

Assessment of the adaptive and phytoremediation potential of *Miscanthus×giganteus* grown in flotation tailings

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Abstract: Mining activities produce enormous amounts of metal-contaminated waste that is the source of ecosystem pollution by metals. Owing to complex adverse environmental conditions, the surface of abandoned flotation tailings is completely devoid of vegetation cover and is therefore very susceptible to fluvial erosion, wind dispersal to neighboring ecosystems and leaching of heavy metals into ground waters. The aim of this study was to estimate the adaptive potential of *Miscanthus×giganteus* (Poaceae) to grow on flotation tailings without any input. In this field experiment, plants were grown for four months in flotation tailings and in unpolluted control chernozem soil. Plants accumulated and retained the major part of metals within their roots, exhibiting their very low transfer to aerial parts, which all define *M.×giganteus* as a phytoexcluder plant species. Plants grown in flotation tailings showed significant reduction in the net CO₂ assimilation rate and growth parameters, and there was no negative impact on pigment content, maximum quantum yield of PSII photochemistry, lipid peroxidation level and total antioxidative capacity in leaves. The obtained results indicate that despite reduced growth, *M.×giganteus* can be cultivated for phytoremediation of flotation tailings.

Keywords: *Miscanthus×giganteus*; chlorophyll *a* fluorescence; lipid peroxidation; heavy metals; photosynthesis

Abbreviations: 1,1-diphenyl-2-picrylhydrazyl (DPPH); 2-thiobarbituric acid (TBA); net CO₂ assimilation rate (A); concentration of chlorophyll (Chl); concentration of total carotenoids (Car); malondialdehyde (MDA); maximum quantum efficiency of PSII photochemistry (Fv/Fm); stomatal conductance (g_s); reactive oxygen species (ROS); transpiration rate (E)

INTRODUCTION

Mining activities and ore processing produce large amounts of fine-grained waste material that is discarded with water as mud into the flotation tailing pond. Flotation tailings that remain after the abandonment of a mine are distinguished by multiple unfavorable physical and chemical properties, such as surface mobility, poor granulometric structure and sturdiness, absence of organic matter and macronutrients and high metal contamination, which altogether seriously aggravate plant growth [1,2]. As a result, its surface is devoid

of vegetation cover and is extremely prone to fluvial erosion and wind dispersal. Its impact on the environment can be summarized as: (i) run-off of heavy metals into the surface and ground waters; (ii) precipitation of fine-grained particles on adjacent cultivated and grazing land and forests; (iii) bioabsorption of metals and their transfer between trophic levels; (iv) overall loss of ecosystem productivity and biodiversity.

The ecological restoration of damaged and destroyed ecosystems involves the introduction and cultivation of suitable plant species with the aim of

initiating natural processes of re-establishment of an ecosystem's form and functions [3]. Ecological restoration of a specific ecosystem requires evaluation of the local climate, the physical and chemical properties of the substrate and the selection of the most appropriate plant species. Sustainable managements of technosols, such as flotation tailings, can be achieved by phytostabilization, an economically acceptable and eco-friendly phytoremediation procedure. Phytostabilization reduces the risk of heavy metal contamination of natural ecosystems by immobilizing the pollutants within the soil and decreasing their bioavailability [4]. It is achieved by chelation of metals in the rhizosphere by root exudates, or metal absorption and their accumulation in roots. In phytostabilization, plant roots mechanically bind substrate particulates and render surface layers less prone to erosion, reducing water percolation and metal leaching to underground water. The effectiveness of phytostabilization depends on the plant's tolerance to metal contamination and its ability to accumulate metals in its underground organs. Only plant species tolerant to the prolonged and extreme multiple stress conditions that dominate at flotation tailings can form a self-sustainable vegetation cover and improve the ecological conditions in its surface layers by their presence and biological activity. Plants suitable for phytostabilization are those that develop strong roots with high efficiency in erosion control and heavy metal absorption. Effective biological restoration and phytostabilization of heavy metal polluted soils in temperate climates have already been achieved with grasses belonging to genera *Festuca*, *Poa* and *Agrostis* and legumes such as *Medicago* and *Vicia* [5].

Heavy metals cause a series of harmful effects on physiological processes, from gene transcription to photosynthesis and respiration [6-8]. They induce disorder in nutrient uptake and lead to a deficiency of some essential microelements in a plant's organism or inhibit the activity of different enzymes substituting their optimal metal cofactor [8]. When present in higher concentrations, transition metals increase ROS generation in Heber-Weiss and Fenton reactions. The formed ROS induce oxidative damage to membrane lipids, proteins, pigments and nucleic acids, thus disrupting the structure of various cellular components and their related processes. Oxidative damage to membrane unsaturated fatty acids results in the formation of MDA, which is therefore a good indicator

of ROS-induced lipid membrane oxidative damage. In order to remove ROS, plants have developed an efficient antioxidative system whose nonenzymatic component is involved in direct ROS detoxification.

The photosynthetic process is very sensitive to heavy metal stress because it induces changes in the photosynthetic pigment content, activities of enzymes involved in CO₂ fixation, structure of photosystem II and chloroplast ultrastructure [9,10]. As a result, both the CO₂ assimilation rate and chlorophyll *a* fluorescence parameters are disturbed by heavy metal stress and therefore are very good indicators of elemental disorders in a plant organism.

Both light-harvesting pigments, chlorophylls and carotenoids, are very sensitive to environmental stress and variations in their amounts and ratios represent a reliable marker of plant stress. Heavy metals can affect the pigment content in leaves directly, causing inhibition of activities of enzymes involved in their biosynthetic pathways or substitution of the central Mg ion in the chlorophyll molecule by other divalent metal cations, or indirectly, causing ROS-induced oxidative damage [11]. Carotenoids are a large group of pigments that perform different functions in the plant cell, and are involved in direct ROS detoxification.

Miscanthus×giganteus J. M. Greef & Deuter (Poaceae) is a triploid hybrid grass native to southeastern Asia originating from the natural crossing of *M. sinensis* and *M. sacchariflorus* [12]. This highly productive, perennial, rhizomatous sterile hybrid is vegetatively propagated by rhizome cuttings. It has a rapidly growing and extensive root system, which is very efficient in nutrient and water supply. Owing to its tolerance to heavy metals, organic pollutants, nutrient deficiency, drought and low temperatures, it remains highly productive in different climatic conditions and on contaminated, degraded and marginal lands [13-17]. It displays large biomass yield and is successfully cultivated worldwide for renewable energy production [18,19]. Comprehensive investigations into its heavy metal accumulation and its potential use for aided phytoremediation of heavy metal polluted soils, fly ash or technosols have been performed [20-26]. However, there is little information on its growth in the flotation tailings and its possible cultivation for their phytostabilization [27,28].

On the basis of its tolerance to various environmental stress conditions, we hypothesized that *M. × giganteus* possesses a good capability to grow in flotation tailings and provide phytostabilization effects. The high heavy metal content in flotation tailings is expected to change the amounts of chlorophyll and carotenoids, induce ROS generation, increase membrane lipid peroxidation and reduce photosynthetic efficiency and growth of *M. × giganteus* plants. The aims of this field study were to (i) determine metal accumulation in the plant's underground and above-ground organs; (ii) assess the adaptive response of *M. × giganteus* to heavy metal stress, and (iii) evaluate its capability for phytostabilization of flotation tailings.

MATERIALS AND METHODS

Site description

A microplot field experiment was carried out in 2016. It was set on two localities: (i) in the flotation tailings of the mine "Rudnik", and (ii) at an unpolluted site at the Institute for Application of Nuclear Energy (INEP), Zemun, Serbia (Supplementary Fig. S1A).

The mine "Rudnik" is located on Mt. Rudnik in central Serbia (44°06'N, 20°29'E). During the last fifty years, over 80% of the estimated ore has been excavated, which is more than 10 million tons. Lead, zinc and copper are extracted after the mechanical crushing of the polymetallic ore into small-sized particles ($\phi=75\ \mu\text{m}$) and the flotation process of their minerals. The resulting flotation tailings are discharged with water as mud into a tailing pond, bordered by natural birch forest and grazing fields (Supplementary Fig. S1B). The section of the flotation tailings that is temporarily abandoned was used as an experimental field for plant cultivation (Supplementary Fig. S1C, D). The control plants were cultivated at INEP (44°51'N, 20°22'E) in unpolluted, high quality carbonate chernozem soil [29].

Temperatures and precipitation at each experimental site during the growth period [30] are shown in Supplementary Table S1.

Plant material

The planted rhizomes derived from the clonal *M. × giganteus* grown in the field at INEP. The material

is the clone of *M. × giganteus* purchased as rhizomes from Walter Kellner (Austria) in 2007, since when it has been continuously cultivated at INEP.

Experimental design

Pre-grown *M. × giganteus* rhizomes 7-10 cm long were planted at the beginning of March 2016. They were planted at 10-cm depth with a planting density of 2 rhizomes per m^2 (equivalent to 20000 plants ha^{-1}). The experimental plants were organized in four plots (2.5m×2.0m=5 m^2 each) with a 2-m distance from each other. The substrate and plant material were sampled for analysis in June 2016.

Determination of the chemical properties of the substrate

The control soil and flotation tailings were sampled up to 20-cm depth, which corresponds to the depth at which the major portion of plant roots was present at the time of plant sampling.

For determination of pH, actual ($\text{pH}_{\text{H}_2\text{O}}$) and exchangeable (pH_{KCl}), 25 mL of double distilled water or 25 mL 1M KCl were added to 10 g of soil and stirred for 30 min. The pH values were measured directly in the suspensions (Iskra MA 5730, Slovenia).

Organic carbon was measured [31] and was used for calculation of organic matter [32].

The total contents of elements (Pb, Cu, Zn) in the soil were determined after the digestion of sieved soil samples (<200 μm) in HCl:HNO₃ (3:1, v/v) according to method 3051 [33]. The total nitrogen content was determined by Kjeldahl digestion [34].

In order to determine the content of the available forms of metals in the soil, dried and sieved soil samples were mixed continuously for 2 h in 1 M ammonium acetate and 0.01 M EDTA mixture (pH 7), and then filtered (Sartorius filter paper No. 391) [35]. Available forms of phosphorus and potassium were analyzed using the standard AL-method [36].

Concentrations of metals in samples were determined using an atomic absorption spectrophotometer (Shimadzu AA 7000, Japan) by comparing the obtained absorption values with those of known standards.

Determination of metal accumulation in the plant

Concentrations of metals were determined in roots, rhizomes, stems and leaves. After sampling, the plant material was thoroughly washed in tap water; roots and rhizomes were also washed using an ultrasonic cleaner in order to avoid surface contamination of samples by small substrate particulates. All plant material was washed in deionized water. Air-dried plant material was ground with a ceramic mortar and pestle and dried at 105°C to constant weight. Powdered plant material was digested in HCl:HNO₃ (3:1, v/v) [33]. Concentrations of metals in samples were determined with an atomic absorption spectrophotometer by comparison of their absorption values with those of known standards.

Assessment of plant phytostabilization potential

The phytostabilization potential of *M. × giganteus* was estimated using the bioconcentration factor (BCF) and translocation factor (TF) [37], and calculated as follows:

$$\text{BCF} = \frac{\text{metal concentration in roots}}{\text{available metal concentration in the substrate}}$$

$$\text{TF} = \frac{\text{metal concentration in leaves}}{\text{metal concentration in roots}}$$

Gas exchange rates and chlorophyll *a* fluorescence

Measurements of gas exchange rates and chlorophyll *a* fluorescence were performed on an intact penultimate leaf using an infrared gas analyzer (CIRAS 2, PP System, USA) equipped with a chlorophyll fluorescence module (CFM). The net CO₂ assimilation rate (*A*), transpiration rate (*E*) and stomatal conductance (*g_s*) were measured on the penultimate leaf enclosed in a universal automatic PLC (U) cuvette. Conditions in the leaf chamber were as follows: leaf temperature 25°C, relative air humidity 60%, 380 μmol mol⁻¹ CO₂. The light response curves (*A/PPFD*) were obtained at descending photosynthetic photon flux density (PPFD) values from 2000 to 0 μmol m⁻² s⁻¹.

The CFM provides measurements of chlorophyll fluorescence using the pulse amplitude modulation (PAM) technique. For measuring fluorescence parameters, leaves were pre-darkened for 30 min before ex-

posure to the saturation pulse of actinic light. Before the saturating pulse was applied, the minimal level of fluorescence in the dark (*F_o*) was measured. A saturating flash of 5100 μmol photon m⁻² s⁻¹ and duration of 0.7 s was then applied to the leaf in order to obtain the maximal fluorescence yield (*F_m*). At the *F_m* level, all PSII electron acceptors were fully reduced, preventing photochemistry. The parameters *F_o* and *F_m* were used to calculate the maximum quantum efficiency of PSII photochemistry (*F_v/F_m*). All measurements were performed in the midmorning.

Pigment quantification

The amounts of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids (Car) in leaves were detected [38]. The absorption of Chl *a*, Chl *b* and Car were determined by UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) at 663 nm, 645 nm and 480 nm, respectively. The pigment contents were calculated [39,40] and their amounts were expressed as mg g⁻¹ of the leaf dry weight (dw).

Lipid peroxidation

The lipid peroxidation in the penultimate leaf was determined by measuring the amount of MDA produced in the reaction with TBA [41]. The absorbance of the extract was measured spectrophotometrically at 450 nm, 532 nm and 600 nm. The level of lipid peroxidation was expressed as μmol MDA g⁻¹ dw.

Determination of total antioxidant capacity

The total antioxidant capacity of the penultimate leaf was determined using the stable free radical DPPH [42]. Spectrophotometric measurements were performed at 517 nm. The total antioxidant capacity of samples was expressed as relative to control plants (100%).

Plant biometric parameters

Before the harvest, the following plant biometric parameters were measured: number of shoots per rhizome, number of leaves, stem height, width of the stem at 10 cm from the ground, penultimate leaf length and width. The percentage of senescent leaves per plant was calculated.

Statistical analysis

All data are expressed as the mean \pm standard deviation (SD) of six replicates per treatment. The obtained results between two groups were compared using the Mann-Whitney U Test ($p < 0.05$). Spearman's rank correlation coefficient was performed in order to analyze the relationships between measured parameters. Statistical analyses were performed in R (v3.5.1; R Core Team 2018).

RESULTS

Chemical characteristics of the control soil and flotation tailings

The pH values of the control soil (C) and flotation tailings (FT) were very similar, both being neutral, as shown in Table 1. The $\text{pH}_{\text{H}_2\text{O}}$ of the control soil was higher by more than a half of a unit than pH_{KCl} , whereas $\text{pH}_{\text{H}_2\text{O}}$ and pH_{KCl} of the FT were very similar. Although C- and FT- substrates contained similar percentages of organic matter, they showed pronounced differences in N, P and K concentrations. Total available N and K were 60-fold and 42-fold lower in FT than in C soil, respectively, whereas available P in the FT was under the detection limit. The total amounts of Pb, Cu and Zn in the FT were 81-, 43- and 43-fold higher in comparison with their concentrations in control soil, respectively (Table 1). Although their available forms represent a small portion of their total amounts in the FT, their concentrations were far higher in comparison with those detected in the control soil.

Table 1. Chemical characteristics of the control soil and flotation tailings with total and EDTA-available metal concentrations.

Chemical properties	Control	Flotation tailings
$\text{pH}_{\text{H}_2\text{O}}$	6.78 ± 0.00 a	6.58 ± 0.00 b
pH_{KCl}	6.04 ± 0.01 a	6.47 ± 0.02 b
Total N (%)	0.12 ± 0.02 a	0.002 ± 0.0001 b
Available P_2O_5 (mg / 100g soil)	11.1 ± 0.2 a	< DL b
Available K_2O (mg /100 g soil)	23.7 ± 0.1 a	0.56 ± 0.07 b
Organic matter (%)	3.47 ± 0.06 a	2.78 ± 0.07 b
Total concentrations (mg kg^{-1})		
Pb	23.3 ± 0.9 a	1892 ± 4 b
Cu	20.8 ± 0.5 a	893 ± 12 b
Zn	82.7 ± 0.5 a	3544 ± 104 b
Available concentrations (mg kg^{-1})		
Pb_{EDTA}	6.02 ± 0.24 a	525 ± 0.4 b
Cu_{EDTA}	6.90 ± 0.45 a	83.8 ± 2.9 b
Zn_{EDTA}	2.70 ± 0.3 a	541 ± 8 b

< DL – lower than detection limit. The same letters indicate no significantly different values according to Mann-Whitney U test ($p \leq 0.05$).

Metal accumulation by plants

Plants cultivated in the flotation tailings accumulated significantly higher concentrations of Pb, Cu and Zn in all plant organs compared to the control plants (Table 2). The highest concentrations of all metals in both C and FT plants were detected in roots. In FT plants, $\text{BCF} > 1$ was detected only for Cu, whereas the BCF for Zn was slightly lower than 1, indicating a high level of accumulation in roots. TFs for all analyzed metals were significantly below 1.

Table 2. Metal accumulation in *M. x giganteus* grown in control soil (C plants) and the flotation tailings (FT plants), and BCF and TF of plants grown on the flotation tailings.

	Pb		Cu		Zn	
	C plants	FT plants	C plants	FT plants	C plants	FT plants
Root (mg kg^{-1})	4.00 ± 1.47 a	222 ± 0.35 b	8.22 ± 0.78 a	124 ± 16 b	24.7 ± 0.7 a	511 ± 17 b
Rhizome (mg kg^{-1})	<DL a	38.8 ± 7.4 b	5.32 ± 1.24 a	17.4 ± 6.6 b	11.8 ± 0.7 a	47.8 ± 0.7 b
Stem (mg kg^{-1})	<DL a	42.1 ± 10.3 b	3.47 ± 0.64 a	7.81 ± 0.63 b	14.6 ± 0.4 a	43.3 ± 1.2 b
Leaves (mg kg^{-1})	<DL a	47.9 ± 0.9 b	6.23 ± 0.66 a	9.14 ± 0.16 b	18.1 ± 1.2 a	64.8 ± 2.6 b
BCF		0.42		1.48		0.94
TF		0.22		0.07		0.13

<DL – lower than detection limit; BCF – bioconcentration factor; TF – translocation factor. The same letters indicate no significantly different values according to Mann-Whitney U test ($p \leq 0.05$).

Physiological and biochemical parameters

Plants grown in the flotation tailings exhibited a significantly reduced net CO₂ assimilation rate (A) in comparison with control plants, which was especially pronounced at higher light intensities, as described by the light response curves (A/PPFD) (Fig. 1). Control plants reached their highest mean photosynthetic activity (A=16 μmol CO₂ m⁻² s⁻¹) at the highest applied PPFD of 2000 μmol photon m⁻² s⁻¹, whereas FT plants reached their maximum A (9.2 μmol CO₂ m⁻² s⁻¹) at far lower light intensity (1000 μmol photon m⁻² s⁻¹).

The parameters of gas exchange (A, E, g_s) at a light intensity of 1000 μmol photon m⁻² s⁻¹ were significantly lower in FT plants than in the control ones, as shown in Table 3. The maximum quantum yield of PSII photochemistry (Fv/Fm) was similar between treatments. Moreover, no statistically significant difference was found in pigment contents (Chl a, Chl b, Chl a+b, Car) between control and FT plants. However, the slightly higher concentration of Chl a in FT plants led to a significantly higher Chl a/b ratio in comparison to that measured in the control. There were no statistically significant differences in MDA concentration and total antioxidative capacity between the two treatments (Table 3).

Biometric parameters

Plants grown on the flotation tailings showed significantly smaller plant dimensions: stem height and stem width, penultimate leaf length and width (Table 4). Also, they had a significantly higher percentage of senescent leaves per plant.

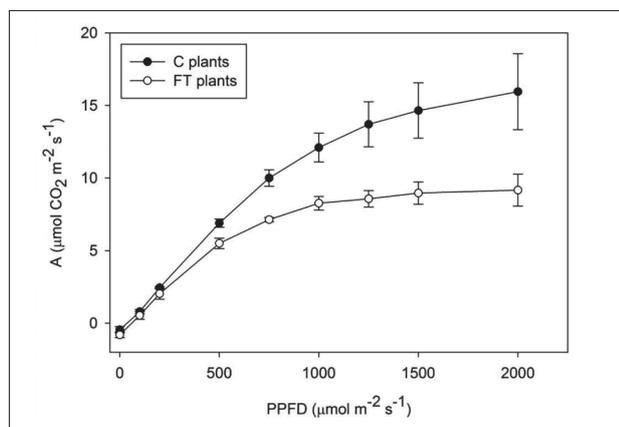


Fig. 1. Light response curves (A/PPFD) of *M. x giganteus* plants cultivated in the control soil (C plants) and flotation tailings (FT plants).

Table 3. Physiological and biochemical parameters of leaves in *M. x giganteus* cultivated in control soil (C plants) and flotation tailings (FT plants).

Parameters	C plants	FT plants
Gas exchange		
A (μmol CO ₂ m ⁻² s ⁻¹)	12.1 ± 1.0 a	8.27 ± 0.47 b
g _s (mmol m ⁻² s ⁻¹)	110 ± 33 a	63.7 ± 10.5 b
E (mmol m ⁻² s ⁻¹)	1.90 ± 0.42 a	1.37 ± 0.31 b
Chlorophyll a fluorescence		
Fv/Fm	0.79 ± 0.01 a	0.79 ± 0.01a
Pigment content		
Chl a (mg g ⁻¹ dw)	5.04 ± 0.42 a	5.55 ± 1.04 a
Chl b (mg g ⁻¹ dw)	1.08 ± 0.13 a	1.06 ± 0.16 a
Chl a+b (mg g ⁻¹ dw)	6.12 ± 0.54 a	6.61 ± 1.20 a
Chl a/b	4.67 ± 0.23 a	5.22 ± 0.24 b
Car (mg g ⁻¹ dw)	1.32 ± 0.29 a	1.23 ± 0.19 a
Lipid peroxidation		
MDA (μmol g ⁻¹ dw)	1.98 ± 0.13 a	1.61 ± 0.20 a
Total antioxidant capacity		
DPPH (%)	100 a	94.5 a

A – net CO₂ assimilation rate; g_s – stomatal conductance; E – transpiration rate; Fv/Fm – maximum quantum efficiency of PSII photochemistry; Chl – chlorophyll; Car – total carotenoids; MDA – the amount of formed malondialdehyde; DPPH – capacity of leaf extract to reduce DPPH, expressed as relative to the control plants. The same letters indicate no significantly different values according to Mann-Whitney U test (p≤0.05).

Table 4. Biometric parameters of *M. x giganteus* plants grown in control soil (C plants) and the flotation tailings (FT plants).

Parameter	C plants	FT plants
Survival (%)	100 a	100 a
No. shoots per plant	1.63 ± 0.77 a	1.81 ± 0.75 a
Stem height (cm)	23.9 ± 6.1 a	12.0 ± 5.8 b
Stem width (mm)	7.5 ± 1.7 a	5.6 ± 1.2 b
No. leaves per plant	7.7 ± 1.2 a	5.6 ± 1.0 a
Dry leaves (%)	0 a	3.6 b
Penultimate leaf length (cm)	53.8 ± 6.3 a	29.6 ± 7.0 b
Penultimate leaf width (mm)	14.3 ± 1.5 a	12.9 ± 3.4 b

Survival – percentage of plants survived from the initial 100% of planted rhizomes. The same letters indicate no significantly different values according to Mann-Whitney U test (p≤0.05).

DISCUSSION

Flotation tailings represent a specific substrate classified as technosol. Having in mind its origin, we expected to detect very high concentrations of heavy metals acquired from the parent rock, predominantly Pb, Cu and Zn, which were the main metals extracted from the ore. The total Pb, Cu and Zn concentrations in the flotation tailings were in the range characteristic for soils heavily contaminated by sewage sludge, metal

smelting processes and industrial sources of pollution, and were far higher than in unpolluted soils [43]. Their available concentrations were greater than those considered toxic to most plants ($>30 \text{ mg kg}^{-1}$ for Pb, $>20 \text{ mg kg}^{-1}$ for Cu, $>300 \text{ mg kg}^{-1}$ for Zn) and background values detected in different soils in the world ($5\text{-}90 \text{ mg kg}^{-1}$ for Pb, $14\text{-}109 \text{ mg kg}^{-1}$ for Cu, $60\text{-}89 \text{ mg kg}^{-1}$ for Zn) [43]. Flotation tailings are characterized by the complete absence of vegetation cover, mosses and lichens on the surface. Therefore, it can be inferred that the relatively high percentage of organic matter detected in the flotation tailings derives from the used organic collector xanthate that remained after the flotation process.

In this real-field experiment, *M. x giganteus* was shown to be capable of developing and growing in flotation tailings loaded with heavy metals. By far the largest portions of accumulated Pb, Cu and Zn were retained in the roots, which corresponded to data reported for different *Miscanthus* species grown on metal polluted substrates [29,44-46]. Such pronounced retention of metals by the roots is due to their effective immobilization, mostly within the cell walls of the cortex parenchyma cells, and efficiently prevented transport toward the stele and aerial parts by the Casparian strip [47,48]. Although metal concentrations in roots exceeded those that are toxic to shoots in most plants, their critical concentrations in roots are expected to be much higher, mostly due to their immobilization in the extracellular spaces of the root cortex. As a result of the relatively low transfer of all three metals to leaves, their translocation factors were far below 1, being 0.07 for Cu, 0.13 for Zn, and 0.22 for Pb. Owing to their low translocation to aboveground organs, the concentrations of Cu and Zn in both stems and leaves were under the critical values considered toxic to various plants ($20\text{-}100 \text{ mg kg}^{-1}$ for Cu, $100\text{-}200 \text{ mg kg}^{-1}$ for Zn) [43,49,50]. In contrast to Cu and Zn, which are necessary in small amounts for normal plant functioning, Pb has no physiological role in plant organisms and at even very low concentrations it can disturb metabolic processes and be harmful to normal cell functioning. The detected concentration of Pb in leaves is similar to that reported for *M. x giganteus* grown in soil severely contaminated with Pb [27].

After several months of plant growth, there was no difference in the amounts of photosynthetic pigments

between control plants and those cultivated in the flotation tailings. However, the light response curves showed prominent reduction in the net CO_2 assimilation rate in FT plants, which is evident at higher light intensities ($500\text{-}2000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$). In control plants, A continuously increased in parallel with increasing light intensity up to $2000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, whereas in FT plants, A reached the maximum values at far lower PPFD ($1000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$), which points to the limitation of photosynthesis. Elevated Pb concentrations in *M. x giganteus* leaves negatively affected several parameters of gas exchange: it caused a significant decline in A ($\rho=-0.502$), g_s ($\rho=-0.986$) and increased respiration in the dark ($\rho=0.477$). Similarly, a decrease in A and g_s was reported for *M. x giganteus* plants grown in Zn contaminated land [51], and in As, Pb, and Sb highly contaminated technosols [27]. Investigations related to Pb toxicity in different plant species have shown that it inhibits photosynthesis by causing negative effects on various components of the photosynthetic machinery; the authors detected Pb-induced distortions of chloroplast ultrastructure, obstruction of electron transport rate, decreased stomatal conductance and gas exchange, inhibition of the activities of enzymes involved in the Calvin cycle, etc. [9,52-55].

Several parameters that are very useful in the assessment of plant stress, such as Fv/Fm, lipid peroxidation level and total antioxidative capacity in leaves, were not negatively affected by elevated Pb in *M. x giganteus* plants grown in the flotation tailings. Namely, their Fv/Fm values were as in the control, and within the optimal range for plants (0.75-0.85) [56], indicating the absence of photoinhibition of Photosystem II. The obtained results point to the high efficiency of mechanisms involved in the acclimation process against metal stress, such as metal immobilization in leaves, and effective antioxidative protection of the photosynthetic system and membrane lipids against oxidative damage [56]. Several authors found that in the case of Pb, its complexation with pectin carboxyl groups in the cell wall and its retention in the extracellular spaces represent the main mechanism of Pb immobilization and Pb tolerance in plants [48,58], although Pb can also be sequestered in the cell vacuole [48] and endoplasmatic reticulum [59].

As the consequence of prolonged exposure of roots and shoots to metal stress and reduced net

photosynthetic rate during plant growth, FT plants showed significantly reduced growth, represented by the smaller dimensions of their stems and penultimate leaves. It is known that heavy metals alter metabolism and impede plant growth after prolonged metal-stress exposure [60]. The higher percentage of non-functional senescent leaves per plant may also have had a negative impact on overall plant growth.

CONCLUSION

The results of this real-field experiment showed that *M. × giganteus* can (i) efficiently adapt to the hostile environmental conditions of flotation tailings for growth and development, and (ii) form a sustainable plant cover at flotation tailings with relatively low metal transfer to aboveground parts. Plants accumulated and retained the major portion of metals within their roots, restricting their further transfer to aboveground organs and their negative impact on the leaf biochemical functions. Based on heavy metal accumulation and distribution in the plant, *M. × giganteus* can be considered a phytoexcluder plant species. The low metal transfer to aerial parts is of high importance because of the relatively low probability of metal entry into the food web, which makes this plant suitable for cultivation for both bioenergy production and the restoration of heavy metal degraded land. Since only Pb was translocated to shoots in amounts that are normally toxic to plants, the detected significant reduction in net CO₂ assimilation rate and biometric parameters can be attributed to Pb-induced metabolic disorders. Due to reduced growth on the flotation tailings, this bioenergy crop cannot be used for satisfactory biomass production. However, our results recommend *M. × giganteus* for the design of a suitable technical solution for the phytostabilization of Pb-Zn-Cu-rich flotation tailings. The plant cover formed with *M. × giganteus* plants could mitigate water erosion and the wind-blow of small particulates from its surface, improve the chemical, physical and biological properties of its surface layers and thus reduce the risk of pollution of the neighboring environment and health risks, as well as provide a hiding place for local wildlife and aesthetically complement the landscape.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Andrejic%20et%20al_4468_Supplementary%20Material.pdf