INTERNATIONAL JOURNAL OF PHYTOREMEDIATION
2023, VOL. 25, NO. 10, 1371–1383
https://doi.org/10.1080/15226514.2022.2158783

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Effects of urban wastewater application on growth, biomass, nutrition, and heavy-metal accumulation of *Populus nigra* L. "62/154," *P. alba* L. "20/45," *P. euramericana* (Dode) Guinier "92/40," and *Salix excelsa* S.G. Gmel grown in heavy-metal contaminated soil

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ABSTRACT

Iran is located in a dry climate zone, and climate change has substantially reduced its precipitation and water resources. Reusing wastewaters from urban communities can meet some requirements for irrigation and fertilization of tree plantations in arid environments, leading to sustainable wastewater recycling, enhanced biomass production, and reduced land degradation. The objective of this study was to test the growth, biomass, nutrition, and heavy-metal accumulation of poplars [*Populus nigra* L. "62/154," *P. alba* L. "20/45," *P. euramericana* (Dode) Guinier "92/40"], and willow (*Salix excelsa* S.G. Gmel) in a pot experiment at four and eight months after planting when grown in soils irrigated with tap water (SITW) and wastewater (SIWW). After four months, SIWW treatment had no significant effect on growth, biomass, nor absorption of macronutrients. After eight months, SIWW treatment of poplars and willow significantly ($p = 0.000$) increased: (1) height, (2) leaf area, (3) root, stem, leaf, and total biomass, and (5) phytoextraction and phytoaccumulation of macro-/micro-nutrients and heavy metals in tree tissues, over trees receiving the SITW treatment. There were significant differences in growth, biomass, and accumulation of micronutrients and heavy metals in poplar versus willow tissues, with the highest biomass production and tissue-specific content of heavy metals in *P. nigra* trees, and the greatest total concentrations of heavy metals in *P. alba* and *S. excelsa* trees. In contrast, uptake of Fe, Cu, Ni, Cr and Pb were similar between poplar and willow, and phytoaccumulation of these elements was primarily in the roots. Leaf concentrations were highest for Zn and Mn. While *P. nigra* outperformed all other species overall, tolerance index (TI; defined as the tolerance to the heavy metals as calculated by the ratio of the biomass of SIWW trees relative to SITW trees) values exceeding 100% for all one-year-old poplar and willow trees demonstrated that they can be considered for planting in soil affected by urban wastewaters with similar contaminant profiles as in the current study.

NOVELTY AND SIGNIFICANCE OF RESEARCH

Since the species would differ in their growth, biomass, and phytoremediation responses to the nutrient and heavy metal concentrations of the wastewater over time, this research is important for the development of silvicultural prescriptions of these fast-growing trees that support effective wastewater reuse strategies throughout heterogeneous landscapes and across variable human community resources and needs.

KEYWORDS
Heavy metals; irrigation; phytoaccumulation; phytoextraction; phytoremediation; phytotechnologies; poplar; wastewater; willow

Introduction

Increasing urban populations and associated anthropogenic activities throughout the world has led to the degradation of ecosystem services such as a global scarcity of fresh water (Costanza *et al.* 2014). As a result, an abundance of high-quality water is not available for woody biomass production systems in many urban and rural landscapes, despite the need for woody feedstocks for bioenergy, biofuels, and bioproducts. This situation is especially prevalent in most arid and semi-arid countries that have faced scarcity of water resources for agriculture and forestry production (Evett *et al.* 2011; Morante-Carballo *et al.* 2022). Given that many urban activities result in wastewater production, their use for herbaceous crops and trees has attracted worldwide attention. In particular, wastewater recycling and reuse for irrigation and fertilization is one possible mechanism to reduce pressure on freshwater resources (Zalesny *et al.* 2011; Monteverdi *et al.* 2014; Ahmad *et al.* 2020). Appropriate wastewater use reduces surface water pollution problems and preserves beneficial water resources while providing organic matter and beneficial amounts of nutrients required for plant growth, biomass production, and soil improvement (Toze 2006; Ungureanu *et al.* 2020). There is

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concern about the possible risks to human and environmental health associated with agricultural wastewater reuse given entry of physical, chemical, and microbial contaminants into the human food chain (Leonel and Tonetti 2021), whereas disposal or reuse of wastewaters for woody biomass production systems do not have those concerns and, therefore, may be one of the most sustainable management options (Singh *et al.* 2022).

Utilization of wastewater in forestry and tree plantations has many benefits to communities and the environment, regardless of where they are used geographically and across the landscape. Such benefits include: (1) safe disposal and suitable management of wastewater, (2) reduced discharge of wastewater into freshwater resources (El-Moussaoui and Quazzani 2019), (3) amelioration of heavy-metal impacted soil conditions as trees are long-lived organisms with no direct link to the food chain, (4) reduced ecological degradation of the soil by pollutants given the potential for phytoremediation with the absorption of harmful trace elements from the soil via extensive tree root systems and associated long-term uptake and accumulation into aboveground tissues (Madejon *et al.* 2017; Pilipović *et al.* 2019), and (5) opportunities for commercial biomass production and sequestration of excess minerals in the plant system through nutrient-rich wastewater (Lachapelle *et al.* 2019).

A review of previous studies indicated that trees from the *Khaya*, *Robinia*, *Pinus*, *Eucalyptus*, *Populus*, and *Salix* genera did not exhibit substantial disruption of plant growth and biomass accumulation when grown with irrigation from untreated and treated wastewater (Zalesny and Bauer 2007; Tabari and Salehi 2009a, 2009b; Singh *et al.* 2010; Dimitriou and Aronsson 2011; Ali *et al.* 2013; Aryal and Reinhold 2015; Chandra and Kang 2015; Sas *et al.* 2021). However, selection of suitable tree species and superior genotypes within those species that tolerate and thrive when grown in wastewater-contaminated soils depends on their phytoremediation ability, growth patterns and ecophysiological responses, biomass production potential, and adaptability toward soil or wastewater contaminants (Maaloul *et al.* 2019; Singh *et al.* 2022).

Poplars (*Populus* spp.) and willows (*Salix* spp.) of the Salicaceae family are characterized by rapid growth, high biomass production, elevated coppicing ability and associated propagability, efficient photosynthesis, low nutrient demands, tolerance of high plantation densities, and adaptability for ecological niches such as nutrient-poor, dry, wet or metal-contaminated environments (Kuzovkina and Volk 2009; Mukherjee and Coleman 2020; Salehi *et al.* 2022). Likewise, poplars and willows are very useful for reforestation and rehabilitation (Pilipović *et al.* 2021; Zalesny *et al.* 2021) with considerable economic importance as a wood resource (Marmiroli *et al.* 2011; Ciadamidaro *et al.* 2013; Hu *et al.* 2014). Also, poplars and willows are the most commonly used trees for phytoremediation (Salehi *et al.* 2016; Zalesny *et al.* 2016), with numerous studies reporting their successful remediation of both inorganic and organic contaminants of soil and water (Dickinson and Pulford 2005; Pajević *et al.* 2009; Hu *et al.* 2021; Pilipović *et al.* 2021; Zalesny *et al.* 2021).

Despite these advantages of poplars and willows, there exist challenges for plantation establishment and subsequent growth in arid and semi-arid environments with limited water availability, such as those present in Iran. In response to these needs, utilization of wastewaters has been cost-effective and sustainable alternatives for irrigation and fertilization of poplar and willow production systems in similar environments (Evett *et al.* 2011; Zalesny *et al.* 2011). Given the broad genetic diversity of both genera, poplars and willows represent a rich resource for wastewater production systems requiring species and genotypes with elevated metal resistance and high or low metal uptake (Marmiroli *et al.* 2011). The objective of this study was to test the growth, biomass, nutrition, and heavy-metal accumulation of *Populus nigra* L. "62/154," *P. alba* L. "20/45," *P. euramericana* (Dode) Guinier "92/40," and *Salix excelsa* S.G. Gmel in a pot experiment at four and eight months after planting when grown in soils irrigated with tap water (SITW) and wastewater (SIWW). We hypothesized that the species would differ in their growth, biomass, and phytoremediation responses to the nutrient and heavy metal concentrations of the wastewater over time. This information is important for the development of silvicultural prescriptions of these fast-growing trees that support effective wastewater reuse strategies throughout heterogeneous landscapes and across variable human community resources and needs.

Materials and methods

Soils and wastewater

Two soil types were tested in this experiment. First, soils were collected from agricultural lands located south of Tehran, Iran (35° 29′ 59″ N, 51° 29′ 56″ E) that have been irrigated with urban wastewater for many years. The second soil type was sourced from the Alborz Research Center of the Research Institute of Forests and Rangelands, Tehran, Iran (35° 48′ 21.7″ N, 50° 54′ 10.9″ E) that was irrigated with tap water. The characteristics of the two soil types used in this study are given in Table 1. Based on these data, the mean values of heavy metals in the soil irrigated by urban wastewaters are higher than world's average value. Likewise, previous studies have shown an increasing trend of heavy metal contamination in the soil of agricultural lands in the south of Tehran due to wastewater irrigation and the historic use of fertilizers and pesticides during the past decades (Hani and Pazira 2011).

Urban wastewater used as the irrigation in this experiment was collected from a source located south of Tehran, Iran (35° 32′ 35″ N, 51° 30′ 58″ E). The wastewater contained a mixture of mountain and urban effluents as well as domestic and industrial wastewater, and it is categorized as raw and untreated wastewater. Table 2 shows the physicochemical properties of the tap water and wastewater used in this study. According to limits of wastewater for agriculture reuse (the standards of FAO, EPA), this wastewater falls within the regulated limits for reuse in agriculture for pH,

Table 1. Physico-chemical properties of soil irrigated with tap water (SITW) and wastewater (SIWW).

Parameters	SITW	SIWW	World-soil average[a]
Soil texture	Clay loam	Clay loam	–
pH	7.9	8	–
EC (ds/m)	1.4	1.61	–
Organic matter (%)	0.95	1.23	–
N (%)	0.07	0.11	–
Fe (g/kg)	17.93	28.42	–
	mg/kg		
P	32	50	–
K	202	256	–
Zn	78.10	183.32	70
Cu	36.30	146.72	38.90
Mn	322.13	981.40	488
Ni	22.10	41.81	29
Cr	33.13	88.10	59.5
Pb	20.78	48.01	27

[a]Kabata-Pendias 2011.

Table 2. Physico-chemical properties of tap water and wastewater.

			Limits of wastewater for agricultural reuse (mg/l)	
Parameters	Tap water	Wastewater	FAO	US EPA
pH	7.32	7.13	6–8.5	6–8.5
EC (ds/m)	0.63	1.12	0–3	0.7–3
	mg/l			
N	10.12	24.92	0–15	5–30
P	0.77	1.96	0–2	0.04–0.4
K	5.06	12.4	–	10–20
Ca	33.4	64.6	–	40–70
Mg	3.13	9.22	–	5–20
Na	40.12	82.7	–	90–200
Fe	0.024	0.080	5	5
Zn	0.032	0.088	2	2
Cu	0.009	0.026	0.2	0.2
Mn	0.007	0.030	0.2	0.2
Ni	0.005	0.030	0.2	0.2
Cr	0.003	0.015	0.1	0.1
Pb	0.013	0.093	5	5

EC, the level of nutrients and heavy metals. However, since there are no possibilities for wastewater treatment, it is advisable to avoid the irrigation of agricultural crops with this urban wastewater, especially vegetable crops.

Plant material and experimental design

One-year-old whips of three registered poplar clones (*Populus nigra* "62/154," *P. alba* "20/45," *P. euramericana* "92/40") and a genotype of *Salix excelsa* were collected from the Alborz Research Center in February 2017 and processed into dormant, hardwood cuttings ranging in length from 15 to 20 cm and in diameter from 1.0 to 1.5 cm. Cuttings were stored at 4 °C, removed from cold storage, and soaked for 12 h over running tap water before planting into 25-cm-high plastic pots (7.5 L). One cutting was planted in each pot. The cuttings were planted in an experimental layout consisting of a completely randomized design (CRD) with two factors: (1) soils in two levels [soil irrigated with tap water (SITW); soil irrigated with urban wastewater (SIWW)] and (2) species in four levels (*P. nigra*, *P. alba*, *P. euramericana* and *S. excelsa*). Twelve trees were tested for each soil treatment × species combination at each harvest schedule (i.e., four and eight months after planting). The study was conducted in greenhouse conditions under a photoperiod of 15 h light and 9 h dark with average temperature 25–30 °C which was controlled with an air conditioner at the Research Institute of Forests and Rangelands. Following establishment, SITW and SIWW treatments were applied twice weekly throughout the remainder of the experiment. Specifically, the pots were watered to field capacity of the soils with tap water (SITW) or urban wastewater (SIWW).

Measurement of growth and biomass parameters

At four and eight months after planting, half of the trees were harvested and analyzed for growth, biomass, and phytoremediation traits. First, the trees were carefully excavated from the pots and washed with tap and distilled water to eliminate soil particles from the roots. Then, height, diameter, leaf area, and dry biomass of the roots, stems, and leaves were determined. Shoot:root ratio was calculated based on aboveground (stem + leaf) and root biomass. Leaf area was measured using a Gate House Model 4cht Aok leaf area meter (UK). Plant tissues were oven-dried at 70 °C until constant mass and dry mass was determined for each tissue. Metal resistance of each species was calculated as tolerance index (TI) on the basis of total dry biomass of shoot and root according to Landberg and Greger (2002; formula 1):

$$\text{TI} = \frac{\text{Dry biomass treated}}{\text{Dry biomass control}} \times 100 \quad (1)$$

Chemical analyses of waters, soils, and plant tissues

Chemical analysis of tap water and wastewater was conducted according to standard methods for water and wastewater outlined by the American Public Health Association (APHA *et al.* 2012). The chemical composition of the soils was analyzed before the experiment. Soil samples were collected in paper bags and then air-dried and passed through a 2-mm sieve. The hydrometer method of Gee and Balder (1986) was used for soil texture analyses. Saturated extracts were analyzed for electrical conductivity (EC) and pH by the method of Jackson (1973). The total N, available P and K, and organic matter content of soil was determined by the Kjeldahl method (Bremner 1996), Olsen method (Olsen and Sommers 1982), ammonium acetate method (Page *et al.* 1982), and the Walkley and Black method (Nelson and Sommers 1996), respectively. Total micro-nutrients (Fe, Zn, Mn, Cu) and heavy metals (Ni, Cr, Pb) were measured using ICP-MS (PerkinElmer ICP-MS: Elan 9000 DRC-E, USA) after acid digestion in a microwave digestion system (Multiwave 3000, Anton Paar, GmbH, Austria) using a mix of HCl (37%) and HNO_3 (65%) at a 3:1 ratio.

The same method was used for the determination of macro-/micro-nutrients and heavy metals in plant tissues (roots, stems, leaves). Certified standards [CRM059-TraceMetals-Loamy Clay 2 (Sigma-Aldrich, USA), GSB-11- Citrus leaves (NCS ZC73018, China National Analysis Center for Iron and Steel) and CD281-Rey Grass (Institute for Reference Materials and Measurements, European Commission)], with known element concentration, were analyzed with the soil and plant samples in order to confirm the correctness of the procedure.

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) using SPSS software version 27 to examine significant differences for soil treatments and species main effects as well as their interactions. The parameters were tested separately at four and eight months after planting. Separation of means was performed using Tukey's HSD test at a 0.05 level of probability.

Results and discussion

Plant growth parameters and biomass production

After four months of growth, soil treatments had no significant effect on growth and biomass parameters of poplars and willow ($p > 0.05$ for all the parameters). Relative to the control soil (SITW), treatment of poplars and willow with SIWW significantly ($p = 0.000$) increased height, leaf area, and root, stem, leaf, and total biomass at eight months after planting, with differences at eight months between soil treatments being negligible for diameter ($p = 0.064$) and shoot:root ratio ($p = 0.173$) (Table 3; Figure 1).

Table 3. Probability values from analyses of variance (ANOVA) testing the effects of soil, species, and soil × species interaction on growth and biomass parameters at two periods.

		Source of variation				Source of variation		
Period	Parameter	Soil	Species	Soil × species	Parameter	Soil	Species	Soil × species
4	Diameter	0.052	0.000**	0.971	Root mass	0.855	0.111	0.703
8	Diameter	0.064	0.000**	0.392	Root mass	0.000**	0.000**	0.057
4	Height	0.066	0.000**	0.351	Stem mass	0.110	0.000**	0.936
8	Height	0.000**	0.034*	0.216	Stem mass	0.000**	0.000**	0.059
4	Leaf Area	0.070	0.000**	0.439	Leaf mass	0.090	0.014*	0.203
8	Leaf Area	0.000**	0.000**	0.091	Leaf mass	0.000**	0.000**	0.594
4	S:R ratio	0.140	0.000**	0.247	Total mass	0.121	0.000**	0.587
8	S:R ratio	0.173	0.24	0.140	Total mass	0.000**	0.000**	0.363

Significant differences are indicated with $^{*}p < 0.05$ and $^{**}p < 0.01$.

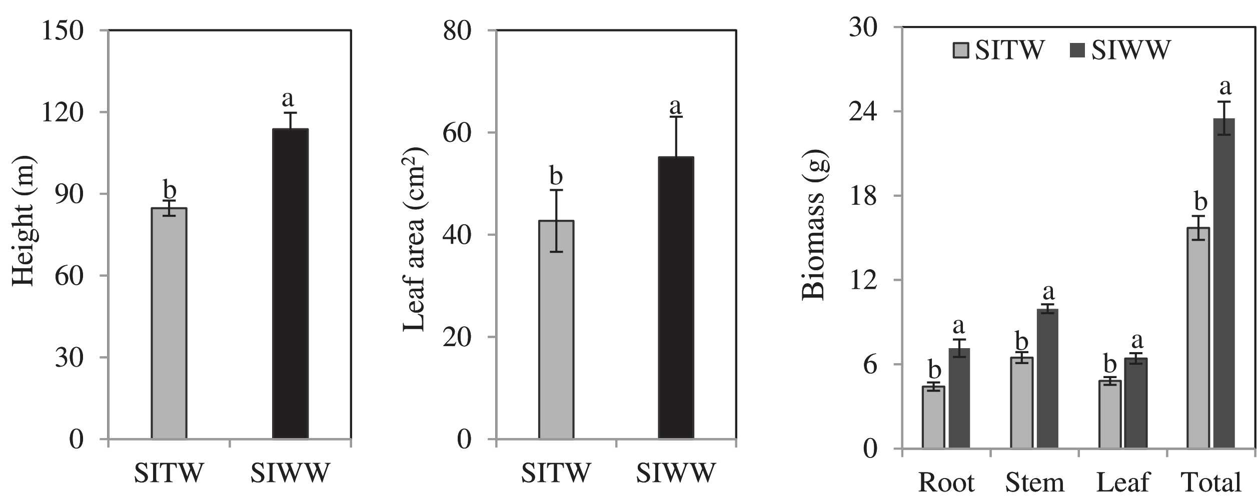

Figure 1. Soil main effect for height, leaf area, and root, stem, leaf, and total biomass at eight months after planting; Each value represents the mean (± SE) of all species (24 replications) in soil irrigated by tap water (SITW) and in soil irrigated by wastewater (SIWW); Different letters indicate significant differences between two soil treatments at each parameter.

Compared to their respective controls, SIWW treatment enhanced total plant biomass of *P. nigra*, *P. alba*, *P. euramericana*, and *S. excelsa* by 44.7, 36.9, 62.1 and 58.0%, respectively. With the exception of shoot:root ratio at eight months and root mass at four months, the species main effect was significant for all plant growth and biomass parameters. *P. nigra* exhibited the greatest increase in diameter and tissue biomass, while *S. excelsa* had the tallest plant height (Table 3; Figures 3 and 4). The soil treatment × species interaction was non-significant for all growth and biomass traits ($p > 0.05$ for all the parameters) (Table 3). Nevertheless, all four species had tolerance indices above 100% that were not different at either harvest time (Figure 2).

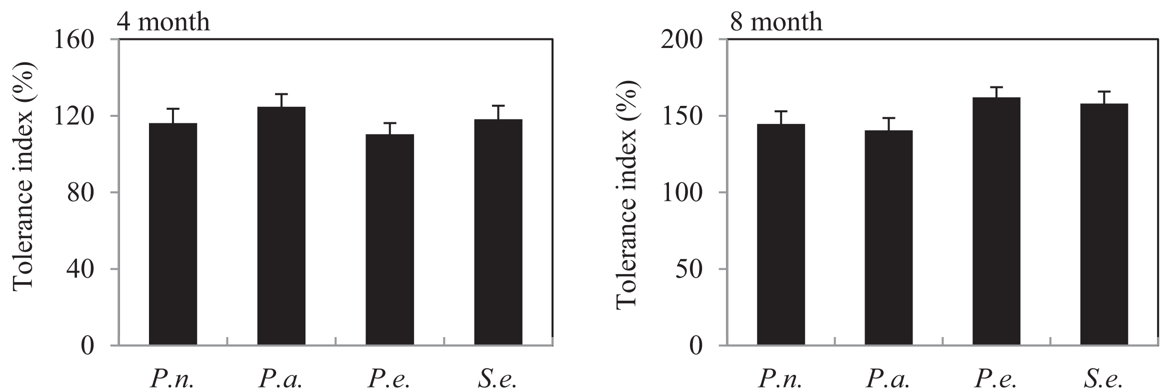

Figure 2. Tolerance index (%) of *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*), and *S. excelsa* (*S.e.*) grown in soil irrigated by wastewater after 4 and 8 months; Values are mean of 6 replications (± SE).

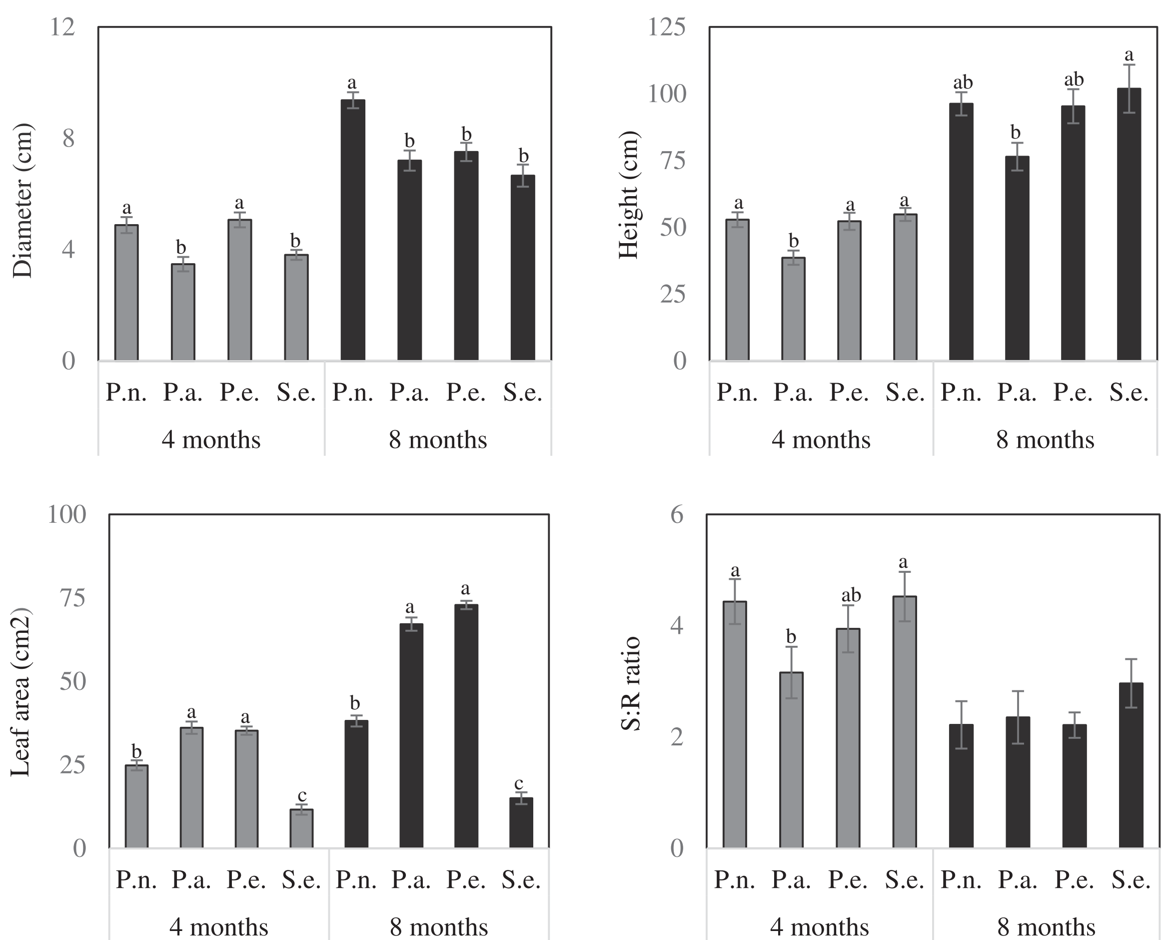

Figure 3. Species main effect for diameter, height, leaf area, and S:R (shoot:root biomass) ratio in *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*), and *S. excelsa* (*S.e.*); Each value represents the mean (± SE) of two soil treatments (SITW and SIWW) for each species (12 replications); Different letters indicate significant differences between species at each period.

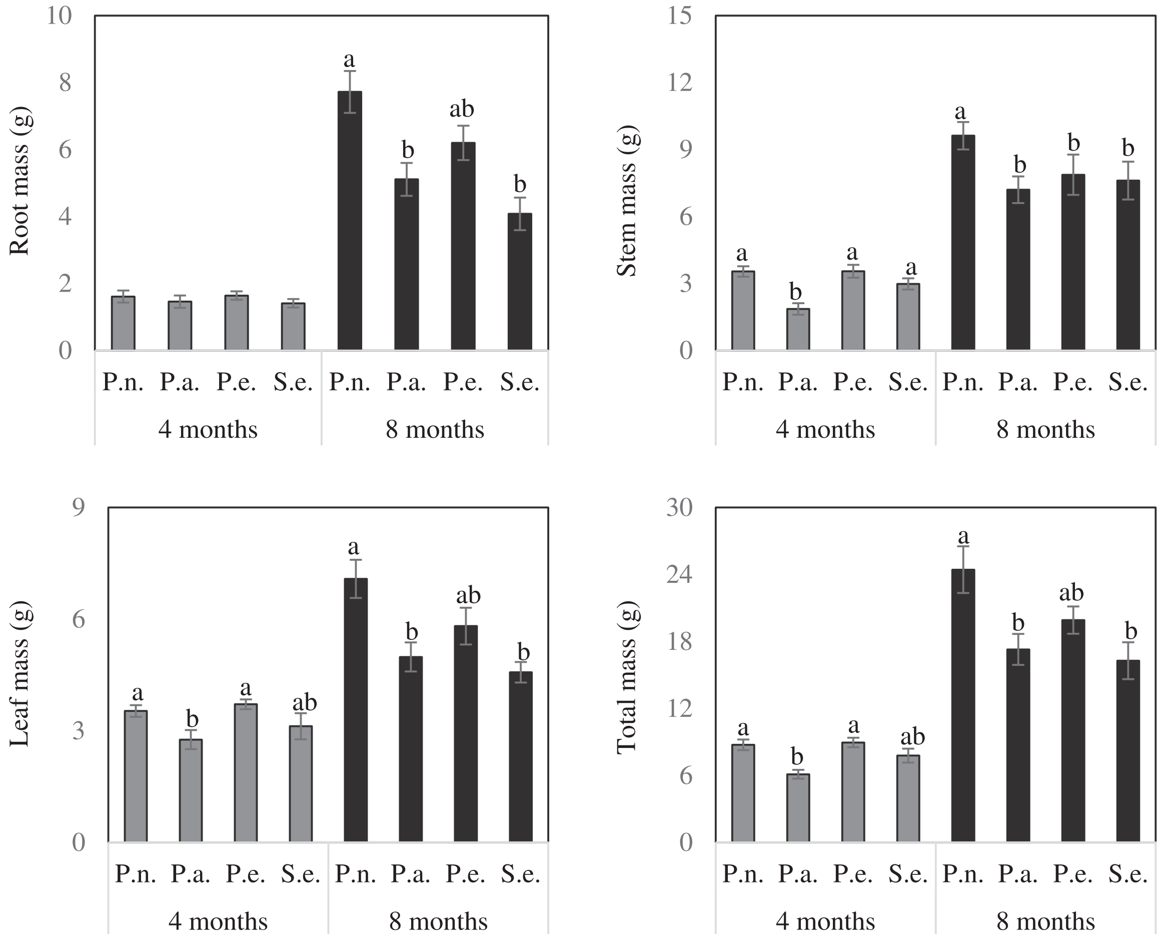

Figure 4. Species main effect for root mass, stem mass, leaf mass, and total mass in *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*), and *S. excelsa* (*S.e.*); Each value represents the mean (± SE) of two soil treatments (SITW and SIWW) for each species (12 replications); Different letters indicate significant differences between species at each period.

The results of the present study indicating that *P. nigra* "62/154," *P. alba* "20/45," *P. euramericana* "92/40," and *S. excelsa* irrigated with wastewater (SIWW treatment) had significantly higher growth and biomass rates compared to control trees irrigated with tap water (SITW treatment) corroborated several studies reporting the positive effects of wastewater irrigation on tree growth and biomass production, indicating fertilizing potential of wastewater (Zalesny *et al*. 2009; Justin *et al.* 2010; Herteman *et al*. 2011; Rasheed *et al*. 2019). For example, Tsakou et al. (2003) for *P. euramericana*, Guo *et al*. (2006) for *Eucalyptus sp*., Justin *et al*. (2010) for *P. deltoides* Bartr. ex Marsh., *S. viminalis* L. and *S. purpurea* L., Ali *et al*. (2013) for *Khaya senegalensis* (Desr.) A. Juss., Houda *et al*. (2016) for *P. alba*, Hussain et al. (2021) for four agroforestry tree species (*Morus alba* L., *Acacia nilotica* (L.) P.J.H. Hurter ex. Mabb., *Acacia ampliceps* Maslin, and *Azadirachta indica* A. Juss.), and Sas *et al*. (2021) for *S. miyabeana* Seemen reported improvement of growth characteristics under wastewater irrigation. Since providing sufficient plant nutrients for optimal growth play a vital role in increasing plant biomass production (Guo and Sims 2003), the observed positive responses following wastewater irrigation is likely due to the high presence of essential plant nutrients like nitrogen and phosphorus in the wastewater (Libutti *et al*. 2018). Nitrogen, phosphorus, and other nutrients improve soil fertility for better biomass production and physiological mechanisms (Larchevêque *et al*. 2006; Lundberg *et al*. 2011). Our studied poplars and willow showed different responses in terms of biomass production, with *P. nigra* exhibiting the greatest total biomass and *S. excelsa* the least biomass. These results are consistent with previous studies reporting broad variability among poplar and willow species for growth and biomass parameters under wastewater irrigation (Justin *et al*. 2010; Houda *et al*. 2016), with differences in growth traits due to species characteristics in resource use and growth (Singh *et al*. 2010). Tolerance index is a useful measure to assess the ability of poplars and willows to grow in heavy-metal contaminated soils. Our findings for tolerance index showed values above 100% for all poplars and willow studied (Figure 2) (i.e., dry biomass of wastewater-irrigated trees was universally greater than for tap water-irrigated control trees), which was similar to other results for poplars and willows (Dos Santos Utmazian et al. 2007), *P.* × *canescens* (Dai *et al*. 2013), and *P. alba* (Houda *et al*. 2016).

Macro- and micro-nutrient concentrations in plant tissues

Despite non-significant effects after four months of growth, continual treatment with wastewater (SIWW) for eight months after planting significantly ($p = 0.000$) increased macronutrient concentrations, including nitrogen (N), phosphorus (P) and potassium (K), in roots, stems and leaves of *P. nigra* "62/154," *P. alba* "20/45," *P. euramericana* "92/40," and *S. excelsa* compared with tap water treatment (SITW). The main effect of species and the soil treatment × species interaction were not significant for macronutrient concentrations of plant tissues after four nor eight months ($p > 0.05$ for all parameters) (Table 4; Figure 5). With the exception of copper (Cu) uptake into roots after four months, SIWW treatment significantly increased ($p = 0.010$ for stem Fe and $p = 0.019$ for leaf Fe at four months and $p = 0.000$ for the rest of the parameters) tissue-specific uptake and accumulation along with total content of micronutrients, including iron (Fe), cupper (Cu), zinc (Zn), and manganese (Mn), in roots, stems and leaves of poplars and willow, relative to control trees after four and eight months ($p = 0.000$). Similarly, except for total Zn content after four months ($p = 0.675$), the species main effect was significant for tissue-specific concentrations and total content of micronutrients in tree tissues (roots + stems + leaves) after four and eight months, while the soil treatment × species interaction was negligible for all combinations ($p > 0.05$ for all parameters) (Tables 5 and 6).

Table 4. Probability values from analyses of variance (ANOVA) testing the effects of soil, species, and soil × species interaction on macronutrient concentrations of plant tissues at two periods.

		Source of variation					Source of variation		
Period	Parameter	Soil	Species	Soil × species	Period	Parameter	Soil	Species	Soil × species
4	Root N	0.184	0.525	0.482	8	Root P	0.000**	0.495	0.738
4	Stem N	0.103	0.133	0.455	8	Stem P	0.000**	0.726	0.610
4	Leaf N	0.215	0.517	0.340	8	Leaf P	0.000**	0.794	0.135
8	Root N	0.000**	0.758	0.928	4	Root K	0.222	0.339	0.536
8	Stem N	0.000**	0.076	0.092	4	Stem K	0.116	0.316	0.502
8	Leaf N	0.000**	0.759	0.240	4	Leaf K	0.158	0.453	0.563
4	Root P	0.155	0.355	0.603	8	Root K	0.000**	0.283	0.653
4	Stem P	0.233	0.536	0.440	8	Stem K	0.000**	0.446	0.730
4	Leaf P	0.144	0.244	0.322	8	Leaf K	0.000**	0.563	0.537

Significant differences are indicated with $^{**}p < 0.01$.

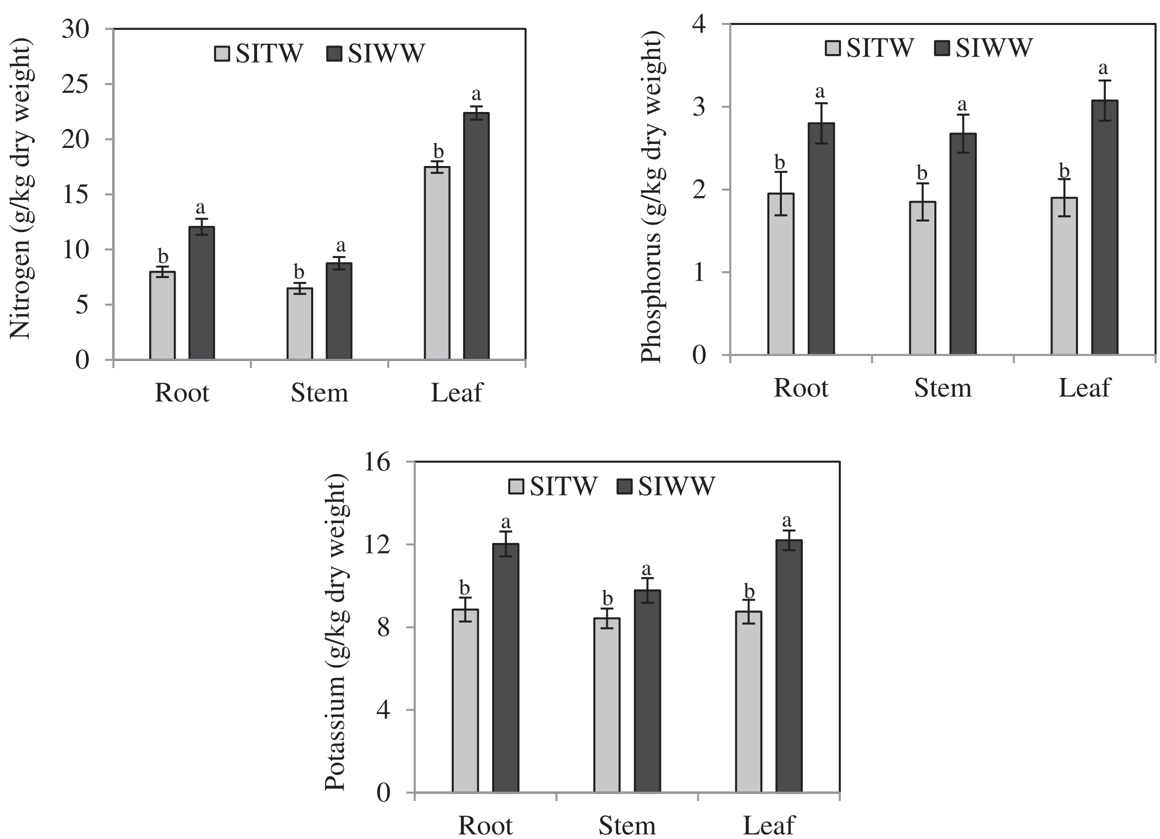

Figure 5. Soil main effect for concentrations of N, P, and K (g/kg) in the roots, stems, and leaves at eight months after planting; each value represents the mean (± SE) of all species (24 replications) in soil irrigated by tap water (SITW) and in soil irrigated by wastewater (SIWW); Different letters indicate significant differences between two soil treatments at each tissue.

Table 5. Effect of wastewater application on micronutrient concentrations in plant tissues.

		Periods					
		4 month			8 month		
		Root	Stem	Leaf	Root	Stem	Leaf
		Fe (mg/kg)					
Soil treatment	SITW	407.69 ± 25.45b	90.58 ± 7.72b	144.31 ± 8.24b	741.03 ± 32.67b	123.37 ± 14.54b	361.98 ± 32.97b
	SIWW	501.32 ± 37.10a	119.75 ± 10.72a	208.45 ± 7.57a	1105.26 ± 50.94a	191.59 ± 19.77a	615.35 ± 53.39a
Species	P.n.	317.15 ± 42.96b	91.87 ± 8.85b	201.13 ± 18.17a	790.53 ± 61.92b	131.04 ± 16.38b	590.22 ± 79.43a
	P.a.	514.13 ± 46.50a	93.45 ± 7.85b	194.59 ± 14.54ab	1048.97 ± 105.16a	146.46 ± 15.85ab	565.15 ± 66.22a
	P.e.	494.68 ± 34.92a	116.58 ± 8.52ab	156.68 ± 19.24b	850.61 ± 70.82b	170.79 ± 18.35a	454.12 ± 48.66b
	S.e.	501.45 ± 37.50a	120.11 ± 7.61a	164.42 ± 15.94ab	1006.78 ± 103.33a	181.63 ± 21.34a	448.91 ± 45.94b
p Values	Soil	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
	Species	0.000**	0.000**	0.032*	0.000**	0.007**	0.000**
	Soil × species	0.383	0.469	0.522	0.105	0.153	0.053
		Zn (mg/kg)					
Soil treatment	SITW	101.60 ± 6.32b	95.59 ± 7.41b	167.78 ± 16.31b	94.34 ± 5.88b	88.47 ± 6.52b	145.63 ± 15.03b
	SIWW	149.02 ± 10.23a	117.78 ± 12.56a	218.73 ± 18.49a	175.52 ± 10.68a	137.85 ± 10.31a	239.13 ± 19.41a
Species	P.n.	114.86 ± 12.29ab	95.47 ± 9.27b	164.35 ± 10.48b	128.36 ± 18.72ab	108.97 ± 15.73b	169.96 ± 12.46b
	P.a.	143.60 ± 17.69a	128.63 ± 10.41a	276.56 ± 28.81a	151.10 ± 24.24a	136.14 ± 16.61a	264.85 ± 29.49a
	P.e.	105.31 ± 10.35b	99.45 ± 7.69b	180.61 ± 22.21b	117.81 ± 14.34b	106.77 ± 9.86b	175.92 ± 20.55b
	S.e.	131.25 ± 12.73a	103.19 ± 8.65ab	166.85 ± 18.38b	142.46 ± 20.28a	100.76 ± 8.84b	158.78 ± 18.95b
p Values	Soil	0.000**	0.010*	0.019*	0.000**	0.000**	0.000**
	Species	0.009**	0.048*	0.01*	0.029*	0.005**	0.003**
	Soil × species	0.407	0.781	0.809	0.396	0.164	0.468
		Cu (mg/kg)					
Soil treatment	SITW	11.94 ± 0.541	8.34 ± 0.315b	8.60 ± 0.234b	9.53 ± 0.214b	8.24 ± 0.218b	8.99 ± 0.354b
	SIWW	12.77 ± 0.561	10.09 ± 0.334a	10.29 ± 0.298a	13.94 ± 0.366a	10.93 ± 0.329a	12.22 ± 0.448a
Species	P.n.	14.94 ± 0.799a	10.48 ± 0.636a	10.80 ± 0.496a	13.52 ± 0.856a	10.60 ± 0.710a	11.73 ± 0.740a
	P.a.	12.84 ± 0.733ab	9.24 ± 0.630ab	9.19 ± 0.430ab	11.89 ± 0.870b	10.03 ± 0.791a	11.03 ± 0.643a
	P.e.	10.91 ± 0.502b	8.60 ± 0.465b	8.87 ± 0.365b	10.98 ± 0.830b	8.84 ± 0.477b	9.79 ± 0.708b
	S.e.	10.74 ± 0.454b	8.55 ± 0.434b	8.94 ± 0.376b	11.01 ± 0.880b	8.89 ± 0.508b	9.87 ± 0.598b
p Values	Soil	0.053	0.000**	0.000**	0.000**	0.000**	0.000**
	Species	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
	Soil × species	0.991	0.186	0.514	0.088	0.101	0.847
		Mn (mg/kg)					
Soil treatment	SITW	111.33 ± 5.75b	68.49 ± 4.98b	115.9 ± 8.85b	99.26 ± 8.95b	59.12 ± 5.85b	95.05 ± 7.52b
	SIWW	136.67 ± 10.71a	84.26 ± 5.42a	145.02 ± 11.23a	134.18 ± 11.99a	80.90 ± 6.72a	140.67 ± 10.43a
Species	P.n.	117.21 ± 6.22ab	71.18 ± 4.33bc	147.23 ± 11.72a	112.43 ± 8.52ab	65.10 ± 5.36b	133.58 ± 12.41a
	P.a.	140.77 ± 8.68a	87.17 ± 5.70a	126.10 ± 7.75ab	130.71 ± 10.39a	80.85 ± 6.76a	114.35 ± 10.17b
	P.e.	98.34 ± 6.56b	67.33 ± 3.30c	119.44 ± 8.25b	90.09 ± 7.39b	61.33 ± 4.47b	110.86 ± 7.06b
	S.e.	138.68 ± 9.07a	83.82 ± 4.56ab	121.76 ± 7.54b	133.65 ± 12.11a	72.75 ± 5.21ab	116.64 ± 7.21b
p Values	Soil	0.000**	0.000**	0.001**	0.000**	0.000**	0.000**
	Species	0.000**	0.000**	0.004**	0.000**	0.001**	0.003**
	Soil × species	0.101	0.818	0.123	0.103	0.655	0.112

Value of each soil treatment represents the means ± SE of all species and value of each species represents the means ± SE of two soil treatments; SITW: Soil irrigated by tap water; SIWW: Soil irrigated by wastewater; *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*) and *S. excelsa* (*S.e.*); Significant differences are indicated with $^{*}p < 0.05$ and $^{**}p < 0.01$; Different letters indicate significant differences.

Continual wastewater irrigation for eight months after planting of *P. nigra* "62/154," *P. alba* "20/45," *P. euramericana* "92/40," and *S. excelsa* enhanced total (roots + stems + leaves) concentrations of all macronutrients relative to trees irrigated with tap water only. Specifically, SIWW treatment increased concentrations of N by 31.8, 40.2, 29.0, and 40.2%, P by 62.3, 43.9, 45.0 and 50.0%, and K by 35.1, 34.9, 26.4, and 26.7% of these four species, respectively. Similarly, after eight months, plants of *P. nigra, P. alba, P. euramericana*, and *S. excelsa* grown in soils irrigated with wastewater (SIWW) exhibited higher total content for micronutrients relative to their SITW counterparts: 128.9, 114.4, 136.9, and 140.0% for Fe, 132.4, 136.3, 163.8, and 175.7% for Zn, 99.5, 95.3, 117.6, and 118.6% for Cu, and 111.1, 89.2, 111.4, and 117.9% for Mn, respectively. Given expected relationships between growth and phytoremediation capacity, trees of the current study with higher growth and biomass rates in SIWW treatment were positively correlated with increased uptake and accumulation of macro- and micro-nutrients in tree tissues. Increasing the concentration and content of nutrients likely was due to the constant addition of fertility through nutrient-rich wastewater. These concentrations were major factors enhancing tree growth and biomass, comprising many metabolically important compounds and serving a fundamental role in a suite of physiological processes necessary for phytoextraction and phytoaccumulation (de Bang *et al.* 2021). These results from the present study corroborated those from previous researchers reporting substantial benefits of wastewater application for increasing nutrients in plant tissues and thereby enhancing plant growth and biomass production: Guo and Sims (2000) for *Eucalyptus globulus* Labill., Patterson *et al.* (2008) for *P. deltoides* × *P. petrowskyana* L., Singh *et al.* (2010) for *Acacia nilotica* (L.) P.J.H. Hurter ex. Mabb., *Dalbergia sissos* Roxb., and *Eucalyptus camaldulensis* Dehnh., and Dimitriou and Aronsson (2011) for a range of poplars and willows.

Our results demonstrated that the studied poplars and willow were significantly different in terms of concentrations and total content of micronutrients in tree tissues (Tables 5 and 6). The greatest concentration of Fe in roots, stems, and leaves, respectively, were measured in *P. alba*, *S. excelsa,* and

Table 6. Effect of wastewater application on total content of micronutrients.

		Periods	
		4 months	8 months
		Total Fe (mg)	
Soil treatment	SITW	4.65 ± 0.532b	18.91 ± 1.18b
	SIWW	6.96 ± 0.654a	43.35 ± 2.69a
Species	P.n.	5.17 ± 0.467ab	37.36 ± 4.68a
	P.a.	4.82 ± .580b	31.15 ± 5.16ab
	P.e.	6.89 ± 0.577a	29.42 ± 5.58b
	S.e.	6.34 ± 0.679a	26.58 ± 5.08b
p Values	Soil	0.000**	0.000**
	Species	0.000**	0.001**
	Soil × species	0.799	0.325
		Total Zn (mg)	
Soil treatment	SITW	2.59 ± 0.118b	5.09 ± 0.330b
	SIWW	4.02 ± 0.250a	12.65 ± 0.970a
Species	P.n.	3.25 ± 0.328	10.34 ± 2.01a
	P.a.	3.40 ± 0.428	9.83 ± 1.81ab
	P.e.	3.45 ± 0.401	8.35 ± 1.70b
	S.e.	3.13 ± 0.274	6.94 ± 1.47b
p Values	Soil	0.000**	0.000**
	Species	0.675	0.001**
	Soil × species	0.674	0.662
		Total Cu (mg)	
Soil treatment	SITW	0.201 ± 0.023b	0.420 ± 0.038b
	SIWW	0.296 ± 0.033a	0.867 ± 0.063b
Species	P.n.	0.316 ± 0.328a	0.883 ± 0.051a
	P.a.	0.192 ± 0.328b	0.579 ± 0.042b
	P.e.	0.255 ± 0.328ab	0.610 ± 0.070ab
	S.e.	0.221 ± 0.328b	0.502 ± 0.039b
p Values	Soil	0.000**	0.000**
	Species	0.000**	0.000**
	Soil × species	0.943	0.051
		Total Mn (mg)	
Soil treatment	SITW	2.02 ± 0.185b	3.92 ± 0.430b
	SIWW	3.18 ± 0.254a	8.13 ± 0.604a
Species	P.n.	3.04 ± 0.259a	7.82 ± 0.608a
	P.a.	2.15 ± 0.234b	5.68 ± 0.591b
	P.e.	2.53 ± 0.134ab	5.28 ± 0.415b
	S.e.	2.69 ± 0.215ab	5.32 ± 0.445b
p Values	Soil	0.000**	0.000**
	Species	0.000**	0.000**
	Soil × species	0.101	0.818

Value of each soil treatment represents the means ± SE of all species and value of each species represents the means ± SE of two soil treatments; SITW: Soil irrigated by tap water; SIWW: Soil irrigated by wastewater; *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*) and *S. excelsa* (*S.e.*); Significant differences are indicated with $^{**}p < 0.01$; Different letters indicate significant differences.

P. nigra, while the greatest concentrations of Zn and Cu in all plant tissues were observed in *P. alba* and *P. nigra*, respectively. *P. nigra* had the most leaf Mn, while *P. alba* had the greatest root and stem Mn (Table 5). After four and eight months, *P. nigra* had the greatest total content of most micronutrients, with one exception: *P. euramericana* had the greatest total Fe content after four months. In contrast, there were no significant differences among species for total Zn content after four months of growth (Table 6). These results agreed with studies of Castiglione *et al.* (2009), Baldantoni *et al.* (2014) and Đelić *et al.* (2018) for different poplar species. Likewise, Zalesny and Bauer (2007) evaluated growth and phytoremediation potential of poplars and willows irrigated with landfill leachate, and they reported that uptake was genotype-specific for most elements. In the present study, uptake of micronutrients was more tissue-specific for Fe and Cu (i.e., mainly being accumulated in roots), whereas Zn and Mn showed higher concentrations in leaves (Table 5). These results are consistent with the findings of many other poplar studies (Laureysens *et al.* 2004; Todeschini *et al.* 2007; Lingua *et al.* 2008; Hu *et al.* 2013).

Heavy metal concentrations in plant tissues

At four and eight months after planting, continual treatment with wastewater (SIWW) significantly ($p = 0.001$ for stem and leaf Pb at four months and $p = 0.000$ for the rest of the parameters) increased heavy metal concentrations, including nickel (Ni), chromium (Cr) and lead (Pb), in roots, stems and leaves of *P. nigra* "62/154," *P. alba* "20/45," *P. euramericana* "92/40," and *S. excelsa* compared with tap water treatment (SITW) (Table 7). After eight months, relative to their SITW counterparts, SIWW treatment enhanced total (roots + stems + leaves) concentrations of Ni by 47.7, 44.4, 42.1, and 47.1%, Cr by 45.4, 42.9, 44.3, and 45.2%, Pb by 33.3, 30.8, 34.5, and 35.2%, respectively, for *P. nigra, P. alba, P. euramericana*, and *S. excelsa*. Similarly, at eight months after planting, trees of *P. nigra, P. alba, P. euramericana*, and *S. excelsa* irrigated with wastewater exhibited higher total content of 113.9, 96.9, 129.8, and 128.7% for Ni, 105.2, 95.0, 133.4, and 129.1% for Cr, and 91.6, 78.7, 121.9, and 113.8% for Pb compared to those irrigated with tap water, respectively. These results corroborated previous findings demonstrating that phytoextraction and phytoaccumulation of heavy metals were higher in poplars and willows grown in heavy-metal contaminated soils compared to control plants (Baldantoni *et al.* 2014; Chandra and Kang 2015; Pilipović *et al.* 2019). In the current study, Ni, Cr and Pb concentrations were higher in roots than leaves and stems, suggesting a functional plant defense strategy to combat heavy metal stress (Viehweger 2014). In this regard, other studies also showed root tissues accumulated greater concentrations of metals than shoot tissues (Shi *et al.* 2011; Baldantoni *et al.* 2014; Pilipović *et al.* 2019). Moreover, our poplars and willow exhibited significant differences in the concentration of heavy metals in roots, stems, and leaves, except stem Pb at four and eight months. At four and eight months after planting, *P. alba* had the highest concentration of Ni and Cr in all plant tissues and Pb in roots, followed by *S. excelsa* having the highest Pb concentration in leaves (Table 7). In contrast, total Ni, Cr, and Pb content at four and eight months was largest for *S. excelsa* and *P. nigra*, respectively (Table 8). Previous studies also confirmed the broad genetic variability of the Salicaceae family for uptake and accumulation of heavy metals under different experimental conditions (Dos Santos Utmazian et al. 2007; Zalesny and Bauer 2007; Castiglione *et al.* 2009; Baldantoni *et al.* 2014).

Conclusions

Increases in heavy metal concentrations in roots, stems, and leaves of *P. nigra* "62/154," *P. alba* "20/45," *P. euramericana* "92/40," and *Salix excelsa* grown in soil irrigated with wastewater did not reduce growth and biomass of the trees after eight months of growth. In contrast, the wastewater application used as irrigation and fertilization resulted in beneficial

Table 7. Effect of wastewater application on heavy metal concentrations in plant tissues.

		Periods					
		4 month			8 month		
		Root	Stem	Leaf	Root	Stem	Leaf
		Ni (mg/kg)					
Soil treatment	SITW	9.25 ± 0.540b	4.09 ± 0.222b	5.53 ± 0.294b	8.28 ± 0.346b	2.69 ± 0.189b	4.05 ± 0.234b
	SIWW	12.67 ± 0.831a	5.13 ± 0.293a	7.04 ± 0.283a	11.86 ± 0.486a	4.03 ± 0.256a	5.96 ± 0.359a
Species	P.n.	10.03 ± 0.90b	4.17 ± 0.341b	5.74 ± 0.290b	9.12 ± 0.810b	2.92 ± 0.400b	4.48 ± 0.346b
	P.a.	12.14 ± 0.92a	5.07 ± 0.272a	7.08 ± 0.287a	11.54 ± 0.970a	3.82 ± 0.320a	5.69 ± 0.458a
	P.e.	9.56 ± 0.652b	4.19 ± 0.243b	5.57 ± 0.284b	8.46 ± 0.650b	2.94 ± 0.280b	4.57 ± 0.397b
	S.e.	12.12 ± 0.781a	5.02 ± 0.331a	6.98 ± 0.315a	11.11 ± 0.810a	3.76 ± 0.370a	5.48 ± 0.462a
p Values	Soil	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
	Species	0.000**	0.004**	0.000**	0.000**	0.004**	0.000**
	Soil × species	0.667	0.663	0.263	0.321	0.663	0.104
		Cr (mg/kg)					
Soil treatment	SITW	10.86 ± 0.702b	1.34 ± 0.091b	2.03 ± 0.130b	11.89 ± 0.740b	1.46 ± 0.107b	2.41 ± 0.129b
	SIWW	15.77 ± 1.18a	2.33 ± 0.131a	3.09 ± 0.292a	16.67 ± 1.25a	2.79 ± 0.125a	3.59 ± 0.126a
Species	P.n.	10.45 ± 0.790b	1.81 ± 0.291ab	2.64 ± 0.281ab	11.47 ± 0.731b	2.01 ± 0.301ab	3.04 ± 0.322ab
	P.a.	16.53 ± 1.44a	2.13 ± 0.232a	2.85 ± 0.240a	17.68 ± 1.42a	2.25 ± 0.241a	3.29 ± 0.262a
	P.e.	10.05 ± 0.770b	1.45 ± 0.210b	1.94 ± 0.271b	10.54 ± 0.931b	1.43 ± 0.224b	2.39 ± 0.288b
	S.e.	16.16 ± 1.23a	2.06 ± 0.215a	2.83 ± 0.222a	17.21 ± 1.22a	2.23 ± 0.220a	3.26 ± 0.241a
p Values	Soil	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
	Species	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
	Soil × species	0.053	0.456	0.743	0.06	0.483	0.570
		Pb (mg/kg)					
Soil treatment	SITW	8.72 ± 0.490b	2.49 ± 0.210b	3.54 ± 0.370b	8.07 ± 0.349b	2.79 ± 0.313b	3.71 ± 0.256b
	SIWW	10.80 ± 0.562a	4.29 ± 0.360a	5.39 ± 0.550a	9.86 ± 0.594a	4.09 ± 0.430a	5.59 ± 0.450a
Species	P.n.	9.48 ± 0.391ab	3.17 ± 0.301	4.02 ± 0.360b	8.61 ± 0.462ab	3.22 ± 0.304	4.06 ± 0.363b
	P.a.	10.88 ± 0.570a	3.68 ± 0.322	3.93 ± 0.242b	9.70 ± 0.421a	3.93 ± 0.382	4.21 ± 0.415b
	P.e.	8.84 ± 0.341b	3.25 ± 0.404	4.66 ± 0.356ab	7.89 ± 0.420b	3.29 ± 0.384	4.88 ± 0.481b
	S.e.	10.81 ± 0.592a	3.26 ± 0.231	5.66 ± 0.460a	9.46 ± 0.405a	3.31 ± 0.309	5.44 ± 0.501a
p Values	Soil	0.000**	0.001**	0.001**	0.000**	0.000**	0.000**
	Species	0.000**	0.104	0.004**	0.000**	0.114	0.001**
	Soil × species	0.777	0.998	0.775	0.944	0.988	0.180

Value of each soil treatment represents the means ± SE of all species and value of each species represents the means ± SE of two soil treatments; SITW: Soil irrigated by tap water; SIWW: Soil irrigated by wastewater; *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*) and *S. excelsa* (*S.e.*); Significant differences are indicated with $^{**}p < 0.01$; Different letters indicate significant differences.

Table 8. Effect of wastewater application on total content of heavy metals.

		Periods	
		4 months	8 months
		Total Ni (mg)	
Soil treatment	SITW	0.136 ± 0.008b	0.230 ± 0.009b
	SIWW	0.208 ± 0.011a	0.498 ± 0.02a
Species	P.n.	0.173 ± 0.019b	0.436 ± 0.051a
	P.a.	0.149 ± 0.017b	0.364 ± 0.041b
	P.e.	0.175 ± 0.013b	0.331 ± 0.044b
	S.e.	0.191 ± 0.019a	0.345 ± 0.032b
p Values	Soil	0.000**	0.000**
	Species	0.017*	0.031*
	Soil × species	0.661	0.690
		Total Cr (mg)	
Soil treatment	SITW	0.100 ± 0.015b	0.239 ± 0.018b
	SIWW	0.176 ± 0.021a	0.511 ± 0.0021a
Species	P.n.	0.131 ± 0.015b	0.416 ± 0.056a
	P.a.	0.133 ± 0.018b	0.398 ± 0.031ab
	P.e.	0.122 ± 0.014b	0.288 ± 0.025b
	S.e.	0.166 ± 0.021a	0.385 ± 0.021ab
p Values	Soil	0.000**	0.000**
	Species	0.007**	0.000**
	Soil × species	0.482	0.305
		Total Pb (mg)	
Soil treatment	SITW	0.119 ± 0.007b	0.224 ± 0.009b
	SIWW	0.175 ± 0.010a	0.447 ± 0.021a
Species	P.n.	0.159 ± 0.010a	0.401 ± 0.041a
	P.a.	0.122 ± 0.011b	0.312 ± 0.032b
	P.e.	0.160 ± 0.013a	0.329 ± 0.035b
	S.e.	0.169 ± 0.014a	0.307 ± 0.042b
p Values	Soil	0.000**	0.000**
	Species	0.001**	0.000**
	Soil × species	0.913	0.143

Value of each soil treatment represents the means ± SE of all species and value of each species represents the means ± SE of two soil treatments; SITW: Soil irrigated by tap water; SIWW: Soil irrigated by wastewater; *P. nigra* (*P.n.*), *P. alba* (*P.a.*), *P. euramericana* (*P.e.*) and *S. excelsa* (*S.e.*); Significant differences are indicated with $^{*}p < 0.05$ and $^{**}p < 0.01$; Different letters indicate significant differences.

effects on these traits while enriching the soil with important essential nutrients. Likewise, due to the uptake and accumulation of heavy metals in plant tissues, these poplars and willow could be planted for phytoremediation to improve the properties of heavy-metal contaminated soils. Therefore, wastewater could be considered as an alternative source of irrigation that may solve challenges associated with disposal while reducing risks of environmental concerns. The current results corroborated those previously reported that poplars and willows have considerable potential for planting in soils impacted by urban wastewaters. Given the variability in the responses across the four species tested, future studies testing a greater number of genotypes and conducted in the field for longer periods of time are warranted. Such studies may be useful for corroborating current trends where *P. nigra* had the greatest biomass production and total heavy metal content, while phytoextraction and phytoaccumulation, which were tissue-specific, were best for *P. alba* and *S. excelsa*.

Disclosure statement

The authors report there are no competing interests to declare.

Funding

This work was funded by the Research Institute of Forests and Rangelands of Iran (RIFR); and Iran's National Elites Foundation.

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