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A Field Evidence of Cd, Zn and Cu Accumulation in Soil and Rice Grains after Long-Term (27 Years) Application of Swine and Green Manures in a Paddy Soil

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Abstract: Although inorganic and organic manures with high concentrations of heavy metals can lead to accumulation or contamination of heavy metals in soils, there are few reports on the effects of long-term application of swine and green manures on the accumulation of heavy metals in rice grains in paddy soils. A long-term field experiment, which was established in 1990 in paddy soil in Hangzhou, China, was used to investigate the effects of inorganic and organic manures on the availability and accumulation of heavy metals in soil and uptake by rice plant. The results showed that long-term application of nitrogen, phosphorus and potash (NPK) plus green manure or swine manure, and swine manure only increased 202%, 146%, and 100% for total Cd, and 5.5%, 7.6%, and 6.6% for total Cu in rice grains, respectively compared to the control without fertilization. Total Zn in rice grain was significantly increased by 13.9% for the treatment of NPK plus green manure. The accumulation of Cd, Zn, and Cu in rice grains after long-term application of swine and green manures is due to the combined effects of the increased concentrations of total and EDTA extractable Cd, Zn, and Cu in soil and the changes of soil properties. Furthermore, the highest bioconcentration factor for Cd was found in the treatment of NPK plus green manure while for Zn and Cu it was observed in NPK treatment. Thus, it may be concluded that green manure and manure with increased Cd, Zn, and Cu in rice grain results in a potential risk of metal accumulation in paddy soils.

Keywords: long-term fertilizers; heavy metals; paddy soil; rice grain

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1. Introduction

Cadmium (Cd) is a toxic heavy metal. Although copper (Cu) and zinc (Zn) are biological elements, excessive amounts of Cu and Zn can also be detrimental to crop growth and can be added to the environment through atmospheric deposition, agro-chemicals and inorganic fertilizers, animal manure, and sewage sludge, etc. [1,2]. Fertilization is a traditional method to improve soil fertility and increase crop yield, but many studies have reported that fertilization causes heavy metal accumulation in soil. Some fertilizers containing a certain amount of heavy metals, such as Cd, were found in phosphate fertilizers [3,4]. While phosphate rocks also contain many other components, such as uranium, and the accumulation of uranium in fertilizers needs to be investigated [5,6]. However, due to the higher accumulation of Cd, Zn, and Cu in food crops, our analysis was focused primarily on these elements. The application of N fertilizers has been shown to increase Cd concentrations in durum wheat by 50% [7]. Luo et al. [8] reported that ~63% of Cd input to agricultural land occurs through mineral and organic fertilizers. The application of green manure can also increase heavy metal solubility since it contains soluble compounds that form complexes with metals [9,10]. In two long-term experimental sites that had received mineral fertilizers and swine manure for 17 or 16 years on black and red soils in northeast and south China, respectively, the plots treated with swine manure contained a higher amount of Cd compared with control plots [2].

Long-term experiments not only provide information about changes in available Cd over decades but also on the fertility, soil quality, and productivity effects of fertilizers [11–13], which are useful for predictions of future metal accumulation and soil environment interactions. In a 22-year fertilization study, swine manure significantly enhanced Cd (average 1.1 mg kg^{-1}) and Cu (average 81.5 mg kg^{-1}) accumulation in soil, which was higher than the national soil standards (0.3 and 50 mg kg^{-1}, respectively) [14]. Some researchers reported that long-term usage of chemical fertilizers contributes to substantially regional soil acidification which results in increasing heavy metal mobility and availability in soils [15,16]. Moreover, agrichemicals have been extensively used in paddy soils to improve rice production and quality in the past three decades. For example, the concentrations of Cd, Cu, Pb, and Zn in fertilizers are in the ranges of 0.0005−0.5, 0.41−11.6, 0.0008−0.93, and 4.87−348.2 mg kg^{-1}, respectively [17]. Hence the application of agrochemicals for long-term purposes may result in the accumulation of heavy metals in the paddy soils. Mainly, the mean concentration of Cd was greater than twice the background value [18]. In China, many long-term experiments have also been conducted [19,20]. In these experiments, most of the treatments contained mineral fertilizers as well as organic manure but their heavy metal concentrations have not been studied in detail [2,14]. Therefore, more attention should be given to long-term fertilization in order to control and monitor the accumulation, availability, and uptake of heavy metals by rice. It was hypothesized that the application of organic and green manure may decrease heavy metal concentrations in paddy fields, however, their effect on crop growth and accumulation may vary depending on the type and amount of applied fertilizers. Based on the long-term rice localization test of different inorganic and organic fertilizer treatments for 27 years, the changes of heavy metals in soil were investigated. The aims of the present work were to (1) determine the effects of long-term chemical fertilization and manure on the accumulation of Cd, Zn, and Cu in soil and rice grain in paddy soils in Hangzhou, Zhejiang province, China and (2) find out the effect of long-term fertilization on the available contents of heavy metals in soil and their relation with the concentration of heavy metals in rice grain.

2. Materials and Methods

2.1. Site Description

The long-term experiment was initially started in 1990 with a plot area of 300 m^2. It is located in Hangzhou city of Zhejiang province of southeast China (120°24′22″ E, 30°26′07″ N), at an altitude of 3~4 m. The mean annual temperature is 16−17 °C (the highest temperature in historical records is 40.2 °C and the lowest temperature is −7.9 °C). The accumulated temperature at or above 10 °C is 4800−5200 °C and the annual precipitation is 1500~1600 mm, the annual evaporation is 1000−1100 mm, the frost-free period is 240−250 d, the annual sunshine time is 1900–2000 h and the annual solar radiation is 100−115 J cm^{-2}. The soil belongs to paddy soil (Haplaquept world soil map, Inceptisol-anthraquic soil-USDA). The soil parent material is shallow marine sediments in lake and marine facies and the terrain belongs to the alluvial plain. The drainage condition is good and the plow layer is silty clay loam.

2.2. Experimental Design and Fertilization Treatments

The experimental treatments were controlled without fertilizers (CK), inorganic fertilizers (NPK: N as urea 375 kg hm^{-2}, P_2O_5 as superphosphate 187.5 kg hm^{-2}, K_2O as potassium chloride 187.5 kg hm^{-2}), swine manure (M: 22,500 kg hm^{-2}), inorganic fertilizers with green manure (NPK(GM): NPK plus green manure return), and inorganic fertilizers with swine manure (NPKM, NPK with swine manure 22,500 kg hm^{-2}). All the treatments were replicated three times. The crop rotations for CK, NPK, M, and NPKM were barley-rice-rice-barley—rice-rice per two years before 2000, changed to barley-rice-barley—rice per two years, then changed to rape-rice-rape-rice per two years in 2011. NPK(GM) was barley-rice-rice—Chinese milk vetch (*Astragalus sinicus* L.)—rice-rice per two years before 2000, changed to barley-rice- Chinese milk vetch (*Astragalus sinicus* L.)—rice per two years before 2010, then changed to rape-rice—Chinese milk vetch—rice per two years The Chinese milk vetch as green manure was all returned after harvest. The swine manure contained N 0.5%, P 0.6%, K 0.7% and was used in each season on average in one year. Before 2000, the rates of N, P, and K are 75, 37.5, and 37.5 kg hm^{-2} during the barley period and are 150, 75, and 75 kg hm^{-2} during the rice period, respectively. then are 120, 60, and 60 kg hm^{-2} during the barley period and are 255,127.5 and 127.5 kg hm^{-2} during the rice period, respectively, after 2011 changed to 135, 67.5, and 67.5 kg hm^{-2} during the rape period and are 240, 120, and 120 kg hm^{-2} during the rice period, respectively.

2.3. Sample Collection

Topsoil samples (0−20 cm) from the paddy soil were collected in November 2017. Samples from all five treatments from each replicate plot were stored separately. The soil samples were air-dried and stored at room temperature. The rice plants were harvested after reaching the full maturity stage. The plants were threshed and grains samples were collected, then the husks were removed from grains with a thresher. Finally, the samples were crushed into a fine powder with a grinder and the samples were ready for heavy metal determination.

2.4. Analytical Procedures and Measurement of Heavy Metal Dynamics in Paddy Field

Soil samples were air-dried at room temperature, the visible non-soil objects such as stones and roots, stems, leaves of the plant body were removed from the soil samples, and then all of the soil samples were ground in an agate mortar and passed through a 0.15 mm nylon mesh for analysis of total heavy metals.

Soil pH was measured by pH meter in a soil water proportion of 1:2.5 [21]. Organic matter concentration was analyzed by the potassium dichromate wet combustion method [22]. Total N was estimated by titration of distillations after Kjeldahl test readiness and examination [23]. Total P in soil was measured by the perchloric corrosive assimilation strategy technique [24]. Available P was analyzed by molybdenum blue colorimetry [25]. While available K was estimated by utilizing atomic absorption spectrophotometry. The content of soil organic matter was measured by the method of [26], with the following formula:

$$\text{Organic carbon\%} = \frac{(\text{mLblank} - \text{mLsample})\left(\text{MFe}^{2+}\right)(0.003)(100)}{\text{wt.water} - \text{free soil (g)}} \times \text{f} \quad (1)$$

where, mLsample is the sample titration value, mLblank is the titration value of heated (boiled) blank sample, 0.003 is the correction factor. Then by using Equation (1) we calculated organic matter content as:

$$\text{Organic matter (g kg}^{-1}) = 0.35 + 1.80 \times \text{\%organic C}$$

where, 1.80 is the appropriate factor for organic soils.

Cation exchange capacity (CEC) was determined by extraction with 1 M NH_4OAc [27] with the following formula:

$$\text{CEC cmol kg}^{-1} = \text{meq L}^{-1}\ \text{Na (from calibration curve)} \times \frac{\text{A}}{\text{Wt}} \times \frac{100}{1000}$$

where, A is the total volume of the extract (mL), and wt. is the weight of the air-dry soil (g).

The soil total Cd, Zn, and Cu were extracted from the soil samples using a combined approach of wet acid digestion in a sealed microwave digestion system (9 mL HNO_3 and 3 mL HF), and the acidic digestion solutions were transferred to a fume hood prior to dilution with 50 mL ultra-pure water containing 5% HNO_3. The total Cd, Zn, and Cu concentrations in the digestion solutions were determined via ICP-MS. For the analysis of plant samples, the plant samples were rinsed with tap water followed by deionized water, and then the rice grain with the shell removed was dried at 100 °C before cooling in a desiccator. The heating process was repeated until the weight remained constant. The plant samples were then digested with 6 mL HNO_3 and 3 mL H_2O_2 in a high pressure, sealed micro-wave digestion oven in accordance with the determination of heavy metals in foods. After the digestion procedure, samples were diluted with ultra-pure water containing 5% HNO_3 to a final volume of 50 mL. Then the heavy metal concentrations in washed digest solutions were measured via inductively coupled plasma mass spectrometry (ICP-MS) [28].

Samples were replicated thrice and reagent blanks were used to ensure quality control for the metal analyses. Standard reference material for soil (GBW07605) and plant (GBW08502) was obtained from the National Research Center for Standard of China. The accuracy of digestion procedure and analytical method were tested with certified reference material and percentage recoveries of heavy metals from soil and plant samples were 96.5–103.8% and 97–115% respectively.

2.5. Statistical and Data Analysis

Data were analyzed by one-way analysis of variance using XLSTAT software (XLSTAT 2013). Differences in the mean concentration of heavy metals were compared using Duncan's multiple range test at a 5% level of significance. The data was checked by binomial distribution before performing a one-way ANOVA analysis. The formula used for binomial distribution was as:

$$\text{Binomial distribution} = \text{BINOM.DIST (n, x, STDEV.S, FALSE)}$$

where, n is the number (data), x is the mean values, STDEV.S is the standard deviation and the no cumulative was FALSE.

The bioconcentration factor (BCF) of the heavy metals in rice was calculated using the following formula [29]:

$$BCF = \frac{M_{grain}}{M_{soil}}$$

where M_{grain} is the total concentration of heavy metals in rice grains and M_{soil} is the total concentration of heavy metals in soil.

3. Results

3.1. Soil Properties and Crop Yield Parameters

The soil properties showed a significant difference between long-term fertilization treatments. Compared with CK, soil pH, soil organic matter (SOM) and cation exchange capacity (CEC) were increased NPK(GM), M, and NPKM treatments compared with CK, while they were decreased by NPK application. Compared to CK, soil total N, P, and K were increased by NPK, NPK(GM), M, and NPKM treatments. Also, soil available N, P, and K were increased by NPK, NPK(GM), M, and NPKM treatments (Table 1).

Grain yield and straw yield were higher in order in NPK, NPK(GM), M, and NPKM treatments as compared to CK. The highest grain and straw yield, 8893 kg ha^{-1} and 16,950 kg ha^{-1} were observed in NPK(GM) and NPKM treatments, respectively (Table 2).

Table 1. Effect of long-term application of inorganic fertilizers, swine manure, and green manure on soil physcio-chemical properties. Soil samples from each plot were collected after harvesting of crop and then the following parameters were measured. SOM was soil organic matter; CEC was cation exchange capacity; TN, TP, TK were total nitrogen, phosphorus, and potassium; AN, AP, and AK were available nitrogen, phosphorus, and potassium. Mean values followed by different letters are significantly different using Duncan's multiple range test at 5% level.

Treatments	pH	SOM (g kg^{-1})	CEC (cmol kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	TK (g kg^{-1})	AN (mg kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})
CK	6.42 c	23.4 e	14.8 a	1.16 e	0.94 e	10.8 e	111 e	47.0 e	46 e
NPK	6.09 e	22.9 d	12.5 d	1.34 d	1.61 d	11.9 a	117 d	87.6 d	59 c
NPK(GM)	6.32 d	30.4 c	12.9 c	1.77 c	1.91 c	11.5 c	128 c	99.9 c	58 d
M	6.70 a	32.6 b	14.8 a	1.91 b	2.06 b	10.9 d	174 b	136 b	110 b
NPKM	6.63 b	33.1 a	14.7 b	2.00 a	2.16 a	11.7 b	179 a	157 a	123 a

Table 2. Effect of long-term application of inorganic fertilizers, swine manure, and green manure on grain yield, straw yield, and percentage (%) of NPK in grain and straw as affected by long-term application of fertilizers and pig manure. Rice plants from each plot were harvested and measured the following parameters: GN, GP, and GK were nitrogen, phosphorus, and potassium in grain (g kg^{-1}); SN, SP, and SK were nitrogen, phosphorus, and potassium in straw (g kg^{-1}). Mean values followed by different letters are significantly different using Duncan's multiple range test at 5% level.

Treatments	Grain Yield (kg ha^{-1})	GN (g kg^{-1})	GP (g kg^{-1})	GK (g kg^{-1})	Straw Yield (kg ha^{-1})	SN (g kg^{-1})	SP (g kg^{-1})	SK (g kg^{-1})
CK	5665 e	11.5 c	3.37 b	1.90 c	10,335 e	6.24 e	1.44 c	19.4 b
NPK	5763 d	12.5 a	3.36 b	2.50 b	14,850 c	8.02 a	1.65 b	19.3 b
NPK(GM)	8893 a	12.3 ab	3.50 a	2.85 a	15,082 b	7.45 b	2.04 a	18.7 c
M	8353 b	11.5 c	2.72 c	1.85 d	11,273 d	6.32 d	1.41 c	20.7 a
NPKM	8075 c	12.3 ab	3.54 a	2.45 b	16,950 a	6.72 c	1.64 b	20.8 a

3.2. Total Heavy Metals Accumulation in Soil

Soil total Cd content was found to be significantly different with chemical fertilizer, manure, and green manure treatments. Compared with CK (0.2 mg kg^{-1}), total Cd was increased in NPK, NPK(GM), M, and NPKM to 0.33, 0.75, and 0.85, and 0.85 mg kg^{-1} treatments, respectively, which was increased by 63.2%, 266%, 315%, and 317% in NPK, NPK(GM), M, and NPKM treatments, respectively (Figure 1a). The total Zn content of soil was higher in NPK(GM) (134.9 mg kg^{-1}), M (143.3 mg kg^{-1}), and NPKM (160.4 mg kg^{-1}) treatments than that of CK (91.5 mg kg^{-1}), it seems that higher soil total Zn was related to higher organic manure and green manure return (Figure 1b). The total Zn in soil was increased by 47.4%, 56.6%, and 75.3% under NPK(GM), M, and NPKM treatments, respectively. While Zn content in the soil treated with long-term application of NPK fertilizer showed a non-significant difference compared to CK. As compared to CK (29.1 mg kg^{-1}), NPK(GM), M, and NPKM treated plots significantly enhanced total Cu in soil to 45.1, 49.4, and 54.3 mg kg^{-1}, respectively. The amount of soil total Cu in NPK(GM), M, and NPKM treatments were increased by 54.9%, 68.5%, and 86.5% than CK, respectively. Whereas in NPK treatment the total Cu was significantly decreased by 10.1% than CK (Figure 1c), probably due to plant uptake and leaching.

3.3. EDTA Extractable Metals in Soil

The long-term application of green manure and organic manure treatments markedly affected the EDTA extractable heavy metal contents in soil (Figure 2). All the treatments NPK, NPK(GM), M, and NPKM significantly enhanced EDTA extractable Cd from 0.02 mg kg^{-1} (CK) to 0.04, 0.12, 0.16, and 0.15 mg kg^{-1}, respectively. Compared to CK, EDTA extractable Cd was significantly increased by 77.4%, 517%, 693, and 664% in NPK, NPK(GM), M, and NPKM treatments, respectively (Figure 2a). The EDTA extractable Zn content in soil increased through long-term application of NPK(GM), M, and NPKM (3.1, 5.9, and 6.5 mg kg^{-1}, respectively) as compared to CK (2.3 mg kg^{-1}). Compared to CK, it significantly enhanced by 33.7%, 157%, and 182% under NPK(GM), M, and NPKM treatments, respectively. While EDTA extractable Zn in soil was reduced to 1.3 mg kg^{-1} than CK. EDTA Zn was significantly reduced in NPK treatment by 45.4% NPK treatment than CK (Figure 2b). Whereas NPK(GM), M, and NPKM treated plots showed an increase of 7.2, 9.23, and 9.33 mg kg^{-1}, respectively, compared to CK (4.21 mg kg^{-1}). Soil EDTA extractable Cu was non-significantly decreased by 5.1% under NPK treatment after long-term fertilization (Figure 2c). While EDTA-Cu was significantly increased by 70.9%, 119%, and 121% in NPK(GM), M, and NPKM treatments than CK, respectively.

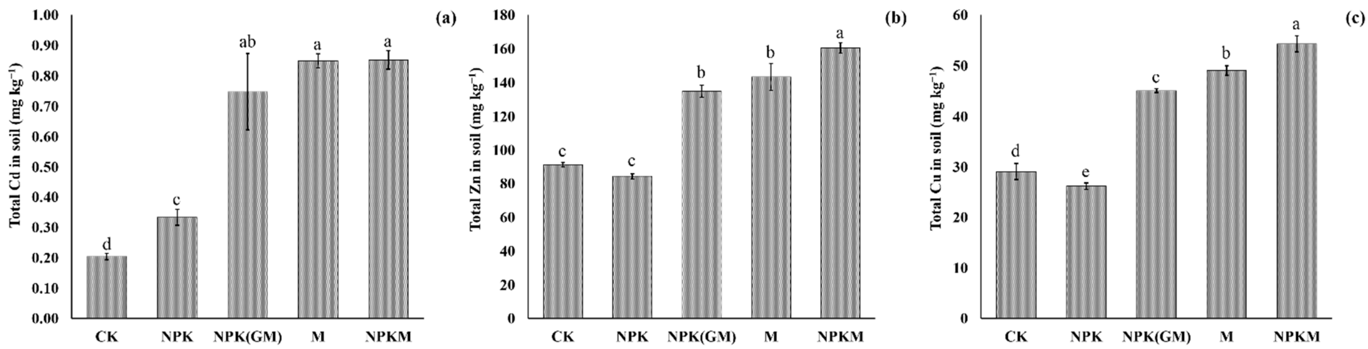

Figure 1. Concentration of total soil (**a**) Cd, (**b**) Zn, and (**c**) Cu extracted by acid digestion in topsoil under different long-term fertilizer treatments for paddy soil. Total treatments were five: control (CK), M (pig manure), NPK (N, P, and K fertilizer), NPK(GM) (N, P, and K fertilizer + green manure), and NPKM (NPK fertilizer + pig manure). The data presented are mean ± standard deviation (n = 3). Mean values followed by different letters are significantly different using Duncan's multiple range test at 5% level.

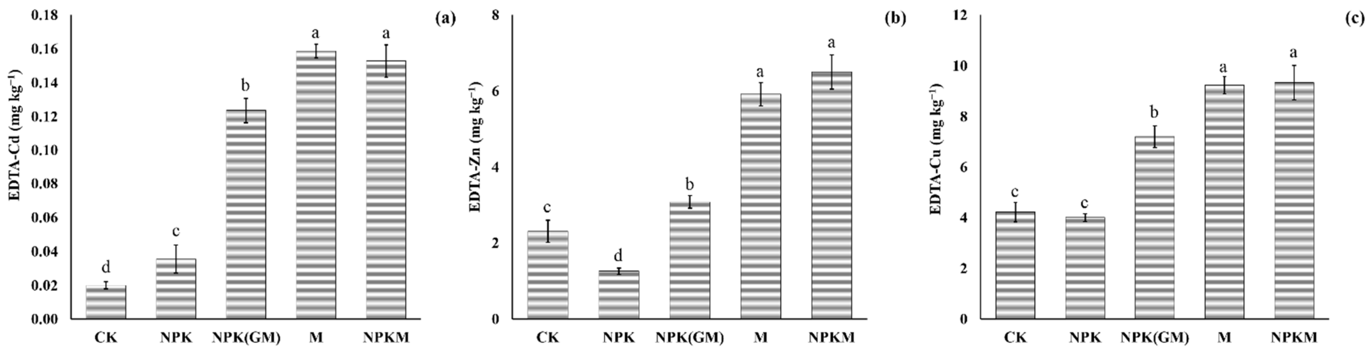

Figure 2. EDTA extractable (**a**) Cd, (**b**) Zn, and (**c**) Cu in topsoil under different long-term fertilizer treatments for paddy soil. The data presented are mean ± standard deviation (n = 3). Mean values followed by different letters are significantly different using Duncan's multiple range test at 5% level.

3.4. Heavy Metals Accumulation in Rice Grain

Long-term fertilization showed a significant effect on grain metal contents. Compared to CK (0.13 mg kg^{-1}), NPK(GM)-, M-, and NPKM-treated plots presented a remarkable increase in Cd in rice grains to 0.40, 0.26, and 0.32 mg kg^{-1}, respectively. The total Cd in rice grain was significantly increased by 202%, 100%, and 146% under NPK(GM), M, and NPKM treatments, respectively (Figure 3a). The total Cd in rice grain was 0.15 mg kg^{-1} under NPK treatment which was non-significantly different ($p < 0.05$) compared to CK. The Zn in rice grain was 8.34 mg kg^{-1} compared to CK (7.32 mg kg^{-1}). Compared with CK, total Zn in rice grain was significantly higher under NPK(GM), which increased by 13.9%. The treatments NPK, M, and NPKM were non-significantly different ($p < 0.05$) than CK (Figure 3b). The results showed that green manure could promote the absorption of Zn in rice, but there was no difference between chemical fertilizer and organic fertilizer with CK. As compared to CK (1.74 mg kg^{-1}), there were increases in total Cd in rice grain under NPK(GM), M, and NPKM treatments as 1.84, 1.86, and 1.88 mg kg^{-1}, respectively. The total grain Cu was significantly higher in NPK(GM), M, and NPKM treatments than CK, which increased by 5.5%, 6.6%, and 7.6%, respectively. While total Cu in rice grain was significantly decreased by 0.8% under NPK treatment (Figure 3c).

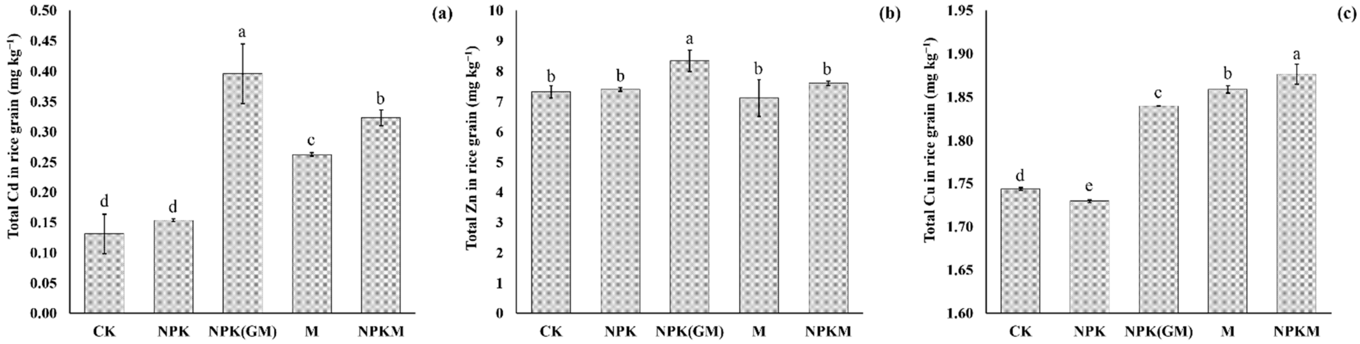

Figure 3. Concentration of total (**a**) Cd, (**b**) Zn, and (**c**) Cu in rice grain extracted by acid digestion under different fertilizer treatments for paddy soil. The data presented are mean ± standard deviation (n = 3). Mean values followed by different letters are significantly different using Duncan's multiple range test at 5% level.

3.5. Heavy Metal Transfer from Soil to Rice Grain

The BCFs of different heavy metals after the long-term application of chemical fertilizers and manure are listed in Table 3. The BCFs for heavy metals were ranked as Cd > Zn = Cu. This shows that the accumulation capacity of Cd was higher in rice grain. The highest BCF values were found in CK, NPK(GM), and NPK treatments for Cd. While BCF for Zn and Cu were found in NPK treatment. Compared to CK, the application of NPK(GM) treatments significantly increased the BCF of Cd in rice by 0.17%, while it significantly decreased by 454% and 343% under M and NPKM treatments, respectively. There was a non-significant difference found between CK and NPK treatments. Relative to CK, the BCF for Zn significantly increased by 10% in NPK. Reductions in the BCF for Zn by 21.2%, 36.2%, and 41.2% were induced by the application of NPK(GM), M, and NPKM treatments, respectively. Compared to CK, the BCF for Cu decreased by 31.6%, 36.6%, and 41.6% under NPK(GM), M, and NPKM treatments, respectively, while it increased by 10% in NPK treatment (Figure 4).

Table 3. Pearson correlation between soil metals content in rice grain and metals content in soil as well as soil pH and SOM.

	pH	SOM	SCd	ECd	GCd	SZn	EZn	GZn	SCu	ECu	GCu
pH	1										
SOM	0.773	1									
SCd	0.651	0.980 **	1								
ECd	0.718	0.985 **	0.994 **	1							
GCd	0.317	0.803	0.837	0.777	1						
SZn	0.768	0.988 **	0.955 *	0.955 *	0.807	1					
EZn	0.918 *	0.914 *	0.845	0.886 *	0.516	0.921 *	1				
GZn	−0.303	0.212	0.269	0.165	0.747	0.248	−0.14	1			
SCu	0.782	0.992 **	0.959 *	0.961 **	0.8	0.999 **	0.927 *	0.23	1		
ECu	0.82	0.992 **	0.966 **	0.983 **	0.722	0.978 **	0.952 *	0.088	0.984 **	1	
GCu	0.759	0.997 **	0.974 **	0.974 **	0.825	0.996 **	0.908 *	0.256	0.997 **	0.984 **	1

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

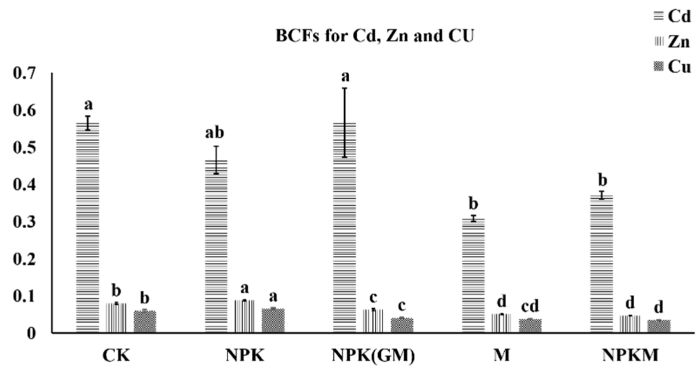

Figure 4. Bioconcentration factor of heavy metals in rice grain with long-term application of fertilizers and pig manure (mean ± S.D., n = 3). Different letters in vertical columns represent significant differences ($p < 0.05$) between the fertilizer and manure treatments.

4. Discussion

4.1. Availability and Accumulation of Cd, Zn, and Cu in Soil

There was a significant accumulation of soil total Cd, Zn, and Cu with all fertilizers treatments after 27 years of long-term experiment (Figure 1). The content of organic matter, total and available N, P, K increased with the application of chemical fertilizers and manures in the soil (Table 1). This is consistent with the fact that pig manure contains essential nutrients and organic carbon [30]. Also, the application of chemical fertilizers and manures markedly enhanced the grain and straw yield of rice plants (Table 2). The results are consistent with the fact that the supply of chicken and swine manure increased the rice grain yields more than the control [31]. However, the application of NPK, NPK(GM), M, and NPKM markedly increased total Cd in soil. While total Zn and Cu also increased by all the treatments except NPK treatment (Figure 1a,b). It was reported that long-term application of chemical fertilizers and manure after 20 years increased the accumulation of Cd in soil [32]. In another study, it was shown that 32 years of long-term application of chemical fertilizer and organic matter markedly enhanced total and available Cd in soil [33]. Zhou et al. [14] reported that long-term 22 years of fertilization, especially with swine manure, resulted in increased Cd accumulation (average 1.1 mg kg^{-1}) in soil. This is due to the presence of Cd-containing feed additives in swine manure [2]. A wide range of heavy metal concentrations has been found in the swine manure depending upon the age of the pigs and the quantity of metal supplements added to their diet [34]. The soil total Zn and Cu showed the same trends in the treatments; for instance, greater soil totals of Zn and Cu were observed in NPK(GM), M, and NPKM treatments. It was confirmed that the manure fertilization significantly increased the relative activity of Zn and Cu because manure could promote soil pH and SOM thus resulting in the activation of Zn and Cu by complexation, or Zn and Cu might exist in the available form (0.1 M HCl extractable) in fresh swine manure [14,35]. However, there are few reports on the long-term application of manure on the concentration of metals in rice grains, except for [36], who found that the average Cd concentrations in rice grown in green manure and swine manure plots were higher than rice grown in chemical fertilizers plot ($p < 0.05$) and significantly exceeded 0.2 mg Cd DW kg^{-1} grain, the maximum permissibility according to the Hygienic Standards for grains (GB-2712-2005) of China. The root system of green manure is widely distributed in the soil, and the huge root system of green manure plants extends into the lower soil to absorb heavy metals in a wide range [36].

The principal component analysis (PCA) showed that pH presented a positive but weak relationship with GCd and GZn (Figure 5). The Pearson correlation coefficients between heavy metals contents in the rice grain and the soil pH, SOM, soil total metals, EDTA extractable metals, and grain total metals are shown in Table 3. It was further confirmed from a study that the increase in soil pH resulted in more consumption of protons and enhanced desorption of metals, subsequently increased release of metal contents in soil [37]. Furthermore, dissolved Cd, Pb, As, and Cr were not significantly influenced by soil pH in slightly alkaline soil [38].

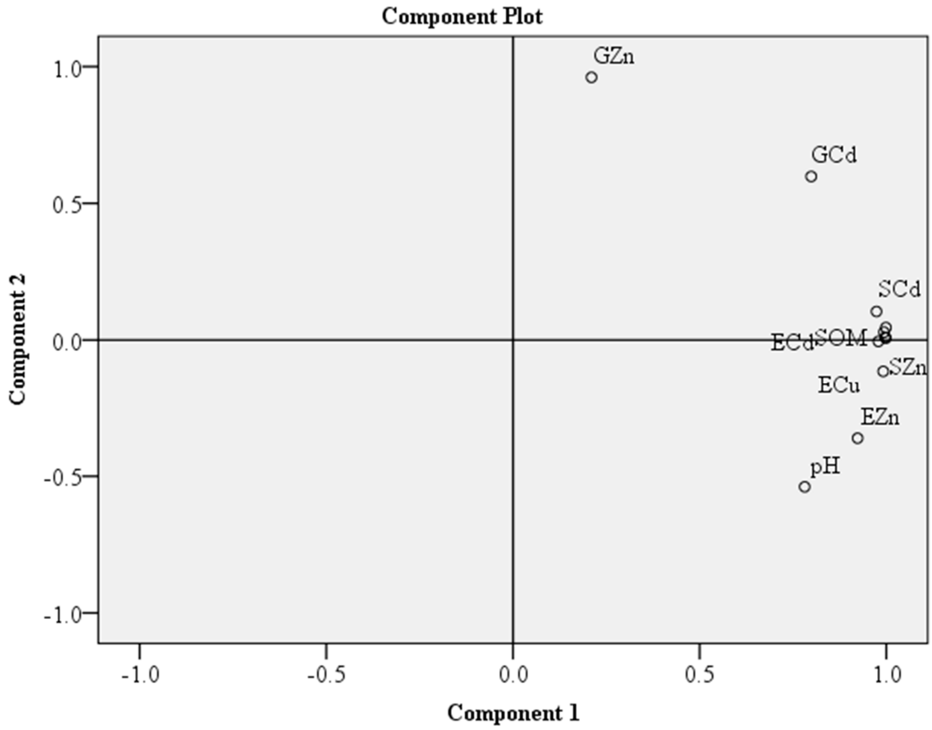

Figure 5. Biplot for the first two principle components (PC1 and PC2 accounting for 81.91% and 15.71% of total variance, respectively) for 11 variables. pH = soil pH; SOM = soil organic matter; SCd = total Cd content in soil; ECd = EDTA extractable Cd; GCd = total Cd content in rice grain; SZn = total Zn content in soil; EZn = EDTA extractable Zn; GZn = total Zn content in rice grain; SCu = total Cu content in soil; ECu = EDTA extractable Cu; GCu = total Cu content in rice grain.

Generally, the available form of Cd rather than the total Cd determines the mobility and toxicity of Cd [39]. Cd in soil solution extracted by using EDTA extractants is considered the bioavailable form of Cd in soil [40,41]. EDTA extractable heavy metals in soil were differentially affected by the application of chemical fertilizers, green manure, and pig manure. The EDTA extractable and total Cd, Zn, and Cu contents were significantly increased by the combined application of chemical fertilizers with green manure or swine manure (Figure 2). Generally, the available form of Cd rather than the total Cd determines the mobility and toxicity of Cd [39]. Soil Cd extracted using EDTA extractants is considered as bioavailable forms [40,41]. Application of swine manure or chemical fertilizer combined with swine manure resulted in significant accumulation of total and EDTA extractable Cd in soil, but only chemical fertilizer treatment had little effect on the accumulation of Cd in soil (Figure 2a). Pig manure from extensive pig farming contains a remarkable amount of total and bioavailable metals because of their application as feed additives to commercial pig diets [42]. On the other hand, Huang et al. [43] stated that the annual soil Cd accumulation rates were 0.0007−0.032 mg kg^{-1} and 0.005−0.022 mg kg^{-1} after four years of phosphate fertilization and swine manure application, respectively. In addition, Qaswar et al. [44] reported that long-term application of high swine manure combined with chemical fertilizers increased the soil available and total Cd content. The increase in EDTA extractable metals in soil might be due to this phenomenon in the manure application treatments. Meanwhile, in the present study, the EDTA extractable Zn and Cu increased under NPK(GM), M, and NPKM treatments compared to CK (Figure 2b,c). While EDTA Zn and Cu decreased in NPK treatment. It was confirmed that the application of farmyard manure alone or in combination with chemical fertilizers increased DTPA Zn more than the control after 41 years of fertilization [45]. While a decrease in DTPA Zn in chemical fertilizer treatments was found compared to the fallow plot [20]. Other studies also reported that the application of N alone or in combination with K decreased available Zn [46]. This might be due to DTPA Zn being taken up and removed by crops [20,46]. An increase in EDTA Cu was also reported when using a combination of NPK and manure [20]. It was suggested that it might be due to high Cu contents in the organic fertilizer applied [20]. In addition, manure promoted SOM, Olsen P, and even soil pH, and thus increased the activation of Cu and Zn by complexation [35,47] or Cu and Zn mainly existed in the available form (0.1MHCl-extractability) in fresh pig manure.

The green manure crops used in this study were the Chinese milk vetch. Chinese milk vetch is a legume crop used as green manure in rice fields in Southern China [48]. The mechanism behind this is the accumulation of metal^{2+} in the nodules of Chinese milk vetch because of tri-peptide glutathione (GSH) in the nodules [49]. In addition, GSH is the precursor for phytochelatins and contains heavy metal binding peptides that can make bonds with metal^{2+} and are involved in metal sequestrations [50]. Therefore, a high level of Cd, Zn and Cu may be release in soil and consequently, higher uptake by rice plants occurred. In another study, it was noted that the green manure crop (red clover and sunflower) enhanced DTPA Zn in soil. This may be due to the organic carbon and amino acids released from green manure which could make chelates with Zn and other metals [51].

4.2. Accumulation of Cd, Zn, and Cu in Rice Grains

The heavy metal concentrations in rice grains showed greatly that the higher level of Cd concentration in rice grains was found with NPK(GM), M, and NPKM treatments (Figure 3a). Some researchers reported that heavy metal mobility in soils increased by the application of organic materials due to the potential decrease in soil pH and increase in DOM, which increased metal-DOM soluble complexes [52,53]. Due to the presence of functional groups or ligands, organic constituents in soil can form chelates with metals. The alcoholic, phenolic, carbonyl, and carboxyl functional groups dissociate by increasing soil pH, as a result, ligand ion affinity towards the metals increases [54,55]. For instance, for heavy metals such as Cd, Pb, and Zn, desorption increased and dissolution in soil solution occurred from soil constituents with decreased soil pH [56]. Thus organic matter addition in soils caused either positive or negative impacts on metal mobility depending on the characteristics of the material and soil properties (soil pH, organic matter, and clay content) [36]. Long-term application of inorganic fertilizers increased the risk of Cd accumulation due to soil acidification with higher relative activity and enrichment coefficient of Cd. In addition to this, long-term application of pig manure also resulted in higher accumulation and availability of Cd in red soils and exceeding national standards (0.3 mg kg^{-1}) for Cd in wheat as well as in rice grains in China [14,57]. Due to the decomposition of OM, Zn forms labile organic mineral complexes, as a result, Zn content in soil increased, and subsequently, its uptake by plants increased [45,58]. The interesting result shows that Cu in rice grain was reduced by the NPK treatment (Figure 3c). It was found that the long-term application of NPK fertilizers could enhance the availability of Cu in soil and the Cu contents in wheat [59]. Moreover, it was reported that the application of inorganic fertilizers (NP, NK, PK) for more than 45 years substantially increased Cd, Cu, Cr, and Ni in the soil and in rice grains [60]. Furthermore, Zhou et al. [14] reported that more metal was removed by wheat and maize crop under manure application especially for NPKM treatment, due to its higher biomass, with about 5, 8, and 10 fold higher uptakes of Zn, Cu, and Cd, respectively than CK. Wan et al. [31] demonstrated that swine manure remarkably increased DTPA-Zn contents in soil and enhanced Zn accumulation in rice grain by 2.6–30.9% [31].

Only a few studies have been conducted on the effects of green manure on heavy metal accumulation and uptake by crops. Interestingly in our study, the highest soil total and EDTA Cd, Zn, and Cu were found in NPK(GM) treated plots rather than CK. The concentrations of Cu and Cd in rice grain in Chinese milk vetch treatment were higher than in any other amendment treatments [61]. It was reported that large amount of dissolved organic matter released from the disintegration of pig manure and green manure may cause Cd mobility and activation, which acts as a carrier of Cd and increased bioavailability and mobility of Cd in soil and then promoted Cd taken up by rice [36]. These may also be a reason for increased uptake of Zn and Cu under chemical fertilizers combine with swine manure and green manure treatments.

The results of BCF exhibited that the bioavailability potential of Cd was higher than Zn and Cu in rice grain. The BCF for Cd showed that the NPK(GM) treatment promotes Cd transformation from soil to grains. Whereas, Zn and Cu were bioavailability were induced by NPK treatment (Figure 4). It was stated that the Cd had greater bioavailability and soil-plant translocation as compared to other heavy metals and it is easily uptake by plants [62]. Some studies showed that organic matter had a limited impact on the bioavailability of Cu [31]. Meanwhile, Qaswar et al. [44] demonstrated that compared to CK, biological accumulation coefficients of Zn and Cu were increased by 17% and 69% in NPK-treated plots, respectively. These results showed that inorganic fertilizer, and combining the application of inorganic and green manure fertilizers, promoted the bioavailability of heavy metals in rice grains. This is because of the higher decrease in soil pH under NPK fertilization than the combined application of manures and chemical fertilizers (Table 1). Furthermore, the variation in bioaccumulation coefficients might induce changes in soil pH due to long-term fertilization [58] because acidic soil promotes bioavailability [63].

5. Conclusions

Long-term application of organic fertilizer, the combination of organic fertilizer and chemical fertilizer, and the combination of green manure and chemical fertilizer can increase soil EDTA Cd, Zn, and Cu. Long-term application of organic manure and combining the application of chemical fertilizers with green manure and organic manure increases the accumulation of heavy metal elements in rice grains. It is found that planting green manure can significantly increase the Cd, Zn, and Cu content in rice, which provides a strong basis for correcting the wrong practice of metal contaminated soil fallow-planting with green manure. Meanwhile, inorganic fertilizer and combining the application of inorganic and green manure fertilizers promote the bioavailability of heavy metals in rice grains. Furthermore, this research would help establish plans for minimizing heavy metal contribution to agricultural land and effectively implement policies to preserve the soil ecosystem from the long-term accumulation of heavy metals.

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References

1. Jalloh, M.A.; Chen, J.; Zhen, F.; Zhang, G. Effect of different N fertilizer forms on antioxidant capacity and grain yield of rice growing under Cd stress. *J. Hazard. Mater.* **2009**, *162*, 1081–1085. [CrossRef] [PubMed]
2. Wu, L.; Tan, C.; Liu, L.; Zhu, P.; Peng, C.; Luo, Y.; Christie, P. Cadmium bioavailability in surface soils receiving long-term applications of inorganic fertilizers and pig manure. *Geoderma* **2012**, *173*, 224–230. [CrossRef]
3. Grant, C.A.; Sheppard, S.C. Fertilizer impacts on cadmium availability in agricultural soils and crops. *Hum. Ecol. Risk Assess. Int. J.* **2008**, *14*, 210–228. [CrossRef]
4. Sebastian, A.; Prasad, M.N.V. Cadmium minimization in rice. A review. *Agron. Sustain. Dev.* **2014**, *34*, 155–173. [CrossRef]
5. Shang, D.; Geissler, B.; Mew, M.; Satalkina, L.; Zenk, L.; Tulsidas, H.; Barker, L.; El-Yahyaoui, A.; Hussein, A.; Taha, M.; et al. Unconventional uranium in China's phosphate rock: Review and outlook. *Renew. Sustain. Energy Rev.* **2021**, *140*, 110740. [CrossRef]
6. Ye, Y.; Al-Khaledi, N.; Barker, L.; Darwish, M.S.; El Naggar, A.M.; El-Yahyaoui, A.; Hussein, A.; Hussein, E.-S.; Shang, D.; Taha, M.; et al. Uranium resources in China's phosphate rocks—Identifying low-hanging fruits. *IOP Conf. Series: Earth Environ. Sci.* **2019**, *227*, 052033. [CrossRef]
7. Mitchell, L.; Grant, C.; Racz, G. Effect of nitrogen application on concentration of cadmium and nutrient ions in soil solution and in durum wheat. *Can. J.Soil Sci.* **2000**, *80*, 107–115. [CrossRef]
8. Luo, L.; Ma, Y.; Zhang, S.; Wei, D.; Zhu, Y.-G. An inventory of trace element inputs to agricultural soils in China. *J. Environ. Manag.* **2009**, *90*, 2524–2530. [CrossRef] [PubMed]
9. Almås, A.R.; Singh, B.; Salbu, B. Mobility of Cadmium-109 and Zinc-65 in Soil Influenced by Equilibration Time, Temperature, and Organic Matter. *J. Environ. Qual.* **1999**, *28*, 1742–1750. [CrossRef]
10. Shuman, L.M. Organic Waste Amendments Effect on Zinc Fractions of Two Soils. *J. Environ. Qual.* **1999**, *28*, 1442–1447. [CrossRef]
11. Mustafa, A.; Minggang, X.; Shah, S.A.A.; Abrar, M.M.; Nan, S.; Baoren, W.; Zejiang, C.; Saeed, Q.; Naveed, M.; Mehmood, K.; et al. Soil aggregation and soil aggregate stability regulate organic carbon and nitrogen storage in a red soil of southern China. *J. Environ. Manag.* **2020**, *270*, 110894. [CrossRef]
12. Abrar, M.M.; Xu, M.; Shah, S.A.A.; Aslam, M.W.; Aziz, T.; Mustafa, A.; Ashraf, M.N.; Zhou, B.; Ma, X. Variations in the profile distribution and protection mechanisms of organic carbon under long-term fertilization in a Chinese Mollisol. *Sci. Total Environ.* **2020**, *723*, 138181. [CrossRef]
13. Richter, D.D.; Hofmockel, M.; Callaham, M.A.; Powlson, D.S.; Smith, P. Long-term soil experiments: Keys to managing earth's rapidly changing ecosystems. *Soil Sci. Soc. Am. J.* **2007**, *71*, 266–279. [CrossRef]
14. Zhou, S.; Liu, J.; Xu, M.; Lv, J.; Sun, N. Accumulation, availability, and uptake of heavy metals in a red soil after 22-year fertilization and cropping. *Environ. Sci. Pollut. Res.* **2015**, *22*, 15154–15163. [CrossRef] [PubMed]
15. Debreczeni, K.; Kismányoky, T. Acidification of soils in long-term field experiments. *Commun. Soil Sci. Plant Anal.* **2005**, *36*, 321–329. [CrossRef]
16. Guo, J.H.; Liu, X.J.; Zhang, Y.; Shen, J.L.; Han, W.X.; Zhang, W.F.; Christie, P.; Goulding, K.W.T.; Vitousek, P.M.; Zhang, F.S. Significant acidification in major chinese croplands. *Science* **2010**, *327*, 1008–1010. [CrossRef]
17. Wang, Q.; Ma, Z. Heavy metals in chemical fertilizer and environmental risks. *Rural Eco-Environ.* **2004**, *20*, 62–64. (In Chinese)
18. Zhao, K.; Liu, X.; Xu, J.; Selim, H. Heavy metal contaminations in a soil–rice system: Identification of spatial dependence in relation to soil properties of paddy fields. *J. Hazard. Mater.* **2010**, *181*, 778–787. [CrossRef]
19. Shah, S.A.A.; Xu, M.; Abrar, M.M.; Mustafa, A.; Fahad, S.; Shah, T.; Yang, X.; Zhou, W.; Zhang, S.; Nan, S.; et al. Long-term fertilization affects functional soil organic carbon protection mechanisms in a profile of Chinese loess plateau soil. *Chemosphere* **2021**, *267*, 128897. [CrossRef] [PubMed]
20. Li, B.-Y.; Huang, S.-M.; Wei, M.-B.; Zhang, H.; Shen, A.-L.; Xu, J.-M.; Ruan, X.-L. Dynamics of soil and grain micronutrients as affected by long-term fertilization in an aquic inceptisol. *Pedosphere* **2010**, *20*, 725–735. [CrossRef]
21. Lu, R.K. *Soil and Agro-Chemical Analysis Methods*; Agricultural Science and Technology Press: Beijing, China, 1999; pp. 255–266. (In Chinese)
22. Nelson, D.W.; Sommers, L.E. Total Carbon, organic carbon, and organic matter. In *Methods of Soil Analysis*; Wiley Online Library: Hoboken, NJ, USA, 1996; pp. 961–1010. [CrossRef]
23. Bremner, J.; Mulvaney, C. Total nitrogen. In *Methods of Soil Analysis*, 2nd ed.; Wiley Online Library: Hoboken, NJ, USA, 1983. [CrossRef]
24. Sommers, L.E.; Nelson, D.W. Determination of total phosphorus in soils: A rapid perchloric acid digestion procedure. *Soil Sci. Soc. Am. J.* **1972**, *36*, 902–904. [CrossRef]
25. Bray, R.H.; Kurtz, L.T. Determination of total, organic, and available forms of phosphorus in soils. *Soil Sci.* **1945**, *59*, 39–46. [CrossRef]
26. Nelson, D.W.; Sommers, L.E. Total carbon, organic carbon, and organic matter. *Methods Soil Anal.* **1983**, *9*, 539–579. [CrossRef]
27. Ross, D.S.; Ketterings, Q. Recommended methods for determining soil cation exchange capacity. *Recomm. Soil Test. Proced. Northeast. USA* **1995**, *493*, 62.

28. Da Silva, Y.J.A.B.; Nascimento, C.W.A.D.; Biondi, C.M. Comparison of USEPA digestion methods to heavy metals in soil samples. *Environ. Monit. Assess.* **2014**, *186*, 47–53. [CrossRef]
29. Wan, Y.; Huang, Q.; Wang, Q.; Yu, Y.; Su, D.; Qiao, Y.; Li, H. Accumulation and bioavailability of heavy metals in an acid soil and their uptake by paddy rice under continuous application of chicken and swine manure. *J. Hazard. Mater.* **2020**, *384*, 121293. [CrossRef] [PubMed]
30. Song, C.; Shan, S.; Müller, K.; Wu, S.; Niazi, N.K.; Xu, S.; Shen, Y.; Rinklebe, J.; Liu, D.; Wang, H. Characterization of pig manure-derived hydrochars for their potential application as fertilizer. *Environ. Sci. Pollut. Res.* **2017**, *25*, 25772–25779. [CrossRef]
31. Wan, Y.; Huang, Q.; Wang, Q.; Ma, Y.; Su, D.; Qiao, Y.; Jiang, R.; Li, H. Ecological risk of copper and zinc and their different bioavailability change in soil-rice system as affected by biowaste application. *Ecotoxicol. Environ. Saf.* **2020**, *192*, 110301. [CrossRef] [PubMed]
32. Wang, Q.; Zhang, J.; Zhao, B.; Xin, X.; Zhang, C.; Zhang, H. The influence of long-term fertilization on cadmium (Cd) accumulation in soil and its uptake by crops. *Environ. Sci. Pollut. Res.* **2014**, *21*, 10377–10385. [CrossRef]
33. Xu, Y.; Tang, H.; Liu, T.; Li, Y.; Huang, X.; Pi, J. Effects of long-term fertilization practices on heavy metal cadmium accumulation in the surface soil and rice plants of double-cropping rice system in Southern China. *Environ. Sci. Pollut. Res.* **2018**, *25*, 19836–19844. [CrossRef]
34. Nicholson, F.; Smith, S.; Alloway, B.; Carlton-Smith, C.; Chambers, B. An inventory of heavy metals inputs to agricultural soils in England and Wales. *Sci. Total Environ.* **2003**, *311*, 205–219. [CrossRef]
35. Fan, J.; Ding, W.; Chen, Z.; Ziadi, N. Thirty-year amendment of horse manure and chemical fertilizer on the availability of micronutrients at the aggregate scale in black soil. *Environ. Sci. Pollut. Res.* **2012**, *19*, 2745–2754. [CrossRef]
36. Wang, G.; Zhou, L. Application of Green Manure and Pig Manure to Cd-Contaminated Paddy Soil Increases the Risk of Cd Uptake by Rice and Cd Downward Migration into Groundwater: Field Micro-Plot Trials. *Water Air Soil Pollut.* **2016**, *228*, 29. [CrossRef]
37. Yamaguchi, N.; Nakamura, T.; Dong, D.; Takahashi, Y.; Amachi, S.; Makino, T. Arsenic release from flooded paddy soils is influenced by speciation, Eh, pH, and iron dissolution. *Chemosphere* **2011**, *83*, 925–932. [CrossRef] [PubMed]
38. Wan, Y.; Huang, Q.; Camara, A.Y.; Wang, Q.; Li, H. Water management impacts on the solubility of Cd, Pb, As, and Cr and their uptake by rice in two contaminated paddy soils. *Chemosphere* **2019**, *228*, 360–369. [CrossRef] [PubMed]
39. Vig, K. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: A review. *Adv. Environ. Res.* **2003**, *8*, 121–135. [CrossRef]
40. Gupta, A.K.; Sinha, S. Assessment of single extraction methods for the prediction of bioavailability of metals to *Brassica juncea* L. Czern. (var. Vaibhav) grown on tannery waste contaminated soil. *J. Hazard. Mater.* **2007**, *149*, 144–150. [CrossRef] [PubMed]
41. Zhang, S.; Song, J.; Gao, H.; Zhang, Q.; Lv, M.-C.; Wang, S.; Liu, G.; Pan, Y.-Y.; Christie, P.; Sun, W. Improving prediction of metal uptake by Chinese cabbage (*Brassica pekinensis* L.) based on a soil-plant stepwise analysis. *Sci. Total Environ.* **2016**, *569*, 1595–1605. [CrossRef] [PubMed]
42. Meng, J.; Liang, S.; Tao, M.; Liu, X.; Brookes, P.C.; Xu, J. Chemical speciation and risk assessment of Cu and Zn in biochars derived from co-pyrolysis of pig manure with rice straw. *Chemosphere* **2018**, *200*, 344–350. [CrossRef] [PubMed]
43. Huang, Q.; Yu, Y.; Wan, Y.; Wang, Q.; Luo, Z.; Qiao, Y.; Su, D.; Li, H. Effects of continuous fertilization on bioavailability and fractionation of cadmium in soil and its uptake by rice (*Oryza sativa* L.). *J. Environ. Manag.* **2018**, *215*, 13–21. [CrossRef]
44. Qaswar, M.; Yiren, L.; Jing, H.; Kaillou, L.; Mudasir, M.; Zhenzhen, L.; Hongqian, H.; Xianjin, L.; Jianhua, J.; Ahmed, W.; et al. Soil nutrients and heavy metal availability under long-term combined application of swine manure and synthetic fertilizers in acidic paddy soil. *J. Soils Sediments* **2020**, *20*, 2093–2106. [CrossRef]
45. Shahid, M.; Shukla, A.K.; Bhattacharyya, P.; Tripathi, R.; Mohanty, S.; Kumar, A.; Lal, B.; Gautam, P.; Raja, R.; Panda, B.B.; et al. Micronutrients (Fe, Mn, Zn and Cu) balance under long-term application of fertilizer and manure in a tropical rice-rice system. *J. Soils Sediments* **2015**, *16*, 737–747. [CrossRef]
46. Wei, X.; Hao, M.; Shao, M.; Gale, W.J. Changes in soil properties and the availability of soil micronutrients after 18 years of cropping and fertilization. *Soil Tillage Res.* **2006**, *91*, 120–130. [CrossRef]
47. Wang, K.; Peng, N.; Wang, K.; Xie, X. Effects of long-term manure fertilization on heavy metal content and its availability in paddy soils. *J. Soil. Water. Conserv.* **2008**, *22*, 105–108. (In Chinese)
48. Chen, W.; Li, G.; Qi, Y.; Wang, E.; Yuan, H.L.; Li, J. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *Int. J. Syst. Evol. Microbiol.* **1991**, *41*, 275–280. [CrossRef]
49. Moran, J.F.; Iturbe-Ormaetxe, I.; Matamoros, M.A.; Rubio, M.C.; Clemente, M.R.; Brewin, N.J.; Becana, M. Glutathione and homoglutathione synthetases of legume nodules. cloning, expression, and subcellular localization. *Plant Physiol.* **2000**, *124*, 1381–1392. [CrossRef]
50. Ullah, A.; Mushtaq, H.; Ali, H.; Munis, M.F.H.; Javed, C.H.; Chaudhary, H.J. Diazotrophs-assisted phytoremediation of heavy metals: A novel approach. *Environ. Sci. Pollut. Res.* **2015**, *22*, 2505–2514. [CrossRef] [PubMed]
51. Aghili, F.; Gamper, H.; Eikenberg, J.; Khoshgoftarmanesh, A.H.; Afyuni, M.; Schulin, R.; Jansa, J.; Frossard, E. Green Manure Addition to Soil Increases Grain Zinc Concentration in Bread Wheat. *PLoS ONE* **2014**, *9*, e101487. [CrossRef]
52. Houben, D.; Evrard, L.; Sonnet, P. Mobility, bioavailability and pH-dependent leaching of cadmium, zinc and lead in a contaminated soil amended with biochar. *Chemosphere* **2013**, *92*, 1450–1457. [CrossRef]

53. Khokhotva, O.; Waara, S. The influence of dissolved organic carbon on sorption of heavy metals on urea-treated pine bark. *J. Hazard. Mater.* **2010**, *173*, 689–696. [CrossRef]
54. Hartley, W.; Edwards, R.; Lepp, N.W. Arsenic and heavy metal mobility in iron oxide-amended contaminated soils as evaluated by short- and long-term leaching tests. *Environ. Pollut.* **2004**, *131*, 495–504. [CrossRef]
55. Park, J.H.; Lamb, D.; Paneerselvam, P.; Choppala, G.; Bolan, N.; Chung, J.-W. Role of organic amendments on enhanced bioremediation of heavy metal(loid) contaminated soils. *J. Hazard. Mater.* **2011**, *185*, 549–574. [CrossRef]
56. Bang, J.; Hesterberg, D. Dissolution of trace element contaminants from two coastal plain soils as affected by pH. *J. Environ. Qual.* **2004**, *33*, 891–901. [CrossRef]
57. Li, P.; Wang, X.-X.; Zhang, T.-L.; Zhou, D.-M.; He, Y.-Q. Distribution and accumulation of copper and cadmium in soil–rice system as affected by soil amendments. *Water Air Soil Pollut.* **2008**, *196*, 29–40. [CrossRef]
58. Behera, S.; Singh, M.; Singh, K.; Todwal, S. Distribution variability of total and extractable zinc in cultivated acid soils of India and their relationship with some selected soil properties. *Geoderma* **2011**, *162*, 242–250. [CrossRef]
59. Li, B.; Zhou, D.; Cang, L.; Zhang, H.; Fan, X.; Qin, S. Soil micronutrient availability to crops as affected by long-term inorganic and organic fertilizer applications. *Soil Tillage Res.* **2007**, *96*, 166–173. [CrossRef]
60. Kuppusamy, S.; Yoon, Y.-E.; Kim, S.Y.; Kim, J.H.; Lee, Y.B. Long-term inorganic fertilization effect on the micronutrient density in soil and rice grain cultivated in a South Korean paddy field. *Commun. Soil Sci. Plant Anal.* **2017**, *48*, 1603–1615. [CrossRef]
61. Li, P.; Wang, X.; Zhang, T.; Zhou, D.; He, Y. Effects of several amendments on rice growth and uptake of copper and cadmium from a contaminated soil. *J. Environ. Sci.* **2008**, *20*, 449–455. [CrossRef]
62. Ouyang, J.; Liu, Z.; Zhang, L.; Wang, Y.; Zhou, L. Analysis of influencing factors of heavy metals pollution in farmland-rice system around a uranium tailings dam. *Process. Saf. Environ. Prot.* **2020**, *139*, 124–132. [CrossRef]
63. Zeng, F.; Ali, S.; Zhang, H.; Ouyang, Y.; Qiu, B.; Wu, F.; Zhang, G. The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. *Environ. Pollut.* **2011**, *159*, 84–91. [CrossRef] [PubMed]