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Efficacy of EDTA and Olive Mill Wastewater to Enhance As, Pb, and Zn Phytoextraction by *Pteris vittata* L. from a Soil Heavily Polluted by Mining Activities

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Abstract: A pot experiment was conducted to evaluate the effect of Na₂-EDTA 0.01 M (E) and olive mill wastewater 15% (OMW) on As, Pb, and Zn uptake by *Pteris vittata* L. grown in a soil highly contaminated by mining activities. A two-factor experimental design was followed; 3 treatments (E, OMW, and E + OMW) × 2 batches (single or double dose). Six weeks after the *P. vittata* transplanting, all pots received the selected dose of each treatment (Batch I). At 8 weeks, in half of the pots, a second dose of the same treatments was added (Batch II). Plants were harvested after 10 weeks and As, Pb, and Zn concentrations were determined in fronds and roots. Depending on the element, both treatment and batch effects were significant. In Batch II, EDTA application resulted in a 55% increase of As and 9- and 4-fold of Pb and Zn concentrations in the fronds, while OMW treatment substantially reinforced plant uptake when combined with EDTA. Roots to fronds translocation of the metal(loid)s highly increased in Batch II. After harvest, composite soil samples of all treatment–batch combinations were subjected to sequential extraction, but no significant differentiations of As, Pb, and Zn partitioning in soil phases were detected.

Keywords: enhanced phytoextraction; *Pteris vittata* L.; arsenic; lead; zinc; soil fractionation

1. Introduction

Human activities, such as metalliferous mining and metallurgical processes, could pose a severe threat to the local environment. The weathering impact of air and water on the produced metal-rich tailings and rock wastes results in a continuous release of heavy metals and metalloids in the surrounding soils. When metal(loid) concentrations in soils reach or exceed the corresponding threshold limits for humans, animals, or plants, cleanup measures must be implemented [1].

Various physical, chemical, and biological techniques have been used in order to remediate metal-polluted soils [2,3]. Because of the increased cost and the danger of irreversible changes in soil properties and biodiversity, conventional remediation methods (e.g., vitrification, landfilling, soil washing, electrokinetic systems, etc.) are not applicable for the decontamination of large field sites and, therefore, phytoremediation can be considered as an alternative, green, cost-efficient, and more sustainable remediation technology [4,5].

Phytoextraction is a phytoremediation method based on the use of plants that can hyperaccumulate metals in the aboveground biomass, which then can be harvested and removed [6]. Nevertheless, soil metal remediation with the phytoextraction technique is a relatively slow process due

to the low bioavailability of the metals in the soil system, as they are mostly occurring in less soluble chemical forms and therefore unavailable for plant uptake [7,8]. To enhance phytoextraction, several chelating and reducing agents have been used as soil amendments in order to increase metal solubility in soils. EDTA is amongst the most commonly used chelating agent, as it is capable of desorbing metals from the soil constituents, forming soluble metal complexes [9]. For the same purpose, organic materials originated from agricultural activities have been applied to soils for increasing their fertility and, at the same time, increasing or decreasing metals' availability, according to the soil remediation strategy [10]. Olive mill wastewaters (OMWs) (byproduct of the olive oil processing industry) have increased organic load, an acidic nature, and contain large amounts of soluble phenols. Due to these characteristics, implementation of OMWs in soils can influence the soil redox potential and promote metal chelation [11,12]. Although a large body of literature has demonstrated the high effectiveness of EDTA on metal mobilization and uptake by plants, the effect of EDTA on soil quality is a complex issue [13]. The low biodegradability of EDTA can pose an elevated risk of adverse effects on soil microorganisms and metal leaching. Moreover, the application of OMW as a soil amendment represents a controversial discussion. Recent studies found that the disposal of untreated OMW in soil causes serious environmental problems due to the acidic pH and the high content of potentially antimicrobial compounds, such as phenols [14].

To characterize a plant as a metal hyperaccumulator, it should demonstrate certain features, such as good adaptation, easy propagation, extensive root system, high aboveground biomass production, tolerance to metal toxicities, and efficient translocation factor for the targeted metals [15,16]. Chinese brake fern *Pteris vittata* L. is a hardy, versatile, and fast-growing plant that is well known for its efficiency in hyperaccumulating arsenic [17–20]. Ma et al. [21] first conducted a greenhouse experiment and observed that *P. vittata* had the ability to accumulate As in its fronds, up to 2.3% and 0.7% dry weight, after 6 weeks of growing in an artificially and a chromated copper arsenate contaminated soil, respectively. Nevertheless, according to the contamination sources, the soil types, and land uses, As distribution in the soil fractions differs, while mine-affected sites are usually co-polluted with other heavy metals. Thus, it is very important to investigate the efficiency of *P. vittata* to tolerate or even accumulate other metals simultaneously with As.

It has been reported that the potential use of OMW as a soil amendment, had positive effects on soil properties (total organic C, total N, available P, exchangeable K, and available trace elements) [10,22], while EDTA addition was found to be efficient in increasing the availability of As and heavy metals in soils [23,24].

The objectives of the present study were to (a) investigate the ability of *P. vittata* to extract As, Pb, and Zn from a soil polluted by mining activities; (b) assess the effect of EDTA, olive mill wastewaters, and their combination on As, Pb, and Zn uptake by *P. vittata*; (c) discuss the effect of the repeated application of these soil amendments on As, Pb, and Zn uptake by *P. vittata*; and (d) evaluate the effect of EDTA and OMW on the redistribution of As, Pb, and Zn between the soil reactive phases.

2. Materials and Methods

2.1. Soil Sampling and Soil Properties

An area of 10 × 10 m at a depth of 0.15 m was excavated from a mine-affected area in the outskirts of Lavrion, Central Greece (X: 503825.35, Y: 4175571.92/EGSA87) and the bulk soil obtained was transferred to the laboratory facilities. The soil is classified as Cambisol (according to FAO) and it is characterized as loam (L) with an alkaline pH (Table 1). From 3500 BC until 1989 AD, the wider area of Lavrion was intensively mined for silver, lead, and zinc. During this period, mining and metallurgical byproducts were scattered all over the Lavreotiki peninsula, polluting the surrounding soils with toxic elements [1].

Table 1. Main soil physicochemical properties.

Clay g kg ⁻¹	Silt g kg ⁻¹	Sand g kg ⁻¹	pH (1:1)	Total CaCO ₃ g kg ⁻¹	OM ¹ g kg ⁻¹	CEC ² cmol _c kg ⁻¹	Total As mg kg ⁻¹	Total Pb mg kg ⁻¹	Total Zn mg kg ⁻¹
198	301	501	7.72	69	45	18	822	5677	4428

¹ OM (organic matter); ² CEC (cation exchange capacity).

The obtained topsoil was passed through a 1 cm sieve and homogenized for the pot filling. A portion of the bulk soil was air-dried, crushed, and grounded to pass through a 2 mm and a 0.5 mm sieve for analysis. The Bouyoucos hydrometer method [25] was followed for the determination of the particle-size distribution, pH was estimated in a 1:1 (*w/v*) soil/water slurry, total calcium carbonate (CaCO₃) was measured according to the Bernard calcimeter method [26], organic matter (OM) content was estimated by the Walkley–Black procedure [27], and the sodium acetate method [28] was followed to determine the cation exchange capacity (CEC). Soil samples were digested with aqua regia and analyzed for total metal concentrations. Due to the incomplete destruction of silicates, aqua regia digestion provides the “pseudo-total” metal concentrations [29], termed for simplicity as “total” in this study. The soil physicochemical properties are summarized in Table 1.

2.2. Greenhouse Experiment

Ten-month old ferns with approximately 30 cm fronds in length were transplanted to 2 L plastic pots (1 fern per pot) filled with 2 kg of soil polluted by mining activities and transferred to a greenhouse. A total of 56 pots were placed in random order and the ferns were watered once or twice a week in order to keep soil moisture at ~60% of the water-holding capacity. After 3 weeks of cultivation, fertilization with 0.4 g N/kg as (NH₄)₂SO₄ was applied to enhance plant growth. Six weeks after transplant, additions of the treatments were performed. Each treatment was replicated eight times, including pots treated only with deionized water (DW) as control. The treatments applied were: (i) Na₂-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 organic carbon (TOC): 26 ± 2.4 g L⁻¹, total N: 0.9 ± 0.1 g L⁻¹, P: 0.21 ± 0.02 g L⁻¹, K: 6.1 ± 0.2 g L⁻¹, total phenolics: 8.8 ± 0.3 mg mL⁻¹, chemical oxygen demand (COD): 48 ± 2.1 g L⁻¹, total suspended solids (TSSs): 42 ± 3.2 g L⁻¹ [30], and (iii) a combination of EDTA 0.01 M and OMWs 15%. All amendments were added in a volume equal to the ~60% (500 mL) of the soil water-holding capacity. After 8 weeks from transplant, the treated ferns were divided into two batches. Batch I contained ferns that no other treatments were performed on until harvest, and Batch II contained ferns to which a second dose of the exact same treatments was applied. After 10 weeks of cultivation, all ferns were harvested and four of the eight replicates were completely destroyed to obtain the roots. Three soil subsamples from the pots of each treatment and batch were used for the sequential extraction of the studied elements. During the experimental period, air temperature and relative humidity in the greenhouse ranged between 20 and 35 °C and 70% and 90%, respectively. The experimental design is presented in Figure 1.

Figure 1. Experimental design.

2.3. Plant Analysis

Total metal(loid)s' concentrations in fronds and roots were determined following a wet digestion procedure that utilizes concentrated nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2). Plant fronds and roots were thoroughly washed with deionized water to discard any impurities and oven dried at 60 °C for 2 days. Then, all samples were weighed and grounded using a mixer mill in order to produce a fine powder with a particle size less than 0.2 mm. In 0.5 g of pulverized tissue, 5 mL of 65% HNO_3 was added in conical flasks covered with watch glasses and left overnight. Plant samples were then digested over a heat plate for 1 h at 125 °C and, after cooling the samples, were mixed with 2 mL of 30% H_2O_2 and re-digested at 80 °C repeatedly until the digests became colorless. The digests were then filtered and diluted to a volume of 25 mL for analysis [31]. The translocation factor (TF) was calculated as the ratio of metal(loid) concentration in the fronds to those in the roots [32].

2.4. Sequential Extraction Procedures

To investigate the distribution of the studied elements into the different soil chemical phases, two sequential extraction procedures were performed. The well-established three-step sequential extraction procedure proposed by BCR (Community Bureau of Reference) [33] was used for the partitioning of Pb and Zn, and a specifically designed scheme was used for As forms, recommended by Wenzel et al. [34]. The operationally defined chemical fractions and working conditions for both partitioning schemes are analytically presented in Table 2.

Table 2. The BCR (Community Bureau of Reference) and Wenzel partitioning schemes after Kalyvas et al. [35].

Step	Fraction	Extractant	Extraction Conditions	Extractant Volume (mL) for 1 g of Soil
BCR ^a				
B1	Exchangeable/acid soluble	Acetic acid 0.11 mol L ⁻¹	16 h shaking at 22 ± 5 °C	40
B2	Bound to Fe/Mn oxides (reducible)	Hydroxylammonium chloride 0.5 mol L ⁻¹ pH = 1.5	16 h shaking at 22 ± 5 °C	40
B3	Bound to organic matter (oxidizable)	Hydrogen peroxide 8.8 mol L ⁻¹ Ammonium acetate 1.0 mol L ⁻¹ , Ph = 2 ± 0.1	1 h digestion at 85 ± 2 °C 16 h shaking at 22 ± 5 °C	10 + 10 50
BRF ^b	Residual	Aqua regia (HCl/HNO ₃)	16 h digestion	25
WENZEL ^c				
W1	Non-specifically sorbed	(NH ₄) ₂ SO ₄ 0.05 mol L ⁻¹	4 h shaking at 20 °C	25
W2	Specifically sorbed	NH ₄ H ₂ PO ₄ 0.05 mol L ⁻¹	16 h shaking at 20 °C	25
W3	Amorphous and poorly crystalline hydrous oxides of Fe and Al	NH ₄ ⁺ -oxalate buffer 0.2 mol L ⁻¹ , pH = 3.25	4 h shaking in the dark at 20 °C	25
W4	Well-crystallized hydrous oxides of Fe and Al	NH ₄ ⁺ -oxalate buffer 0.2 mol L ⁻¹ + ascorbic acid 0.1 mol L ⁻¹ , pH = 3.25	30 min shaking in the light at 96 °C	25
WRF	Residual	Aqua regia (HCl/HNO ₃) ^d	16 h digestion ^d	25

^a Rauret et al. [33]; ^b not included in the BCR specifications; ^c Wenzel et al. [34]; ^d modification of the original method.

2.5. Analytical Determinations

Arsenic, Pb, and Zn concentrations were determined by flame atomic absorption spectrophotometry (F-AAS) using a Varian SpectrAA-300 system. For the determination of As at low concentrations, the spectrophotometer was equipped with a hydride generator Varian model VGA 77 (HG-AAS), with working conditions previously reported by Kalyvas et al. [35]. A control sample was analyzed for every 10 samples and reproducibility was tested by reanalyzing 30% of the samples. The analytical precision, estimated as relative standard deviation, was less than 5%. To verify the accuracy of aqua regia procedure that followed for total element determination, ERM-CC141 European Reference Material (loam soil) was used. The results showed that mean As, Pb, and Zn recovery was 98, 95, and 104%, respectively. At all stages of the sample preparation and analysis, stringent precautions were taken to minimize contamination through air, glassware, and reagents. All reagents used in this study were of analytical grade and supplied from Merck Millipore (Darmstadt, Germany).

2.6. Statistical Analysis

Statistical analysis was carried out by using STATISTICA (StatSoft, Inc., Tulsa, OK, USA, 1984–2011, version 10) and IBM SPSS Statistics 20 software (IBM, Armonk, NY, USA).

3. Results

3.1. Plant Growth

As indicated by the ANOVA results, treatment effect on *P. vittata* growth was significant, while batch effect was not. No interaction between the two factors was observed (Figure 2). Compared to control, both EDTA and OMW addition significantly reduced plant growth, leading to up to 20% lower aboveground biomass production in the case of EDTA first addition (Figure 2). However, no visual toxicity or nutrient deficiency symptoms were observed. The second addition of OMW alleviated this phenomenon, suggesting reduced plant stress. It is well known that OMW addition in soils may result in anaerobic soil conditions and toxicity stress to plants and the soil microcosms due to its high phenolic content [36]. On the other hand, OMWs contain substantial nutrient concentrations that can promote plant growth. It seems that adverse effects appeared after the first addition of OMW, while, afterward, nutrient load of OMW assisted the healthier growth of the plants and balanced the initially negative impact of OMW. Moreover, the observed increased biomass production with the second OMW application suggests a rapid adaptation of both soil microbial communities and plants to the adverse OMW effect on the soil ecosystem [37]. EDTA alone or combined with OMW disturbed the rhizosphere soil environment and stressed plants, possibly due to the release of high amounts of toxic elements, for example, Pb. *Pteris vittata* is a well-known As hyperaccumulator plant that can tolerate high As soil concentrations, but it is probably less tolerant in the case of abrupt increase of available concentrations of other toxic elements in the soil solution [38]. Additionally, EDTA may have influenced several plant physiological functions responsible for nutrient uptake and thus immobilization–mobilization nutrient balance that could have led to the lower *P. vittata* growth [39].

Figure 2. Aboveground biomass (dry weight) of *P. vittata*. Data shown are the mean values of eight replicates. Comparisons were performed by Tukey's HSD test ($p \leq 0.05$) and are demonstrated with lowercase letters. The presence of a common letter implies no significant difference. ANOVA results presenting the treatment and batch effects on dry weight of *P. vittata* are also included (*, $p < 0.05$).

3.2. Elements Concentrations and Translocation in *P. vittata*

The effects of treatment and batch on metal(loid) concentrations in the aboveground biomass of *P. vittata* are presented in Figure 3 and the accompanying ANOVA results table. While for all elements the treatment effect was significant, batch effect was significant only for Pb plant uptake. Since, for all elements, interaction between the two factors was observed, post hoc comparisons between treatments are valid within each batch, and differences for the same treatment between the two batches were evaluated by *t*-test. Regarding As, in Batch I, only the combined application of EDTA and OMW exhibited a significant As concentration increase compared to control, while in Batch II, both EDTA and EDTA + OMW applications promoted As uptake by *P. vittata*. Moreover, the second application of EDTA significantly increased As concentration in plant fronds compared to the first EDTA application.

ANOVA RESULTS					
Effect	SS	DF	MS	F	p
As					
Treatment	108×10^4	3	359×10^3	10.71	0.000 *
Batch	231×10^2	1	231×10^2	0.69	0.410
Treatment $\times$ Batch	788×10^3	3	263×10^3	7.84	0.000 *
Pb					
Treatment	499×10^4	3	166×10^4	63.80	0.000 *
Batch	202×10^3	1	202×10^3	7.73	0.007 *
Treatment $\times$ Batch	245×10^3	3	817×10^2	3.13	0.033 *
Zn					
Treatment	779×10^3	3	260×10^3	135.7	0.000 *
Batch	6381	1	6381	3.3	0.073
Treatment $\times$ Batch	317×10^2	3	106×10^2	5.5	0.002 *

Figure 3. Treatment effect on As, Pb, and Zn accumulation in the fronds of *P. vittata*. Data shown are the mean values of eight replicates. Comparisons between treatments within Batch I and II were performed by Tukey's HSD test ($p \leq 0.05$) and are demonstrated with lowercase letters, while the differences between the same treatments that belong to different batches were checked by *t*-test ($* p \leq 0.05$) and are presented by *. The presence of a common letter implies no significant difference. ANOVA results presenting the treatment and batch effects on As, Pb, and Zn uptake by *P. vittata* are also presented ($*, p < 0.05$).

Considering the treatment effects on Pb and Zn, it is apparent that only EDTA application similarly enhanced plant uptake of both metals. Additionally, the second application of EDTA further significantly increased Pb and Zn concentrations in *P. vittata* fronds.

Both EDTA and OMW treatments and their combination significantly affected root to fronds translocation (TF) of Pb and Zn but did not influence the TF of As. Batch effect was significant for As and Zn TFs, while no interaction between the two factors was noticed (Table 3). Compared to control, the second application of EDTA significantly increased Pb and Zn TFs (Table 4). A similar but not significant pattern was also observed for As TF. Arsenic TFs were higher than those of the other elements and several times above unity, verifying the well-known ability of *P. vittata* to behave as an As hyper-accumulating plant [40].

Table 3. ANOVA results presenting the treatment and batch effects on As, Pb, and Zn TFs (*, $p \leq 0.05$).

ANOVA RESULTS					
Effect	SS	DF	MS	F	p
As					
Treatment	61.1	3	20.4	0.662	0.583
Batch	138.7	1	138.7	4.511	0.044 *
Treatment $\times$ Batch	70.9	3	23.6	0.769	0.523
Pb					
Treatment	0.110	3	0.037	6.705	0.002 *
Batch	0.014	1	0.014	2.633	0.118
Treatment $\times$ Batch	0.013	3	0.004	0.794	0.509
Zn					
Treatment	0.296	3	0.099	4.234	0.015 *
Batch	0.130	1	0.130	5.584	0.027 *
Treatment $\times$ Batch	0.157	3	0.052	2.251	0.108

Table 4. Translocation factor values of As, Pb, and Zn in *Pteris vittata* L. among the different treatments in Batch I and II. Data shown are the mean values of four replicates. Comparisons of TFs 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.

TRANSLOCATION FACTOR (TF)								
	BATCH I				BATCH II			
	CONTROL	EDTA	OMW	EDTA + OMW	CONTROL	EDTA	OMW	EDTA + OMW
As	6.91 a	6.30 a	7.99 a	5.11 a	6.91 a	14.45 a	11.18 a	10.44 a
Pb	0.08 abc	0.14 abc	0.04 b	0.14 abc	0.08 abc	0.25 c	0.06 b	0.18 abc
Zn	0.16 a	0.20 a	0.12 a	0.19 a	0.16 a	0.57 b	0.16 a	0.29 ab

3.3. Distribution of Arsenic and Metals in Soil Fractions

Mean percentages and concentrations of As, Pb, and Zn into the different soil fractions after the plant harvest are presented in Figure 4 and the accompanying tables. The mean percentages of As, Pb, and Zn into the various soil fractions were in the order $W1 < W2 < WRF \leq W4 < W3$, $B1 < B3 < BRF < B2$, and $B3 < B1 < BRF < B2$, respectively. The metal(loid) distribution was not affected either by the first or by the second application of the treatments. The only exception was observed in treatment EDTA/Batch II, where the As residual fraction (WRF) was higher than the reducible fraction (W4). Arsenic was found to be mostly adsorbed in the amorphous and crystalline Fe oxides [35]. Addition of EDTA and OMW and their combination, single or double dose, had no significant result in increasing the most available fractions of As, i.e., non-specifically (W1) and specifically sorbed (W2). The BCR partitioning scheme demonstrated the affinity of Pb and Zn for Fe oxides, exhibiting an average percent value of 64.9% and 41.5%, respectively, in the reducible fraction (B2) [2].

Figure 4. Mean percentages and values of As, Pb, and Zn, into the different soil fractions (numbers in tables are in mg kg^{-1}). The error bars represent the standard error of the mean at the 95% of confidence level ($p \leq 0.05$) ($n = 3$). I (Batch I), II (Batch II), C (control), O (olive mill wastewater), E (EDTA), W1 (non-specifically 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).

4. Discussion

4.1. Effect of Added Amendments on As and Metal Accumulation in *P. vittata* Fronds

Regarding As and metal uptake by *P. vittata*, three different patterns can be distinguished. Arsenic concentration in the aboveground biomass was increased by the second application of EDTA (Batch II), while the first application of OMW enhanced the extracting ability of EDTA (Batch I). Pb and Zn concentrations increased only by the EDTA application. As indicated by the effect of combined EDTA + OMW addition (Figure 3), OMW reduced the uptake of both Pb and Zn.

The observed higher As, Pb, and Zn concentrations in the fronds of *P. vittata* due to EDTA application in the heavily polluted soil used in this study can be attributed to the increased availability of these elements in the soil environment. Indeed, because of its strong complexing properties, EDTA acts as a strong competitor to the soil reactive surfaces and thus is capable to extract metal(loid)s from the different soil chemical fractions, such as exchangeable carbonates, Fe/Mn amorphous oxides, and organic matter [9]. Many authors have demonstrated EDTA's effectiveness to mobilize As, Pb, and Zn in metal-polluted soils. Meers et al. [41] studied Zn mobilization in a metal-contaminated dredged sediment that was amended with EDTA, and they found that Zn mobility was significantly increased. Similarly, increased As availability in an industrially As-polluted soil after the addition of several EDTA concentrations was observed by Abbas and Abdelhafez [42]. Mühlbachová [43] found that 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.

However, while for Pb and Zn this EDTA effect is clearly demonstrated from the first dose (Batch I), for As, the second dose (Batch II) was necessary to trigger the above-explained mechanism.

It seems that the rather low dosage of 2.5 mmol EDTA kg⁻¹ of soil was not enough to liberate As bound to different soil phases (primarily from amorphous oxides and inner-sphere complexes where As was strongly retained), whereas the doubling of the EDTA dose succeeded in increasing As solubility and, correspondingly, significantly enhancing As plant uptake. Compared to Batch I, the application of a second dose of EDTA also significantly increased Pb and Zn concentrations in *P. vittata* fronds. Surprisingly, Pb and Zn concentration in *P. vittata* fronds increased up to 9 and 4 times, respectively, when compared to control, suggesting that, under EDTA application, *P. vittata* can function as a Pb hyperaccumulator for the decontamination of soils heavily polluted with As and Pb [44]. On the contrary, the relatively smaller effect of all amendments on As uptake can be directly attributed to the ability of *P. vittata* to absorb high amounts of As under common soil conditions. Though the EDTA concentration used in this study is considered as environmental and plant-safe according to Vamerali et al. [45] (<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 metal solubility in soils due to (i) the reductive dissolution of metal oxides as a result of the organic matter mineralization from the microorganisms that consume free oxygen, and the oxidation of phenolic compounds, and (ii) the formation of organometallic complexes of the soluble organic substances and phenols that inhibit metal sorption into the soil solid phases [46,47]. Following this, increased availability and higher plant uptake should be expected for all elements. Depending on the element, OMW application produced different plant uptake patterns. In relation to control, OMW resulted in significantly higher concentrations of As, Pb, and Zn in *P. vittata* fronds only when combined with EDTA (Figure 3). Excluding As, this can be attributed to the properties of EDTA and not to those of OMW, since the EDTA + OMW treatment resulted in lower Pb and Zn concentrations in plant fronds than EDTA alone. It seems that OMW did not effectively dissolve metal oxides of the studied soil and did not promote the formation of organometallic complexes subsequently. This is in line with De la Fuente et al. [47], who conducted a pot experiment using a calcareous agricultural soil with increased concentrations of Pb and Zn, and found that the addition of the water-soluble fraction of fresh solid olive husk had no significant result on Pb/Zn solubility. Although this may be true for Pb and Zn, it cannot explain the increased As concentration in the aboveground biomass with the OMW treatment. Though this increase is not statistically significant, a clear increasing trend was apparent when OMW and OMW + EDTA applications are compared to control. Unlike Pb and Zn, As exists in soils as an oxyanion and, therefore, it competes with phosphates for sorption sites [48]. Considering that OMWs contain significant amounts of phosphorous, it is highly possible that the phosphates PO₄³⁻ added in the soil via OMW replaced arsenates AsO₄³⁻ in the sorption sites of the soil colloids and, thus, As concentration in the soil solution increased, as well as plant uptake [49].

4.2. Arsenic, Lead, and Zinc Extraction per Pot

Another way to express the results of the study is by considering the biomass production per pot in order to determine the actual amounts of As, Pb, and Zn extracted by *P. vittata*. Table 5 summarizes the corresponding mg pot⁻¹ values. Since EDTA and OMW addition reduced *P. vittata* growth (Figure 2), the use of the extracted amounts of As, Pb, and Zn may better portrait the dynamics of the tested systems and can provide reliable information to be used for the reduction of As, Pb, and Zn load in contaminated soils. According to the ANOVA results in Table 6, treatment effect is significant for all elements, batch effect is significant only for Pb, and interaction between the two factors was noticed only for As. Arsenic extracted by the second addition of EDTA almost doubled when compared to control, reinforcing the hyperaccumulating effectiveness of *P. vittata*. Considering that each pot contained 2 kg of soil and that 135.24 mg of As was extracted, a promising prospective for site decontamination is emerged by the *P. vittata*–EDTA system. Nevertheless, a similar value that was significantly higher than the control was obtained by the first application of EDTA + OMW, pointing to

synergistic action of EDTA and OMW to extract As from the soil constituents. For Pb and Zn, though EDTA dramatically increased the extracting ability of *P. vittata*, especially the second dose, the 83.43 and 39.02 mg of Pb and Zn extracted from the soil cannot be evaluated as adequate for successful site remediation when soil concentration of Zn especially is extremely high. However, these results indicate that in soils with high and particularly high As, Pb, and Zn concentrations, the *P. vittata*–EDTA system can serve for the drastic reduction of As load and for a considerable Pb decrease.

Table 5. Total extraction of As, Pb, and Zn expressed as mg pot^{−1}. Data shown are the mean values of eight replicates. For Pb and Zn, post hoc comparisons performed by Tukey’s HSD test ($p \leq 0.05$) are demonstrated with lowercase letters and are valid within each element. For As, comparisons between treatments within Batch I and II were performed by Tukey’s HSD test ($p \leq 0.05$) and are demonstrated with lowercase letters, while the differences between the same treatments that belong to different batches were checked by *t*-test ($p \leq 0.05$) and are demonstrated by capital letters. The presence of a common letter implies no significant difference.

mg pot ^{−1}								
BATCH I					BATCH II			
	CONTROL	EDTA	OMW	EDTA + OMW	CONTROL	EDTA	OMW	EDTA + OMW
As	73.29 a/A	102.45 ab	106.08 ab/A	123.11 b/A	73.29 a/A	135.24 b/A	76.87 a/B	114.17 b/A
Pb	9.87 a	56.95 bc	5.90 a	48.73 b	9.87 a	83.43 c	6.74 a	62.17 bc
Zn	9.48 a	31.62 bc	9.60 a	26.11 b	9.48 a	39.02 c	6.91 a	26.65 b

Table 6. ANOVA results, presenting the treatment and batch effects on As, Pb, and Zn mg pot^{−1} (*, $p \leq 0.05$).

ANOVA RESULTS					
Effect	SS	DF	MS	F	p
As					
Treatment	238 × 10 ²	3	7932	11.89	0.000 *
Batch	29	1	29	0.04	0.836
Treatment × Batch	8004	3	2668	4.00	0.012 *
Pb					
Treatment	498 × 10 ²	3	166 × 10 ²	53.52	0.000 *
Batch	1662	1	1662	5.36	0.024 *
Treatment × Batch	1869	3	623	2.01	0.123
Zn					
Treatment	8382	3	2794	80.92	0.000 *
Batch	27	1	27	0.80	0.376
Treatment × Batch	222	3	74	2.14	0.105

4.3. Element Partitioning

The increased concentrations of As, Pb, and Zn in the fronds of *P. vittata* strongly suggest that the applied treatments increased the mobility of these elements in the soil environment. However, this was not demonstrated by the results of both Wenzel and BCR sequential extraction protocols, since no significant effect of the applied treatments on element partitioning in the reactive soil phases was observed. Single harvest of *P. vittata* fronds resulted in the extraction of small amounts of As, Pb, and Zn in relation to the total concentrations of these elements in the tested soil. Thus, it is assumed that EDTA and OMW applications resulted in little redistribution of the studied elements between the soil phases that did not allow for the detection of sensitive significant alterations in element partitioning that could be related to plant uptake of the As, Pb, and Zn.

5. Conclusions

The results of the present study clearly demonstrated that Na₂-EDTA 0.01 M application to a soil heavily polluted with As, Pb, and Zn enhanced plant uptake of these elements by *P. vittata* and significantly promoted the translocation of Pb and Zn from the roots to the above ground biomass. Compared to control, *P. vittata* extracted almost double the amount of As from the soil with the double EDTA dose, suggesting enhanced phytoextraction that may lead to relatively time-effective soil decontamination. The same amendment greatly assisted plant uptake of both Pb and Zn. According to the extracted amounts of Pb and Zn, the EDTA–*P. vittata* system can efficiently reduce the load of these elements in soils, especially that of Pb. Though the EDTA concentration used in this study is rather low (single EDTA application failed to mobilize As) 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. Single diluted OMW application alone or combined with EDTA highly increased As extraction by *P. vittata* (106 and 123 mg pot^{−1}, respectively). Considering that Pb and Zn were not mobilized by OMW application and that high quantities of OMWs are produced in olive oil-producing countries, further detailed study is needed to assess the potential of this agroindustry byproduct to enhance As uptake by *P. vittata*, and to be used subsequently for the decontamination of As-polluted soils.

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