Phytoextraction of Cadmium and Zinc from Contaminated Soils by *Salix spp*.

Dissertation zur Erlangung des akademischen Grades

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1 ZUSAMMENFASSUNG

Im Zeitraum von 2002 – 2006 war ich in eine Reihe von Projekten involviert, die Phytoextraktion von Schwermetallen aus kontaminierten Böden mithilfe von Weiden (*Salix ssp.*) zum Thema hatten. Die vorliegende Arbeit besteht im Wesentlichen aus zwei Publikationen in denen die Hauptergebnisse aus zwei Projekten präsentiert werden, die sich mit verschiedenen Aspekten der Phytoextraktion mit *Salix ssp.* befassten. Daneben habe ich in diesem Zeitraum als Co-Autorin noch zu weiteren Publikationen, die sich mit Phytosanierung beschäftigen, in SCI-gelisteten Journalen beigetragen (siehe Kapitel 5).

Die Verunreinigung von landwirtschaftlich genutzten Oberböden mit Schwermetallen, insbesondere mit Cadmium, ist ein weltweites Problem. Derzeit verfügbare Sanierungsmethoden sind zumeist teuer und/oder relativ invasiv, außerdem wird dabei oft die Bodenstruktur sowie das mikrobielle Bodenleben stark beeinträchtigt oder zerstört.

Die Extraktion von Schwermetallen aus dem Boden durch Anreicherung in der oberirdischen Biomasse von Pflanzen (Phytoextraktion) wurde daher als sanfte, *in-situ* Sanierungsmethode von kontaminierten Oberböden vorgeschlagen. Sogenannte „Hyperakkumulator"-Pflanzen (z.B. *Thlaspi caerulescens*) sind dabei in der Lage extrem hohe Metallkonzentrationen in der oberirdischen Biomasse aufzunehmen. Das langsame Wachstum sowie die überwiegend geringe Biomassebildung dieser meist krautigen Pflanzen stellen jedoch gravierende Nachteile dar, weil dadurch das Erreichen deutlicher Reduktionen von Schwermetallkonzentrationen im Boden aufgrund des relativ geringen Totalentzuges pro Vegetationsperiode eine sehr lange Zeitspanne in Anspruch nehmen würde.

Zur Weiterentwicklung der Phytoextraktion waren daher Pflanzen gefragt, die einerseits die Fähigkeit besitzen, Schwermetalle aus dem Boden in den Sproß aufzunehmen, und anderseits eine hohe Biomasse ausbilden. Es ist seit längerem bekannt, dass Gattungen der Familie *Salicaceae* (u.a. Weiden, Pappeln, Birken) erhöhte Schwermetallkonzentrationen im Sproß aufweisen können. Zudem sind einige *Salix*-Arten schnellwüchsig und bilden eine hohe Biomasse aus. Der Einsatz von metallakkumulierenden Weiden wurde daher als kostengünstige, nachhaltige und ökologisch verträgliche Lösung zur Sanierung von kontaminierten Böden vorgeschlagen.

Neben Daten aus Feldscreenings, in denen vor allem *Salix viminalis* untersucht wurde, sind in der Literatur Ergebnisse aus einer Reihe von Nährlösungsversuchen mit verschiedenen *Salix*-Arten verfügbar. Da sich viele entscheidende Faktoren (z.B. Ausbildung von Wurzelhaaren oder Mykorrizen) maßgeblich unterscheiden, lassen Ergebnisse aus Nährlösungsversuchen leider nur sehr eingeschränkt auf das Verhalten von Weiden im Boden schließen. Es wurden aber bisher nur einige wenige Arten von Weiden in Experimenten mit langzeitkontaminierten Böden untersucht, wobei noch keine bemerkenswerten Entzugsraten gezeigt werden konnten.

Die Ziele dieser Dissertation waren folgende:

1) Untersuchung von verschiedenen *Salix*-Arten hinsichtlich ihres Phytoextraktionspotentials auf langzeitkontaminierten Böden
2) Bestimmung von Phytoextraktionsraten von Cd und Zn nach drei Vegetationsperioden für vier *Salix*-Arten auf drei moderat kontaminierten, landwirtschaftlichen Böden
3) Bestimmung der vertikalen Verteilung von Schwermetallen in verschiedenen Geweben von Weiden
4) Evaluierung von Effekten des Intercroppings von *Salix caprea* mit der Hyperakkumulator-Pflanze *Arabidopsis halleri* auf die Biomasseproduktion und Metallakkumulation
5) Vergleich von Effekten eines kommerziell erhältlichen Mykorrhiza-Rhizobakterien-Inokulates und eines bodenbürtigen Bakterien-Inokulates auf die Metallakkumulation und die Biomasseproduktion von drei Weidenarten (*Salix caprea*, *Salix fragilis*, *Salix x rubens*) über zwei Vegetationsperioden

6) Bestimmung von saisonalen und jahresabhängigen Schwankungen der Schwermetall- und Nährstoffkonzentrationen in Blättern von Weiden

Zur Erreichung dieser Ziele wurden zwei Gefäßversuche im Freiland ausgeführt. In Versuch Nr. 1 wurden vier Weidenarten (*Salix caprea*, *Salix fragilis*, *Salix x smithiana*, *Salix x dasyclados*) auf drei verschiedenen, moderat kontaminierten, landwirtschaftlichen Böden (A, B, C) gepflanzt. Auf Boden A und B wurde zusätzlich ein Intercropping von *Salix caprea* und *Arabidopsis halleri* realisiert. Die Versuchsdauer betrug drei Jahre.

Im zweiten Experiment wurden die Effekte von zwei mikrobiellen Behandlungen (1. MycorTree™, 2. metalltolerante, bodenbürtige Bakterien) auf die Biomassebildung und die Metallakkumulation in Blättern von drei Weidenarten (*Salix caprea*, *Salix fragilis* und *Salix x rubens*) auf zwei Böden mit hohen Cd- und Zn-Konzentrationen (ARN, ABW) untersucht. Ein nicht kontaminierter, landwirtschaftlicher Boden (HIR) diente als Kontrolle bezüglich Biomasseausbildung der Weiden. Im zweiten Versuchsjahr wurden ausserdem saisonale Schwankungen von Konzentrationen an Makronährstoffen (Ca, Mg, K, P, N) und Eisen in den Blättern der Weiden untersucht. Die Versuchsdauer betrug zwei Jahre.

In Experiment Nr. 1 wurden auf Boden B, der 13.4 mg kg^{-1} Cd und 955 mg kg^{-1} Zn enthielt, außergewöhnlich hohe Konzentrationen an Cd (250 mg kg^{-1}) und Zn (3300 mg kg^{-1}) in Blättern von *Salix x smithiana* bestimmt. Dies resultierte in Bioakkumulationsfaktoren von 27 für Cd und 3 für Zn. Im Laufe der drei Versuchsjahre wurde der weitaus größte Anteil an Schwermetallen in den Blättern, gefolgt von den Wurzeln, gespeichert, während nur ein sehr geringer Anteil im Holz akkumuliert wurde. Auf Böden, die mit *Salix x smithiana* bepflanzt waren, wurde nach drei Vegetationsperioden ein Gesamtentzug von bis zu 20% Cd und 5% Zn pro Topf bestimmt. *S. caprea*, *S. fragilis* und *S. dasyclados* lagen nur knapp hinter diesen Werten. Während Totalkonzentrationen an Cd im Boden deutlich reduziert wurden, haben sich die 1 M NH_4NO_3-extrahierbaren Metallkonzentrationen nach drei Jahren nicht signifikant verringert. Durch das Intercropping von *S. caprea* und *A. halleri* konnte im Vergleich zu Einzelpflanzungen von *S. caprea* nach zwei Vegetationsperioden zum Teil eine Erhöhung des Gesamtentzuges an Zn erreicht werden, während der Gesamtentzug an Cd keine Änderung zeigte. Reduzierte, individuelle Schwermetallaufnahmen von *S. caprea* und *A. halleri* gemeinsam mit einer geringeren Biomassebildung können auf Konkurrenz um Nährstoffe und Wasser zurückgeführt werden. Um das dauerhafte Wachstum von *A. halleri* im vorliegenden Versuch zu ermöglichen, war eine kontinuierliche, manuelle Beikrautregulierung notwendig. Es kann daher angenommen werden, dass sich der Einsatz von Intercropping mit krautigen Hyperakkumulator-Pflanzen in der Praxis auch hinsichtlich einer nachhaltigen Kultivierung im Feld als schwierig erweisen wird.

Im zweiten Versuch wurden ebenfalls außergewöhnlich hohe Konzentrationen an Cd und Zn in Blättern von *S. fragilis* und *S. caprea* (bis zu 380 mg kg^{-1} Cd und 4400 mg kg^{-1} Zn) bestimmt, während sich *S. x rubens* als weniger schwermetalltolerant herausstellte und auch deutlich geringere Metallkonzentrationen in den Blättern aufwies. Die hohen Schwermetallkonzentrationen in den Böden ARN und ABW zeigten im Vergleich zum nicht kontaminierten Boden HIR keinen Einfluß auf die Biomasseausbildung von *S. caprea*, wodurch Berichte über eine besonders hohe Metalltoleranz dieser Weide bestätigt wurden. Die mikrobiellen Behandlungen zeigten zwar auf keinem der Böden einen Einfluß auf die Biomasseausbildung der Weiden, erhöhten aber die Metallkonzentrationen in den Blättern von *S. caprea* und teilweise *S. x rubens* auf dem Boden ABW für zwei Vegetationsperioden. Effekte der mikrobiellen Behandlungen stellten sich daher als Weiden- und bodenabhängig heraus. Obwohl die Böden nicht sterilisiert wurden, konnte der Anstieg der Metallkonzentrationen in Blättern von *S. caprea* und teilweise *S. x rubens* nach den mikrobiellen Behandlungen auf dem Boden ABW für zwei Vegetationsperioden nachgewiesen werden. Dennoch bleibt zu beantworten, warum die mikrobiellen Behandlungen die Aufnahme von Cd in die Blätter von *S. caprea* und *S. x rubens* auf Boden ABW, aber nicht auf Boden ARN, beeinflusst haben. Ausserdem bleibt zu untersuchen, welche Komponenten des MycorTree™ die Metallaufnahme der Weiden beeinflusst haben. Signifikante saisonale Unterschiede wurden für Cd und Zn in den Blättern aller Weiden festgestellt, wobei aber diese Variationen nicht mit saisonalen Schwankungen von

Makronährstoffen (Ca, Mg, K, P, N) oder Eisen korrelierten. Dies deutet darauf hin, dass die Metallakkumulation in den Blättern unabhängig von einer pflanzeninternen Nährstoffumlagerung vor dem Laubabfall stattfand und/oder Konkurrenzmechanismen zwischen Schad- und Nährelementen abläuft.

Die in beiden Experimenten bestimmten hohen Schwermetallkonzentrationen in den Blättern und Bioakkumulationsfaktoren von *S. caprea*, *S. fragilis* und *S. x. smithiana* waren im Bereich der höchsten in der Literatur beschriebenen Werte. Zudem wurde in der vorliegenden Arbeit zum ersten Mal eine effektive Phytoextraktion von Cd durch den Einsatz von Weiden nachgewiesen. Im Gegensatz dazu waren die Phytoextraktionsraten von Zn weniger erfolgreich. Im Kontext einer möglichen Metallakkumulation in Futter- oder Nahrungspflanzen müssen erhöhte Cd-Konzentrationen im Boden im Vergleich zu Zn als weitaus problematischer eingestuft werden. Es wäre daher in vielen Fällen möglicherweise ausreichend, vor allem Cd kostengünstig aus kontaminierten, landwirtschaftlichen Böden zu entfernen. Die deutliche Korrelation von Cd- und Zn-Konzentrationen in den Blättern der Weiden lassen auf ähnliche Translokationsmechanismen für diese Elemente und eventuell auf ähnliche Verteilungsmuster in den Blättern schließen. In beiden Experimenten wurde ein Anstieg der Metallkonzentrationen in den Blättern gegen Ende der Vegetationsperiode gezeigt. Dieser Effekt ist vermutlich auf eine Umlagerung von Metallen in den Geweben von Weiden vor dem Laubabfall zurückzuführen, was eventuell auf einen aktiven Detoxifikationsmechanismus metalltoleranter Weiden hinweisen könnte. Dieser signifikante Alterungseffekt der Blätter zeigt, dass durch die Ernte der Blätter nach dem natürlichen Blattabfall die größte Menge an Schwermetallen von einer Fläche entfernt werden kann.

Aus diesen Ergebnissen kann geschlosen werden, dass die Phytoextraktion von Cadmium aus kontaminierten, landwirtschaftlichen Böden mit effizienten, metalltoleranten und metallakkumulierenden Weiden wie z.B. *S. fragilis*, *S. x smithiana* oder *S. caprea* eine erfolgversprechende Methode darstellt. Der nächste wichtige Schritt zur Umsetzung von Phytoextraktion in die Praxis, ist die Ausführung von Feldversuchen, in denen Metallentzugsraten unter realen Bedingungen bestimmt und Managementtechniken von

Weidenplantagen untersucht werden, die zu einer Maximierung der Extraktionsraten führen. Zudem stellt die Anwendung von mikrobiellen Behandlungen im Feld einen vielversprechenden Ansatz zur Steigerung der Effizienz von Phytoextraktion mit *Salix ssp*. dar. Da sich herausstellte, dass Hyperakkumulatorpflanzen wenig geeignet sind, sind weitere Untersuchungen notwendig, um Pflanzen zu identifizieren, die sich als Unterwuchs von Weiden eignen und sich zudem eventuell positiv auf die Metallaufnahme von Weiden auswirken.

2 ABSTRACT

From 2002 to 2006 I was involved in several R & D projects dealing with the remediation of heavy metal contaminated soils by phytoextraction using willows (*Salix ssp.*). This thesis consists in principle of two publications presenting the major outcomes of two projects on different aspects of phytoextraction by *Salix ssp.*. Additionally I have contributed as co-author to several other publications dealing with phytoremediation in SCI-listed journals (see Chapter 5).

Contamination of agricultural topsoils with heavy metals - especially Cd - above guideline values is of concern in many countries throughout the world. Currently available remediation technologies are often expensive and/or rather invasive methods, which harm or destroy soil structure or soil biology.

The extraction of heavy metals from soils by accumulation in aboveground plant parts (phytoextraction) has been suggested as soft, *in-situ* remediation technology of contaminated top soils. So called „hyperaccumulator“ plants (e.g. *Thlaspi caerulescens*) are capable of accumulating extraordinary large concentrations of heavy metals in the aboveground biomass. Unfortunately hyperaccumulators are usually small plants with slow growth and low biomass, taking a long period of time until significant reductions of metal concentrations in soils can be achieved.

To further develop phytoextraction technologies plants were needed, that on the one hand are capable of accumulating heavy metals in the above ground biomass and on the other hand produce large biomass. It is known, that species of the *Salicaceae* (e.g. *Salix*, *Populus*, *Betula*) can have enhanced heavy metal concentrations in the above ground biomass. Some *Salix* species are fast growing and produce large biomass. Hence *Salix ssp.* have been suggested as low cost, sustainable and ecologically sound solution to rememdiate contaminated soils.

Apart from data on some field screenings, results on a number of hydroponic experiments estimating the phytoextraction potential of willows are available in the literature. However, it is known, that results achieved in hydroponic studies cannnot be directly converted to soil conditions, as many crucial factors (e.g. formation of mycorrhiza or root hairs) are different. Yet only a few different *Salix* species have been tested on their phytoextraction potential on long term contaminated soils, but reasonable metal extraction rates remain to be demonstrated.

The objectives of this thesis were

1) to test different *Salix* species on their phytoextraction potential on long term contaminated soils
2) to assess the Cd and Zn phytoextraction rates of four *Salix* species (*Salix caprea*, *Salix fragilis*, *Salix x smithiana*, *Salix x dasyclados*) on three moderately contaminated, agricultural soils over three vegetation periods
3) to investigate the vertical distribution of heavy metals in different tissues of *Salix ssp.*
4) to evaluate the effects of intercropping *Salix caprea* with the hyperaccumulator *Arabidopsis halleri* on biomass production and metal accumulation
5) to compare effects of a commercially available mycorrhiza-rhizobacteria inoculate to an indigenous bacteria inoculate on foliar metal concentrations and biomass production of three willow species (*Salix caprea*, *Salix fragilis*, *Salix x rubens*) over two vegetation periods
6) to assess seasonal and annual variations of heavy metal- and nutrient concentrations in leaves of willows

To meet these objectives two outdoor experiments were conducted. In experiment Nr. 1 four willow species (*Salix caprea*, *Salix fragilis*, *Salix x smithiana*, *Salix x dasyclados*) were planted on three different, moderately contaminated, agricultural soils (A, B, C). Additionally an intercropping treatment of *Salix caprea* and *Arabidopsis halleri* was carried out on soil A and B. This experiment was attended for three vegetation periods.

In experiment Nr. 2 we investigated the effects of two microbial treatments (1. MycorTree^{TM}, 2. metal tolerant, indigenous soil bacteria) on biomass production and metal accumulation in leaves of three *Salix* species (*S.* x *rubens*, *S. fragilis*, *S. caprea*) on two soils with large Cd and Zn concentrations (ARN, ABW). A non-contaminated agricultural soil (HIR) served as control in terms of biomass production. Biomass production and foliar metal concentrations were monitored for two vegetation periods. In the second year seasonal variations of foliar concentrations of macronutrients and Fe were determined.

In experiment Nr. 1 large concentrations of Cd (250 mg kg^{-1}) and Zn (3300 mg kg^{-1}) were determined in leaves of *Salix x smithiana* grown on soil B, containing 13.4 mg kg^{-1} Cd and 955 mg kg^{-1} Zn, resulting in bioaccumulation factors of 27 (Cd) and 3 (Zn). The major proportion of metals was stored in leaves, followed by the roots, whereas only minor amounts were accumulated in the wood. Total removal of up to 20 % Cd and 5 % Zn after three vegetation periods were shown for *Salix x smithiana* closely followed by *S. caprea*, *S. fragilis* and *S. x dasyclados*. While total Cd concentrations in soils were reduced by up to 20 %, 1 M NH_4NO_3-extractable metal concentrations did not significantly decrease within three years. Intercropping of *S. caprea* and *A. halleri* partly increased total removal of Zn, but did not enhance total Cd extraction compared to single plantings of *S. caprea* after two vegetation periods. Reduced individual metal uptake in *A. halleri* and *S. caprea* along with less biomass production can be related to competition for water or nutrients and possibly also metal sequestration into plants. In practice, limitations for successful intercropping of willows with small herbaceous hyperaccumulators may rather arise in terms of sustained establishment, maintaining and harvest of the hyperaccumulator understorey. In our experiment continuous manual weed control was critical to maintain *A. halleri*.

In experiment Nr. 2 large concentrations of Cd and Zn were determined in leaves of *S. fragilis* and *S. caprea* (up to 380 mg kg^{-1} Cd and 4400 mg kg^{-1} Zn), whereas *S. x rubens* was found to be less tolerant and to accumulate less metals in the foliage. Large heavy metal concentrations of the soils ARN and ABW did not affect leaf biomass production of *S. caprea* compared to the non-contaminated HIR soil confirming reports on particularly large metal tolerance of this species. Both microbial treatments showed no effect on biomass production of willows on either soil, but significantly increased foliar metal concentrations of *S. caprea* and partly *S.* x *rubens* grown on soil ABW for two vegetation periods. Hence effects of microbial treatments proved to be species and soil dependent. Although soils were not sterilised, the increase of foliar metal concentrations after microbial treatments was demonstrated for *S. caprea* and partly *S. x rubens* over two vegetation periods. However, it remains to be answered why microbial treatments influenced the Cd uptake of *S. caprea* and *S. x rubens* on soil ABW, but not on soil ARN. Furthermore it needs to be investigated, which compounds of the MycorTree™ product affected the metal uptake of willows. Significant seasonal differences were found for Cd and Zn concentrations in leaves of all willows, but variations were found to be not correlated to foliar concentrations of macronutrients (Ca, Mg, K, P, N) or Fe. This indicates that metal accumulation in leaves occurred independently from plant internal nutrient relocation at leaf senescence and competition/or mechanisms between elements.

Foliar metal concentrations and bioaccumulation factors of *S. caprea*, *S. fragilis* and *S. x smithiana* in both experiments were among the largest reported in the literature. To our knowledge, effective phytoextraction of Cd using willows, indicated by the substantial decrease in *aqua regia* extractable soil Cd, has been shown by this study for the first time. In contrast to Cd, effective phytoextraction of Zn using willows still remains to be demonstrated. However, regarding metal accumulation in crops, enhanced Cd concentrations in agricultural topsoils are considered to be more problematic than Zn. Hence a cost-extensive removal of primarily Cd using phytoextraction by willows may be appropriate for many contaminated, agricultural topsoils. The clear correlation of foliar Cd and Zn concentrations on individual soils suggests similar translocation mechanisms for these elements to the leaves and possibly similar sequestration characteristics in the leaves. An increase of foliar metal concentrations towards the end of the vegetation period was shown in both studies, which is most likely due to shunting of metals in plant tissues prior to senescense, possibly an active detoxification mechanism of metal tolerant willows. This significant leaf age effect suggests that harvesting the leaves after the natural leaf fall will remove most metals from the contaminated site.

According to our results phytoextraction using efficient, metal tolerant and metal accumulating willows such as *S. fragilis*, *S. x smithiana* or *S. caprea* seems promising. The next important step to approach practical application of phytoextraction is the establishment of field experiments determining metal extraction rates under real conditions and investigating management practices of willow stands to maximise extraction rates. The application of microbial treatments in the field may be a suitable approach to further improve the efficiency of phytoextraction using *Salix ssp.*. As the intercropping of hyperaccumulator plants proved to be not successful, further research is needed to identify appropriate understorey plants with possibly beneficial effects on metal extracting willows.

3 Publication # 1 – Phytoextraction of Cd and Zn from agricultural soils by *Salix ssp*. and intercropping of *Salix caprea* and *Arabidopsis halleri*

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ABSTRACT

Contamination of agricultural topsoils with Cd above guideline values is of concern in many countries throughout the world. Extraction of metals from contaminated soils using high-biomass, metal-accumulating *Salix sp.* has been proposed as a low-cost, gentle remediation strategy, but reasonable phytoextraction rates remain to be demonstrated. In an outdoor pot experiment we assessed the phytoextraction potential for Cd and Zn of four willow species (*Salix caprea*, *S. fragilis*, *S.* x *smithiana*, *S.* x *dasyclados*) and intercropping of *S. caprea* with the hyperaccumulator *Arabidopsis halleri* on three moderately contaminated, agricultural soils. Large concentrations of Cd (250 mg kg^{-1}) and Zn (3300 mg kg^{-1}) were determined in leaves of *Salix* x *smithiana* grown on a soil containing 13.4 mg kg^{-1} Cd and 955 mg kg^{-1} Zn, resulting in bioaccumulation factors of 27 (Cd) and 3 (Zn). Total removal of up to 20 % Cd and 5 % Zn after three vegetation periods were shown for *Salix* x *smithiana* closely followed by *S. caprea*, *S. fragilis* and *S.* x *dasyclados*. While total Cd concentrations in soils were reduced by up to 20 %, 1 M NH_4NO_3-extractable metal concentrations did not significantly decrease within three years. Intercropping of *S. caprea* and *A. halleri* partly increased total removal of Zn, but did not enhance total Cd extraction compared to single plantings of *S. caprea* after two vegetation periods.

INTRODUCTION

Long-term application of heavy metal containing fertilisers, sewage sludges, pesticides and emissions from industrial activities have contaminated agricultural topsoils above guideline values either locally or over large areas. Current available remediation techniques are typically expensive, ex-situ physico-chemical methods of extraction. Furthermore, they destroy the soil structure and leave it biologically inactive (McGrath et al., 2001). The extraction of heavy metals by accumulation in plants (phytoextraction) has been suggested as a gentle, *in-situ* remediation strategy for contaminated topsoils. Hyperaccumulator plants are capable of accumulating exceptionally large concentrations of metals in their aboveground biomass without showing phytotoxicity symptoms (Baker et al., 2000).

However, such hyperaccumulator plants are typically slow growing and produce only small amounts of biomass, thus requiring many years for decontamination of polluted sites (Cherian and Oliveira, 2005).

Trees have been proposed as an alternative due to their extensive root system, high transpiration rates, rapid growth and large biomass production. Some species of the genera *Salix* and *Populus* have been reported to accumulate elevated concentrations of heavy metals - particularly Cd and Zn - in their above ground biomass (Pulford and Dickinson, 2005; Pulford and Watson, 2002). In field/screening experiments, mostly *S. viminalis* has been investigated for its metal accumulating potential, probably because this species is widely used in short-rotation forestry in North-West Europe (Hammer et al., 2003; Keller et al., 2003; Klang-Westin and Eriksson, 2003; Rosselli et al., 2003; Vervaeke et al., 2003; Kayser et al., 2000; Riddell-Black et al., 1997; Landberg and Greger, 1996). The largest shoot Cd concentration in field-grown *S. viminalis* (22 mg kg-1) was found by Felix (1997) on a soil containing 6.6 mg kg-1 Cd, resulting in a Cd - bioaccumulation factor (BCF; foliar metal concentration : total soil metal concentration) of 3.7, while a Zn-BCF of > 1 was not reported in any of the published data on field experiments (Pulford and Dickinson, 2005). The metal uptake behaviour of several *Salix* species or clones has been estimated in a range of hydroponic studies (e.g. Dos Santos Utmazian et al., 2007), whereas only a limited number of species other than *S. viminalis* have been investigated in pot experiments using long-term-contaminated soils (Dos Santos Utmazian and Wenzel, 2007; Fischerova et al., 2006; Vyslouzilova et al., 2006; Vandecasteele et al., 2005; Granel et al., 2002; Klang-Westin and Perttu, 2002). However, effective reductions of total metal concentrations in contaminated soils resulting from accumulation by *Salix ssp*. remain to be demonstrated.

Intercropping of plants is mainly applied as agricultural technique and aims at interspecific below-ground interactions, which may result in improved nutrient availability and increased yield of crops. It has been shown, that intercropping of the hyperaccumulator *Thlaspi caerulescens* can increase the Cd uptake of non-accumulating plants such as barley

(Gove et al., 2002), while no reports are available on metal accumulation by *Salix ssp.* planted in an intercropping system with a hyperaccumulator.

The objectives of this work were to 1) assess the Cd and Zn phytoextraction rates of four willow species (*Salix caprea*, *Salix fragilis*, *Salix x smithiana* and *Salix x dasyclados*) in a 3 year outdoor pot trial that used 3 different, moderately contaminated, agricultural soils, and 2) to investigate the effect of intercropping *Salix caprea* with the Cd/Zn hyperaccumulator *Arabidopsis halleri* on metal accumulation in leaves/shoots.

MATERIAL & METHODS

Experimental soils

Three (A, B and C) agricultural topsoils (0-20 cm) were collected from the vicinity of an abandoned Zn/Pb smelter at Arnoldstein (Carinthia, Austria). The smelter had been active since 1882 resulting in contamination of surrounding agricultural soils with heavy metals. Smelter and metal processing activities were terminated in 1991. Experimental soils had similar textures (loamy sand), but differed in pH values (at a 1 : 2.5 soil : H_2O ratio), $CaCO_3$ content, cation exchange capacity (CEC) and heavy metal concentrations (Table 1). The Austrian guideline values of 1 mg kg^{-1} total Cd and 300 mg kg^{-1} total Zn in agricultural soils (ÖNORM L 1075) were exceeded in all three soils (Table 1).

Table 1. Selected physical and chemical properties of three experimental soils (A, B, and C).

Soil	pH H_2O	$CaCO_3$	Sand	Silt	Clay	CEC	Cd AR[1)]	Zn AR[1)]	Cd NH_4NO_3[2)]	Zn NH_4NO_3[2)]
		g kg^{-1}	g kg^{-1}			$mmol_c$ kg^{-1}	mg kg^{-1}		mg kg^{-1}	
A	7.4	302	418	480	102	204	13.4	955	0.21	1.68
B	6.5	0.00	412	472	116	79	4.75	500	0.13	2.76
C	7.4	35.0	380	490	130	166	4.03	490	0.03	0.23

[1)] Aqua regia-extractable, “total”

[2)] 1 M NH4NO3-extractable, “phytoavailable”

Experimental plants

Salix caprea L. (clone BOKU 01 AT-004), *S. fragilis* L. (clone BOKU 01 CZ-001), *S. x smithiana* Willd. (*S. caprea* L. x *S. viminalis* L., clone BOKU 03 CZ-001), *S. x dasyclados* Wimm. (*S. caprea* L. x *S. cinerea* L. x *S. viminalis* L., clone BOKU 03 CZ-002), and *Arabidopsis halleri subsp. halleri* L. (seeds obtained from plants growing in the surroundings of the former smelter at Arnoldstein mentioned above) were used in this trial. All cuttings of willows were selected according to equal diameter and length before they were pre-grown in a sand-soil mixture under controlled environmental conditions (day/night temperature: 20 / 15°C, 60 % air moisture, 16 hrs light day^{-1}) for six months. Initial Cd concentrations of the cuttings were below the detection limit, while Zn concentrations were between 62 and 93 mg kg^{-1} DW. Seeds of *A. halleri* were germinated and pre-grown in a sand-soil mixture in a greenhouse (environmental conditions see above) before they were transplanted to the pot experiment.

Pot experiment setup

Soils were homogenised and sieved to < 15 mm before they were filled into 60 L plastic pots (70 x 34 x 30 cm, surface area = 0.24 m^2). The amounts of soil per pot were 25 kg of soil A, 22 kg of soil B and 27 kg of soil C, which corresponded to 16.5, 15.6, and 21.2 kg, respectively, in the soil fraction < 2 mm. To maintain aerobic conditions a 5 cm thick quartz gravel layer was placed as a drainage beneath the soil, separated from the soil by a PE-fleece. A tube was connected to a hole at the bottom of the pot and lechate was collected in PE-canisters. Each pot was planted with one specimen of willow or 20 seedlings (corresponding to 80 plants m^{-2}) of *A. halleri*. Soil A and B were planted with *S. fragilis*, *S. x smithiana*, *S. caprea*, *A. halleri*, and with a combination of the latter two species (intercropping treatment, IC). Soil B was additionally planted with *S. x dasyclados*. Soil C was planted with *S. caprea* and *S. fragilis* as the amount of soil available was limited. All treatments were carried out in triplicate and non-planted control soils were included. Pots were positioned in the field (Experimental Station of BOKU, 2301 Großenzersdorf, Austria) in a randomised design in 4 rows (at 1 m distance between pots and 2 m distance between rows) and the experiment was maintained for 3 growing seasons (2003 – 2005). Pots were not affected by shading of any other trees. Plants were watered using an automated irrigation system depending on weather conditions. Shoots of *Arabidopsis halleri* were cut in October 2003 and plants were left to regrow for a second year. Leaves of willows were harvested in autumn 2003 and 2004 shortly before the natural leaf fall. On 17th of May and 2nd of August 2005 a third of new shoots length (grown in 2005) was cut to stimulate leaf biomass production. Biomass and metal concentrations of the harvested shoot material were determined. In September 2005 the whole plants were harvested and divided into leaves, new twigs (produced in the current vegetation period), old twigs, and roots. Cuttings could not be separated from the wood after three years, because they were either degraded or completely incorporated in the stem. The biomass of the initial cutting (on average 2.7 g DW) was neglectable compared the wood biomass at harvest.

Plant and soil analysis

All plant parts were cleaned in an ultrasonic bath using deionised water, dried at 80 ^{o}C until constant weight and finely ground in a stainless steel mill (MF 10, IKA® Werke, Staufen, Germany). Subsamples of 0.5 g were digested in 4 ml HNO_3 (puriss. p.a., Sigma-Aldrich Handels GmbH, Vienna, Austria) and 1 ml $HClO_4$ (puriss. p.a., Sigma-Aldrich Handels GmbH, Vienna, Austria) at 225 ^{o}C using an automated heating block (Digester DK 42/26, Velp Scientifica, Milano, Italy). After roots had been removed from the soils in autumn 2005, soils were sieved to < 2 mm and thoroughly homogenised. Fresh subsamples were analysed for 1 M NH_4NO_3 – extractable metal concentrations at a soil : solution ratio of 1 : 2.5 (ÖNORM L 1094). A subset of samples was dried at 105 ^{o}C to constant weight and digested with aqua regia (HCl and HNO_3 puriss. p.a., Sigma-Aldrich Handels GmbH, Vienna, Austria; ÖNORM L 1085). Plant and soil samples were analysed for Cd and Zn by Graphite Furnace and Flame AAS, respectively (Perkin Elmer 2100). Chemical analyses were validated by including reference material (BCR-482, LGC Promochem, Teddington, UK; mean recovery rates of Cd and Zn: 99.1% and 99.7%, respectively), blanks and replicate samples at a rate of not less than 10%.

RESULTS

Biomass production and tolerance to metals

The cumulative total biomass produced by the willow species during three vegetation periods ranged between 400 and 900 g DW plant^{-1} (Figure 1). Generally, the biomass production followed the order roots > leaves > new twigs > old twigs. New twigs and leaves together accounted for 49 - 58 % of the total biomass produced. The biomass production of *S. x smithiana* and *S. fragilis* varied notably between the experimental soils. Despite diminished biomass production of *S. x smithiana* and *S. fragilis* on soil A in comparison to soil B, they were vital and showed no visible signs of phytotoxicity such as necroses or intercostal stippling of leaves (Vollenweider and Günthardt-Goerg, 2005). Biomass production of *S. caprea* proved to be independent of soil properties and contamination levels, indicating high metal tolerance (Figure 1, Figure 2). During the three years of the experiment (2003-2005), we observed clear annual increases of leaf biomass production of the willows (Figure 2), but marginal changes for the herbaceous *A. halleri* (2003-2004, Figure 6). The shoot biomass of *A. halleri* was in the same range as in a field experiment (up to 25 g DW m^{-2} for 25 plants) reported by McGrath et al. (2006) and exceeded the leaf biomass produced by the willow species in the first growing season (2003) per pot, but represented only a fraction of willow leaf biomass in the subsequent year of the experiment (Figure 2, Figure 6).

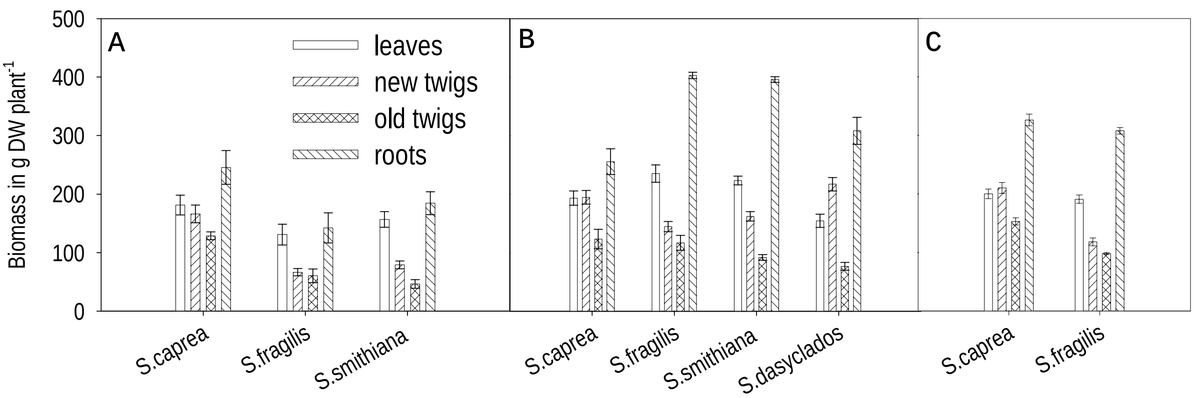

Figure 1. Total biomass of leaves, new and old twigs, and roots produced by individual willow species during three vegetation periods (2003-2005) on the experimental soils A, B and C (error bars indicate standard errors of the mean, n = 3).

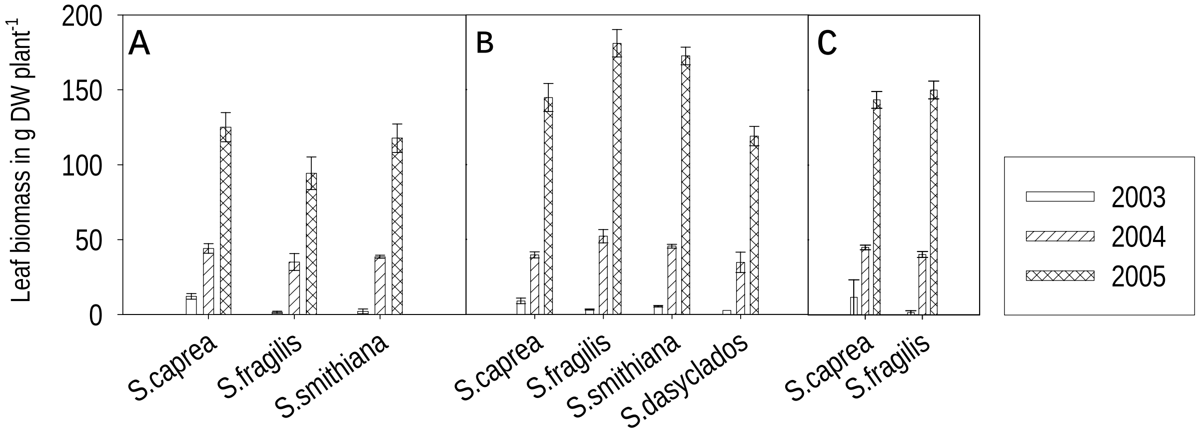

Figure 2. Annual leaf biomass production of willow species during three vegetation periods (2003-2005) on the experimental soils A, B and C (error bars indicate standard errors of the mean, n = 3).

Metal concentrations and bioaccumulation factors (BCF) in plant tissues

Large concentrations of Cd were determined in leaves of *S. x smithiana* on soil A (250 mg kg^{-1}) and soil B (130 mg kg^{-1}) after the first vegetation period (2003). The corresponding bioconcentration factors (BCF) for Cd of 19 on soil A and 27 on the more acidic soil B demonstrate a good Cd accumulation potential of this species. This was further substantiated by similiar Cd concentrations in *S. x smithiana* leaves and *A. halleri* shoots (Figure 3, Figure 6). Foliar Cd of *S. fragilis* and *S. caprea* was in the range of 140 mg kg^{-1} Cd on soil A, while on the less contaminated soil B *S. fragilis* had still > 100 mg kg^{-1} Cd in the leaves. Foliar Cd concentrations of the other willows on soil B and C were < 100 mg kg^{-1}, with bioaccumulation factors > 7 (Figure 3). The Cd concentrations (>100) and - BCF (>1) obtained for these willows exceeded the threshold values for hyperaccumulation (Baker et al., 2000) and are among the highest reported for willows in the literature (Dos Santos Utmazian and Wenzel, 2007).

The largest Zn concentrations in leaves on soil A were accumulated by *S. x smithiana* (3300 mg kg^{-1} Zn, BCF = 3) followed by *S. fragilis* and *S. caprea* (2020 mg kg^{-1} Zn) after the first vegetation period. Previously, foliar Zn concentrations of > 3000 mg kg^{-1} in willows were obtained for *S. x dasyclados* and *S. viminalis* on a soil freshly spiked with 2087 mg kg^{-1} Zn

(Vyslouzilova et al., 2003). In this case the fraction of available Zn was unusually large as compared to long - term contaminated soils. The Zn concentrations in leaves showed only little variation among willow species on soil B (1500 – 1720 mg Zn kg^{-1}; BCF = 3) and C (~ 1000 mg Zn kg^{-1}; BCF = 2 - 3, Figure 3). The Zn concentrations in shoots of *A. halleri* were 2-fold (BCF = 7) and 3-fold (BCF = 10) higher than in leaves of the best performing willow on soil A and soil B, respectively (Figure 3, Figure 6). In contrast to Cd none of the investigated willows achieved the Zn accumulation capacity of the hyperaccumulator plant.

Declining metal concentrations in foliage were recorded for subsequent years, whereas the BCFs of Cd were still in the range of 2 – 7 and those of Zn between 1 and 2 (Figure 3). On average foliar Cd concentrations were reduced by 30% in the second and by 70% in the third compared to the first vegetation period. Zn concentrations in leaves did not show a decrease in the second compared to the first year, while in the third year a decrease of 40% was determined. After three vegetation periods, we found similar metal concentrations in new and old willow twigs. Metal concentrations in leaves were 2- to 4-fold (Cd) and 3- to 6-fold (Zn) larger than in twigs (Figure 4). Leaf : root translocation factors (TF) were in the range of 1 - 3 for Cd and 2 - 4 for Zn, fulfilling the criterion of TF > 1 for plants suitable for phytoextraction according to Baker et al. (2000).

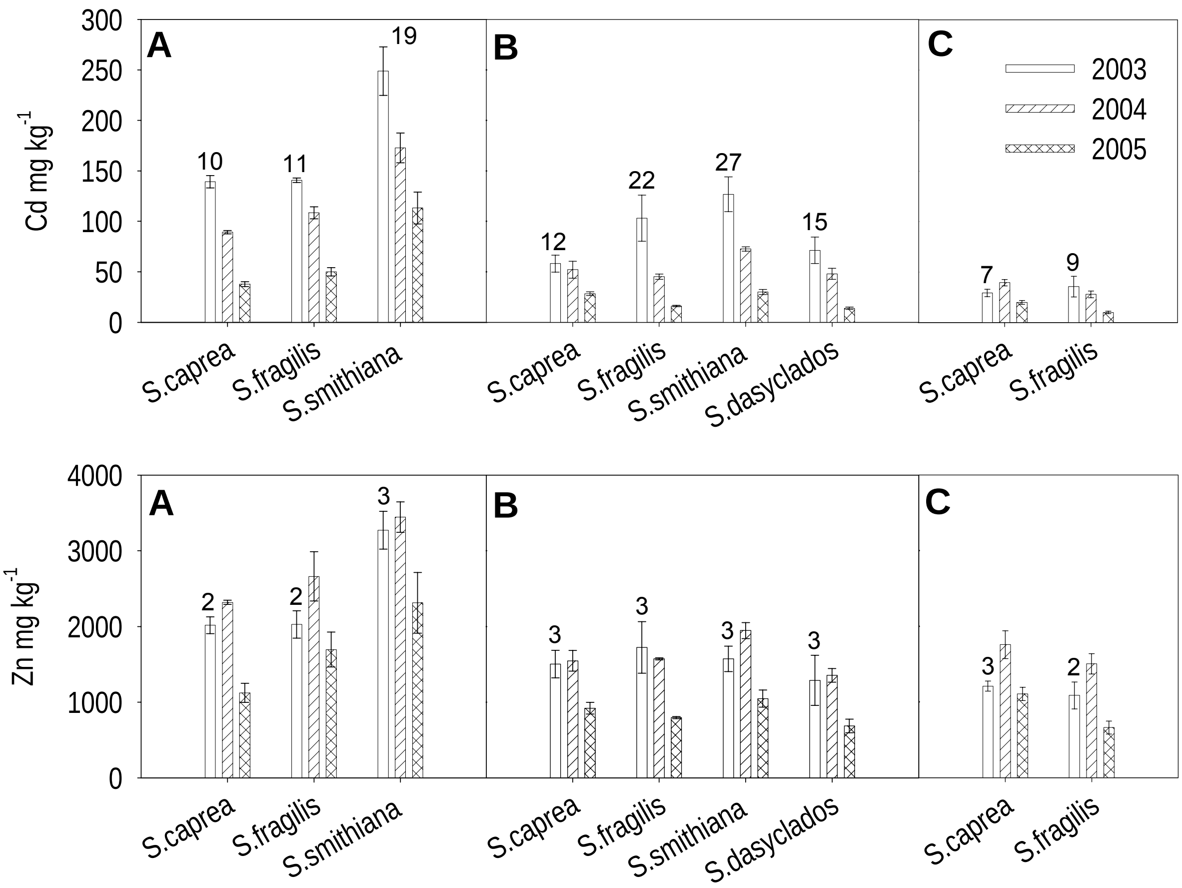

Figure 3. Annual Cd and Zn concentrations in leaves of *Salix ssp.* on the experimental soils A, B and C (error bars indicate standard errors of the mean, n = 3). Numbers above columns refer to metal bioconcentration factors (metal in leaves : total metal in soils) after the first vegetation period.

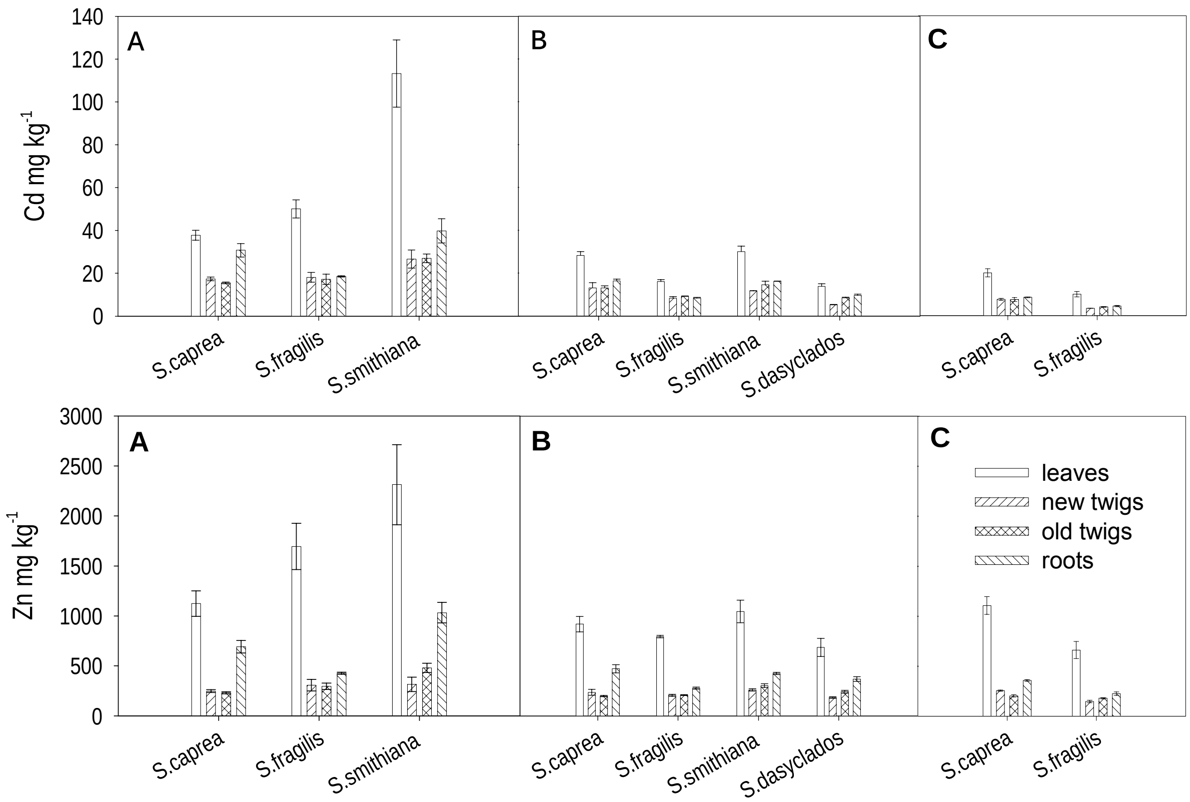

Figure 4. Cd and Zn concentrations in different tissues of willows on the experimental soils A, B and C after the third vegetation period (error bars indicate standard errors of the mean, n = 3).

Metal contents in willows and removal from soil

Figure 5 shows the Cd and Zn contents (= biomass x metal concentrations) accumulated in different willow tissues over three vegetation periods (2003-2005). The major proportion of metals was stored in leaves, followed by the roots, whereas only minor amounts were accumulated in the twigs. The Cd accumulation in leaves and twigs over the course of the experimental period accounted for 4 – 20 % of the initial total (aqua regia extractable) Cd contents in the experimental soils per pot. Broadly, these total extraction rates corresponded with reductions of the total Cd contents measured in the experimental soils (Table 2). Despite partly reduced biomass production on soil A, most Cd was extracted from this soil, reflecting the largest metal concentrations in tissues due to larger metal concentrations in the soil. Relative to total Cd in soil, the largest Cd proportions were extracted from soil B (Figure 5), which is in line with the largest bioconcentration factors measured on this soil. Low soil pH and CEC (Table 1) obviously contributed to a greater plant availability of Cd in this soil.

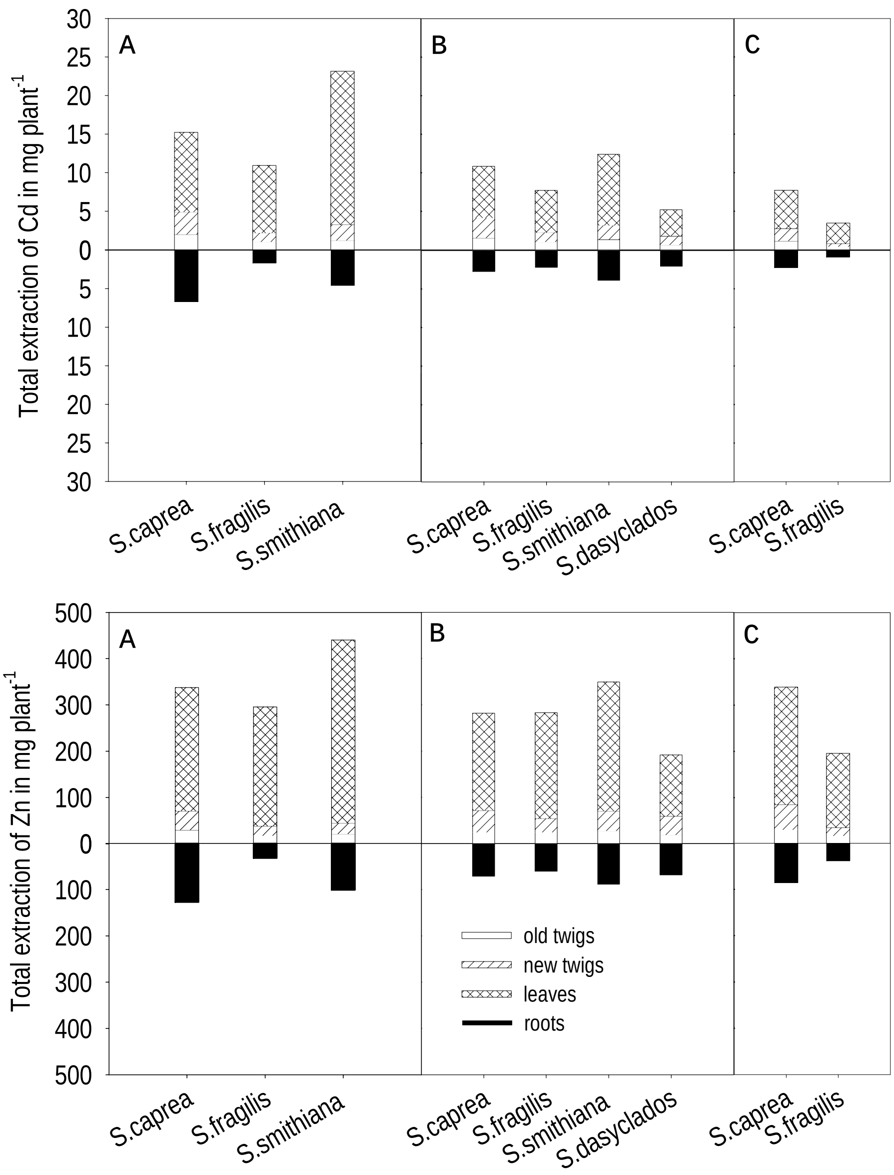

Figure 5. Total metal extraction by accumulation in tissues of *Salix ssp*. after 3 growing seasons on the experimental soils A, B and C.

The proportion of total Zn in soil extracted by accumulation in leaves and shoots during the three years experiment was generally lower (≤ 5 %) than for Cd. *S. x smithiana* was the best performing species in terms of extraction of both metals, followed by *S. caprea* and *S. fragilis*. However, reductions of total (*aqua regia* extractable) Zn concentrations in planted soils compared to those in the non-planted control soils were found to be not significant (Table 2). Despite large metal removal rates of willows, determination of “phytoavailable”

Cd/Zn fractions according to standard procedures (1M NH_4NO_3-extraction) showed no substantial decrease in planted versus non planted soils after three vegetation periods in our experiment (Table 2).

Intercropping of *Salix caprea* and *Arabidopsis halleri*

The intercropping experiment was stimulated by our observation of a native *A. halleri* understory in *S. caprea* stands on metal-contaminated sites at Arnoldstein (southern Austria). The Cd- and Zn-hyperaccumulating *A. halleri* (Küpper et al., 2000; Brooks, 1998) might provide a suitable understory for willows over the phytoextraction period. During the first two experimental years, we tested the effect of intercropping (IC) *A. halleri* with *S. caprea* on plant biomass production, Cd and Zn concentrations in leaves/shoots and metal removal from the soil.

In 2003 leaf/shoot biomass of *S. caprea* and *A. halleri* did not significantly differ between IC treatment and single plantings, while in 2004 leaf biomass production of S. caprea decreased by 60 % in the IC treatment compared to single plantings on soil A and B (Figure 6a). In 2003 we also observed reduced Cd concentrations in leaves of *S. caprea* on soil A (-53 %) and reduced Zn concentrations on soil B (-42 %) in the IC treatment versus single plantings, while intercropping had no influence on Cd and Zn concentrations in shoots of *A. halleri* (Figure 6b). In 2004 reduced Cd concentrations in leaves of *S. caprea* were confirmed on soil A. In the same year lower Cd and Zn concentrations were also determined in shoots of *A. halleri* on soil A, while Zn concentrations on soil B were higher in the IC treatment compared to respective single planting (Figure 6b). Except for Zn on soil B, total metal removal after two cropping cycles in the IC treatment was not significantly enhanced, compared to individual plantings of *S. caprea* (Figure 6c).

Table 2. „Total“ and „phytoavailable“ Cd / Zn concentrations in the experimental soils A, B and C versus Cd and Zn extracted via leaves, twigs and roots after 3 growing seasons (SE = standard error of the mean, n = 3).

SOIL	WILLOW	CADMIUM						ZINC					
		"total" [1)] in soil in mg pot^{-1}	SE	"phytoavailable" [2)] in soil in mg pot^{-1}	SE	total in plant parts[3)] in mg pot^{-1}	SE	"total" [1)] in soil in mg pot^{-1}	SE	"phytoavailable" [2)] in soil in mg pot^{-1}	SE	total in plant parts[3)] in mg pot^{-1}	SE
A	-	268	9.88	2.19	0.05	-	-	17800	537	16.6	0.14	-	-
	S. caprea	249	25.8	2.05	0.05	21.9	2.72	17900	74.5	17.5	0.77	465	56.9
	S. fragilis	252	1.64	2.13	0.10	12.6	2.38	18300	125	16.5	0.71	327	80.1
	S. x smithiana	242	4.68	1.96	0.02	27.7	0.76	18500	176	18.8	1.35	541	31.1
B	-	87.2	0.68	0.31	0.00	-	-	9500	44,6	2.73	0.23	-	-
	S. caprea	69.2	1.58	0.24	0.02	13.6	1.47	9300	23.5	3.64	0.57	353	29.7
	S. fragilis	76.7	2.02	0.27	0.00	9.89	0.39	9500	356	3.25	0.52	343	10.3
	S. x smithiana	71.6	1.16	0.27	0.01	15.3	0.80	8900	166	3.25	0.34	405	13.7
	S. x dasyclados	76.6	2.12	0.31	0.01	7.23	1.15	8600	176	2.73	0.23	260	46.7
C	-	95.5	0.09	0.28	0.02	-	-	11300	485	2.65	0.31	-	-
	S. caprea	91.2	3.21	0.25	0.01	9.95	1.05	11300	408	2.65	0.31	425	40.2
	S. fragilis	91.4	2.62	0.29	0.01	4.34	0.34	12300	358	3.45	0.22	234	26.6

[1)] A*qua regia*- extractable; [2)] 1M NH_4NO_3-extactable; [3)] = leaves, twigs and roots

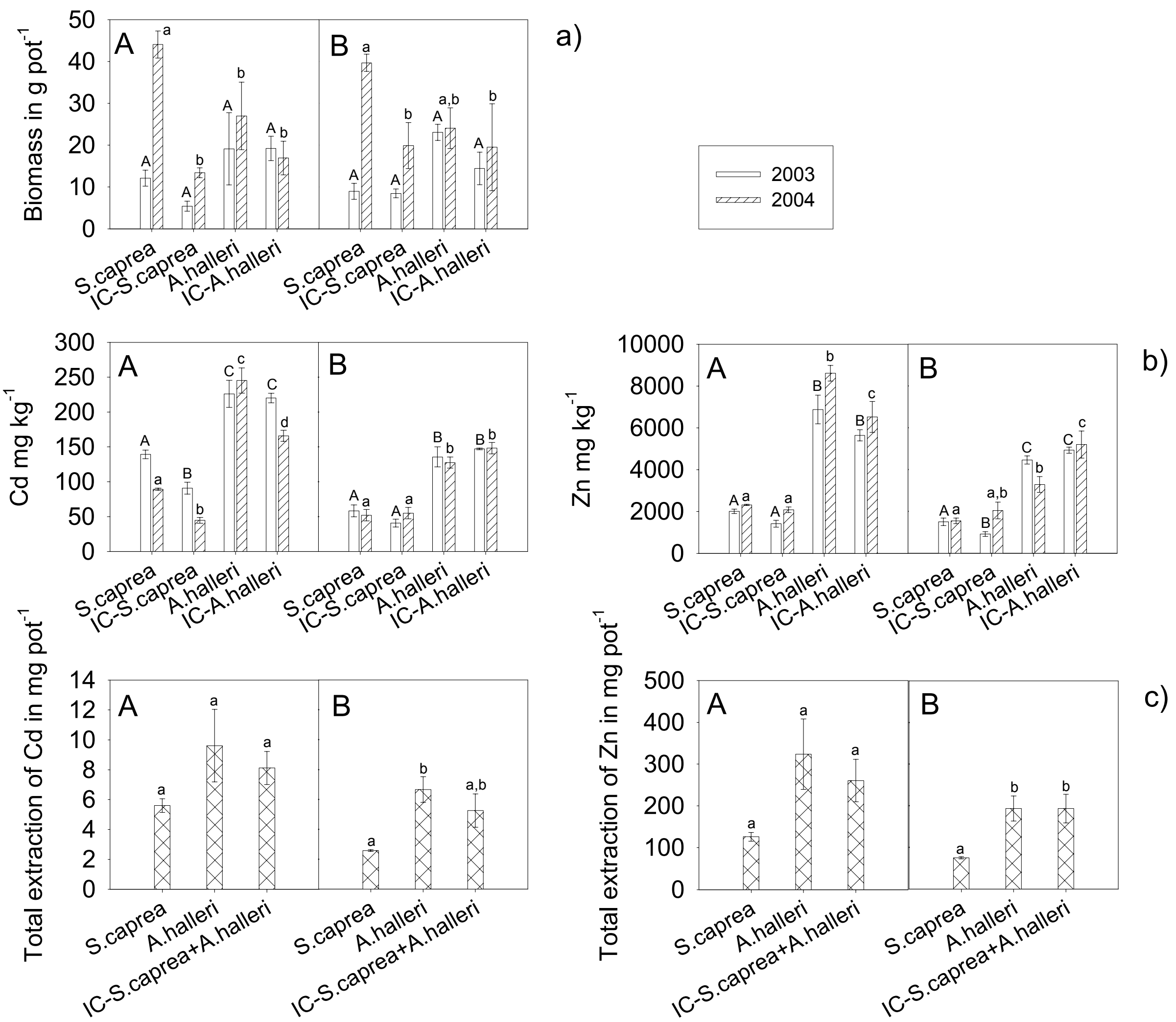

Figure 6. Biomass (a) and Cd/Zn concentrations (b) in leaves of *S. caprea* and shoots of *A. halleri* for two vegetation periods (2003-2004) in single plantings compared to the intercropping (IC) on soil A and B (different letters above columns indicate significant differences (LSD-test, p < 0.05) for 2003 and 2004, respectively). (c) Total extraction of Cd/Zn within two vegetation periods by accumulation in leaves of *S. caprea* and shoots of *A. halleri* in single plantings compared to the intercropping (IC) on soil A and B (different letters above columns indicate significant differences (Tukey HSD-test, p < 0.05). Error bars indicate standard errors of the mean for n = 3.

DISCUSSION

Foliar metal concentrations and bioaccumulation factors after the first vegetation period of willows were among the largest reported in the literature. However, metal concentrations in leaves declined in subsequent years, while leaf biomass clearly increased. This effect was much more pronounced for Cd than for Zn. Significant annual variations of metal concentrations in leaves of willows confirm results of other authors (Pulford and Dickinson, 2005) and could be explained by a shortened accumulation period, as in this study shoots had been cut in August of the third year, or more likely by dilution effects due to increasing leaf biomass. In spite of declining Cd and partly Zn concentrations with time, a substantial fraction of extracted metals was accumulated in leaves. To our knowledge this is the first study to show effective phytoextraction using willows, indicated by the substantial decrease in aqua regia extractable soil Cd (Table 2).

Although phytoextraction using effective metal accumulating willows such as S. x smithiana on moderately contaminated, agricultural soils seems promising, it has to be considered, that extrapolating data obtained in a pot experiment to field performance of willows can be problematic. For example root morphology and soil Cd distribution in the field are known to differ from pots filled with homogenised soils (Keller et al., 2003; Klang-Westin and Perttu, 2002). In our study soils were densely rooted after three vegetation periods. It can be assumed that in the field roots would in fact explore a larger volume of soil, but with lower density. Hence Cd removal rates obtained in this study certainly need validation in the field including consideration of management practices. We suggest to harvest not only twigs, but also the leaves after the natural abscission in autumn. This would have two major advantages 1) metal containing foliage can simply be collected from the ground using a leaf hoover and 2) the natural redistribution of nutrients within the tree can be ensured, e.g. the export of nitrogen from senescing leaves for storage in stem tissues during the dormant period to be relocated in the subsequent season.

In contrast to Cd, effective phytoextraction of Zn using willows still remains to be demonstrated. However, regarding metal accumulation in crops, enhanced Cd concentrations in agricultural topsoils are considered to be more problematic than Zn. Hence a cost-extensive removal of primarily Cd using phytoextraction by willows may be appropriate for contaminated, agricultural topsoils.

On the other hand the "phytoavailable" Cd/Zn fractions according to standard procedures (1M NH_4NO_3-extraction) did not decline in planted versus non planted soils after three vegetation periods. Similar findings have been reported for other phytoextraction studies targeting heavy metals or As (Pulford and Dickinson, 2005; Fitz et al., 2003). Although standard procedures using single extractions with neutral salt solutions in some cases correlate well with plant uptake of metals (Menzies et al., 2007), they clearly underestimate the total phytoavailable metal fraction per a determined mass of soil and neglect resupply of metals from strongly bound fractions. There is general agreement, that total concentrations in soils are an inappropriate measure for pollutant bio (pyhto-) availability. Thus it might not be necessary to reduce pollutant concentrations down to threshold values, but a sustained decrease in phytoavailable pollutant concentrations could be sufficient. This risk based approach would substantially reduce the time required to clean up polluted soil, while minimizing ecological hazards (Fitz et al., 2003). Effective methods to determine phytoavailable metal fractions are needed, as approaching sustained removal of ecologically relevant, bioavailable metal fractions in soils in a reasonable period of time, could represent a milestone in the acceptance and practical implementation of phytoextraction using metal accumulating willows.

Results from the intercropping treatment showed, that the total Cd removal could not be enhanced by planting *S. caprea* with *A. halleri*. This can be explained by partly reduced individual biomass production and decreased metal concentrations in leaves/shoots of both IC partners. Reduced Cd and Zn uptake in *A. halleri* and *S. caprea* along with less biomass production can be related to competition for water or nutrients and possibly also metal sequestration into plants. Limited water availability for each of the species is also expected to result in lower passive influx of dissolved metals. Competition for water and metal uptake may be less critical in the field where plants can explore larger volumes of soil, where willows can grow into deeper soil layers than herbaceous hyperaccumulator species such as A. halleri or Thlaspi caerulescens (Keller et al., 2003). In practice, limitations for successful intercropping of willows with small herbaceous hyperaccumulators may rather arise in terms of sustained establishment, maintaining and harvest of the hyperaccumulator understorey. In our experiment continuous manual weed control was critical to maintain *A. halleri* and we question if this would work in practice.

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4 Publication # 2 – Effects of microbial treatments on foliar Cd and Zn concentrations of *Salix ssp.*: Seasonal and annual variations

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ABSTRACT

Willows (*Salix sp*.) have been reported to accumulate elevated concentrations of Cd and Zn in their leaves. Phytoextraction using *Salix* holds promise due to large biomass production, deep root system and fast growth. In this study we investigated the effects of microbial treatments (MycorTree^{TM}, metal tolerant soil bacteria) on biomass production and metal accumulation in leaves of three *Salix* species (*S.* x *rubens*, *S. fragilis*, *S. caprea*) on two soils with large Cd and Zn concentrations. Effects on biomass production and foliar metal concentrations were monitored for two vegetation periods. A non-contaminated agricultural soil served as control in terms of biomass production. Large concentrations of Cd and Zn were determined in leaves of *S. fragilis* and *S. caprea*, whereas *S. x rubens* was found to be less tolerant and to accumulate less metals in the foliage. Microbial treatments showed no effect on biomass production of willows, but significantly increased foliar metal concentrations of *S. caprea* and partly *S.* x *rubens* grown on soil ABW for two vegetation periods. Significant seasonal differences were found for Cd and Zn concentrations in leaves of all willows, but variations were found to be not correlated to foliar concentrations of macronutrients (Ca, Mg, K, P, N) or Fe, indicating that metal accumulation in leaves occurred independently from plant internal nutrient relocation at leaf senescence.

INTRODUCTION

Several willow species (*Salix sp.*) have been reported to accumulate heavy metals, particularly Cd and Zn, in their above ground biomass (Pulford & Dickinson, 2005; Pulford & Watson, 2002). Exceptionally large concentrations of > 200 mg kg^{-1} Cd and > 3000 mg kg^{-1} Zn in leaves of *Salix ssp*. have been reported for pot experiments (Wieshammer et al., 2007; Vyslouzilova et al., 2003). The large metal accumulation potential of some willow species and additional properties such as easy propagation, fast growth, high biomass production and deep root systems, suggest their implementation in phytoextraction technologies to remediate contaminated soils.

Willows are associated with endo- and/or ectomycorrhizal fungi (Smith & Read, 1997). Among soil microorganisms only mycorrhizal fungi provide a direct link between soil and roots and are therefore deemed to affect metal availability and toxicity to plants (Leyval & Joner, 2001; Leyval et al., 1997). Effects of mycorrhizal associations on metal accumulating willows are still poorly understood and only few publications on this topic are available. In studies of Sell et al. (2006) and Dos Santos Utmazian et al. (2007) the tested fungi showed no influence on foliar Cd and/or Zn concentrations of *S. viminalis*, *S. caprea* or *S.* x *smithiana*, whereas Baum et al. (2006) reported, that a certain strain of *Paxillus involutus* enhanced the uptake of Cd and Zn by *Salix* x *dasyclados* on a soil with large phytoavailable metal concentrations. Apart from mycorrhizal associations, the bacterial community is an integral active part of the soil biota. It has been shown, that the inoculation of herbaceous hyperaccumulators such as *Thlaspi caerulescens* or *Alyssum murale* with certain rhizosphere bacteria can promote biomass and/or increase Zn or Ni uptake (Whiting et al. 2001, Abou-Shanab et al., 2003). However, most observations on rhizosphere processes regarding heavy metal accumulation in shoots have been made with crop plants (DeSouza et al., 1999; Burd et al., 1998). Hence information on effects of microbial treatments on metal accumulation in willows is still scarce. Production of organic acids by soil bacteria, including rhizobacteria, may promote solubilisation, mobility and bioavailability of metals by lowering the pH and supplying metal-complexing organic acids ligands (e.g. siderophores, Goldstein et al., 1999).

We hypothesise that the promotion of biomass production and/or an improvement of foliar metal concentrations by the application of microbial treatments in the rhizosphere could enhance the phytoextraction potential of *Salix ssp*..

The objectives of this work were (1) to determine the metal accumulation potential of three willow species (*Salix x rubens, Salix fragilis* and *Salix caprea*) in a pot experiment using two highly polluted soils, (2) to compare effects of a commercially available mycorrhiza-bacteria-inoculate to an indigenous bacteria inoculate on foliar metal concentrations and biomass production, and (3) to assess seasonal and annual variations of heavy metal- and nutrient-concentrations in leaves of willows.

MATERIAL & METHODS

Plants

Cuttings of *Salix x rubens* Schrank (*S. alba x S. fragilis,* clone BOKU 01 CZ-003) were obtained from trees growing in Kutna Hora (CZ), an area contaminated with up to (mg kg^{-1}) 3150 Zn, 21 Cd and 6560 Pb due to long term emissions of a lead smelter. Additional contamination derived from polluted sediments, which were deposited on the site during floodings of the river Litavka. Cuttings of *Salix fragilis* L. (clone BOKU 01 CZ-001) derived from Přibram (CZ), where one of the largest silver mines in Europe had been active since the 13^{th} century. Until termination in 1992, large amounts of Zn ores were processed. At present the area is contaminated with up to (mg kg^{-1}) 9590 Zn, 70 Cd and 6560 Pb. Cuttings of *Salix caprea* L. (clone BOKU 01 AT-004) were obtained from a tree nursery in Vienna (Austria). Cuttings of equal length (~ 20 cm) and diameter (~ 1 cm) were propagated in a sand-soil mixture in the greenhouse under controlled environmental conditions (day/night temperature: 20/15°C, 60 % air moisture, 16 hrs light day^{-1}), until they had developed sufficient roots (~ six months). Initial cuttings contained on average 0.56 mg kg^{-1} Cd and 79.3 mg kg^{-1} Zn.

Soils

Three soils were included in the experiment. Soil HIR, an agricultural soil ($pH_{in\ H2O}$: 8.1; CEC: 249 $mmol_c$ kg^{-1}; texture (g kg^{-1}): 367 sand, 425 silt, 208 clay) served as non-contaminated control in terms of biomass production and metal accumulation of the investigated willows. Two other soils (ARN, ABW) had been contaminated due to long term industrial activities. The ARN soil ($pH_{in\ H2O}$: 6.4; CEC: 231 $mmol_c$ kg^{-1}; texture (g kg^{-1}): 668 sand, 240 silt, 92 clay; Cd_{total}: 76.6 mg kg^{-1}; Zn_{total}: 3440 mg kg^{-1}; Cd_{labile} 6.67 mg kg^{-1}; Zn_{labile} 179 mg kg^{-1}) was excavated in the vicinity of an abandoned Zn-Pb smelter in Arnoldstein (Carinthia, Austria). The smelter had been active since 1882 and was expanded to recycling of storage-batteries and metal-chemistry in 1945 resulting in soil pollution with Cd, Zn and Pb in the surroundings. Smelter and metal processing actvities were terminated in 1991. The ABW soil (pH $_{in\ H2O}$: 8.0; CEC: 156 mmol$_c$ kg^{-1}; texture (g kg^{-1}): 505 sand, 377 silt, 118 clay; Cd$_{total}$: 25.3 mg kg^{-1}; Zn$_{total}$: 3840 mg kg^{-1}; Cd$_{labile}$ 0.21 mg kg^{-1}; Zn$_{labile}$ 15.1 mg kg^{-1}) was obtained from an abandoned gaswork site in Vienna (Austria). Both soils have large total metal concentrations, whereas the labile metal concentrations (1M NH_4NO_3-extractable) are considerably lower in the ABW than in the ARN soil.

Microbial treatments

Willows were treated with MycorTree^{TM} (MT), a commercially available root dip for tree seedlings (Plant Health Care, Inc., Pittsburgh) containing spores of the ectomycorrhizal fungi *Pisolithus tinctorius* and rhizosphere bacteria (*Bacillus thuringiensis, B. megaterium, B. licheniformis, B. subtilis, B. polymyxa and Paenibacillus azotofixans*). Following the directions for use of the product, a MT-slurry was produced, into which roots were dipped until they were completely covered with the gel, before planting.

The second microbial treatment consisted of an inoculum of bacteria occuring naturally at the Arnoldstein site. 10 g of soil were extracted with 100 ml 0.8 % NaCl solution for 30 min by gentle shaking. Dilutions of the suspension were plated on R2A-agar + 1mM $Cd(NO_3)_2$ + 1mM $Pb(NO_3)_2$ + 1mM $Zn(NO_3)_2$ and incubated at room temperature for 7 days. Colonies were selected for further experiments. Identification of the bacterial strains was done by PCR amplification of the 16S-rDNA with primers 16S0008f (AGAGTTTGATCCTGGCTCAG; Edwards et al. 1989) and 16S1512r (ACGGTTACCTTGTTACGAC; Lane 1991), by RFLP analysis with the restriction enzyme *Hin*6I and sequencing of representative PCR products (Table 1). The strains were further characterised by determining their minimal inhibitory concentration (MIC) of Cd, Pb and Zn. The strains were plated on tryptic soy agar (TSA) amended with nitrate salts of the respective heavy metals in concentrations of 2, 4, 6, 8 and 10 mM. Plates were incubated at room temperature and growth was recorded after two days and after two weeks (Table 1). For inoculum production, bacterial strains were grown separetely in 5 ml tryptic soy broth (TSB) for three days at 30 °C and 150 rpm. Then 100 ml

TSB were inoculated with the whole preculture and incubated over night at 30 °C and 150 rpm. At the end of the growth period, the optical density at 595 nm (OD_{595}) was determined and cells were then harvested by centrifugation. Cells were resuspended in 5 % glycerol / 10 mM sodium phosphate (pH 7) and mixed. Final volume of the inoculum was 500 ml with roughly 1.7×10^{11} cells (calculated from the OD_{595}). Due to unequeal growth, the individual strains were present in different amounts in the inoculum. The composition of the inoculum was as follows: ~ 84 % *Pseudomonas* sp (PR04), ~ 12 % *Janthinobacterium lividum* (PR02, PR13), ~ 2,6 % *Flavobacterium* sp. (PR01) and ~ 1,7 % Flexibacteraceae bacterium (PR05).

Table 1. Bacterial strains isolated from the metal contaminated ARN soil. Strains were identified and tested on their metal tolerance before application to the ABW soil.

Strain	Closest described relative[a] (Accession nr.) (homology)	MIC[b] (mM) Zn	Cd
PR01	*Flavobacterium frigidimaris* (AB183888) (99%)	10	< 2
PR02	*Janthinobacterium lividum* (AF174648) (99%)	> 10	4
PR13	*Janthinobacterium lividum* (AF174648) (99%)	10	4
PR04	*Pseudomonas sp.* BE3 dil (AY263472) (100%)	> 10	4
PR05	*Flexibacteraceae bacterium* (AY065626) (100%)	n.d.	n.d.

[a] determined by sequencing of the 16S rDNA gene
[b] minimal inhibitory concentration

Experimental setup

In August of year 1 rooted cuttings were planted in pots containing 6.75 kg soil (DW, < 2 mm) mixed with 2.25 kg washed quartz gravel with a grain size of 2 mm to alleviate soil compaction. Willows growing on the ABW and ARN soils were treated with MycorTree™ (+MT) prior to planting. A second set of willows grown on the ABW soil was treated with 30 ml of the bacteria inoculum (+B). As bacteria were isolated from the ARN soil, the inoculum was not applied to willows on this soil. Non-treated willows were planted in HIR, ARN and ABW soils. Every treatment was carried out in five replicates and pots were positioned outdoors in a randomised design. To achieve results close to real world conditions, soils were not sterilised prior to the treatments. Willows were watered as required during the growth period. Leaf samples were collected in August and October of year 1 and 2 to monitor seasonal and annual variations in metal accumulation. The overall leaf biomass was determined by collecting the foliage at senescence using a net wrapped around each willow.

Plant and soil analyses

Leaves were washed using deionised water in an ultrasonic bath. Samples were dried at 80 ^{o}C until constant weight and the dry weight was recorded before they were finely ground in a stainless steel mill (MF 10, IKA® Werke, Staufen, Germany). Aliquots of 0.2 g were digested using a mixture of 0.5 ml H_2O_2, 6 ml HNO_3, and 1 ml $HClO_4$ (all puriss. p.a., Sigma-Aldrich Handels GmbH, Vienna, Austria) in a closed microwave system (MLS Mega 240 S/N 121 398).

Soils were analysed for pH (in a 1 : 2.5 soil : water extract), cation exchange capacity at soil pH (CEC, in a 0.1 M $BaCl_2$-extract at a 1 : 20 soil : solution ratio), texture, total metal concentrations (*aqua regia* extractable; HCl and HNO_3 puriss. p.a., Sigma-Aldrich Handels GmbH, Vienna, Austria) and labile metal concentrations (in a 1 M NH_4NO_3-extract at a 1 : 2.5 solution ratio). All metal analyses were performed using an ICP-MS (Elan 5000, Perkin-Elmer). Chemical analyses were validated by including reference materials, blanks and replicate samples at a rate of at least 10%.

RESULTS

Leaf biomass

On the ARN soil *S. x rubens* developed visible phytotoxic symptoms in leaves soon after planting and finally plants did not survive in this soil. No phytotoxic symptoms were observed on leaves of *S. caprea* and *S. fragilis* on the same soil. In the ABW and HIR soil all three willow species grew well throughout the experiment. While leaf biomass of *S. fragilis* and *S. x rubens* was significantly lower on the contaminated soils in comparison to the HIR soil, leaf biomass of *S. caprea* was not affected by soil contamination (Figure 1). On average leaf biomass produced during two vegetation periods decreased in the following order: *S. caprea* > *S. fragilis* > *S. x rubens.* None of the microbial treatments significantly affected leaf biomass production of *S. caprea*, *S. fragilis* or *S. x rubens* on either soil (Figure 1).

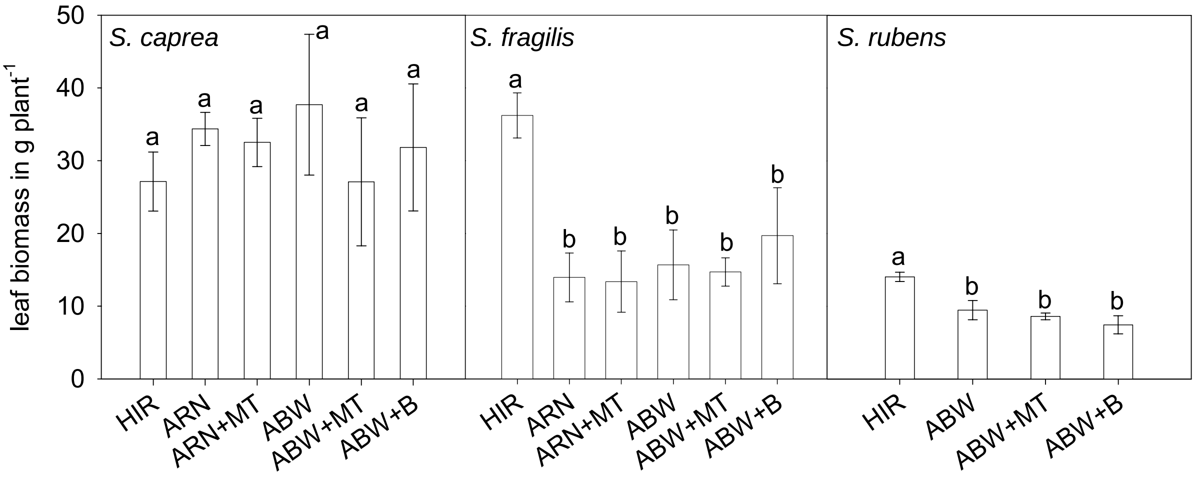

Figure 1. Leaf biomass produced within two years by individual willows on the non contaminated soil HIR in comparison to the contaminated soils ARN and ABW (microbial treatments: MT = MycorTree™, B = bacteria inoculate). Error bars indicate standard errors of the mean and different letters above columns show significant differences (LSD-test, $p < 0.05$).

Metal accumulation in leaves

Foliar Cd and Zn concentrations showed substantial interspecific differences. On average largest metal concentrations were determined in leaves of *S. fragilis* followed by *S. caprea*. Clearly lower foliar Cd and Zn concentrations were found in *S. x rubens* (Figure 2). Unusually large metal concentrations in leaves predominantly occurred towards the end of the first growing season (up to 380 mg kg^{-1} Cd and 4400 mg kg^{-1} Zn) and differences between metal accumulation in leaves of *S. caprea* and *S. fragilis* became smaller towards the end of the second year (Figure 2). While foliar Zn concentrations of individual willow species were similar on both contaminated soils, foliar Cd concentrations of willows grown on the soil ARN were significantly larger as compared to the same species grown in the ABW soil (Figure 2).

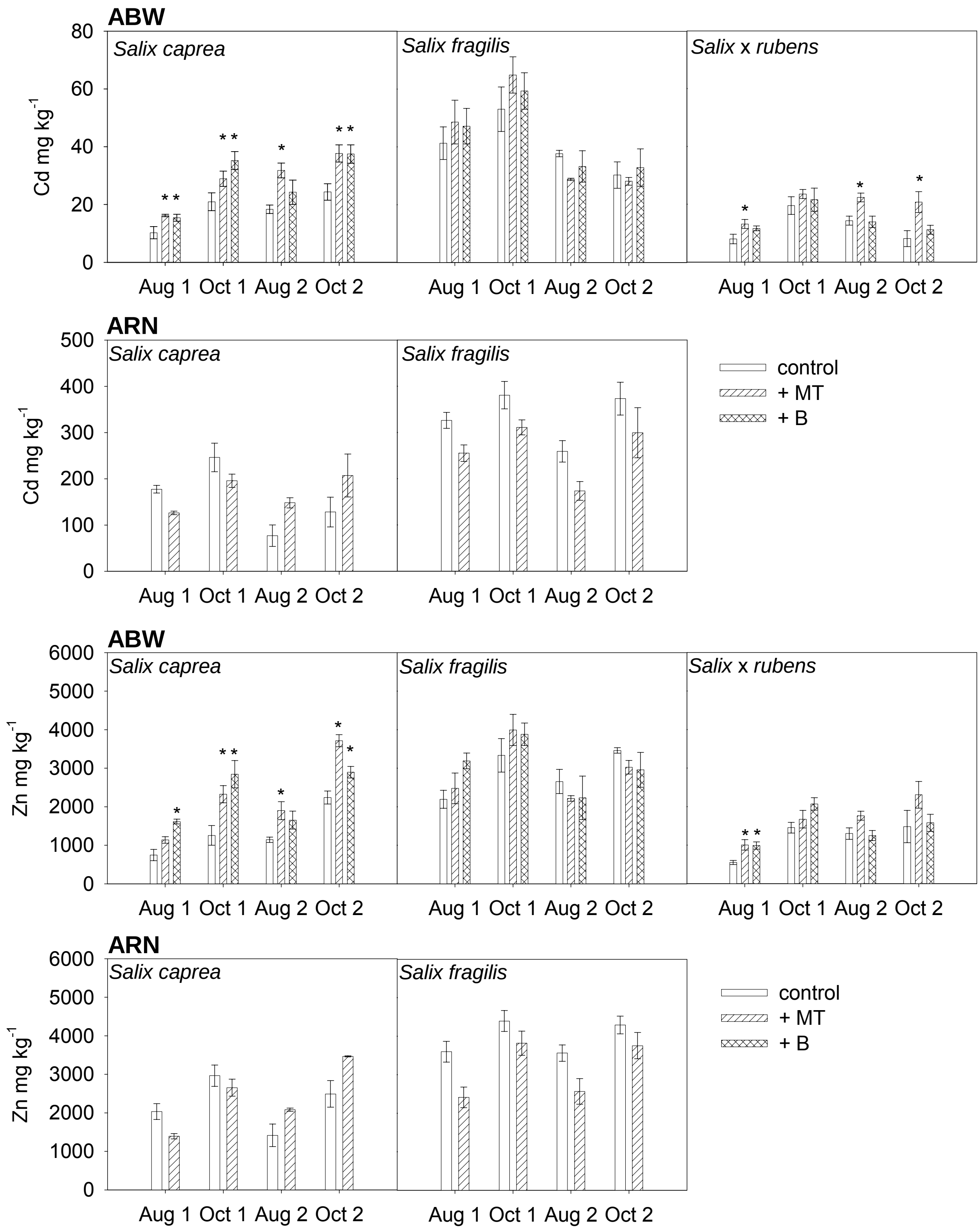

Figure 2. Cd – and Zn-concentrations in leaves of *S. caprea*, *S. fragilis* and *S. x rubens* grown on the contaminated soils ABW and/or ARN for four samplings in two years (MT = MycorTree™, B = bacteria inoculate). Error bars indicate standard errors of the mean and asterices indicate significant differences in comparison to respective non treated control plants (LSD-test, p < 0.05).

However, Cd concentrations in leaves generally increased with increasing foliar Zn concentrations (Figure 3). Foliar background concentrations of willows grown on the non-contaminated soil HIR were between 1.4 - 2.0 mg kg^{-1} Cd and 530 - 820 mg kg^{-1} Zn. For most soil x willow combinations metal concentrations increased from August to October in both years (Figure 2). Bioaccumulation factors (BCF, foliar metal concentration : *aqua regia* extractable soil metal concentration) for Cd were consistently larger than those for Zn, while *S. x rubens* had cleary lower BCFs (0.3 - 1 for Cd; 0.4 - 0.6 for Zn) than the other willows (1.0 – 5.0 for Cd; 0.4 – 1.3 for Zn; Figure 4).

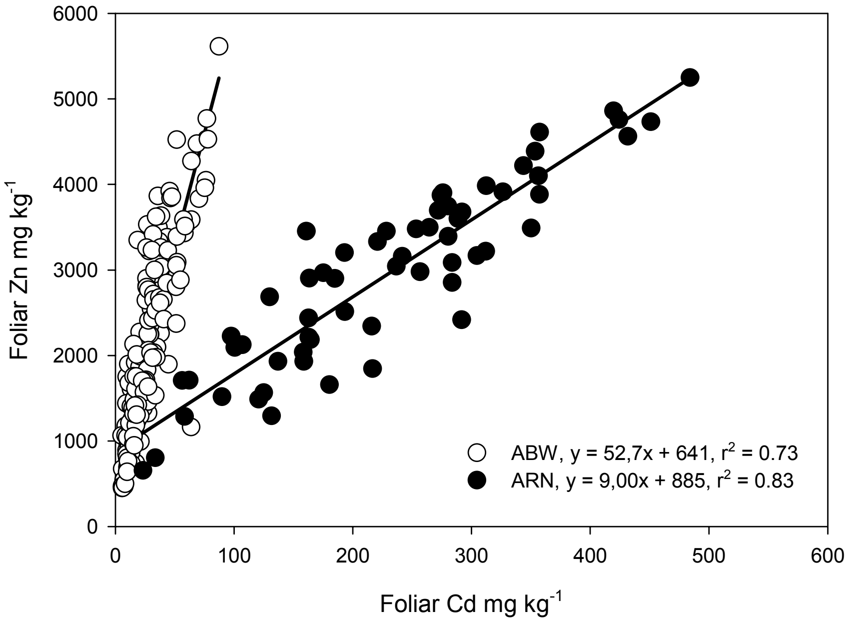

Figure 3. Correlation between foliar Cd and Zn concentrations of *Salix ssp.* harvested in August and October of two vegetation periods grown on two contaminated soils (ABW, ARN).

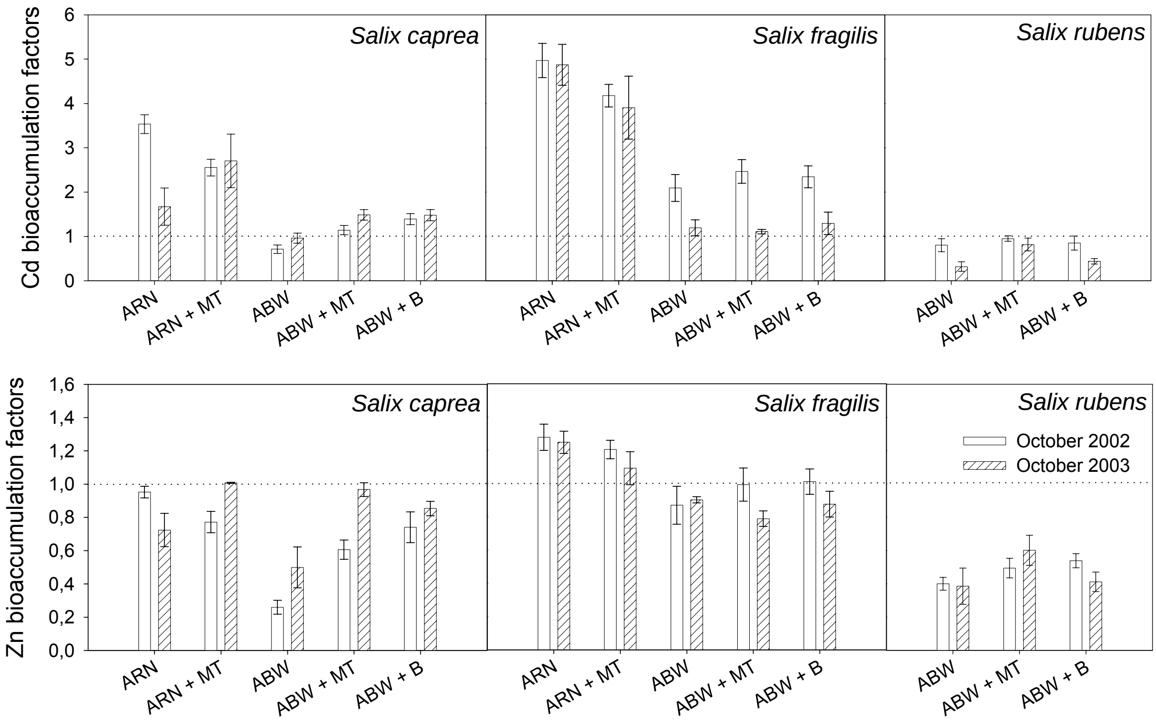

Figure 4. Cd- and Zn- bioaccumulation factors (metal concentration in leaves / aqua regia extractable metal concentrations in soil) of *S. caprea*, *S. fragilis* and *S. x rubens* grown on the contaminated soils ABW and/or ARN for samplings in October of two years (MT = MycorTree™, B = bacteria inoculate). Error bars indicate standard errors of the mean.

Effect of microbial treatments on foliar metal concentrations

None of the microbial treatments had a significant effect on foliar metal concentrations of either willow grown on the ARN soil (Figure 2). No effect was found as well for *S. fragilis* grown on the ABW soil, while foliar Cd concentrations of *S. x rubens* on soil ABW were found to be significantly enhanced after application of MT in October of both growing seasons. Significantly increased Zn concentrations in leaves of *S. x rubens* after treatment with B or MT in October of the first year could not be confirmed in the following year. On the ABW soil foliar Cd concentrations of *S. caprea* increased by 68 % after application of MT and by 51 % after application of B determined in October of the first year. Zn concentrations in leaves of *S. caprea* increased by 130 %, when MT and by 85 %, when B was applied. This significant increase in foliar metal concentrations of *S. caprea* after microbial treatments was confirmed in October of the second year (Figure 2).

Total metal accumulation in leaves

Corresponding to larger Cd concentrations in leaves, total Cd accumulation (concentration in leaves x leaf biomass) of *S. caprea* and *S. fragilis* after two vegetation periods was clearly larger on the ARN compared to the ABW soil (Figure 5). Total foliar accumulation of Zn was similar for *S. fragilis* on soil ARN and ABW. *Salix caprea* accumulated more Zn on the ARN soil than on the ABW soil. Significant effects of microbial treatments on foliar metal concentrations of *S. caprea* were not reflected in increased total metal uptake. Due to little biomass and low foliar metal concentrations total metal uptake of *S.* x *rubens* was only fractional compared to the other willows (Figure 5).

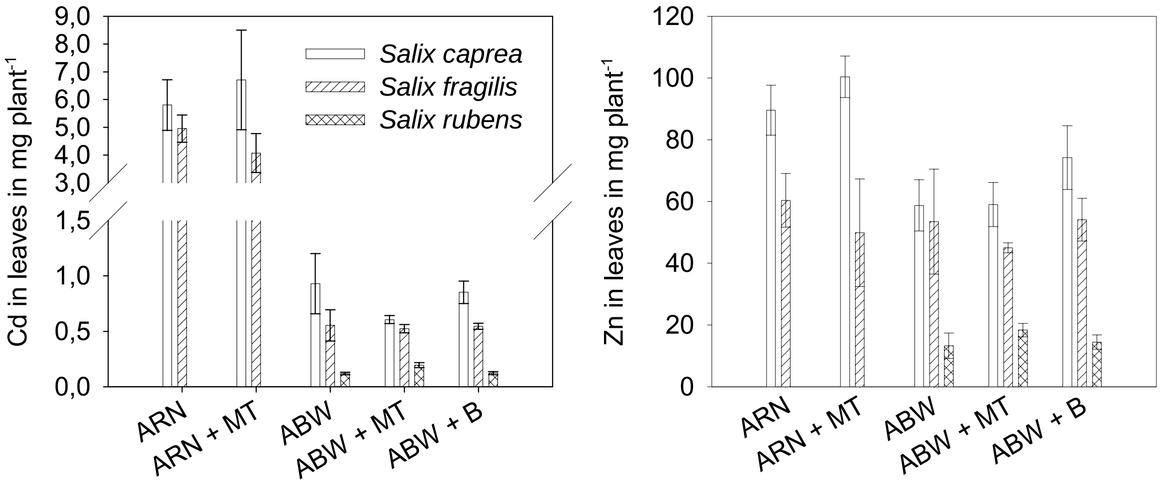

Figure 5. Total amounts of Cd and Zn accumulated in leaves of *S. caprea*, *S. fragilis* and *S. x rubens* during two years on the contaminated soils ABW and/or ARN (MT = MycorTree^{TM}, B = bacteria inoculate). Error bars indicate standard errors of the mean.

Foliar nutrient concentrations

Mean concentrations of macronutrients (N, P, K, Mg, Ca) and Fe determined in mature leaves in August and October decreased in the following order Ca > K > N > Mg > P >> Fe. Microbial treatments had no consistent effect on nutrient concentrations in leaves of either willow (Figure 6). Foliar Ca concentration increased from August to October, but values were within the normal range for *Salix ssp.* according to Bergmann (1993). Ca in leaves of *S.*

fragilis and *S.* x *rubens* on the ABW soil was significantly larger than on the HIR soil, but did not correlate with foliar Cd or Zn concentrations on the ABW soil. Concentrations of K significantly decreased from August to October (on average to 63 %) in leaves of all investigated willows on either soil. On the other hand foliar Fe concentrations consistently doubled from August to October, whereas Mg and P concentrations in leaves were within usual ranges for willows (Bergmann, 1993) and not affected by season.

A significant decrease in foliar N concentration from August to October was determined for *S. caprea* and partly for *S. fragilis*. In general seasonal variations of nutrients in leaves did not correlate with foliar Cd or Zn concentrations.

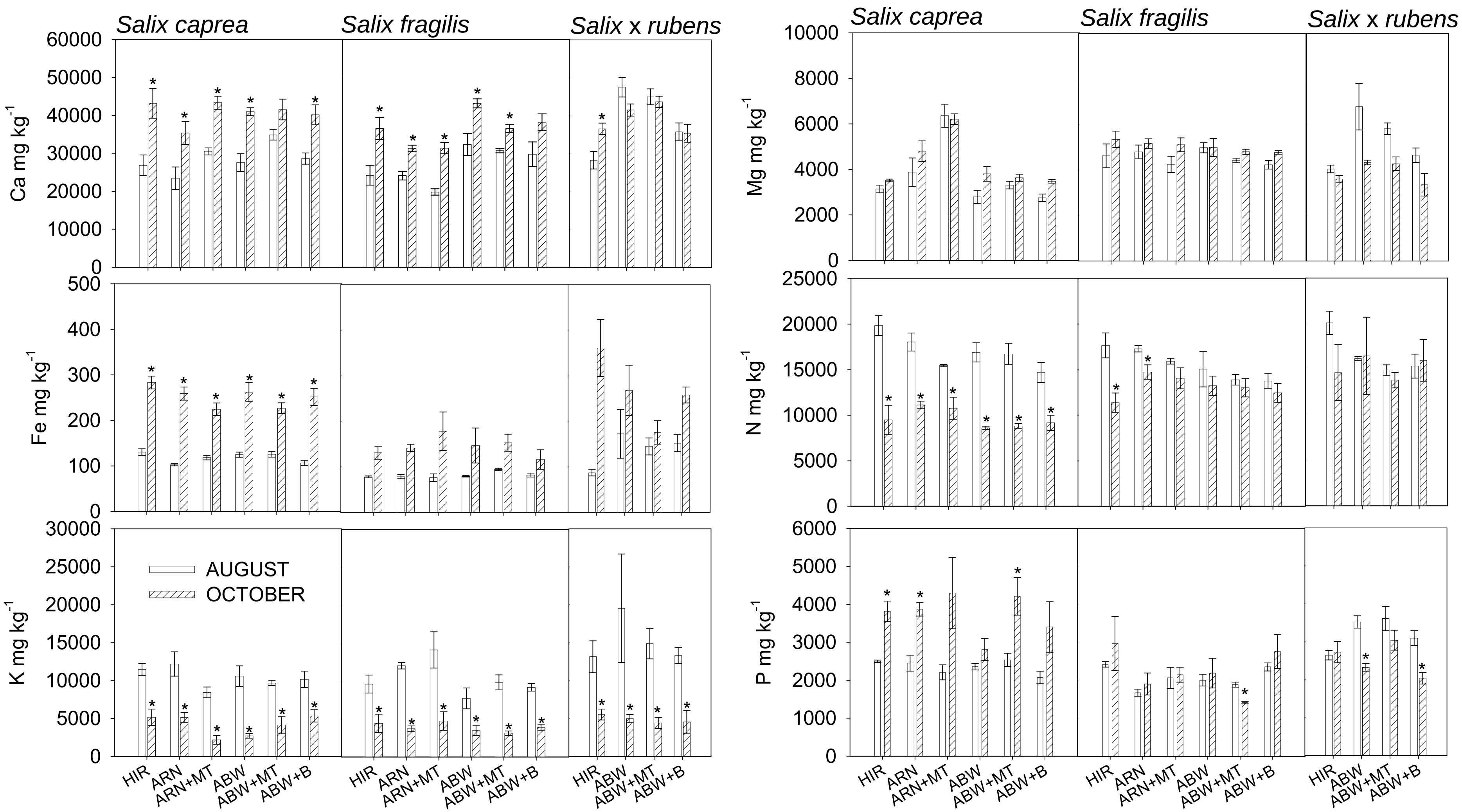

Figure 6. Concentrations of macronutrients and Fe in mature leaves of *S. caprea*, *S. fragilis* and *S. x rubens* grown on the contaminated soils ABW and/or ARN and on the non contaminated soil HIR (MT = MycorTree^{TM}, B = bacteria inoculate) in August and October of year 2. Error bars indicate standard errors of the mean and asterices indicate significant differences in October in comparison to August (LSD-test, $p < 0.05$).

DISCUSSION

Phytoextraction requires highly metal-tolerant plants and plants with large metal accumulation in harvestable tissues. In this context the investigated clone of *S. x rubens* (BOKU 01 CZ-003) proved to be not suitable for phytoextraction because (1) foliar Cd and Zn concentrations were not particularly large, especially in comparison to the other species investigated; (2) leaf biomass was significantly reduced on the contaminated compared to the non contaminated soil; and (3) *S. x rubens* produced significantly less leaf biomass than *S. caprea* or *S. fragilis* not only on the contaminated soil ABW, but as well on the non contaminated soil HIR. However, significant differences in yield and foliar Cd accumulation between two clones of *S. x rubens* were shown by Vysloužilová et al. (2006), so other clones of *S. x rubens* might be more efficient than the one investigated here.Large heavy metal concentrations of the soils ARN and ABW did not affect leaf biomass production of *S. caprea* compared to the non-contaminated HIR soil confirming reports on particularly large metal tolerance of this species (Dos Santos and Wenzel, 2007; Wieshammer et al., 2007). Although leaf biomass of *S. fragilis* decreased in the contaminated soils compared to the HIR soil, large Cd concentrations in leaves compensated for this and results for the total Cd uptake during two vegetation periods were similar to those of *S. caprea*. In general foliar Cd and Zn concentrations of *S. caprea* and *S. fragilis* in this study largely exceeded values reported in the literature so far (Dos Santos Utmazian and Wenzel, 2007). Despite approximately 10-fold lower labile Zn concentrations in the ABW soil compared to the ARN soil, *S. caprea* and *S. fragilis* accumulated similar amounts of Zn in their leaves on both soils, indicating a high Zn extraction potential of these trees. The clear correlation of foliar Cd and Zn concentrations on individual soils as shown in Figure 3 suggests similar translocation mechanisms for these elements to the leaves and possibly similar sequestration characteristics in the leaves (Meertens et al., 2006).

In a study of Lepp and Madejón (2007) foliar Zn and Cd concentrations of *S. caprea* decreased in October compared to earlier in the season. The same was shown for Cd and Zn in leaves of non-accumulating *Fagus sylvatica* (Tyler and Olsson, 2006). The opposite trend of an increase of foliar metal concentrations towards the end of the vegetation period as found in our study is in accordance to findings of Laureysens et al. (2004) for *Populus ssp*. and of Meertens et al. (2006) for *Salix ssp.*. These results were attributed to either seasonal patterns of metal availability in soil or shunting of metals in plant tissues prior to senescense. Seasonal patterns of metal availability in soil expressed in an increase of labile metals towards the end of the season could not be confirmed in our study (data not shown). Hence, an active detoxification mechanism of metal tolerant willows as suggested by Vollenweider et al. (2006) by shunting of metals into leaves at the end of the vegetation period seems to be most likely. The significant leaf age effect suggests that harvesting the leaves after the natural leaf fall will remove most metals from the contaminated site. Annual variations of metal concentrations in leaves, which mostly results in a decrease of foliar Cd and Zn in the course of several vegetation periods due to dilution by the increase of biomass as reported by some authors (Pulford and Dickinson, 2005) could not be confirmed consistently by our results. Despite increasing leaf biomass, annual changes in foliar metal concentrations were mostly not significant.

Effects of microbial treatments proved to be species and soil dependent. In our study no influence of microbial treatments on leaf biomass production of either willow was determined, which is in line with findings of Sell et al. (2005), but contrary to results reported by Dos Santos Utmazian et al. (2007) and Baum et al. (2005). Pot experiments dealing with microbial treatments are commonly conducted with sterilized soils to (1) eliminate competition of native microorganisms and to (2) connect observed effects to specific treatments. However, sterilization of soils not only kills microorganims, but can also substantially alter soil characteristics such as decrease the organic matter or change metal availability (Egli et al. 2006; Luo et al. 2001). Hence, sterilization of soils can have a significant influence on plant growth as reported by Dos Santos Utmazian et al. (2007) for *Salix ssp.*. This shortfall has to be considered and addressed, when discussing practical application of apparently effective treatments. We have decided to conduct our experiments with non sterilized soils to maintain conditions as close as possible to the field. Although soils were not sterilised, effects of microbial treatments in combination with *S. caprea* and partly *S. x rubens* could be demonstrated for two vegetation periods. However, it remains to be answered why microbial treatments influenced the Cd uptake of *S. caprea* and *S. x rubens* on soil ABW, but not on soil ARN. Furthermore it needs to be investigated, which compounds of the MycorTree™ product affected the metal uptake of willows.

Our findings on seasonal variations in nutrient concentrations are partly in line with findings of Von Fircks et al. (2001), who reported retranslocation of N, P and partly K, but no seasonal trend from senescing leaves for Ca and Mg of *S.* x *dasyclados*. *A* negative correlation of Ca or Mg concentrations with Cd or Zn concentrations in shoots as reported for *Thlaspi caerulescens* (Saison et al., 2004), indicating competition between Cd or Zn and Ca or Mg uptake, was not confirmed by our results. The lack of correlation of nutrients with metal concentration in leaves suggests that metal accumulation in leaves of the investigated willows occurred independent of seasonal plant-internal nutrient cycling and competition mechanisms.

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6 CURRICULUM VITAE

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Education

2004 – 2007	PhD at the University of Natural Resources and Applied Life Sciences, Department of Forestry and Soil Science, Insitute of Soil Science, Working group „Rhizosphere Ecology and Biogeochemistry“ Title of Phd-thesis: Phytoextraction of Cadmium and Zinc from Contaminated Soils by *Salix ssp*.
1993 – 2002	Diploma study „Landscape Architecture and Landscape Planning“ at the University of Natural Resources and Applied Life Sciences, Vienna Title of diploma-thesis: *In-Situ* Immobilisation of Heavy Metals and Arsenic in Highly Polluted Soils by Applying Iron Bearing Industrial By-Products and Phosphorus: Chemical and Ecotoxicological Assessment

Professional Experience

since June 2001

Research assistant at the University of Natural Resources and Applied Life Sciences, Department of Forestry and Soil Science, Institute of Soil Science, Working group „Rhizosphere Ecology and Biogeochemistry“

Involvement in the following R & D projects:

- since 2006 ACULTURE - The Re-establishment of Human Resources, Curricula, Systems and Institutions at the Agricultural Faculty of the Syiah Kuala University in Aceh (funded by the ASIA LINK Program EuropeAid – European Commission)
- since 2006 MUBIL II - Auswirkungen unterschiedlicher Düngungsvarianten auf die Pflanzenverfügbarkeit von Phosphor und Kalium im biologischen Landbau (funded by the Federal Ministry of Agriculture, Forestry, Environment and Water Management)
- 2006 GENOMETALIX - Genomforschung für eine bessere Umwelt: die molekularen Mechanismen der Schwermetallaufnahme im System Weidenwurzeln-Mikroorganismen (funded by the Wiener Wissenschafts-, Forschungs- und Technologiefonds, WWTF)
- 2003-2006 INTERLAND - Innovative Technnolgies for Remediation of Landfills and Contaminated Soils (funded by the Federal Ministry of Agriculture, Forestry, Environment and Water Management; project development by Kommunalkredit Austria Public Consulting)
- 2004-2005 Entwicklung eines innovativen Verfahrens zur Sanierung von schwermetallkontaminierten Böden mit Hilfe von Pflanzen (*Salix ssp.*) (funded by the Austrian Federal Economic Chamber)

- 2004-2005 Wärmepumpen, Erdkollektoren, Garten- und Wohnqualität: Untersuchungen der Auswirkungen auf Wurzelwachstum und Bodenmikrobiologie (funded by Niederösterreichische Wohnbauforschung, Verein für Konsumenteninformation and Leistungsgemeinschaft Wärmepumpe Austria)
- 2001-2002 Rhizosphere processes in phytoremediation: assessment, modeling and management (funded by the Federal Ministry for Education, Science and Culture)
- 2001 Phytosanierung und Phytoprävention im urbanen Raum: Kombinierte Verfahren zur Bodendekontamination und Reinigung von Abwässern (funded by the City of Vienna)

Feb 2000 – May 2001	Research assistant at Rothamsted Research, Soil Protection and Remediation Group (Harpenden, Hertfordshire, UK) R & D project: ▪ IMPRIMIS - International Project for the Remediation and Inactivation of Metals *In Situ* (funded by the „International Lead Zinc Research Organization“, Inc., North Carolina, USA)
June – Sep 1999	Alpine farming in Fusch / Glocknerstrasse
April – May 1999	Project assistant at the Office for Landscape Planning Stöckl, Zell/Pram, Upper Austria
June – Sep 1998	Vounteer at the National Park Hohe Tauern, Großkirchheim, Carinthia
Apr 1998, July-Sep 1997	Employee at the Company for Landscape Gardenening Hauer, Raab, Upper Austria
Apr-Oct 1996, Nov 1995	Field work in the course of the soil survey (BZI) in Carinthia
1994-1999	Part time assistant at the Technisches Büro für Bodenkultur Wieshammer, Vienna

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