

Article

# Acid-Modified and Unmodified Natural Clay Deposits for In Situ Immobilization and Reducing Phytoavailability of Molybdenum in a Sandy Loam Calcareous Soil

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**Abstract:** Molybdenum (Mo) in basic soils has high bioavailability and plant toxicity. This study aimed to investigate the effect of increasing Mo concentration on its availability and toxicity threshold in alfalfa plants grown in sandy loam calcareous soils, and the potential use of raw and acid-modified clay deposits as soil additives to immobilize Mo and reduce its phytoavailability. Raw clay deposits (RCD) were treated with H<sub>2</sub>SO<sub>4</sub> to produce acid-modified clay deposits (AMCD). The first experiment was performed using soils treated with 0, 0.1, 1, 10, 50, and 100 mg Mo kg<sup>-1</sup>. The second experiment was conducted with soils treated with 10 or 50 mg Mo kg<sup>-1</sup> and amended with RCD and AMCD at application rates of 0, 2.5, 5, and 10% (*w/w*). After harvesting, water-soluble Mo, ammonium bicarbonate-diethylenetriaminepentaacetic acid (AB-DTPA)-extractable Mo, and shoot Mo content as well as dry matter were measured. The results showed that water-soluble Mo, AB-DTPA-extractable Mo, and shoot Mo concentration increased at higher Mo soil addition. AMCD had a stronger influence on Mo immobilization and reduction effect on plant shoots compared to RCD, depending on soil Mo concentration and application rate. Applying AMCD decreased soil pH but increased salinity levels. The shoot dry matter significantly increased in soils amended with RCD and/or AMCD compared to control soils; with the highest improvement recorded for RCD at 10%. It was concluded that AMCD is an efficient immobilizing agent to reduce Mo mobility and its phytoavailability in calcareous soils. Additionally, both AMCD and especially RCD were able to create favorable conditions for plant growth.

**Keywords:** remediation; toxic trace element; activation; clay minerals; alkaline soils

## 1. Introduction

With the continually-rising global population, the increased demand for farmland is becoming an urgent need, yet soil pollution has become a major global environmental issue. Trace elements are found in the environment and may have beneficial or detrimental impacts. These elements are introduced to soils from various sources, either natural or anthropogenic [1,2]. Among the essential trace elements, molybdenum (Mo) can be introduced into the soils from various sources including naturally occurring (with the average concentrations of 1–5 mg kg<sup>-1</sup> in worldwide soils) and anthropogenic activities (e.g., mining activities, atmospheric deposition near smelters, or application of biosolid and fertilizers) [3,4]. Mo plays an important role in plant growth and development, given its influence on plant metabolism (enzyme catalysis, nitrogen assimilation, potassium absorption, phytohormone synthesis, sulfite detoxification; [5]). Despite this, the presence of Mo in high concentrations and

bioavailability leads to its accumulation within plant tissues, which becomes toxic for the plant at high concentrations and causes molybdenosis in cattle grazing [6,7]. Plant Mo toxicity is related to its availability and the anionic forms in soil solution, depending on the soil properties [8–10]. In this context, plants uptake Mo in the form of molybdate anions, which are more soluble and bioavailable in neutral and alkaline soils compared to acidic soils [8,9,11]. It has been previously reported that the adsorption of Mo in calcite and calcareous soils was very low compared to that of iron and aluminum oxides and clay minerals, indicating that calcium carbonate ( $\text{CaCO}_3$ ) is not considered an important component for preserving and holding Mo in calcareous soils [12].

The available pools of soil Mo mostly measure the actual toxicity for plants and its environmental risk. Therefore, controlling the bioavailable pools of soil Mo is key for soil remediation. Basic soils increase Mo mobility, hence the remediation of Mo-contaminated calcareous soils is necessary to reduce excessive plant Mo uptake and toxicity. One solution for remediation in metal-contaminated soils is in situ immobilization using immobilizing agents to decrease the bioavailability and toxicity of the metal to plants. Among various soil additives, clay minerals have been widely applied as soil immobilizing agents to improve soil properties and reduce metal mobility and bioavailability, hence helping to remediate metal-contaminated soils [13–19]. Clay minerals also adsorb cationic metals due to their permanent negative charge (cationic metal immobilization), reflecting their use in the remediation of polluted soils [20]. Clay minerals also influence the mobilization of anionic metals such as Mo: Neunhäuserer et al. [21] found that the mobile fraction of soil Mo was enhanced significantly by 90% following vermiculite addition compared to the control soil. Contrastingly, He et al. [22] found that applying bentonite, zeolite or dolomite to metal-contaminated soils reduced the bioavailability of arsenic (As), which is mainly found in its anionic forms of arsenate, As(III), and arsenite, As(V).

In the Kingdom of Saudi Arabia, the majority of soils are characterized by a high sand content, high pH, high  $\text{CaCO}_3$  concentration, low cation exchangeable capacity, and low organic matter content. Despite these unfavorable conditions, national agriculture has grown quickly over the past decades, requiring large quantities of agrochemicals which have led to soil pollution with trace elements [1]. In such sandy soils with high pH, phytoavailability and toxicity of Mo are expected. To improve the properties of sandy soils for agriculture, naturally-occurring clay minerals can be applied as soil conditioners, given their environmental benefits and cost-effectiveness, as well as their suitability for large-scale application. Several studies have shown that additions of natural clay deposits can potentially improve sandy soil properties [23–26]. In studies conducted by Al-Omran et al. [23,24], applying natural clay deposits to sandy calcareous soils improved their hydrophysical properties, including available water content, wetting front, relative swelling, infiltration, and water conservation. Sheta et al. [27] found that adding clay-rich deposits increased clay content and cation exchange capacity (CEC) while reducing intermittent evaporation in sandy soils. Adding clay deposits increases the soil's ability to retain water and nutrients due to an increase in the proportion of clay, which in turn leads to an increase in the total size of pores. Despite the increasing attention around the use of naturally-occurring clay minerals in the remediation of heavy metal-contaminated soils, there is lack of up-to-date scientific data on the influence of natural clay minerals/deposits on the alleviation and immobilization of toxic anionic metals such as Mo. As anionic metals and clay minerals both have negative charges, clay minerals cannot efficiently adsorb/immobilize anionic metals. It is, therefore, necessary to modify clay mineral soils to enhance their anionic adsorption properties. Previous studies have shown that the modification of clay minerals and deposits enriched with clay surfaces plays a significant role in the removal of metal anions from aqueous solutions [28–31], suggesting that this method may be used for the in-situ immobilization of anionic metals in contaminated soils, depending on clay properties, activation/modification, anionic metal type, and the soil properties.

Based on the above, this study aimed to investigate the following: (I) the effect of the contamination of calcareous soil with high Mo concentrations on its bioavailability and toxicity threshold for alfalfa plants, and (II) the efficacy of sulfuric acid-activated and non-activated clay deposits on the immobilization of Mo and its bioavailability to alfalfa plants.

## 2. Materials and Methods

### 2.1. Sample Collection, Preparation, Modification, and Characterization

Clay deposits samples were collected from Mahouez Mountain, in the Al-Kharj region, Saudi Arabia. Samples were prepared by grinding the collected clay deposits and passing them through a 50- $\mu\text{m}$  sieve. The clay fraction ( $<2\ \mu\text{m}$ ) was separated from the clay deposit samples using the sedimentation method [32] to identify their mineral composition with X-ray diffraction (XRD-7000; Shimadzu Corp, Kyoto, Japan). The semi-quantitative analyses of the X-ray patterns were carried out to estimate the mineral contents in the clay fractions of the clay deposits [33].

The sieved clay deposits were then treated with  $\text{H}_2\text{SO}_4$  (3 M) at a clay deposit: acid ratio of 1:10 ( $w/v$ ). The suspension was stirred continuously for 24 h, after which it was filtered, washed with deionized water several times, dried, and ground. Similarly, the raw (unmodified) clay deposits were also washed, dried, and ground. The mineralogical analysis of activated and inactivated clay deposits with acid was performed using X-ray diffraction (XRD diffractometer Shimadzu 7000). Changes in the surface functionality of clay deposit surfaces before and after activation were also estimated using FTIR (Nicolet 6700 FTIR).

### 2.2. Soil Sample Collection, Preparation, and Characterization

Sandy calcareous soil samples used in the experiment were collected from farms in Al-Kharj, Saudi Arabia. The collected soil samples were air-dried, ground, and passed through a 2-mm sieve. The soil texture was determined according to Gee and Bauder [34], pH was measured in a suspension of soil:water (1:1,  $w/v$ ) and electrical conductivity (EC) was measured in the extracts [35]. The total content of calcium carbonate ( $\text{CaCO}_3$ ) was measured according to Loeppert and Suarez [36]. The soil organic matter was measured using the Walkley–Black method [37]. The total content of Mo was measured using the inductively coupled plasma optical emission spectroscopy (ICP-OES) after digestion with  $\text{HClO}_4\text{-H}_2\text{SO}_4\text{-HF}$  according to Hossner [38]. The analyzed soil samples indicated that the soil texture was a loamy sand with 78% sand, 10% silt, and 12% clay; while its pH and EC were 7.75 and  $1.87\ \text{dS m}^{-1}$ , respectively. Additionally, the soil samples contained 17.1%  $\text{CaCO}_3$ , 0.11% organic matter, and  $1.0\ \text{mg kg}^{-1}$  Mo.

### 2.3. Greenhouse Pot Experiments

In this study, two greenhouse pot experiments were conducted. The first experiment was performed in a greenhouse (potted experiment) to study the impact of Mo soil amendments on the availability of soil Mo and its toxicity to alfalfa plants. Specifically, a pot experiment with alfalfa plants was performed using 500 g of soil treated with 0, 0.1, 1, 10, 50, and  $100\ \text{mg Mo kg}^{-1}$ . Meanwhile, a second experiment was performed to investigate the impact of different additives from inactivated and activated clay deposits by  $\text{H}_2\text{SO}_4$  on Mo immobilization and shoot Mo content in alfalfa plants grown in calcareous sandy loam soils. To achieve this objective, a greenhouse pot experiment with alfalfa plants was conducted. Specifically, each pot consisted of 500 g of soil amended with either 0, 2.5, 5, and 10% ( $w/w$ ) of the unmodified and acid-modified clay deposits. The treated and untreated soil samples of the first and second pot experiment were then filled in pots and 50 alfalfa seeds were sown in each pot. After seedlings occurred, the alfalfa plants in each pot were thinned to thirty. During the growth period, pots were frequently irrigated and soil moisture content was maintained at 70% of the field capacity by weight loss. After 35 days of planting, soil samples and alfalfa shoots were collected from each pot and prepared for further analyses.

Soil samples were air-dried and passed through a 2-mm sieve. The soil pH was measured in a (1:1) suspension using a pH-meter and the EC was measured in the extracted suspension of soil:water using an EC meter. Soil samples were extracted using water at a ratio of 1:1 (50 g soil: 50mL distilled water), then shaken for 24 h. Next, the solution was filtered to determine the concentration of soluble Mo in the water extraction. Additionally, soil samples were extracted using AB-DTPA (20 g of soil with

40 mL of AB-DTPA extract solution) according to Soltanpour and Schwab [39]. In the extracts of water and AB-DTPA, Mo concentrations were determined using ICP-OES.

Shoot samples of plants were collected at the end of the experiment. Shoot dry weight was recorded after drying at 70 °C. Then, plant shoots were ground and digested using the dry digestion method described in Chapman and Pratt [40]. In the digestion extracts, shoot Mo content was determined using ICP-OES.

#### 2.4. Statistical Analysis

The mean and standard deviation ( $\pm$ SD) of the obtained data were calculated and the data were statistically analyzed using ANOVA in the TIBCO Statistica 13.5.0 software [41]. Pearson's correlation coefficients ( $r$ ) between Mo plant concentration and Mo bioavailability in soil were measured, as well as those for correlations between Mo concentration and other soil characteristics.

### 3. Results

#### 3.1. XRD and FTIR Analyses of Activated and Inactivated Clay Deposits

The XRD analysis of RCD clay fractions ( $<2 \mu\text{m}$ ) showed peaks at  $d$  spacing of 14.44, 4.29, and 3.13 Å, suggesting the presence of montmorillonite with 41.3% (Figure S1). Meanwhile, we also identified peaks at  $d$  spacing of 7.24 and 3.5 Å, suggesting the presence of kaolinite with 24.3%. Some associated minerals of quartz and feldspar were also detected (9.0 and 5.6%, respectively). These results indicate that montmorillonite is the dominant mineral in clay deposits, followed by kaolinite.

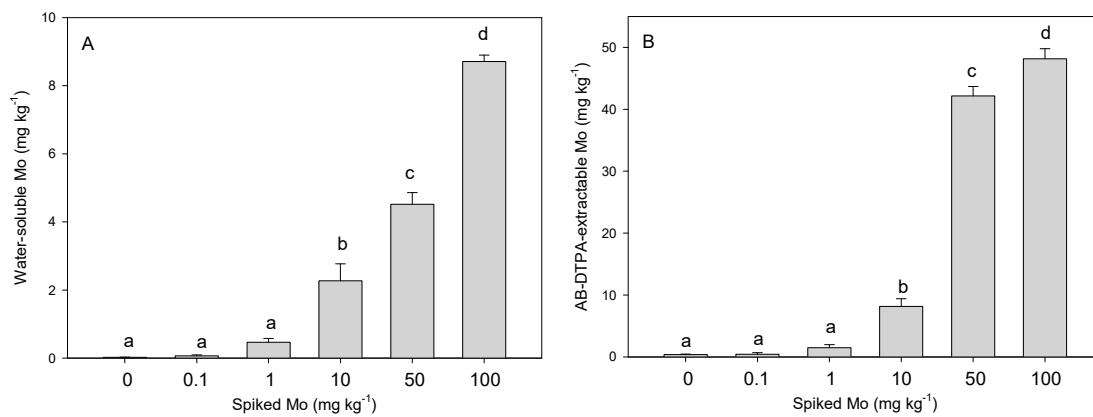
Our XRD results also highlighted differences between the XRD peaks of RCD and AMCD, mainly due to acid modification (Figure S2). It was noted that the mineral composition of raw clay deposits was dominated by smectite, followed by kaolinite and associated minerals (quartz and feldspar), whereas the intensity of peaks in acid-modified clay deposits either decreased, reached their maximum, or disappeared altogether.

The FTIR results showed that the investigated sorbents presented bands at 3621 and 3697  $\text{cm}^{-1}$  for RCD and 3621 and 3698  $\text{cm}^{-1}$  for AMCD (Figure S3). This is generally thought to be because the presence of H–O–H stretching and O–H linked with numerous bonds with Al and Mg (Al/Mg–O–H stretching) between the clay layers. Additionally, both RCD and AMCD exhibited bands at 3429  $\text{cm}^{-1}$  and 3405  $\text{cm}^{-1}$ , respectively, due to the H–O–H stretching. RCD and AMCD sorbents also exhibited bands at 1634  $\text{cm}^{-1}$  and 1626  $\text{cm}^{-1}$ , respectively, as a result of the –O–H bending-deformation of water. Active functional groups expressing the proportions of silicate minerals were also observed in the tetrahedral layers of the clay minerals (Si–O stretching) at 1032  $\text{cm}^{-1}$  for RCD and AMCD. Additionally, the presence of active functional groups of siloxane and aluminol groups (Si–O–Al stretching) was recorded at 534  $\text{cm}^{-1}$  for RCD and 535  $\text{cm}^{-1}$  for AMCD, as well as other active functional groups associated with Si and O bonds in the Si surfaces of the tetrahedral layer within activated and inactivated clay deposits. It was generally observed that the positions of the observed peaks for AMCD showed some deviations at low intensity when they were compared to RCD. Additionally, the observed peak at 1433  $\text{cm}^{-1}$  for RCD disappeared after acid modification. The new peaks were also detected in AMCD samples, which were at 1114, 1148, 2345, and 3549  $\text{cm}^{-1}$ . The newly appeared peaks (at 2345, and 3549  $\text{cm}^{-1}$ ) suggest the presence of free OH sites in acid-modified clay deposits.

#### 3.2. Effect of Mo Addition on Soil Mo Availability and Plant Toxicity

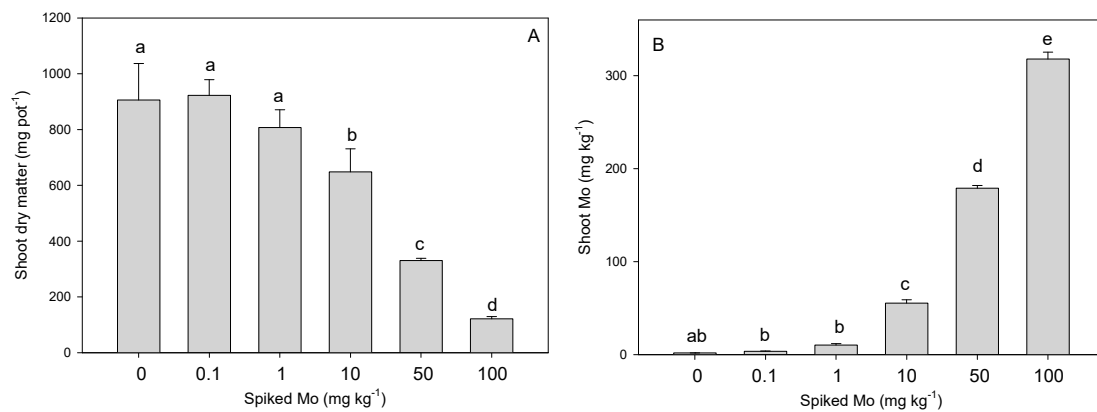
Recently, a significant amount of research has been focused on the extraction of soil-available Mo using various extraction methods to predict its uptake by plants [8,9,21,42]. In this study, two extraction methods (water and AB-DTPA) were applied to measure the solubility and availability of Mo addition to calcareous soils. Our results show that water-soluble and AB-DTPA-extractable Mo concentrations were positively correlated with the concentration of Mo added to the soil (Figure 1). In soils treated with 0, 0.1, 1, 10, 50, and 100 mg Mo/kg, the water-soluble Mo in the soil was 0.025, 0.065, 0.46, 2.27,

4.51, and 8.71 mg kg<sup>-1</sup>, respectively; while the corresponding concentrations when extracted with AB-DTP were significantly higher: 0.39, 0.45, 1.50, 8.2, 42.2, and 48.1 mg kg<sup>-1</sup>.



**Figure 1.** The water-soluble Mo (A) and AB-DTPA-extractable Mo (B) influenced by different contamination levels of Mo. Bars (means  $\pm$  SD,  $n = 3$ ) with different letters within the same Mo treatment is significantly different based on LSD ( $p \leq 0.05$ ).

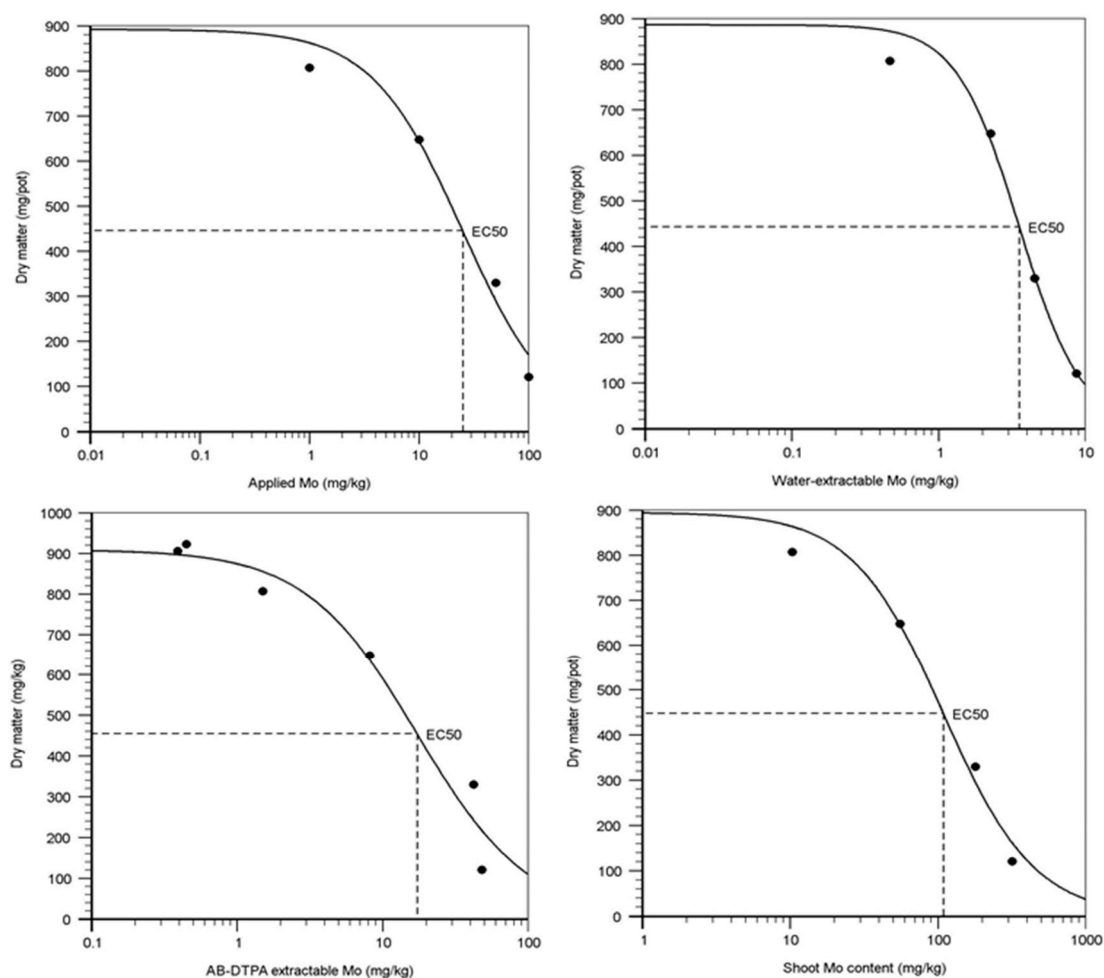
Concerning Mo toxicity to alfalfa plants, the plant dry weight was found to decrease with increasing added Mo concentration in the soil (Figure 2). The plant dry weight accounted for 906 mg pot<sup>-1</sup> in control pots and 923 mg pot<sup>-1</sup> in pots treated with 0.1 mg Mo kg<sup>-1</sup>, decreasing by 10.9% at 1 mg Mo kg<sup>-1</sup>, 28.5% at 10 mg Mo kg<sup>-1</sup>, 63.6% at 50 mg Mo kg<sup>-1</sup>, and 87.6% at 100 mg Mo kg<sup>-1</sup>. In soils artificially contaminated with 0.1, 1, 10, 50, and 100 mg Mo kg<sup>-1</sup>, the shoot Mo content increased from 1.92 mg kg<sup>-1</sup> to 3.72, 10.3, 55.5, 179, and 318 mg kg<sup>-1</sup>, respectively (Figure 2).



**Figure 2.** The shoot dry matter (A) and its concentrations of Mo (B) influenced by different contamination levels of Mo. Bars (means  $\pm$  SD,  $n = 3$ ) with different letters within the same Mo treatment is significantly different based on LSD ( $p \leq 0.05$ ).

Figure 3 shows the toxicity threshold based on applied Mo, water-soluble Mo, AB-DTPA-extractable Mo, and shoot Mo content. The toxicity threshold (EC<sub>50</sub>) values amounted to 24.6 mg kg<sup>-1</sup> (based on added Mo concentration), 3.37 mg kg<sup>-1</sup> (soil water-soluble Mo), 15.5 mg kg<sup>-1</sup> (AB-extractable Mo), and 108 mg kg<sup>-1</sup> (shoot Mo).





**Figure 3.** The toxicity threshold based on applied Mo, water-soluble Mo, AB-DTPA-extractable Mo, and shoot Mo content.

### 3.3. The Effect of Treatments on Soil Properties and Mo Availability

The addition of modified and raw clay deposits increased the content of clay fraction in the soil; with the highest increases at the highest application rate (Table S1). Similarly, the soil sand content tended to decrease after the addition of RCD and AMCD. The results also showed that adding only  $\text{H}_2\text{SO}_4$  acid-modified clay resulted in a significant decrease in soil pH compared to the control (Table 1). In soils contaminated with  $10 \text{ mg Mo kg}^{-1}$ , applying 2.5, 5, and 10% AMCD led to a decrease in soil pH from 7.74 to 7.56, 7.57, and 7.33, respectively. In soils contaminated with  $50 \text{ mg Mo kg}^{-1}$ , applying 2.5, 5, and 10% AMCD led to a decrease in pH from 7.82 to 7.60, 7.56, and 7.37, respectively. These results indicate that the highest decrease in soil pH was achieved by an application rate of 10%. Soil EC increased significantly after AMCD treatments but was not significantly affected by RCD treatments (Table 1). AMCD addition also increased EC values from  $1.89 \text{ dS m}^{-1}$  in the control soil to  $2.34\text{--}3.0 \text{ dS m}^{-1}$  in soils contaminated with  $10 \text{ mg Mo kg}^{-1}$ ; and from  $1.76 \text{ dS m}^{-1}$  to  $2.2\text{--}3.15 \text{ dS m}^{-1}$  in soils contaminated with  $50 \text{ mg Mo kg}^{-1}$ , showing that the highest EC values were generally found at the highest Mo application rate.

Our results showed that water-soluble and AB-DTPA-extractable Mo were significantly influenced by the addition of clay deposit amendments (both modified and unmodified; Figure 4). At  $10 \text{ mg Mo kg}^{-1}$ , the addition of 2.5, 5, and 10% AMCD decreased the soil water-soluble Mo by 33.2, 55, and 63.3% of that in control soil, respectively. We also observed significant decreases in water-soluble Mo with RCD treatments, amounting to 4.24% (2.5% RCD), 30.6% (5% RCD), and 22.3% (10% RCD) compared to untreated soils. In soils amended with  $50 \text{ mg Mo kg}^{-1}$ , water-soluble Mo significantly

decreased by 37.3–43.8% with AMCD treatments compared to the control, with no significant difference between application rates. However, it was not significantly influenced by RCD additions. It was generally observed that the highest immobilization of water-soluble Mo (63.3% compared to the control soil) was recorded at the highest application rate of modified clay deposit treatments and at low Mo concentration.

Table 2 shows Pearson correlation coefficients ( $r$ ) between soil pH or clay content and water-soluble Mo or AB-DTPA-extractable Mo. Our results show a significant positive relationship between soil pH and the availability of soil Mo (extracted with water and/or AB-DTPA). Additionally, a negative relationship was found between the soil clay content and water-soluble Mo or AB-DTPA-extractable Mo.

**Table 1.** Soil pH and EC ( $\text{dS m}^{-1}$ ) influenced by different contamination levels of Mo and amendment additions.

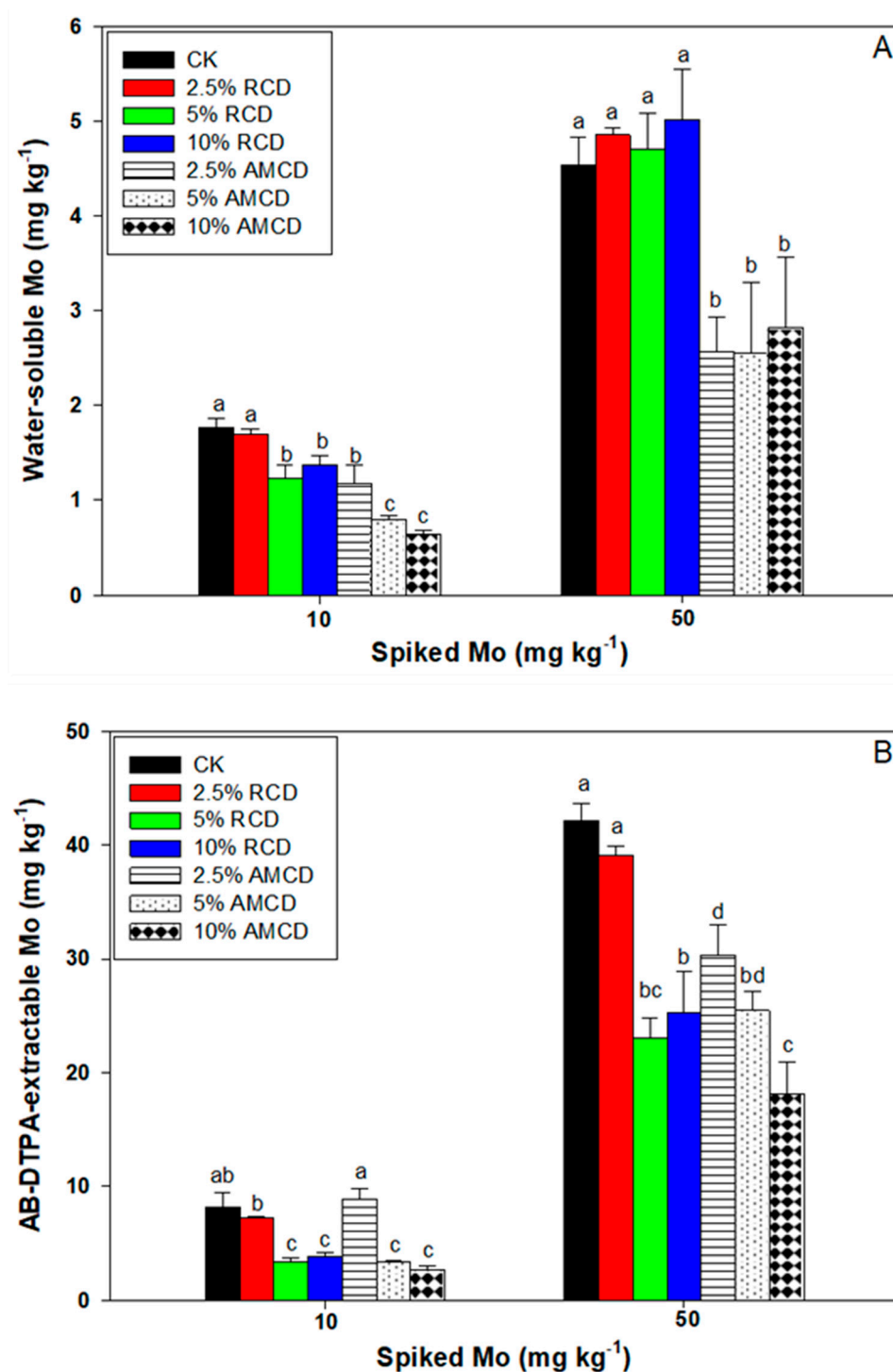
Treatments	Application Rate	pH		EC			
		%	value	$\pm\text{SD}^3$	value	$\pm\text{SD}$	
Soil contaminated with 10 mg Mo $\text{kg}^{-1}$							
CK <sup>1</sup>	0		7.74 a <sup>2</sup>	0.05	1.89 a	0.15	
	RCD	2.5		7.72 a	0.02	1.91 a	0.05
		5		7.74 a	0.06	1.80 a	0.16
		10		7.66 ab	0.05	1.81 a	0.08
AMCD	2.5		7.56 b	0.00	2.34 b	0.13	
	5		7.57 b	0.06	2.70 c	0.16	
	10		7.33 c	0.10	3.00 d	0.08	
	<i>F</i> -value		13.91	-	29.93	-	
	<i>p</i> -value		0.0000	-	0.0000	-	
Soil contaminated with 50 mg Mo $\text{kg}^{-1}$							
CK	0		7.82 a	0.10	1.76 a	0.10	
	RCD	2.5		7.78 a	0.01	1.87 a	0.06
		5		7.74 ab	0.07	1.73 a	0.05
		10		7.62 bc	0.02	1.78 a	0.13
AMCD	2.5		7.60 bc	0.02	2.20 b	0.11	
	5		7.56 c	0.02	2.63 c	0.05	
	10		7.37 d	0.13	3.15 d	0.12	
	<i>F</i> -value		9.73	-	65.21	-	
	<i>p</i> -value		0.0003	-	0.0000	-	

<sup>1</sup> Treatments include CK: control soil, RCD: raw clay deposit, AMCD: acid-modified clay deposits. <sup>2</sup> Different letters within the same Mo treatment are significantly different based on LSD ( $p \leq 0.05$ ). <sup>3</sup> Means  $\pm$  SD.

**Table 2.** Pearson correlation coefficients ( $r$ ) between soil pH or clay content and water-soluble Mo or AB-DTPA-extractable Mo.

Parameters	In the Soil Contaminated with 10 mg Mo $\text{kg}^{-1}$		In the Soil Contaminated with 50 mg Mo $\text{kg}^{-1}$	
	Water-Soluble Mo	AB-DTPA-Extractable Mo	Water-Soluble Mo	AB-DTPA-Extractable Mo
pH	0.7377 ***	0.3259	0.6560 **	0.6471 **
Clay	-0.6425 **	-0.7183 ***	-0.1015	-0.7632 ***

\* Significant at  $p \leq 0.05$ ; \*\* Significant at  $p \leq 0.01$ ; \*\*\* Significant at  $p \leq 0.001$ .



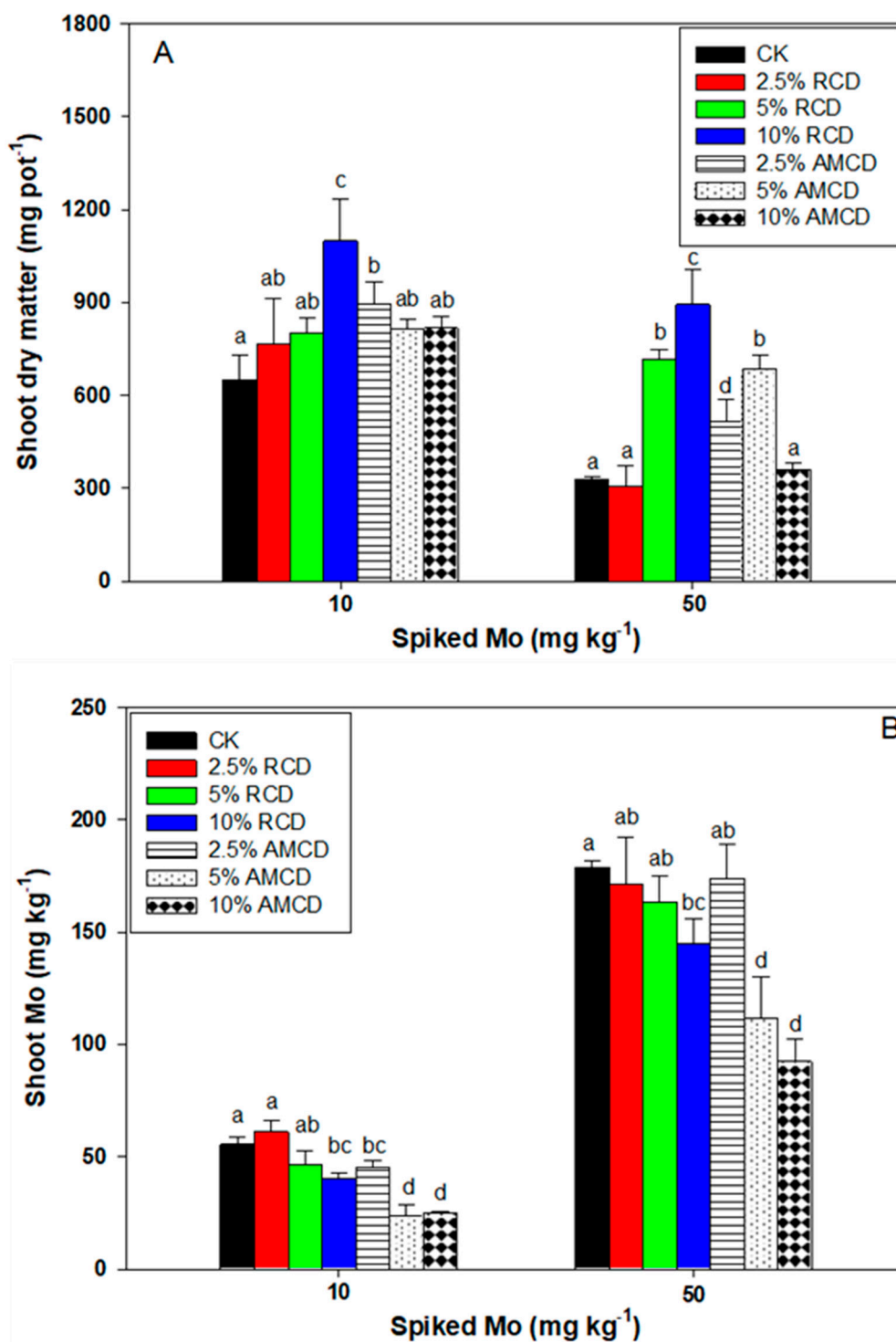
**Figure 4.** The water-soluble Mo (A) and AB-DTPA-extractable Mo (B) influenced by different contamination levels of Mo and amendments additions. Bars (means  $\pm$  SD,  $n = 3$ ) with different letters within the same Mo treatment are significantly different based on LSD ( $p \leq 0.05$ ).

#### 3.4. The Effect of Treatments on Plant Dry Matter and Shoot Mo Concentration

Figure 5 illustrates the effect of adding raw and acid-modified clay deposits on the dry matter weight of alfalfa shoots and their Mo concentration. In soils contaminated with  $10 \text{ mg Mo kg}^{-1}$ , the dry matter weight of alfalfa shoots increased by 17.8% (2.5% RCD), 23.5% (5% RCD), 69.4% (10% RCD), 38.3% (2.5% AMCD), 25.3% (5% AMCD), and 26.1% (10% AMCD) compared to the control. Meanwhile, in soils contaminated with  $50 \text{ mg Mo kg}^{-1}$ , the dry matter weight of alfalfa shoots increased by



117% (5% RCD), 171% (10% RCD), 56.2% (2.5% AMCD), 108% (5% AMCD), and 8.89% (10% AMCD) compared to the control. Among all treatments, in soils contaminated with 10 mg kg<sup>-1</sup>, only 10% RCD and 2.5% AMCD showed significant higher increases in shoot dry matter compared to control soil. However, in soils contaminated with 50 mg Mo kg<sup>-1</sup>, all treatments (except for 2.5% RCD and 10% AMCD) caused significantly higher increases in shoot dry matter compared to control soil.



**Figure 5.** The shoot dry matter (A) and its concentrations of Mo (B) influenced by different contamination levels of Mo and amendments additions. Bars (means  $\pm$  SD,  $n = 3$ ) with different letters within the same Mo treatment are significantly different based on LSD ( $p \leq 0.05$ ).

In soils contaminated with 10 mg Mo kg<sup>-1</sup>, shoot Mo concentration decreased by 15.7% (5% RCD), 27.8% (10% RCD), 18.8% (2.5% AMCD), 57.5% (5% AMCD), and 55.0% (10% AMCD) compared to the control. Meanwhile, in soils contaminated with 50 mg Mo kg<sup>-1</sup>, shoot Mo concentration decreased by 4.29% (2.5% RCD), 8.76% (5% RCD), 19.0% (10% RCD), 2.85% (2.5% AMCD), 37.5% (5% AMCD), and 48.4% (10% AMCD) compared to the control. These results indicate that the addition of acid-activated clay reduced shoot Mo concentration more significantly than RCD.

Significant Pearson's correlation coefficients (*r*) were reported between dry matter or shoot Mo concentration and measured soil parameters (Table 3). The results showed a significant positive correlation between soil pH and Mo concentration in plant tissues, while a significant negative correlation was found between soil clay content and shoot Mo concentration. The water-soluble soil Mo and AB-DTPA-extractable Mo were significantly correlated with Mo plant concentration in soils contaminated with 10 mg Mo kg<sup>-1</sup>, while only the AB-DTPA-extractable Mo was significantly correlated with Mo plant concentration in soils contaminated with higher Mo concentrations (50 mg kg<sup>-1</sup>).

**Table 3.** Pearson correlation coefficients (*r*) between soil properties or the extractability of Mo and dry matter or shoot Mo content.

Parameters	In the Soil Contaminated with 10 mg Mo kg <sup>-1</sup>		In the Soil Contaminated with 50 mg Mo kg <sup>-1</sup>		Combined Data	
	Dry Matter	Shoot Mo	Dry Matter	Shoot Mo	Dry Matter	Shoot Mo
pH	-0.0309	0.6697 ***	-0.0902	0.7258 ***	-0.1014	0.3649 *
EC	-0.0643	-0.7633 ***	-0.2170	-0.7728 ***	-0.0952	-0.3618 *
Clay	0.4840 *	-0.6670 ***	0.4121	-0.6919 ***	0.3266 *	-0.2538
Water-soluble Mo	-0.1593	0.8519 ***	0.1396	0.4085	-0.4692 **	0.8582 ***
AB-DTPA-Mo	-0.2571	0.6498 **	-0.4816 *	0.6894 ***	-0.6915 ***	0.9345 ***
Shoot Mo	-0.2310	1.0000	-0.1489	-	-0.6012 ***	1.0000

\* Significant at  $p \leq 0.05$ ; \*\* Significant at  $p \leq 0.01$ ; \*\*\* Significant at  $p \leq 0.001$ .

#### 4. Discussion

The obtained results clearly show that increasing the concentration of added Mo results in an increase in the availability of soil Mo and its concentration in plant tissues (inducing toxicity), and a corresponding decrease in plant dry matter (Figures 1 and 2). This suggests the toxicity of Mo and its adverse impact on plant biological processes and resulting growth. In Axelon [43,44], the author showed that Mo concentrations exceeding 5 mg kg<sup>-1</sup> dry matter were found in 38 out of 95 plant samples. The highest Mo content observed in forage plants was 58.7 mg kg<sup>-1</sup>, and the animals on that farm suffered from molybdenous. In another study, Kubota and colleagues [6] observed an increase in Mo concentration in forage plants with increasing calcareous soil Mo concentration but no such consistent increase in plant Mo concentration in acidic soils. They also found that Mo concentration in the plant tissues was still within the toxic range (10 to 20 ppm) for grazing animals. In this context, the results obtained in this study also show that shoot Mo content of alfalfa plants increased with increasing soil Mo content and the plant toxicity to ruminants may be significant at soil Mo of  $\geq 1$  mg kg<sup>-1</sup>.

The toxicity threshold (EC50) values in this study were assessed based on the concentration of spiked Mo, water-soluble Mo, AB-DTPA-extractable Mo, and shoot Mo by determining the effective concentration that occurs when a 50% reduction in the dry matter of plant shoots (EC50) occurs (Figure 3). The EC50 values obtained in this study, which were 24.6 mg kg<sup>-1</sup> (based on added Mo concentration), 3.37 mg kg<sup>-1</sup> (soil water-soluble Mo), 15.5 mg kg<sup>-1</sup> (AB-extractable Mo), and 108 mg kg<sup>-1</sup> (shoot Mo), are significantly lower than those obtained from previous studies, mainly due to the high alkalinity of soils used in this experiment. For example, McGrath et al. [8] showed that the EC50 values based on shoot Mo concentrations of oilseed rape, red clover, ryegrass, and tomato amounted respectively

to 525–6815, 780–2058, 1008–4270, 680–6833 mg kg<sup>-1</sup> in soils having pH < 7.0 but they were in the range of 603–1348, 499–1285, 863–1670, and 631–1740 mg kg<sup>-1</sup> in soils having pH > 7.0. They also indicated that EC<sub>50</sub> for Mo in neutral and basic soils is much lower than that in acid soils, suggesting more pronounced phytotoxicity in basic and calcareous soils. Unlike cationic toxic trace elements, the solubility, bioavailability, and phytotoxicity of Mo generally increases with increasing soil pH (increasing alkalinity). Previous studies have indicated that the retention of Mo on the surfaces of calcareous soils and calcite is low, suggesting that calcium carbonate is not an important sink for Mo to restrict its solubility, bioavailability, and plant toxicity in calcareous soils [12]. Goldberg et al. [45] further reported that Mo adsorption onto soils is heavily influenced by pH, with the greater adsorption capacity recorded at pH 2–5. The adsorption capacity then starts to decrease as pH increases to 8.0 and becomes very low at pH > 9. In another study, McGrath et al. [9] stated that Mo concentration in the soil solution and the corresponding plant uptake increased when soil pH was artificially raised, as Mo sorption to amorphous oxides is greatly reduced at high pH. However, they also reported the influence of other factors (e.g., organic C or ammonium oxalate-extractable Fe) in predicting the toxicity threshold of Mo. In this study, therefore, the high calcium carbonate content and pH value of the soil used might explain the increased solubility and availability of added Mo and its high toxicity to alfalfa plants. Therefore, soil properties need to be taken into account to assess the risk of Mo exposure.

The toxicity threshold based on AB-DTPA-extractable Mo (EC<sub>50</sub> = 15.5 mg kg<sup>-1</sup>) is relatively close to that based on added Mo concentration (EC<sub>50</sub> = 24.6 mg kg<sup>-1</sup>), suggesting the importance of using AB-DTPA to predict the bioavailability and toxicity of Mo in alkaline soils. Zhang et al. [46] similarly reported that using AB-DTPA to extract potentially available metals and nutrients is an efficient method in alkaline soils. Another study by Wang et al. [47] also suggested that the AB-DTPA extraction method was suitable to evaluate the availability of soil Mo and its potential phytotoxicity.

The effect of treatments on some soil properties (including soil texture, EC and pH) and Mo availability was also investigated in the current study. As expected, applying modified and raw clay deposits increased the content of clay fraction in the soil. Additionally, applying AMCD decreased soil pH but increased EC values. The effect of acid-modified clay deposit addition on decreasing pH could be attributed to the acid components of the chosen amendment. The amendment increases ion release from the added clay deposits after being activated by sulfuric acid, which would explain the increasing soil EC values. Therefore, the addition of clay deposits after this activation may lead to an increase in soil salinity. The concentration of soluble salts in the soil can be controlled by the mineral's solubility. Therefore, the low pH values obtained after the addition of modified clay may be the driving force increasing the EC values by increasing mineral solubility.

In terms of Mo immobilization by using soil additives, our results clearly showed that water-soluble and AB-DTPA-extractable Mo were significantly influenced by both modified and unmodified clay deposits (Figure 4). It was generally observed that the immobilization of water-soluble and AB-DTPA-extractable Mo was significantly higher in soils with added modified clay deposits compared to those with raw clay deposits, regardless of Mo treatment concentration. Past research successfully used AB-DTPA to predict Mo availability in polluted and unpolluted soils [47,48]. In Kirby et al. [49], a good relationship was found between the labile pool (MoO<sub>4</sub><sup>2-</sup>) and AB-DTPA-extractable Mo. The results showed that applying RCD and AMCD amendments can potentially have a significant effect on the immobilization of AB-DTPA-extractable Mo. In soils contaminated with 10 mg Mo kg<sup>-1</sup>, the concentrations of AB-DTPA-extractable Mo were significantly reduced (11.4–59.1%) in soils treated with RCD and by 8.85–67.0% in soils treated with AMCD compared to the control. Contrastingly, in soils contaminated with 50 mg Mo kg<sup>-1</sup>, the AB-DTPA-extractable Mo reduced by 7.21–45.3% in RCD treatments and by 28.0–56.9% in AMCD treatments compared to the control. Previous studies used clay minerals as immobilizing agents to reduce metallic ion mobility and bioavailability to remediate contaminated soils [13–17]. Applying clay minerals can reduce the labile and available pools of potentially toxic metals in contaminated soils, converting them into more stable, less toxic fractions. The surface sites of clay minerals are usually negatively charged, indicating their potential

use in the remediation of heavy metal-polluted soils through the immobilization of cationic metals [20]. Ou et al. [14] used two clay minerals (maifan stone and illite/smectite clay) to immobilize in situ potentially toxic metals. They found that these clays significantly reduced DTPA-extractable Cd, Ni, Cr, Zn, Cu, and Pb in alkaline soils. In this context, they suggested several mechanisms for soil metal immobilization using clay minerals, including physical and chemical adsorption, ion exchange and specific adsorption, complex formation, isomorphic substitution, and diffusion [14,20,50]. Additionally, the high pore density in clay could be responsible for providing new available adsorption sites for metal immobilization in soils [14]. By contrast, Neunhäuserer et al. [21] found that soil  $\text{NH}_4\text{OAc}$ -extractable Mo increased significantly (90%) following vermiculite (clay mineral) addition compared to the control soil. The increase in the extractable amount of soil Mo after vermiculate application could be attributed to an increase in the number of negatively charged sites in soils, enhancing the release of molybdate through exchange with other soil anions [21,51]. In this study, however, clay deposits play a key role in Mo in situ immobilization, depending on Mo concentration, application rate, and activation method. For instance, in soils contaminated with a relatively low level of Mo ( $10 \text{ mg kg}^{-1}$ ), a higher immobilization rate of AB-DTPA-extractable Mo was recorded for both RCD and AMCD at an application rate of 5 and 10%, with no significant difference between them. Meanwhile, in Mo-contaminated soils ( $50 \text{ mg kg}^{-1}$ ), the highest immobilization capacity of Mo extracted using AB-DTPA was found in 10% AMCD, followed by 5% RCD or 10% RCD or 5% AMCD. In most cases, the lowest water-extractable and AB-DTPA-extractable amount of Mo were found in soils treated with AMCD (except those contaminated with  $10 \text{ mg Mo kg}^{-1}$ ); the addition of AMCD showed almost similar efficiency to RCD in immobilizing AB-DTPA-extractable Mo. This suggests that acid-modified clay deposits have a strong influence on reducing the extractability of soil Mo compared to unmodified clay deposits. This could be explained by the activation of clay using sulfuric acid, which enables its use as an immobilizing agent to adsorb anionic metals. The organic and inorganic acid-activation of mineral surfaces was found to improve their binding capacity to heavy metals [52,53]. Moradi et al. [30] found that clay modified with sulfuric acid enhanced the porous external surface, providing more active sites and inducing a positive charge on the clay surface, leading to higher adsorption of Cr(VI) anions via the electrostatic attraction mechanism. It may be that using acid for clay modification might neutralize a portion of the negatively-charged sites onto the clay surface and result in an increase in the number of positively charged sites and the functionalization of clay surfaces with effective groups. This in turn leading to the increased immobilization capacity of clay minerals for anionic metals (e.g., molybdate). This mechanism might explain the low amount of water-extractable and AB-DTPA-extractable Mo as an anionic metal in soils treated with AMCD in our experiment.

The solubility and availability of soil Mo are influenced by a variety of factors including soil pH, soil texture, CEC, clay minerals, oxides, inorganic carbon content, and organic matter content [9,54,55]. The efficiency of Mo immobilization in RCD- or AMCD-amended soils relies mainly on the effects of soil amendments, which strongly influence Mo availability. In this study, the decrease in soil pH after AMCD amendments increases Mo immobilization more significantly than RCD. We found a significant positive relationship between soil pH and the availability of soil Mo (extracted with water and/or AB-DTPA; Table 2), suggesting that Mo availability is dependent upon soil pH. Previous studies also suggested that alkaline soils increase Mo availability [8,9,56,57]. Another study by Rutkowska et al. [57] also found a significant positive correlation between Mo availability and soil pH in the soil, which further supports this interpretation. They suggested that this relationship was mainly caused by an increase in free negative charges onto the soil colloidal surfaces, which led to electrostatic repulsion between Mo anions and soil particles [56,57]. Additionally, the excess amount of hydroxyl anions in alkaline soils can compete strongly with Mo anions onto the soil adsorption sites, resulting in more available soil Mo concentration [57,58].

Our results showed a negative relationship between the soil clay content and water-soluble Mo or AB-DTPA-extractable Mo, suggesting that the clay fraction might act as an important sink for Mo in alkaline soils, lowering soil Mo availability through adsorption onto the exchange sites of the soil

colloidal surfaces. Previous studies have also shown that soil clay is an important factor in controlling Mo adsorption and bioavailability [54,57], however, the extent to which soil pH and clay content affect the partitioning of Mo between different soil pools remains unclear.

The dry weight of plant shoots was generally lower in soils contaminated with 50 mg Mo kg<sup>-1</sup> compared to that contaminated with 10 mg Mo kg<sup>-1</sup> (Figure 5), mainly because of the toxicity effect induced by higher Mo concentration. When examining the effect of soil amendments, the dry weight of plant shoots showed significant increases in soils treated with RCD and/or AMCD compared to the control; no negative impacts on plant growth were observed. Overall, our results indicate that the highest application rate of RCD had the greatest enhancing effect on plant growth. For example, in soils contaminated with 10 mg Mo kg<sup>-1</sup>, the shoot dry matter increased significantly by 69.4% and 38.3% in soils treated with 10% RCD and 2.5% AMCD, respectively. Meanwhile, in soils contaminated with 50 mg Mo kg<sup>-1</sup>, the shoot dry matter increased significantly by 117% for 5% RCD, 171% for 10% RCD, 56.2% for 2.5% AMCD, and 108% for 5% AMCD compared to the control soil. The effect of applying soil amendments on accelerating plant growth might be influenced by changes in the soil's physical and chemical properties such as pH, EC, clay content, CEC, fertility, and water holding capacity. Several studies have shown that clay additions have the potential to improve sandy soil properties [23,24,26,27]. Applying clay deposits increases the soil's nutrient absorption and retention capacity, thus enhancing plant growth. Previous research also suggested that clay amendments to sandy soils increase crop yield [25,59,60], mainly due to improved water and nutrient retention capacity [26]. In this experiment, plant growth significantly increased following the addition of vermiculite (56% compared to control soil).

We also observed that the highest AMCD application rate (10% *w/w*) led to lower shoot dry weight than the 2.5 and 5% AMCD as well as the 5 and 10% RCD treatments, but was not significantly different compared to the control. This may be because of a higher increase in the soluble salts induced by the high application rate of acid-modified clay deposits combined with the high Mo contamination level. Increased levels of soluble salts in soils are usually associated with decreased plant biomass [61,62].

The results indicated that the addition of acid-activated clay reduced shoot Mo concentration more significantly than RCD (Figure 5). The magnitude of this decrease is strongly influenced by Mo availability in the soil. Previous research has shown that applying amendment-based minerals to contaminated soils was effective in reducing soil metal availability and their concentration in plant shoots, resulting in increased plant growth [17,63]. He et al. [22] found that bentonite addition at 4% (*w/w*) to metal-contaminated soils reduced the exchangeable form of As by 4.05% and shoot As concentration by 36.2% in *Brassica chinensis* L. compared to the control. They also found that the addition of bentonite enhanced the dry matter weight of plants by 289% compared to that in control soils. In this study, despite the high application rate of AMCD leading to a greater reduction in shoot Mo content, the amendment did not significantly improve plant growth in Mo-contaminated soils. This suggests that metal immobilization might have an interactive effect with another factor on plant growth, such that its effects on reducing toxicity and metal uptake by plants, as well as the nature and modification of soil additives and application rate, might play a considerable role in accelerating plant growth.

It is clear from our findings that the decrease in the shoot Mo concentration in alfalfa plants is due to a reduction in soil available Mo due to the addition of AMCD and RCD, and that this relationship is further influenced by soil pH and clay content, subsequently enhancing the capacity of alkaline soil to retain Mo anions onto the soil exchange sites.

## 5. Conclusions

This study investigated the effects of Mo application on phytotoxicity in alfalfa plants grown on sandy loam calcareous soils, as well as the influence of natural clay deposits and its acid-activation form on Mo immobilization and bioavailability. Our findings show that AMCD treatment is more efficient for Mo immobilization and reducing shoot Mo content than RCD. However, the RCD amendment



(particularly at higher application rates) increased the maximum dry matter in plant shoots. Our results suggest that acid-activated clay deposits are ideal soil amendments to increase Mo immobilization and its uptake by plants in contaminated agricultural calcareous soils. Additional long-term research is required to understand the behavior of acid-activated and non-activated natural clay deposits in Mo immobilization.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2071-1050/12/19/8203/s1>, Figure S1. XRD analysis for clay fraction (<2 µm) of raw unmodified clay deposits. Figure S2. XRD analysis for raw (RCD) and acid-modified clay deposits (AMCD). Figure S3. FTIR analysis for raw (A: RCD) and acid-modified clay deposits (B: AMCD). Table S1. The overall changes in soil sand, silt and clay following the addition of modified and unmodified clay deposits, regardless of levels of added Mo.

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