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Effects of Sewage Sludge Application on Biomass Production and Concentrations of Cd, Pb and Zn in Shoots of *Salix* and *Populus* Clones: Improvement of Phytoremediation Efficiency in Contaminated Soils

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Abstract Fast-growing clones of *Salix* and *Populus* species have been studied for phytoremediation of soils contaminated by risk elements (REs) using short-rotation coppice plantations. Biomass yield, accumulation and removal of RE (Cd, Pb and Zn) by highly productive willow (S1—(*Salix schwerinii* × *Salix viminalis*) × *S. viminalis*, S2—*Salix* × *smithiana*) and poplar (P1—*Populus maximowiczii* × *Populus nigra*, P2—*P. nigra*) clones were investigated with and without sewage sludge (SS) application. The precise field experiment was established in April 2008 on moderately Cd-, Pb- and Zn-contaminated soil. Initially, shoots were harvested after four seasons in February 2012 and then after two more seasons in February 2014. The application of SS limited plant growth during the first years of the experiment in the majority of treatments, mainly due to weed competition and higher concentrations of available soil nutrients causing lower yields than those of control (C) treatments. Well-developed roots were able to take advantage of SS applications, and shoot yield was mainly higher in SS treatments in the second harvest, reaching up to 15 t dry matter (DM) ha^{-1}. Willows performed better than poplars. Application of SS reduced RE shoot concentrations compared to the C treatment. The removal of RE was significantly higher in the second harvest for all clones and elements (except the P2 clone), and the biomass yield was the major driving force for the amount of RE removed by shoots. Well-developed plantations of fast-growing trees showed better suitability for the phytoextraction of moderately contaminated soils for Cd and partly for Zn but not for Pb, which was less available to plants. From the four tested clones, S2 showed the best removal of Cd (up to 0.94 %) and Zn (up to 0.34 %) of the total soil element content, respectively, and this clone is a good candidate for phytoextraction. SS can be a suitable source of nutrients for *Salix* clones without any threat to the food chain in terms of biomass contamination, but its application to the soil can result in an increased incidence of some weeds during the first years of plantation.

Keywords Cadmium · Lead · *Populus* spp. · *Salix* spp. · Sewage sludge · Zinc

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Introduction

Sustainable use of environmentally friendly remediation methods of contaminated sites is among the major environmental issues [1]. Therefore, the interest in use of suitable methods to decontaminate soils is still increasing [2]. Phytoextraction, removing RE from the soil by their accumulation in plant tissues, is a very challenging technique [3]. Many plant species have been tested for their ability to accumulate elements in their aboveground biomass. According to Pulford and Dickinson [4] and also from our previous experience [5–7], the plants suitable for phytoextraction of metals are fast-growing trees; mainly species of willows and poplars perform well in medium and moderately contaminated soils [8]. Their advantages include their ability to accumulate and translocate metals to the aerial parts, fast growth and extended root systems [9]. Willows and poplars have the added advantage of producing biomass that can be used for energy production [4].

Phytoextraction potential of *Salix* and *Populus* clones has been tested, for example, using hydroponic screening [10]. Also, the results of pot experiments with Cambisol moderately contaminated with RE (5.46 mg Cd kg^{-1}, 956 mg Pb kg^{-1}, 279 mg Zn kg^{-1}) revealed that *Salix* clones are able to accumulate Cd and Zn well (*Salix dasyclados*, 41 mg Cd kg^{-1}, 591 mg Zn kg^{-1}), whereas *Populus* clones prefer to accumulate more Pb (*Populus trichocarpa*, 17.3 mg Pb kg^{-1}) than do *Salix* clones. Remediation factors (RFs) of these clones were comparable to hyperaccumulators [7]. Evaluation of phytoextraction effectiveness is usually based on the results of pot and laboratory experiments, but due to long plant stands at a specific site and unlimited root growth, it is vital to test plants in field conditions within a longer period of time on a global scale. Maxted et al. [11] performed field experiments with *Salix* clones. The best *Salix* clones removed within 4 years 15–20 % of the available Cd content in soil. Laureysens et al. [12] carried out field experiments with *Populus* clones on slightly contaminated soil. The best remediation efficiency was shown with clones with high numbers of shoots and with high ability of Cd and Zn accumulation. Vysloužilová et al. [5] confirmed in pot experiments the phytoextraction potential of clones in moderately contaminated soil (RF for Cd = 20 % and for Zn = 4 %), but on heavily contaminated soil, the RF for Zn was less than 1 %. In this context, the methods of RE immobilisation in the soil are tested to decrease RE concentration in the soil solution and, subsequently, to decrease the phytotoxic effect of the extreme soil RE contents. The correct choice of the suitable RE immobilisation agent is a crucial point of the successful remediation of such a soil.

Disposal of SS poses a serious threat to our environment. It is assumed that the amount of SS produced in Europe will increase in the near future, mainly due to increasing demands for quality of clean sewage water [13]. At present, there is growing pressure to minimise or forbid landfilling of SS; therefore, there are mainly two ways of its disposal: application on agricultural land and incineration. In economic terms, the application of SS onto the soil is considered to be an advantageous method due to the significant portion of nutrient recycling. However, this solution involves severe risks with respect to the occurrence of organic and inorganic contaminants, mainly some pathogens and RE present in the sludge. Both organic and inorganic contaminants could be accumulated in the soil, taken up by plants and thereby transferred to humans via the food chain [14]. On the other hand, the application of SS has a positive effect on soil fertility and its physical characteristics, such as increasing porosity of the soil and stability of soil aggregates [15], and the sludge is a good source of N, P and other nutrients. Application of organic fertilisers can decrease mobility and availability of these elements to plants, especially for Cu [16, 17] less for Pb and Cd [18, 19]; conversely, Zn mobility can be increased [18, 19]. Thus, SS can be a significant source of organic matter for potential RE immobilisation. However, the application of SS can result in the addition of the toxic organic and inorganic compounds into soil together with the fertilisation effect [20]. Chaney et al. [21] proved that organic fertilisation and other agronomic activities (e.g., liming) can limit RE uptake by plants. Behaviour of RE in the sludge-treated soil and their plant uptake are difficult to generalise because they are strongly dependent on the nature of the metal, sludge, soil properties and crop planted [22].

At our experimental location, the amounts of most RE in contaminated soils are much higher (with the exception of Zn) than the obvious contents of the RE in SS. Therefore, the application of local SS should not increase the soil contamination but rather should dilute the soil RE content and enrich it with nutrients. Investigation of SS application has not been studied under the mentioned conditions so far, but SS amendment can help plants develop higher biomass amounts and close a loop of on-site safe recycling and utilisation of SS. The application of SS for biomass production can also reduce total cost of remediation and SS utilisation.

In our field study, the biomass production and phytoremediation potential of four clones of fast-growing trees were investigated as follows:

(i) Clone (*Salix schwerinii* × *Salix viminalis*) × *S. viminalis*, called Tordis SW 960299, belongs among the registered varieties of 'Swedish willows', which were mostly hybrids of *S. viminalis* [23]. Clone Tordis achieved high biomass yields [24, 25] and responded positively to the application of different fertilisers containing N [25]. Clone Tordis was tested for phytoextraction of As, Cd, Pb and Zn in field conditions at project KBBE-266124 [26] and in the study of Zárubová et al. [27] (only for Cd, Pb and Zn), in which this clone achieved high concentrations of Cd (148–171 mg kg^{-1} in bark, 50–73 mg kg^{-1} in wood) and especially Zn (1104–1388 mg kg^{-1} in bark, 338–723 mg kg^{-1} in wood) but very low biomass yield (0.26 t DM ha^{-1} $year^{-1}$).

(ii) Clone *Salix* × *smithiana*, S-218 is a spontaneous hybrid of *S. viminalis* and *Salix caprea* [28] and is among the best-performing clones in the Czech Republic. This clone reached biomass yields of more than 14 t ha^{-1} $year^{-1}$ [29]. Simultaneously, the S-218 clone demonstrated a high ability to accumulate Cd (76.8 mg kg^{-1} in leaves, 41.9 mg kg^{-1} in twigs) and Zn

(2105 mg kg^{-1} in leaves, 592 mg kg^{-1} in twigs) in pot experiments in soil with an addition of 100 mg Cd kg^{-1}, 2000 mg Pb kg^{-1} and 2000 mg Zn kg^{-1} [6]. Also, in the pot experiment [28], clones of *S. smithiana* (S-218 and S-150) demonstrated higher phytoremediation potential compared to the remaining ones, where S-218 and S-150 clones were characterised by the highest biomass yield (up to 15 t DM ha^{-1} $year^{-1}$).

(iii) Clone *Populus nigra* × *Populus maximowiczii*, J105 is among the most productive clones in the Czech Republic, according to Weger [30] and Weger and Bubeník [31]. Hybrid *P. nigra* × *P. maximowiczii*, tested by [7], was able to accumulate Cd (17.3 mg kg^{-1}), Zn (344 mg kg^{-1}) and especially Pb (16.7 mg kg^{-1}). For this clone, the Pb RF was 0.025 % higher than for hyperaccumulating plants. However, according to Komárek et al. [32], this hybrid is not suitable for remediation of moderately and highly contaminated soils (4.86 mg Cd kg^{-1}, 1360 mg Pb kg^{-1}, 266 mg Zn kg^{-1}), because the RF for Pb was only 0.02 %.

(iv) Clone *P. nigra*, Wolterson was tested in a field experiment on a former waste disposal site slightly polluted by RE (0.4–0.8 mg Cd kg^{-1}, 39–52 mg Pb kg^{-1}, 103–161 mg Zn kg^{-1} [33]) for biomass production and for accumulation of RE in studies by [12]. In this study, clone Wolterson was among clones with the best remediation potential (removed 47 g Cd ha^{-1} and 2400 g Zn ha^{-1} during 2 years in a second rotation) and with best biomass yield (9 t DM ha^{-1} $year^{-1}$).

The main objectives of the study were (i) to evaluate the potential immobilisation effects of SS application on plant growth and/or RE uptake and (ii) to compare the RE (Cd, Pb and Zn) phytoremediation efficiency of individual willow and poplar clones.

Materials and Methods

Study Site and Field Experiment

The field experiment was established in April 2008 on multi-RE (mostly Cd, Pb and Zn)-contaminated agricultural soil in Podlesí (49° 42′ 24″ N, 13° 58′ 32″ E), near the town of Příbram, 58 km south of Prague. The altitude of the study site is 500 m above sea level, with a mean annual precipitation of 700 mm and mean annual temperature of 6.5 °C. On this experimental area were 64 rows (experimental units) [34], each row contained one clone and one treatment. Each row was 7.5 × 1.3 m, and the intra-row distance among plants was 0.25 m. Experimental units were arranged in a split-plot randomisation. Treatments [control (C) and sewage sludge (SS)] were whole plots arranged in a completely randomised design with eight replicates. Each whole plot contained four sub-plots, corresponding to two *Salix* and two *Populus* clones [34].

Two promising *Salix* clones, allochthonous ((*S. schwerinii* × *S. viminalis*) × *S. viminalis*) hybrid Tordis and autochthonous *S.* × *smithiana* clone S-218 (hereafter denoted S1 and S2, respectively), were selected. Among *Populus* clones, we selected the most widely planted hybrid clone in the Czech Republic, *P. maximowiczii* × *P. nigra* J-105, also known as Max-4, as well as *P. nigra* clone Wolterson (hereafter denoted P1 and P2, respectively). These clones were grown in contaminated soil (especially Cd, Pb and Zn) in the C treatments and in treatments with the application of SS.

The soil type is a weakly acidic modal Cambisol, with a cation exchange capacity of 166 $mmol_{H+}$ kg^{-1}, C_{org} of 4.1 %, C/N ratio of 9, humus horizon thickness of 26 cm and soil bulk density of 1.35 t m^{-3}. The mean soil pH_{H2O} is 5.66 and pH_{KCl} 5.27. Pseudo-total (*Aqua regia*-soluble) concentrations of elements in the soil are as follows: 7.3 mg Cd kg^{-1}, 218 mg Zn kg^{-1} and 1368 mg Pb kg^{-1} [27]. Czech legislation limits for pseudo-total concentrations of elements in agricultural soils are 1.0, 140 and 200 mg kg^{-1} of Cd, Pb and Zn, respectively [35]. Plant-available Mehlich III [36] concentrations of P, K, Ca and Mg in the top horizon were 14, 84, 4441 and 324 mg kg^{-1}, respectively. Plant-available concentrations of Cd, Pb and Zn in the top horizon were 4.92, 705 and 37 mg kg^{-1}, respectively, determined in Mehlich III [36], as well.

Application of Sewage Sludge

Fresh SS was applied to all 32 experimental units of the SS treatment for the first time in April 2008 before planting of willow and poplar clones at rate 7.5 kg m^{-2}, and a second application was done in May 2012 at rate 3.5 kg m^{-2}. SS was ploughed into the soil immediately after application. The main characteristics of the both sludges are summarised in Table 1.

Harvesting of Plant Material

Shoots of cuttings were harvested in February 2012 after four vegetative seasons and again in February 2014 after the next two seasons. The shoots were cut 20 cm above the soil surface. Harvested shoots were dried at 60 °C and weighed.

Laboratory Analyses

Dry biomass samples were ground using a stainless steel Retsch friction mill (Retsch, Haan, Germany; particle size 0–1 mm). The total concentrations of elements in the biomass of shoots were determined using inductively coupled plasma with optical emission spectroscopy (ICP-OES; VARIAN VistaPro, Australia), where dry ashing procedures [37] were applied for sample decomposition.

Table 1 Dry matter content (DM), concentration (in DM) and amount of elements applied by sewage sludge in 2008 and 2012

Sewage sludge		DM (%)	N	P	K	Ca	Mg	Cd	Pb	Zn
2008	Content (mg kg^{-1})	12	40,800	7950	2602	17,484	4053	2.9	102	913
	Rate (kg ha^{-1})		367	71	23	157	36	0.03	0.92	8
2012	Content (mg kg^{-1})	18	51,200	10,531	8621	8348	2126	2.10	46	571
	Rate (kg ha^{-1})		323	66	54	53	13	0.01	0.29	4

Remediation Factor

Phytoextraction potential of the examined clones was expressed as an RF per cent, which indicates the proportion of elements removed by harvested biomass from the total contents of elements at the site. The RF was calculated as follows (1):

$$RF(\%) = \frac{C_{plant} DM_{plant}}{C^{soil} W^{soil}} 100 \quad (1)$$

where C_{plant} is the concentration of a metal in the plant dry biomass (g t^{-1}), DM_{plant} the dry matter plant biomass yield (t), C_{soil} the total concentration of the metal in soil (g t^{-1}) and W_{soil} the amount of soil in the top horizon (t ha^{-1}), modified according to Komárek et al. [32].

Data Analyses

All statistical analyses were performed using the Statistica 10.0 (www.statsoft.com) and CANOCO 5 [38] programs. All data were checked for homogeneity of variance and normality (Levene and Shapiro–Wilk tests). Collected data did not meet assumptions for the use of analysis of variance (ANOVA) and were thus evaluated by the non-parametric Kruskal–Wallis test. Principal component analysis (PCA), in the Canoco 5 program, was applied to all collected data together (concentrations of elements in plants as well as biomass yield). We used the standardisation of species data because data of a different character were analysed together. The results were visualised in the form of a bi-plot ordination diagram in the CanoDraw program 5. The PCA is a multivariate method, useful for data presentation, because of overview formation over the correlations among all the analysed data and because of showing general trends visible in one ordination diagram.

Results

Biomass Yield and Mortality

With the exception of the S1 clone, biomass yield was higher in the C than in all SS treatments in the first harvest in 2012 (Tables 2 and 3). In the C treatment, the mean yield decreased in the order of P1 > S2 > P2 ≫ S1 clones, whereas in the SS treatment, the yield decreased in the order of S2 > P1 > S1 > P2 clones calculated per plant. The order was changed to S2 > S1 > P1 > P2 if the mortality was included into the calculation, and results were expressed on a per hectare basis (Table 4).

With the exception of the P2 clone in the C treatment, biomass yield in 2014 was substantially higher than in 2012, in which higher biomass yield was recorded in the SS compared to the C treatment (with the exception of the P1 clone). In 2014, the biomass yield in the C treatment decreased in the order of P1 > S2 > S1 ≫ P2 clones and in the SS treatment S2 > S1 > P1 ≫ P2. No changes in the clone order were observed after recalculation of the yield per hectare (Table 4). Biomass yield adversely corresponded with the plant mortality. In the C treatment, mortality increased in the order of P1 (15 %) < S2 (23 %) < P2 (25 %) < S1 (48 %) clones and in the SS treatment increased in the order of S2 (40 %) < S1 (41 %) < P1 (50 %) < P2 (51 %) clones in the second harvest.

Concentrations of Elements

In 2012, the concentration of RE in shoots was higher in C treatments than in SS treatments (with the exception of the P2 clone with higher Zn concentration in the SS compared to the C treatment). In the first harvest, the highest Cd and Zn concentrations were recorded in the S1 clone in the C treatment, and the highest Pb concentration was recorded in the P1 clone in the C treatment, where the higher RE contents were mainly associated with the lowest biomass yield (Table 2). A similar pattern was observed in 2014. In 2014, however, in the SS treatment, the concentration of Pb was lower than in 2012 (with the exception of the P1 clone). In 2014, concentrations of Pb in clones S1 and S2 were higher in the SS than in the C treatment (Table 2). Thus, the results showed a higher effect of clone rather than SS application on the concentrations of investigated RE.

Removal of Elements from the Soil

In 2012, the removal of Cd and Zn per plant in the C treatment was the highest for clone S2 and in the SS treatment for clone S1, and the remaining clones decreased in the order of S2 > P1 > P2. Removal of Pb per plant by clones harvested in the C treatment decreased in the order of P1 > S2 > P2 > S1, and this

Table 2 Mean (±SE) standing biomass and concentrations of elements (Cd, Pb and Zn) in shoots (wood + bark) of *Salix* (S1—(*S. schwerinii* × *S. viminalis*) × *S. viminalis*, S2—*S. smithiana*) and *Populus* (P1—*P. maximowiczii* × *P. nigra*, P2—*P. nigra*) clones grown in control (C) and in sewage sludge (SS) treatments

Variable	Time	Treatment	Clones			
			S1	S2	P1	P2
Dry biomass (g plant^{-1})	2012	C	38.8 ± 6.9^{Aa}	132 ± 16^{Bab}	197 ± 43.4^{Bab}	102 ± 29.1^{ABab}
		SS	97.4 ± 22^{Aab}	119 ± 22.9^{Aa}	108 ± 13.6^{Aa}	56.4 ± 12.4^{Aa}
	2014	C	403 ± 183^{ABbc}	498 ± 137^{Bab}	541 ± 104^{Bb}	71.2 ± 16.6^{Aab}
		SS	516 ± 135^{ABc}	715 ± 203^{Bb}	496 ± 118^{ABb}	148 ± 15.8^{Ab}
Cd (mg kg^{-1})	2012	C	55 ± 6.83^{Bc}	44.4 ± 7.9^{ABb}	23.6 ± 2.91^{Ac}	25.3 ± 3.36^{Ab}
		SS	48.2 ± 5.17^{Cbc}	32.9 ± 2.7^{BCab}	18.2 ± 1.89^{Abc}	25.1 ± 1.82^{ABb}
	2014	C	26.7 ± 1.99^{Cab}	22.4 ± 1.51^{BCa}	11 ± 0.68^{Aab}	14.4 ± 1.63^{ABa}
		SS	24.1 ± 2.2^{Ba}	19.8 ± 2.56^{Ba}	8.63 ± 1.46^{Aa}	13.7 ± 1.8^{ABa}
Pb (mg kg^{-1})	2012	C	28.8 ± 4.02^{Ab}	24.5 ± 3.57^{Aa}	30.1 ± 2.89^{Aa}	19.5 ± 3.20^{Aa}
		SS	23.1 ± 2.79^{Aab}	20.6 ± 2.07^{Aa}	22.7 ± 2.23^{Aa}	19.4 ± 1.82^{Aa}
	2014	C	14.8 ± 0.97^{Aa}	14.4 ± 1.25^{Aa}	27.3 ± 2.74^{Ba}	16.9 ± 1.74^{Aa}
		SS	16.4 ± 1.27^{ABab}	18.7 ± 2.42^{ABa}	24.2 ± 2.04^{Ba}	15.1 ± 1.04^{Aa}
Zn (mg kg^{-1})	2012	C	506 ± 66.2^{BCb}	343 ± 38.9^{Bb}	207 ± 23.5^{ABb}	158 ± 21.5^{Aa}
		SS	457 ± 42.5^{Bb}	306 ± 24.4^{ABb}	176 ± 14^{Ab}	174 ± 15.7^{Aa}
	2014	C	251 ± 24^{Bab}	187 ± 16^{ABa}	115 ± 7.81^{Aa}	120 ± 14.3^{Aa}
		SS	224 ± 16.4^{Ba}	181 ± 22.7^{ABa}	109 ± 6.79^{Aa}	136 ± 25.7^{Aa}

Differences between clones and treatments were evaluated by Kruskal–Wallis tests. Clones with the same capital letter for each treatment in each harvest year were not significantly different. In each clone during both harvest years together, treatments with the same lowercase letter were not significantly different

order was in accordance with biomass yield. Removal of Pb per plant in the SS treatment was similar to that in the C treatment, and clone S1 removed a higher amount of Pb than clone P2 (Table 3).

In 2014, all the investigated clones removed significantly more RE than in 2012. The only exception was in the case of clone P2 in the C treatment, with a lower amount of removed RE than in 2012. Among the elements, the highest removal was reported for Zn. In 2014, *Salix* clones, in comparison to *Populus* clones, removed higher amounts of Zn and Cd per plant in both treatments. The most apparent differences were observed especially for clone S1 in the C and clone S2 in the SS treatments. In the C treatment, Pb was removed predominantly by clone P1 and in the SS treatment by clone S2 (Table 3).

Table 3 Mean (±SE) amount of Cd, Pb and Zn in shoots of individual plants grown in control (C) and in sewage sludge (SS) treatments

Removal	Time	Treatment	Clones			
			S1	S2	P1	P2
Cd (mg plant^{-1})	2012	C	2.06 ± 0.45^{Aa}	5.76 ± 0.98^{Ba}	4.26 ± 1.17^{ABab}	2.43 ± 0.79^{Aa}
		SS	4.24 ± 1.01^{Bab}	3.5 ± 0.54^{Ba}	1.66 ± 0.1^{ABa}	1.27 ± 0.22^{Aa}
	2014	C	12.2 ± 6.2^{Bb}	11.4 ± 3.09^{Ba}	6.13 ± 1.33^{Bb}	1 ± 0.23^{Aa}
		SS	11.4 ± 3.13^{Bb}	12.8 ± 3.51^{Ba}	3.51 ± 0.69^{ABab}	2.08 ± 0.41^{Aa}
Pb (mg plant^{-1})	2012	C	1 ± 0.18^{Aa}	3.12 ± 0.38^{Bab}	5.44 ± 1.18^{Bab}	1.9 ± 0.57^{ABa}
		SS	2.11 ± 0.47^{Aab}	2.15 ± 0.36^{Aa}	2.21 ± 0.3^{Aa}	1.01 ± 0.20^{Aa}
	2014	C	6.30 ± 2.98^{ABb}	6.88 ± 1.8^{ABab}	16.1 ± 4.39^{Bb}	1.27 ± 0.42^{Aa}
		SS	7.77 ± 1.79^{ABb}	11.3 ± 2.71^{Bb}	11.0 ± 2.35^{Bb}	2.24 ± 0.27^{Aa}
Zn (mg plant^{-1})	2012	C	18.1 ± 3.5^{Aa}	45.4 ± 6.21^{Ba}	38.4 ± 9.48^{ABab}	16.9 ± 6.11^{Aa}
		SS	36.4 ± 7.53^{Bab}	35 ± 6.67^{Ba}	17.2 ± 1.6^{ABa}	9.75 ± 2.49^{Aa}
	2014	C	125 ± 69.4^{Bb}	90.3 ± 24.1^{Ba}	65.4 ± 15.9^{Bb}	8.51 ± 2.08^{Aa}
		SS	112 ± 32.1^{Bb}	119 ± 35.8^{Ba}	52.6 ± 13.6^{ABb}	21.2 ± 5.61^{Aa}

Differences between clones and treatments were evaluated by Kruskal–Wallis tests. Clones with the same capital letter for each treatment in each harvest year were not significantly different. In each clone during both harvest years together, treatments with the same lowercase letter were not significantly different. Abbreviations of clones and treatments are given in Table 1

Table 4 Mean (±SE) dry matter yield and amount of Cd, Pb and Zn removed by harvested shoot biomass of plants grown in control (C) and in sewage sludge (SS) treatments (30,769 plants ha^{-1} minus mortality)

Variable	Time	Treatment	Clones			
			S1	S2	P1	P2
Dry biomass (t ha^{-1})	2012	C	0.67 ± 0.18^{Aa}	3.26 ± 0.57^{Ba}	5.14 ± 1.08^{Bab}	2.58 ± 0.91^{ABa}
		SS	1.93 ± 0.62^{Aab}	2.61 ± 0.74^{Aa}	1.68 ± 0.53^{Aa}	1.06 ± 0.45^{Aa}
	2014	C	6.28 ± 2.86^{ABb}	11.7 ± 3.10^{Ba}	14.1 ± 2.82^{Bb}	1.70 ± 0.46^{Aa}
		SS	9.99 ± 3.19^{Ab}	15.1 ± 4.94^{Aa}	8.64 ± 3.70^{Aab}	2.43 ± 0.67^{Aa}
Cd (g ha^{-1})	2012	C	32.6 ± 9.58^{Aa}	133 ± 20.5^{Ba}	108 ± 24.8^{ABb}	62.8 ± 24.7^{ABa}
		SS	76.2 ± 18.4^{Aab}	74.2 ± 17.6^{Aa}	25 ± 6.2^{Aa}	23.4 ± 8.9^{Aa}
	2014	C	186 ± 96.7^{ABab}	268 ± 70.6^{Ba}	158 ± 34^{ABb}	23.6 ± 6.16^{Aa}
		SS	215 ± 65.2^{Bb}	265 ± 88.9^{Ba}	58.5 ± 22.7^{ABab}	31.5 ± 9.21^{Aa}
Pb (g ha^{-1})	2012	C	15.5 ± 3.94^{Aa}	72.8 ± 8.36^{Bab}	141 ± 28^{Bab}	48.7 ± 17.3^{ABa}
		SS	37.8 ± 8.64^{Aab}	45.5 ± 11.4^{Aa}	32.4 ± 9.08^{Aa}	19.1 ± 7.55^{Aa}
	2014	C	95.2 ± 46.6^{ABab}	163 ± 41^{ABab}	429 ± 128^{Bb}	30.7 ± 11.7^{Aa}
		SS	146 ± 44.7^{ABb}	235 ± 69.3^{Bb}	186 ± 74.7^{ABab}	35.2 ± 9.79^{Aa}
Zn (g ha^{-1})	2012	C	288 ± 76.7^{Aa}	1090 ± 185^{Ba}	984 ± 208^{Bab}	441 ± 191^{ABa}
		SS	675 ± 164^{Aab}	769 ± 210^{Aa}	262 ± 69.9^{Aa}	197 ± 88.1^{Aa}
	2014	C	1930 ± 1080^{ABab}	2160 ± 585^{Ba}	1720 ± 453^{Bb}	204 ± 58.4^{Aa}
		SS	2140 ± 697^{ABb}	2540 ± 938^{Ba}	943 ± 420^{ABab}	341 ± 119^{Aa}

Differences between clones and treatments were evaluated by Kruskal–Wallis tests. Clones with the same capital letter for each treatment in each harvest year were not significantly different. In each clone during both harvest years together, treatments with the same lowercase letter were not significantly different. Abbreviations of clones and treatments are given in Table 1

In 2012, removal of RE per hectare in the C treatment displayed a similar pattern as removal of RE per plant. In 2012, in the SS treatment, removal of Cd per hectare displayed a similar pattern as removal of Cd per plant, but the order of clones according to removed Zn and Pb per hectare was different: S2 > S1 > P1 > P2 (Table 4).

In 2014, the S2 clone in the C treatment removed higher amounts of Cd and Zn per hectare than did the S1 clone. Removal of Pb per hectare in the C treatment and removal of Cd, Pb and Zn per hectare in the SS treatment reflected the removal of these elements per plant (Table 4).

Results of Principal Component Analysis

The first axis of the PCA of RE concentrations and DM biomass yield explained 59 % of the data variability, and the first two axes together explained 80 % (Fig. 1). The length and direction of the vectors indicate the strength of the vector effect and correlation between vectors respectively. A long vector for a particular variable indicates that it highly affected the results of the analysis, while the opposite is the case for a short vector. For example, DM yield of biomass was clearly negatively related to concentrations of all RE, as their vectors were directed into opposite parts of the diagram. The concentration of Zn in plant biomass was positively correlated with the concentration of Cd, as indicated by the angle smaller than 90° between vectors for Zn and Cd.

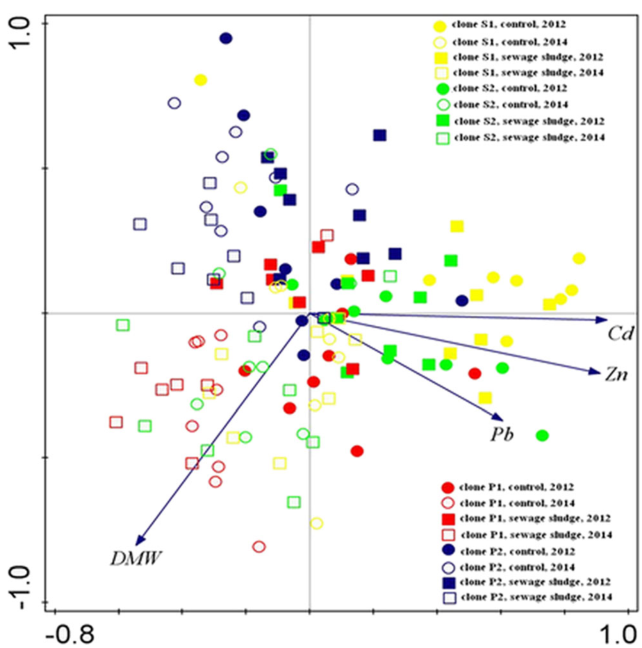

Fig. 1 Ordination diagram showing results of principal component analysis of dry matter yield of shoots per plant (DMW) and concentrations of Cd, Pb and Zn in shoots of plants grown in control and sewage sludge treatments in harvest years 2012 and 2014 (based on data provided in Table 2). Clone abbreviations: S1—(*Salix schwerinii* × *S. viminalis*) × *S. viminalis*, S2—*S. smithiana*, P1—*Populus maximowiczii* × *P. nigra*, P2—*P. nigra*

Markers for the SS treatment in 2014 (empty squares) and markers for the C treatment in 2014 (empty circles) were located on the right side of the diagram, while markers for the SS treatment in 2012 (filled squares) and markers for the C treatment in 2012 (filled circles) were located on the left side of the diagram. This indicates higher biomass yields and lower concentrations of RE in 2014 compared to 2012.

Salix clones, especially S1, were characterised by higher concentrations of Cd and Zn compared to *Populus* clones.

Remediation Factors

The ability of plants to remove relative portions of metals showed significant differences between individual harvests for Cd and Zn (Table 5). In both cases, RF increased several times in the second harvest compared to the first one. Willows showed better removal of Cd and Zn, reaching up to 0.94 % for Cd and 0.34 % for Zn in the S2 clone. RF of poplars was lower. The RF for Pb was negligible for all tested clones, reaching less than 0.01 %.

Table 5 Remediation factor (RF, %) indicates the proportion of elements (Cd, Pb and Zn) removed by harvested biomass (S1, S2, P1 and P2 clones grown in control (C) and in sewage sludge (SS) treatments) from the total contents of elements in the arable layer (upper 25 cm of the soil)

RF	Time	Treatment	Clones			
			S1	S2	P1	P2
Cd %	2012	C	0.11^{Aa}	0.46^{Ba}	0.38^{ABb}	0.22^{ABa}
		SS	0.27^{Aab}	0.26^{Aa}	0.09^{Aa}	0.08^{Aa}
	2014	C	0.65^{ABab}	0.94^{Ba}	0.55^{ABb}	0.08^{Aa}
		SS	0.75^{Bb}	0.93^{Ba}	0.20^{ABab}	0.11^{Aa}
Pb %	2012	C	0.0004^{Aa}	0.002^{Bab}	0.003^{Bab}	0.001^{ABa}
		SS	0.0009^{Aab}	0.001^{Aa}	0.0008^{Aa}	0.0005^{Aa}
	2014	C	0.002^{ABab}	0.004^{ABab}	0.01^{Bb}	0.0007^{Aa}
		SS	0.004^{ABb}	0.006^{Bb}	0.004^{ABab}	0.0008^{Aa}
Zn %	2012	C	0.04^{Aa}	0.14^{Ba}	0.13^{Bab}	0.06^{ABa}
		SS	0.09^{Aab}	0.1^{Aa}	0.03^{Aa}	0.03^{Aa}
	2014	C	0.26^{ABab}	0.29^{Ba}	0.23^{Bb}	0.03^{Aa}
		SS	0.28^{ABb}	0.34^{Ba}	0.13^{ABab}	0.05^{Aa}

Differences between clones and treatments were evaluated by Kruskal–Wallis tests. Clones with the same capital letter for each treatment in each harvest year were not significantly different. In each clone during both harvest years together, treatments with the same lowercase letter were not significantly different. Abbreviations of clones and treatments are given in Table 1

Discussion

Biomass Yield and Mortality

The estimated yields of clones in our experiment in the C treatment ranged from 0.7 (S1) to 5.1 (P1) t DM ha^{-1} in the first harvest (2012), provided 4 years after the establishment of the experiment. P1 and S2 clones were the most productive ones with the lowest mortality. These clones also were listed among the most productive clones in the Czech Republic by Weger [30] and Weger and Bubeník [31]. The estimated yields of clones treated with SS provided 4 years after the establishment of the experiment were lower, ranging from 1.6 (P2) to 2.6 (S2) t DM ha^{-1} in the first harvest (2012). The yield suppression was probably caused by higher incidence of weeds in the experimental units with the application of SS. There especially was *Rumex obtusifolius*, which is a highly problematic and widely spread weed [39]. It is well established that the occurrence of weeds can reduce the yield of fast-growing trees during the establishment year, but weeds can also have severe effects on yield in subsequent years [40, 41]. The application of SS increased yield of biomass only for the S1 clone, where the yield in the C treatment was the lowest among clones. Also, Sevel et al. [25] cited that the S1 clone positively responded to application of N fertilisers, including SS.

According to Havlíčková et al. [23], the biomass yield of the first harvest represented approximately only 30 % of the biomass yield from the following harvests. This corresponds well with our results, because the yields of the biomass harvested in 2014 were generally substantially higher than those in the first harvest in 2012. In the second harvest (2014), yield in the C treatment ranged from 1.7 (P2) to 14.13 (P1) t DM ha^{-1}, and yield in the SS treatment ranged from 2.43 (P2) to 15.14 (S2) t DM ha^{-1}. High increases in yield of biomass were recorded especially for *Salix* clones (S1 and S2) in the SS treatment. Only the P2 clone in the C treatment had lower biomass yield in 2014 than in 2012. Opposite findings were presented by Laureysens et al. [33], where clone P2 achieved in the first rotation 8 t DM ha^{-1} year^{-1} and in the second rotation 9 t DM ha^{-1} year^{-1} on a former waste disposal site moderately polluted by RE. Similarly, Al Afas et al. [42] characterised the P2 clone by high biomass production throughout the three rotations. These discrepancies can be explained by different conditions of the individual experiments, especially by lower levels of soil pollution (only 0.8 mg Cd kg^{-1}, 52 mg Pb kg^{-1} and 161 mg Zn kg^{-1}) compared to levels in this study. Nevertheless, for *Populus* clones, the rotations longer than 2 years seem to be more effective. Fortier et al. [43] observed the yields of the hybrid *P. maximowiczii* × *P. nigra* clone NM6 (the same parentage as P1 used in our study) reaching up to 12 t DM ha^{-1} year^{-1} in a 6-year rotation in unpolluted soil. Also, Weger [30] found a similar yield (11.7 t DM ha^{-1} year^{-1}) of the P1 clone harvested after 6 years in one rotation. However, in two rotations provided every 3 years, a biomass yield of only 9.2 t DM ha^{-1} year^{-1} was found, and when the P1 clone was harvested every year in six rotations, a biomass yield of only 5.7 t

DM ha^{-1} $year^{-1}$ was found. Weger [29] observed that the yields of *Salix* (including S2) and *Populus* clones in a third harvest were generally higher than in the second one, but the yields especially of some *Populus* clones (*Populus* cf. *deltoides* × *P. trichocarpa* P-NE44B-466; *P. trichocarpa* × *Populus koreana* P-trikor-468; *P.* cf. *deltoides* × *P. koreana* P-delkor-473) were conversely lower. For example, Tlustoš et al. [28] quoted yields of 2–5 t DM ha^{-1} $year^{-1}$ for clone S2 (first rotation) in moderately contaminated soil, and Weger [29] stated that in the ninth year of growth (third rotation), yield of S2 clones was more than 14 t DM ha^{-1} $year^{-1}$ in unpolluted soil. Sevel et al. [25] recorded yield amounts of the clone S1 ranging from 9 to 10 t DM ha^{-1} $year^{-1}$ in the first rotation and from 8.7 to 11.9 t DM ha^{-1} $year^{-1}$ in the second rotation, respectively, depending on the fertiliser application regime. Conversely, Pulford et al. [44], using clone Tora (similar to S1), recorded only 0.09 t ha^{-1} yield after the first year of growth in heavily contaminated soil. Soil at our site was also poor in plant-available P, which may contribute to low yield and explain the favourable response of willow clones to SS application. Obviously, yields are highly dependent not only on the clone but also on the level of soil contamination, nutrient and water availabilities, climate conditions, weed infestation and sequence and period of rotation. All of these factors were manifested in this study.

The relatively high mortality of plants (in the C treatment from 15 % for P1 to 48 % for S1 and in the SS treatment from 41 % for S1 to 51 % for P2) resulted probably from the high density of planting (30,769 plants ha^{-1}), which caused considerable self-thinning. Typical planting densities range between 6000 and 12,000 for *Populus* clones and between 10,000 and 20,000 for *Salix* clones [23, 45]. Although Armstrong et al. [46] and Bullard et al. [47] observed that the increase in clone density results in the increase in biomass yield from the plot, this relationship is not valid at the extremely high plant density. For example, the economic optimum of planting density of *Salix* clones is 15,000 cuttings [23, 47]. *P. nigra* clones are very strong heliophiles [48] and have a high ability of tillering [42]. We have tested a high density of plants because of their mutual low competition, higher positive effect on phytoextraction, better survival of young plants under weed pressure and regular sampling of biomass within the first years of the experiment.

Risk Element Contents in Plants

Generally, the clones of *Salix*, particularly clone S1, showed higher concentrations of Zn and Cd than *Populus* clones, and, conversely, clone P1 accumulated more Pb than did other clones. This is consistent with results from pot experiments of Fischerová et al. [7] and how accumulation ability for different elements by different clones can be an inherent property.

Increased contents of present RE in SS do not result in increased contents of RE in plants. Shoots showed higher contents of RE in the C treatment than in the SS treatment in both harvests. Application of organic fertilisers can limit mobility and availability of RE to plants [21]. Garrido et al. [16] reported that the SS constitutes a good source of organic matter that contributes new sites for the sorption of metals, but Shaheen and Tsadilas [19] showed that the application of SS did not significantly change Cd and Pb sorption compared to the C. The sorption ability seemed to be mainly affected by soil pH, which was revealed by the significant correlations of Cd and Pb sorption with soil pH. The lower metal biomass content could be also explained as a relative dilution of soil metal content in the SS treatments due to application of amendments with higher pH and lower Cd and Pb contents than are present in soil. Conversely, SS contains higher amounts of Zn compared to soil, but higher Zn concentrations in clones grown in the SS treatment compared to clones of the C treatment were not found, with the only exception of the P2 low-productive clone. Zn behaviour confirmed that immobilisation properties of applied SS played a more important role than did the dilution effect of amendment. Tlustoš et al. [18] applied a significantly higher rate of SS and found increased soil mobility of Zn, especially on acid Cambisol, whereas low mobility of Pb was not increased.

The biomass Cd, Pb and Zn concentrations were mostly lower in clones harvested in 2014 compared to clones harvested in 2012. According to Tinker et al. [49], a high growth rate of the plant may cause internal 'dilution' of trace elements. Also, Hejcman et al. [50] recorded that the concentrations of some micro and trace elements in plant biomass were negatively correlated with biomass production, probably due to the dilution effect.

Removal of Elements from the Soil

Our results clearly showed that efficiency of phytoextraction for RE is driven to a large extent by biomass yield, followed by the concentrations of elements in the biomass. Similar results were also presented by Laureysens et al. [51], Lonardo et al. [9] and Zárubová et al. [27]. The removal of Pb from the soil in both treatments and in both harvests exhibited similar trends and increased with higher biomass yield (except in the SS treatment in 2012 per plant and SS treatment in 2014 per hectare). Jensen et al. [8] reported annual removal of Pb (0.4 g ha^{-1}) by *S. viminalis* in field experiments in soil slightly contaminated with RE (2.5 mg Cd kg^{-1}, 400 mg Zn kg^{-1} and 170 mg Pb kg^{-1}) after the first year, whereas the removal of Pb in our experiment ranged from 3.8 g ha^{-1} $year^{-1}$ (15.46 g ha^{-1}) for the S1 clone in the C treatment in 2012 to 241 g ha^{-1} $year^{-1}$ (428.69 g ha^{-1}) for the P1 clone in the C treatment in 2014, especially due to high yield of biomass of 6-year-old clones and high content of Pb in the soil.

Cd and Zn were removed more by *Salix* than by *Populus* clones, due to higher concentrations of these elements in shoots of *Salix* clones. It seems also that application of SS has a positive effect on high biomass production [25] and thus a high removal of RE for *Salix* clones. Generally, the highest mean Cd removal was done by S2 clones in all treatments, mainly due to higher biomass yield, with the exception of S1 in SS treatments in 2012, where the removal was slightly higher due to higher Cd accumulation. The highest Cd removal was done by S2 clones in C treatments in 2014 (267 g ha^{-1}; 134 g ha^{-1} $year^{-1}$), and the lowest Cd removal was done by P2 clones in the SS treatment in 2012 (23.4 g ha^{-1}; 5.85 g ha^{-1} $year^{-1}$). Laureysens et al. [12] recorded for the same 7-year-old P2 clone removal of 47 g Cd ha^{-1} during 2 years. Differences can be explained again by significantly higher biomass production. However, in our experiment, P2 clones had a much higher content of Cd but simultaneously lower yield of biomass than P2 clones in experiments of Laureysens et al. [12]. In a study by Jensen et al. [8], 1-year-old *S. viminalis* achieved removal of 9.5 g Cd ha^{-1} $year^{-1}$. In our experiment, all clones had higher biomass yield and also a higher content of Cd than those in the experiment of Jensen et al. [8].

Removal of Zn was also better performed by *Salix* clones than *Populus* ones in both harvests. The SS application slightly improved Zn removal (grams per hectare) due to higher yield compared to C treatments, with the exception of the S2 treatment in the first harvest due to yield depression. Conversely, application of SS to *Populus* clones had a rather negative effect on biomass production and thus on shoot RE removal from the soil. The highest Zn removal was done by S2 clones in SS treatments in 2014 (2536 g ha^{-1}; 1268 g ha^{-1} $year^{-1}$), and the lowest Zn removal was done by P2 clones in SS treatments in 2012 (197 g ha^{-1}; 49 g ha^{-1} $year^{-1}$). Laureysens et al. [12] recorded for same 7-year-old P2 clone removal of 2400 g Zn ha^{-1} during 2 year. Differences between our results and results of Laureysens et al. [12] can be explained again by high biomass production similar to the case of Cd removal. The reason why P2 clones in the study by Laureysens et al. [12] removed much larger amounts of Cd and Zn than in our study could be different conditions in their field experiment, especially low levels of soil pollution, resulting in high biomass yield and thus high removal of Zn. In a study of Jensen et al. [8], removal of 345 g Zn ha^{-1} $year^{-1}$ by 1-year-old *S. viminalis* was achieved. Conversely, in our experiment, all clones had higher biomass yields and also higher contents of Zn than those in the experiment of Jensen et al. [8]; therefore, in our experiment, all clones had higher removal of Zn compared to clones of their experiment.

Remediation Factors

RF calculated on the basis of total soil contents significantly differed between harvests, individual elements and plant species; only slight differences were found between clones. The calculated Cd, Pb and Zn RF in our study were significantly lower compared to the RF determined in pot experiments with *Salix* and *Populus* clones [5, 7, 32]. Vysloužilová et al. [5] found out in their pot experiment a very high RF for *Salix* clones in moderately contaminated soil after 2 years (RF for Cd = 22.3 % for clone *S.* × *smithiana* S-150; RF for Zn = 4.3 % for clone *Salix* × *rubens* S-394). Also, Fischerová et al. [7] reported very high annual RF for Cd and Zn in their pot experiment with three *Salix* clones (RF for Cd = 3.4–8.1 %; RF for Zn = 1.2–2.2 %; RF for Pb = 0.005–0.012 %) and with two *Populus* clones (RF for Cd = 4.6–5 %; RF for Zn = 1.6–1.8 %; RF for Pb = 0.024–0.025 %). Komarek et al. [32] calculated these RF (Cd = 2.22 %, Zn = 0.48 % and Pb = 0.02 %) for 2-year-old *P. nigra* × *P. maximowiczi* hybrids, which were grown in moderately RE-contaminated soil.

Nevertheless, our RF are comparable with RF from field experiments of other authors. Jensen et al. [8] found RF of 0.13 % for Cd, 0.029 % for Zn and 0.001 % for Pb, respectively, in a field study on moderately polluted soil. According to Schmidt [52], in field experiments, we can assume only 20 % RF compared to controlled pot experiments. There are several reasons for different results coming from pot and field experiments: (i) The roots of plants in pots penetrate properly only a limited volume of soil, but in the field, they can easily avoid a contaminated top soil layer; (ii) a portion of removed RE is stored in the leaves that fall down and are not harvested in the field; and (iii) concentrations of elements are almost significantly higher in the roots than in aboveground tissues, and the roots are not harvested until the plantation was terminated [53].

RF calculations in our experiment confirmed that natural phytoextraction of Pb from this contaminated soil is not a suitable remediation method. According to Komarek et al. [32], the Pb RF values are very low (≤0.001 %), due to the high total concentration of Pb in the soil, its stable binding to the oxide and organic fractions of the soil. Better results were obtained for Zn (up to 0.34 %) and especially for Cd (up 0.94 %) by S2 clones in the second harvest. Even though these percentages are small, the removal by plants will most likely reduce the amount that can be leached, representing the most readily available fraction in the soil [8].

Conclusions

The results from the 6-year experiment showed that high biomass production was found in the second harvest (last 2 years) of the experiment either in C or SS treatments when sufficient roots were developed. The application of SS limited plant growth in the first years of the experiment but significantly increased biomass yield in willows in treatments, reaching up to 15 t DM ha^{-1} for S2 clones in the second harvest. The biomass yield was the major driving force for the RE amount removed by shoots. The removal was significantly higher by the second harvest for all clones and elements, with the exception of the P2 clone, in the C treatment. The *Salix* clones were characterised by higher removal of Cd and Zn compared to *Populus* clones. The S2 clone showed the best removal of Cd (up to 0.94 %) and Zn (up to 0.34 %); removal of Pb was negligible. SS can be a suitable amendment for willow plantations. Very promising phytoextraction efficiency presented in pot experiments was not confirmed in the field due to lower density of roots per volume of soil.

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