Article

Enhanced As, Pb and Zn Uptake by *Helianthus annuus* from a Heavily Contaminated Mining Soil Amended with EDTA and Olive Mill Wastewater Due to Increased Element Mobilization, as Verified by Sequential Extraction Schemes

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Abstract: Soils close to former mines or metallurgical facilities often contain extreme concentrations of potentially toxic elements (PTEs), and among soil remediation actions needed, enhanced phytoextraction techniques using chelating agents could be a very promising option. In this context, a pot experiment was conducted to evaluate the effect of Na_2-EDTA, olive mill wastewater (OMW), and their combination (EDTA + OMW) on As, Pb and Zn uptake by *Helianthus annuus* (sunflower), grown in a heavily contaminated soil from Lavrio, Greece. After harvest, aboveground and belowground biomass and the concentrations of As, Pb and Zn were determined. Additionally, composite soil samples of all treatments were subjected to appropriate sequential extraction procedures for each PTE. Results showed that although nonstatistically significant effects on plant growth and As concentration were observed, Pb and Zn plant concentrations increased by the EDTA addition, alone or combined with OMW. When the total bioavailability parameter was assessed, EDTA treatment was also found to significantly enhance As uptake (50% increase, $p \leq 0.05$). These results were further supported by the respective sequential extraction schemes in which both Pb and As showed increased concentration in the corresponding more soluble soil fractions, probably as an outcome of partial Fe–Mn amorphous oxide dissolution. Our study thus suggests that the applied treatments of EDTA, OMW or their combined application could play a key role as reductive agents in enhancing the mobility of these PTEs in the soil environment, triggering their increased uptake by *H. annuus*.

Keywords: enhanced phytoextraction; sunflower; potentially toxic elements (PTEs); soil fractionation

1. Introduction

Understanding the anthropogenic and natural sources of potentially toxic elements (PTEs) in soils, their soil–plant relationship and the mechanisms that control their bioavailability has always been a major issue for the environment, human health and agricultural production [1]. Numerous anthropogenic activities play a key role in the continuous PTEs contamination of terrestrial ecosystems [2]. One of them, which causes intense dispersion and deposition of PTEs in the environment with consequent degradation of surrounding areas, is mining and metallurgical processes [3,4]. The management of degraded lands and the effort to restore them, while adapting to sustainable models of natural resource management, is one of the greatest challenges of the current decade. Various techniques have been proposed for the removal of PTEs from soil environments, especially concerning agricultural soils, such as vitrification, soil washing and land filling [5], but for the most part these are costly techniques that damage natural biodiversity, destroy the natural soil

structure and are not feasible to apply to large areas [6]. Instead, an alternative remediation technique that is gaining ground due to its low cost and environmentally friendly approach is phytoremediation. The typical method of phytoremediation is phytoextraction, a technique in which plants that accumulate heavy metals are grown in contaminated soils, take up high amounts of the heavy metals in their aboveground biomass, and subsequently are harvested and removed from the field [7]. Because the phytoextraction technique is a slower process compared to other remediation techniques, many studies have been done to understand and find factors that could enhance the intensity of PTEs uptake by the accumulator plants, and therefore accelerate the process of remediation [8]. As pointed out by Ali et al. (2013) [9], the main factor determining the amount of PTEs taken up by plants, beyond evidently the plant species, is the concentration of the bioavailable fraction. This fraction depends on total concentration, soil physicochemical properties such as pH/Eh, clay quantity and mineralogy, organic matter content and the adsorptive capacity of the soil [10].

Thus, crucial points in the application of phytoextraction techniques are, on the one hand, the complete understanding and assessment of soil properties and PTEs concentrations per soil fraction and, on the other hand, the attempt to find possible mechanisms that will increase the plant-available concentration fraction in the soil environment [11]. A very commonly used chelating agent that desorbs PTEs from soil colloids, making them available for plant uptake, is EDTA. The impactful effects of EDTA in the rhizosphere environment regarding metal mobilization are well established in the literature [12]; however, the potential risk of its use is also highlighted owing to its low biodegradability that can lead to leaching phenomena [13].

Many studies emphasize the need to find alternative soil amendments that have a similar effect in terms of mobilizing metals in the soil solution, with olive mill wastewaters (OMWs) being considered as a candidate alternative. OMWs have a high organic content, acidic pH and are rich in soluble phenols, and thus are able to affect the redox potential and promote metal chelation [14]. To qualify a plant as a PTEs hyperaccumulator, it must have certain characteristics that vary depending on the approach chosen, but it must also grow naturally in contaminated soils, have good root system development, high PTEs uptake and a good translocation factor for targeted PTEs [15]. *Helianthus annuus* is a plant known to grow in environments with elevated concentrations of PTEs and resistance to toxicity, while the review study by Jadia and Fulekar (2009) [16] reported an increased ability of the plant to accumulate Pb, Ni, Zn, Cr, As, Cd and Cu. However, as far as we know, systematic studies emphasizing on how the application of different chelating agents affect the particular redistribution of targeted PTEs between different soil fractions and which of them contributes the most on its phytoextraction ability, are limited. We consider this a research gap that needs further investigation.

Therefore, the aim of the present study was (i) to investigate the ability of *H. annuus* plant to be used for removing As, Zn and Pd from contaminated mine-affected soils; (ii) to evaluate the effect of EDTA and OMW application on the uptake of As, Zn and Pd by *H. annuus* plants; and (iii) to examine the effect of EDTA and OMW application on the redistribution of targeted PTEs between different soil fractions through the determination of sequentially extracted chemical forms.

2. Materials and Methods

2.1. Soil Selection and Characteristics

From an area of $10 \times 10 \text{ m}^2$ and a depth of 20 cm, in the area of Lavrion, central Greece, soil heavily contaminated by mining activity was excavated and transported to the facilities of the Agricultural University of Athens. A soil sample was air-dried, ground and passed through a 2 mm sieve, in order to carry out a complete set of soil analyses for the determination of its physicochemical properties, which are summarized in Table 1. The remaining quantity of the soil was transferred to the greenhouse, homogenized, passed through a 1 cm sieve and subsequently filled into pots. Further information concerning the

natural characteristics of the study area and the coordinates of the soil sampling sites are provided by Kalyvas et al. (2018) [3].

The determination of the particle-size distribution was carried out using the Bouyoucos hydrometer method [17], while electrical conductivity and pH were determined in 1:1 (*w/v*) soil/water slurry [18]. The Bernard calcimeter method [19] was followed for the determination of total calcium carbonate ($CaCO_3$), Walkley–Black procedure was used to estimate the organic matter (OM) content [20] and the sodium acetate method [21] was chosen for the determination of cation exchange capacity (CEC). The total concentrations of metals in the soil samples were determined after digestion with aqua regia in microwave tubes and measured by atomic absorption spectroscopy [22].

2.2. Greenhouse Experiment

Three to four sunflower seeds (*H. annuus*) were planted into plastic pots filled with the As, Pb and Zn contaminated soil from mining activities. Two weeks after emergence, one plant per pot was kept with the criterion that all selected plants had similar biomass and the pots were placed in a greenhouse in a randomized pattern. At the same time, fertilizer with 0.4 g N/kg as $(NH_4)_2SO_4$ was applied to enhance plant growth. Plants were watered 2 to 3 times per week (same irrigation rates for all treatments) to maintain soil moisture at about 60% of the water-holding capacity. During the experimental period, relative humidity and air temperature in the greenhouse ranged between 70% and 90% and 20 and 35 °C, respectively. Fourteen days after the selection of plants, macroscopic control was repeated for homogeneous plant growth, and the three following treatments were applied in six replications: (i) Na_2-EDTA 0.01 M (or 2.5 mmol kg^{-1} of soil, referred in the text as EDTA); (ii) OMWs 15% (diluted in deionized water) with the following characteristics: total N: 0.9 ± 0.1 g L^{-1}, total organic carbon (TOC): 26 ± 2.4 g L^{-1}, K: 6.1 ± 0.2 g L^{-1}, P: 0.21 ± 0.02 g L^{-1}, chemical oxygen demand (COD): 48 ± 2.1 g L^{-1}, total suspended solids (TSSs): 42 ± 3.2 g L^{-1}, total phenolics: 8.8 ± 0.3 mg mL^{-1}; and (iii) a combination of OMWs 15% and EDTA 0.01 M. Six pots treated only with deionized water, which served as control. After 4 weeks from the application of treatments, the plants were harvested and transferred to the laboratory where the analyses for the determination of metal concentrations in the plant tissues were carried out. At the same time, the soil from two pots per treatment was homogenized and thus three mixed samples were taken to carry out the sequential extraction schemes.

2.3. Plant Analysis

Total metals concentrations were determined in aboveground plant tissues and roots following a wet digestion protocol suggested by Jones et al. [23] that utilizes concentrated nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2). After harvesting, both the aboveground part of the plants and the root system were transferred to the laboratory, thoroughly washed with deionized water, and dried in an oven at 60 °C until a stable weight was obtained (desiccation). Because of the limited belowground biomass of roots, only one composite sample per treatment was analyzed. Subsequently, all plant samples were weighed and then ground using a mixer mill, producing a fine powder less than 0.2 mm particle size. Then, 0.5 g of the plant samples were placed together in conical flasks with 5 mL of 65% HNO_3, covered with watch glasses, and left overnight. This was followed by digestion at 125 °C for one hour on a hot plate, cooling of the samples, and after the addition of 2 mL of 30%, H_2O_2 redigestion at 80 °C until a white or colorless solution was recovered. Digests were filtered and diluted to a volume of 10 mL for analysis. Translocation factor (TF), which describes the respective ability of a plant to translocate the targeted PTEs from roots through shoots and leaves, was calculated as the ratio of metals concentration in the aboveground part of the plants to those in the roots [24].

2.4. Sequential Extraction Procedures

Two sequential extraction procedures were performed to investigate the distribution of As, Pb and Zn into the different chemical phases of the soil. The Wenzel et al. [25] scheme was used for As fractionation, while BCR (Community Bureau of Reference) [26] was used to determine the Pb and Zn partitioning. The operationally defined chemical fractions and analytical stages for both sequential extraction protocols are presented in detail in Kalyvas et al. [13].

2.5. Statistical Analysis

The data of each variable were checked for homogeneity with the standardized skewness and standardized kurtosis criterion, and when values of these statistics were outside the expected range, appropriate transformations were applied to make them more normal. Analysis of variance (ANOVA) was conducted to compare the means among different treatments and respective soil fractions, and then Tukey's honestly significant difference (HSD) studentized range test was applied for evaluating significant differences among means at $p \leq 0.05$. Principal component analysis (PCA) was applied to identify the variation in the parameters studied, and interrelationships between each PTE uptake and respective distribution patterns among different soil fractions were checked with Pearson correlation tests. For all statistical analyses, the Statgraphics software was used (STATGRAPHICS, Centurion XVI, version 16.1.11, StatPoint Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. Physical and Chemical Properties of Soil Samples

The contaminated soil used for the pot experiment classified as Cambisol (according to WRB), loam in texture (L) and with an alkaline reaction [27]. In addition, it was calcareous with generally moderate levels of $CaCO_3$ content and cation exchange capacity (CEC), but contrarily with high content of organic C. As expected, the levels of As, Zn and Pb total contents determined were much above the upper toxicity limits referred to in the literature for EU agricultural soils (Table 1) [28,29].

Table 1. Basic soil properties of the studied soil.

Clay	Silt	Sand	OM [1]	Total CaCO$_3$	pH	CEC [2]	Total As	Total Zn	Total Pb
		g kg^{-1}			**1:1**	**cmol$_c$ kg^{-1}**		**mg kg^{-1}**	
198	301	501	45	69	7.7	18.0	822 (55)	5677 (720)	4428 (530)

[1] OM: organic matter; [2] CEC: cation exchange capacity. Dutch intervention values of respective PTEs (Netherlands MHPPE, 2000) [29] are given in parentheses.

3.2. Effects of EDTA and Olive Mill Wastewater Treatments on Plant Growth, Aboveground Biomass PTEs Content and Respective Roots-to-Shoots Translocation Factor

Analysis of variance showed that different treatments of selected chelating agents did not cause any statistically significant effect on plant growth, and thus the aboveground biomass of *H. annuus*, practically remained unaffected as compared with the control in which no extraction promoter was applied (Figure 1a). The nonsignificant effect was also the case with the concentration of As in the aboveground biomass of *H. annuus* when possible treatments were investigated (Figure 1c). In contrast, this result did not stand concerning Zn and Pb contents of aboveground biomass, where respective changes were observed.
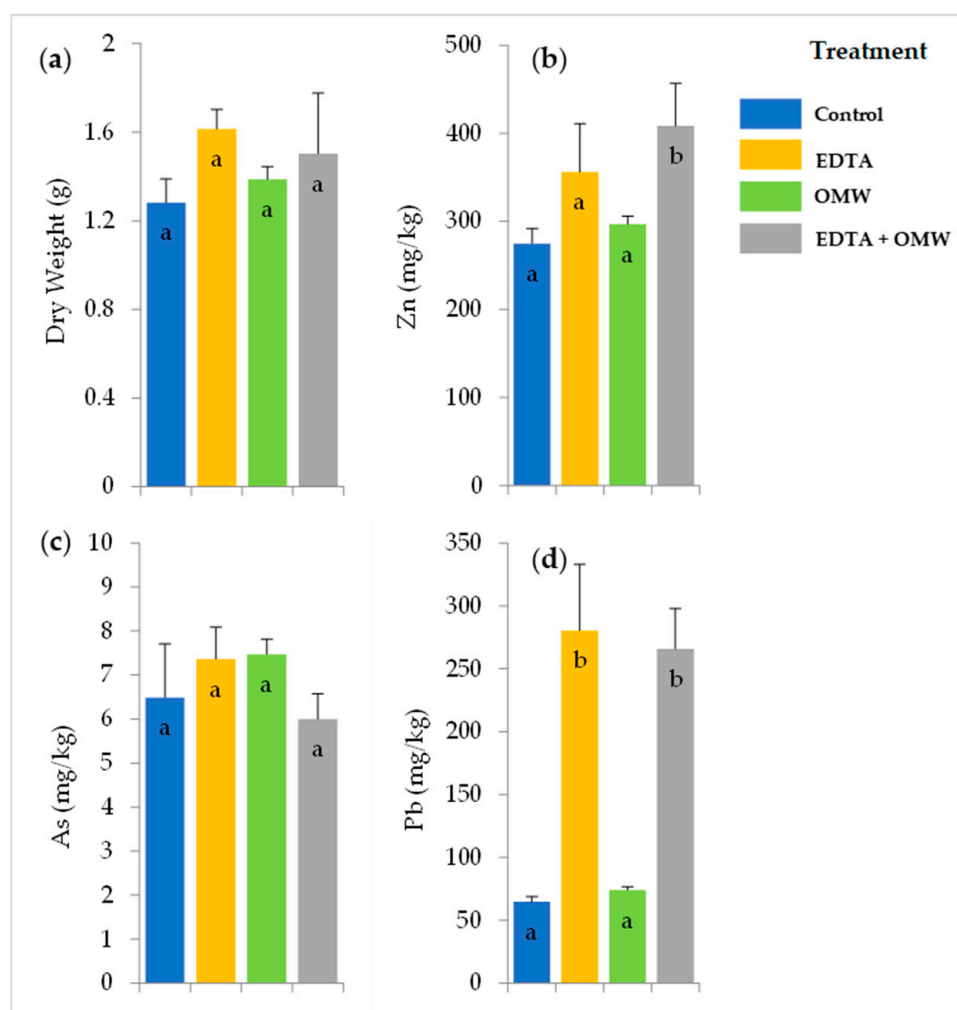
Figure 1. (**a**) Aboveground biomass (dry weight) of *H. annuus* and respective (**b**) Zn, (**c**) As and (**d**) Pb concentrations among different treatments. The data shown are the mean values of six replicates. The error bars represent the standard error of the mean at the 95% confidence level ($p \leq 0.05$). Comparisons were performed by Tukey's HSD test ($p \leq 0.05$) and are demonstrated with lowercase letters. The presence of a common letter between each column implies no significant difference. OMW: olive mill wastewater.

More specifically, the combined treatment with EDTA and OMW managed to significantly increase Zn concentration as compared to control or the other treatment schemes of EDTA or OMW when applied alone (Figure 1b). In addition, the results in the case of Pb concentration were clearer, which were accompanied by significant positive effects in both EDTA and the combined EDTA + OMW treatment (Figure 1d).

Since no significant changes in the DW of the aboveground biomass were observed, these results concerning the concentration of the studied PTEs were also reflected in the respective uptake parameters, assessed as the total concentration of each element taken up by sunflower plants per pot (Table 2). However, as seen from the respective results shown in the table, in the case of As with EDTA treatment, the nonsignificant increase trend on its concentration gave significant uptake results, assessed as the total amount of As phytoextracted by *H. annuus* per pot. The same was also the case for Zn, which despite the marginally nonsignificant differences in its concentration for the OMW treatment, the total Zn uptake per pot significantly increased for this specific treatment.

Table 2. Total uptake of As, Zn and Pb by *H. annuus* for different treatments. Comparisons performed by Tukey's HSD test ($p \leq 0.05$) are demonstrated with lowercase letters and are valid within each element. The presence of a common letter implies no significant difference. OMW: olive mill wastewater. The standard errors of means are given in parentheses.

	As	Zn	Pb
	mg pot^{-1}		
Control	0.008 (0.001) a	15.5 (1.8) a	4.6 (0.5) a
EDTA	0.012 (0.002) b	12.0 (2.1) a	14.6 (2.6) b
OMW	0.010 (0.001) ab	24.2 (1.5) b	5.4 (0.6) a
EDTA + OMW	0.009 (0.002) ab	24.1 (4.7) b	14.9 (2.3) b

Roots-to-shoots translocation factor (TF) of As, Zn and Pb is presented in Figure 2. However, these results could only be evaluated as trends since the limited belowground biomass of roots led to one composite root sample, which was a constrain parameter for the appropriate statistical analysis and respective means comparison. Nevertheless, as shown in Figure 2, in the case of As or Pb, it can be clearly indicated that the TF value of the control was not found to be smaller as compared with other treatments, while concerning Zn, this trend was valid only for the EDTA and the combined EDTA + OMW treatment. Additionally, and for all cases, also clear was the finding that the roots-to-shoots translocation factor was <1, a fact which points to a limited phytoextraction potential of the specific plant species to extract large amounts of the PTEs studied.
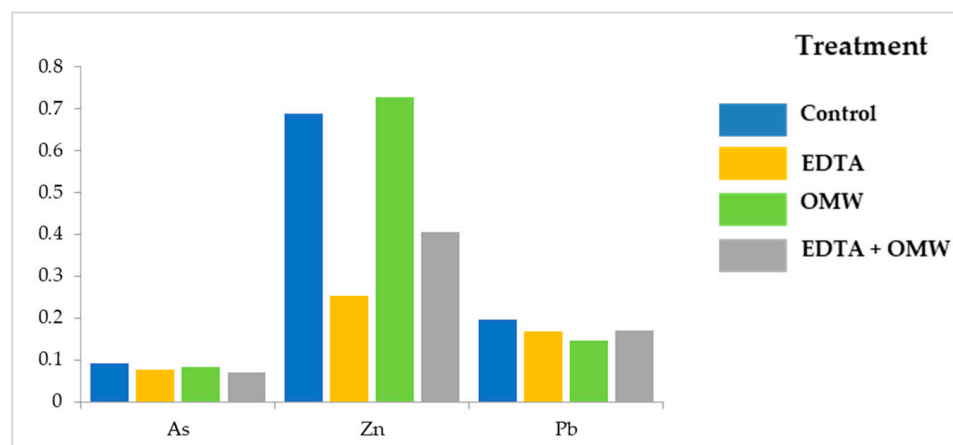
Figure 2. Roots-to-shoots translocation factor (TF) of *H. annuus* for As, Zn and Pb for different treatments. OMW: olive mill wastewater.

3.3. Distribution of As, Zn and Pb in Soil Fractions

Distribution patterns of corresponding PTEs in different soil fractions are presented in Figure 3. The mean percentages of As, Zn, and Pb into the various soil fractions were in the order W1 < W2 < WRF ≤ W4 < W3, B3 < B1 < BRF < B2 and B1 < B3 < BRF < B2, respectively. As expected, arsenic was found to be mostly adsorbed in the amorphous and crystalline Fe oxides [30] with an average percent value of 68.2%, while the BCR partitioning scheme demonstrated the affinity of Zn and Pb for Fe oxides, exhibiting an average percent value of 35.6% and 54.8%, respectively, in the reducible fraction (B2) [31].

As

	Control	EDTA	OMW	EDTA+OMW
■ WRF	196	130	133	139
■ W4	150	175	164	197
■ W3	377	364	357	345
■ W2	86	91	88	95
■ W1	6	8	8	7

Zn

	Control	EDTA	OMW	EDTA+OMW
■ BRF	918	841	819	872
■ B3	681	675	765	667
■ B2	1757	1709	1790	1766
■ B1	902	891	899	894

Pb

	Control	EDTA	OMW	EDTA+OMW
■ BRF	1428	1413	1404	1309
■ B3	306	296	303	279
■ B2	3622	3575	3741	3608
■ B1	285	419	299	368

Figure 3. Mean percentages and values of As, Zn and Pb in the different soil fractions, between different treatment schemes (numbers in tables are in mg kg^{-1}). The error bars represent the standard error of the mean at the 95% confidence level ($p \leq 0.05$) (n = 3). W1: nonspecifically sorbed; W2: specifically sorbed; W3: amorphous hydrous-oxide-bound; W4: crystalline hydrous-oxide-bound; WRF: residual fraction; B1: exchangeable/weak acid soluble; B2: reducible; B3: oxidizable; BRF: residual fraction.

3.4. Effects of Different Chelating Agents on PTE Uptake by H. annuus and Soil Fraction Distribution

3.4.1. Arsenic

PCA revealed that slightly over 70% of the total variance of As concentration between different treatments and among different soil fractions could be grouped into two components, in the first of which the specifically sorbed (W2) and well crystallized Fe and Al hydrous oxides (W4) fractions contributed the most, whereas in the second, As uptake in the aboveground biomass and the nonspecifically sorbed fraction (W1) were the dominant parameters (Figure 4).

In addition, Pearson correlation tests confirmed these findings and further showed that As uptake was significantly and positively correlated with the nonspecifically sorbed W1 fraction, while positive correlations were also observed between the specifically sorbed and well-crystallized Fe and Al hydrous oxides (Table 3a). In contrast, the above soil fractions (W2, W4) were found to be negatively correlated with the amorphous and poorly crystalline hydrous oxides of Fe and Al (W3), at $p < 0.05$.
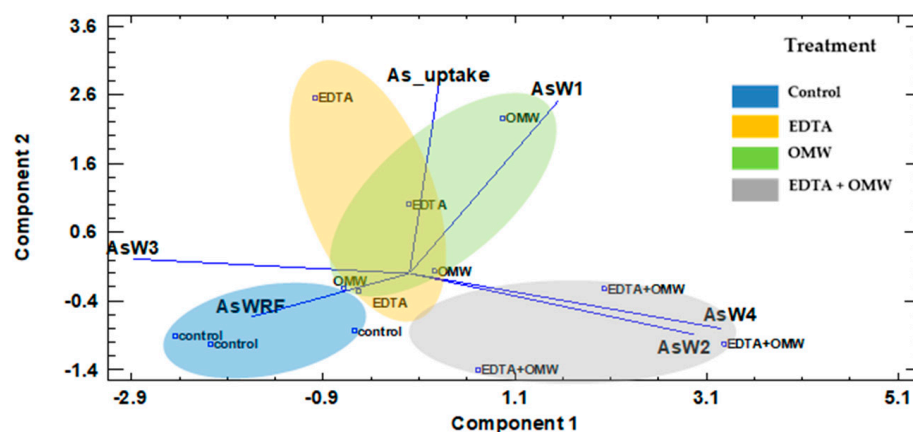
Figure 4. Biplot of the first two principal components regarding As uptake by *H. annuus* and different soil fractions among different treatments. W1: nonspecifically sorbed; W2: specifically sorbed; W3: amorphous hydrous-oxide-bound; W4: crystalline hydrous-oxide-bound; WRF: residual fraction; OMW: olive mill wastewater.

Table 3. (**a**) Pearson correlation matrix between As uptake by *H. annuus* and the respective soil fractions, as extracted by the Wenzel sequential extraction protocol. (**b**) As distribution between different soil fractions as determined by the Wenzel sequential extraction protocol, among different treatments. Comparisons performed by Tukey's HSD test ($p \leq 0.05$) are demonstrated with lowercase letters and are valid within each soil fraction. The presence of a common letter implies no significant difference. *, *** indicate significant r values at $p < 0.05$ and 0.001, respectively ($n = 3$).

(a)	As Uptake	AsW1	AsW2	AsW3	AsW4
AsW1	**0.66 ***				
AsW2	−0.09	0.14			
AsW3	−0.03	−0.29	**−0.62 ***		
AsW4	−0.12	0.16	**0.82 *****	**−0.65 ***	
AsWRF	−0.02	−0.33	−0.12	0.15	−0.42

	AsW1	AsW2	AsW3	AsW4	AsWRF
(b)		mg kg^{-1}			
Control	6.04 a	86.08 a	376.5 ab	150.2 a	196.4 a
EDTA	8.32 b	91.35 ab	364.0 b	174.8 a	130.3 a
OMW	7.58 a	87.72 a	357.4 ab	163.8 a	132.8 a
EDTA + OMW	6.88 a	95.32 b	345.2 a	196.7 b	138.7 a

As far as the potential interrelationships between soil fractions and the different chelating treatment effects are concerned, the multiple comparisons between the means showed a significant positive effect of EDTA treatment in As concentration of the W1 fraction as compared with the control (Table 3b). In addition, although marginally nonsignificant, an opposite trend with a respective decrease was observed in the EDTA treatment regarding the W3 fraction. In the case of OMW treatment, ANOVA results showed no significant effects. Nevertheless, the combination of EDTA and OMW treatments was found to exert a more significant effect on W2 and W4 fractions, showing respective increases in As concentration.

3.4.2. Zinc

Although significant responses were recorded in Zn concentration with the combined EDTA + OMW application, as well as in the total Zn uptake parameter per pot with both combined or OMW treatment alone, this was not reflected with corresponding rearrangements in the soil distribution patterns among different Zn soil fractions (Table 4). In contrast,

Zn uptake was not significantly correlated with any of the soil fractions that were extracted by the BCR sequential extraction protocol, while no respective affinity was observed by the PCA that was conducted.

Table 4. Zn distribution between different soil fractions as determined by the BCR sequential extraction protocol, among different treatments. Comparisons performed by Tukey's HSD test ($p \leq 0.05$) are demonstrated with lowercase letters and are valid within each soil fraction. The presence of a common letter implies no significant difference ($n = 3$).

	Zn B1	**Zn B2**	**Zn B3**	**Zn BRF**
	mg kg^{-1}			
Control	902.1 a	1757 a	681.3 a	917.7 c
EDTA	890.6 a	1709 a	675.0 a	840.6 ab
OMW	899.0 a	1790 a	764.6 a	818.8 a
EDTA + OMW	893.8 a	1766 a	666.7 a	871.9 b

Nevertheless, a statistically significant positive correlation was observed between the reducible and oxidizable soil fraction bound to Fe/Mn oxides (B2) or to organic matter (B3), respectively (r = 0.7, $p \leq 0.01$), while the Zn concentration for the residual soil fraction was found to be significantly decreased, mainly for the OMW treatment, and secondarily for the combined application with EDTA + OMW (Table 4). However, the decrease was not somehow accompanied by corresponding redistributions with any of the other soil fractions extracted by the BCR protocol.

3.4.3. Lead

Results regarding PCA conducted between Pb distribution among different soil fractions and its uptake by *H. annuus* were even more clear, with the first two components managing to explain almost 88% of the total variance (Figure 5). Component weights of the PCA, as well as Pearson correlations, indicated a respective affinity of the exchangeable fraction with Pb uptake (B1), and, on the contrary, a correspondingly strong and negative correlation between uptake parameter and the oxidizable soil fraction where Pb cations are bound to organic matter (B3) (Table 5a). The latter was also found to be negatively correlated with the residual fraction (BRF) while concerning the reducible soil fraction, where Pb is bound to Fe/Mn oxides (B2), strong and negative correlations were observed with the exchangeable fraction.
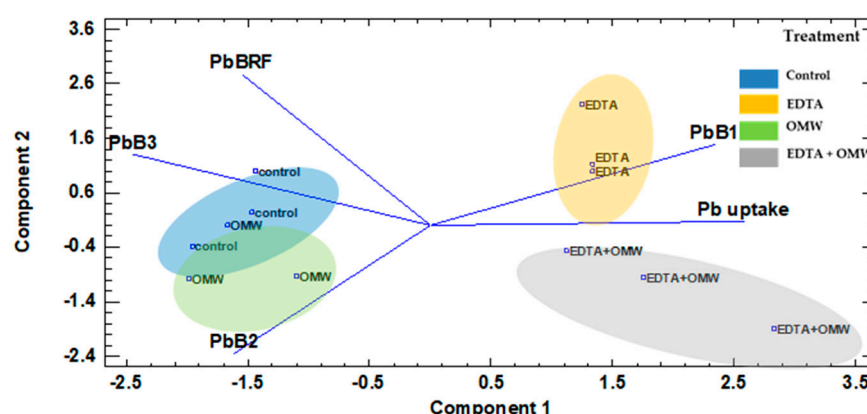

Figure 5. Biplot of the first two principal components regarding Pb uptake by *H. annuus* and different soil fractions among different treatments. B1: exchangeable/weak acid soluble; B2: reducible; B3: oxidizable; BRF: residual fraction; OMW: olive mill wastewater.

Table 5. (**a**) Pearson correlation matrix between Pb uptake by *H. annuus* and the respective soil fractions, as extracted by the BCR sequential extraction procedure. (**b**) Pb distribution between different soil fractions as determined by the BCR sequential extraction protocol, among different treatments. Comparisons performed by Tukey's HSD test ($p \leq 0.05$) are demonstrated with lowercase letters and are valid within each soil fraction. The presence of a common letter implies no significant difference. *, **, *** indicate significant r values at $p < 0.05$, 0.01 and 0.001, respectively ($n = 3$).

(a)	Pb Uptake	PbB1	PbB2	PbB3
PbB1	0.85 ***			
PbB2	−0.43	−0.65 *		
PbB3	−0.78 **	−0.55	0.33	
PbBRF	−0.45	−0.13	−0.10	0.76 **

(b)	Pb B1	Pb B2	Pb B3	Pb BRF
	mg kg^{-1}			
Control	285.4 a	3622 abc	305.9 c	1428 b
EDTA	419.4 c	3575 ab	296.3 b	1413 b
OMW	298.8 a	3741 c	303.3 c	1404 b
EDTA + OMW	368.0 b	3608 b	279.4 a	1309 a

Tukey's multiple comparison test conducted between different Pb soil distribution patterns and respective treatments applied for promoting PTEs solubility indicates a clear significant increase, mainly in the case of EDTA, and in the second place for the combined application with EDTA + OMW (Table 5b). In contrast, an opposite decrease condition was observed in the reducible fraction where Pb is bound to Fe/Mn oxides for the EDTA, or the EDTA + OMW combined addition. On the other hand, regarding the oxidizable fraction where Pb is bound to organic matter, a significant decrease in Pb concentration was observed mainly in the combined EDTA + OMW application, and secondly, in the EDTA treatment when added alone. The efficacy of the EDTA + OMW application to trigger a respective significant decrease in Pb content was also observed in the residual fraction.

4. Discussion

As shown by the ANOVA results, the treatment effect on *H. annuus* growth was non-significant compared to control, indicating that both EDTA and OMW or their combined addition practically left unaffected the plant's growth. Thus, possible plant stress, which might occur by hyperaccumulation of trace elements due to increased mobilization caused by chelating agents, was not the case in this studied plant species. In contrast, for the given dosage of respective treatments, *H. annuus* managed to show tolerance to both potentially toxic phenolic content of OMW [32], and to potential rhizosphere soil environment disturbance that might be caused by an abrupt increase in available concentrations of toxic elements in the soil solution by EDTA addition [33]. This is in line with previous findings reported in the literature that indicate the high tolerance of *H. annuus* to heavy metals [34–39].

In addition, the increased uptake of As, Pb and Zn by *H. annuus* strongly suggests that the applied treatments played a key role in increasing the mobility of these elements in the soil environment. These findings were also confirmed by the results of both Wenzel and BCR sequential extraction protocols, especially with respect to As and Pb, in which respective affinity patterns between PTEs uptake by *H. annuus* and their increased concentration by different chelating treatments, were also observed.

As previously reported in the Introduction section, EDTA is amongst the most commonly used chelating agents, as it is capable of desorbing metals from the soil constituents, forming soluble metal complexes [12]. The above assumption was verified in the case of the *H. annuus* phytoextraction experiment since its addition managed to significantly increase As and Pb total uptake. Furthermore, our results show that this increase was

also accompanied by redistributions that occurred in their contents as distributed to the respective soil fractions.

This result is in line with the findings of other authors who have demonstrated EDTA's effectiveness to mobilize As and Pb in metal-polluted soils. For instance, Abbas and Abdelhafez [40] observed an increased As availability in an industrially As-polluted soil after the addition of several EDTA concentrations, whereas Mühlbachová [41] found that the addition of EDTA in long-term contaminated arable and grassland soils led to increased NH_4NO_3-extractable amounts of Pb up to 600 and 122 times, respectively.

Though the EDTA concentration used in this study is considered environmental and plant-safe according to Vamerali et al. [42] (<3 mmol EDTA kg^{-1} of soil), and no leaching from the pots was observed, the possible negative effects of EDTA on soil ecosystem and on metal leaching should always be evaluated.

The addition of fresh OMW in soils can enhance plant uptake of metals by promoting their solubility due to (i) the chelating capacity of the containing soluble phenols that act as leaching promoters, and thus blocking metal sorption in the solid phase [43], or (ii) their ability to influence redox processes in soils resulting in the release of reduced elements that are highly soluble [44–46]. However, this was not the case when Pb was investigated, whereas, in contrast, Zn total uptake by *H. annuus* was significantly increased with both OMW application alone or in combination with EDTA.

This Zn increase, although not directly associated with a corresponding increase in the most soluble exchangeable soil fraction (B1), was, however, accompanied by a respective decrease in the less soluble residual fraction (BRF). Possible surface complexation of the residual Zn form by organic ligands offered by the OMW addition, and possible inhibition of Fe and Al crystallization providing reactive surfaces and effective sinks for Zn, thus enhancing further dissolution of residual Zn, might offer an explanation for this phenomenon. Nonetheless, the fact that the residual Zn soil fraction was significantly decreased by OMW application, may also indicate that these Zn forms are not associated with resistant minerals [47].

Arsenic, which is a metalloid, is usually thought to be slightly affected by EDTA applications to soil [48]. However, contradictory results have been also reported in the literature [49] revealing that its application to the growth media could increase the availability and uptake of As(III) and As(V) in different plant species such as alfalfa or maize [40]. In our case, although *H. annuus* did not respond significantly to different treatments applied, and correspondingly the same occurred with the As concentration in the aboveground biomass when the total As uptake per pot was examined, a significant increase was observed under the EDTA treatment.

As seen from the multiple comparisons made between soil fractions and the different chelating agents applied, the increase noted above could be attributed to the respective increase observed in the nonspecifically sorbed W1 fraction. The latter is in line with the PCA conducted or the Pearson correlations that were made between As uptake parameter and As distribution in different soil fractions, from which a respective affinity of As uptake and As content in the W1 fraction was also observed. Although nonsignificant, the decreasing trend that was recorded, as compared to the control and for the amorphous and poorly crystalline hydrous oxides of Fe and Al (W3) under EDTA treatment, could be speculated as the main contributor responsible for As increase in the W1 fraction.

Moreover, as also conducted from the multiple comparison tests, the combined application of EDTA + OMW managed to significantly increase both specifically sorbed As from mineral surfaces or the As bound to well-crystallized hydrous oxides of Fe and Al. Unlike Pb and Zn, As exists in soils as an oxyanion and therefore competes with phosphates for sorption sites [50]. Considering that OMWs contain significant amounts of phosphorus, it is highly possible that the phosphates PO_4^{3-} added to the soil via OMW replaced arsenates AsO_4^{3-} in the sorption sites of the soil colloids and thus enhanced EDTA chelating capacity for the W2 and W4 soil fractions [51].

Wenzel et al. (2001), when introducing the respective sequential extraction scheme, reported that the easily exchangeable soil fraction (W1) has been shown to correlate well with As in field-collected soil solutions and hence can be used for predicting readily available As [25]. In addition, and in line with our results, they suggested that fractions W2-W4 may provide information on the potential lability of As from different solid phases as a result of soil remediation or alteration in soil (e.g., redox, pH) and environmental factors.

Regarding Pb, the higher concentrations observed in the stems and leaves of *H. annuus* due to EDTA application in the heavily polluted soil used in this study can be attributed to the increased availability of Pb in the soil environment. Indeed, because of its strong complexing properties, EDTA acted as a strong competitor to the soil reactive surfaces and thus managed to extract Pb mainly from exchangeable/acid-soluble soil fraction [12]. The latter is in line with the findings of the PCA conducted or the respective correlations that were made between Pb uptake parameter and Pb distribution among different soil fractions, from which a clear affinity of Pb uptake and Pb content in B1 fraction was observed. Moreover, the fact that except for the EDTA treatment, the total uptake of Pb by *H. annuus* was also more than tripled in the combined EDTA + OMW application, may be fundamentally attributed to the EDTA chelating effect. In the case of Pb, the OMW application had practically no effect, neither in uptake nor in Pb aboveground biomass concentration; thus, any potential synergistic act of OMW could not be speculated.

As seen from respective multiple comparison tests conducted between different Pb soil distribution patterns and respective treatments applied for promoting PTEs solubility, the increase in B1 fraction noted above could be clearly attributed to the significant decrease observed in the reducible fraction where Pb is bound to Fe/Mn oxides. Hence, a partial Fe–Mn amorphous oxides dissolution due to the chelating properties of EDTA could be assumed as the main contributor to the increased Pb availability. On the other hand, regarding the oxidizable fraction where Pb is bound to organic matter, the significant decrease in Pb content was mainly observed in the combined EDTA + OMW application, and, secondly, in the EDTA treatment when added alone. The efficacy of the EDTA + OMW application to trigger a respective significant decrease in Pb content was also observed in the residual fraction. Therefore, a potential enhancing contribution of the fresh OMW, due to a similar mechanism as suggested for the Zn, could also be a possible explanation.

The amelioration effect of EDTA application in Pb uptake or a respective significant improvement in Pb content of the plants' aboveground biomass has also been demonstrated by other authors [52,53]. Corroborating our results, the basic scenario usually suggested as a possible mechanism is EDTA's effect on increasing the absorption and solubility of Pb–EDTA complex by plants [54].

Results concerning the roots-to-shoots translocation factor, although could not be subjected to appropriate statistical analysis due to the limited belowground biomass, revealed, however, some discouraging trends for the adoption of *H. annuus* as an efficient hyperaccumulator for the studied PTEs. More specifically, our study showed that in the case of As or Pb, the TF value of control was not found to be smaller as compared with other treatments, while regarding Zn, this trend was valid only for the EDTA and the combined EDTA + OMW treatment.

Thus, with one possible exception of Zn in the OMW addition, none of the treatments managed to further trigger the PTEs translocation from roots to shoots, a condition that is required for their accumulation in the aboveground biomass. Additionally, and for all cases, also clear was the finding that the roots-to-shoots translocation factor was <1, a fact that points to a limited phytoextraction potential for the specific plant species to extract high amounts of the PTEs studied. The latter also corroborates the findings of other authors such as Alaboudi et al. (2018), who by investigating the potential use of *H. annuus* as a heavy metal hyperaccumulator, suggested that, unlike Cd, the specific plant was unable to be used for desirable Pb accumulation in its upper parts [55].

5. Conclusions

Our study showed that the applied treatments of EDTA, OMW or their combined application triggered an increased uptake of As, Pb and Zn by *H. annuus*, a fact that strongly suggests that they played a key role as reductive agents in enhancing the mobility of these elements in the soil environment. These findings were also confirmed by the results for both the Wenzel and BCR sequential extraction protocols, especially with respect to As and Pb, in which respective affinity patterns between PTE uptake by *H. annuus* and their increased concentration by different chelating treatments were also observed. This is particularly supported for Pb by the results of BCR, which for the EDTA or/and EDTA + OMW treatments showed increased Pb concentration in the B1 fraction and lower Pb concentration in the B2 fraction. In addition, and expressing the results as mg/pot, both amendments significantly increased As plant uptake, in line with the increased As concentrations in the W1 fraction and the lower As concentration in the W3 fraction of the Wenzel scheme. Following these results, the observed higher Pb and As availability due to EDTA and OMW addition was probably the outcome of a partial Fe–Mn amorphous oxides dissolution. However, for all the studied PTEs, roots-to-shoots translocation factor (TF) was <1, pointing to the limited potential of sunflower to serve for the decontamination of the mining-affected Lavrio soils.

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