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Impact of pyrometallurgical slags on sunflower growth, metal accumulation and rhizosphere microbial communities

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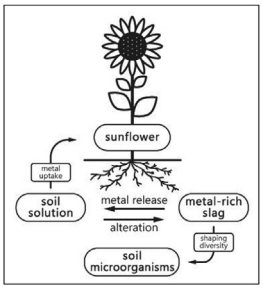
HIGHLIGHTS

- Soil solution enhances the leaching of Cu from metallurgical slags.
- Cu is the most bioavailable metal in the soil/slag mix, followed by Zn and Pb.
- Sunflower was able to grow in the soil/slag mix, tolerating metal rich slags.
- Sunflower accumulated Cu, Pb and Zn in the above-ground tissues.
- Slags have a role in shaping the diversity of soil microbial communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Metallurgical exploitation originates metal-rich by-products termed slags, which are often disposed in the environment being a source of heavy metal pollution. Despite the environmental risk that this may pose for living organisms, little is known about the impact of slags on biotic components of the ecosystem like plants and rhizosphere microbial communities.

In this study, metal-rich (Cu, Pb, Zn) granulated slags (GS) derived from Cu production process, were used for a leaching test in the presence of the soil pore solution, showing that soil solution enhanced the release of Cu from GS. A pot experiment was conducted using as growing substrate for sunflower (*Helianthus annuus*) a 50% w/w mix of an agricultural soil and GS. Bioavailability of metals in soil was, in increasing order: Pb < Zn < Cu. Sunflower was able to grow in the presence of GS and accumulated metals preferentially in above-ground tissues. Microbial diversity was assessed in rhizosphere and bulk soil using community level physiological profiling (CLPP) and 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE) analyses, which demonstrated a shift in the diversity of microbial communities induced by GS.

Overall, these results suggest that metallurgical wastes should not be considered inert when dumped in the soil. Implications from this study are expected to contribute to the development of sustainable practices for the management of pyrometallurgical slags, possibly involving a phytomanagement approach.

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1. Introduction

Since ancient times the extraction of metallic elements from their ores through smelting processes has been carried out in order to obtain pure enough metals for specific human applications (Bodsworth, 1994). However, besides the extraction of valuable metals, the smelting industry also produces huge amounts of by-products termed slags, which still may contain large amounts of residual metallic elements (Ettler, 2000; Jarošíková et al., 2018; Piatak, 2018; Piatak et al., 2015). In what way these secondary products are managed by metallurgical industries is a concerning issue because the safety of the environment may be compromised in case of inadequate management practices (Dudka and Adriano, 1995; Tyszka et al., 2018). Unattended disposal of slags in dumping sites in the neighboring area where metallurgical exploitation took place has been a common practice in the past because the environmental risk that this could pose was often neglected or underestimated (Ettler, 2016; Kierczak et al., 2013; Lottermoser, 2002). When slags are merely disposed in the environment with any confinement they are exposed to a number of physical (e.g. wind, rain, temperature), chemical (e.g. pH, redox potential, organic compounds) and biological factors (i.e. microorganisms, plants). These factors can be at the basis of slag physical (*e.g* cracking, fragmentation) and/or biochemical (*e.g* acid-base, hydrolysis, reduction-oxidation, complexation reactions) alteration (Potysz et al., 2018, 2016a, 2015). As a consequence of these interactions, a labile fraction of metals can be mobilized and released from slags threatening the ecosystem (Sobanska et al., 2016; Tyszka et al., 2018).

Not only environmental factors alter slags but also the presence of slags affects their surrounding environment. Soil properties in slag dumping sites can be severely modified. Slags in soil surface can interfere with the soil-forming process and modify the way in which soil particles aggregate altering its structure as a result. Slags may also modify other soil characteristics like the pH, the content of organic matter and nutrients. In addition, slags are a source of an elevated content of metals, which can be toxic for plants and soil microorganisms. Besides, metal toxicity is not only related to the total soil concentration but to the fraction that is actually bioavailable in the soil solution (van Hullebusch et al., 2005a). As a result of a combination of the factors that alter soil properties, the establishment, development and maintenance of a vegetation cover in slag dumping sites might be challenging (Ettler et al., 2004). Similarly, soil microbial biodiversity may be compromised threatening in turn the ecosystem services provided by the soil biota (Adhikari and Hartemink, 2016).

It is well described in the literature that plants can contribute to the cleaning-up of a metal contaminated soil by removing or reducing the toxicity of metals in soils through phytoextraction and phytostabilization processes (Agnello et al., 2016; Bolan et al., 2011; Farid et al., 2017; McGrath and Zhao, 2003). However, questions have been raised about the feasibility to apply such phytoremediation approaches at real field scale and in a reasonable timeframe (Ernst, 2005). Moreover, phytoremediation experiments giving rise to contradictory findings, in which both effective and unsuccessful results were achieved, did not consent to draw definite conclusions about the efficiency of phytoremediation (Bell et al., 2014; Evangelou et al., 2007; Luo et al., 2016). While the debate still continues about the suitability of phytoremediation strategies, the notion of phytomanagement emerged. The term phytomanagement is a relatively new concept for producing economic revenue from biomass growing in contaminated land (Robinson et al., 2009). The main objective in phytomanagement is to use contaminated areas as a resource to get an economic profit (e.g. production of bioenergy, biochar, fodder), while simultaneously mitigating the risk deriving from contaminated land (Evangelou and Deram, 2014). Furthermore, the development of a vegetation cover in degraded sites could result in other benefits such as controlling soil erosion and particle dispersion, creating a niche for rhizosphere microorganisms and improving aesthetics of the place. In conclusion, the development of a vegetation cover in degraded sites such as slag disposal places is sought, either to conduct phytoremediation, phytomanagement and/or additional improvement actions.

Among all plant species, there are two main reasons why sunflower (*Helianthus annuus* L.) has been widely used in phytoremediation: high biomass production and accumulation of heavy metals (Farid et al., 2018; Rizwan et al., 2016). Some studies found a primary role for sunflower to translocate some metals (Cd, Pb and Zn) from roots to the above-ground tissues, which is desired for phytoextraction (Adesodun et al., 2010; Laporte et al., 2015). On the contrary, other studies found major accumulation of metals (e.g. As, Cd, Cu and Pb) in their roots, which is required for phytostabilization (Laporte et al., 2015; Madejón et al., 2003). Besides, research on merely classical phytoremediation studies has now been challenged by recent studies using sunflower in the context of phytomanagement of degraded soils due to the possibility to obtain economical revenue from this crop. Angelova et al. (2016) demonstrated that it could be feasible to use the seeds of sunflower plants growing in a soil contaminated with Cd, Pb and Zn for edible oil production as the content of metals demonstrated to be below the regulated limits. In addition, other uses that have already been envisaged for sunflower growing in metal-contaminated agricultural land or even marginal urban land include the production of alternative fodder fortified with Zn, green manure for micronutrient-deficient soils biofuel feedstock, or ornamental plants (Fässler et al., 2010; Hao et al., 2012; Zhao et al., 2014).

A review of the literature shows many studies dedicated to unravel how physico-chemical (Jarošíková et al., 2018; Sobanska et al., 2016; Yin et al., 2016) and biological (Potysz et al., 2016a; van Hullebusch et al., 2015) factors alter slags. On the contrary, much less is known about the direct impact of slags on biotic components of the ecosystem like plants and soil microbial communities.

The central hypothesis of this paper is that pyrometallurgical slags are not inert in the environment as their presence could affect plant growth, metal uptake and shape soil microbial communities. Consequently, the aim of this study was to explore the behavior of Cu-rich pyrometallurgical slags in the complex weathering system involving plants (*i.e.* sunflower) and soil (*i.e.* rhizosphere, bulk) microorganisms. The specific objectives of this study were: i) to investigate if soil and sunflower plants influence the release of metals from slags, ii) to ascertain the influence of slags on sunflower growth, metal accumulation and soil microbial diversity and iii) to generate insights into the development of safe disposal strategies for slags, possibly involving a phytomanagement plan.

2. Materials and methods

2.1. Soil and slag samples

The agricultural soil (AS) used in this study was collected from a site in Italy where agricultural activities have been carried out. The soil was homogenized, air dried and sieved to 2 mm. Granulated slags (GS) used in this study are by-products derived from metallurgical exploitation, in particular copper production process. The slag is composed of amorphous glass characterized by the frequent occurrence of intermetallic droplets, which contain more than 90% of Cu and minor elements like Pb and Zn. Full structural characterization of GS was performed by Potysz et al. (2016b) and a

Table 1
Physico-chemical characterization of soil samples.

Parameter	AS	AS + GS $_{50\%\ wt.}$
pH (H_2O) $_{NF\ ISO\ 10390}$	8.0	8.4
Cation exchange capacity at pH 7 (mEq/100 g) $_{NF\ X\ 31-130}$	23.5	11.5
Organic matter (g kg^{-1} DW) $_{NF\ ISO\ 14235}$	42.7	29.9
Organic carbon (g kg^{-1} DW) $_{NF\ ISO\ 14235}$	24.7	17.3
Total Nitrogen (g kg^{-1} DW) $_{NF\ ISO\ 13878}$	0.74	1.4
C/N ratio	33.4	12.3
P_2O_5 (g kg^{-1} DW) $_{Joret\text{-}Hebert\text{-}NF\ X\ 31-161}$	0.21	0.16
K_2O (g kg^{-1} DW) $_{NF\ X\ 31-108}$	0.42	0.26
MgO (g kg^{-1} DW) $_{NF\ X\ 31-108}$	0.92	0.61
CaO (g kg^{-1} DW) $_{NF\ X\ 31-108}$	13.1	10.2
Fe (mg kg^{-1} DW) $_{DTPA\text{-}NF\ X\ 31-121}$	25.5	80.6
Mn (mg kg^{-1} DW) $_{DTPA\text{-}NF\ X\ 31-121}$	20.1	103
B (mg kg^{-1} DW) $_{DTPA\text{-}NF\ X\ 31-122}$	0.26	0.31
Sand (%) $_{NF\ X\ 31-107}$	8.0	58.7
Silt (%) $_{NF\ X\ 31-107}$	50.5	23.1
Clay (%) $_{NF\ X\ 31-107}$	41.5	18.2
Texture	Silty clay	Sandy loam
Cd (mg kg^{-1} DW) $_{NF\ X\ 31-147+ICP-MS}$	0.19 (± 0.08)	4 (± 1)
Cr (mg kg^{-1} DW) $_{NF\ X\ 31-147+ICP-MS}$	87.4 (± 31.7)	149 (± 48)
Cu (mg kg^{-1} DW) $_{NF\ X\ 31-147+ICP-MS}$	19.4 (± 3.8)	16505 (± 594
Hg (mg kg^{-1} DW) $_{Internal\ method\ MA7-82\ v5}$	0.015 (± 0.005)	0.065 (± 0.018)
Ni (mg kg^{-1} DW) $_{NF\ X\ 31-147+ICP-MS}$	43.2 (± 6.5)	387 (± 24)
Pb (mg kg^{-1} DW) $_{NF\ X\ 31-147+ICP-MS}$	34.1 (± 6.5)	23297 (± 234)
Zn (mg kg^{-1} DW) $_{NF\ X\ 31-147+ICP-MS}$	85.4 (± 12.4)	8732 (± 219)

AS: Agricultural soil; AS + GS $_{50\%\ wt.}$: Agricultural Soil + Granulated Slag; DTPA: Diethylenetriaminepentaacetic acid; DW: Dry Weight; ICP-MS: Inductively Coupled Plasma Mass Spectrometry; ISO: International Organization for Standardization; NF: French Norm; NF X 31-147: Total mineralization with fluorhydric acid (HF).

summary can be found in supplementary data (Fig. S1, Tables S1 and S2). Physico-chemical characterization of AS and the mix AS + $GS_{50\%\ wt.}$ was performed by SADEF (France), which included the following determinations: pH (electrometric procedure in water, according to NF ISO 10390), cation exchange capacity (measurement of cations displaced by ammonium acetate at pH 7, according to NF ISO 10390), organic carbon (sulfochromic oxidation, according to NF ISO 14235), organic matter content calculation, total N (dry combustion, according to NF ISO 13878), P_2O_5 (Joret-Hebert method for the determination of soluble phosphorus in ammonium oxalate, according to NF X 31-16), K_2O, MgO and CaO (agitation method for the determination of ammonium acetate extractable cations, according to NF X 31-108), Fe, Mn and B (DTPA extraction for exchangeable metal determination, according to NF X 31-12), particle size determination (sedimentation-pipette method, according to NF X 31-107), texture (soil texture calculator from the United States Department of Agriculture), metals (total Cd, Cr, Cu, Ni, Pb and Zn solubilized by HF attack, according to NF X 31-147 and quantification by ICP-MS; Hg by internal method MA7-82 v5). Results can be found in Table 1.

2.2. Bioavailable metals in soil

A metal fractionation scheme was carried out following the first two steps of a modified BCR (Bureau Communautaire de Reference) protocol for metal sequential extraction in order to quantify the metals present in most bioavailable soil fractions of the samples AS and the mix AS + $GS_{50\%\ wt.}$ (Mossop and Davidson, 2003; van Hullebusch et al., 2005b). First, soils (1 g) were shaken (30 rpm) at 20 °C for 16 h with 40 mL of 0.11 M CH_3COOH adjusted to pH 1.5 with concentrated NaOH. Second, the resulting residue was shaken (30 rpm) at 20 °C for 16 h with 40 mL of 0.5 M $NH_2OH{\cdot}HCl$ adjusted to pH 1.5 with concentrated HNO_3. Between the first and second step residue was washed with 25 mL ultrapure water (UPW). An aliquot of the extracted samples was filtered with acetate cellulose filters (pore size 0.45 μm), acidified using concentrated HNO_3 to reach a concentration of 1% v/v and stored at 4 °C until elemental analysis. Single extraction protocol was carried out by SADEF (France) following the method described by Lindsay and Norvell (1978), in which the extractant consists of 0.005 M DTPA (diethylenetriaminepentaacetic acid), 0.1 M triethanolamine, and 0.01 M $CaCl_2$, with a pH of 7.3. Briefly, 10 g of air-dry soil are mixed with 20 mL of extractant for 2 h. The leachate is filtered, and Zn, Fe, Mn, and Cu are quantified in the filtrate.

2.3. Plants

Sunflower seeds (*Helianthus annuus* v. Sunspot) were surface disinfected by immersion in 2% (v/v) hydrogen peroxide for 8 min (Qu et al., 2011), in order to avoid the addition of non-indigenous microorganisms to the system. Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiments.

2.4. Leaching solution collection and leaching test

Plastic pots (15 cm diameter) were fulfilled with 1.1 kg of agricultural soil (AS) placing 9 Rhizon Flex (Rhizosphere Research Products©) samplers per pot (5 cm porous part, OD 2.5 mm, nylon wire, PVC/PE 13 cm tubing, female luer lock for creating a vacuum with a syringe and 7 cm wooden retainers) to collect soil solution in a non-destructive way. The experimental design comprised two conditions: (a) non-planted AS as control and (b) AS planted with 15 sunflower seeds. Pots were placed in a growth chamber (Sanyo Versatile Environmental Test Chamber MLR-352) under a photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C and a photosynthetic photon flux density (PPFD) of 130 μmol m^{-2} s^{-1}. Pots received tap water daily to adjust soil moisture and location of pots was randomly changed every day as well. After 108 d of experiment, the collection of soil solution from humid soil was done overnight using Rhizon Flex samplers. GS was then submitted to a leaching test using as leaching solution the soil solution collected from unplanted and planted pots. The leaching experiment was carried out in 50 mL polypropylene batches, in which 20 mL of leaching solution were put in contact with 2 g of GS to keep a liquid to solid ratio of 10 (volume (mL)/weight (g)). Batches were performed in triplicates and a control with UPW and a blank in the absence of GS were also included. Batches were kept at room temperature (20 °C) on a horizontal shaker at 160 rpm and the leachates were sampled after 72 h. The pH of the leaching solution was measured at the beginning and at the end of the experiment leaving it at free drift. Solutions were filtered on acetate cellulose syringe filters (pore size 0.45 μm), then acidified using concentrated HNO_3 to reach a concentration of 1% v/v and stored at 4 °C until elemental analysis. This study focused on the behavior of three elements: Cu, Pb and Zn, which are the major metals found in GS (Table 1).

2.5. Pot experiment

Sunflower seeds (12) were sown in plastic pots (15 cm diameter) filled with 800 g of substrate. The experimental design comprised two conditions: (a) AS as control and (b) mixture of 50% wt. GS and 50% wt. AS (AS + $GS_{50\%\ wt.}$). To limit the level of heavy metals present in GS and test plant tolerance, in a previous experiment GS were mixed in different proportions (from 5 to 50% wt.) with AS. Sunflower was able to grow and performed well in the presence of GS up to a level of 50% wt. (data not shown). As a result, this proportion was chosen for the present study.

Pots were placed in a growth chamber (Sanyo Versatile

Environmental Test Chamber MLR-352) under a photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C and a photosynthetic photon flux density (PPFD) of 130 μmol m^{-2} sec^{-1}. Pots received tap water daily to adjust soil moisture and location of pots was randomly changed every day as well.

Plants were harvested after 90 d of growth. Plants were removed from pots and biometric parameters such as plant height, root length, number of leaves and number of flowers per plant were recorded. Afterwards, roots, shoots and flowers were separated. Two types of soil samples were collected from pots: the external soil detached to roots (bulk soil) and the soil in the immediate surface/vicinity of roots (rhizosphere soil). Afterwards, roots were washed with distilled water and blotted with tissue paper. Bulk and rhizosphere soil samples were kept at 4 °C until further analyses of microbial diversity whereas plant material was put at 70 °C for 3 d (Tabatabai, 1998) and dry weights of shoots, flowers and roots were then recorded. Prior to elemental analysis in plant tissues, dried plant material was wet digested as described by Tabatabai (1998). Briefly, plant material was mixed with 5 mL concentrated HNO_3 and 2 mL 30% H_2O_2 in a digestion block (LabTech DigiBlock Digester ED16S) at 125 °C for 1 h. Heating cycles and hydrogen peroxide additions were repeated three times to obtain a clear digest. To remove residual particles, mineralized samples were filtered through cellulose filters (pore size 2.5 μm) and brought to a final volume of 20 mL. Samples were additionally filtered through nitrocellulose syringe filters (pore size 0.45 μm) and stored at 4 °C until heavy metals were analysed.

2.6. Elemental analysis in leachate, soils and plant tissues

Metal quantification in leachate solutions, soil sequential extraction fractions and digested plants was carried out with an Inductively Coupled Plasma-Optical Emission Spectrometer (PerkinElmer Optima 8300 ICP-OES Spectrometer). Cu, Pb and Zn were analysed at the respective wavelengths of 324.752 nm, 220.353 nm and 213.857 nm. Calibration curves with the same matrix as the sample solutions were prepared accordingly. Accuracy of method was verified using soil (Standard Reference Material©, Montana I soil 2710a) and plant reference material (Polished Certified Reference Material, Oriental Basma tobacco leaves INCT-OBTL-5).

2.7. Microbial diversity in soils

2.7.1. Community level physiological profiling (CLPP)

Microbial metabolic diversity of bulk and rhizosphere soil samples was assessed by analysing the utilization pattern of 31 carbon sources in Biolog EcoPlates™ (Biolog, Inc., Hayward, CA, USA) (Campbell et al., 1997; Garland and Mills, 1991). Briefly, soil samples (1 g) were shaken at 80 rpm in 100 mL of sterile 0.85% NaCl solution for 2 h at 28 °C, 140 μl of soil suspension were inoculated in Biolog EcoPlates™ and incubated at 28 °C for one week. Absorbance of formazan was determined at 590 nm and recorded daily over one week (Biotek Power Wave XS). Microbial activity in each microplate was expressed as the average well color development (AWCD) as described by Garland (1996) and Hitzl et al. (1997). Further methodological details can be obtained from supplementary materials and the evolution of AWCD over the incubation period can be found in supplementary data (Fig. S2). Besides, diversity indices (*i.e.*, Shannon diversity and evenness, substrate richness) were calculated with Diversity add-in in Microsoft Excel 2011 and Principal Component Analysis (PCA) (Garland, 1996; Zak et al., 1994) was performed with Statistica 7.0 software (Stat. Soft. Inc. UK, USA).

2.7.2. DNA extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

The extraction of genomic DNA from bulk and rhizosphere soil samples (0.5 g) was done using PowerSoil® DNA Isolation kit (MoBio Laboratories, Inc.) following manufacturer's instructions. The amplification of 16S rRNA gene of the bacterial genomic DNA was done by PCR using universal primers 337F and 518R. PCR amplification was performed in 50 μl reaction volume (5 μl genomic DNA, 0.25 μM of forward and reverse primers, 0.25 mM of each dNTP (Qiagen), 2.5 U Taq DNA polymerase (Sigma), the manufacturers' recommended buffer as supplied with the polymerase enzyme and nuclease-free water) in a thermocycler (Agilent Sure Cycles 8800). PCR products were analysed for its expected size (200 bp) on 1% agarose gel stained with ethidium bromide. An ultraviolet (UV) transilluminator (Universal Hood III, Bio-Rad, USA) was used for gel visualization and image acquisition. DGGE was performed in DcodeTM universal mutation detection system (Bio-Rad, USA). PCR products were loaded in a 8% (w/v %) acrylamide:bis-acrylamide (37.5:1) gel containing a vertical linear denaturing gradient (40–70%) of denaturant solution (100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. Electrophoresis (60 V, 32 mA) was carried out for 16 h at 60 °C using 1× TAE as running buffer. The gel was then stained for 20 min in ethidium bromide solution (0.75 mg L^{-1} in 0.5 × TAE buffer) and gel images were acquired on a UV transilluminator. For gel images analysis and construction of single linkage phylogenetic tree, PyElph 1.4 software was used (Pavel and Vasile, 2012). In addition, the clustering matrix obtained from DGGE banding pattern was used to calculate diversity indices (Shannon Diversity and Richness) (Supplementary data, Table S4). Full methodological details can be found in supplementary materials.

2.8. Statistical analysis

The experiment was arranged in a completely randomized design. Data reported were averaged values of three independent replicates. StatPlus Add-In in Excel 2011 software was used to assess for significant differences between treatments using unpaired *t*-tests or one way Analysis of Variance (ANOVA) coupled with Tukey's post-hoc test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Leaching test

The most widespread approach to study the reactivity of slags in the environment is performing laboratory-leaching tests that mimic the external conditions to which slags might be exposed (Piatak et al., 2015). In this study unaltered soil solution obtained from undisturbed pots fulfilled with AS, planted or not with sunflower plants, was used as leaching solution. The aim was to estimate the potential of metal release from slags in a vegetated and non-vegetated soil context.

Results of leaching tests expressed as absolute concentrations of metals in the solutions (Table 2) give insight into the slag reactivity potential by way of the metal content that can be released into the environment. As can be seen from Table 2, the leaching of metals occurred at a pH close to neutrality. For the three leaching solutions used for the experiment it was observed a pH shift to alkalinity of approximately one unit within 72 h. Metals were released from slags in the following decreasing order: Cu > Pb > Zn. Soil solution (coming both from planted and non-planted pots) significantly increased the leaching of Cu as compared to the UPW control. In contrast, no significant increase in Pb and Zn release was detected.

Table 2
pH shift and amounts (mg kg^{-1}) of metals leached out from GS after 72 h of leaching experiment. Different letters (a, b) indicate significant differences in mean values between treatments. Mean values with the same letters were similar and no statistically significant differences between treatments were observed for these samples.

Leaching solution	$pH_{initial}$	pH_{final}	Cu	Pb	Zn
UPW	5.78 (±0.05) a	7.23 (±0.05) a	1.2 (±0.1) a	1.1 (±0.0) a	1.0 (±0.1) a
Soil solution (- sunflower)	6.83 (±0.05) b	7.75 (±0.03) b	12.5 (±1.7) b	2.1 (±0.4) a	1.0 (±0.2) a
Soil solution (+sunflower)	6.92 (±0.05) b	7.72 (±0.03) b	10.6 (±0.7) b	1.5 (±0.1) a	0.9 (±0.1) a

UPW: ultrapure water.

Finally, no significant differences were found for any metal between the leaching with soil solution obtained from pots vegetated with sunflower and that from non-vegetated pots.

Potysz et al. (2017) identify three factors affecting the release of metals from metallurgical wastes: i) the surrounding pH, ii) the intrinsic susceptibility of wastes to be altered and iii) the exposure to organic molecules with binding-metal ability. These main factors will be taken into consideration to explain the results obtained in the present leaching test.

Firstly, in what concerns the pH, extensive research has shown that acidic conditions enhance the leaching of metals from slags (Ettler et al., 2009; Jarošíková et al., 2018; Manz and Castro, 1997; Piatak et al., 2015; Saikia et al., 2018). A recent leaching study with GS samples found intense release of metals under strong acidic conditions (pH values between 2 and 4): 1700–6000 mg kg^{-1} for Cu, 900–6000 mg kg^{-1} for Pb and 400–4000 mg kg^{-1} for Zn. On the contrary, at pH 8.1 the amounts of metals released from GS were <5 mg kg^{-1} for Cu and <0.01 mg kg^{-1} for Pb and Zn (Potysz et al., 2016b). The values reported herein are in agreement with these previous results: under intermediate values of pH (*i.e.,* close to neutrality) and using UPW as leaching solution, approximately 1 mg kg^{-1} of Cu, Pb and Zn were released. The higher release of metals under the present conditions may be due to the leachate lower pH, despite the use of larger slag grains. Moreover, during the experiment, the pH of the leachates shifted from initial values to approximately one unit above, possibly putting in evidence the acid neutralization potential of the slags (Hageman et al., 2015). These results seem to be consistent with other research which found a similar trend of pH increase, but with a greater pH change (Potysz et al., 2017). The smaller pH shift of leaching solution observed herein may be attributed to the slag fraction size. The slag grains used in this study were larger (above 80% of the grains correspond to fraction > 0.5 mm, data available as supplementary data, Table S2) as compared to that study (100% fraction <0.3 mm), which surely rendered lower neutralization/buffering potential.

Secondly, the observed trends in metal leachability may be related to the inherent mineralogical composition of GS. The main metal-bearing phase component of this waste is copper droplet embedded in amorphous glassy matrix (supplementary data, Fig. S1). According to data obtained from electron microprobe (Potysz et al., 2016b) these intermetallic blebs contain high quantity of above-mentioned elements that occur in the following decreasing order of concentration: Cu ≫ Pb > Zn (90.2, 3.25 and 0.14% wt., respectively; supplementary data, Table S1). Furthermore, the liberation of copper droplets from GS was previously reported regardless of simulated environmental conditions. The latter includes slag exposure to UPW, mineral salt medium, mineral medium inoculated with heterotrophic bacteria, soil organic acids and artificial root exudates (Potysz et al., 2017, 2016c; 2016a). However, it has to be noted that dissolution of intermetallic components under circumneutral conditions has undoubtedly been not sole source of these metallic elements. Amorphous glass being volumetrically major mineral phase is known to be generally susceptible to weathering (Ettler et al., 2002, 2001; Vítková et al., 2010). Therefore, it is expected that its dissolution has also appeared in the studied weathering system and accounted for resulting metal content in the leachate. As quantified by Potysz et al. (2016a,b,c), these elements are incorporated in glass structure as well (Cu: 2.31, Pb: 4.15 and Zn: 1.17% wt.; supplementary data, Table S1). It is interesting to note that both distinct phases that compose GS (*i.e.,* droplets and glass) may behave differently when exposed to a leaching solution and may be altered in a particular way. Besides, metal speciation in the slag solid phase is possibly a factor conditioning elements release in the leachates. Further research should be undertaken to discern the contribution of metal release proceeding from each individual phase.

Finally, the complex composition of soil solution can be expected to influence metal release from slags as well. Soil solution is the water content of soil. This liquid part of soil is a complex mix that contains various dissolved substances: gases (e.g., O_2, N_2, CO_2), organic matter (e.g., organic acids, sugars, humic acids, amino acids) and minerals (e.g., free salts and ions of Ca, Na, K, Mg) (Baetz and Martinoia, 2014; Montiel-Rozas et al., 2016; Wolt, 1994). Likewise, the release of organic compounds by soil microorganisms and plants (*i.e.*, rhizodeposition), may be an additional input source of a number of organic compounds: simple and complex sugars, amino acids, organic acids, phenolics, alcohols, polypeptides and proteins, hormones and enzymes (Nguyen, 2003; Steinauer et al., 2016). According to calculations of modal phase composition and approximated metal distribution (Table S3, Supplementary materials) glass hosts more than 98% of Zn and Pb bulk content, while it contains just 62% of Cu. Intermetallic droplets are responsible for the remaining 38% of Cu bulk content. It has already been demonstrated that glass is susceptible to dissolution under near-neutral conditions (Ettler et al., 2002). It may be then assumed that under near-neutral conditions (*i.e.*, UPW) metals bound in glass are more easily liberated than metals bound in Cu droplets. Therefore, release of Zn and Pb bound to glass was similar for UPW and soil solution, whereas liberation of Cu from intermetallic droplets accounted for higher content of Cu in soil solution as compared to UPW likely due to the presence of soil organic compounds. Organic ligands with binding ability capable of forming organometallic complexes may enhance metal release from metallurgical wastes. In addition, an enhanced release of Cu in soil solution relative to UPW could be explained as a result of Cu binding by dissolved organic matter (Karlsson et al., 2006; Ma, 2000; Matijevic et al., 2014; Nierop et al., 2002). In contrast, no significant increase in Pb and Zn release was detected suggesting weak complex formation with organic ligands present in soil solution. For example, Pandey et al. (2000) demonstrated higher stability constants of humic acid with Cu as compared to Zn and Pb. Furthermore, Potysz et al. (2017) observed considerable release of Cu, Pb and Zn from GS after 72 h of a leaching test using a mix of artificial root exudates at pH 4.4 as leaching solution. These values are considerably higher than those obtained in the present work using a real soil solution coming from pots vegetated and non-vegetated with sunflower. As that experiment was conducted under more acidic conditions, it is probable that pH has been the most relevant factor driving metal release. Leaching of GS in the presence of humic and fulvic acids proceeded under pH conditions similar to

these applied in the present study, *i.e.*, circumneutral that was caused by the neutralization potential of slags. 72 h long leaching with these compounds resulted in lower metal quantities as compared to these achieved during leaching with real time soil leachate used in the present study (Potysz et al., 2017). Interestingly, this higher metal release from slag immersed in real soil solution was attained in spite of the use of larger slag grains (*i.e.,* above 0.5 mm). Therefore, it can be assumed that a long-term presence of GS in a real soil solution could potentially result in even higher metal release. Besides, there is a potential bias from comparing artificial root exudates and a real soil solution.

Contrary to expectations, this study did not find a significant difference in the leaching ability of the two types of soil solutions tested. When comparing the results of metal release by soil solution obtained from vegetated and non-vegetated pots, it can be seen that the presence of plants did not have a net contribution in what respects the property of soil solution to enhance metal release. To understand this result it would be necessary to make a full characterization of the chemical composition of both soil solutions to see if a significant difference between their components is found. Hence, it could conceivably be hypothesized that organic compounds found in soil solution as a consequence of rhizodeposition by plants are rapidly biodegraded in the non-sterile conditions in which the leaching test was performed (Cotrufo et al., 2013; Sollins et al., 1996). Such observation has already been proven by Banks et al. (1994) who demonstrated that microbial degradation of organic acids present in the leachate resulted in a lower metal extraction yield. On the other hand, the presence of microorganisms in the non-sterile soil solution should not be kept behind as a biotic factor responsible for GS weathering, especially considering the extent of its contribution in a long-term perspective (Uroz et al., 2009). What is clear from these results is that certain components present in the soil solution and/or microbial activity contributed to the release of Cu as observed by the significant increase in Cu release relative to the control with UPW. It can be suggested that the selectivity for Cu is the result of specific molecules (e.g. siderophores) with high affinity to coordinate this metal (Cornu et al., 2014). The presence of siderophores and other organic compounds associated with the activity of bacteria has already been found to be a main factor accounting for enhanced Cu release from GS (Potysz et al., 2016c, 2016a). Despite no difference between soil solution and UPW treatments in terms of Zn and Pb release from slag, it is possible that these elements are less easily liberated as compared to Cu. This hypothesis is made based on previous study that demonstrated clearly enhanced release of Cu from GS within 14 d of the experiment with bacteria, whereas microbial impact on Zn and Pb appeared to be marginal at this stage of the experiment (Potysz et al., 2016a). Even though occurrence of bacteria in the batches, it is possible that longer contact time with GS grains would be needed for elucidating its impact on Zn and Pb release in terms of quantity and rates.

3.2. Total and bioavailable metals in soil samples

Due the fact that total metal concentration does not necessarily reflect the actual amount of mobile metals that really represent a threat for the environment (e.g. by bio-accumulating in living organisms or leaching to the groundwater), it is essential to take into consideration laboratory techniques that allow assessing metal bioavailability by distinguishing between chemical species associated with variable strength to different soil fractions. For this reason, two distinct extraction methodologies were applied to AS and AS + $GS_{50\%\ wt.}$ samples: i) DTPA acid single extraction protocol (Lindsay and Norvell, 1978) and ii) the first and second steps of BCR sequential extraction procedure (Mossop and Davidson, 2003).

Table 1 presents the data of the total content of metals in the soil samples AS and in the mix AS + $GS_{50\%\ wt.}$. The level of Cu, Pb and Zn found in AS sample is 19.4, 34.1 and 85.4 mg kg^{-1} soil, respectively. The comparison of these values with the maximum allowable concentrations (MAC) of trace metals fixed for agricultural soils reveals that Cu and Zn are below the MAC range (60–150 mg kg^{-1} for Cu; 100–300 mg kg^{-1} for Zn) while the values of Pb are within the established MAC limit of 20–300 mg kg^{-1} for Pb (Kabata-Pendias, 2011). For this reason, this soil was used as the control. When metal-rich slags are mixed with the AS in the sample AS + $GS_{50\%\ wt.}$ the total content of metals evidently rose to a large extent reaching 16505, 23297 and 8732 mg kg^{-1} for Cu, Pb and Zn, respectively. Such extraordinary high levels are considerably above the trigger action values (TAV) established for Cu, Pb and Zn in agricultural soils (60–500 mg kg^{-1} for Cu; 50–300 mg kg^{-1} for Pb and 200–1500 mg kg^{-1} for Zn), which necessarily requires human intervention to be restored (Kabata-Pendias, 2011).

In what concerns DTPA acid extraction (Table 3), the methodology was developed exclusively for the extraction of Cu, Fe, Mn and Zn, thus data is not available for Pb. Relative percentages of extracted Cu and Zn were higher in AS than in the mix AS + $GS_{50\%\ wt.}$ However, due to the higher content of metals in the mix AS + $GS_{50\%\ wt.}$ such percentages account for larger concentrations of potentially bioavailable metals (up to 49.4 mg kg^{-1} for Cu and 10.5 mg kg^{-1} for Zn).

The first step of BCR sequential extraction (CH_3COOH at pH 7) did not account for the extraction of metals in any of the samples, as observed by the values that are below de detection limits. The metals that could be extracted in this step encompass the most mobile metals, *i.e.,* metals bound to carbonates and constitute the exchangeable, water and acid soluble phase. In contrast, in the second step of BCR sequential extraction, which makes use of stronger extracting conditions ($NH_2OH \cdot HCl$ at pH 1.5), metals were released from the mix AS + $GS_{50\%\ wt.}$ in the following decreasing order: Cu > Zn > Pb. Once again, low percentages of the total metal content (0.20%, 0.46% and 0.76% for Pb, Cu and Zn, respectively) translate into high amounts of extracted metals (up to 46.9 mg kg^{-1} for Pb, 62.8 mg kg^{-1} for Zn and 75.3 mg kg^{-1} for Cu). Metals extracted in this step are those supposed to be bound to iron and manganese oxides and constitute the reducible phase. In addition, both absolute and relative values were higher in AS + $GS_{50\%\ wt.}$ than in AS.

Differences in the behavior of extracting agents among AS and AS + $GS_{50\%\ wt.}$ samples may be influenced by the fact that metals are associated to a complex matrix in the sample AS + $GS_{50\%\ wt.}$ (*i.e.*, interstitial glass and Cu droplets in slags, soil particles), which could led to different kinetics of metal release.

The fact that Cu, Pb and Zn are extracted in the decreasing order of: Cu > Zn > Pb by DTPA acid single extraction and by the second step of BCR sequential extraction is in agreement with the results obtained in the leaching test herein and in line with metal distribution among mineral phases in GS: prevalent Cu in droplets (Cu: 90.19%, Pb: 3.25% and Zn: 0.14%), which is prone to dissolution, whereas prevalent Pb and Zn in interstitial glass (as oxides) are less available (Potysz et al., 2016b). Moreover, the release of metals by both extraction methodologies indicates that these metals could be phyto-available; suggesting that plants could uptake metals in slag dumping sites. Nevertheless, this assumption should be confirmed by the corresponding plant analyses because a direct correlation between the results obtained from soil chemical extractions and plant metal uptake is not always found, which means that it may be difficult to predict metal accumulation by plants only based on chemical extraction analyses of soil (Zhang et al., 2016b). Interestingly, Marguí et al. (2007) applied both extraction procedures: DTPA extraction protocol and first and second steps of BCR

Table 3
Total and extractable metals (Cu, Pb and Zn) in soil samples (mg kg^{-1}, % of the total values) using simple DTPA acid extraction and BCR sequential extraction procedures.

Extraction procedure	Units	Cu		Pb		Zn	
		AS	AS + GS $_{50\% wt.}$	AS	AS + GS $_{50\% wt.}$	AS	AS + GS $_{50\% wt.}$
DTPA	mg $(kg soil)^{-1}$	4.2	49.4	n.d.	n.d.	3.2	10.5
	%	21.6	0.30	n.d.	n.d.	3.8	0.12
BCR (Step 1)	mg $(kg soil)^{-1}$	<0.04	<0.04	<0.40	<0.40	<0.04	<0.04
	%	<0.21	<0.0002	<1.17	<0.0017	<0.05	<0.0005
BCR (Step 2)	mg $(kg soil)^{-1}$	<0.04	75.3 (± 3.6)	<0.40	46.9 (± 9.2)	<0.04	62.8 (± 0.8)
	%	<0.21	0.46	<1.17	0.20	<0.05	0.72

AS: Agricultural soil; AS + GS $_{50\% wt.}$: Agricultural Soil + Granulated Slag; BCR: Bureau Communautaire de Reference; DTPA: Diethylenetriaminepentaacetic acid extraction, according to French Norm X 31-121; n.d.: no data available.

procedure in a soil from a mining waste site in order to study Pb and Zn mobility and likely bioavailability to *Betula pendula* growing on the same mining spoils. They found suitable the use of DTPA extraction as indicator of plant bioavailability, while the extractable metal fractions determined by employing the first two steps of BCR sequential extraction did not determine clearly relationships between extracted metals and metal concentrations in the vegetation samples studied.

3.3. Plant biometric parameters

One of the approaches to assess plant tolerance to contaminants consists in making plants grow in the presence of the concerned contaminants, evaluate a number of biometric parameters and compare the results to control plants growing in the absence of contaminants. The results of biometric parameters of sunflower plants growing for 90 d in the absence (AS) and in the presence of metal-rich granulated slags (AS + $GS_{50\% wt.}$) are set out in Fig. 1.

Fig. 1a provides an overview of plant survival during the time that lasted the pot experiment. Different phases of plant growth, which follow a similar trend in both treatments, are observed. Firstly, there was an initial rapid increase in the number of plants after sowing. The germination rates achieved after six days were 83% and 94% in AS and AS + $GS_{50\% wt.}$ treatments, respectively. The

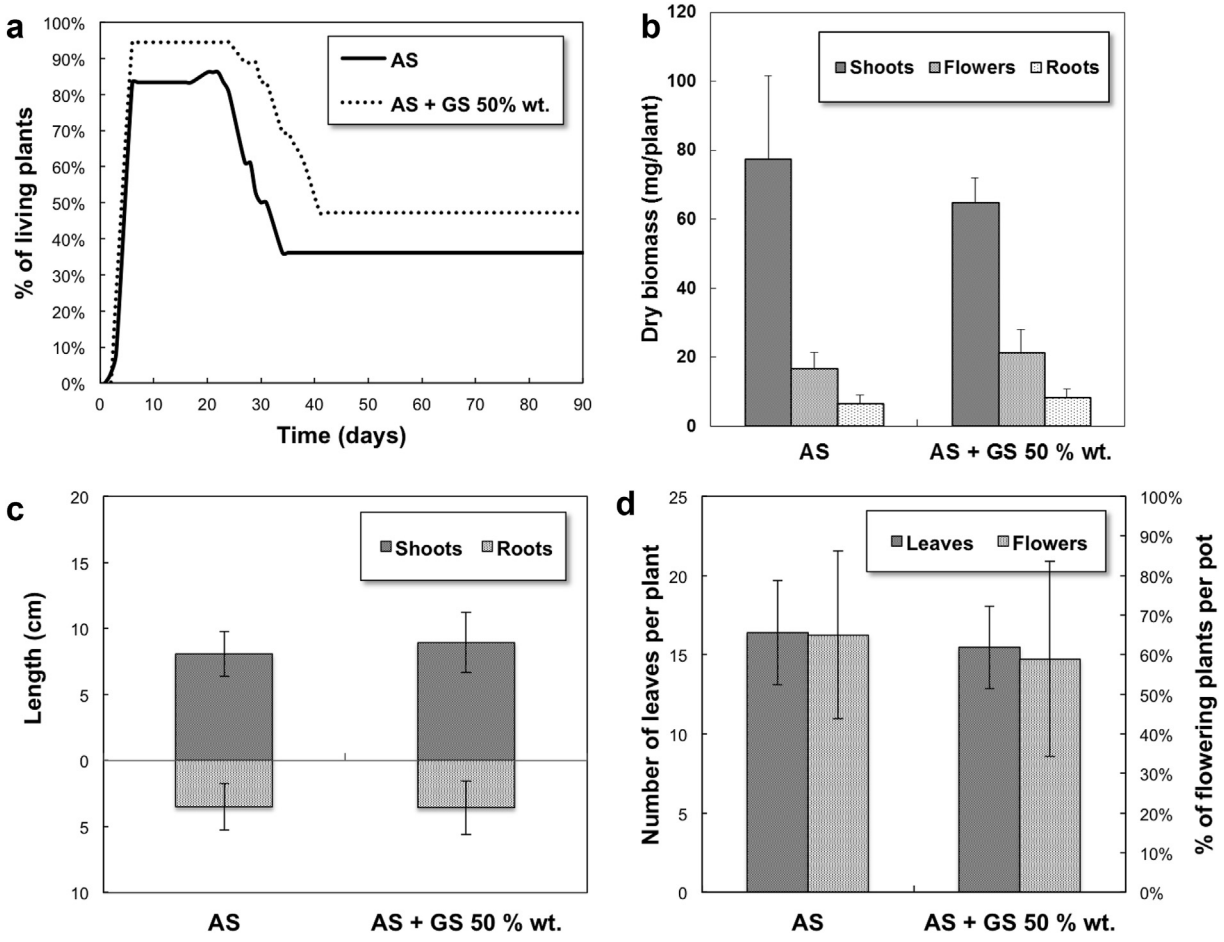

Fig. 1. Biometric parameters of sunflower after 90 d: a) Percentage of living plants throughout the experiment; b) Dry biomass of shoots, flowers and roots; c) Length of shoots and roots and d) Number of leaves per plant and percentage of flowering plants per pot. AS: Agricultural soil; AS + $GS_{50\% wt.}$: Agricultural Soil + Granulated Slag (No significant differences were found between AS and AS + $GS_{50\% wt.}$ treatments).

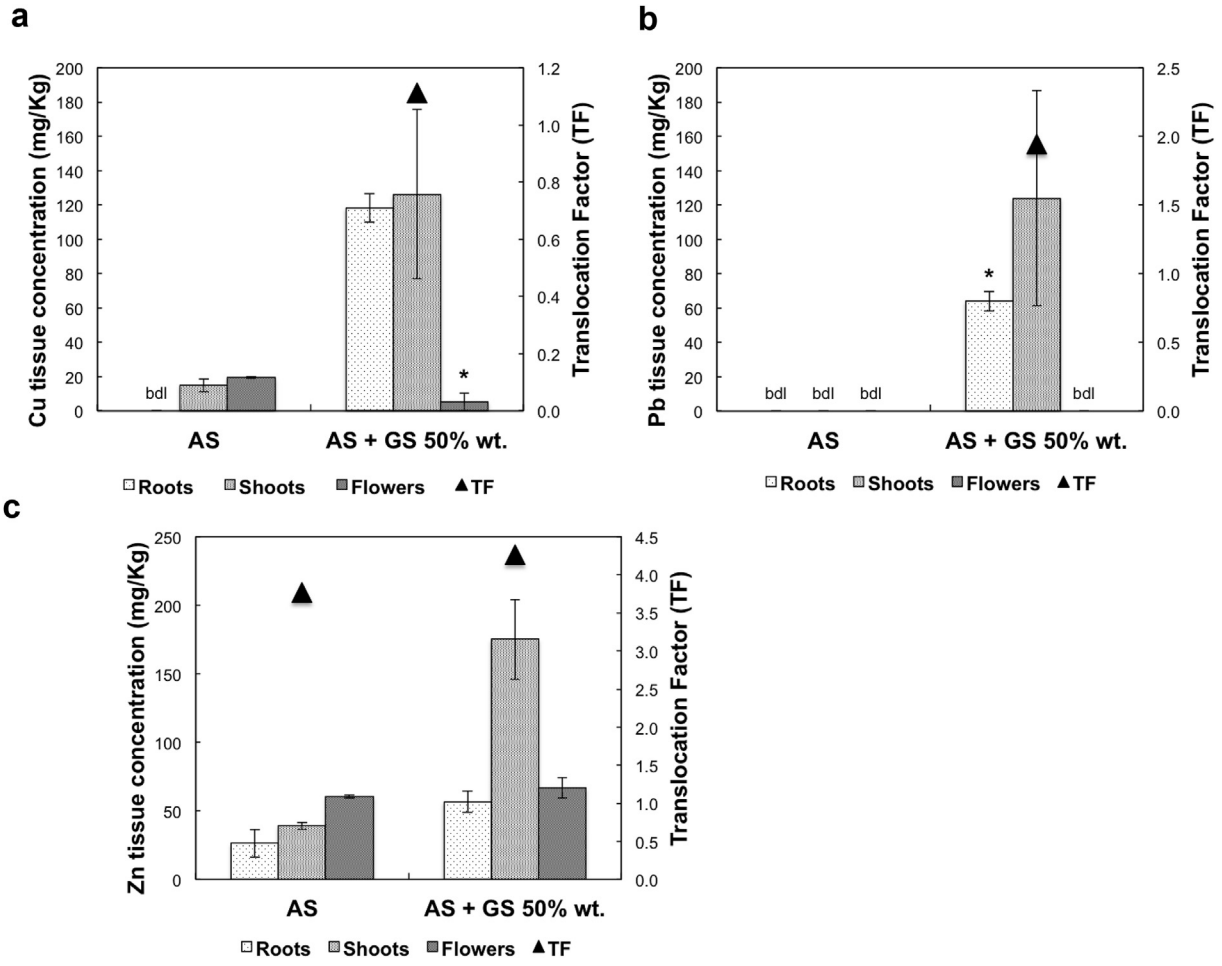

Fig. 2. Metal (a) Cu, b) Pb and c) Zn) concentration in sunflower tissues (roots, shoots and flowers) and translocation factors after 90 d. AS: Agricultural soil; AS + $GS_{50\%\ wt.}$: Agricultural Soil + Granulated Slag; TF: Translocation Factor; bdl: below detection limit; the symbol * indicates a significant difference between AS and AS + $GS_{50\%\ wt.}$ treatments.

number of living plants was then maintained constant for about 15 d. However, after the initial establishment and over the successive 15 d, some symptoms of plant toxicity (*i.e.*, abnormal development of growing points, foliar chlorosis, dieback of terminals, leaves curling downwards) were manifested in plants growing both in AS and AS + $GS_{50\%\ wt.}$ substrates and a considerable number of plants died. Due the fact that symptoms of phytotoxicity and plant mortality affected both treatments and to a similar extent, they cannot be directly attributable to the presence of metal rich GS. It could be hypothesized then that some of the soil properties may be a limiting factor for plant growth. Among other soil oligo-elements, B is an essential element needed for the growth and development of plants (Kabata-Pendias, 2011). The critical level of soluble forms of B for optimal sunflower growth is established at 0.5 mg kg^{-1} soil (Scott, 1933). As shown in Table 1, the amounts of extractable B in both samples are below this limit. As a result of soil characterization and considering the above-mentioned visual toxicity symptoms, sunflower sensitivity to B deficiency appears to be at the basis of poor plant performance (Ahmad et al., 2012). Application of soil B fertilizers at the time of sowing or foliar application of B during the vegetation period could have been a solution to prevent or minimize deleterious effects and improve plant yield. However, B fertilizers were not used herein in order to mimic conditions closer to reality as 73% of Polish soils exhibit a negative balance of B, 26% display moderate B abundance and only in 1% of soils is highly abundant (Grześkowiak, 2013). Undoubtedly, this variable must be taken into consideration if the experiment is sought to be up-scaled and/or plant yield increased. In spite of this, after the critical phase, the number of living plants remained unchanged for the rest of the experiment in both treatments. The number of living plants was stabilized leaving 36% and 47% of living plants in AS and AS + $GS_{50\%\ wt.}$ substrates, respectively until the end of the experiment. It may be the case therefore that after some adaptation time a number of sunflower plants could withstand B limitations and grow in spite of this deficiency.

Fig. 1 also shows dry biomass (Fig. 1b) and length (Fig. 1c) of plant tissues, number of leaves and rates of flowering plants (Fig. 1d). Statistical tests (*t*-test) found no significant difference between the two groups (*i.e.*, plants growing in AS and AS + $GS_{50\%\ wt.}$ substrates) for any of these parameters, suggesting that sunflower was able to grow and tolerate the presence of metal rich slags.

Previous studies have reported a number of deleterious effects of heavy metals on sunflowers: alteration of seed dormancy time and germination rates (Kranner and Colville, 2011), visual toxicity symptoms such as necrosis and chlorosis (Lopes Júnior et al., 2014), decrease in plant growth and biomass yield (Ahmad et al., 2011), decrease in the rates of photosynthesis (Carlson et al., 1975), alteration in the uptake of mineral nutrients (Ahmad et al., 2011; Lopes Júnior et al., 2014), impairment of proteasome functionality (Pena et al., 2008). In contrast, sunflower has also been demonstrated to withstand the presence of heavy metals through a

number of mechanisms: i) enhancement of antioxidation systems by boosting antioxidative enzymes (e.g. ascorbate peroxidase, glutathione reductase) (Nehnevajova et al., 2012) or synthesizing molecules with free radical scavenging activity (e.g. carotenoids, flavonoids) (Yadav et al., 2016), ii) improvement of metal detoxification machinery by synthesizing molecules able to sequestrate metals (e.g. phytochelatins, metallothioneins) (Raab et al., 2005; Tomas et al., 2014), iii) development of metal tolerance mechanisms involving plant growth regulators (Tassi et al., 2008). It is likely that one or several of the above-mentioned tolerance mechanisms were implicated in the present study to explain, at least partially, sunflower ability to grow in the presence of GS. Besides, taking into consideration the results of DTPA acid single extraction and BCR sequential extraction it may be the case that the amounts of bioavailable metals (*i.e.*, low fraction of available metals regarding total metal content in the sample AS + $GS_{50\%\ wt.}$) were below the threshold toxicity limits that sunflower is able to withstand. In any case, these results support previous research, which also demonstrated sunflower ability to grow in multi-metal contaminated soils (Herrero et al., 2003). In general, therefore, it seems that plant response to heavy metals is usually irregular and complicated to predict because it depends not only in intrinsic plant characteristics (*i.e.*, species, cultivars) (Laporte et al., 2015), but also on several external variables such as: metal type, metal concentration, metal bioavailability, growth medium or exposure time (Rizwan et al., 2016). As a result, the net effect of metals on plants will be the resulting balance between metal phytotoxic effects and plant tolerance mechanisms. Performing experiments in each particular scenario is, in most cases, inevitable.

3.4. Metals in plants

The concentration of metals found in plant tissues is in close association with the chemical composition of the media where plants are growing (Kabata-Pendias, 2011). Therefore, investigating metal uptake by plants in a system that involves a metal-rich growing environment is crucial. Likewise, investigating the distribution of metals within the plant tissues is also relevant.

Fig. 2 presents the concentration of Cu, Pb and Zn in sunflower tissues (*i.e.*, roots, shoots and flowers) as well as the translocation factors (TF) for these metals. In the case of sunflowers grown in the control AS soil, metals are preferentially located in the flowers, followed by shoots and roots. The amounts of Cu and Zn found in shoots of these plants are considered sufficient or normal when compared to the expected values in mature leaf tissue for these essential elements: 5–30 mg kg^{-1} for Cu and 27–50 mg kg^{-1} for Zn, which have been generalized for various plant species by Kabata-Pendias (2011). While Cu and Zn are essential micronutrients for plant life, Pb has not been shown to play any essential role in plant metabolism. Thus, deficiency limits are not established for this metal (Kabata-Pendias, 2011).

By contrast, metal content in plant parts of sunflowers growing in the presence of metals in the AS + $GS_{50\%\ wt.}$ substrate was considerably higher. The concentration reached in shoots was: 126, 124 and 175 mg kg^{-1} for Cu, Pb and Zn, respectively. Although such values of Cu, Pb and Zn in leaf tissue are generally considered excessive or toxic for plants (Kabata-Pendias, 2011) they were not translated in a deterioration of plant biometric parameters, which is likely to be related to metal detoxification and tolerance mechanisms in sunflower. Besides, these values are above the limit concentration tolerable in leaf tissue of agronomic crops, which is fixed at a maximum of 20, 10 and 100 mg kg^{-1} for Cu, Pb and Zn, respectively (Kabata-Pendias, 2011). As a result, some of the issues emerging from this finding relate specifically to the phytomanagement practices envisaged for sunflower plants growing in degraded soils. Although performing full correlating analyses between extracted metals using DTPA/BCR procedures and accumulated metals in plant leaves were beyond the scope of this paper, it was not found a straightforward association of these variables, suggesting that other factors govern plant metal uptake. While Cu was the prevalent element released in the leaching test, extracted with DTPA and also in the second step of BCR sequential extraction, Zn was the most translocated and abundant metal found in sunflower shoots. A possible explanation for these results may be related to metal speciation. Schijf et al. (2015) demonstrated that the stability constant of the complexes formed by metals and a model siderophore produced by soil actinomycetes is higher for Cu than for Zn. It is possible, therefore, that if metals occur as analogous siderophore complexes, Cu was less available for plant uptake than Zn, and thus a higher content of Zn in the plant shoots as compared to Cu was found. Another possible explanation for this might be associated with the differential efficiency by which plants obtain and distribute Cu and Zn through specific metal transporters (Grotz and Guerinot, 2006; Printz et al., 2016).

In what concerns the distribution of metals within sunflower plants growing in AS + $GS_{50\%\ wt.}$ sample, the most favored tissues for metal accumulation were the shoots, followed by roots and flowers. Moreover, considering that shoots account for more plant biomass than roots and flowers, then shoots stand as the principal metal host in the plant. The preferential location of metals in the above-ground tissues with respect to roots is reflected in the values of TF, which were >1 for all the metals: 1.1 for Cu, 1.9 for Pb and 4.3 for Zn. These results are in agreement with those of Angelova et al. (2016) who also obtained TF > 1 for Pb and Zn in sunflowers growing in multi-metal contaminated soil, demonstrating that metals were easily transferred from roots to shoots. It is likely that metal detoxification machinery was activated in sunflower, allowing plant adaptation to the excess of metals. In order to effectively transport metals from roots to above parts a number of processes are required: chelation, compartmentalization, biotransformation, and cellular repair mechanisms (Salt et al., 1998).

This study at laboratory level, although preliminary, has already some implications for future studies at bigger scale (*i.e.*, plots, field) implementing agronomic practices with sunflower.

In a recent study by Fässler et al. (2010) investigating the long-term effectiveness of phytomanagement in moderately metal-contaminated agricultural land, sunflower exhibited low levels of metal accumulation enabling the production of valuable biomass as safe fodder or green manure. On the contrary, the present study showed that metal concentration in aerial parts of sunflower exceeds the acceptable levels for agronomic crops, which would invalidate the use of sunflower by-products for human consumption (e.g. edible seeds and oil). In addition, the use of sunflower leaves as feed for livestock would pose a risk directly to animals and indirectly to humans and other living organisms if such undesirable compounds enter in the food chain. Alternative commercial uses of oil, other than food products (paints, varnishes, lubricants, soap, candles), could be then considered. Other application of sunflower that could be contemplated is the use of vegetable oil as alternate source to produce biodiesel fuel. This possibility has recently been explored by Zhao et al. (2014) in a study in urban marginal land, which include energy balance calculations with favorable results. A note of caution is due here since, independently of ultimate oil application, heavy metal content in the final oil product should always be quantified to verify that it is compatible with the foreseen purpose. Another interesting study by Hao et al. (2012) demonstrated that the production of ornamental sunflower with economic interest could be feasible in Cd and Zn contaminated soil to maximize the profit that could be obtained from degraded land resources. Overall, the common aim of these practices is finding the

balance between environmental and economic interests.

3.5. Soil microbial diversity

3.5.1. Community level physiological profiling (CLPP)

CLPP is a technique widely used to study functional diversity of microbial communities. It consists in determining the capability of soil microbial communities to metabolize a variety of sole carbon sources that are placed in specially designed microplates, *i.e.*, Biolog EcoPlates™. The extent of well color change over the incubation period indicates the richness of the functional diversity of the soil microbial community. As a result, a characteristic pattern of carbon source utilization by microbial communities present in a particular soil sample is obtained (Garland and Mills, 1991).

Shannon diversity and evenness, and substrate richness indices based on carbon source utilization pattern on Biolog EcoPlates™ (Table 4) tended to be higher in the bulk soil of AS + $GS_{50\%\ wt.}$ sample as compared to the remaining samples. Moreover, while no statistical significant difference was found between bulk and rhizosphere samples of AS soil, there was a significant difference in Shannon indices between these two conditions of AS + $GS_{50\%\ wt.}$ sample, being higher in bulk soil. (Further information of complete AWCD profiles from Biolog EcoPlates™ during one week can be found in supplementary data, Fig. S2).

Thus far, previous studies have examined the association between heavy metal stress and soil microbial communities. Teng et al. (2008) evaluated the effect of different proportions of Cu mine tailings on soil functional microbial diversity, observing significant differences in the richness of the soil microbial community, with greater richness in the control having no mine tailings. This differs from the findings presented here, where (although not statistical significant) diversity tended to be higher in the presence of metal rich slags. This could be explained by the fact that the presence of GS tended to select a population of microbes able to metabolize a wide variety of substrates, which was translated in higher richness and also more diversity and evenness Shannon indices.

In addition, the functional diversity of soil microbial community has been used as indicator for evaluating the effect of vegetation restoration on soil quality. In this sense, restoration of degraded soil by vegetation has shown to improve soil conditions: analyses of CLPP data indicated that vegetation restoration tended to result in higher AWCD, substrate richness, and functional diversity (Wei et al., 2011). In addition, Teng et al. (2008) demonstrated that particular forage grasses have distinct impact on soil microbial functional diversity when growing in the presence of Cu mine tailings, indicating that specific plant species have a key role restoring abandoned mining areas. However, the current study has been unable to demonstrate a net improvement of microbial functional diversity as a consequence of the presence of plants. By contrast, higher functional diversity was found in the bulk soil than in the rhizosphere soil when slags were present. It could be argued that rhizosphere exudates somehow minimized or repressed the effect of GS resulting in decreased Shannon diversity and evenness indices, but not in substrate richness. In other words, while the number of substrates that were being metabolized was not influenced by plants, the extent to which they were used did change.

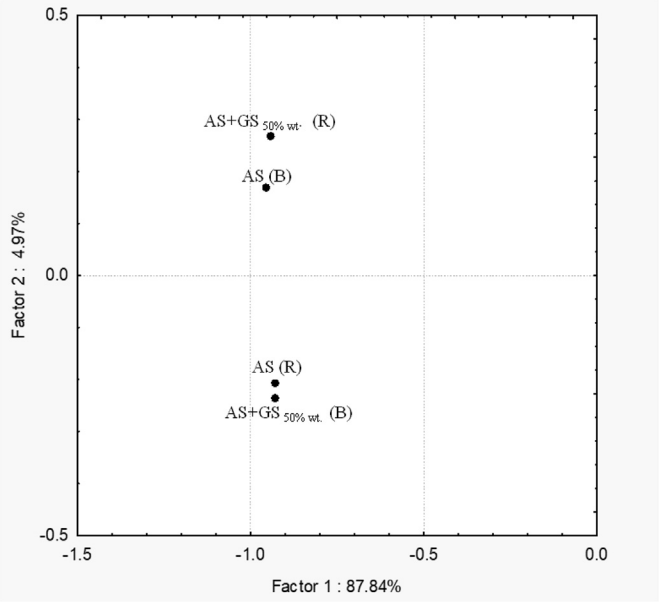

Fig. 3. Principal Component Analyses (PCA) from substrate utilization profiles of soil samples based on 6 d incubation data of Biolog EcoPlates™. The percentage of total variation accounted for by each principal component is specified for both factors in each axe. AS: Agricultural soil; AS + $GS_{50\%\ wt.}$: Agricultural Soil + Granulated Slag; B: Bulk soil; R: Rhizosphere soil.

Finally, the Principal components (PC) score plots of carbon source utilization pattern on Biolog EcoPlates™ (Fig. 3) revealed the clustering of two distinct groups for the utilization of carbon sources. Samples AS (B) and AS + $GS_{50\%\ wt.}$ (R) were close together in one group while samples AS (R) and AS + $GS_{50\%\ wt.}$ (B) were grouped in another cluster. Factor 1 and factor 2 together accounted for 92.8% of the total variability in these analyses. This demonstrates that both the presence of GS and sunflower are factors that changed the diversity pattern in soils.

3.5.2. Denaturing gradient gel electrophoresis (DGGE)

The profiles of bacterial communities extracted from soil samples were generated using the molecular fingerprinting technique: denaturing gradient gel electrophoresis (DGGE) of 16S rDNA. This independent-culture technique allows estimating the diversity of microbial communities by the comparison of characteristic banding patterns.

The different DGGE banding patterns of soil samples are shown in Fig. 4a, which allowed the construction of a clustered matrix to obtain a phylogenetic tree (Fig. 4b). The dendrogram made based on PCR-DGGE analysis showed a close gathering for at least two independent replicates of each treatment. The fact that frequently

Table 4
Diversity indices calculated based on 6 d incubation data of Biolog EcoPlates™ Different letters (a, b) indicate significant differences in mean values between treatments. Mean values with the same letters were similar and no statistically significant differences between treatments were observed for these samples.

Sample	Shannon Diversity	Shannon Evenness	Substrate Richness
AS (B)	3.203 (±0.020) ab	0.942 (±0.006) ab	26.333 (±1.155) a
AS + $GS_{50\%\ wt.}$ (B)	3.278 (±0.039) b	0.964 (±0.011) b	28.667 (±1.155) a
AS (R)	3.187 (±0.063) ab	0.937 (±0.019) a	25.500 (±2.121) a
AS + $GS_{50\%\ wt.}$ (R)	3.186 (±0.014) a	0.937 (±0.004) a	27.667 (±1.528) a

AS: Agricultural soil; AS + $GS_{50\%\ wt.}$: Agricultural Soil + Granulated Slag; B: Bulk soil; R: Rhizosphere soil.

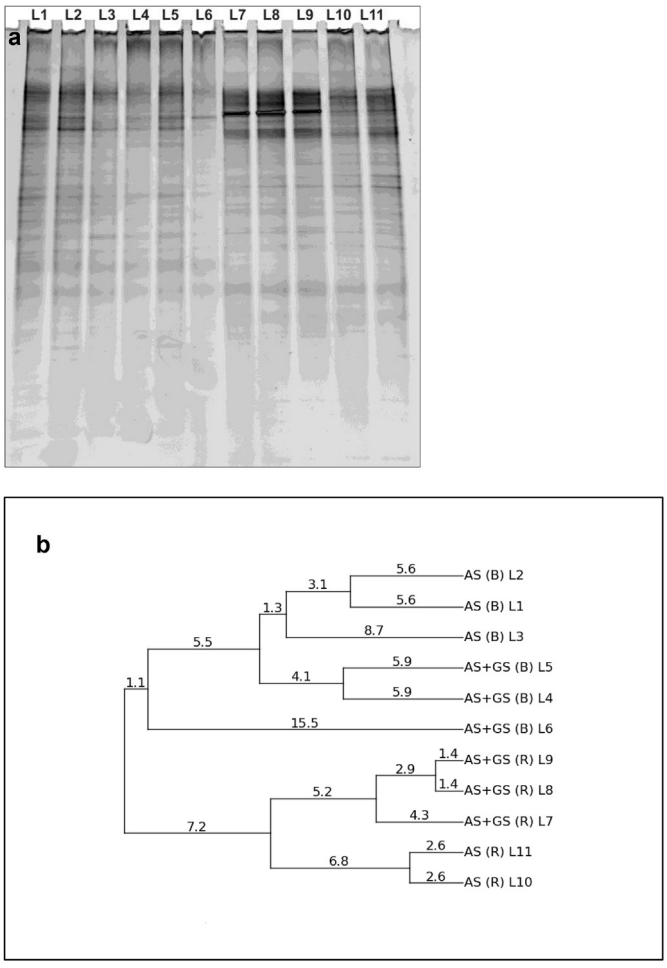

Fig. 4. a. Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA gene of the bacterial genomic DNA extracted from soil samples. L1-3: AS (B), L4-6: AS + $GS_{50\%\ wt.}$ (B), L7-9: AS + $GS_{50\%}$ (R), L10-11: AS (B) b. Hierarchical clustering dendrogram representing genetic similarity of microbial-community profiles obtained with PCR-DGGE banding pattern. Clustering analysis was performed using single linkage method. AS: Agricultural soil; AS + $GS_{50\%\ wt.}$: Agricultural Soil + Granulated Slag; B: Bulk soil; R: Rhizosphere soil; L: line.

one of the replicates diverged to some extent, probably reflects soil sampling heterogeneity. In addition, the dendrogram showed a distinct clustering between two main groups: bulk soil and rhizosphere samples, highlighting the rhizosphere effect. The presence of vegetation has demonstrated to have an effect on microbial communities of degraded soils. Results reported here reveal a positive trend of the rhizosphere in increasing bacterial richness and diversity in the presence of GS, although it was not statistically significant (Richness and Shannon diversity indices calculated from DGGE banding pattern data are available in supplementary data, Table S4). Similarly, Chen et al. (2008) observed that the colonization of Cu mine tailings by reed (*Phragmites communis*) significantly changed the bacterial community of tailings and 16S rRNA gene sequencing method demonstrated that bacterial diversity in the bare mine tailing was lower than that of the mine tailing vegetated with reed.

Apart from rhizosphere/bulk grouping, also subgroups caused by the presence of GS were made. Besides, a trend to decrease bacterial richness and diversity in the presence of GS was also observed (supplementary data, Table S4). Lower diversity in the presence of metal-rich slags could be indicative of an association between metal stress and lower microbial diversity, which is supported by previous research. A recent study demonstrated that the diversity of the microbial community of post-mined sites was significantly lower than the undisturbed sites (de Quadros et al., 2016). Likewise, inoculation of Cu-Pb-Zn tailings with native soil boosted the establishment of native microbial communities and initiated the rehabilitation of biogeochemical processes in the degraded soil. Besides, this approach could be used before the establishment of native plant species (Li et al., 2015). To date, a number of studies have put the focus on how soil metal pollution and changes in soil microbial communities are linked (Thavamani et al., 2012). For instance, in a recent study, Zhang et al. (2016a) showed that heavy metal pollution (*i.e.*, Cd, Cu, Cr and Pb) is a principal determining factor shaping wetland bacterial community structure. In this study, PCR-DGGE analysis of bacterial community composition indicated a clustering among several soil samples that could be associated with different soil pollution levels, although the numbers of bands were not dependent on the level of contamination with heavy metals (Kozdrój and Van Elsas, 2001). Interestingly, Uroz et al. (2015) have recently defined the concept of mineralosphere as the microbial habitat in which physicochemical properties of minerals are key drivers of the microbial communities.

Discrepancies in soil microbial diversity between CLPP and PCR-DGGE techniques could be attributed to the fact that results from culture-independent techniques can be quite different (and usually complementary) from those obtained from enrichment cultures (Chien et al., 2008). While CLPP data is based on cultivable species with ability to grow on different substrates, PCR-DGGE also takes into consideration non-cultivable species broadening the information that can be obtained. This is of capital importance given the fact that it is estimated that only 1% of existing microorganisms can be cultured *in vitro* (McCaig et al., 2001).

4. Conclusion

To conclude, this study supports the idea that reactivity of slags dumped in the environment is an aspect that cannot be neglected since metallurgical wastes must not be considered inert when disposed in the soil. The results of this investigation show that:

- Soil solution influences metal leaching from slags, Cu being the most bioavailable metal in a soil/slag mix and the most released metal from slags.
- The presence of metal rich slags in soil was tolerated by sunflower, which accumulated Cu, Pb and Zn, preferentially in above-ground tissues. Moreover, slags shaped the diversity of soil microbial communities.
- Sunflower could be a candidate for the phytomanagement of soils, enabling that slag-dumping sites become a potential resource for profitable biomass production.

Finally, it must be noted that presented results herein derived from observations at laboratory level. Plot and/or field experiments with sunflower growing in soils altered by metallurgical wastes assessing mid to long-term effects should be conducted in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.06.038.

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