control condition).

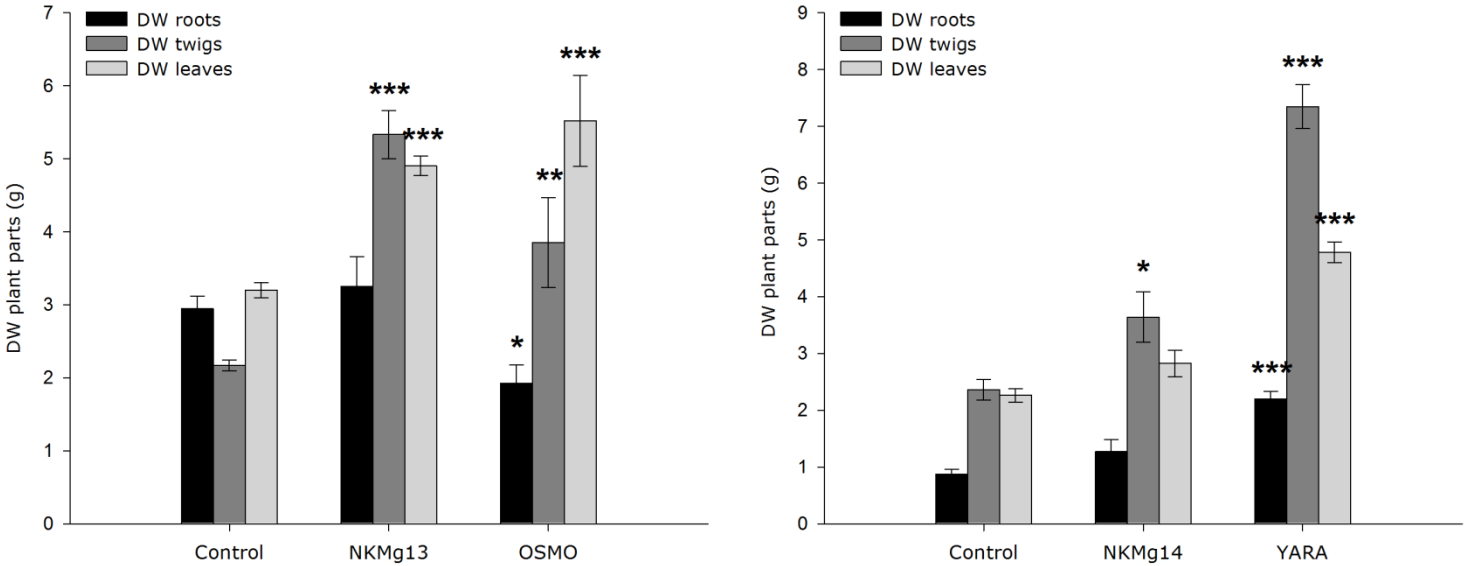

Figure 5.7 *S. alba x alba* fertilization experiments: Biomass production. Left graph: Fertilization experiment 2013, right graph: Fertilization experiment 2014. Bars represent mean dry weight (DW in g) production of roots, twigs and leaves of control (2013: N = 20, 2014 N = 12) and fertilized (2013: N = 10, 2014 N = 6) cuttings after 90 (2013) or 70 (2014) days of growth in pots. Error bars are standard errors. Significance levels: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (compared to the control condition).

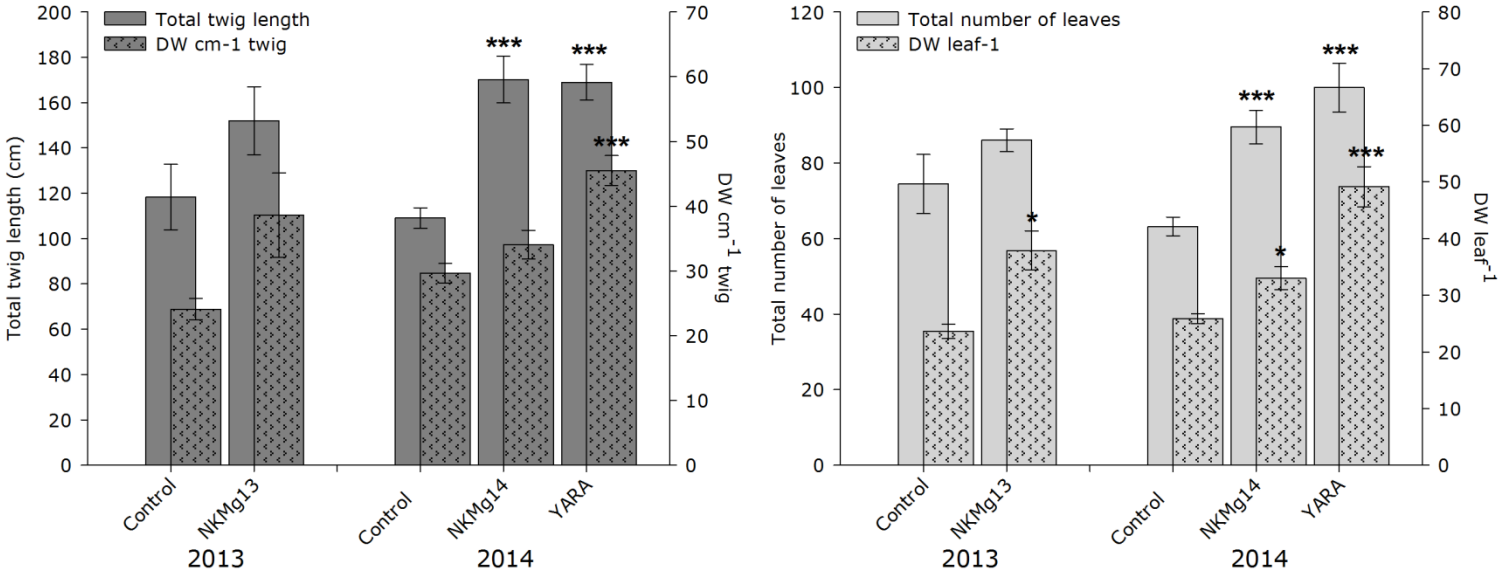

Figure 5.8 *S. viminalis* fertilization experiments 2013 and 2014: Twig and leaf production. Left graph: Total twig length (cm) per plant (even bars) and DW cm^{-1} twig (arrowed bars). Right graph: Total number of leaves per plant (even bars) and DW $leaf^{-1}$ (arrowed bars). Bars represent mean data of control (2013: N = 20, 2014 N = 12) and fertilized (2013: N = 10, 2014 N = 6) cuttings after 90 (2013) or 70 (2014) days of growth in pots. Error bars are standard errors. Significance levels: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (compared to the respective control condition).

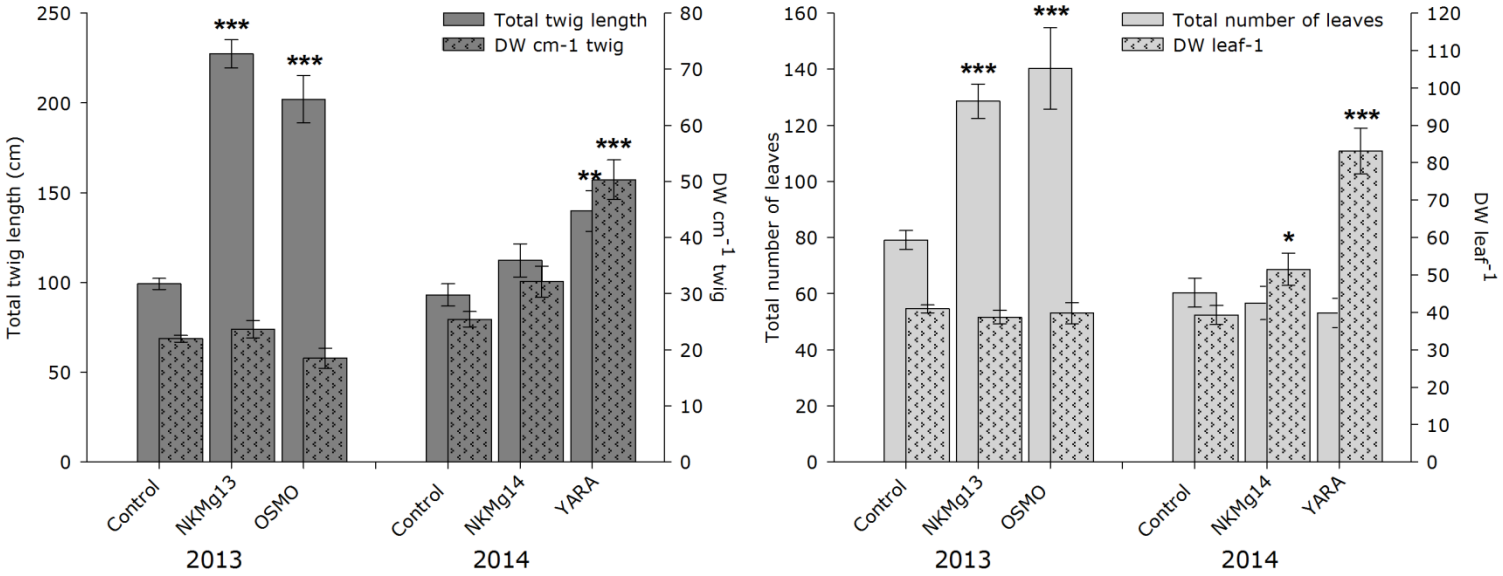

Figure 5.9 *S. alba x alba* fertilization experiments 2013 and 2014:Twig and leaf production. Left graph: Total twig length (cm) per plant (even bars) and DW cm^{-1} twig (arrowed bars). Right graph: Total number of leaves per plant (even bars) and DW $leaf^{-1}$ (arrowed bars). Bars represent mean data of control (2013: N = 20, 2014 N = 12) and fertilized (2013: N = 10, 2014 N = 6) cuttings after 90 (2013) or 70 (2014) days of growth in pots. Error bars are standard errors. Significance levels: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (compared to the respective control condition).

5.III.3.3 *In planta* metal concentrations, bioconcentration factor and translocation factor

Cadmium, Zn and Pb concentrations in roots, cutting, twigs and leaves of control and fertilized conditions for both fertilization experiments are summarized in Tables 5.17 (*S. viminalis*) and 5.18 (*S. alba x alba*). Lead concentrations in leaves and twigs were frequently (depending on experiment and evaluated clone) below the detection limit of 0.05 mg L^{-1} and were not shown. For the ***S. viminalis*** clone, in both fertilization experiments, Cd and Zn concentrations in roots, cutting and leaves mostly tended to increase slightly after fertilization while the concentration in the twigs tended to decrease (slightly) compared to control plants (Table 5.17). In fertilization experiment 2013, Zn accumulation in twigs of the NKMg13 condition was significantly lower ($p < 0.05$) than values found for the control condition. Regarding Pb, the YARA treatment in experiment 2014 tended to increase Pb concentrations in roots and cuttings while other effects of fertilization on Pb accumulation were inconsistent. In both fertilization experiments with the ***S. alba x alba*** cuttings, fertilization in general seemed to increase Cd and Zn concentrations in all plant parts with significant increases in roots and leaves (Table 5.18). The OSMO treatment (experiment 2013) showed highest increases in Cd, Zn and Pb accumulation of all fertilization treatments. Again, regarding Pb, other fertilization effects were inconsistent.

The importance of changes in *in planta* Cd and Zn concentrations as a result of fertilization for metal movement was estimated using the bioconcentration factor (BCF) and translocation factor (TF) (Table 5.19). In general, for ***S. viminalis*** plants, the translocation of Cd and Zn from soil to twigs (BCF) and roots to twigs (TF) tended to decrease after fertilizer treatment with a significant decrease in BCF of Zn in the NKMg13 treatment compared to control conditions in experiment 2013. For the ***S. alba x alba*** clone, in both fertilization experiments, the BCFs tended to increase after fertilization with a significant difference between control and the OSMO condition in 2013. On the other hand, the TFs were (significantly) lower after fertilization in experiment 2013 while they were similar to the control ones in the experiment of 2014.

Table 5.17 Metal (Cd, Zn, Pb) concentrations (mg kg^{-1} DW) in roots, cutting, twigs and leaves of *S. viminalis* control and fertilized plants for both fertilization experiments.

S. viminalis		**Cd (mg kg^{-1} DW)**				**Zn (mg kg^{-1} DW)**				**Pb (mg kg^{-1} DW)**	
Fertilization experiment	**Condition**	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**
2013	**Control**	20.70 ± 3.33	14.94 ± 1.64	16.51 ± 2.44	26.81 ± 4.77	958.39 ± 148.84	420.82 ± 55.75	503.05 ± 86.53	1763.74 ± 298.50	68.67 ± 15.41	5.87 ± 1.34
	NKMg13	21.12 ± 4.25	16.07 ± 2.16	16.42 ± 0.68	29.53 ± 0.57	896.77 ± 266.80	423.51 ± 19.84	340.95 ± 47.04 *	1331.60 ± 121.93	102.33 ± 64.58	5.51 ± 1.63
2014	**Control**	36.88 ± 5.27	18.14 ± 2.21	13.35 ± 1.18	20.16 ± 1.61	1440.30 ± 172.55	527.69 ± 23.15	458.07 ± 41.16	1246.34 ± 125.20	123.22 ± 27.49	6.23 ± 2.07
	NKMg14	38.38 ± 3.01	22.82 ± 3.87	12.38 ± 1.01	21.30 ± 1.74	1577.56 ± 116.71	604.55 ± 48.36	383.43 ± 44.27	1339.86 ± 129.24	123.56 ± 7.98	6.01 ± 2.60
	YARA	37.59 ± 7.08	18.05 ± 2.16	11.41 ± 0.85	23.36 ± 2.09	1547.58 ± 93.45	543.71 ± 31.13	425.67 ± 29.94	1492.08 ± 104.87	153.70 ± 17.41	7.44 ± 3.47

Values are mean ± standard deviation of 5 (10 for control) (2013) or 6 (12 for control) (2014) biological replicates. Stars indicate a significant different metal concentration in a plant part after fertilization compared to the respective control plant part (significance level: * $p < 0.05$).

Table 5.18 Metal (Cd, Zn, Pb) concentrations (mg kg^{-1} DW) in roots, cutting, twigs and leaves of *S. alba x alba* control and fertilized plants for both fertilization experiments.

S. alba x alba		**Cd (mg kg^{-1} DW)**				**Zn (mg kg^{-1} DW)**				**Pb (mg kg^{-1} DW)**	
Fertilization experiment	**Condition**	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**	**Twigs**	**Leaves**	**Roots**	**Cutting**
2013	**Control**	5.94 ± 0.70	5.68 ± 1.10	8.90 ± 1.11	12.10 ± 1.31	458.00 ± 33.58	257.53 ± 47.21	345.13 ± 32.95	945.75 ± 75.64	67.57 ± 16.65	3.78 ± 1.48
	NKMg13	8.66 ± 1.31**	5.88 ± 1.27	10.38 ± 1.61	20.35 ± 1.15***	509.21 ± 92.08	274.66 ± 62.82	337.65 ± 45.09	1324.84 ± 104.47***	62.01 ± 16.65	4.05 ± 0.80
	OSMO	15.59 ± 2.23***	6.73 ± 0.81	11.78 ± 1.02**	21.63 ± 1.04***	743.37 ± 142.19***	292.65 ± 24.18	398.69 ± 56.70	1642.33 ± 142.32***	82.14 ± 8.84	5.93 ± 0.26*
2014	**Control**	12.21 ± 1.39	4.97 ± 0.71	3.28 ± 0.59	7.68 ± 0.73	865.90 ± 37.60	249.75 ± 26.72	203.14 ± 22.81	671.28 ± 82.19	128.39 ± 9.06	5.53 ± 1.41
	NKMg14	16.40 ± 1.39**	5.47 ± 0.20	4.05 ± 0.89	10.11 ± 0.95*	957.00 ± 87.64	243.05 ± 1.74	235.19 ± 77.39	1010.09 ± 129.12**	179.24 ± 29.18	5.05 ± 1.11
	YARA	14.42 ± 1.66	5.83 ± 0.39	3.88 ± 0.46	10.80 ± 0.58**	951.39 ± 81.88	268.35 ± 27.74	229.66 ± 27.70	1120.01 ± 79.39***	113.92 ± 27.27	4.66 ± 0.91

Values are mean ± standard deviation of 5 (10 for control) (2013) or 6 (12 for control) (2014) biological replicates. Stars indicate a significant different metal concentration in a plant part after fertilization compared to the respective control plant part (significance level: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Table 5.19 Bioconcentration factors (BCFs) and translocation factors (TFs) of Cd and Zn for *S. viminalis* and *S. alba x alba* control and fertilized plants for both fertilization experiments. The BCF is defined as the ratio of metal concentration in twigs to total soil metal content while the TF is defined as the metal concentration in twigs to the metal concentration in roots.

Fertilization experiment	Condition	*S. viminalis*		*S. alba x alba*	
		BCF Cd	**BCF Zn**	**BCF Cd**	**BCF Zn**
2013	**Control**	4.11 ± 0.61	2.25 ± 0.39	2.21 ± 0.28	1.55 ± 0.15
	NKMg13	4.09 ± 0.17	1.53 ± 0.21*	2.58 ± 0.40	1.51 ± 0.20
	OSMO			2.93 ± 0.25**	1.79 ± 0.25
2014	**Control**	1.93 ± 0.17	1.07 ± 0.10	0.47 ± 0.09	0.47 ± 0.05
	NKMg14	1.79 ± 0.15	0.89 ± 0.10	0.58 ± 0.13	0.55 ± 0.18
	YARA	1.65 ± 0.12	0.99 ± 0.07	0.56 ± 0.07	0.54 ± 0.06
		TF Cd	**TF Zn**	**TF Cd**	**TF Zn**
2013	**Control**	0.79 ± 0.11	0.52 ± 0.09	1.52 ± 0.31	0.77 ± 0.14
	NKMg13	0.79 ± 0.13	0.39 ± 0.06	1.24 ± 0.36	0.69 ± 0.18
	OSMO			0.79 ± 0.08**	0.55 ± 0.13
2014	**Control**	0.37 ± 0.06	0.32 ± 0.05	0.27 ± 0.04	0.23 ± 0.03
	NKMg14	0.32 ± 0.03	0.24 ± 0.03	0.25 ± 0.06	0.25 ± 0.07
	YARA	0.31 ± 0.04	0.26 ± 0.02	0.27 ± 0.06	0.24 ± 0.03

Values are mean ± standard deviation of 5 (10 for control) (2013) or 6 (12 for control) (2014) biological replicates. Stars indicate a significant different BCF/TF after fertilization compared to the respective control plant part (significance level: * $p < 0.05$, ** $p < 0.01$).

5.III.3.4 Twig metal extraction

The extracted amounts of Cd and Zn by twigs of *S. viminalis* and *S. alba x alba* control and fertilized plants for both fertilization experiments are summarized in Table 5.20. All fertilizer treatments increased metal extraction by twigs considerably, and, besides the combination of *S. viminalis* with the YARA treatment, all clone-fertilizer combinations increased the extraction of Cd more than the extraction of Zn. Furthermore, the *S. viminalis* twigs extracted always obviously more Cd and Zn than *S. alba x alba* twigs. For the ***S. viminalis*** clone, in experiment 2014, best results were obtained with fertilizer YARA (more than doubling Cd and Zn extraction compared to the control condition). Highest Cd and Zn extractions after fertilizing the ***S. alba x alba*** clone were achieved with fertilizer treatment NKMg13 in experiment 2013 (more than doubling Cd and Zn extraction compared to control plants) while highest extractions were achieved with the YARA treatment in experiment 2014 (more than tripling Cd and Zn extraction compared to the control condition).

5.III.3.5 Physico-chemical soil characteristics (including soil metal concentrations) at the end of fertilization experiment 2013

After growing *S. viminalis* and *S. alba x alba* control and fertilized (NKMg13, OSMO) plants for 90 days in pots in experiment 2013, physico-chemical characteristics of the soil (mixture of bulk and rhizosphere soil) were determined (Table 5.21). For ***S. viminalis***, the soil of NKMg13-fertilized plants did not differ from soil of unfertilized plants in actual (pH-H_2O) or potential (pH-KCl) acidity. However, the EC increased significantly ($p < 0.01$) after NKMg13 fertilization. Differences in CEC_e as well as in pseudo-total or $CaCl_2$-exchangeable Cd, Zn and Pb concentrations between NKMg13 fertilization and the control condition were not found or could not be detected due to a lack of data. In case of ***S. alba x alba***, no (significant) differences were observed between the soil from NKMg13-fertilized plants and soil from control plants although the EC tended to increase. Three months (90 days) of OSMO fertilization however, significantly decreased soil pH compared to control soil while the EC increased ($p < 0.001$) tremendously. Also $CaCl_2$-exchangeable Cd, Zn and Pb concentrations increased

significantly. Differences in pseudo-total metal concentrations could not be detected.

Table 5.20 Cadmium and Zn extracted by twigs (mg plant^{-1} 70 or 90 days^{-1}) of fertilized and control plants of *S. viminalis* (left columns) and *S. alba x alba* (right columns) for both fertilization experiments.

Fertilization experiment	Condition	*S. viminalis*		*S. alba x alba*	
		Extracted Cd (mg plant^{-1} 90 days^{-1})	**Extracted Zn (mg plant^{-1} 90 days^{-1})**	**Extracted Cd (mg plant^{-1} 90 days^{-1})**	**Extracted Zn (mg plant^{-1} 90 days^{-1})**
2013	**Control**	0.046 ± 0.015	1.390 ± 0.486	0.019 ± 0.004	0.748 ± 0.143
	NKMg13	0.095 ± 0.010 (+108%)	1.967 ± 0.336 (+42%)	0.055 ± 0.014 (+187%)	1.800 ± 0.426 (+141%)
	OSMO			0.045 ± 0.019 (+135%)	1.536 ± 0.682 (+105%)
		Extracted Cd (mg plant^{-1} 70 days^{-1})	**Extracted Zn (mg plant^{-1} 70 days^{-1})**	**Extracted Cd (mg plant^{-1} 70 days^{-1})**	**Extracted Zn (mg plant^{-1} 70 days^{-1})**
2014	**Control**	0.043 ± 0.009	1.466 ± 0.297	0.008 ± 0.002	0.479 ± 0.133
	NKMg14	0.071 ± 0.010 (+66%)	2.193 ± 0.366 (+50%)	0.015 ± 0.005 (+90%)	0.856 ± 0.381 (+79%)
	YARA	0.088 ± 0.017 (+106%)	3.282 ± 0.627 (+124%)	0.029 ± 0.005 (+268%)	1.688 ± 0.297 (+252%)

Values are mean ± standard deviation. Grey shades indicate a higher Cd and Zn extraction compared to respective controls (light grey: less than double of control, middle grey: more than double of control and dark grey: more than triple of control).

Table 5.21 Physico-chemical characteristics of the soil (mixture of bulk and rhizosphere soil) in pots of control and fertilized plants of *S. viminalis* and *S. alba x alba* at the end of fertilization experiment 2013.

Clone		pH-H_2O	pH-KCl	CEC_e (meq 100 g^{-1})	EC (µS cm^{-1})	Pseudo-total metal concentrations (mg kg^{-1} dry soil)			$CaCl_2$-exchangeable metal concentrations (mg kg^{-1} dry soil)		
						Cd	Zn	Pb	Cd	Zn	Pb
S. viminalis	**Control**	6.45 ± 0.09	5.43 ± 0.22	4.97 ± 0.11	72.52 ± 3.83	2.67 ± 0.09	184.80 ± 4.10	131.85 ± 0.14	0.17 ± 0.02	7.32 ± 1.32	0.19 ± 0.03
	NKMg13	6.41 ± 0.12	5.40 ± 0.25	5.12	84.20 ± 2.97**	2.76	195.19	147.14	0.18 ± 0.01	8.04 ± 0.80	0.23 ± 0.00
S. alba x alba	**Control**	6.59 ± 0.14	5.75 ± 0.24	5.60 ± 0.12	65.42 ± 7.88	2.62 ± 0.16	181.14 ± 12.84	128.50 ± 10.08	0.17 ± 0.01	7.05 ± 0.61	0.14 ± 0.03
	NKMg13	6.51 ± 0.14	5.68 ± 0.13	n.d.	76.47 ± 7.51	2.55	179.33	126.74	0.18 ± 0.01	8.14 ± 0.69	0.17 ± 0.02
	OSMO	5.72 ± 0.48***	4.99 ± 0.51**	n.d.	349.67 ± 90.01***	2.38	169.26	121.38	0.40 ± 0.09***	26.33 ± 7.04***	0.32 ± 0.10**

Values are mean ± standard deviation of 6 (control) or 3 (NKMg13 and OSMO) independent replicates. n.d. = not detected. Stars indicate a significant different mean value after fertilization compared to control (significance levels: ** $p < 0.01$, *** $p < 0.001$).

5.III.4 Discussion

A third strategy to improve metal phytoextraction using SRC of willow is the application of fertilizers. Fertilization is reported to increase willow biomass production, but the effects of fertilization on metal accumulation in willow are unknown. The amount and type of fertilizer play a prominent role. In this research, 2 commercially available NPK-fertilizers were tested (Substral Osmocote (OSMO) and Yara Opticrop (YARA)) as well as a self-composed fertilizer of nitrogen (N), potassium (P) and magnesium (Mg) (NKMg13 and NKMg14). Amounts applied were based on recommendations of the manufacturer (OSMO) or specified for hardwood cultivation after analysis of the soil from the metal-contaminated field in Lommel (YARA, NKMg13 and NKMg14). The fertilizer treatments in pot experiments 2013 and 2014 indeed increased biomass production of *S. viminalis* and *S. alba x alba* cuttings. For the *S. viminalis* clone, all fertilizers improved growth of roots, twigs and leaves compared to control plants (Figure 5.6). The twigs did not only increase length but also weight (more DW cm^{-1} twig) and the leaves were more numerous as well as bigger (higher DW $leaf^{-1}$) (Figure 5.8). Also Merilo *et al.* (2006) found the leaf area of a *S. viminalis* clone to increase considerably after fertilization (rather than an increased photosynthetic capacity per leaf through higher leaf 'photosynthetic N' concentrations). Fertilizing *S. alba x alba* cuttings mainly improved aboveground productivity in comparison to control plants (Figure 5.7). However, a remarkable difference between experiments 2013 and 2014 was observed (Figure 5.9). While in 2013 (NKMg13 and OSMO treatment) supplied nutrients seemed to be allocated to develop longer twigs and more leaves, however not heavier/bigger than those of control plants, in 2014 (NKMg14 and YARA treatments), fertilized plants were not necessarily bigger but mass/size of twigs and leaves per unit increased. Although both patterns, allocation of supplied resources either to existing leaves or to the production of more leaves, result in an increased whole-plant photosynthesis, it is not clear why this distinction exists for a certain clone between experiments.

The fertilization response allows comparing different fertilizer treatments based on the amount of N added (Table 5.16). Since these calculations assume that

the increment in biomass is solely due to the addition of N, thereby ignoring the possible contribution in biomass increments of other added nutrients, care should be taken with the validity of the results. For both clones, NKMg13 clearly seemed to be the best fertilizer in the experiment of 2013 while YARA was double as efficient as NKMg14 in the experiment of 2015. Fertilization of selected willow clones with Substral Osmocote (OSMO, 12 g pot^{-1}) was lethal in case of *S. viminalis* and only caused a minor fertilization response in case of *S. alba x alba* (although production of twigs and leaves increased significantly). Probably a combination of a too high dose and the relatively high temperature in the black soil in black pots (compared to common garden soil) might have caused a too high availability of nutrients, mainly N, in a short time span (the Osmocote manufacturer mentions a faster release of nutrients with increasing temperature). The very high EC values measured in OSMO soil at the end of the experiment indeed confirm high soluble salt concentrations in the pots (Table 5.21). Since salty compounds retain a lot of water, plants might desiccate due to osmotic stress. Moreover, 'new' cuttings are reported to be highly sensitive to this extent (personal communication Stan Deckers, Soil Service of Belgium). The wilting twig tops observed seem to support the hypothesis of 'salt stress', usually called fertilization burn, after OSMO over-fertilization.

The effect of fertilization on the accumulation of Pb in roots and cutting was not clear. In case of Cd and Zn, besides for twigs of *S. viminalis*, *in planta* concentrations increased slightly (*S. viminalis*) or obviously to significantly (*S. alba x alba*) after all fertilizer applications (Tables 5.17 and 5.18). The soil from NKMg-fertilized plants, evaluated after experiment 2013, did however not show to be different from soil of control plants regarding pH or pseudo-total and $CaCl_2$-exchangeable metal concentrations (Table 5.21). The (highly) significant increments in *in planta* Cd, Zn and Pb concentrations after OSMO fertilization are very likely the result of acidification of the soil, increasing available metal concentrations in the soil (Kayser *et al.* 2000; Zaccheo *et al.* 2006; Iqbal *et al.* 2012). The curly, chlorotic leaves observed after about 8 weeks of growth in pots might reveal some indication of phytotoxicity (Van Assche and Clijsters 1990; Robinson *et al.* 2000; Benavides *et al.* 2005; Rout and Das 2009). The potential influence of changes in EC and CEC_e as a result of fertilization on metal

availability could unfortunately not be determined with the data available. Furthermore, little information was found in literature since the role of soil properties related to fertility (except pH) on uptake and accumulation of metals by willow was rarely evaluated (Cloutier-Hurteau *et al.* 2014). Moreover, regarding CEC in general, contrasting results were reported (Vamerali *et al.* 2010; Cloutier-Hurteau *et al.* 2014). It is however strongly suggested to investigate correlations between these parameters and metal availability in soil and uptake by plants.

As a result of fertilization effects on metal concentrations in twigs and roots, the efficiency of transporting Cd and Zn from soil to twigs (BCF) and from roots to twigs (TF) is considered to be slightly lower than for control plants in case of *S. viminalis* plants (Table 5.19). For the *S. alba x alba* clone, a possible increased accumulation of Cd and Zn in twigs relative to soil (BCF) was observed after fertilization while the transport from roots to twigs (TF) decreased or remained the same.

Fertilizer applications clearly affect biomass production and Cd and Zn accumulation. However, both clones respond in a different way. The fertilized *S. alba x alba* clone allocates disproportionally more energy to twig biomass production than production of roots and leaves, exposed an overall increased *in planta* metal concentration and a (slightly) increasing bioconcentration of Cd and Zn, all in contrast to the *S. viminalis* clone. As a result, fertilizing the *S. alba x alba* clone improves metal phytoextraction more than fertilizing *S. viminalis* (tripling *vs.* doubling of twig Cd and Zn extraction compared to control plants; Table 5.20), but the extraction of Cd and Zn by twigs of the latter one is still more efficient. These results strongly suggest that fertilization regimes can be exploited to enhance the efficiency of metal phytoextraction.

Since these first results are very promising but comparing results of both pot trials is not possible due to different experimental conditions (duration, soil contamination level, climate and water supply) (see also PART II: 'Discussion' section), more research is needed to indicate the optimal fertilization treatment in relation to metal phytoextraction. It is moreover highly recommended to evaluate both the self-composed, to the local soil conditions adjusted NKMg-

fertilizer as well as Yara Opticrop, a commercial NPK-fertilizer often used in SRC willow agricultures in Sweden (personal communication SLU, Uppsala, Sweden) in field conditions. It was illustrated previously (PART II) that performance of the selected willow clones on field-scale could not be predicted based on results in short-term pot trials (the clonal differences in pot trials and comparison with field data, described in PART II: 'Discussion' section, do also fully apply here). Field experiments are not only strongly advised to evaluate longer-term biomass increments and metal accumulation, but also to exclude a possible dilution of the latter one as a result of the former one, an observation reported in literature for Cd concentrations in fertilized wheat (Landberg and Greger 2003) and willow (Klang-Westin and Perttu 2002). Furthermore, a profound knowledge of the behavior of N fertilizers in soils is required for the development of fertilizer application as a strategy to improve metal phytoremediation (Zaccheo *et al.* 2006). Several chemical and biological processes (*i.e.* microbial immobilization of N, urea hydrolysis, nitrification, denitrification,…) are involved to determine N availability to plants and subsequent changes in the soil, particularly pH, as a result of N uptake. Although the addition of ammonium-N (NH_4^+-N) is favorable in terms of metal phytoextraction, this form of N is generally less preferred by plants and can be deleterious to growth, depending on plant species (Haynes 1990; Lasa *et al.* 2001). Finally, field research should reveal the environmental impact of fertilizing willow SRC. Fertilization has an impact in terms of global warming, acidification, eutrophication and energy ratio (González-García *et al.* 2012). Although a higher biomass production is reported to increase soil organic matter, improve soil quality, with positive implications also for carbon storage in soils, site-specific field measurements to unravel possible nutrient and metal leaching in willow SRC are needed.

Conclusion

The *in situ* evaluation of all SRC clones on the Cd-Zn-Pb-contaminated field led to the selection of the 'experimental' willow clones *Salix viminalis* and *Salix alba x alba* which revealed to improve respectively stem metal accumulation and stem biomass production compared to best performing commercial and other experimental clones. These naturally occurring superiority strongly suggest that *in situ* selection of clones should be the first step in the process to improve metal phytoextraction efficiency in a certain area.

There is no conclusive evidence for improved Cd and Zn phytoextraction of the selected willow clones by exploiting the selected 17 *in vitro* promising bacterial strains that are evaluated in this study. Moreover, effects were highly depending on the clone-bacteria combination, the evaluated plant part and the experiment. It can however not be excluded that other strains from the available collection or from promising inoculation experiments reported in literature might positively affect metal phytoextraction of the selected willow clones. Further research should focus on proper phenotypic characterization methods for strains selection (whether or not *in vitro*) as well as on colonization efficiency and effects of (re-) inoculation on the longer term.

Fertilizing the selected willow clones in pot trials increased biomass production considerably, and in case of *S. alba x alba*, also beneficially influenced Cd and Zn accumulation in the plant. The highly improved extractions of Cd and Zn with self-composed fine-tuned as well as with commercial fertilizer applications do suggest that fertilization can definitely contribute to enhance the efficiency of metal phytoextraction using SRC of willow in the metal-contaminated area of interest. Field experiments using both fertilizer strategies are highly recommended to evaluate fertilization responses of both clones in field conditions.

Twig concentrations of Pb were very low and phytoextraction of this element is considered insufficient.

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Supplementary information

Supplementary table 5.1 Genotypic identification, abundance and phenotypic traits of all isolated strains from rhizosphere soil, root and twig samples of *S. viminalis*. The abundance of every strain is expressed as colony forming units (CFU) per g of rhizosphere soil or per g of root or twig fresh weight (FW) and as abundance (%) relative to the total amount of CFU isolated per compartment. Results of the *in vitro* phenotypic traits testing (+ = positive, - = negative) are based on the 2011 phenotypic screening. For every genus, the total number of CFU, the total relative abundance and the total relative (based on relative abundances) positive test results are highlighted in bold under the collection of strains in that genus. Pie charts representing diversity and relative abundances per compartment and total percentages of positive phenotypic test results per compartment can be found in Chapter 5: Figure 5.1 and Table 5.4.

***S. viminalis*: Rhizosphere isolates**								**(mM)**								
N°	**Genotypic identification**	**CFU g^{-1} rhizosphere soil**	**Abundance (%)**	**IAA**	**ACC deam**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
1	Arthrobacter	1414301	4.86	+	+	-	-	-	+	-	-	+	-	-	+	-
2	Arthrobacter	1414301	4.86	+	-	-	-	-	+	-	-	+	-	-	-	-
3	Arthrobacter	1414301	4.86	+	+	-	-	+	+	+	+	+	-	+	-	-
4	Arthrobacter	1414301	4.86	+	+	-	-	-	+	-	-	+	-	-	-	-
5	Arthrobacter	988490	3.40	+	+	-	+	+	+	+	+	+	+	-	+	-
6	Arthrobacter	988490	3.40	+	-	-	-	+	+	+	+	+	-	-	+	-
7	Arthrobacter	988490	3.40	+	+	-	-	+	+	+	+	+	+	-	-	-
8	Arthrobacter	76038	0.26	+	-	-	+	+	+	+	+	-	-	-	-	-
9	Arthrobacter	38019	0.13	+	+	-	-	-	+	-	-	-	-	-	-	-
10	Arthrobacter	988490	3.40	+	+	+	-	+	+	+	-	-	-	-	-	-
	Total	**9.72×10^6**	**33.42**	**33.42**	**24.90**	**3.40**	**3.66**	**18.71**	**33.42**	**18.71**	**15.31**	**29.63**	**6.79**	**4.86**	**11.65**	**0.00**
11	Bacillus	152076	0.52	-	-	+	-	-	-	-	-	-	-	-	-	-
12	Bacillus	988490	3.40	-	+	+	+	+	+	+	+	+	+	-	+	-
	Total	**1.14×10^6**	**3.92**	**0.00**	**3.40**	**3.92**	**3.40**	**3.40**	**3.40**	**3.40**	**3.40**	**3.40**	**3.40**	**0.00**	**3.40**	**0.00**
13	Bosea	106453	0.37	-	-	-	-	-	-	-	-	-	-	-	-	-
14	Bosea	212906	0.73	+	-	-	-	-	+	-	-	+	-	-	-	-
15	Bosea	106453	0.37	+	-	-	-	-	-	-	-	-	-	-	-	-
16	Bosea	212906	0.73	+	-	-	-	-	-	-	-	-	-	-	-	-
17	Bosea	212906	0.73	+	-	-	-	-	-	-	-	-	-	-	-	-
18	Bosea	76038	0.26	+	-	+	-	-	+	-	-	+	-	-	-	-

19	Bosea	76038	0.26	+	-	+	-	-	+	-	-	+	-	-	-	-
20	Bosea	1414301	4.86	-	+	-	-	-	-	-	-	-	-	-	-	-
21	Bosea	53227	0.18	+	+	-	-	-	-	-	-	-	-	-	-	-
22	Bosea	76038	0.26	+	+	-	-	+	+	+	+	+	-	-	+	-
23	Bosea	76038	0.26	+	-	-	-	-	+	-	-	+	-	-	+	-
	Total	**2.62×10^6**	**9.01**	**3.79**	**5.30**	**0.52**	**0.00**	**0.26**	**1.78**	**0.26**	**0.26**	**1.78**	**0.00**	**0.00**	**0.52**	**0.00**
24	Caulobacter	106453	0.37	+	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**1.06×10^5**	**0.37**	**0.37**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
25	Duganella	76038	0.26	+	+	-	+	+	+	-	+	+	-	+	+	-
26	Duganella	76038	0.26	-	-	-	-	-	+	-	-	+	-	-	-	-
27	Duganella	273736	0.94	+	-	-	+	+	-	-	-	-	-	-	-	-
	Total	**4.26×10^5**	**1.46**	**1.20**	**0.26**	**0.00**	**1.20**	**1.20**	**0.52**	**0.00**	**0.26**	**0.52**	**0.00**	**0.26**	**0.26**	**0.00**
28	Dyadobacter	152076	0.52	-	-	-	-	+	+	+	+	+	-	-	+	-
29	Dyadobacter	76038	0.26	-	+	-	+	+	+	+	+	+	-	-	+	-
	Total	**2.28×10^5**	**0.78**	**0.00**	**0.26**	**0.00**	**0.26**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.00**	**0.00**	**0.78**	**0.00**
30	Mesorhizobium	988490	3.40	+	+	-	+	-	+	-	-	+	-	-	+	-
31	Mesorhizobium	988490	3.40	+	-	-	+	-	-	-	-	-	-	-	-	-
	Total	**1.98×10^6**	**6.79**	**6.79**	**3.40**	**0.00**	**6.79**	**0.00**	**3.40**	**0.00**	**0.00**	**3.40**	**0.00**	**0.00**	**3.40**	**0.00**
32	Nocardioides	988490	3.40	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**9.88×10^5**	**3.40**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
33	Paenibacillus	707151	2.43	+	+	-	+	+	+	+	-	+	-	-	+	-
34	Paenibacillus	707151	2.43	+	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**1.41×10^6**	**4.86**	**4.86**	**2.43**	**0.00**	**2.43**	**2.43**	**2.43**	**2.43**	**0.00**	**2.43**	**0.00**	**0.00**	**2.43**	**0.00**
35	Pseudomonas	152076	0.52	+	+	-	+	-	+	-	-	+	-	-	+	-
36	Pseudomonas	547471	1.88	+	+	-	+	-	-	-	-	-	-	-	-	-
37	Pseudomonas	547471	1.88	+	+	-	+	-	-	-	-	-	-	-	-	-
38	Pseudomonas	152076	0.52	+	+	+	+	+	-	-	+	-	-	-	-	-
39	Pseudomonas	152076	0.52	+	-	-	-	-	+	+	-	+	-	-	-	-
40	Pseudomonas	1414301	4.86	+	+	-	+	-	-	-	-	-	-	-	-	-
	Total	**2.97×10^6**	**10.19**	**10.19**	**9.67**	**0.52**	**9.67**	**0.52**	**1.05**	**0.52**	**0.52**	**1.05**	**0.00**	**0.00**	**0.52**	**0.00**
41	Rahnella	76038	0.26	+	-	-	+	-	-	-	-	-	-	-	-	-
42	Rahnella	273736	0.94	+	-	-	+	+	-	-	+	-	-	-	-	-
43	Rahnella	152076	0.52	+	+	+	+	+	+	+	+	+	-	+	+	-
44	Rahnella	547471	1.88	+	+	-	+	-	-	-	-	-	-	-	-	-
45	Rahnella	547471	1.88	+	+	-	+	-	-	-	-	-	-	-	-	-
	Total	**1.60×10^6**	**5.49**	**5.49**	**4.29**	**0.52**	**5.49**	**1.46**	**0.52**	**0.52**	**1.46**	**0.52**	**0.00**	**0.52**	**0.52**	**0.00**
46	Ralstonia	1414301	4.86	+	-	-	-	+	+	+	+	+	+	+	+	-
	Total	**1.41×10^6**	**4.86**	**4.86**	**0.00**	**0.00**	**0.00**	**4.86**	**4.86**	**4.86**	**4.86**	**4.86**	**4.86**	**4.86**	**4.86**	**0.00**
47	Sphingobacterium	136868	0.47	+	+	-	-	+	+	+	-	+	+	-	+	-
48	Sphingobacterium	136868	0.47	-	+	-	-	-	+	-	-	+	-	-	-	-
	Total	**2.74×10^5**	**0.94**	**0.47**	**0.94**	**0.00**	**0.00**	**0.47**	**0.94**	**0.47**	**0.00**	**0.94**	**0.47**	**0.00**	**0.47**	**0.00**
49	Staphylococcus	76038	0.26	-	-	-	-	-	-	+	-	-	-	-	-	-

	Total	**7.6 x 10^4**	**0.26**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.26**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
50	Stenotrophomonas	76038	0.26	+	+	+	+	+	+	-	+	+	-	-	-	-
51	Stenotrophomonas	136868	0.47	+	-	-	+	-	-	-	-	-	-	-	-	-
52	Stenotrophomonas	547471	1.88	+	+	-	+	-	+	-	-	+	-	-	+	-
	Total	**7.60 x 10^5**	**2.61**	**2.61**	**2.14**	**0.26**	**2.61**	**0.26**	**2.14**	**0.00**	**0.26**	**2.14**	**0.00**	**0.00**	**1.88**	**0.00**
53	Streptomyces	1414301	4.86	-	-	-	+	-	-	-	-	-	-	-	-	-
54	Streptomyces	1414301	4.86	-	-	-	+	+	+	-	+	+	-	-	+	-
	Total	**2.83 x 10^6**	**9.72**	**0.00**	**0.00**	**0.00**	**9.72**	**4.86**	**4.86**	**0.00**	**4.86**	**4.86**	**0.00**	**0.00**	**4.86**	**0.00**
55	Variovorax	76038	0.26	-	-	-	-	-	+	-	-	-	-	-	-	-
56	Variovorax	136868	0.47	+	-	-	+	+	+	-	+	-	-	-	-	-
57	Variovorax	152076	0.52	-	-	-	+	-	-	-	-	-	-	-	-	-
58	Variovorax	38019	0.13	+	+	-	+	+	+	-	+	+	-	+	+	-
59	Variovorax	152076	0.52	-	-	-	+	-	-	-	-	-	-	-	-	-
	Total	**5.55 x 10^5**	**1.91**	**0.60**	**0.00**	**0.00**	**1.65**	**0.60**	**0.86**	**0.00**	**0.60**	**0.13**	**0.00**	**0.13**	**0.13**	**0.00**

***S. viminalis*: Root isolates**

N°	**Genotypic identification**	**CFU g^{-1} FW root**	**Abundance (%)**	**IAA**	**ACC deam**	**OA**	**SID**	**(mM) 0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
60	Alcaligenes	28086	0.16	+	+	-	-	+	+	+	+	-	-	+	-	-
	Total	**2.81 x 10^4**	**0.16**	**0.16**	**0.16**	**0.00**	**0.00**	**0.16**	**0.16**	**0.16**	**0.16**	**0.00**	**0.00**	**0.16**	**0.00**	**0.00**
61	Arthrobacter	23148	0.13	+	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.31 x 10^4**	**0.13**	**0.13**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
62	Bacillus	84259	0.48	+	-	-	+	-	+	+	-	+	+	-	-	-
63	Bacillus	44445	0.25	-	+	+	-	+	+	+	+	+	+	-	+	-
64	Bacillus	481481	2.72	+	-	+	+	+	+	+	-	+	-	-	-	-
65	Bacillus	3704	0.02	+	-	-	+	-	+	-	-	+	-	-	-	-
66	Bacillus	180556	1.02	+	-	-	-	-	+	-	-	+	-	-	-	-
67	Bacillus	83334	0.47	-	-	-	+	+	+	+	+	+	+	-	+	-
	Total	**8.78 x 10^5**	**4.95**	**4.23**	**0.25**	**2.97**	**3.68**	**3.44**	**4.95**	**3.91**	**0.72**	**4.95**	**1.20**	**0.00**	**0.72**	**0.00**
68	Bosea	92593	0.52	-	+	-	+	-	-	-	-	-	-	-	-	-
69	Bosea	27778	0.16	-	+	-	-	-	+	+	-	+	+	-	+	-
70	Bosea	84259	0.48	+	+	-	-	+	+	+	+	+	+	-	+	-
	Total	**2.05 x 10^5**	**1.15**	**0.48**	**1.15**	**0.00**	**0.52**	**0.48**	**0.63**	**0.63**	**0.48**	**0.63**	**0.63**	**0.00**	**0.63**	**0.00**
71	Caulobacter	166667	0.94	+	+	-	-	+	+	+	+	+	-	-	+	-
72	Caulobacter	83334	0.47	+	+	-	-	+	+	+	+	+	-	-	+	-
73	Caulobacter	166667	0.94	-	-	-	-	-	+	-	-	+	-	-	+	-
74	Caulobacter	435185	2.46	-	+	-	+	-	+	-	-	+	-	-	+	-
75	Caulobacter	84259	0.48	+	+	-	-	-	+	-	-	+	-	-	-	-

76	Caulobacter	26852	0.15	-	+	-	+	+	+	+	+	+	+	-	+	-
77	Caulobacter	3704	0.02	-	+	-	-	+	+	-	+	+	-	+	+	-
78	Caulobacter	185185	1.04	+	-	-	-	+	+	+	+	-	-	-	-	-
79	Caulobacter	185185	1.04	+	-	-	-	-	-	-	-	-	-	-	-	-
80	Caulobacter	166667	0.94	-	-	-	-	-	-	-	-	-	-	-	-	-
81	Caulobacter	435185	2.46	-	-	-	-	-	-	+	-	-	-	-	-	-
82	Caulobacter	84259	0.48	+	-	-	-	-	+	-	-	+	-	-	+	-
83	Caulobacter	23148	0.13	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.05 x 10^6**	**11.55**	**4.45**	**4.51**	**0.00**	**2.61**	**2.63**	**6.97**	**5.06**	**2.63**	**5.45**	**0.15**	**0.02**	**5.45**	**0.00**
84	Chryseobacterium	212963	1.20	+	+	-	-	+	+	+	+	+	+	+	+	-
	Total	**2.13 x 10^5**	**1.20**	**1.20**	**1.20**	**0.00**	**0.00**	**1.20**	**1.20**	**1.20**	**1.20**	**1.20**	**1.20**	**1.20**	**1.20**	**0.00**
85	Curtobacterium	28086	0.16	+	-	-	+	-	+	-	-	-	-	-	-	-
86	Curtobacterium	180556	1.02	+	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.09 x 10^5**	**1.18**	**1.18**	**0.00**	**0.00**	**0.16**	**0.00**	**0.16**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
87	Dyadobacter	14815	0.08	-	+	-	-	+	+	+	+	+	+	-	+	-
88	Dyadobacter	11111	0.06	-	+	-	-	+	+	+	+	+	+	-	+	-
89	Dyadobacter	11111	0.06	-	+	-	+	+	+	+	+	+	-	-	+	-
90	Dyadobacter	11111	0.06	-	+	-	+	-	+	-	-	+	-	-	+	-
91	Dyadobacter	42130	0.24	+	+	-	+	+	+	+	+	+	-	-	+	-
	Total	**9.03 x 10^4**	**0.51**	**0.24**	**0.51**	**0.00**	**0.36**	**0.45**	**0.51**	**0.45**	**0.45**	**0.51**	**0.15**	**0.00**	**0.51**	**0.00**
92	Mesorhizobium	435185	2.46	+	+	-	-	+	+	+	+	+	-	-	+	-
93	Mesorhizobium	84259	0.48	+	-	-	-	-	-	-	-	-	-	-	-	-
94	Mesorhizobium	88889	0.50	+	+	-	+	+	+	+	+	+	+	-	-	-
	Total	**6.08 x 10^5**	**3.43**	**3.43**	**2.96**	**0.00**	**0.50**	**2.96**	**2.96**	**2.96**	**2.96**	**2.96**	**0.50**	**0.00**	**2.46**	**0.00**
95	Microbacterium	55556	0.31	+	+	-	-	+	+	-	-	-	-	-	-	-
96	Microbacterium	212963	1.20	+	+	-	-	+	+	+	+	+	+	-	+	-
97	Microbacterium	217593	1.23	+	+	+	+	+	+	+	+	+	-	-	+	-
	Total	**4.86 x 10^5**	**2.74**	**2.74**	**2.74**	**1.23**	**1.23**	**2.74**	**2.74**	**2.43**	**2.43**	**2.43**	**1.20**	**0.00**	**2.43**	**0.00**
98	Paenibacillus	23148	0.13	+	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.31 x 10^4**	**0.13**	**0.13**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
99	Phaeospirillum	27778	0.16	+	-	-	+	-	-	-	-	-	-	-	-	-
	Total	**2.78 x 10^4**	**0.16**	**0.16**	**0.00**	**0.00**	**0.16**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
100	Plantibacter	11111	0.06	-	+	-	-	+	+	+	+	+	+	-	+	-
	Total	**1.11 x 10^4**	**0.06**	**0.00**	**0.06**	**0.00**	**0.00**	**0.06**	**0.06**	**0.06**	**0.06**	**0.06**	**0.06**	**0.00**	**0.06**	**0.00**
101	Polaromonas	92593	0.52	-	-	-	-	-	-	-	-	-	-	-	-	-
102	Polaromonas	185185	1.04	-	-	-	-	-	-	-	-	-	-	-	-	-
103	Polaromonas	481481	2.72	-	+	-	-	-	+	-	-	+	-	-	-	-
104	Polaromonas	84259	0.48	+	+	-	+	-	+	+	-	+	+	-	+	-
105	Polaromonas	9259	0.05	+	+	-	+	-	+	-	-	+	-	-	-	-
106	Polaromonas	44445	0.25	+	+	-	+	-	+	-	-	+	-	-	+	-
107	Polaromonas	231482	1.31	-	+	-	+	-	-	-	-	-	-	-	-	-
108	Polaromonas	88889	0.50	-	-	-	-	-	+	-	-	+	-	-	+	-

109	Polaromonas	120370	0.68	+	-	-	-	-	-	-	-	-	-	-	-	-
110	Polaromonas	44445	0.25	+	+	-	-	+	+	+	+	+	+	+	+	-
111	Polaromonas	88889	0.50	-	-	-	-	-	+	+	-	+	+	-	+	-
112	Polaromonas	88889	0.50	-	-	-	-	-	-	+	-	-	-	-	-	-
113	Polaromonas	88889	0.50	-	-	-	-	-	-	-	-	-	-	-	-	-
114	Polaromonas	84259	0.48	-	-	-	-	-	-	+	-	-	-	-	-	-
	Total	**1.73 x 10^6**	**9.78**	**1.71**	**5.05**	**0.00**	**2.08**	**0.25**	**4.75**	**2.20**	**0.25**	**4.75**	**1.23**	**0.25**	**1.98**	**0.00**
115	Pseudomonas	14815	0.08	+	+	-	-	+	+	+	+	+	-	-	+	-
116	Pseudomonas	9259	0.05	+	+	+	+	+	-	-	-	-	-	-	-	-
117	Pseudomonas	14815	0.08	+	+	-	+	+	-	-	+	-	-	-	-	-
118	Pseudomonas	14815	0.08	+	+	-	+	+	+	+	-	+	-	-	-	-
	Total	**5.37 x 10^4**	**0.30**	**0.30**	**0.30**	**0.05**	**0.22**	**0.30**	**0.17**	**0.17**	**0.17**	**0.17**	**0.00**	**0.00**	**0.08**	**0.00**
119	Rahnella	11111	0.06	+	+	-	+	-	+	-	-	+	-	-	+	-
120	Rahnella	11111	0.06	+	+	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.22 x 10^4**	**0.13**	**0.13**	**0.13**	**0.00**	**0.06**	**0.00**	**0.06**	**0.00**	**0.00**	**0.06**	**0.00**	**0.00**	**0.06**	**0.00**
121	Rhizobium	53704	0.30	+	+	-	+	+	+	+	-	+	-	-	+	-
122	Rhizobium	53704	0.30	+	+	-	-	-	+	-	-	+	-	-	-	-
123	Rhizobium	4630	0.03	-	-	-	+	-	-	-	-	-	-	-	-	-
124	Rhizobium	462963	2.61	+	-	-	+	-	-	-	-	-	-	-	-	-
125	Rhizobium	435185	2.46	-	-	-	-	+	+	+	+	+	+	-	+	-
126	Rhizobium	4630	0.03	+	+	-	+	+	+	+	+	+	+	-	+	-
127	Rhizobium	3704	0.02	+	+	-	+	-	+	-	-	+	-	-	+	-
128	Rhizobium	5556	0.03	+	-	-	+	-	-	-	-	-	-	-	-	-
129	Rhizobium	435185	2.46	+	-	-	+	+	-	+	+	-	+	+	-	+
130	Rhizobium	46297	0.26	-	+	-	-	-	-		-	-	-	-	-	-
	Total	**1.51 x 10^6**	**8.49**	**5.75**	**0.91**	**0.00**	**5.48**	**5.24**	**3.11**	**5.24**	**4.94**	**3.11**	**4.94**	**2.46**	**2.81**	**2.46**
131	Sphingobacterium	425926	2.40	-	+	+	+	+	+	+	-	+	-	-	+	-
132	Sphingobacterium	240741	1.36	-	+	-	+	+	+	+	+	+	-	-	+	-
133	Sphingobacterium	425926	2.40	-	+	-	+	+	+	+	-	+	-	-	+	-
134	Sphingobacterium	92593	0.52	-	+	-	+	+	+	+	+	+	+	-	+	-
135	Sphingobacterium	88889	0.50	+	+	-	+	-	-	-	-	-	-	-	-	-
136	Sphingobacterium	435185	2.46	-	+	-	+	+	+	+	+	+	+	-	+	-
137	Sphingobacterium	240741	1.36	-	+	-	+	+	+	+	+	+	+	-	+	-
138	Sphingobacterium	83334	0.47	-	-	-	+	+	+	+	+	+	-	-	+	-
139	Sphingobacterium	92593	0.52	-	+	+	+	+	+	+	+	+	+	+	+	-
140	Sphingobacterium	185185	1.04	+	+	+	+	+	+	+	+	+	+	+	+	+
141	Sphingobacterium	115741	0.65	-	+	-	+	+	+	+	+	+	-	-	+	-
	Total	**2.43 x 10^6**	**13.69**	**1.55**	**13.22**	**3.97**	**13.69**	**13.19**	**13.19**	**13.19**	**8.38**	**13.19**	**5.90**	**1.57**	**13.19**	**1.04**
142	Sphingomonas	481481	2.72	-	-	-	-	-	-	-	-	-	-	-	-	-
143	Sphingomonas	481481	2.72	-	-	-	-	-	-	-	-	-	-	-	-	-
144	Sphingomonas	462963	2.61	-	-	-	+	-	-	-	-	-	-	-	-	-
145	Sphingomonas	46297	0.26	-	+	-	-	-	-	-	-	-	-	-	-	-

146	Sphingomonas	9259	0.05	-	+	+	+	-	-	-	-	-	-	-	-	-
147	Sphingomonas	185185	1.04	-	+	-	-	-	-	-	-	-	-	-	-	-
148	Sphingomonas	9259	0.05	+	-	-	-	-	+	-	-	-	-	-	-	-
149	Sphingomonas	55556	0.31	+	-	-	-	-	+	+	-	+	-	-	+	-
	Total	**1.73 x 10^6**	**9.77**	**0.37**	**1.36**	**0.05**	**2.66**	**0.00**	**0.37**	**0.31**	**0.00**	**0.31**	**0.00**	**0.00**	**0.31**	**0.00**
150	Staphylococcus	462963	2.61	-	+	+	+	+	+	+	-	+	-	-	+	-
151	Staphylococcus	462963	2.61	-	+	+	+	+	+	+	-	+	-	-	+	-
152	Staphylococcus	435185	2.46	-	-	-	-	-	+	-	-	+	-	-	-	-
153	Staphylococcus	44445	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**1.06 x 10^6**	**5.97**	**0.00**	**2.61**	**2.61**	**2.61**	**2.61**	**5.07**	**2.61**	**0.00**	**5.07**	**0.00**	**0.00**	**2.61**	**0.00**
154	Stenotrophomonas	14815	0.08	+	+	+	+	+	+	+	+	+	+	-	-	-
155	Stenotrophomonas	28086	0.16	+	+	-	-	+	+	+	-	+	-	-	+	-
156	Stenotrophomonas	53704	0.30	+	+	-	-	+	+	+	-	+	-	-	+	-
	Total	**9.66 x 10^4**	**0.55**	**0.55**	**0.55**	**0.08**	**0.08**	**0.55**	**0.55**	**0.55**	**0.08**	**0.55**	**0.08**	**0.00**	**0.46**	**0.00**
157	Uncultured bacterium	9259	0.05	+	-	-	+	-	+	-	-	-	-	-	-	-
158	Uncultured bacterium	120370	0.68	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**1.30 x 10^5**	**0.73**	**0.05**	**0.00**	**0.00**	**0.05**	**0.00**	**0.05**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
159	Variovorax	83334	0.47	+	-	-	-	+	+	+	-	-	-	-	-	-
160	Variovorax	425926	2.40	+	-	-	-	+	+	-	+	+	-	-	-	-
161	Variovorax	92593	0.52	+	-	-	-	+	+	-	+	+	-	-	-	-
162	Variovorax	217593	1.23	-	-	-	-	-	-	-	-	-	-	-	-	-
163	Variovorax	11111	0.06	+	+	-	+	-	+	-	-	+	-	-	-	-
164	Variovorax	5556	0.03	+	-	-	+	-	+	+	-	+	-	-	-	-
165	Variovorax	88889	0.50	+	+	-	-	-	+	-	-	-	-	-	-	-
166	Variovorax	88889	0.50	+	-	-	-	-	+	-	-	+	-	-	-	-
167	Variovorax	361111	2.04	+	+	-	+	+	+	+	+	+	-	-	+	-
168	Variovorax	361111	2.04	-	-	-	-	-	-	-	-	-	-	-	-	-
169	Variovorax	180556	1.02	-	-	-	+	-	-	-	-	-	-	-	-	-
170	Variovorax	361111	2.04	-	-	-	-	-	-	-	-	-	-	-	-	-
171	Variovorax	361111	2.04	+	-	+	-	-	-	-	-	-	-	-	-	-
172	Variovorax	361111	2.04	+	-	+	-	-	+	-	-	+	-	-	-	-
173	Variovorax	361111	2.04	-	-	+	+	-	-	-	-	-	-	-	-	-
174	Variovorax	92593	0.52	+	+	-	+	-	-	-	-	-	-	-	-	-
175	Variovorax	92593	0.52	+	+	-	-	-	-	-	-	-	-	-	-	-
176	Variovorax	435185	2.46	-	+	-	-	-	-	-	-	-	-	-	-	-
177	Variovorax	55556	0.31	+	-	-	+	-	-	-	-	-	-	-	-	-
	Total	**4.04 x 10^6**	**22.78**	**11.96**	**6.10**	**6.11**	**6.02**	**5.43**	**8.57**	**2.54**	**4.96**	**7.60**	**0.00**	**0.00**	**2.04**	**0.00**
178	Xanthomonas	26852	0.15	+	+	-	+	+	+	-	+	+	-	-	-	-
179	Xanthomonas	53704	0.30	+	+	-	+	+	+	-	+	+	-	+	+	-
	Total	**8.06 x 10^4**	**0.45**	**0.45**	**0.45**	**0.00**	**0.45**	**0.45**	**0.45**	**0.00**	**0.45**	**0.45**	**0.00**	**0.30**	**0.30**	**0.00**

***S. viminalis*: Twig isolates**								(mM)								
N°	**Genotypic identification**	**CFU g^{-1} FW twig**	**Abundance (%)**	**IAA**	**ACC deam**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
180	Curtobacterium	331	34.62	+	+	-	+	+	+	-	+	+	-	-	-	-
181	Curtobacterium	331	34.62	+	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**6.62 x 10^2**	**69.25**	**69.25**	**34.62**	**0.00**	**34.62**	**34.62**	**34.62**	**0.00**	**34.62**	**34.62**	**0.00**	**0.00**	**0.00**	**0.00**
182	Pseudomonas	147	15.38	+	+	-	+	-	-	-	-	-	-	-	-	-
	Total	**1.47 x 10^2**	**15.38**	**15.38**	**15.38**	**0.00**	**15.38**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
183	Sphingomonas	147	15.38	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**1.47 x 10^2**	**15.38**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

IAA = production of indole-3-acetic acid, ACC deam. = production of 1-aminocyclopropane-1-carboxylate deaminase, OA = production of organic acids, SID = production of siderophores.

Supplementary table 5.2 Genotypic identification, abundance and phenotypic traits of all isolated strains from rhizosphere soil, root and twig samples of *S. alba x alba*. The abundance of every strain is expressed as colony forming units (CFU) per g of rhizosphere soil or per g of root or twig fresh weight (FW) and as abundance (%) relative to the total amount of CFU isolated per compartment. Results of the *in vitro* phenotypic traits testing (+ = positive, - = negative) are based on the 2011 phenotypic screening. For every genus, the total number of CFU, the total relative abundance and the total relative (based on relative abundances) positive test results are highlighted in bold under the collection of strains in that genus. Pie charts representing diversity and relative abundances per compartment and total percentages of positive phenotypic test results per compartment can be found in Chapter 5: Figure 5.1 and Table 5.4.

***S. alba x alba*: Rhizosphere isolates**								(mM)								
N°	**Genotypic identification**	**CFU g^{-1} rhizopshere soil**	**Abundance (%)**	**IAA**	**ACC deam**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
184	Arthrobacter	434783	1.06	-	-	+	-	-	-	-	-	-	-	-	-	-
185	Arthrobacter	782609	1.90	+	+	-	-	+	+	-	-	+	-	-	-	-
186	Arthrobacter	434783	1.06		+	-	-	-	+	-	-	+	-	-	-	-
187	Arthrobacter	840580	2.04	+	+	-	-	-	+	-	-	+	-	-	-	-
188	Arthrobacter	434783	1.06	-	-	-	-	-	+	-	-	+	-	-	-	-
	Total	**2.93 x 10^6**	**7.11**	**3.94**	**5.00**	**1.06**	**0.00**	**1.90**	**6.05**	**0.00**	**0.00**	**6.05**	**0.00**	**0.00**	**0.00**	**0.00**
189	Bacillus	391305	0.95	-	-	-	-	-	-	-	-	-	-	-	-	-
190	Bacillus	434783	1.06	-	-	-	-	-	+	-	-	+	-	-	-	-
191	Bacillus	434783	1.06	+	+	-	-	+	+	+	+	+	+	-	+	-
192	Bacillus	434783	1.06	-	-	-	+	-	-	-	-	-	-	-	-	-
193	Bacillus	217392	0.53	-	-	-	+	-	-	-	-	-	-	-	-	-
194	Bacillus	217392	0.53	-	-	-	+	-	+	-	-	+	-	-	+	-
195	Bacillus	840580	2.04	-	-	-	-	-	+	-	-	+	-	-	-	-
196	Bacillus	2521739	6.12	+	-	+	+	-	+	-	-	+	-	-	-	-
197	Bacillus	630435	1.53	+	-	-	+	+	+	-	-	+	-	-	-	-
198	Bacillus	1043478	2.53	-	-	-	+	-	+	-	-	+	-	-	+	-
199	Bacillus	434783	1.06	-	-	-	-	-	-	-	-	-	-	-	-	-
200	Bacillus	434783	1.06	-	-	-	-	-	-	-	-	-	-	-	-	-
201	Bacillus	579710	1.41	-	-	-	-	-	-	-	-	-	-	-	-	-
202	Bacillus	1739130	4.22	-	-	-	-	-	-	-	-	-	-	-	-	-
203	Bacillus	1739130	4.22	+	-	-	-	+	+	+	+	+	+	-	-	-
204	Bacillus	1739130	4.22	+	+	-	+	+	+	-	+	+	-	-	+	-
205	Bacillus	434783	1.06	-	-	-	-	+	-	-	+	-	-	-	-	-
206	Bacillus	434783	1.06	-	-	-	-	+	-	-	+	-	-	-	-	-

	Total	**1.47 x 10^7**	**35.70**	**17.16**	**5.28**	**6.12**	**16.52**	**13.14**	**23.32**	**5.28**	**11.61**	**23.32**	**5.28**	**0.00**	**8.34**	**0.00**
207	Bosea	434783	1.06	-	+	-	-	-	-	-	-	-	-	-	-	-
	Total	**4.35 x 10^5**	**1.06**	**0.00**	**1.06**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
208	Caulobacter	144928	0.35	+	-	-	-	-	+	-	-	-	-	-	-	-
	Total	**1.45 x 10^5**	**0.35**	**0.35**	**0.00**	**0.00**	**0.00**	**0.00**	**0.35**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
209	Chryseobacterium	782609	1.90	-	+	-	-	+	+	+	+	+	+	-	+	-
210	Chryseobacterium	782609	1.90	-	-	-	-	+	+	+	+	+	+	-	+	-
	Total	**1.57 x 10^6**	**3.80**	**0.00**	**1.90**	**0.00**	**0.00**	**3.80**	**3.80**	**3.80**	**3.80**	**3.80**	**3.80**	**0.00**	**3.80**	**0.00**
211	Paenibacillus	1043478	2.53	-	+	-	-	-	+	-	-	-	-	-	-	-
212	Paenibacillus	434783	1.06	+	+	-	-	-	+	-	-	+	-	-	-	-
213	Paenibacillus	1043478	2.53	-	+	-	-	-	+	-	-	+	-	-	-	-
214	Paenibacillus	869565	2.11	+	+	-	-	+	+	+	+	-	-	+	-	-
215	Paenibacillus	869565	2.11	+	+	-	-	-	-	-	-	-	-	-	-	-
216	Paenibacillus	434783	1.06	+	-	-	-	-	+	-	-	+	-	-	-	-
217	Paenibacillus	434783	1.06	-	+	-	-	+	+	+	+	+	+	-	+	-
	Total	**5.13 x 10^6**	**12.46**	**6.33**	**11.40**	**0.00**	**0.00**	**3.17**	**10.35**	**3.17**	**3.17**	**5.70**	**1.06**	**2.11**	**1.06**	**0.00**
218	Polaromonas	434783	1.06	+	-	-	-	-	-	-	-	-	-	-	-	-
219	Polaromonas	1260870	3.06	-	-	-	+	-	-	-	-	-	-	-	-	-
220	Polaromonas	1260870	3.06	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.96 x 10^6**	**7.18**	**1.06**	**0.00**	**0.00**	**3.06**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
221	Rhizobium	391305	0.95	-	-	-	+	-	-	-	-	-	-	-	-	-
222	Rhizobium	72464	0.18	-	-	-	+	-	-	-	-	-	-	-	-	-
223	Rhizobium	434783	1.06	+	-	-	+	-	-	-	-	-	-	-	-	-
224	Rhizobium	434783	1.06	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**1.33 x 10^6**	**3.24**	**1.06**	**0.00**	**0.00**	**2.18**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
225	Sphingobacterium	391305	0.95	-	+	-	+	+	+	+	+	+	+	+	+	-
226	Sphingobacterium	391305	0.95	-	+	-	+	+	+	+	-	+	-	-	+	-
	Total	**7.83 x 10^5**	**1.90**	**0.00**	**1.90**	**0.00**	**1.90**	**1.90**	**1.90**	**1.90**	**0.95**	**1.90**	**0.95**	**0.95**	**1.90**	**0.00**
227	Staphylococcus	630435	1.53	-	+	+	-	-	-	-	-	-	-	-	-	-
	Total	**6.30 x 10^5**	**1.53**	**0.00**	**1.53**	**1.53**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
228	Stenotrophomonas	144928	0.35	+	+	-	-	-	+	+	-	-	-	-	-	-
229	Stenotrophomonas	144928	0.35	+	+	-	-	-	+	-	-	-	-	-	-	-
	Total	**2.90 x 10^5**	**0.70**	**0.70**	**0.70**	**0.00**	**0.00**	**0.00**	**0.70**	**0.35**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
230	Variovorax	630435	1.53	-	-	-	+	-	+	-	-	-	-	-	-	-
231	Variovorax	217392	0.53	+	-	-	+	-	+	-	+	+	-	-	-	-
232	Variovorax	1260870	3.06	+	+	-	+	-	+	-	-	+	-	-	+	-
233	Variovorax	434783	1.06	-	-	-	-	-	-	-	-	-	-	-	-	-
234	Variovorax	434783	1.06	+	+	-	+	+	+	-	+	+	-	-	-	-
235	Variovorax	217392	0.53	-	-	-	+	-	-	-	-	-	-	-	-	-
236	Variovorax	217392	0.53	-	-	-	-	-	-	-	-	-	-	-	-	-
237	Variovorax	434783	1.06	+	+	-	-	+	+	-	-	+	-	-	+	-
238	Variovorax	217392	0.53	-	-	-	+	-	-	-	-	-	-	-	-	-

239	Variovorax	72464	0.18	-	-	-	+	-	-	-	-	-	-	-	-	-
240	Variovorax	72464	0.18	-	-	-	+	-	-	-	-	-	-	-	-	-
241	Variovorax	1260870	3.06	+	-	-	+	+	+	-	-	-	-	-	-	-
242	Variovorax	579710	1.41	-	-	-	-	-	+	-	-	-	-	-	-	-
243	Variovorax	840580	2.04	+	-	-	+	-	-	-	-	-	-	-	-	-
244	Variovorax	217392	0.53	-	-	-	+	-	-	-	-	-	-	-	-	-
245	Variovorax	217392	0.53	-	-	-	+	-	-	-	-	-	-	-	-	-
	Total	**7.33 x 10^6**	**17.79**	**10.80**	**5.17**	**0.00**	**13.74**	**5.17**	**11.70**	**0.00**	**1.58**	**5.70**	**0.00**	**0.00**	**4.12**	**0.00**
246	Xanthomonas	434783	1.06	-	+	-	-	-	+	-	-	+	-	-	-	-
247	Xanthomonas	434783	1.06	-	-	-	-	-	+	-	-	+	-	-	-	-
248	Xanthomonas	1043478	2.53	+	+	-	-	-	+	-	-	+	-	-	-	-
249	Xanthomonas	1043478	2.53	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**2.96 x 10^6**	**7.18**	**2.53**	**3.59**	**0.00**	**0.00**	**0.00**	**4.65**	**0.00**	**0.00**	**4.65**	**0.00**	**0.00**	**0.00**	**0.00**

***S. alba x alba*: Root isolates**								(mM)								
N°	**Genotypic identification**	**CFU g^{-1} FW root**	**Abundance (%)**	**IAA**	**ACC deam**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**
250	Alcaligenes	78740	1.24	-	-	+	-	-	-	-	-	-	-	-	-	-
251	Alcaligenes	39370	0.62	+	-	+	-	-	-	-	-	-	-	-	-	-
	Total	**1.18 x 10^5**	**1.86**	**0.62**	**0.00**	**1.86**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
252	Arthrobacter	39370	0.62	+	+	-	-	+	+	+	+	+	+	-	+	-
253	Arthrobacter	19685	0.31	+	+	-	-	-	+	-	-	+	-	-	-	-
	Total	**5.90 x 10^4**	**0.93**	**0.93**	**0.93**	**0.00**	**0.00**	**0.62**	**0.93**	**0.62**	**0.62**	**0.93**	**0.62**	**0.00**	**0.62**	**0.00**
254	Bacillus	157480	2.48	+	-	+	-	-	-	-	-	-	-	-	-	-
255	Bacillus	39370	0.62	-	-	-	-	-	-	-	-	-	-	-	-	-
256	Bacillus	3937	0.06	-	+	-	+	+	+	-	+	+	-	+	+	-
257	Bacillus	157480	2.48	-	-	-	-	-	+	-	-	+	-	-	+	-
258	Bacillus	78740	1.24	+	+	-	+	+	+	+	+	+	+	-	+	-
	Total	**4.37 x 10^5**	**6.89**	**3.73**	**1.30**	**2.48**	**1.30**	**1.30**	**3.79**	**1.24**	**1.30**	**3.79**	**1.24**	**0.06**	**3.79**	**0.00**
259	Bosea	228347	3.60	+	-	-	-	+	+	+	+	+	+	-	+	+
260	Bosea	9449	0.15	+	-	-	-	-	+	-	-	+	-	-	-	-
261	Bosea	33465	0.53	-	+	-	+	+	+	+	-	+	+	-	+	-
262	Bosea	78740	1.24	+	-	-	-	+	-	-	+	-	-	+	-	-
	Total	**3.50 x 10^5**	**5.52**	**4.99**	**0.53**	**0.00**	**0.53**	**5.37**	**4.28**	**4.13**	**4.84**	**4.28**	**4.13**	**1.24**	**4.13**	**3.60**
263	Caulobacter	456693	7.20	+	-	-	-	-	-	-	-	-	-	-	-	-
264	Caulobacter	456693	7.20	-	-	-	+	-	-	-	-	-	-	-	-	-
265	Caulobacter	78740	1.24	-	-	-	-	-	-	-	-	-	-	-	-	-
266	Caulobacter	39370	0.62	-	-	-	-	-	+	+	-	+	-	-	+	-

267	Caulobacter	39370	0.62	+	+	+	+	+	+	+	+	+	+	+	+	+
268	Caulobacter	228347	3.60	+	-	-	-	-	+	-	-	-	-	-	-	-
269	Caulobacter	228347	3.60	+	-	-	-	-	+	-	-	-	-	-	-	-
270	Caulobacter	39370	0.62	+	-	-	-	-	+	-	-	+	-	-	-	-
271	Caulobacter	78740	1.24	+	-	-	-	-	-	-	-	-	-	-	-	-
272	Caulobacter	78740	1.24	+	-	-	-	-	-	-	-	-	-	-	-	-
273	Caulobacter	78740	1.24	-	+	-	+	+	+	-	+	+	-	+	+	-
274	Caulobacter	9449	0.15	+	-	-	-	-	+	-	-	+	-	-	-	-
275	Caulobacter	9449	0.15	+	-	+	-	-	+	-	-	+	-	-	-	-
	Total	**1.82 x 10^6**	**28.73**	**18.43**	**1.86**	**0.77**	**9.06**	**1.86**	**10.60**	**1.24**	**1.86**	**3.40**	**0.62**	**1.86**	**2.48**	**0.62**
276	Chryseobacterium	81102	1.28	-	+	-	+	+	+	+	+	+	+	+	+	-
277	Chryseobacterium	27034	0.43	-	+	-	+	+	+	+	-	+	+	-	+	-
	Total	**1.08 x 10^5**	**1.71**	**0.00**	**1.71**	**0.00**	**1.71**	**1.71**	**1.71**	**1.71**	**1.28**	**1.71**	**1.71**	**1.28**	**1.71**	**0.00**
278	Duganella	66929	1.06	-	+	-	+	+	+	-	+	+	-	+	+	-
	Total	**6.69 x 10^4**	**1.06**	**0.00**	**1.06**	**0.00**	**1.06**	**1.06**	**1.06**	**0.00**	**1.06**	**1.06**	**0.00**	**1.06**	**1.06**	**0.00**
279	Mesorhizobium	78740	1.24	+	-	-	-	-	+	-	-	+	-	-	-	-
280	Mesorhizobium	78740	1.24	+	+	-	-	+	+	+	+	+	-	-	+	-
281	Mesorhizobium	78740	1.24	+	-	-	-	-	-	-	-	-	-	-	-	-
282	Mesorhizobium	39370	0.62	-	-	-	+	-	+	-	-	+	-	-	-	-
	Total	**2.76 x 10^5**	**4.35**	**3.73**	**1.24**	**0.00**	**0.62**	**1.24**	**3.10**	**1.24**	**1.24**	**3.10**	**0.00**	**0.00**	**1.24**	**0.00**
283	Mycobacterium	78740	1.24	-	-	-	+	-	-	-	-	-	-	-	-	-
284	Mycobacterium	19685	0.31	-	-	-	-	+	-	-	-	-	-	-	-	-
	Total	**9.84 x 10^4**	**1.55**	**0.00**	**0.00**	**0.00**	**1.24**	**0.31**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
285	Paenibacillus	15748	0.25	+	-	-	-	-	-	-	-	-	-	-	-	-
286	Paenibacillus	47244	0.75	-	-	-	-	-	-	-	-	-	-	-	-	-
287	Paenibacillus	39370	0.62	+	-	+	-	-	+	-	-	+	-	-	-	-
288	Paenibacillus	66929	1.06	-	-	-	+	+	-	-	-	-	-	-	-	-
289	Paenibacillus	66929	1.06	+	-	-	-	-	-	-	-	-	-	-	-	-
290	Paenibacillus	81102	1.28	-	-	-	-	+	+	-	+	+	-	+	-	-
	Total	**3.17 x 10^5**	**5.00**	**1.92**	**0.00**	**0.62**	**1.06**	**2.33**	**1.90**	**0.00**	**1.28**	**1.90**	**0.00**	**1.28**	**0.00**	**0.00**
291	Polaromonas	157480	2.48	-	-	-	-	-	-	-	-	-	-	-	-	-
292	Polaromonas	157480	2.48	-	-	-	-	-	-	-	-	-	-	-	-	-
293	Polaromonas	23622	0.37	+	+	-	+	+	+	-	+	+	-	+	+	-
294	Polaromonas	78740	1.24	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**4.17 x 10^5**	**6.58**	**0.37**	**0.37**	**0.00**	**0.37**	**0.37**	**0.37**	**0.00**	**0.37**	**0.37**	**0.00**	**0.37**	**0.37**	**0.00**
295	Pseudomonas	7874	0.12	-	-	-	+	+	+	-	-	+	-	-	+	-
	Total	**7.87 x 10^3**	**0.12**	**0.00**	**0.00**	**0.00**	**0.12**	**0.12**	**0.12**	**0.00**	**0.00**	**0.12**	**0.00**	**0.00**	**0.12**	**0.00**
296	Rahnella	78740	1.24	+	+	-	-	+	+	+	-	+	-	-	+	-
	Total	**7.87 x 10^4**	**1.24**	**1.24**	**1.24**	**0.00**	**0.00**	**1.24**	**1.24**	**1.24**	**0.00**	**1.24**	**0.00**	**0.00**	**1.24**	**0.00**
297	Rhizobium	81102	1.28	+	-	-	-	-	+	-	-	+	-	-	-	-
298	Rhizobium	66929	1.06	+	+	-	+	+	-	-	+	-	-	-	-	-
299	Rhizobium	78740	1.24	-	-	-	-	-	-	-	-	-	-	-	-	-

300	Rhizobium	7874	0.12	+	+	+	+	+	+	+	+	-	-	-	-	-
301	Rhizobium	66929	1.06	+	+	-	+	+	+	-	+	+	-	-	+	-
302	Rhizobium	157480	2.48	+	+	-	+	+	-	-	+	-	-	-	-	-
303	Rhizobium	66929	1.06	+	+	+	+	+	+	+	+	-	-	-	-	-
304	Rhizobium	39370	0.62	+	+	-	-	-	-	-	-	-	-	-	-	-
	Total	**5.65 x 10^5**	**8.92**	**7.67**	**6.39**	**1.18**	**5.77**	**5.77**	**3.51**	**1.18**	**5.77**	**2.33**	**0.00**	**0.00**	**1.06**	**0.00**
305	Sphingobacterium	33465	0.53	+	+	-	+	+	+	+	+	+	+	+	+	-
	Total	**3.35 x 10^4**	**0.53**	**0.53**	**0.53**	**0.00**	**0.53**	**0.53**	**0.53**	**0.53**	**0.53**	**0.53**	**0.53**	**0.53**	**0.53**	**0.00**
306	Sphingomonas	157480	2.48	+	-	-	-	-	-	-	-	-	-	-	-	-
307	Sphingomonas	157480	2.48	-	+	-	+	+	+	+	+	+	-	-	+	-
308	Sphingomonas	78740	1.24	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	**3.94 x 10^5**	**6.21**	**2.48**	**2.48**	**0.00**	**2.48**	**2.48**	**2.48**	**2.48**	**2.48**	**2.48**	**0.00**	**0.00**	**2.48**	**0.00**
309	Staphylococcus	9449	0.15	+	-	-	-	-	+	-	-	+	-	-	-	-
	Total	**9.45 x 10^3**	**0.15**	**0.15**	**0.00**	**0.00**	**0.00**	**0.00**	**0.15**	**0.00**	**0.00**	**0.15**	**0.00**	**0.00**	**0.00**	**0.00**
310	Stenotrophomonas	78740	1.24	-	-	-	-	-	-	-	-	-	-	-	-	-
311	Stenotrophomonas	39370	0.62	-	+	-	-	+	+	+	-	-	-	-	-	-
312	Stenotrophomonas	39370	0.62	+	+	-	-	-	+	-	-	+	-	-	-	-
313	Stenotrophomonas	19685	0.31	+	+	-	-	+	+	-	-	+	-	-	-	-
	Total	**1.77 x 10^5**	**2.79**	**0.93**	**1.55**	**0.00**	**0.00**	**0.93**	**1.55**	**0.62**	**0.00**	**0.93**	**0.00**	**0.00**	**0.00**	**0.00**
314	Variovorax	3937	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-
315	Variovorax	3937	0.06	+	-	-	+	+	+	-	-	+	-	-	+	-
316	Variovorax	3937	0.06	-	-	-	-	+	+	-	+	+	-	+	+	-
317	Variovorax	23622	0.37	+	-	-	-	-	-	-	-	-	-	-	-	-
318	Variovorax	23622	0.37	-	-	-	+	-	+	-	-	+	-	-	+	-
319	Variovorax	7874	0.12	-	-	-	-	+	+	+	+	+	+	-	-	-
320	Variovorax	66929	1.06	-	-	-	+	+	+	+	+	+	+	-	-	-
321	Variovorax	3937	0.06	-	-	-	-	+	+	+	+	+	-	+	+	-
322	Variovorax	81102	1.28	+	-	-	-	-	+	-	-	+	-	-	-	-
323	Variovorax	27034	0.43	-	-	-	-	+	+	-	+	+	-	+	-	-
324	Variovorax	40551	0.64	-	-	-	-	+	+	+	+	+	+	+	-	-
325	Variovorax	81102	1.28	+	+	-	+	+	+	+	+	+	+	-	+	-
326	Variovorax	81102	1.28	-	-	-	-	+	+	-	+	+	-	-	-	-
327	Variovorax	23622	0.37	-	-	-	+	+	+	+	-	+	-	-	-	-
328	Variovorax	27034	0.43	-	-	-	+	+	+	+	+	+	+	+	+	-
329	Variovorax	228347	3.60	-	-	-	-	-	+	-	-	+	-	-	-	-
330	Variovorax	3937	0.06	-	-	-	+	-	-	-	-	-	-	-	-	-
331	Variovorax	23622	0.37	+	-	-	+	+	+	+	+	+	-	-	+	-
332	Variovorax	3937	0.06	-	-	-	-	+	+	+	+	+	+	-	-	-
333	Variovorax	15748	0.25	-	-	-	-	+	+	+	+	+	+	+	-	-
334	Variovorax	15748	0.25	+	-	-	-	+	+	+	+	+	+	+	+	-
335	Variovorax	3937	0.06	-	-	-	-	+	+	+	+	+	+	+	+	-
336	Variovorax	19685	0.31	+	-	-	+	-	-	-	-	-	-	-	-	-

337	Variovorax	66929	1.06	+	-	-	-	-	-	-	-	-	-	-	-	-	-
338	Variovorax	39370	0.62	-	+	-	-	+	+	+	-	+	+	-	+	-	
	Total	**9.21 x 10^5**	**14.52**	**4.98**	**1.90**	**0.00**	**4.31**	**7.40**	**12.65**	**5.57**	**6.35**	**12.65**	**4.77**	**2.18**	**3.57**	**0.00**	
339	Xanthomonas	81102	1.28	+	+	-	-	-	+	-	-	+	-	-	+	-	
340	Xanthomonas	3937	0.06	-	+	-	+	-	+	-	-	+	-	-	+	-	
	Total	**8.50 x 10^4**	**1.34**	**1.28**	**1.34**	**0.00**	**0.06**	**0.00**	**1.34**	**0.00**	**0.00**	**1.34**	**0.00**	**0.00**	**1.34**	**0.00**	

***S. alba x alba*: Twig isolates**

								(mM)									
N°	**Genotypic identification**	**CFU g^{-1} FW twig**	**Abundance (%)**	**IAA**	**ACC deam**	**OA**	**SID**	**0.4 Cd**	**0.6 Zn**	**0.4 Cd + 0.6 Zn**	**0.8 Cd**	**1.0 Zn**	**0.8 Cd + 1.0 Zn**	**1.6 Cd**	**2.5 Zn**	**1.6 Cd + 2.5 Zn**	
341	Alcaligenes	14	0.59	-	-	-	-	+	+	-	+	+	-	+	+	-	
342	Alcaligenes	142	5.88	+	+	+	+	-	+	-	-	+	-	-	-	-	
343	Alcaligenes	14	0.59	-	-	-	-	+	+	-	+	+	-	+	-	-	
344	Alcaligenes	14	0.59	-	-	-	-	+	-	-	+	+	-	+	+	-	
	Total	**1.85 x 10^2**	**7.66**	**5.88**	**5.88**	**5.88**	**5.88**	**1.78**	**7.06**	**0.00**	**1.78**	**7.66**	**0.00**	**1.78**	**1.19**	**0.00**	
345	Arthrobacter	142	5.88	+	-	+	-	+	+	-	-	-	-	-	-	-	
346	Arthrobacter	43	1.78	+	+	-	-	+	+	-	+	+	-	-	+	-	
	Total	**1.85 x 10^2**	**7.66**	**7.66**	**1.78**	**5.88**	**0.00**	**7.66**	**7.66**	**0.00**	**1.78**	**1.78**	**0.00**	**0.00**	**1.78**	**0.00**	
347	Caulobacter	142	5.88	+	+	-	-	-	+	-	-	+	-	-	-	-	
	Total	**1.42 x 10^2**	**5.88**	**5.88**	**5.88**	**0.00**	**0.00**	**0.00**	**5.88**	**0.00**	**0.00**	**5.88**	**0.00**	**0.00**	**0.00**	**0.00**	
348	Curtobacterium	142	5.88	+	+	-	+	-	+	-	-	+	-	-	-	-	
349	Curtobacterium	142	5.88	+	+	+	+	+	+	-	+	-	-	-	-	-	
350	Curtobacterium	71	2.94	+	+	-	+	-	+	-	-	+	-	-	-	-	
351	Curtobacterium	71	2.94	+	-	+	+	+	+	-	+	-	-	-	-	-	
	Total	**4.26 x 10^2**	**17.63**	**17.63**	**14.70**	**8.82**	**17.63**	**8.82**	**17.63**	**0.00**	**8.82**	**8.82**	**0.00**	**0.00**	**0.00**	**0.00**	
352	Flavobacterium	52	2.13	-	-	-	+	+	-	-	+	-	-	-	-	-	
353	Flavobacterium	43	1.78	+	-	+	+	-	-	-	-	-	-	-	-	-	
354	Flavobacterium	43	1.78	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Total	**1.38 x 10^2**	**5.69**	**1.78**	**0.00**	**1.78**	**3.91**	**2.13**	**0.00**	**0.00**	**2.13**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	
355	Frigoribacterium	71	2.94	+	-	-	-	-	+	-	-	-	-	-	-	-	
356	Frigoribacterium	103	4.26	+	-	-	-	-	-	-	-	-	-	-	-	-	
357	Frigoribacterium	26	1.07	+	-	-	-	-	+	-	-	+	-	-	-	-	
358	Frigoribacterium	103	4.26	+	-	-	-	-	-	-	-	-	-	-	-	-	
359	Frigoribacterium	103	4.26	-	-	-	-	-	-	-	-	-	-	-	-	-	
360	Frigoribacterium	103	4.26	-	-	-	-	-	-	-	-	-	-	-	-	-	
361	Frigoribacterium	114	4.70	+	-	-	-	-	-	-	-	-	-	-	-	-	
	Total	**6.22 x 10^2**	**25.76**	**17.23**	**0.00**	**0.00**	**0.00**	**0.00**	**4.00**	**0.00**	**0.00**	**1.07**	**0.00**	**0.00**	**0.00**	**0.00**	
362	Microbacterium	43	1.78	+	-	-	-	+	+	+	+	+	+	-	+	-	

		Total	**4.30 x 10^1**	**1.78**	**1.78**	**0.00**	**0.00**	**0.00**	**1.78**	**1.78**	**1.78**	**1.78**	**1.78**	**1.78**	**0.00**	**1.78**	**0.00**
363	Rahnella		142	5.88	+	-	+	+	+	+	-	+	-	-	-	-	-
		Total	**1.42 x 10^2**	**5.88**	**5.88**	**0.00**	**5.88**	**5.88**	**5.88**	**5.88**	**0.00**	**5.88**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
364	Uncultured bacterium		22	0.89	+	+	-	-	-	+	-	-	+	-	-	+	-
365	Uncultured bacterium		114	4.70	+	+	-	-	-	-	-	-	-	-	-	-	-
		Total	**1.35 x 10^2**	**5.59**	**5.59**	**5.59**	**0.00**	**0.00**	**0.00**	**0.89**	**0.00**	**0.00**	**0.89**	**0.00**	**0.00**	**0.89**	**0.00**
366	Xanthomonas		142	5.88	+	+	-	+	-	+	-	-	+	-	-	-	-
367	Xanthomonas		142	5.88	+	+	-	+	-	-	-	-	-	-	-	-	-
368	Xanthomonas		71	2.94	+	+	-	-	+	+	+	-	+	+	-	+	-
369	Xanthomonas		43	1.78	+	-	-	-	+	+	-	+	-	-	-	-	-
		Total	**3.98 x 10^2**	**16.48**	**16.48**	**14.70**	**0.00**	**11.76**	**4.72**	**10.60**	**2.94**	**1.78**	**8.82**	**2.94**	**0.00**	**2.94**	**0.00**

IAA = production of indole-3-acetic acid, ACC deam. = production of 1-aminocyclopropane-1-carboxylate deaminase, OA = production of organic acids, SID = production of siderophores.

Supplementary table 5.3 Loss of *in vitro* re-cultivability and phenotypic traits (%) when comparing the second screening (2013 screening) with the first screening (2011 screening) of in total 103 isolates.

		S. viminalis	***S. alba x alba***	**Total**
Number of isolates tested a second time	**Rhizosphere**	21	10	**103**
	Roots	38	18	
	Twigs	2	14	
Unsuccessful re-cultivation (in 1/10 diluted 869 medium and in the other media) (%)				**9**

		S. viminalis	***S. alba x alba***	**Total**
Loss of **IAA** production capacity (%)	**Rhizosphere**	26	100	**46**
	Roots	52	11	
	Twigs	0	78	
Loss of **ACC deam.** production capacity (%)	**Rhizosphere**	50	100	**74**
	Roots	87	71	
	Twigs	0	67	
Loss of **OA** production capacity (%)	**Rhizosphere**	50	-	**62**
	Roots	40	100	
	Twigs	-	75	
Loss of **SID** production capacity (%)	**Rhizosphere**	47	50	**43**
	Roots	41	35	
	Twigs	0	56	
Loss of tolerance to **0.8 mM Cd** (%)	**Rhizosphere**	100	100	**83**
	Roots	95	67	
	Twigs	-	63	
Loss of tolerance to **1 mM Zn** (%)	**Rhizosphere**	50	50	**41**
	Roots	46	15	
	Twigs	-	43	
Loss of tolerance to **1.6 mM Cd** (%)	**Rhizosphere**	100	100	**94**
	Roots	100	83	
	Twigs	-	100	
Loss of tolerance to **2.5 mM Zn** (%)	**Rhizosphere**	78	80	**52**
	Roots	45	38	
	Twigs	-	33	

IAA = indole-3-acetic acid, ACC deam. = 1-aminocyclopropane-1-carboxylate deaminase, OA = organic acids, SID = siderophores.

Chapter 6

General discussion, conclusions and perspectives

6.1 Thesis overview

In the northeast of Belgium (the Campine region), an area of about 280 km^2 is historically contaminated with mainly cadmium (Cd), zinc (Zn) and lead (Pb). The negative impacts on inhabitants and the environment in general as well as economic losses in the farming industry urged regional policy makers to strongly recommend the remediation of the metal-contaminated soil. Given the vastness of the area and the diffuseness, moderation and shallowness of the contamination, phytoextraction, using plants to extract metals out of the soil and accumulate them in harvestable biomass, is proposed as a good remediation strategy. More specifically, cultivating non-food high biomass crops with moderate metal accumulation capacity reveals promising for this area.

In this thesis, phytoextraction potentials of the high biomass crops tobacco (*Nicotiana tabacum* L.), sunflower (*Helianthus annuus* L.), hemp (*Cannabis sativa* L.) and short rotation coppice (SRC) of willow (*Salix*) and poplar (*Populus*) were evaluated and compared by cultivating them over a 4-year period on a metal-contaminated field in the northeast of Belgium. The main conclusions of this research are summarized in part 6.1.

In parallel with the field evaluations of the above-mentioned high biomass crops, the research focused on metal phytoextraction using SRC of willow. SRC of willow is an abundantly studied high biomass crop in the framework of metal phytoextraction but there still are 2 main problems using this (and almost all other) remediating crop(s), seriously hampering the implementation of this

remediation technology. Firstly, the phytoextraction efficiency of SRC of willow on the longer term is highly uncertain, but remediation of Cd to legal threshold limits will likely take longer than the period considered a threshold for phytoextraction as a stand-alone technology. Secondly, there is up till now in Belgium no sustainable, environmentally sound biomass disposal or conversion route for metal-contaminated wood. While the second problem was behind the scope of this thesis, estimating remediation time of metal phytoextraction using SRC of willow based on longer-term field observations (part 6.2) and improving its phytoextraction potential by means of clone selection, bioaugmentation and fertilization (part 6.3) are addressed.

Finally, in part 6.4, the reliability of information that is obtained when performing short-term pot experiments with SRC of willow is discussed.

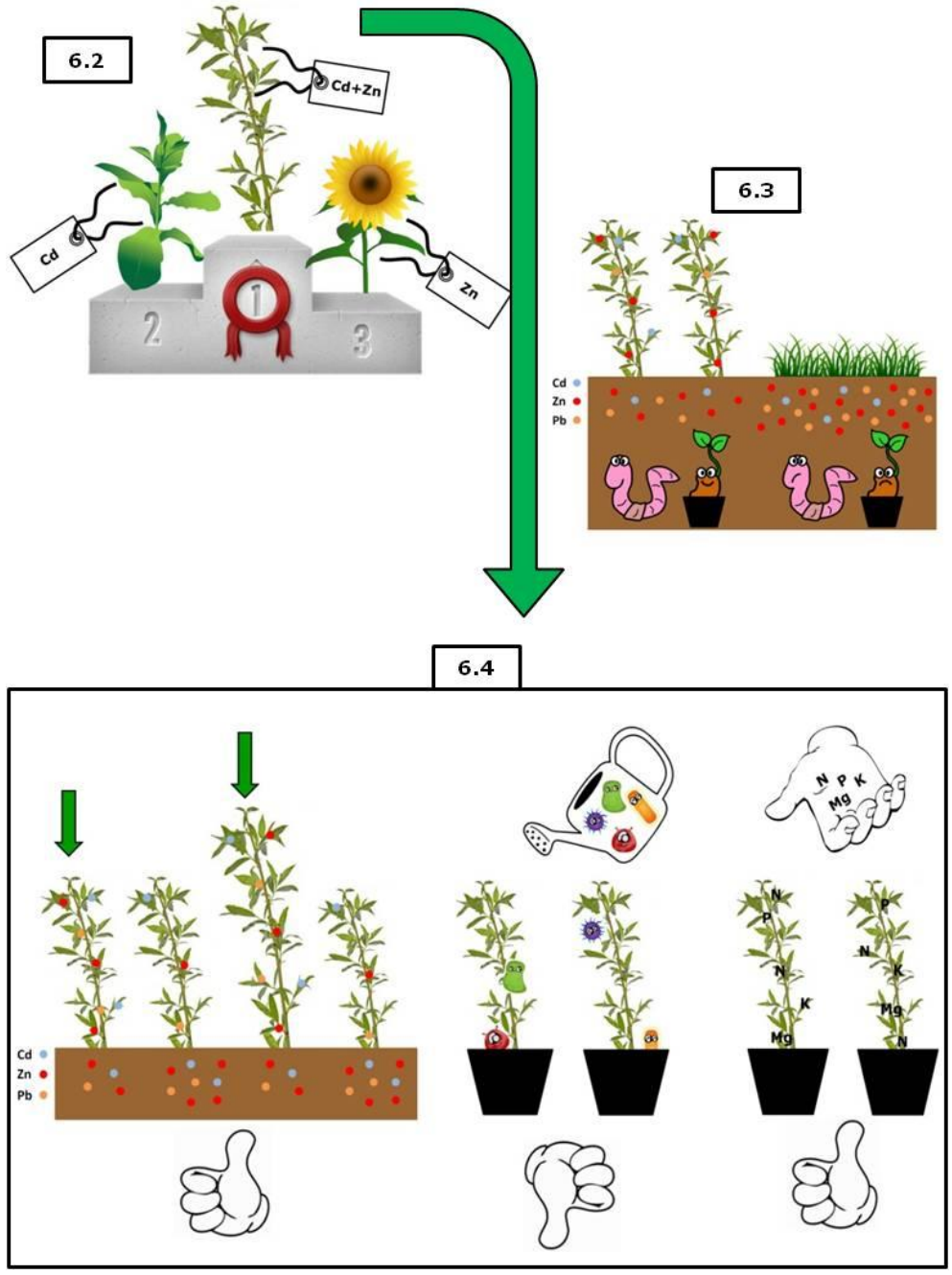

Figure 6.2 Schematic overview of the main results of this thesis. Note: the results are highly simplified and only additional to the discussion in the text (numbers coincide with subtitles in Chapter 6).

6.2 Four years of metal phytoextraction using high biomass crops: a stronger case for SRC

The commercial and experimental poplar and willow clones in SRC, planted on the field in 2006, were evaluated for stem biomass production and stem metal accumulation in 2009 after the first 4 growing seasons (defined the first cutting cycle). Selected tobacco clones and sunflower mutants were cultivated and evaluated every summer from 2011 till 2014 and growth and phytoextraction potential of hemp were investigated in 2013.

All evaluated tobacco clones, sunflower mutants and SRC clones proved to be efficient accumulators of Cd and Zn when cultivated on the metal-contaminated field (bioconcentration factors > 1 (Dickinson and Pulford 2005; Kötschau *et al.* 2014)) (Table 3.8). Hemp did not fulfill this criterion. The extraction potential for Cd and Zn, defined as the amount of metals removed from the soil by aboveground/stem harvest, together with bioconcentration values tend to indicate sunflower as a highly efficient extractor of Zn, tobacco as a more pronounced extractor of Cd and most poplar and willow clones as the crops with the highest combined extraction of Cd and Zn (Figure 3.4). The hypothetical remediation times calculated, suggest the commercial willow clone Zwarte Driebast and the experimental poplar clone D x (T x M) to be respectively the best and second best option for Cd and Zn phytoextraction in this area (Table 3.9). However, the variation on the estimated extraction potentials (and remediation times) is too high to conclude with great certainty that SRC is the best option for metal phytoextraction in this area. Analyzing the source of this variation and the extent of its perseverance might be helpful in order to draw proper conclusions when comparing the phytoextracting crops.

The productivity of tobacco clones varied significantly over the 4 tested years, while metal concentrations in aboveground biomass showed a considerable year-to-year variation for all tobacco clones as well as almost all sunflower mutants (Tables 3.4 and 3.5). Since both crops were cultivated every year in summer on adjacent plots containing the same levels of contamination, causes for these yearly variations are believed to be related to climatological conditions, nursing and management practices and/or generation number and quality of the seeds.

The fact that both species are non-native to Belgium, probably enhances their sensitivity to certain environmental factors. As a result of these yearly fluctuations in biomass productivity and/or metal accumulation levels, phytoextraction using tobacco clones and sunflower mutants can be considered quite effective but, by the same token, very inefficient (Tables 3.4 and 3.5). Optimizing nursing and management practices might reduce these variations to some extent, but other factors are difficult (seed characteristics) or impossible (climatological conditions) to control. It is therefore concluded that when implementing phytoextraction in the Campine area using the selected tobacco clones and sunflower mutants, (large) variations seem to be inherently present.

In case of the SRC species (Table 3.1), the variations observed are related to the field heterogeneity (a certain clone is planted in replicates in different plots on the experimental field and data are a mix of all plots) and to clonal differences (the experimental groups represent a collection of several clones with the same crossing type). The variation can very likely be reduced when evaluating a clone in a certain plot and construing the experimental groups. Although the possibility to reduce this variation (exposed in the first cutting cycle) argues in favor of SRC, changes in biomass production and metal accumulation in later cutting cycles, which determine the phytoextracting stability of this crop over the longer term, are likely to occur (Hammer *et al.* 2003; Labrecque and Teodorescu 2003; Mertens *et al.* 2006; Aronsson *et al.* 2014; Van Slycken *et al.* 2015). As a consequence, the leading role of SRC can be considered to not have become more pronounced when analyzing naturally occurring variation. There is however another argument supporting the case of SRC. While the tobacco clones and sunflower mutants were already the result of respectively *in vitro* breeding and chemical mutagenesis followed by continuous breeding and selection for improved phytoextraction efficiencies (Herzig *et al.* 1997, 2014; Guadagnini 2000, Nehnevajova *et al.* 2005, 2007, 2009), in case of the poplar and willow clones, the large range of (combined) Cd and Zn extraction covered (Figure 3.4), provides considerable cause for optimism that clone selection and/or conventional breeding approaches may provide additional clones with a high combined extraction of Cd and Zn. Clone selection as a

strategy to improve metal phytoextraction using SRC was also addressed in this research (see further).

When implementing phytoextraction as a stand-alone remediation technology, the remediation time should preferably not exceed a period of around 10 years to render the technology economically feasible in itself (Blaylock and Huang 2000). Since the evaluated high biomass crops here (as well as almost all evaluations of this matter in literature) exceed this term (by far), it is necessary to crosscut this remediation technology with other opportunities. Synergies with economic as well as (other) environmental agendas seem to be indispensible for the justification, advancement and eventual implementation of metal phytoextraction (Dickinson *et al.* 2009). However, to be clear, although these beneficial externalities should be present for the implementation of metal phytoextraction using high biomass crops, their incorporation seems far from realistic nowadays. There are still major problems to be encountered when converting metal-contaminated herbaceous or woody biomass into bioenergy (metal emissions, disposal of 'waste', rather limited supply of biomass, *etc.*), and economic revenues are non-existent so far. Regarding environmental benefits, although these are not negligible (risk reduction, soil improvements, biodiversity increase, carbon sequestration, *etc.*), there is up till now no system to compensate for these ecosystem services.

If assumed that there will be progress in rewarding either or both economic and environmental externalities, the implementation of metal phytoextracting systems using high biomass crops should, besides its remediation potential and stability, additionally be evaluated for possible economic revenues and environmental benefits. In this case, the leading role of SRC can further be enhanced. Indeed, the native SRC species will very likely expose a more stable (and probably increasing) biomass production over the years compared to the non-native herbaceous annuals, increasing possible economic revenues. Furthermore, a SRC plantation is much less labor-intensive (no nursing, no (yearly) weed control/fertilization/harvest/storage of biomass, no irrigation, crop rotation, *etc.*), seriously reducing the economic input needed. The bigger advantages of SRC over tobacco and sunflower as regards environmental issues mainly arise from the presence of a year-round vegetation cover implying higher

reductions of risks and higher carbon sequestration and soil improving potential. In addition, soil depletion and compaction will be lower. Finally, the natural occurrence in the temperate regions of the Northern hemisphere of most of the SRC willow and poplar species is another environmentally important argument in favor of SRC.

Biomass production of the evaluated commercial hemp was highly promising (almost 75% more than the 10 tons per hectare and year generally reported in Europe (Citterio *et al.* 2003)) (Table 3.7). However, hemp revealed not to be an efficient accumulator of Cd, Zn or Pb on the metal-contaminated field (Table 3.7). Most other studies with hemp also report a BCF < 1 and a low phytoextraction capacity of aboveground biomass (Linger *et al.* 2002; Citterio *et al.* 2003; Shi *et al.* 2012). Therefore, further investigating hemp for metal phytoextraction purposes is only interesting if cultivars with metal accumulating capacities will become available. Thereafter, the high biomass productivity level as well as its stability over the years has to be confirmed on a larger scale, (high-quality) biomass conversion routes must be developed for metal-contaminated hemp and possible environmental (dis)advantages of hemp cultivation in this area need to be assessed.

Of all evaluated high biomass crops for metal phytoextraction in the Campine region, SRC species seem the most promising to reach the shortest remediation time taking into account that variation in biomass production and/or metal accumulation can be reduced and that clone selection or breeding will provide additional clones with combined high extractions of Cd and Zn. When (other) environmental agendas join the picture, the leading role of SRC seems only to be strengthened. A major drawback up till now however, is the lack of a sustainable, environmentally sound, economically profitable crop conversion route.

6.3 Longer-term metal phytoextraction using SRC of willow: uncertainties and the importance of long-term field trials

A phytoextraction system is composed of 4 main entities: the soil, the soil solution, microorganisms and the plant (Lasat 2000; Landberg and Greger 2002; Koopmans *et al.* 2007; Mench *et al.* 2009). The system components together with their complex interactions determine the overall remediation efficiency under prevailing conditions, while future extraction efficiencies also depend on evolutions in components and their interactions. Given the complexity of the system, extrapolations from hydroponics, pot trials or short-term/small-scale field experiments are unreliable and no modeling or simulation can possibly include all variables, interactions, their evolutions and uncertainties (moreover, a lot is still unknown). As a consequence, there is no other way to determine phytoextraction efficiencies than to evaluate such systems in practice. On the metal-contaminated field, a unique opportunity exists to this concern since SRC is cultivated for a longer term (since 2006). Field soil remediated by 2 rotation cycles (8 years) of willow (SRC of Tora; *Salix schwerinnii x Salix viminalis*) management and field soil without remediation management were analyzed for pseudo-total Cd, Zn and Pb concentrations and soil toxicity. It revealed that more than 3 mg Cd, 159 mg Zn and 89 mg Pb per kg^{-1} dry soil were removed/accumulated by 8 years of Tora cultivation (Table 4.1) which significantly reduces the hypthesized remediation times based on metals exported from the field by determining stem biomass production and stem metal accumulation of Tora after the first rotation cycle (Table 4.2). Furthermore, the lower (bio)availability of Cd, Zn and Pb, determined by standardized chemical extractions (Table 4.1), and all ecotoxicity tests, accredited plant and invertebrate ecotoxicity assays (Tables 4.3-4.5, Figure 4.2), unanimously indicated the willow-managed soil to be less toxic compared to the unmanaged soil, seriously constricting environmental risks.

Comparable research on soils remediated by other SRC clones as well as proceeding soil analyses (later growing cycles) can be very valuable for a thorough evaluation of the concept of metal phytoextraction using SRC. In further research, eventually not all tests need to be performed since a smaller

selection of chemical and ecotoxicological tests with a significant share in the risk assessment evaluation of phytomanaged soils was proposed by Kumpiene *et al.* (2014). For selected clones, additionally evaluating (stem) biomass production and (stem) metal uptake and accumulation, is recommended. On the one hand, this will reveal evolutions in biomass production and metal issues. On the other hand, it may unravel the relation between observed reductions of soil metal concentrations (and toxicity) and estimated exported metal levels as well as offer explanations for possible dissimilarities.

6.4 Improving metal phytoextraction using SRC of willow: clone selection and fertilization appear to be promising strategies

Since the state of the art reveals that until now economic revenues from converting metal-contaminated woody biomass do not exist and environmental benefits are not rewarded, phytoextraction using SRC in the Campine area is still addressed only as a remediation technology. By consequence, even when taken into account the highly promising decontamination rate observed in Chapter 4, the hypothesized remediation times for Cd are still considered to be too long (> 10 years) to render the remediation feasible on itself. Therefore, the further research in this thesis focused on improving biomass production and/or metal accumulation of SRC by means of clone selection, bioaugmentation with plant-associated bacteria and fertilization.

The *in situ* selection of best performing clones revealed highly valuable (Table 5.1). Based on stem biomass production and metal accumulation after the first 4 growing seasons as well as health and uniformity performance *in situ*, the experimental willow clones *Salix viminalis* and *Salix alba x alba* were selected and revealed to improve respectively stem metal accumulation and stem biomass production compared to best performing commercial and other experimental clones. In comparison with willow, the poplar clones were in general less successful after the first cutting cycle but, since biomass production and metal accumulation will likely change over time, a re-evaluation of the planted poplar clones imposes itself. Although clone testing in the field is invaluable and very reliable, it is a time consuming and expensive methodology (Weih and Nordh 2002) and practically often not feasible. The metal-

contaminated field in Lommel, with more than 200 different clones cultivated since 2006, offers a unique opportunity to this concern. For the same reason as for poplars, it is highly recommended to also re-evaluate the different willow clones. The performance of clones in the third cutting cycle will be very instructive and rather exceptional in the framework of estimating longer-term phytoextraction effectiveness.

The effect of inoculation or fertilization on the phytoextraction potential of both selected willow clones was assessed by means of 2-3 months pot trials using the soil from the field site as a substrate. It was shown that plant-associated bacteria have potential to improve plant growth (Lodewyckx *et al.* 2002), increase metal uptake and accumulation (Gadd 2004; Sessitsch *et al.* 2013), and/or reduce metal phytotoxicity (Bruins *et al.* 2000; Lodewyckx *et al.* 2001; Sessitsch and Puschenreiter 2008; Haferburg and Kothe 2010). Fertilizer applications are known to improve plant growth but their effect on metal availability and uptake is rather unknown in case of metal phytoextraction using SRC of willow.

In the inoculation experiments, 17 bacterial strains, associated with the willow clones on the field and selected based on their *in vitro* beneficial traits (Table 5.5), were evaluated. There was however no conclusive evidence for improved metal phytoextraction of the selected willow clones by inoculating these promising bacterial strains (Table 5.11). The failure of beneficial effects in this research is suggested to be related to 2 major considerations. Firstly, it is brought to mind that it is not evident to successfully inoculate a non-sterile tree cutting growing in field soil. Since the ultimate goal is to improve metal phytoextraction using SRC of willow on field scale and in the longer-term, for a (re-)inoculation to be successful, the situation is not very likely going to improve. Secondly, the selection of bacterial strains based on the *in vitro* qualitative tests performed in this research seems unsatisfactory. Nevertheless, it cannot be excluded that other strains from the available willow-associated collection or from successful inoculation experiments reported in literature might positively affect metal phytoextraction of the selected willow clones. Further research could focus on more extended phenotypic characterization methods for strains selection (whether or not *in vitro*) as well as on colonization efficiency

and effects of (re-)inoculation on the longer term. However, before proceeding inoculation-related investigations, it might be interesting (and maybe even critical) to evaluate the feasibility of bioaugmenting trees under field conditions with regard to practical, economic and environmental aspects.

The fertilization experiments revealed that very promising improvements could be achieved for Cd and Zn extraction due to a considerably increased biomass production (*S. viminalis*) or a combination of both a higher biomass productivity and metal accumulation (*S. alba x alba*) (Table 5.20). There is potential for both to the local soil conditions adjusted NKMg-fertilizer as well as for Yara Opticrop, a commercial NPK-fertilizer often used in SRC willow agricultures in Sweden. Moreover, applying fertilizer to SRC on field scale could rather easily be conducted after each harvest. Nonetheless, a thorough study of fertilizing SRC of willow on the metal-contaminated field is required for several reasons. Firstly, to assess alterations in phytoextraction potential, longer-term biomass increments and metal accumulation in stem should be evaluated as well as a possible dilution of the latter one as a result of the former one. Secondly, to exploit fertilizer applications as a strategy to improve metal phytoremediation, the behavior of fertilizers in soils (*i.e.* availability of nutrients, form of nutrients, effects on metal availability and eventual metal leaching) and the response of willow clones should be unraveled as much as possible in order to optimize the amount and type of fertilizer added. Finally, field research should reveal the environmental impacts (in terms of global warming, acidification, eutrophication and energy ratio) of fertilizing SRC of willow. To this extent, it is also of importance to assess the amount of nutrients removed from the site with each harvest. In this way, fertilizer treatments cannot only be soil-adjusted but also site- and even clone-adopted which is crucial when aiming a sustainable fertilization plan on the longer term (to avoid nutrient leaching). A long way is still ahead and not much is known from fertilizing SRC of willow in the framework of phytoremediation. However, this rather simple management optimization might be exploited to reduce remediation times. In addition, fertilization has the potential to significantly increase the environmental benefits that come along with a vegetation cover on metal-contaminated soil and, if

sustainable biomass conversion processes are developed in the future, might positively affect the economic profitability of the cultivation.

6.5 Metal phytoextraction using SRC of willow: pot trials *vs.* field experiments

For the evaluation of strategies to improve phytoextraction using SRC of willow, in this research (for reasons of time constraints and overall feasibility), pot experiments were performed. To incorporate all 4 entities of a phytoextraction system and as much as possible of their interactions, soil from the contaminated experimental field was used as a substrate and most experiments were performed in an outdoor, open environment (30 km from the field). Although this can be considered a big step in the direction of a field-scale implementation, striking differences between results obtained in pot and on the field were observed for both *Salix* clones. Whether these differences are attributed to the term of the experiments (in pots: 2-3 months, on the field: 4 years), the volume for root system development (in pot: horizontally and vertically restricted, on the field: possibly horizontally restricted but not vertically restricted) and functioning (nutrient/water/metal uptake,…), a combination of both or even other factors was not determined. Nevertheless, the clonal differences in biomass production and metal accumulation observed in the field could by no means be predicted from extrapolation of values obtained in pot trials. This clearly illustrates the unreliability of extrapolations as described in part 6.2. It also emphasizes once again that clone selection should be done *in situ*.

Although this consideration may appear quite discouraging, pot experiments performed with field soil and in a natural environment, do have potential to produce valuable information. It is believed that effects of treatments in pot trials (differences between a treated and a control condition) will also, to a certain extent, be exposed in field situations of a similar term. It is concluded that pot trials might be useful for the evaluation of some strategies/treatments but do not allow to draw conclusions regarding clonal behavior, and by consequence phytoextraction potential, in the field on the longer term.

6.6 Overall conclusion

Of all evaluated high biomass crops for metal phytoextraction in the Campine area, a leading role is reserved for SRC. Its superiority could even be further enhanced if additional environmental benefits would be acknowledged and rewarded.

Focusing on metal phytoextraction in this region using SRC of willow, the decontamination rate observed on the longer term revealed to be much higher than previously predicted. Moreover, *in situ* clone selection and adjusted fertilization applications seem very promising strategies to further reduce remediation time of this crop.

In every part of this research, it was clear that long-term, large-scale field studies are the only reliable way of evaluating the efficiency of a highly complex soil – soil solution – microorganism – plant phytoextraction system. Therefore, the longer-term field studies on the Lommel soil, performed before, during and hopefully after this thesis, are unique and extremely valuable. The field trial with more than 200 different SCR clones (willow and poplar), already in the middle of their third rotation cycle, offers an unprecedented opportunity for further evaluation of the phytoextraction efficiency as well as the entities controlling it.

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