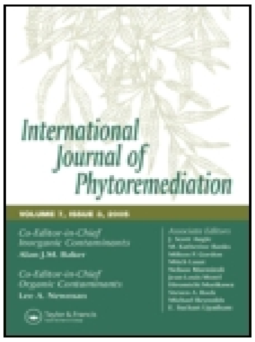

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Zn, Cd, S and trace metal bioaccumulation in willow (*Salix* spp.) cultivars grown hydroponically

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ABSTRACT

Willows (*Salix spp.*) can be used to phytoremediate soils contaminated by Zn and Cd under certain conditions. In this study, the ability of 14 Salix cultivars to concentrate Cd, Zn and S in leaves was measured in hydroponic culture with 10 and 200 μM Cd and Zn, respectively, in the nutrient medium. The cultivars showed a wide range of biomass yields, tolerance to metals, and foliar concentrations of Zn and Cd, with some cultivars accumulating up to 1000 mg kg^{-1} Zn, 70 mg kg^{-1} Cd and 10,000 mg kg^{-1} S with only mild phytotoxicity symptoms attributable to excess Zn. Cultivars with higher foliar Zn concentrations tended to have higher foliar Cd concentrations as well, and competition between Zn and Cd for uptake was observed. Exposure of Salix cultivars to Cd and Zn did not affect foliar concentrations of secondary metabolites such as polyphenols, but trace metal concentrations in leaves were significantly reduced (Fe and Cu) or increased (Mn) by exposure to excess Zn and Cd. Sulfur-XANES spectroscopy showed foliar S to be predominantly in highly oxidized (sulfate plus sulfonate) and reduced (thiol) forms, with oxidized S more prevalent in willows with the highest total S content.

KEYWORDS
Phytoremediation; phytotoxicity; polyphenols; sulfur oxidation state; S-XANES spectroscopy

Introduction

Various willow species and cultivars (*Salix* spp.) have a wide range of abilities to bioaccumulate heavy metals such as Cd and Zn in leaves and stems with a moderately high degree of tolerance to metal toxicity (Landberg and Greger 1996; Vysloužilová *et al.* 2003; Klang-Westin and Eriksson 2003; Mertens *et al.* 2006, Dos Santos Utmazian *et al.* 2007; Marmiroli *et al.* 2011). This characteristic, combined with a high biomass yield potential, makes *Salix* spp. potentially useful in phytoremediation of Zn- or Cd-contaminated soils and sediments (Pulford *et al.* 2002; Hammer *et al.* 2003; Meers *et al.* 2007). Plants can produce glutathione and phytochelatins in response to heavy metal exposure, a response that may serve to detoxify chalcophilic metals by chelation and contribute to Zn and Cd tolerance (Babula *et al.* 2008). Furthermore, *Salix* spp. tend to accumulate relatively high foliar concentrations of S compared to other species even in uncontaminated natural environments (McBride 2007; Reimann *et al.* 2003). Ohlson and Staaland reported (2001) willow (*Salix aurita*) to be compositionally unique among browse plants favored by moose in that it was highest in Zn and Cd, and also has the most consistently high (Mo/Cu) x (S) index, a measure of risk for induced Cu deficiency in ruminants. Thus, willows could pose a health risk to ruminants and possibly other wildlife in contaminated environments from Cd toxicity, Cu deficiency and sulfur toxicity (Nolet *et al.* 1994; Larison *et al.* 2000; McBride 2007).

The naturally high foliar S concentrations in some *Salix* spp. raises the possibility that thiol groups, possibly in the form of phytochelatins, contribute to Cd and Zn tolerance in willows. However, studies of S and phytochelatin levels in willows as affected by metal exposure do not generally support the hypothesis of a phytochelatin defense against toxic metals. For example, Landberg and Greger (2004) detected no phytochelatins in *Salix viminalis* regardless of whether this willow was or was not exposed to heavy metals. Furthermore, thiols measured in the tissues were neither increased nor decreased by exposure of *S. viminalis* to the metals. Zacchini *et al.*(2011) found that exposure of *Salix alba* to Cd in nutrient solution enhanced cysteine but reduced glutathione concentrations in roots, with no enhancement of these thiol-containing compounds in the leaves. Harada *et al.* (2010) found evidence using micro X-ray fluorescence that Cd in leaves of *Salix* spp. was coordinated to O rather than S ligands. In support of the thiol protection hypothesis, Konlechner *et al.*(2013) found that a metallicolous genotype of *Salix caprea* (collected from a metal-contaminated site) expressed a higher activity of the cysteine biosynthesis gene than the nonmetallicolous genotype. Overall, the evidence supporting the hypothesis of a constitutive phytochelatin defense against heavy metal toxicity in *Salix* spp. is weak.

Other mechanisms of metal detoxification not involving complexation by thiol groups could be important in willows. Polyphenols, including tannic acids, form complexes with trace metals and convert them to less soluble and less toxic forms (McDonald *et al.* 1996; Haslam 1996), and also have antioxidative properties that may protect against toxic oxidative stress effects caused by some metals (Morina *et al.* 2008; Babula *et al.* 2008). Therefore, the very high tissue levels of polyphenols found in *Salix* spp. (Nyman and Julkunen-Tiitto 2000) may have some role in metal sequestration and detoxification, as some studies have shown that

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polyphenol (condensed tannins) levels in willows increase in response to Cd stress (Vollenweider *et al.* 2006).

The present study investigated the response to Zn and Cd exposure in a number of willow cultivars. The hypotheses to be tested were

1. Uptake of and tolerance to Zn and Cd is strongly dependent on species and cultivar in willow.
2. Uptake of S is regulated physiologically and therefore dependent on species and cultivar.
3. Exposure to high Zn or Cd leads to higher S in leaf tissues due to induced metallothionein production.
4. Exposure to high Zn or Cd alters the foliar concentrations of other trace elements, specifically Cu, Fe and Mn.
5. Exposure to high Zn or Cd leads to increased formation of secondary metabolites such as polyphenols, in leaf tissues as a mechanism of metal detoxification and sequestration.

Materials and methods

Experiment 1 : Screening for Zn+Cd tolerance

Cuttings of 14 willow cultivars (12 of which were obtained from Landsaga Biogeographical Inc., Ontario, Canada) were first rooted in pure water, and then planted into 2 sets of 14 pots (3 replicate plants/pot) filled with perlite as a support medium. The two sets of pots were placed into separate hydroponic trays supplied with continuously flowing nutrient solution (0.25-strength Hoagland's, including all micronutrients with Zn at 0.40 μM) circulated by continuous pumping from 10-liter reservoirs. The cuttings were allowed to develop roots and grow for 17 days in the absence of heavy metals. After this period, Zn (200 μM) and Cd (10 μM) sulfate salts were added to the nutrient solution for one tray of willows, while the other tray continued to receive nutrient solution without the added Zn or Cd. This hydroponic screening method for willow, including the choice of Zn and Cd exposure concentrations, was adapted from Watson *et al.* (2003). The metal-exposed and unexposed (control) willows were then grown for a further 18 days, after which all new leaf and stem growth was harvested and weighed after drying. Leaf tissue was further separated from the stems, dried, ground and microwave-digested using concentrated HNO_3. The leaf digests were analyzed for Zn, Cd, Fe, Cu, Mn and S by ICP-OES (inductively coupled plasma-optical emission spectrometry) spectrometry.

A second crop from the same rooted cuttings was grown after the first willow coppicing. This second crop was grown in the same nutrient solutions with and without Cd and Zn as described for the first crop, but was harvested after a longer period (40 days of growth), over which time more severe symptoms of phytotoxicity became evident. The leaf and stem tissues were again harvested, dried and weighed, although growth was so poor that replicate samples needed to be pooled to obtain enough plant material for analysis. The foliar tissue was analyzed for total Zn and Cd by flame atomic absorption after digestion in concentrated HNO_3.

Experiment 2 : Screening for tolerance to Zn and Cd

Based on the relative vigor, metal tolerance and Zn/Cd accumulation shown by the cultivars tested in Experiment 1, two cultivars with high metal-uptake potential ("Pseudo" and "301") and one with low potential ("Hotel") were selected for exposure to Zn and Cd separately. Rooted cuttings of these cultivars were first established in 0.25-strength Hoagland's solution for about 2 weeks to develop roots and tops, and then grown hydroponically as described in Experiment 1. In this experiment, however, the replicated ($n = 3$) treatments consisted of three growth media, 0.25-strength Hoagland's, 0.25-strength Hoagland's plus 200 μM Zn, and 0.25-strength Hoagland's plus 10 μM Cd. The growing period during which the willows were exposed to these solutions was 20 days, after which leaves and stems were harvested, dried and weighed. Separated leaf tissues were acid-digested and analyzed for Zn, Cd, Fe, Cu, Mn and S as described for Experiment 1 using microwave digestion and ICP-OES.

The oxidation state profiles of sulfur present in several foliar tissue samples from this hydroponic experiment were measured using sulfur-XANES (X-ray absorption near edge structure) spectroscopy and compared to those of shrub willows collected from the field in Mt. Horeb, WI (McBride 2007). The S K-edge (2472 eV) XANES spectra were collected at Beamline X-19A of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, under standard operating conditions. A Si (111) double-crystal monochromator was detuned by 70% to eliminate higher-order harmonics of the X-ray beam, and fluorescence spectra were collected using a solid-state passivated implanted planar silicon (PIPS) detector. Willow leaf samples were air-dried and ground before pressing them into a 0.5-mm-thick acrylic holder with a 2.5-μm-thick Mylar film (Chemplex Industries, NY) window and mounted in a helium-purged sample chamber. Although air-drying of plant tissue prior to analysis might be expected to alter the S oxidation state, a comparison of spectra from air-dried and fresh willow leaf tissues showed no difference in XANES spectra. The elemental S K-edge spectrum (assigned a value of 2472 eV) was used for energy calibration. Scans ranged from 20 eV to 50 eV for the S absorption edge with 0.2 eV step size. S-XANES data analyses are described by Martínez *et al.* (2007). Briefly, the experimental S-XANES spectrum of each sample was fitted with six Gaussian peaks and two arctangent steps using a non-linear least-squares fitting routine to estimate the fraction of the total sulfur present in each major oxidation state. We report electronic oxidation states, rather than formal oxidation states, that reflect the actual electronic density in the valence shell of sulfur. The accuracy limit of the estimates obtained by fittings of S-XANES spectra has been reported to be 5–10% (Gorbaty *et al.* 1990; George *et al.* 1991; Szulczewski *et al.* 2001).

Experiment 3: Secondary metabolite analysis

The same three *Salix* cultivars selected for Experiment 2 were used in this hydroponic experiment, but cuttings were divided into three groups, exposed to 0.25-strength Hoagland's nutrient solution only (control), 0.25-strength Hoagland's plus 100 μM Zn and 5 μM Cd (half-metal treatment), and 0.25-strength Hoagland's plus 200 μM Zn and 10 μM Cd (full-metal treatment). Metal exposure in this case began 7 days after the experiment began, and continued for 14 days before the foliar tissue was harvested, dried and analyzed for polyphenols, condensed tannins and leucoanthocyanin according to the methods described in Price and Butler (1977) and Julkunen-Tiitto (1985). Specifically, polyphenols were extracted by mixing 1.0 g of dried ground leaf tissue with 20 ml of pure methanol in a flask, covering and agitating for 1 hour at room temperature. After filtration through Whatman 42 paper, gross polyphenol levels were determined by the Prussian Blue assay. This involved pipetting 1 ml of the tissue extract into a test tube and adding 2 ml of 0.008 M $FeCl_3$ in 0.008 N HCl along with 10 ml of 0.0015 M $K_3Fe(CN)_6$. After mixing, absorbance at 720 nm was measured on a UV-visible spectrophotometer 30 seconds after adding the last reagent. Each plant sample was analyzed in triplicate, and the average of the measured polyphenol concentrations was taken. Blank determinations were made by preparing a NaCl solution sample in the same manner and subtracting the blank absorbance from the plant sample readings. The standard curve was prepared by adding the reagents to 1.0, 0.1, 0.05 and 0.01 mM solutions of reagent-grade tannic acid. Final polyphenol results for the willows are reported in tannic acid equivalents (mmoles) per ml of leaf extract.

Statistical analysis of data

The replicated measurements of willow biomass yields and foliar tissue Zn, Cd, Fe, Cu, Mn and S concentrations were subjected to statistical testing using Student's t-test for paired data and one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's all-pairs comparison. These tests allowed cultivar and metal exposure effects on yields and foliar elemental compositions to be assessed for statistical significance at the $p = 0.05$ level.

Results and discussion

Experiment 1 : Zn and Cd uptake and tolerance

In the initial hydroponic experiment, in which cultivars were exposed to Zn+Cd for an 18-day period before harvest, some cultivars (notably "Pseudo" and "Charlie") attained foliar concentrations approaching 1000 mg kg^{-1} Zn and 70 mg kg^{-1} Cd in the leaves with no significant growth reduction (see Table 1), but some visual evidence of mild

Table 1. Zn, Cd and S concentrations (mg kg^{-1}) in *Salix* cultivars grown hydroponically with and without excess Zn and Cd in the nutrient solution. Standard deviations are in parentheses.

		Foliar concentrations (mg kg^{-1})			
Cultivar	Treatment	Zn	Cd	Cu	S
"Alpha" *S. viminalis*	Control	79.2 (50.1)	0.90 (0.27)	8.56 (1.41)	4613 (73)
	Zn+Cd	826 (97.7)	51.1 (10.1)	6.99 (0.50)	6695 (438)
"Amy" *S. amygdaloides*	Control	21	0.72	4.56	4423
	Zn+Cd	467 (167)	16.4 (3.1)	7.82 (3.42)	4463 (144)
"Beta" *Salix* unknown	Control	28.6 (1.2)	0.47 (0.03)	6.30 (0.24)	4755 (252)
	Zn+Cd	742 (94.4)	46.2 (7.5)	5.17 (0.41)	7013 (138)
"Cascadilla" *S. exigua (?)*	Control	45.4 (11.1)	0.47 (0.20)	4.60 (1.52)	7764 (966)
	Zn+Cd	632 (56.5)	46.9 (6.9)	4.01 (0.51)	9920 (489)
Charlie *S. alba x glatfelteri*	Control	44.5 (5.0)	0.55 (0.11)	7.17 (1.04)	7918 (398)
	Zn+Cd	971 (51.4)	68.8 (3.3)	6.64 (0.92)	9787 (310)
"Disc" *S. discolor*	Control	72.8 (28.9)	1.30 (0.65)	10.2 (1. 9)	4552 (647)
	Zn+Cd	403 (59.1)	29.7 (6.2)	6.77 (1.27)	5589 (965)
"Elba" *S. purpurea*	Control	59.1 (15.1)	0.56 (0.30)	7.96 (0.62)	3796 (262)
	Zn+Cd	812 (108)	67.2 (14.5)	7.49 (1.90)	4147 (365)
"Exigua" *S. exigua*	Control	43.8 (13.0)	0.61 (0.03)	5.33 (0.90)	7091 (366)
	Zn+Cd	578 (98.6)	41.9 (10.0)	5.34 (0.91)	10217 (970)
"Hotel" *S. purpurea*	Control	74.1 (6.0)	0.74 (0.14)	8.61 (1.32)	3601 (309)
	Zn+Cd	643 (71.3)	38.0 (11.7)	6.33 (0.71)	4090 (541)
"India" *S. dasyclados*	Control	30.4 (7.5)	0.62 (0.22)	5.27 (0.40)	4928 (229)
	Zn+Cd	689 (206)	60.2 (25.3)	4.20 (0.43)	7737 (776)
"Juliet" *S. eriocephala*	Control	51.7 (14.9)	0.59 (0.10)	4.22 (0.14)	7019 (817)
	Zn+Cd	369 (42.0)	25.5 (5.1)	3.45 (0.17)	7588 (370)
"Nigra" *S. nigra*	Control	54.0 (17.8)	0.70 (0.14)	6.73 (1.44)	4116 (293)
	Zn+Cd	448 (58.0)	20.4 (6.4)	7.16 (0.57)	4426 (385)
"Pseudo" *S. alba*	Control	55.7 (8.7)	0.92 (0.02)	6.59 (0.80)	7692 (200)
	Zn+Cd	950 (60.3)	66.1 (7.5)	6.61 (0.11)	10131 (298)
"301" *S. eriocephala* x exigua	Control	26.2 (3.3)	0.79 (0.12)	5.29 (0.55)	5819 (306)
	Zn+Cd	713 (23.3)	64.1 (3.0)	4.83 (0.61)	9085 (653)

Table 2. Biomass yields for *Salix* cultivars grown hydroponically with and without Zn + Cd added to the nutrient solution. Standard deviations are in parentheses.

Cultivar	Treatment	Leaf and stem biomass yields (g)	
		Crop1	Crop2
"Alpha" *S. viminalis*	Control	9.7 (5.8)	5.2
	Zn+Cd	14.0 (1.0)	1.0
"Amy" *S. amygdaloides*	Control	5	3.0
	Zn+Cd	0.55 (0.64)	0.7
"Beta" *Salix* (unknown)	Control	13.0 (1.0)	9.3
	Zn+Cd	15.7 (6.4)	0.6
"Cascadilla" *S. exigua*	Control	2.0 (1.7)	3.3
	Zn+Cd	2.7 (0.58)	2.2
Charlie *S. alba x glatfelteri*	Control	6.3 (1.5)	15.6
	Zn+Cd	7.3 (2.5)	6.2
"Disc" *S. discolor*	Control	5.4 (8.4)	6.4
	Zn+Cd	4.7 (3.8)	1.7
"Elba" *S. purpurea*	Control	9.3 (2.5)	10.0
	Zn+Cd	9.7 (3.1)	4.7
"Exigua" *S. exigua*	Control	1.0 (0.0)	4.8
	Zn+Cd	0.55 (0.64)	0.9
"Hotel" *S. purpurea*	Control	2.3 (1.2)	4.2
	Zn+Cd	3.3 (1.5)	2.9
"India" *S. dasyclados*	Control	10.3 (1.5)	n.s.*
	Zn+Cd	8.0 (3.6)	1.1
"Juliet" *S. eriocephala*	Control	4.7 (0.58)	10.0
	Zn+Cd	6.0 (1.7)	2.6
"Nigra" *S. nigra*	Control	2.3 (0.58)	5.5
	Zn+Cd	3.7 (2.5)	2.0
"Pseudo" *S. alba*	Control	3.3 (0.58)	17.8
	Zn+Cd	4.0 (1.0)	10.1
"301" *S. eriocephala x exigua*	Control	12.0 (4.4)	14.0
	Zn+Cd	10.7 (3.5)	11.3

*no sample available due to plant failure.

phytotoxicity (leaf chlorosis) in some cultivars. Others, such as *Salix nigra* ("Nigra"), despite reports that cultivars of this species are Cd-tolerant and accumulate high concentrations of Cd (Borišev *et al.* 2009; Kuzovkina *et al.* 2004), had only about 20 mg kg^{-1} Cd and 300–500 mg kg^{-1} Zn. The biomass yields for this trial are reported in Table 2 under the "Crop 1" heading, and generally, there was no relationship between biomass and foliar Cd or Zn concentrations in the different cultivars. Statistical analysis of the yield data for Crop 1 confirmed that cultivar was a highly significant factor in explaining biomass yield ($p < 0.001$) for both the control and the metal-exposed willows. However, overall yield of the cultivars was not significantly impacted by exposure to Zn+Cd in this first short-term trial ($p = 0.406$). In addition, t-tests showed that none of the individual cultivars exposed to Zn+Cd showed a statistically significant reduction in yield compared to the unexposed controls. Cultivar was a barely significant factor ($p = 0.05$) explaining foliar concentrations of Zn in the control treatments, and was not significant for foliar Cd in the controls. Conversely, for the metal-exposed willows, cultivar was a highly significant ($p < 0.0001$) factor in explaining differences in both foliar Zn and Cd concentrations. Cd and Zn concentrations in the leaf tissues of different cultivars were strongly correlated as shown in Figure 1. The link between Zn and Cd uptake among cultivars is possibly explained by a common transporter of these two metals, as Zn and Cd uptake are correlated in a number of species (Cosio *et al.* 2004).

The small effect on biomass yield of the Zn and Cd concentrations used in this hydroponic experiment (200 μM Zn and 10 μM Cd) are consistent with other studies indicating these levels to be relatively nontoxic for 2–4-week willow bioassays (Watson *et al.* 2003). Cd at 5 μM in nutrient solutions has shown little or no toxicity to a number of *Salix* species, but toxicity at 25 μM Cd can be moderate to severe (Kuzovkina *et al.* 2004; Harada *et al.* 2010; Vollenweider *et al.* 2006). Generally, however, Cd and Zn concentrations in hydroponically grown willows increase over time of exposure of at least up to 6 weeks (Watson *et al.* 2003), so that short-term assays may not assess the full extent of toxicity likely to develop over time.

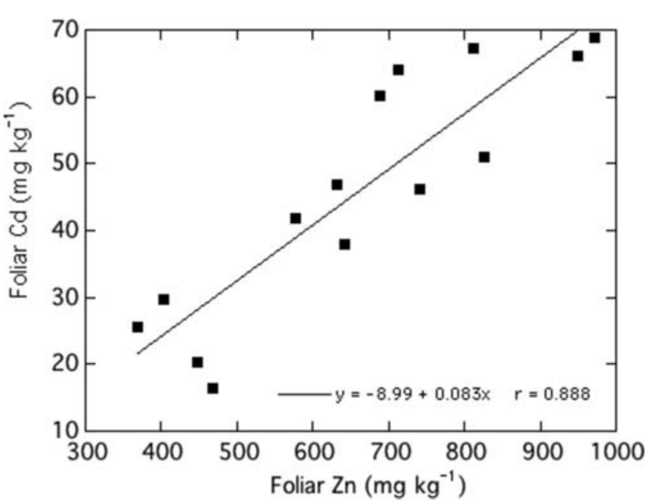

Figure 1. Relationship between foliar Cd and Zn concentrations among 14 Salix cultivars exposed to Cd plus Zn in hydroponic culture.

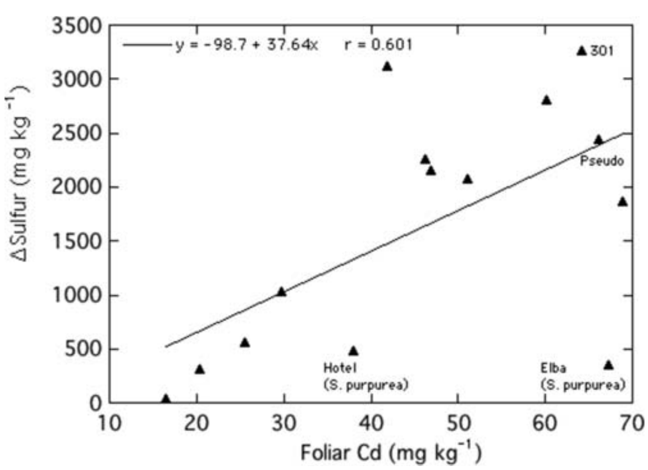

Figure 2. Relationship between ΔS and foliar Cd concentration among 14 Salix cultivars exposed to Cd plus Zn in hydroponic culture.

Table 1 reveals a wide range (4000–10,000 mg/kg) of foliar S concentrations for different *Salix* cultivars grown in the same high Cd/Zn solutions, with *Salix exigua* having one of the highest foliar S concentrations. Statistical analysis showed that cultivar was a highly significant ($p < 0.0001$) factor in explaining the wide range of foliar S concentrations in both the control and the metal-exposed willows.

The foliar S concentrations averaged 5630 ± 1660 and 7320 ± 2330 mg kg^{-1} for all of the control and metal-exposed willow cultivars, respectively. This relatively large (30%) overall increase in foliar S in the metal-exposed relative to the control willows may be explained largely by the fact that the metal-exposed willows were grown in hydroponic solutions with 40% greater sulfate concentrations than the control willows due to the added Zn sulfate. It is therefore not possible to make any firm conclusions about the possible effect of Zn exposure on S uptake by the willows. Nevertheless, Figure 2 shows that the difference in foliar S concentration (ΔS) between the metal-exposed (Cd+Zn) and unexposed (control) cultivars was significantly correlated to the foliar Cd concentrations in the exposed cultivars ($p < 0.05$), whereas the correlation of ΔS to foliar Zn was not significant (correlation not shown). The correlation of ΔS to foliar Cd could indicate that increased uptake of sulfate was provoked by Cd exposure. This effect has been observed in other studies, where it has been suggested to be a defense response involving increased production of S-containing amino acids and phytochelatins (Zacchini *et al.* 2011). It is perhaps suggestive that several of the more metal-tolerant cultivars tested in this experiment, most notably "Pseudo" and "301," had large increases in foliar S in response to Zn+Cd exposure, whereas more metal-sensitive cultivars, such as *S. purpurea*, had much smaller ΔS values (see Figure 2).

The foliar concentrations of essential trace metals, Cu, Mn and Fe measured in the *Salix* cultivars, were affected by exposure of the willows to Zn+Cd in the hydroponic solutions., Only the foliar Cu concentrations are presented in full (see Table 1). The foliar Cu concentrations averaged over all cultivars were 6.71 ± 1.91 and 5.82 ± 1.91 mg kg^{-1} for the controls and metal-exposed treatments, respectively. The Mn concentrations were 38.3 ± 9.64 and 42.4 ± 9.05 mg kg^{-1} for the controls and metal-exposed treatments, respectively. Finally, the Fe concentrations were 92.9 ± 12.4 and 64.6 ± 11.4 mg kg^{-1} for the controls and metal-exposed treatments, respectively. Statistical analysis of these data revealed that the metal-exposed willows had significantly lower Cu ($p = 0.001$) and Fe ($p < 0.0001$), but slightly higher Mn ($p = 0.0027$). The relative effect of Zn and Cd exposure on foliar trace metal concentration was greatest for Fe, and smaller for Cu and Mn. ANOVA revealed cultivar to be a highly significant factor ($p < 0.0003$) in explaining differences in foliar Cu, Mn and Fe in both the control and the metal-exposed willows.

The effects of Cd exposure in reducing Mn, Zn and Fe uptake into *Salix* have been reported by Yang *et al.* (2009), although their study showed little effect of Cd on Cu uptake. Borišev *et al.* (2016) provided evidence that Cd uptake utilizes the same transport pathways as Fe and Mg, possibly competing with these essential metals for uptake.

After the first harvest of all new top growth, the second crop from the same cuttings showed much more severe chlorosis and growth reduction attributable to much higher measured Zn (1000–3000 mg kg^{-1}) and Cd (55–175 mg kg^{-1}) concentrations in leaves relative to the first crop (data not shown). The biomass yields for the second crop exposed to metals, presented in Table 2 under the "Crop 2" column, were reduced on average to 40% of the control yields, a statistically highly significant effect ($p < 0.0001$). Severe toxicity at foliar concentrations of 1000–3000 mg kg^{-1} Zn and 100–200 mg kg^{-1} has been noted for a number of willow species in other studies (Dos Santos Utmazian *et al.* 2007).

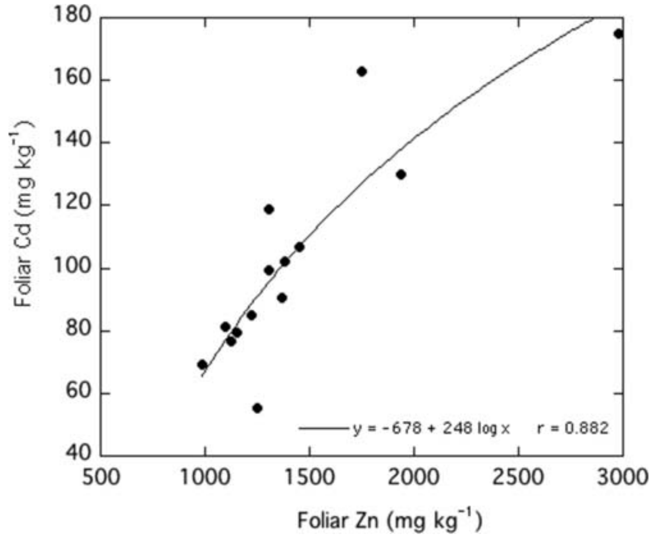

Figure 3. Relationship between foliar Cd and Zn concentrations for the second crop of *Salix* cultivars exposed to Cd plus Zn in hydroponic culture.

The strong correlation between Cd and Zn concentrations in the different cultivars seen in the first crop was again found in the second, as shown in Figure 3 ($r = 0.882$). In addition, there was a weak but statistically significant tendency for cultivars having higher foliar Cd and Zn in the first crop to have higher Cd and Zn in the following crop (data not shown). This pattern among cultivars as shown by linear regression analysis was more consistent for Cd ($r = 0.729$) than for Zn ($r = 0.631$).

The more severe phytotoxicity accompanying greater uptake of Zn and Cd in the second crop of the cultivars may be partly due to the longer (40 day) exposure of the willow cuttings to these metals. However, phytoxicity was visually evident as chlorosis in leaves from the beginning of leaf development in this second crop, indicating that some fraction of the metals accumulated and stored in root and stem tissues of the first crop may have been transferred into the foliar and stem tissues of the second crop. Higher concentrations of trace metals such as Cd, Zn, Ni and Cu accumulate in root than in stem or leaf tissues of many *Salix* species (Kuzovkina *et al.* 2004; Dos Santos Utmazian *et al.* 2007; Watson *et al.* 2003; Cosio *et al.* 2006). Delayed transport of these metals from roots to leaves could account for the higher Zn and Cd as well as more severe Zn toxicity in the second growth, as Zn is a relatively easily translocated metal (Watson *et al.* 2003).

Even higher foliar Zn (>3000 mg kg^{-1}) and Cd (300–600 mg kg^{-1}) concentrations than measured here have been reported in hydroponic assays with willows (Dos Santo Utmazian *et al.* 2007; Cosio *et al.* 2006; Borišev *et al.* 2009). However, those studies used very high soluble concentrations of Zn and Cd (*e.g.,* as high as 100 μM Cd) and resulted in severe

Table 3. Measured Zn, Cd and S foliar concentrations in *Salix* cultivars exposed separately to Cd and Zn added to nutrient growth medium in Experiment 2.

Cultivar	Treatment	Foliar Zn (mg kg^{-1})	Foliar Cd (mg kg^{-1})	Foliar S (mg kg^{-1})
"Hotel" *S. purpurea*	Control	128 (8.2)	6.38 (1.06)	3390 (163)
	+ Cd	63.8 (0.97)	35.2 (4.3)	3240 (42)
	+Zn	1020 (27)	5.52 (0.15)	3590 (45)
"301" *S. eriocephala x exigua*	Control	167 (4.2)	8.59 (1.18)	7180 (170)
	+ Cd	74.1 (6.0)	102 (3.3)	6130 (640)
	+ Zn	874 (65.4)	5.98 (0.32)	7490 (224)
"Pseudo" *S. alba*	Control	151 (2.6)	5.46 (0.33)	8330 (99)
	+ Cd	63.2 (1.3)	55.9 (4.7)	8240 (213)
	+Zn	679 (42.8)	4.00 (0.46)	8220 (110)

phytotoxicity. Such extreme phytotoxicity is not amenable to phytoremendation because of greatly reduced biomass yield. The delayed toxicity observed in Crop 2 could have practical implications for phytoremediation in the field, possibly limiting the ability of repeated coppicing to extract Zn and Cd from contaminated soils as cumulative toxic effects inhibit growth and metal removal in subsequent crops. Short-term hydroponic screening trials could therefore overestimate willow tolerance to high available Zn or Cd encountered in the field.

Experiment 2 : Exposure to Zn and Cd

Because this experiment exposed three cultivars to Zn and Cd separately, the toxic symptoms (foliar chlorosis) observed in Experiment 1 were confirmed to be attributable to Zn, with Cd producing no discernible toxicity. Nevertheless, Zn phytotoxicity was not severe in this 20-day trial, with statistical analysis showing that neither the Zn nor the Cd exposure significantly affected biomass yield ($p > 0.05$). Biomass yields (data not shown) were found not to be statistically different for the three selected cultivars ("301," "Pseudo" and "Hotel") whether exposed to Zn, Cd or no metals (control).

The foliar Cd, Zn and S concentrations for the three cultivars at harvest are given in Table 3. Cultivar was a highly significant factor in explaining differences in foliar S concentration for all treatment comparisons ($p < 0.0001$), and was in most treatment comparisons a significant factor for differences in foliar Cd and Zn concentrations as well ($p < 0.05$). At harvest, the two tolerant cultivars ("301" and "Pseudo") showed no visual toxicity symptoms from Zn, but the less tolerant "Hotel" exposed to the Zn treatment had the highest foliar Zn concentration of 1020 mg kg^{-1} and showed moderate chlorosis in young leaves. Cd exposure significantly suppressed Zn uptake in all three cultivars ($p < 0.0001$), reducing foliar Zn by a factor of approximately two relative to controls (see Table 3). Conversely, Zn exposure significantly reduced Cd in the leaves ($p = 0.017$). However, the three Cd- or Zn-exposed cultivars did not show a statistically significant increase in foliar S compared to the controls. The apparent competition for uptake between Cd and Zn observed in Experiment 2 is known to occur for a number of crop plant species (Hart *et al.* 2002), but has not been shown previously in *Salix* species according to the knowledge of the authors.

The foliar concentrations of essential trace metals, Cu, Mn and Fe measured in the *Salix* cultivars (not shown) were affected differently by separate exposure of the willows to Zn and Cd in the hydroponic solutions. Foliar Cu concentrations were not

Table 4. Calculated foliar S oxidation states based on XANES spectral data obtained from willows collected in the field (MTH) and sampled from hydroponically grown plants in Experiment 2.

	Energy maximum (eV)	Electronic oxidation state	Fraction (% of total S)		Energy maximum (eV)	Electronic oxidation state	Percentageof total S
MTH 14	0.0	−0.1	0.0	Hotel	0.5	0.2	40.1
S. eriocephala (3500)*	0.7	0.3	28.4	*S. purpurea* (3388)	2.7	1.5	13.1
	3.0	1.7	9.3		4.6	2.6	12.3
	8.0	4.7	0.0		6.3	3.6	5.1
	9.4	5.5	25.1		9.2	5.4	24.4
	9.5	5.6	37.3		9.5	5.6	5.0
MTH 15	0.1	−0.1	0.0	Pseudo	0.5	0.2	21.5
S. exigua (5600)	0.6	0.2	19.4	*S. alba* (8326)	3.0	1.7	4.5
	3.1	1.8	7.4		4.9	2.8	6.1
	7.7	4.5	4.2		7.8	4.6	12.0
	9.3	5.5	39.2		9.4	5.5	41.1
	9.9	5.8	29.7		10.0	5.9	14.8
MTH 16	0.0	−0.1	0.0	Pseudo+Zn	0.5	0.2	31.1
S. eriocephala (3600)	0.6	0.3	28.9	*S. alba* (8242)	3.3	1.8	14.3
	3.0	1.7	9.8		4.9	2.8	4.0
	8.0	4.7	0.0		7.5	4.4	0.8
	9.4	5.5	25.1		9.4	5.5	42.1
	9.5	5.6	36.2		12.0	7.0	7.6
MTH 17	0.2	0.0	0.0	Pseudo+Cd	0.5	0.2	39.1
S. exigua (9150)	0.6	0.2	12.9	*S. alba* (8222)	3.0	1.6	11.5
	3.3	1.8	6.9		4.3	2.4	9.2
	8.0	4.7	0.0		7.6	4.4	4.8
	9.4	5.5	46.4		9.4	5.5	28.9
	9.6	5.6	33.9		11.2	6.6	6.6

*Numbers in parentheses are foliar total S concentrations (mg kg^{-1}), determined by a high-T combustion method with IR absorbance (MTH samples) or by microwave digestion and ICP emission.

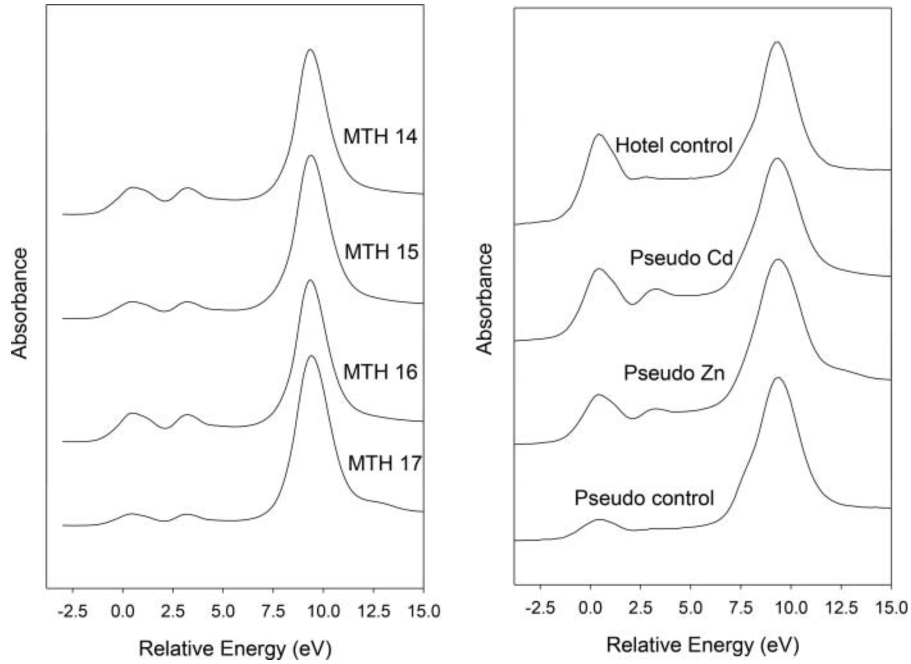

Figure 4. S-XANES spectra of leaf tissue of wild shrub willows (MTH) and hydroponically grown willow cultivars (Hotel and Pseudo).

significantly different in the 3 cultivars, and were not significantly affected by Zn ($p = 0.561$) or Cd ($p = 0.075$) exposure. Mn concentrations were significantly different among the 3 cultivars that were exposed to Cd or Zn ($p < 0.014$), but were not significantly different among the controls ($p = 0.057$). Foliar Mn concentrations were significantly higher in the Zn- ($p = 0.0005$) and Cd-exposed ($p = 0.031$) willows than the controls. Finally, foliar Fe concentrations were significantly different among the three cultivars that were exposed to Cd or Zn ($p < 0.015$), but were not significantly different among the three controls ($p = 0.187$). The foliar Fe concentrations averaged 93.8 ± 14.4, 76.3 ± 12.2 and 77.3 ± 16.8 mg kg^{-1} for the controls, Zn-exposed treatment and Cd-exposed treatment, respectively. Statistical analysis of these data revealed that effect of metal exposure in lowering foliar Fe concentrations were significant for both Zn ($p = 0.0114$) and Cd ($p = 0.0158$). As with Experiment 1, the relative effect of Zn

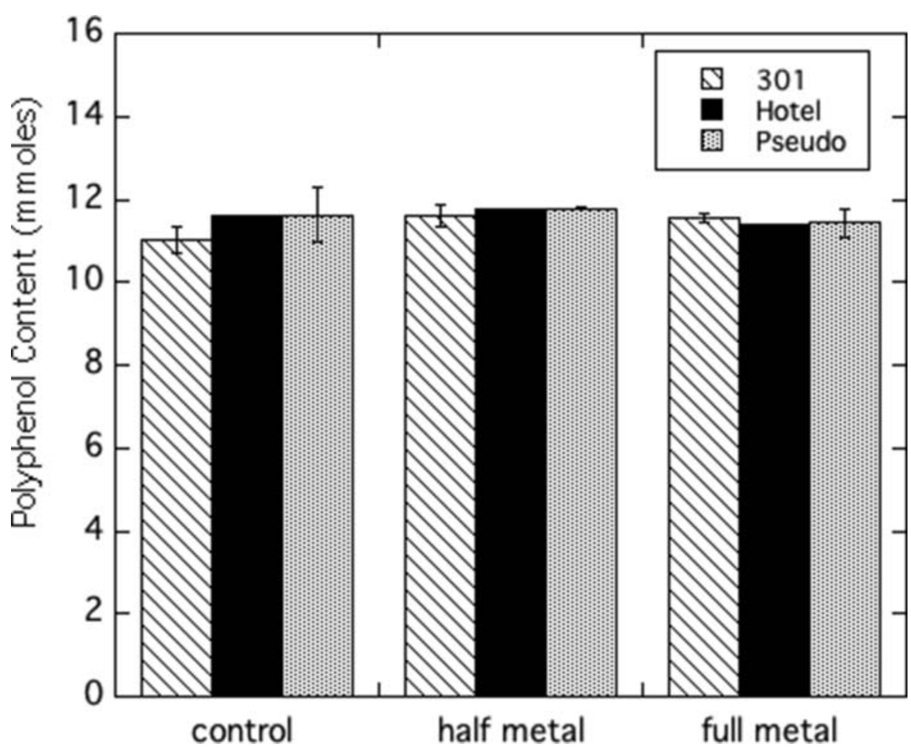

Figure 5. Measured concentrations of extractable polyphenols in *Salix* leaf tissues for three selected willow cultivars exposed to no (control), half and full concentrations of Zn+Cd in hydroponic culture.

and Cd exposure on foliar trace metal concentration was greatest for Fe, and smaller for Cu and Mn.

XANES spectroscopy was employed to determine the profile of S oxidation states in the leaf tissues of two of the *Salix* cultivars grown hydroponically, and for comparison, shrub willows grown under natural conditions. Table 4 summarizes the measured distribution of energy positions (relative energy) for S from the XANES spectra. The most reduced electronic oxidation states, represented by the electronic density of sulfur compounds such as sulfide (R-S-R) and thiol (R-S-H) occur at 0.0–0.7 eV; intermediate electronic oxidation states (representative of S compounds such as sulfoxide, R-S-O-R) occur at 2.7–3.3 eV; the most oxidized forms of S, represented by sulfonate (R-SO_3-H) and sulfate (R-OSO_3-H) occur at 6.3–8.0 and 9.2–10 eV, respectively.

The S-XANES spectra, shown in Figure 4, reveal the dominant peaks to be attributable to oxidized (sulfate + sulfonate) and strongly reduced (largely thiol) forms of S in the hydroponically grown willows. The "Pseudo" cultivar exposed to Cd or Zn had somewhat more pronounced peaks at the 2.7–3.3 (sulfoxide) and 0.0–0.7 (thiol) eV positions than the control "Pseudo." Shrub willows from the field had somewhat less prevalent peaks for the thiol species compared to those hydroponically grown, with the spectra dominated by a somewhat more narrow peak at the position expected for sulfate. The detailed analysis of these spectra, with calculated fractions of S in different oxidation states, is given in Table 4. This analysis revealed a trend for the Mt. Horeb willows that, as total S increased, a larger fraction of the foliar S was in the oxidized (sulfate + sulfonate) form. The Mt. Horeb willow with highest foliar S (9150 mg/kg) had 80.3 % of total sulfur in the sulfate+sulfonate form.

For the hydroponically grown willows, XANES spectroscopy showed "Hotel," a low-S willow, to have a higher fraction of foliar thiol S and lower sulfate/sulfonate S than "Pseudo," a high-S willow (Table 4). Exposure of "Pseudo" to Cd or Zn appeared to raise the fraction of S in the thiol forms relative to the control (Table 4). However, because the XANES spectra were not run on replicate samples, this observation is preliminary and needs confirmation. It would, however, be consistent with greater phytochelatin synthesis in response to metal exposure.

Experiment 3 : Secondary metabolites in foliar tissues

Exposure of the 3 cultivars selected for Experiment 2 for the same "full-metal" treatment with Cd+Zn used in Experiment 1 again revealed signs of metal stress (chlorosis) in all 3 cultivars, with no toxicity symptoms observed in the "half-metal" treatment. The assay for total phenolics conducted on leaf tissues from this hydroponic trial showed little difference among cultivars or metal treatments, as shown in Figure 5. Similarly, condensed tannins and leucoanthocyanins were not affected significantly by exposure to Zn+Cd at the full or half level, although both substrates were significantly higher in cultivar "301" than in "Hotel" or "Pseudo" (data not shown). Therefore, although these secondary metabolites are known to complex toxic metals and potentially reduce their phytotoxicities, exposure to high Cd and Zn did not induce a change in foliar concentrations of these biomolecules.

Conclusions

Based on hydroponics assays, several promising willow cultivars for phytoremediation of soils have been identified. These have the combined desirable properties of high uptake potential for Cd and Zn, considerable tolerance to metal toxicity, and rapid growth. A second exposure of the coppiced willow plants to the same Cd and Zn concentrations in nutrient solution resulted in higher foliar Cd and Zn concentrations and severe phytotoxicity, suggesting a carryover effect of metal ions stored in roots and stems on the following crop. The results indicate that most willows tested here have potential for phytoremediation of Zn- and Cd-contaminated soils as long as foliar Zn concentrations are limited to about 1000 mg kg^{-1} or lower. Higher foliar Zn leads to reduced biomass yield in most cultivars, and less potential for extraction of Cd and Zn from water or soil by phytoremediation. Foliar Cd concentration in the 14 tested cultivars was highly correlated to Zn concentrations, but only weakly correlated to an increase in foliar total sulfur.

Exposure of willow cultivars to Cd and Zn had no consistent effect on foliar concentrations of polyphenols, condensed tannins or leucoanthocyanin. There were, however, concentration differences among species or cultivars in these secondary metabolic products. These results suggest that toxic metal exposure does not typically provoke a response in willows of increasing concentrations of biochemicals capable of complexing and detoxifying these metals. However, exposure to Cd and Zn did significantly decrease the foliar concentrations of Fe and Cu. The very high foliar S concentrations in some *Salix* cultivars (close to 10,000 mg kg^{-1}) combined with relatively low Cu (typically <7.0 mg kg^{-1} in the hydroponic assay) may present a risk of Cu deficiency or even sulfur toxicity (polioencephalomalacia) to wild ruminants in environments where willow is a preferred browse plant.

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