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Auteurs: Authors: Chloé Frédette, Yves Comeau, & Jacques Brisson

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Ecophysiological responses of a willow cultivar (*Salix miyabeana* 'SX67') irrigated with treated wood leachate

Chloé Frédette[a,b] ✉, Yves Comeau[c] & Jacques Brisson[a,b]

[a] Département de sciences biologiques, Université de Montréal, C.P. 6128, succ. Centre-ville, Montreal (QC) Canada H3C 3J7, chloe.fredette@umontreal.ca, jacques.brisson@umontreal.ca

[b] Institut de recherche en biologie végétale, 4101 Sherbrooke East, Montreal (QC) Canada H1X 2B2

[c] Department of Civil, Geological and Mining Engineering, Polytechnique Montréal, 2900 Édouard-Montpetit Boulevard, Montréal (QC) Canada H3T 1J4, yves.comeau@polymtl.ca

✉ **Corresponding author:** Chloé Frédette

4101 Sherbrooke East, Montreal (QC) Canada H1X 2B2

chloe.fredette@umontreal.ca

Abstract

As wood preservatives leach from exposed treated wood, they contaminate soil and water, creating an environmental problem that needs to be addressed. Treating this contamination is particularly challenging since it includes mixed compounds, such as heavy metals and trace elements, as well as xenobiotic organic pollutants like polychlorinated dibenzo-dioxin/furan congeners (PCDD/Fs) that are very toxic and are under very strict discharge regulations. Cultivating fast growing willow shrubs, either in soil or in treatment wetlands, offers a flexible and inexpensive treatment option. The main objective of this study was to evaluate the tolerance of a frequently used willow cultivar (*Salix miyabeana* 'SX67') to irrigation with leachate contaminated with pentachlorophenol (PCP) and chromated chromium arsenate (CCA), two important wood preservatives. We designed a mesocosms experiment with willow grown in three different substrates and irrigated over twelve weeks with three different leachate concentrations. Willow proved to be tolerant to irrigation with the raw leachate, with only leaf area decreasing with increasing leachate concentration. However, the type of growing substrate influenced willow ecophysiological responses and overall performance, and seemed to affect contaminant dynamics in the plant-soil system. All contaminants accumulated in willow roots, and Cu and PCDD/Fs were also translocated to aerial parts. Overall, this study suggests that *Salix miyabeana* 'SX67' could be a good candidate for treating water or soil contaminated with wood preservatives.

Keywords: phytotoxicity, phytoremediation, wood preservatives, pentachlorophenol (PCP), chromated copper arsenate (CCA), polychlorinated dibenzo-dioxins/furans (PCDD/Fs)

1. Introduction

Canada has one of the world's largest wood preservation industries, along with the United States and the United-Kingdom (Morris and Wang, 2006). The nature of wood preservatives has changed over time, and pentachlorophenol (PCP), an oil-borne substance that was commonly used in the 1950s, was gradually replaced by water-borne chemicals such as chromated chromium arsenate (CCA; Environment Canada, 2013), because of its toxicity (WHO, 1987; NTP, 2016). Following public apprehension about the presence of the toxic compound arsenic in the preservatives, CCA was banned from residential use in 2004 in both Canada and the United States (Morrell, 2017). Nonetheless, both CCA and PCP are still permitted for industrial use, including utility wood pole treatment (ATSDR, 2001; Morris and Wang, 2006; Environment Canada, 2013).

During the wood treatment process, or while in use or storage, treated wood exposed to rain events generates leachates that are contaminated with wood preservatives. Although leaching rate and susceptibility over time are often debated, soils at wood treatment facilities and final storage locations have clearly been shown to be contaminated (Bhattacharya *et al*., 2001; Kitunen *et al*., 1987; Stilwell and Gorny, 1997; Valo *et al*., 1984; Zagury *et al*., 2003). Chromium (Cr), copper (Cu) and chlorophenols (CP) seem to be more mobile in the soil, and can potentially reach aquifers of aquatic ecosystems. Arsenic (As) and PCP associated hydrocarbon compounds such as polychlorinated dibenzo-dioxins/furans (PCDD/Fs) are less mobile, but very persistent in the soil (Bhattacharya *et al*., 2001; Kitunen *et al*., 1987).

Phytoremediation has been proposed as a technology with potential to address such soil contamination. Willows and similar fast growing woody species like poplar have been studied specifically for remediation of these types of pollutants (Mills *et al.*, 2006; Önneby, 2006), along with various herbaceous plants. Preventive approaches, such as intercepting the contaminated leachates prior to their release in the soil also represent a sustainable avenue; the intercepted leachates must then be treated to meet water discharge regulations. Treatment wetlands are a proven technology that can be designed to treat various types of wastewaters, including those containing metallic trace elements, chlorinated compounds and hydrocarbons (Kadlec and Wallace, 2008). Recently, an experimental study showed that mixed wood preservatives leachate (PCP and CCA) can be treated successfully with horizontal sub-surface flow wetlands (Lévesque *et al.*, 2017). Designing zero liquid discharge willow wetlands has also been identified as a solution for treating this type of leachate and eliminating the risk of releasing contamination in the environment (Frédette *et al.*, 2019).

If willows are to be used for the treatment of either soil or water contaminated with wood preservatives, it is important to study the effect of those contaminants on willows. Tolerance and toxicity studies have been conducted at laboratory scale in hydroponic solutions for some wood preservative compounds such as As (Purdy and Smart, 2008), Cr (Yu and Gu, 2007; Yu *et al*., 2008) and derivatives of PCP (Clausen *et al*., 2018; Ucisik and Trapp, 2008; Ucisik *et al*., 2007). However, pollutant dynamics are much more complex in soils or substrates, and the presence of mixed contamination could lead to different results than if each contaminant were treated separately. The objective of this mesocosm study was to investigate the potential effects of water contaminated with both ACC and PCP on a willow species frequently used in phytoremediation and treatment wetlands, *Salix miyabeana* 'SX67'. We were particularly interested in physiological parameters associated with biomass production and treatment performance. Furthermore, we wanted to test the influence of different growing media, on the premise that different substrates would demonstrate differences in water holding capacity, nutrient sink in the root zone, and pollutant dynamics, which could in turn influence plant ecophysiological responses.

2. Methods

2.1 Experimental set-up and treatments

This study was conducted in a greenhouse located at the Montréal Botanical Garden (45°33'39.6"N 73°34'19.2"W), in eastern Canada. Each experimental unit consisted of a cylindric lysimeter 0.53 m high and 0.37 m in diameter (0.11 m^2 top area), filled with substrate and planted with one *Salix miyabeana* SX67 individual (Figure 1a). We specifically chose large containers with a depth greater than the expected average root zone (50 cm deep pots compared to an expected average 30 cm root zone for shrub willows). Plant density calculated according to the surface area of our containers was relatively high (10 plants/m^2), but has been observed in willow plantations (Bullard *et al*., 2002). The distance between each pot (Figure 1c) also helped prevent canopy competition for light interception. Six treatments were tested: sand substrate irrigated with various leachate dilutions (S0, S25, S50 and S100), sand topped with a coco fiber substrate layer irrigated with the 25% leachate dilution (C25) and sand topped with an organic substrate layer irrigated with the 25% leachate dilution (O25). Each treatment was replicated three times and one lysimeter filled only with sand remained unplanted to estimate soil evaporation, for a total of 19 lysimeters. Figures 1b and 1c present the experimental treatments and spatial disposition of the 19 lysimeters in the greenhouse. A one-inch wide tube, pierced only in the bottom 5 cm, was placed in the units for irrigation and water sampling (Figure 1a). There was no outflow from the lysimeters, so all water loss could be attributed to evapotranspiration. Willow shrubs were grown in pots from cuttings in the summer of 2017 and transplanted in the lysimeters in August of the same year. Temperature in the greenhouse was adjusted to meet outside temperature but could not be brought below 5°C in winter.

The first layer of the substrate consisted of 8 cm of coarse granitic gravel (16-32 mm) for drainage, topped with either 40 cm of sand or 20 cm of sand topped with one of two other substrates to be tested (*organic* and *coco fiber*), and then covered with 2 cm of fragmented rameal wood as a mulch to limit soil evaporation. The *sand* substrate consisted of washed coarse sand (0.5-1 mm); the *coco fiber* substrate of 80% coconut fiber and 20% coarse sand; and the organic substrate of an assemblage of 60% black earth (Quali Grow, 0.2-0.2-0.1 NPK), 20% potting soil (Fafard, 0.3-0.1-0.4 NPK) and 20% coarse sand. The porosity measurements made in the laboratory for the sand, coco fiber and organic substrates were 36%, 70% and 39% (volume based), respectively.

The raw leachate was collected from a treated wood pole storage site on June 15 (batch 1) and August 6 (batch 2), and stored in 20 L polyethylene tanks at 4°C. Both old PCP treated and new CCA treated wood poles are stored at this specific site. Consequently, chlorophenolic compounds from the PCP (as well as PCDD/Fs that are present in commercial PCP formulations), and As, Cr and Cu from the CCA were expected to be present in the leachate (Lorber *et al*., 2002; Frédette *et al.*, 2019). All the contaminants targeted were present in the leachate, except for pentachlorophenol, which had already begun to degrade into dichlorophenol, but concentrations of this compound were much higher in batch 2 (Table 1). Three lysimeters filled only with sand were irrigated with the raw leachate (100%, S100), three with a first dilution of the leachate (50%, S50), three with a second dilution (25%, S25), and three with tap water only (S0). The six lysimeters filled with *organic* substrate and *coco fiber* were then irrigated with the second dilution (25%, O25 and C25). From the time shrubs were planted in the lysimeters in 2017 to June 17 of 2018, all lysimeters were irrigated manually with tap water one to three times per week, depending on their water consumption. Total irrigation need was determined according to water level prior to irrigation and substrate porosity, with the aim of attaining a water level around 5 to 10 cm below the substrate surface after irrigation. This provided water saturated conditions for the plants, similar to conditions in a horizontal subsurface flow treatment wetland. The first contaminated irrigation took place on June 18, then two and three weeks after (July 2 and 11), and finally two times a week until September 7 for a total of 18 contaminated irrigation events. The amount of leachate provided during those irrigation events was fixed, and tap water was added, if necessary, to complete the total irrigation need. In the end, each lysimeter received 37L of leachate (raw or diluted according to the treatment) except for a few plants that had smaller irrigation needs at the end of the experiment; the contaminant charge applied for each treatment is detailed in Table 1.

A customized fertilizer solution with a nitrogen (N) concentration of 200 ppm and an NPK ratio of 21:7:14 was added to the irrigation water weekly until July 13, after which N concentration was raised to 400 ppm due to notable signs of N deficiency. A mite (*Tetranychus* sp.) infestation was detected in early July, and despite a careful pesticide application every 2 days (Trounce, NFS 176), the infestation caused significant leaf defoliation of several individuals and notable defoliation of neighbors, mainly in bloc 3 (Figure 1c).

2.2 Data collection

All sampling took place over 16 weeks (starting 4 weeks prior to the first leachate irrigation), from May 23 to September 7, 2018. By that date, the damage to shrubs from the mite infestation was so important that we were forced to terminate the experiment.

2.2.1 Plant measurements

Leaf area (LA), proportional growth rate (pRG), biomass production, evapotranspiration rate (ET; total quantity of water loss through ET over a given period of time), photosynthesis rate (Ps), instant transpiration (T; estimated transpiration rate at a specific sampling time) and stomatal conductance ($\bar{G}_s$) were measured. LA was calculated weekly based on direct counting of the number of leaves on each willow and the mean size of one leaf. Throughout the month of June, multiple leaves were randomly collected from the shrubs at different stem heights and development stages to estimate the mean area of one individual leaf using optical software (Mesurim Pro v3.4.4.0). pGR was also calculated once a week using the following equation:

$$pRG = \frac{(H_{t+1} - H_t)}{H_t} \quad \text{(Eq. 1)}$$

Where H_t was the height of the longest stem at the previous measurement, and H_{t+1} the height of the highest stem on the day the measurement was made. Fresh root and stem biomass was collected and weighed at the end of the experiment after residual leaves were removed, and then oven dried at 75°C until constant weight. Leaf biomass could not be measured directly because the plants lost leaves throughout the season and it was impossible to associate the fallen leaves with a plant. Instead, we determined the average weight of one leaf and multiplied it by the number of leaves counted when the LA was maximal, which provided us with an estimate of the minimal amount of leaf biomass produced per plant. The method used to calculate ET rate is detailed in section 2.2.2. Ecophysiological parameters (Ps, T and $\bar{G}_s$) were recorded using a portable measuring instrument (Li-COR 6400XT, Biosciences). Measurements were made one day per week from 10:00 AM to 1:00 PM, and conditions in the leaf chamber of the Li-COR (humidity, temperature, light and CO_2 concentration) were set to match the ambient conditions at the sampling time. Once a week, foliar symptoms of pathology (*e.g.* chlorosis, necrotic spots) were carefully noted and quantified (0 for absence, 1 for weak signs, 2 for present signs, 3 for generalized signs) for every plant.

2.2.2 Evapotranspiration calculation

Before and after every irrigation event, water level in the lysimeters was recorded. The lysimeters were in a greenhouse, so they received no rainfall, and the lysimeters were closed, so no drainage occurred. ET was then calculated as follows:

$$ET = \frac{[\Theta_a(L_{t-1} - L_t)]}{d(t-1)-t} \quad \text{(Eq. 2)}$$

Where ET represents the mean daily lysimeter evapotranspiration (mm/d), Θ_a the effective substrate porosity (unitless), L_t is the water level prior to irrigation (mm) on a given irrigation day, L_{t-1} the water level after irrigation (mm) on the previous irrigation day and $d_{(t-1)-t}$ the number of days between each irrigation events. We used effective (or wet) porosity instead of the theoretical substrate porosity that is measured on completely dry substrate, to avoid overestimating ET. Effective porosity was calculated as follows, every time water level was monitored and irrigation was performed:

$$\theta_a = \frac{I}{A(L_{t+1} - L_t)} \quad \text{(Eq. 3)}$$

Where I is the irrigation volume added (m^3), A is the lysimeter area (m^2), L_t is the water level prior to irrigation (m) and L_{t+1} the water level after irrigation (m).

2.2.3 Water, soil and plant tissue analysis

Every two weeks, hydrogen potential (pH), oxydo-reduction potential (ORP), conductivity (EC) and temperature (T) were measured in the first 15 cm of the substrate using a multiparameter probe (Hanna Instrument, HI98194-6, Smithfield, RI). The substrate measurements were made by collecting a 40 ml composite sample for each treatment, dissolving it in 80 ml of distilled water, letting the particles settle and taking the measurement in the supernatant. Before adding contaminants to the system, the three different substrates (sand, organic and coco) were analyzed for background contamination by PCP and PCDD/F congeners using gas chromatography mass spectrometry (GC-MS), and for As, Cr and Cu by inductively coupled plasma mass spectrometry (ICP-MS).. At the very end of the experiment, the same contaminant analysis was performed on composite samples of the first 20 cm of substrate for the 5 treatments and the control to estimate accumulation (or depletion) of each contaminant in the root zone. To assemble each composite sample, 3 small cylinders of substrate were collected from the 3 lysimeters of each treatment, for a total of 9 sub-samples per treatment, and then mixed together before weighing the mass required for the analysis (30 g). This operation was repeated twice, to yield 2 replicates per treatment. We also performed contaminant analysis for the plant tissues (roots, stems and leaves) to see if any accumulation and/or translocation had occurred. Unfortunately, due to a manipulation error, leaves were not sampled for the control treatment (S0). Root samples were only rinsed with distilled water prior to analysis. All contaminant analyses were performed by an accredited laboratory and sampled according to their protocol (Maxxam Analytique, Montréal, Quebec) and with the lowest detection limit available (from 0.1 to 1.8 pg/g for PCDD/Fs congeners; 0.1 mg/kg for phenolic compounds; 0.5 mg/kg for As, Cr and Cu). Finally, translocation factor (TF) was calculated for the different contaminants by dividing the measured leaf concentration by the measured root concentration.

2.3 Data analysis

We used a type I ANOVA analysis to test the statistical influence of the treatments on plant physiological and morphological variables and on plant tissue accumulation of contaminants. Significant ANOVAs ($\alpha = 0.05$) were followed by a post-hoc Tukey's test to identify the different treatments. Because a mite infestation affected the third bloc of the experiment more severely, we also included the bloc number as a factor in the ANOVAs.. All statistical analyses were performed in R 3.5.1 software. We normalized LA, pGr, ET, Ps, T, and $\bar{G}_s$ results for S25, C25, O25, S50 and S100 treatments by dividing their average value by the average value observed for S0:

$$nX = \frac{\sum_i X_{trait}/i}{\sum_i X_{S0}/i} \qquad \text{(Eq. 4)}$$

Where X represents a given parameter, X_{trait} the value of this parameter measured for a given treatment, X_{S0} the value of this parameter measured for the control treatment, and i the number of replicates. To help with the interpretation of the results regarding PCDDs congeners, they were associated with their relative octanol:water coefficient (K_{ow}), which represents their hydrophobicity (Kim *et al.*, 2019).

3. Results

The leachate concentration had no significant effect on either variable, except for LA, which was significantly lower for the S50 treatment (Table 2). However, there was a bloc effect on LA and ET that was driven by bloc 3 according to the post-hoc analysis. Interestingly, a similar trend was observed for

ET, Ps, T, $\bar{G}_s$ and biomass, where mean values for the S25 treatment were higher than for S0, then decreasing gradually for S50 and S100 to values equal or inferior to S0. The substrate type significantly affected LA, ET and $\bar{G}_s$, and a bloc effect was noticeable only for LA (Table 2). LA increased rapidly during the season and, at the beginning of contaminated irrigation on June 18, the average LA per willow was already 1.4 m^2. Maximal (or peak) LA was generally reached in late July or early August, ranging from 1.2 (S50, mite infestation source) to 5.1 (O25, bloc 1) m2 of leaves per tree. Mean LA was generally lower for the willows growing in sand, followed by those growing in coco fiber, and, finally, much higher in the organic substrate (Table 2). LA for the different leachate concentrations showed a gradual decrease over time when compared to the control treatment (Figure 2). The pGR of the stems was maximal in May, and decreased slowly over the growing season. Shrubs reached a maximal height of 3.2 m on average, and S0 and O25 were the treatments in which pGR was highest (Table 2). Although not significant according to the ANOVA analysis, mean pRG for the different leachate concentrations showed a gradual decrease over time when compared to the control treatment, particularly after week 12 of the experiment (Figure 2). Mean ET rate from May 3 to September 10 was 9.9 ± 4.9 mm/d, while ET of the unplanted lysimeter was 1.0 ± 0.7 mm/d on average, meaning that plant T accounted for about 90% of ET. Willow displayed a higher ET in the coco fiber substrate and even more in the organic substrate (Table 2). Temporal variation of ET showed little difference between the different leachate concentrations, but willow irrigated with the 25% concentration generally had slightly higher ET rate than the control, and the contrary occurred for 50 and 100% concentrations (Figure 2). ET was also consistently higher in coco and organic substrate, but by week 12, ET in coco substrate started to decline and was equal to ET in sand by the end of the experiment. (Figure 2). Ps, T and $\bar{G}_s$ mean values were the highest in O25 and lowest in S0 treatments, although neither leachate concentration nor substrate type seemed to have a significant effect on these variables (Table 2). Until the 10th week of the experiment, mean Ps rate was similar for all treatments (Figure 2). In the 11th week, Ps of the contaminated treatments increased in comparison to the control plants, and remained slightly higher until week 13. Inversely, in the last two weeks of the experiment, Ps of the contaminated treatments was much lower than Ps of the uncontaminated shrubs, except for O25 (Figure 2). Once contaminated irrigation began, T rate and $\bar{G}_s$ began to show more variability depending on the treatment, tending to increase in contaminated treatments (Figure 2). However, by the end of the experiment, mean values of those two parameters were similar to or lower than the control results. Total dry biomass produced was 375 g per tree on average, and stems constituted 80% of total biomass. Biomass production was greater for shrubs growing in coco fiber and organic substrate (Table 2). Some foliar symptoms, such as chlorosis and necrotic spots, were detected throughout the season, but were not very notable and did not seem to be related to the contamination, as they were equally present in control lysimeters and under the different leachate concentrations (data not shown). However, plants growing in the organic and coco fiber substrates showed important signs of nutrient deficiency, even after the fertilizer concentration was doubled. The leachate concentration did not affect soil pH, EC or ORP, which were, respectively and on average, 7.6 ± 0.5, 206 ± 131 μS/cm and 246 ± 32 mV. EC increased throughout the experiment, with an average value of 350 μS/cm at the last measurement, and was always higher in coco fiber and organic substrate compared to sand substrate. Background contamination was observed in the substrate for all contaminants except As (Table 3). An increase in contaminant concentration at the end of the experiment was barely noticeable, and no phenolic compounds or As were detected either before or after the experiment (Table 3). As for the presence of contaminants in the plant tissues, PCDD/Fs and Cu were found in all tissues, while As and Cr were found in roots only, except for a small concentration of Cr detected in the leaves of the S100 treatment (Table 3). No As was found in the roots of the S25 and O25 treatments, and the accumulation in the roots of the control lysimeter (S0) was similar to that in the other treatments. For Cr, accumulation in the roots of the control was higher than in all other treatments. The highest concentrations of PCDD/Fs were found in the leaves, and Cu was more concentrated in the roots. The distribution of the congeners of PCDD/Fs measured in the different compartments of the lysimeters (Figure 3) shows that: 1) the proportion of a congener increased with the number of chlorine atoms, octa-chlorinated dibenzo-dioxin/furan (OcCDD/F) being the most present in the majority of the compartments, 2) the proportion of the different congeners in the substrates changed from the beginning (T0) to the end of the experiment (T1) and 3) *light* dioxin congeners such as Te/Pe/HeCDD were found in plant leaves, but not in stems or roots of the willow. Based on biomass produced and concentration measured, we estimated that willow accumulated up to 0.07 mg of As (S0), 0.7 mg of Cr (S0) and 6 mg of Cu (O25) in their tissues (Figure 4). Since no contaminants were detected in leaves for PCP, As and Cr, no TF was calculated. TF for copper ranged from 0.6 for the S50 treatment to 1.7 for O25 treatment. For total PCDD/Fs, TF ranged from 14 (O25) to 87 (S100) and, for PCDDs, seemed correlated to congener hydrophobicity (K_{ow}; Figure 5).

4. Discussion

Except for a certain LA inhibition, the different concentrations of leachate added to irrigation water had no clear phytotoxic effect on the willows. Furthermore, and although not statistically significant, the most diluted treatment (25%) tended to increase some physiological parameters. We can therefore suggest that *S. miyabeana* 'SX67' is tolerant to irrigation with a leachate contaminated with ACC and PCP under the concentrations tested in this study. At the end of the experiment, all contaminants could be found in/on the willow roots, but only Cu and PCDD/F were detected in aerial parts. The different types of substrate had different background contamination and were associated with significantly different results for most willow parameters measured.

4.1 Willow tolerance, uptake and translocation for PCP derived contaminants

In our samples, the concentration of all phenolic compounds measured, including polychlorinated ones derived from PCP, never exceeded 3.5 μg/L. *Salix* species have previously been found to demonstrate tolerance to a certain range of phenolic compounds; this tolerance decreased with the addition of Cl atoms (Clausen and Trapp, 2017). For example, a concentration of 200 mg/L of phenol was needed to observe a drastic decrease in photosynthetic activity in *S. babylonica* over three days (Li *et al*., 2015), while EC_{50} (*i.e.* concentrations inducing a negative effect in 50% of the organisms observed) of polychlorinated phenols were 5.8 to 37.3 mg/L for *S. viminalis* cuttings over 144 hours or less (Ucisik *et al*., 2007; Ucisik and Trapp, 2008; Clausen and Trapp, 2017; Trapp *et al*., 2000).

An average amount of 141 to 572 pg of PCDD/Fs, depending on the treatment, was provided to the willows, and the highest concentration of PCDD/Fs measured in the soil was 0.47 pg Toxic Equivalents (TEQ)/g (in the C25 treatment at the end of the experiment). To our knowledge, there is very little information on PCDD/Fs toxicity to plants, and even less for willows. However, Urbaniak *et al.* (2017) reported that the application of sewage sludge containing up to 6 pg TEQ/g of PCDD/Fs to a willow plantation (*S. viminalis*) had an overall beneficial effect on the plants, increasing LA, biomass production and chlorophyll content, while the same conditions proved to be phytotoxic for other plant species like *Sinapis alba* and *Sorghum saccharatum*. Moreover, some studies that used PCDD/Fs concentration in plants as a biomonitoring tool reported very high concentrations of those contaminants in trees (up to 2.3×10^5 pg/g of lipids) with no mention of notable tree mortality (Wagrowski and Hites, 2000; Wen *et al.*, 2009). It is therefore no surprise that in the present study, *Salix miyabeana* 'SX67' proved to be tolerant to the raw leachate, because the concentrations of chlorinated phenolic compounds and hydrocarbons derived from the PCP were much lower than estimated phytotoxic concentrations. Concentrations of PCDD/Fs up to 1.4 pg TEQ/kg were found in the willow tissues at the end of the experiment. Concentration in the leaves was 3.4 times higher than in the roots on average, while stem concentration was about 21% of the root concentration. Organic pollutants, including dioxin and furan congeners, can accumulate in plant tissues via either soil or air (Zhang *et al*., 2017). For example, dioxins with 1 to 4 chlorine atoms are likely to volatilize in the air from water or soil and then be deposited on plant leaves or enter them through gas exchange (Bacci *et al*., 1992). PCDD/Fs being hydrophobic molecules, it is sometimes suggested that the major pathway for this contaminant accumulation in plant aerial parts is air-to-plant, because such molecules are not mobile in water and should be strongly bonded to organic matter in the soil (Bacci *et al*., 1992; Zhang *et al*., 2009). However, there is also clear evidence for root adsorption and absorption of PCDD/Fs in the soil, which can be explained by their relatively low molecular mass (below 1000 g) and high hydrophobicity (K_{ow} from 6.8 to 8.2; Zhang *et al*., 2012). Yet, different species have shown different responses to PCDD/Fs (Zhang *et al*., 2009), and some plant families such as the *Cucurbitaceae* have even shown exceptionally high translocation of PCDD/Fs to aerial parts (Inui *et al*., 2011). Based on the analysis of the PCDD/Fs congeners presented in this study, we can state that *S. miyabeana* 'SX67' does accumulate PCDD/Fs, and even translocates them in its aerial tissues. Lighter PCDD/Fs (*e.g.* TeCDD and PeCDD) were found in greater quantities in the leaves than in the roots and stems. At this point, we should also mention that the calculated TF for PCDD/Fs were much higher than those reported in the literature (Inui *et al*, 2001; Nunes *et al*., 2014; Hanano *et al*., 2015), which raises the question of potential aerial deposition. However, while this would be more than plausible under field conditions, due to potentially contaminated rainfall, it seems unlikely that the ambient air in greenhouse contained a high concentration of gaseous PCDD/Fs given the low concentrations used, and the mulch layer and constant soil moisture that should have prevented the transport of aerial dust from the substrate. Furthermore, congeners with 5 or more chloride atoms are usually considered non volatile (Bacci *et al*., 1992).

Theoretically, PCDD/Fs translocation factor should increase with the number of chloride atoms (which increase hydrophobicity or K_{ow}; Zhang *et al.*, 2009; Bacci *et al.*, 1992). However, the inverse trend has been reported for PCDD/Fs hyperaccumulators, with TF decreasing with K_{ow} increase (Inui *et al.*, 2001). We observed the same trend, but only for polychlorinated dibenzo-dioxin congeners with a K_{ow} of 7.6 and higher (hxCDD to OcCDD).

4.2 Willow tolerance, uptake and translocation for CCA derived contaminants

In this study, the highest concentrations of As, Cr and Cu provided to willows were 0.53, 0.07 and 0.16 mg/L respectively, for a total of 14.4, 1.7 and 6.3 mg added in the S100 treatment. Considering that the lysimeter contained roughly 50 kg of soil, this represents a maximal soil concentration of 0.3, 0.035 and 0.13 mg/kg of As, Cr and Cu respectively. This explains why no As was found in the substrate (detection limit of 0.5 mg/kg), and suggests that willow was principally exposed to Cr and Cu from the substrate background concentration (7.3-14 to 5.6-10 mg/kg for Cr and Cu respectively). Although oxidation state of As was not directly measured, we can presume that the arsenite form (AsIII) should have been predominant according to the redox soil conditions (246 mV) and relatively high pH (7.6). The ionic form of chromium was not measured either, but since most of the Cr naturally found in soil is trivalent (Barnhart, 1997), and the hexavalent state was only rarely detected on the industrial site where the leachate was collected (data not published), we can assume that most of the chromium measured in this study was in the Cr^{3+} form.

Tolerance of willows (EC_{50}) to arsenic has been reported to range from 3 to over 20 mg/L in lab tests of over 72 h (arsenate or As(V) form only; Clausen and Trapp, 2017). For *Salix purpurea*, Yanitch *et al.* (2017) reported a toxic effect from as little as 5 mg/L of As(V) in a hydroponic experiment, the effects increasing with increasing concentration of As. According to the Purdy and Smart study (2008), hybrids of *S. viminalis* x *S. miyabeana* and *S. sachalinensis* x *S. miyabeana* were the cultivars most tolerant to

As contamination, with concentrations of As(V) as high as 18.7 mg/L having no effect on plant T and only a slightly deleterious effect on biomass production. In the present study, arsenic was detected in the willow roots only, and concentrations were below the detection limit in the roots of the S25 and O25 treatments. However, at higher As concentrations in water, it has been demonstrated that some willows can translocate As to aerial parts, that TF increases with increasing As concentration, and that the latter is further enhanced in the presence of phosphorus (Purdy and Smart, 2008). In the Purdy and Smart study (2008), *S. viminalis* x *S. miyabeana* was not only the most tolerant cultivar but also the most efficient As accumulator (up to 7000 mg/kg of As in roots, and 200 mg/kg in leaves).

As for chromium, Yu and Gu (2007) and Yu *et al.* (2008) tested the effect of an hydroponic solution of Cr^{3+} and Cr^{6+} (separately) on the T and metabolism of the hybrid *S. viminalis* x *S. alba*. Reduced T occurred at 15 and 4.2 mg/L of Cr^{3+} and Cr^{6+} respectively, but none of the concentrations tested (up to 30 mg/L of Cr^{3+} and 12.6 mg/L of Cr^{6+}) had a significant effect on willow metabolism, apart from slightly reducing soluble protein content in leaves. In a field experiment, *Salix smithiana* was cultivated in soil contaminated with up to 140 mg/kg of chromium (along with significant concentrations of other heavy metals) without showing any visible signs of phytotoxicity (Kacálková *et al.*, 2014). However, most of the Cr in the soil was considered non-available according to a 0.11 mol/L acetic acid extraction method (Kacálková *et al.*, 2014); bioavailability of the contaminants was not determined in the present study. In a pot experiment, a soil Cr concentration of 70 mg/kg was found to have a relative phytotoxic effect on *Salix viminalis*, but *Salix* also proved to be the most tolerant of all the species tested (Ranieri and Gikas, 2014). Chromium was present in the substrate of all treatments, including S0, because of the substrate background concentration, and was consequently detected in the roots in all treatments. Root concentration of Cr was the highest for willows irrigated with tap water only (S0), and was significantly lower in the organic and coco fiber substrates. Cr was not detected in aerial parts, except for a small concentration in leaves of the S100 treatment. While Cr accumulation in willow roots has been reported to be high (up to 15 000 mg/kg; Yu and Gu, 2007), aerial TF seems to be quite low, ranging from 0.03 to 2 (Kacálková *et al*., 2014; Ranieri and Gikas, 2014; Yu and Gu, 2007). However, TF is also thought to increase with initial Cr concentration (Yu and Gu, 2007), which could explain why Cr was detected only in leaves of the willow irrigated with the raw leachate. Chromium has a tendency to bind strongly with organic matter in soil (Fendorf, 1995), and this could explain the lower concentration of this element in willow grown in the organic and coco fiber substrates. Other elements like iron also have the potential to immobilize Cr by forming highly stable complexes (Fendorf, 1995). We can therefore hypothesize that the chemical composition of the leachate could be responsible for the lower Cr accumulation in willow irrigated with the leachate compared to the control.

Finally, the concentration of copper in water, which ranged from 0.25 mg/L to 3.2 mg/L, was previously reported to be sufficient to decrease willow biomass production, although this depended greatly on the cultivar, and did not provoke other visible toxicity symptoms (Punshon *et al.*, 1995; Yang *et al.*, 2014). When considering the concentration of Cu in soil, willow could tolerate concentrations up to 455 mg/kg, again displaying a biomass decrease but no other toxic symptoms (Chen *et al*., 2012). Lastly, copper was found in all plant tissues, with higher concentrations in roots, followed by the leaves and then the stems, except for the O25 treatment, where Cu was more concentrated in aerial parts. Leaf and stem TF were respectively of 0.9 and 0.6 on average, which is higher than the TF reported by Yang *et al.* (2014) for 12 different willow cultivars. Contrary to a study by Chen *et al.* (2012), we did not find that increasing Cu concentration in soil increased willow Cu accumulation. However, in our experiment, only the C25 and O25 treatments provided significantly higher Cu soil concentration, and, at the same time, they provided conditions where Cu could be less mobile (*e.g.* complexion with high organic matter content).

For As, Cr and Cu, it would be expected that the substrate composition and concentration in molecules such as organic matter and other elements (*e.g.* Mn, Fe, Al) would strongly influence bioavailability of those contaminants to a plant. However, based on the data collected in this study and similar examples from the literature, we can hypothesize that, even if a fair amount of the As, Cr and Cu present in the lysimeters at the end of the experiment was available to willows, none of those contaminants were concentrated enough to generate a phytotoxic response in the plant. Therefore, *S. miyabeana* represents a good candidate for treatment of CCA contaminated leachate.

4.3 Influence of the substrate

The two alternative substrates tested had an obvious positive impact on willow performance, and this effect was slightly more evident for the organic than the coco fiber substrate. Apart from the pGR, C25 and O25 treatment willows generally performed better in terms of ET, LA, Ps, T, $\bar{G}_s$ and biomass production. On the one hand, it is most probable that contaminants were less available in the two organic substrates because of their organic matter content, as discussed previously. On the other hand, leachate concentration in sand substrate had little impact on the plants, which suggests that contaminant availability might not be the main explanation for the better performance of C25 and O25. One of the possible causes of this increased performance is the nutrient sink initially present in this substrate compared to sand. However, this in turn increased the nutrient demand from willows, which resulted in signs of important nutrient deficiency throughout the experiment. This means that although the organic substrate initially benefitted the plants, it also increased the need for fertilization following plantation, which can represent substantial costs and manipulations, depending on the intended use of the willows. Root:shoot ratio was significantly decreased in the O25 and C25 treatments, due to higher stem biomass production rather than lower root biomass production. Furthermore, the O25 treatment showed even higher root biomass than S25 and C25, which could in turn increase resource prospection and phytoremediation potential. The willows growing in coco and organic substrate also used much greater quantities of water than those growing in sand, but we cannot confirm whether this is a direct effect of substrate physical properties or a correlated effect of biomass and LA increase. Nevertheless, this result represents an interesting optimization opportunity when using willow ET potential to reduce volumes of contaminated water.

5. Conclusion

Salix miyabeana proved to be tolerant to irrigation with a raw leachate contaminated with ACC and PCP. Based on the concentrations of all contaminants found in the leachate and previous tolerance studies, it is possible that this willow cultivar could sustain a much more concentrated leachate. Even at these low contaminant concentrations, willows have shown a capacity to accumulate all tested contaminants, and potential to translocate PCDD/Fs and Cu. Based on the literature and observed accumulation in roots, we can assume that translocation might have been observed as well for higher concentrations of As and Cr. Finally, the two types of organic substrate tested had significant positive effects on willow growth and physiology. Notably, we observed a change in willow reaction to contaminants that could be attributed to the substrate reducing phytotoxicity of the leachate. However, willow extraction potential was also reduced. This study is the first, to our knowledge, to investigate and evaluate *S. miyabeana* potential to remediate mixed wood preservative contamination in a complex system (mesocosms). Although the mesocosms were designed to mimic in situ conditions, it would be interesting to validate our findings in full-scale remediation systems (i.e. full-scale treatment wetland comprised of phytoremediation plantations). Future research should test the effect of this type of leachate in a longer term experiment and under more concentrated conditions, while investigating the actual availability of the contaminants for the plants after they have reacted with the substrate. Finally, more attention should be given to the risks associated with translocation of highly toxic compounds such as PCDD/Fs, which could be transferred through trophic networks.

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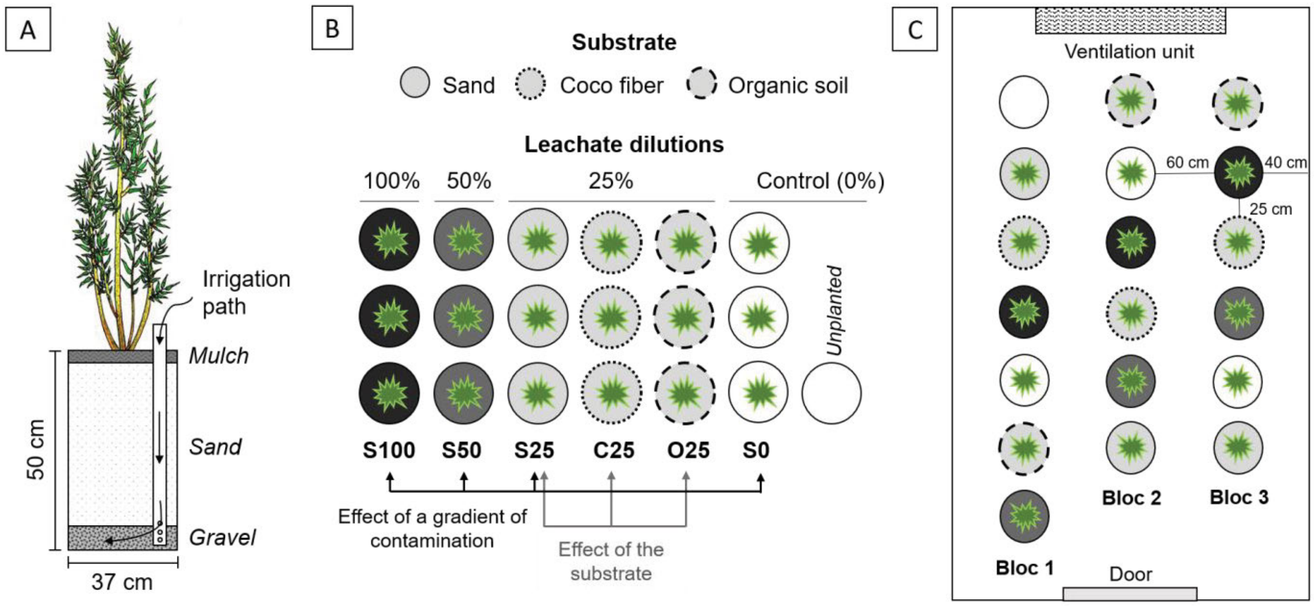

Fig 1 a. sectional view of the lysimeters showing the 3 different substrate layers and the subsurface irrigation path, b. experimental design, c. spatial arrangement of the 19 lysimeters

Graphic program used: MS PowerPoint

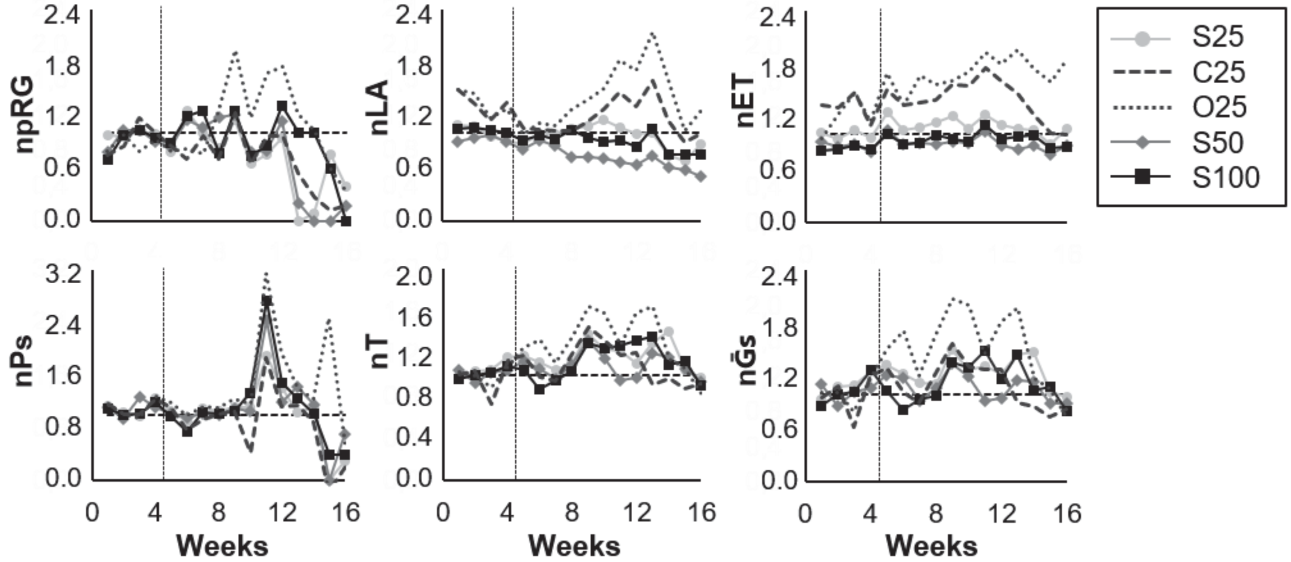

Fig 2 Weekly mean proportional growth rate (pRG), leaf area (LA), evapotranspiration rate (ET), photosynthesis rate (Ps), instant transpiration rate (T) and stomatal conductance ($\bar{G}_s$) of *S. miyabeana* 'SX67' irrigated with different concentrations of leachate (25, 50, 100) contaminated with wood preservatives (PCP and CCA), in different substrate (S, C, O) and normalized to the control (non-contaminated water, S0) observations. Horizontal dashed line represent no difference from the control. Vertical dashed line represent the beginning of contaminated irrigation after the fourth week.

Graphic program used: MS PowerPoint

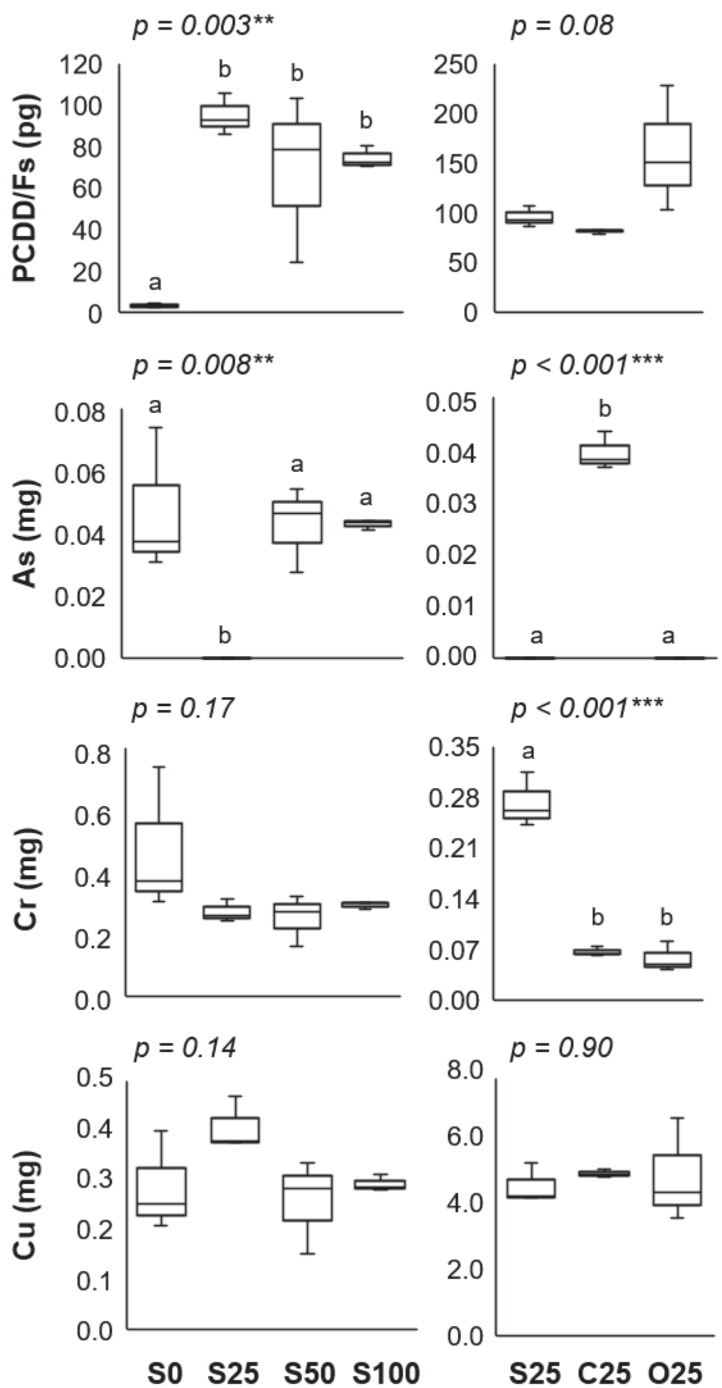

Fig 4 Total contaminant accumulation in *S. miyabeana* 'SX67' tissues after 12 weeks of irrigation with different concentrations of leachate (0%, 25%, 50%, 100%) contaminated with wood preservatives (PCP and CCA), and in different substrates (sand, organic, coco fiber)

Graphic program used: MS PowerPoint

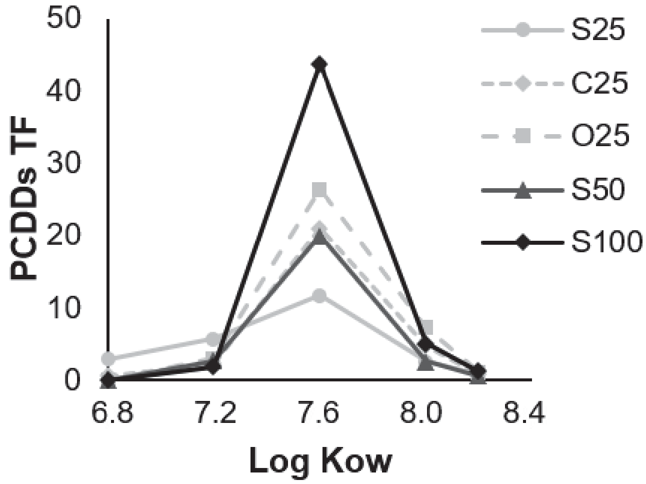

Fig 5 *Salix miyabeana* 'SX67' leaf translocation factor (TF) estimated for different polychlorinated dibenzo-dioxins congeners (PCDDs) and presented according to their octanol:water coefficient (K_{ow})

Graphic program used: MS PowerPoint

Table I. Contaminant concentration in the raw leachate and total mass added per treatment. BDL = below detection limit, TEQ = toxic equivalent; S25, C25 and O25 = sand, coco fiber and organic substrate with 25% leachate dilution, S50 = sand with 50% leachate dilution, S100 = sand with raw leachate (100%).

	Leachate concentration			**Total mass added per treatment**					
Contaminant	*Units*	*Batch 1*	*Batch 2*	*Units*	*S25*	*C25*	*O25*	*S50*	*S100*
PCP	µg/L	BDL	BLD	µg	-	-	-	-	-
3,5-DCP	µg/L	1.2	2.1	µg	14.9	15.3	15.3	27.1	60.4
PCDD/Fs	pg TEQ/L	5.0	27	pg TEQ	141	146	146	251	572
As	µg/L	260	530	mg	3.6	3.7	3.7	6.4	14.4
Cr	µg/L	24	68	mg	0.41	0.42	0.42	0.74	1.7
Cu	µg/L	180	160	mg	1.6	1.6	1.6	2.9	6.3

Table 2. Mean leaf area (LA), relative growth rate (RG), evapotranspiration rate (ET), photosynthesis rate (PS), instant transpiration rate (T) and stomatal conductance ($\bar{g}_s$), as well total dry biomass and root to shoot ratio (± standard deviation) of *S. miyabeana* 'SX67' over 12 weeks of irrigation with different concentrations of leachate contaminated with wood preservatives (PCP and CCA), in different substrates. Exponent letters represent the results of the type I ANOVA analysis, and the post-hoc Tukey analysis; different letters indicate a significant effect of the treatment (α = 0.05) and a capital letters indicate a significant bloc effect.

Willow parameter	**Leachate concentration**				**Substrate type**		
	0% (S0)	*25% (S25)*	*50% (S50)*	*100% (S100)*	*Sand (S25)*	*Coco (C25)*	*Organic (O25)*
Leaf area (m^2)	1.6^{A} ± 0.5	1.5^{A} ± 0.3	1.1^{B} ± 0.5	1.4^{A} ± 0.1	1.5^{A} ± 0.3	$1.9^{A,B}$ ± 0.2	2.3^{B} ± 0.7
Proportional growth rate (m/m)	0.08^{a} ± 0.02	0.06^{a} ± 0.01	0.06^{a} ± 0.01	0.07^{a} ± 0.01	0.06^{a} ± 0.01	0.06^{a} ± 0.01	0.08^{a} ± 0.01
ET rate (mm/d)	10.1^{A} ± 1.8	11.2^{A} ± 0.6	9.1^{A} ± 3.1	9.7^{A} ± 0.2	11.2^{a} ± 0.6	14.5^{b} ± 1.2	17.2^{b} ± 4.3
Photosynthesis (mmol CO_2 m^{-2} s^{-1})	5.3^{a} ± 0.9	5.6^{a} ± 0.1	6.0^{a} ± 0.5	5.6^{a} ± 0.3	5.6^{a} ± 0.1	5.0^{a} ± 0.3	6.5^{a} ± 0.1
Instant T rate (mmol H_2O m^{-2} s^{-1})	2.7^{a} ± 0.5	3.2^{a} ± 0.4	3.0^{a} ± 0.3	3.0^{a} ± 0.5	3.2^{a} ± 0.4	3.1^{a} ± 0.3	3.7^{a} ± 0.3
$\bar{G}_s$ (mmol m^{-2} s^{-1})	0.24^{a} ± 0.06	0.30^{a} ± 0.04	0.26^{a} ± 0.04	0.26^{a} ± 0.07	0.30^{a} ± 0.04	0.27^{a} ± 0.03	0.37^{b} ± 0.06
Total dry biomass (g)	333^{a} ± 98	366^{a} ± 51	267^{a} ± 81	318^{a} ± 29	366^{a} ± 51	444^{a} ± 10	524^{a} ± 160
Root:shoot ratio (g/g)	0.27^{a} ± 0.07	0.29^{a} ± 0.01	0.26^{a} ± 0.01	0.29^{a} ± 0.03	0.29^{a} ± 0.01	0.18^{a} ± 0.02	0.16^{a} ± 0.01

Table 3. Estimated contaminant mass in different substrates before (T0) and after (T1) 12 weeks of irrigation with different concentrations of leachate contaminated with wood preservatives (PCP and CCA), along with mass of the contaminants in the plant tissues at the end of the experiment. All results are based on dry weight of composite samples with 1 (plant tissues) or 2 (substrates T0 and T1) replicates. BDL = below detection limit.

		S0	S25	C25	O25	S50	S100
Soil T0	PCDD/Fs (pg TEQ)	0.23	0.23	14	13	0.23	0.23
	As (mg)	BDL	BDL	BDL	BDL	BDL	BDL
	Cr (mg)	365	365	700	500	365	365
	Cu (mg)	280	280	500	500	280	280
Soil T1	PCDD/Fs (pg TEQ)	0.38	0.11	21	9.8	0.074	0.048
	As (mg)	BDL	BDL	BDL	BDL	BDL	BDL
	Cr (mg)	415	390	750	625	382	427
	Cu (mg)	345	277	700	492	322	330
Roots	PCDD/Fs (pg TEQ)	1.2	2.0	2.3	7.4	1.9	1.1
	As (mg)	0.047	BDL	0.035	BDL	0.043	0.043
	Cr (mg)	0.47	0.27	0.07	0.06	0.25	0.26
	Cu (mg)	1.2	1.2	0.80	0.41	0.90	0.91
Stems	PCDD/Fs (pg TEQ)	2.3	15.0	6.4	0.5	17.5	0.2
	As (mg)	BDL	BDL	BDL	BDL	BDL	BDL
	Cr (mg)	BDL	BDL	BDL	BDL	BDL	BDL
	Cu (mg)	1.6	2.3	2.6	2.9	1.3	1.5
Leaves	PCDD/Fs (pg TEQ)	*	78.0	72.1	152.7	49.2	73.1
	As (mg)	*	BDL	BDL	BDL	BDL	BDL
	Cr (mg)	*	BDL	BDL	BDL	BDL	0.04
	Cu (mg)	*	0.63	0.96	1.0	0.39	0.53

** Not sampled*