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# Effect of Cadmium, Copper and Lead on the Growth of Rice in the Coal Mining Region of Quang Ninh, Cam-Pha (Vietnam)

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Received: 3 May 2018; Accepted: 23 May 2018; Published: 27 May 2018



**Abstract:** The goal of this study was to quantify the mobility and partitioning of trace elements originating from mine waste rocks derived from open pit coal extraction activities. The results showed that native rice plants were adapted to growing in metal contaminated soils, posing a severe health risk to local population. Sequential extraction procedures and bulk soil chemical analyses both suggest enrichment of Cd, Pb and Cu in rice paddy soils. Lead was shown to be evenly partitioned among all mineral and organic phases. Copper was associated with carbonates and organic matter. Smaller fractions of Pb and Cu were also bound to Fe and Mn oxides. Only 25% of Cd, 9% of Pb and 48% of Cu were associated with the exchangeable fraction, considered mobile and thus bioavailable for plant uptake. Effects of Cd, Cu and Pb on local Cam Pha *Nep cai Hoa vang*, and control *Asia* Italian rice, showed marked differences in growth. The local Vietnamese variety grew close to control values, even upon exposure to higher trace metal concentrations. Whereas the development of the control rice species was significantly affected by increasing trace metal concentrations. This result suggests toxic trace elements accumulation in the edible parts of crops.

**Keywords:** open-pit coal mining; *Oryza sativa* L.; rice plant contamination; contaminated paddy soil; metals

## 1. Introduction

Open-pit coal mining in countries such as China and Vietnam, is one of the major causes of freshwater, soil and air pollution [1,2]. Indeed, overburden and acid mine drainage (AMD) from coal mining contaminate fertile soils and induce unproductive wastelands, as shown in Australia [3], Bangladesh [4], China [5,6], India [2,7] and Vietnam [8]. Open-pit coal mining involves excavation of the earthly bound ore, a process which releases large quantities of mine spoil [7]. Subsequent pollution with the characteristic leaching of toxic metal elements (not heavy metals as often read; see details in Pourret and Bollinger [9]) occurs and is of major concern for population cultivating near mining sites (e.g., Cd, Cu, Pb or Zn; [2,7,10]).

Waste rock material derived from open pit coal mining contains significant concentrations of reduced sulfides. These include pyrite ( $\text{FeS}_2$ ), chalcopyrite ( $\text{CuFeS}_2$ ) and other metal sulfides, including covellite ( $\text{CuS}$ ), chalcocite ( $\text{Cu}_2\text{S}$ ) and galena ( $\text{PbS}$ ). The Cd containing sulfide, greenockite ( $\text{CdS}$ ), has been found to be frequently associated with sphalerites and wurtzites [ $(\text{Zn,Fe})\text{S}$ ] [11]. The chemical weathering of these minerals enhances the solubility, mobilization and bioavailability of trace metals [11]. In contaminated areas, these elements may be transported by colloidal or suspended particulates present in water used to irrigate agricultural land [12].

Trace elements in soils have detrimental effects on the growth of staple crops (e.g., rice, barley, garlic, wheat, maize) [13–16], and can accumulate in their edible parts posing a serious health risk to humans [8,17–19]. Cadmium, Cu and Pb have been identified as having adverse effects on rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) growth [16,17,20–22]. In addition, toxic metal tolerance in crop varieties has been shown in rice, sunflower, wheat and leguminous species, leading to an increment in metal uptake and to their concentration in edible plant parts, posing a severe health risk [8,23,24].

In this study, a first approach quantifies metal fractionation and mobility in mine waste rock and rice paddy soil samples, from the open-pit coal mining region of Quang Ninh, (Vietnam). A second part of the study focuses on determining the effect of mobile toxic elements (e.g., Cd, Cu, Pb) on the growth of native and control rice plant varieties.

## 2. Materials and Methods

### 2.1. Study Area

The northeastern province of Quang Ninh hosts the largest anthracite coal reserves in Vietnam (<http://www.eia.gov>). A substantial increase in anthracite production in the past decades has had a detrimental effect on the quality of agricultural soils in this region [8]. In recent years, this problem has worsened since Vietnam became one of the world's major coal exporter (Fong-Sam, 2017). The Coc Sau mine is the largest open pit coal facility and is located near Cam Pha city (Figures 1 and 2) (<http://www.vinacomin.vn/en.html>). This facility has operated in this area for more than 100 years, extracting approximately 1 Mt of anthracite coal annually. During the wet season, the tropical climate of this region enhances chemical weathering of coal-mine tailings. This is known to lead to the oxidation of primary sulfides, the generation of AMD, and the release of trace metals, into aquatic environments such as streams, lakes and agricultural soils [11].



**Figure 1.** Location of Quang Ninh province in NE-Vietnam (A) and the municipalities of Quang Ninh, showing the location of the region of Cam Pha (B).



**Figure 2.** Location of coal, soil and mine tailing sampling sites in the area of Cam Pha in the Quang Ninh province in NE Vietnam. S1, S2 and S4 refer to mine waste rock sampling locations, where samples were collected from each site. S3 refers to the location where coal samples retrieved from the Coc-Sau open pit coal mine. S5 refers to location of rice paddy soil samples, collected and analyzed as described previously in Martinez et al. [8].

### 2.2. Sampling of Coal and Waste Rock Material

Coal and mine waste rock samples were collected for bulk chemical analysis, from the locations shown in Figure 2. Between 10 and 15 hand specimen coal and waste rock samples were collected from these locations and subjected to further analyses. The mine tailing sampling sites were labelled as S1, S2 and S4, and the coal sampling site from inside the Coc-Sau mine, was named: “S3”. “S5” in Figure 2 is the location of rice paddy soil sample collection, as previously described in Martinez et al. [16]. The coal samples were prepared for bulk metal content analysis following the ISO13909-4:2016 protocol (ISO 2016). Both the waste rock and coal samples were dried at 40 °C for 4 h. The samples were then powdered to an approximate grain size of 212 µm for bulk chemical analyses.

### 2.3. Bulk Chemical Analysis of Coal and Mine Waste Rock

Approximately 5 g of powdered coal or mine waste rock samples were dried overnight in an oven at 60 °C. Subsequently, these samples were digested with 60 mL of boiling 2 mol/L HNO<sub>3</sub> for 2 h in a reflux apparatus [25]. The solutions were further allowed to cool before filtration using a Whatman No. 595 filtration device. Thereafter, the filtrate was quantitatively transferred to a clean volumetric flask, diluted to 100 mL with 2 mol/L HNO<sub>3</sub>. The final solution was analyzed for bulk trace metal concentrations using flame (AAS vario-6 analytik Jena) and graphite-furnace (Zeeman Atomic Absorption Spectrometer 4110ZL—Perkin Elmer) atomic absorption spectroscopy (F-AAS and GF-AAS) in the Geochemistry Laboratory at the University of Freiburg, Germany. F-AAS and GF-AAS methods

had a precision better than 5%, and detection limits of 0.029 mg/kg, 0.015 mg/kg and 0.005 mg/kg for Cd, Cu and Pb, respectively [26]. In order to validate F- and GF-AAS methods, a NIST reference material (i.e., SRM 2710) with elevated trace element concentrations, was prepared according to the protocol described by US EPA [25] (Table 1).

**Table 1.** Analysis of NIST Standard Reference Material (SRM) 2710.

Element	* Certified Mass Fractions for SRM 2710 (mg/kg Dry Weight)	AAS Analysis of SRM 2710 (Univ. Freiburg) (mg/kg Dry Weight)
Cd	20.8 ± 0.2	20.6 ± 0.6
Cu	2950 ± 130	2759 ± 3
Pb	5532 ± 80	5367 ± 11

\* From the NIST certification, July 2003.

#### 2.4. Sequential Extraction of Metals from Coal, Waste Rock and Soil Samples

The sequential extraction of metals from coal, waste rock or soil samples was performed as described below (protocol modified from Tessier et al. [27]). The procedure consisted of five steps: (i) *Exchangeable fraction*: To 2 g of coal, waste rock or soil sample, 16 mL of 1 mol/L C<sub>2</sub>H<sub>7</sub>NO<sub>2</sub> (ammonium acetate instead of MgCl<sub>2</sub>, pH = 7 [26]) were added to the sample and mixed with mechanical shaker at 200 rpm for 1 h (VWR<sup>®</sup> Advanced digital shaker—Model 3500). The resulting solution was centrifuged at 4000 rpm for 1 h. The supernatant was filtered through a 0.45 µm cellulose acetate filter, and stored for chemical analysis. The remaining solid sample was then washed with 16 mL of ultrapure water (UPW—18 MΩ) and centrifuged for 30 min. (ii) *Carbonate fraction*: To the solid from Step 1, 16 mL of 1 mol/L C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub> (sodium acetate) in acetic acid (i.e., 12.5 mL of acetic acid (100%) for 500 mL of 1 mol/L C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub>, pH = 5) were added and mixed in a mechanical shaker for 4 h. The solution was then allowed to stand overnight. After this time, the sample was centrifuged at 4000 rpm for 1 h, the supernatant was collected, washed and stored as in step (i). *Fe-Mn oxides fraction*: to the remaining solid, 40 mL of 0.04 mol/L NH<sub>2</sub>OH·HCl (hydroxylammonium chloride) in acetic acid (2.78 g NH<sub>2</sub>OH·HCl in 1 L 25% acetic acid; pH = 1.6) were added to a 50 mL sterile Falcon<sup>®</sup> tube in a water bath set at 96 °C. The sample was tightly closed and allowed to stand overnight. It was then centrifuged for 1 h at 4000 rpm and the supernatant filtered through a 0.45 µm cellulose acetate filter and stored for further chemical analysis. The remaining solid was washed as described above. (iv) *Organic matter fraction*: 6 mL of 0.02 mol/L HNO<sub>3</sub> and 10 mL of 30% H<sub>2</sub>O<sub>2</sub> (pH = 2) were added to the remaining solid at 85 °C for 2 h. After this time, 6 mL of 30% H<sub>2</sub>O<sub>2</sub> (pH = 2) were added at 85 °C for a further 3 h. The solution was allowed to cool and 10 mL of 3.2 mol/L ammonium acetate in 20% HNO<sub>3</sub> were added. The resulting solution was diluted to a maximum volume of 40 mL with UPW and mixed at 200 rpm for 30 min and left standing overnight. The sample was then centrifuged at 4000 rpm for 1 h, the supernatant was filtered through a cellulose 0.45 µm acetate filter and stored for chemical analysis. The remaining solid sample was dried to a constant weight at 40 °C and its weight was noted down. (v) *Residual fraction*: To the remaining solid from (iv), 10 mL of 2 mol/L HNO<sub>3</sub> (instead of a mixture of HF-HClO<sub>4</sub> as in the original protocol [26]) were added to 1 g of the previously dried sample and the resulting mixture was placed in a water bath for 2 h at 90 °C. The solution was then centrifuged at 4000 rpm for 1 h. The supernatant was collected with a 20 mL syringe, filtered through a 0.45 µm cellulose acetate filter and stored in a 15 mL Falcon<sup>®</sup> tube for chemical analysis.

#### 2.5. Experiments of Rice Plant Growth

In order to quantify the effects of trace metals (e.g., Cu, Cd and Pb) on rice plant growth two *Oryza sativa* L. rice cultivars were used. The *Asia* cultivar, used as a control, and the local Vietnamese variety (*Nep cai hoa vang*). *Asia* cultivar whole grains were obtained in collaboration with the Unità

di Ricerca per la Ricoltura (CREA, Vercelli, Italy). Native Vietnamese seeds of the *Nep cai hoa vang* cultivar were acquired from the Quang Ninh Seedlings Joint Stock Company in Cam Pha, Vietnam. For growth experiments sterile rice growth medium (RGM) was prepared. The RGM contained 6 g/L of phyto-agar, 0.049 g/L of Murashige and Skoog (MS) medium basal salt mixture, and 0.050 g/L of MES (2-(N-morpholino) ethanesulfonic acid) biological buffer as described in Martinez et al. [28]. All components were obtained from Duchefa Biochemicals (Haarlem, The Netherlands). The phyto-agar generated a solid medium for the germination of the rice seeds. The RGM pH was adjusted to 5.7 with 0.1 mol/L NaOH prior to the addition of the phyto-agar. The RGM was subsequently sterilized by autoclaving at 121 °C for 20 min. Eventually, the RGM does not contain any trace metals [28].

To test the effect of Cd, Cu and Pb on the growth of rice plants, 200 mL of 0.01 mol/L  $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 0.01 mol/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , and 0.01 mol/L  $\text{Pb}(\text{NO}_3)_2$  were prepared in liquid RGM. Before the addition of the phyto-agar and the pH adjustment, volumes of the trace metal stock solutions were added to the RGM to yield final trace metal concentrations of: 2, 5 and 10  $\mu\text{mol Cd}^{2+}/\text{L}$ ; 2, 5, 10 and 50  $\mu\text{mol Cu}^{2+}/\text{L}$ ; and 50, 150, 300 and 500  $\mu\text{mol Pb}^{2+}/\text{L}$ . These concentrations were chosen based on the labile trace metal content of the paddy soil in Cam Pha reported previously by Martinez et al. [8]. Rice grains from each variety were planted in sterile 15 mL Falcon® tubes, containing 10 mL of solid RGM control, or with a corresponding trace metal content added. Nitrate and sulfate do not have effect on the experiment given the chosen pH (e.g., [8,28,29]).

A total of 120 seeds from each rice cultivar were exposed to the metal concentrations described earlier for a period of 15 days in a greenhouse at the University of Freiburg. A day/night cycle (14 h light/10 h dark) was generated under homogeneous light intensity of 55,000 to 60,000 lx. The temperature ranged from 28 to 32 °C, with a humidity of 85 to 90%. After 15 days, the rice plants were carefully pulled out of the growth medium and any remaining agar on the roots was removed. The plants were then dried overnight, in an oven at 60 °C. Growth was quantified by measuring the dry root biomass, as described previously [8,30,31].

## 2.6. Statistical Analysis

Descriptive statistics were performed on rice plant root dry weights from control (*Asia*) and native (*Nep cai Hoa vang*) rice plant varieties exposed to trace metal concentrations. Normality of data and homogeneity of variances was verified. A one-way ANOVA (Analysis of Variance) tested differences in root dry weight from rice plants grown in the presence of Cd, Cu and Pb concentrations. Significance was defined and represented as follows: \*\*\*:  $p < 0.001$ , \*\*:  $p < 0.01$ , \*:  $p < 0.05$ , NS = non-significant.

## 3. Results and Discussion

### 3.1. Coal and Mine Waste Rock Mineralogy and Bulk Chemical Analysis

The waste rock samples mostly show a massive, fine grained dark groundmass. However, more coarse-grained anthracitic layers displaying a brilliant, almost metallic luster can also be observed. Some samples display fibrous veins on joint surfaces. These coatings primarily consist of chlorite (e.g., clinochlore  $(\text{Mg}_5\text{Al})(\text{AlSi}_3)\text{O}_{10}(\text{OH})_8$ ) and calcite ( $\text{CaCO}_3$ ). Even though bulk chemical analyses of coal samples indicate a significant sulfide content (0.45 wt %), all sulfides were present in small grain sizes. Back-scattered electron imaging revealed fine-grained sub-crystalline to amorphous metal sulfides. This can further indicate an increased surface area and therefore a higher extent of sulfide chemical weathering [1]. In addition, mine waste tailings comprised residues of clastic sediments, intercalated with the commercial coal seams. These sediments show a clast-supported, breccious fabric and a high degree of silica cementation. All clasts are composed of quartz, and show curved cracks running parallel to each other. This indicates the sediments underwent tectonic stress after diagenesis, favoring physical weathering and the kinetics of water-rock interaction. The matrix

between the quartz clasts reveals a darkish color. It can be expected, that it contains impurities of sulfides and organic constituents. As all investigated samples contained coal to various amounts, the release through chemical weathering of associated trace metals is implied. Table 2 shows the results of the sequential extraction analyses of coal and mine waste rock samples collected at the locations indicated in Figure 2. Selected toxic trace metals (e.g., Cd, Pb, Cu) associated with reduced sulfides are reported in Table 2. As mentioned earlier, these metals can be mobilized upon chemical weathering, therefore contaminating freshwater sources and agricultural fields. Enrichment of these elements in Cam Pha agricultural soils, may be suggested by the results of bulk chemical analysis, in this study. In mine waste rocks, average concentrations of  $0.09 \pm 0.01$ ,  $24.5 \pm 0.3$  and  $24.8 \pm 0.8$  mg/kg for Cd, Pb and Cu were determined respectively, whereas for rice paddy soils in Cam Pha, these values increased to  $0.2 \pm 0.1$ ,  $35 \pm 1$ , and  $27 \pm 1$  mg/kg as described in a previous study [8].

**Table 2.** Partitioning of Cu and Pb in mine waste rock (S1, S2 and S4) and coal (S3) samples from the open pit coal-mining region of Cam Pha in NE-Vietnam. Samples collected from the locations indicated in Figure 2.

Sample Location	Fraction **	Cu (mg/kg) +	Pb (mg/kg) +
<b>S1</b>	Exchangeable	0.46	0.43
	Bound to carbonates	1.85	2.67
	Bound to Mn and Fe oxides	1.13	2.33
	Bound to organic matter	7.97	1.68
	Residual ***	2.37	2.40
	Total	13.77	9.51
<b>S2</b>	Exchangeable	0.98	1.43
	Bound to carbonates	3.11	4.25
	Bound to Mn and Fe oxides	2.46	3.36
	Bound to organic matter	5.98	1.44
	Residual ***	2.16	1.61
	Total	14.69	12.09
<b>S3</b> (coal sample)	Exchangeable	0	0.12
	Bound to carbonates	0.77	0.86
	Bound to Mn and Fe oxides	0	1.082
	Bound to organic matter	8.64	1.29
	Residual ***	3.72	1.24
	Total	13.12	4.59
<b>S4</b>	Exchangeable	1.40	0.50
	Bound to carbonates	3.42	4.10
	Bound to Mn and Fe oxides	1.57	7.39
	Bound to organic matter	9.10	2.57
	Residual ***	5.79	6.58
	Total	21.29	21.14

\*\* The "Fraction" corresponds to the steps of the sequential extraction method used described in the material and methods section. \*\*\* The "Residual" fraction signifies the metal concentration in the crystalline structure of primary and secondary minerals not expected to be released in solution. + Error in metal concentration measured by AAS ranges between 2% and 4%.

### 3.2. Trace Metal Sequential Extraction from Mine Waste Rocks

Results of sequential extraction analyses of coal and mine waste rock are shown in Table 2. Sequential extraction procedures revealed that Cu and Pb were associated to different extents with all mineral fractions in these mine waste rocks (Table 2). Cadmium concentrations were found to be below the detection limit. This finding is in good agreement with previous studies where no or little Cd was detected in mine tailings from sequential extraction protocols [32]. Lead was bound to carbonates, Fe- and Mn-oxides and organic matter. For samples collected from locations S1 and S2, 28% to 35% of the total Pb was bound to the carbonate fraction, and up to 28% of the total Pb was

associated to organic matter (samples from location S3). In rocks from location S4, however, 35% of the total Pb concentration was detected in the Fe and Mn oxide fraction. This complex distribution pattern with a fairly even partition of Pb among all mineral phases in all samples suggests these waste rocks constitute a significant Pb source to Cam Pha agricultural soils. However, the release of Pb to aquatic soil environments, would be subjected to conditions resulting from chemical weathering, such as varying pH and  $E_h$ , as well as the presence of autochthonous organo-mineral colloids, capable of controlling the mobilization of trace elements through surface sorption processes [33]. In freshwater irrigation sources, used for Cam Pha rice paddies, pH and  $E_h$  values were measured to be  $2.87 \pm 0.55$  and  $549 \pm 133$  mV respectively [34], indicating an oxidizing environment, arising from the oxidation of reduced sulfide minerals in coal and waste rock particles and appropriate for the release of trace elements. Further support for this process can be obtained from the measured concentrations of Fe and  $SO_4^{2-}$  in these waters, which ranged from 6.5 to 62.5 mg/L and 718 to 1132 mg/L respectively, as compared to control water values of 0 mg/L (Fe) and 173 to 225 mg/L ( $SO_4^{2-}$ ) [34].

Compared to the homogenous partitioning of Pb among the different mineral phases in waste rocks, Cu was preferentially bound to the organic fraction, with a 58% and 65% bound to organic matter in rock samples from locations S1 and S3 and 41% to 43% in rock samples from S2 and S4 respectively (Table 2). It should be noted, however, that 16% to 21% of the Cu was also associated with the carbonates in these two latter samples. Within the heterogeneous nature of the mine waste rocks, the results from the extraction of Pb and Cu, suggest that carbonates and organic matter are the dominant fractions giving rise to enhanced concentrations of these trace metals in paddy soils [8]. Iron- and Mn-oxides were associated with Pb and Cu in waste rock samples, to a lesser extent. Physical and chemical weathering of these mineral fractions can similarly generate low pH waters from the oxidation of sulfides within these rocks. Acidic conditions will lead to the release and mobilization of metal cations such as Cu and Pb into nearby agricultural land, such as rice paddy soils, as observed previously in Martinez et al. [8]. Although not detected through sequential extraction procedures (Table 2), Cd should not be disregarded as a potential contaminant in rice paddy soils, and in freshwater field irrigation sources. The presence of this element in Cam Pha rice paddies as shown by Martinez et al. [8], suggests a continuous accumulation of Cd in organic-rich soils, which can become mobile and thus bioavailable to growing crops, including the local rice plants.

### 3.3. Sequential Extraction of Cd, Cu and Pb from Cam Pha Rice Paddy Soil

Results of sequential extraction analyses of soil sample are shown in Table 3. Sequential extraction experiments were further carried out to understand the distribution of the toxic trace elements in Cam Pha rice paddy soils. As explained earlier, the mobility of an element largely depends on its distribution within geochemical fractions. Generally, it increases as follows: exchangeable > bound to carbonates > bound to Fe and Mg oxides > bound to organic matter >> bound to crystalline stable mineral phases (residual fraction) [35]. In soils, metal cations should be expected in two different pools, where hydrated metal cations may be found as “free” ions, or bound to dissolved organic matter. This dissolved fraction is the source of trace elements bioavailable to rice plant roots, whereas the metals remaining on the less mobile fractions, would not be regarded as being able to affect plant growth. The results in Table 3 show that 25% of the total Cd was bound to the exchangeable fraction, as it has been reported for soil systems with a pH < 8 [36]. Approximately 28% of the extracted Cd was associated with carbonates, 42% with Fe and Mg oxides, and 5% was bound to organic matter. It is consistent with recently published results from Yu et al. [37] and He et al. [38]. Consequently, the mobility of Cd is largely determined by the exchangeable, carbonate and Fe and Mn oxide fractions, which indicates that Cd would be progressively enriched in Cam Pha rice paddy soils.

The results in Table 3 show a distinct pattern of Pb partitioning in Cam Pha rice paddy soils. As suggested by previous studies, 71% of the Pb was found associated to the Fe and Mn oxide fraction [32], whereas only 14%, 9%, and 7% of the Pb was bound to the organic, exchangeable and carbonate fractions, respectively. These results coincide with those of Martley et al. [32], where Pb was

found to be strongly associated with the Fe- and Mn-oxide fractions. As shown by the results in this study, a significant portion the Pb was associated with the carbonates in Cam Pha soils, similarly to what has been described earlier for other areas [8,32,39,40]. The combined binding of Pb to Fe and Mn oxides and the carbonate fraction may be explained by a higher near-neutral soil pH, attributed to the buffering by carbonate minerals found in mine tailings. Lead mobility may be also constrained by its complexation with organic matter [41,42]. Indeed, at slightly acidic pH values (i.e.,  $4 < \text{pH} < 6$ ), a portion of the reactive functional groups within the organic matter matrix can become protonated, generating the release of otherwise bound Pb through a  $\text{H}^+/\text{Pb}^{2+}$  competition reaction, which in turn would depend on acidity and metal sorption constants as indicated previously for Cd [43].

**Table 3.** Partitioning of Cd, Cu and Pb in rice paddy soil samples from the open pit coal-mining region of Cam Pha in NE-Vietnam. Samples collected from location (S5) as indicated in Figure 2.

Sample Location	Fraction **	Cd ( $\mu\text{g}/\text{kg}$ )	Cu ( $\mu\text{g}/\text{kg}$ )	Pb ( $\mu\text{g}/\text{kg}$ )
S5	Exchangeable	$3.6 \pm 0.1$	$29 \pm 24$	$189 \pm 46$
	Bound to carbonates	$3.9 \pm 0.3$	$31 \pm 25$	$168 \pm 67$
	Bound to Mn and Fe oxides	$5.9 \pm 0.4$	nd *	$719 \pm 64$
	Bound to organic matter	$0.7 \pm 0.3$	nd *	$139 \pm 31$
	Total	$14.2 \pm 0.6$	$59.7 \pm 35.6$	$1215 \pm 108$

\* nd means metal concentration below AAS method detection limit (not detected). \*\* The "Fraction" corresponds to the steps of the sequential extraction method used described in the material and methods section. Note: metal concentrations were below detection limit for the residual fraction described previously.

Copper was found associated to the exchangeable fraction (48%) and bound to carbonates (52%), suggesting a pH dependent mobility of Cu in paddy soils. This distribution pattern is consistent with that described as characteristic for highly mobile Cu contamination in soils [32]. However, in cases where the degree of Cu contamination is low, this metal is also present in less mobile forms, such as bound to hydrous Fe–Mn oxides [44,45]. The high-concentration of Cu associated to more exchangeable fraction implies that the Cu would be bioavailable for plant uptake. As further evidence, the results of trace metal sequential extraction in this study are in good agreement with those of chemical equilibrium and metal speciation modeling approaches as shown previously [46–48]. Plants, such as the native rice variety in Cam Pha require Cu as an essential micronutrient for their growth. However, at high concentrations, Cu may have toxic effects as was observed in this study, from growth experiments with Cu concentrations higher than  $10 \mu\text{mol}/\text{L}$ . Thus, of the total extracted metals, 25% of Cd, 9% of Pb and 48% of Cu were considered to be mobile and thus bioavailable in Cam Pha rice paddy soils. These results correlate with previously published results from plant growth experiments in the presence of elevated metal cation concentrations [29,37,49].

### 3.4. Effects of Cd, Cu and Pb on Rice Plant Growth

Cadmium, Pb and Cu were found to be present in Cam Pha rice paddy soils, with concentrations in the range of  $0.2 \pm 0.1 \text{ mg}/\text{kg}$ ,  $35 \pm 1 \text{ mg}/\text{kg}$ , and  $27 \pm 1 \text{ mg}/\text{kg}$  of dry soil, as previously described by Martinez et al. [8]. In order to further understand the effects of toxic trace elements on rice plant growth, hydroponic laboratory experiments were performed, with two rice cultivars, namely: the local *Nep cai hoa vang* and the control *Asia* cultivars obtained as described earlier. The cultivars were exposed to various concentrations of the trace elements Cd, Cu and Pb to test their effects on the growth of the local Cam Pha and the *Asia* control plant varieties. Under laboratory control conditions (i.e., in the absence of added trace metals), the biomass for the *Nep cai hoa vang* and the *Asia* rice cultivars averaged  $7.2 \pm 1.9 \text{ mg}$  and  $3.6 \pm 1.9 \text{ mg}$ , respectively.

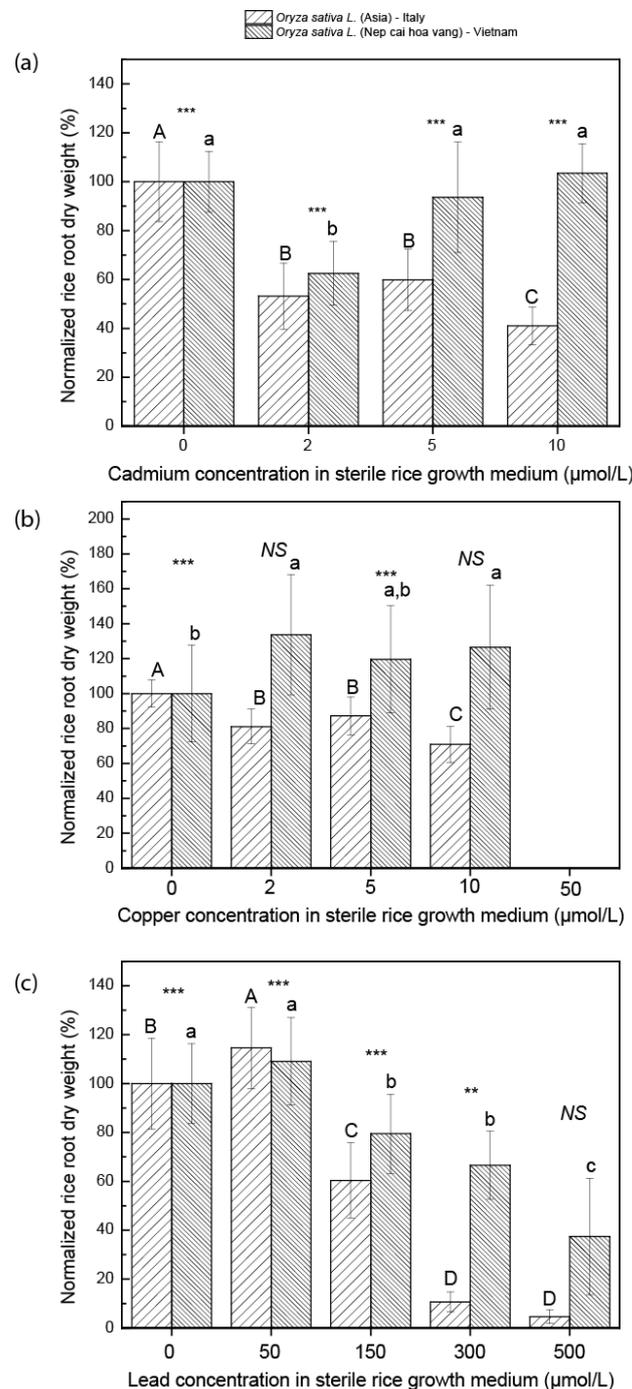
Results from the one-way ANOVA analyses for the two rice varieties, are shown in Figure 3. For each variety, there are no significant differences in rice plant growth between conditions when the same letter is shown [upper case letters for control (*Asia*) and lower case letters for native (*Nep cai Hoa vang*) rice plant varieties]. Figure 3a–c show the effects of Cd, Cu, and Pb concentrations,

respectively on the growth of control *Asia* and native *Nep cai hoa vang* rice plant varieties. Growth was quantified by measuring the root dry weight, as suggested previously [30,31]. The results in Figure 3a show that the local *Nep cai hoa vang* cultivar had a better growth than the *Asia* species at high Cd levels. However, both varieties revealed a significant decrease in root dry weight relative to the control experiments at 2  $\mu\text{mol/L}$  Cd. Under this condition, the biomass of the *Asia* rice root decreased by 53% relative to the control, while the *Nep cai hoa vang* cultivar weight declined by 38%. This suggests the presence of growth stress for the *Asia* variety when exposed to 2  $\mu\text{mol/L}$  Cd concentrations. This coincides with results observed previously by Wang et al. [50]. The difference in growth between the control and native rice cultivars in Cd containing medium could be explained by the divergent selection of cultivars best growing in soil types presenting a high variability of Cd contamination [37,50,51]. This mechanism may be suggested by the results in Figure 3a where the local cultivar (*Nep cai hoa vang*) grows closer to control values in the presence of 5 and 10  $\mu\text{mol/L}$  Cd and may be therefore adapted to high Cd concentrations. Rice plants have been shown to develop trace metal protection mechanisms by sequestering metals in roots or by combining them with deprotonated organic acids, proteins, and polysaccharides [52]. For the 5 and 10  $\mu\text{mol/L}$  Cd conditions, the results in Figure 3a show further a significant decrease in biomass for the *Asia* variety of 41% and 60% respectively with respect to the control at 0  $\mu\text{mol/L}$  Cd. The toxic effect observed on the local variety for the 2  $\mu\text{mol/L}$  Cd and the subsequent growth recovery at higher concentrations (5 and 10  $\mu\text{mol/L}$  Cd) may further suggest the presence of a protective antioxidant mechanism in Cam Pha rice.

Figure 3b illustrates the effect of Cu concentrations (2, 5, 10 and 50  $\mu\text{mol/L}$ ) on the growth of control and local Cam Pha rice plant varieties. Within experimental error, Cu at concentrations ranging from 2 to 10  $\mu\text{mol/L}$  did not significantly affect the growth of the control *Asia* rice species. This result suggests that the resistance to Cu shown by the control variety could be associated to an important nutritional need for Cu as highlighted in a few Cu tolerant plant species [53–55]. However, Cu showed a mild toxic effect for the local *Nep cai Hoa vang* rice cultivar. This metal plays a significant role in cellular metabolism and growth in plants. However, its presence in concentrations higher than those required may impose severe toxic effects [54,56]. At concentrations higher than 3 mg/kg and ranging up to 200 mg/kg (~3000  $\mu\text{mol/L}$ ) Cu has been described to affect the rice root plasma membrane and hinder important physiological processes. Photosynthetic reactions involving photosystem II (PSII) have been proposed to be the principal target of Cu toxicity. In rice plants, Cu has been associated with the production of reactive oxygen species (ROS), leading to oxidative stress [52,55]. For sensitive plant species, such as local rice variety in Vietnam, 3.2 mg/kg (equivalent to 50  $\mu\text{mol/L}$  Cu) were shown to be toxic, yielding decrease of about 10–20% in root biomass as shown in Figure 3b. These results are consistent with those of Xu et al. [57] who report similar effects for Cu induced toxicity in crops. The difference in the response of rice cultivars to Cu may be explained by differences in resistance mechanisms and/or plant nutritional needs. A future perspective will be to examine both the physiological pathways involved in Cu nutrition and oxidative stress in these two rice cultivars.

The results from growth experiments with  $\text{Pb}(\text{NO}_3)_2$  in sterile rice growth medium and two different rice cultivars are presented in Figure 3c. The results suggest a stress-induced growth of roots at 50  $\mu\text{mol/L}$  Pb, as shown in Figures 3c and 4. The sensitivity and tolerance index of rice against Pb stress mainly depends on uptake of Pb from the soil and internal sequestration of the plants [19]. As Pb concentrations in the rice growth medium increased, the growth of both rice cultivars relative to the control condition decreased significantly (Figures 3c and 4). At 150  $\mu\text{mol/L}$  Pb, the *Nep cai hoa vang* rice root dry weight decreased to 82% relative to the control, while the weight of the *Asia* cultivar roots was limited to 63%. At 300  $\mu\text{mol/L}$  Pb, the roots of the *Nep cai hoa vang* variety showed a root dry weight of 69% relative to the control condition, whereas the *Asia* cultivar achieved a weight of only 14%. These results are in good agreement with previous studies suggesting that high Pb concentrations (>300 mg/kg ~ >5000  $\mu\text{mol/L}$ ) in rice paddy soils lead to changes in plant cell physiological pathways and result in rice yield decline and deterioration of rice quality [58]. The growth of the local rice cultivar at highest Pb concentration strongly suggests a higher resistance of the local rice plants to

Pb, which can be explained by its adaptation to trace element contaminated rice paddies in this area, as explained earlier for Cd [8].



**Figure 3.** Root dry weight for the 3 experimental conditions, namely: Asia control and Nep cai Hoa vang native rice plant varieties exposed to increasing, (a) Cd, (b) Cu and (c) Pb, concentrations. A 0  $\mu\text{mol/L}$  metal concentration represents the control growth condition. Control root dry weights represent 100% growth. Error bars = standard deviation. For each variety, there are no significant differences in rice plant growth between conditions when the same letter is shown. For each condition, significance between variety was defined and represented as follow: \*\*\*:  $p < 0.001$ , \*\*:  $p < 0.01$ , NS = non-significant. The results of one-way ANOVA analyses were followed by post-hoc multiple comparison, Tukey HSD test. No results were obtained for plants growing on the 50  $\mu\text{mol/L}$  Cu.



**Figure 4.** Photograph illustrating the effects of increasing Pb concentrations (from 0 to 500 µmol/L in growth medium) on the growth of the Asia control rice plant variety. The roots biomass decreases with increasing metal concentration, however, an increase in root length is observed at the lowest Pb concentration of 50 µmol/L.

The combination of sequential extraction and rice growth experiments shows that mine waste rocks are the source of trace metal bioavailability in Cam Pha rice paddies. Although it has been shown that at low concentrations rice plant growth may be enhanced by these elements, further studies are needed to assess in detail the physiological mechanisms in rice affected by the presence of excess trace metals. Unlike some other higher plants, i.e., maize [59] rice is not considered to be an efficient metal

accumulator, however, the difference in growth observed between rice varieties can cause an artificial selection of plants adapted to growing in soils with high trace metal concentrations. This mechanism would lead to the accumulation of these elements, in edible plant parts (e.g., rice grain endosperm) leading to chronic illnesses in humans, as indicated earlier by Martinez et al. [8], for the Vietnamese rice variety in this study. Exposure of rice plants to trace elements, such as those derived from mine waste rocks in Cam Pha, significantly reduces the root length and shoot length of rice seedlings, in the presence of the highest metal concentrations. The uptake of toxic metal elements by the rice roots may be in part impeded by the presence of root iron plaques, composed mainly of Fe (III) (oxy)hydroxides which heterogeneously cover the root surface. However, further detailed studies are needed to determine the mechanisms of iron plaque formation and quantifying their role in mitigating trace metal uptake by rice roots.

#### 4. Conclusions

Sequential extraction of mine waste tailings from the Cam Pha area show a heterogeneous distribution of trace elements bound to distinct mineral composites (e.g., Pb to Fe-Mn oxides) and organic matter (e.g., Cu). Chemical weathering of waste rocks, especially under tropical climate conditions, leads to an enhanced release of potentially toxic trace elements, which become mobile in agricultural soils and become bioavailable to essential food crops, such as rice. Particles derived from mine waste rocks through weathering are composed of a number of different primary and secondary minerals and organic matter which have different sorption capacities for trace metals. These processes contribute to the accumulation of high levels of Cd, Cu, and Pb within the upper layers of rice paddy soil in excess of the established concentration maxima for healthy agricultural soils as shown by a previous study done on this area. Exposure of control *Asia* and local *Nep cai Hoa vang* rice varieties to increasing concentrations of trace metals (e.g., Cd, Cu and Pb) caused a significant decrease in growth for the *Asia* variety as compared to the local vietnamese rice. This divergence in growth response points to a possible local adaptation of local rice plants to contaminated Cam Pha paddy soils. In the third world, agricultural development in tropical regions with contaminated soils would not be able to discard toxic metal resistant rice varieties to ensure a required rice production, however this implies a danger for human consumption of this major staple crop in these areas.

**Author Contributions:** J.E.M. and R.E.M. conceived and designed the experiments; J.E.M. performed the experiments; J.E.M., O.P., S.W., M.-P.F., T.B.H.H. and R.E.M. analyzed the data and wrote the paper.

**Funding:** This research received no external funding.

**Acknowledgments:** R.E.M. would like to acknowledge funding for this research project provided by the Institute for Earth and Environmental Science and the Faculty of Environment and Natural Resources, at the University of Freiburg. The authors would also like to thank Giampiero Valé of the Consiglio per la Ricerca e la Sperimentazione in Agricoltura—Unità di Ricerca per la Riscicoltura in Vercelli, Italy, for kindly providing model *Oryza sativa* L. whole rice grain varieties. Sigrid Hirth-Walther, of the Institute of Earth and Environmental Sciences, for analysis of soil and plant biomass samples with for AAS and IC. This work was also made possible through a M. Sc. DAAD/CONACYT Scholarship to J.E.M.

**Conflicts of Interest:** The authors declare no conflict of interest.

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