

## Article

# Sequential Extraction Resulted in Similar Fractionation of Ionic Zn, Nano- and Microparticles of ZnO in Acidic and Alkaline Soil

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**Abstract:** The evaluation of nanoparticle bioavailability or the bioavailability of dissolved elements by direct measurement through plant uptake is a strenuous process. Several multi-step sequential extraction procedures, including the BCR sequential extraction procedure, have been created to provide potential accessibility of elements, where real soil-plant transfer can be problematic to implement. However, these have limitations of their own based on the used extractants. For the purposes of our research, we enriched two soils: an untilted forest soil with naturally acidic pH and a tilted agricultural soil with alkaline pH by three Zn forms—ionic Zn in the form of  $\text{ZnSO}_4$ , ZnO nanoparticles (ZnO NP) and larger particles of ZnO (ZnO B)—by batch sorption. We then extracted the retained Zn in the soils by BCR sequential extraction procedure to extract three fractions: ion exchangeable, reducible, and oxidizable. The results were compared among the soils and a comparison between the different forms was made. Regardless of the difference in soil pH and other soil properties, ZnO NP, ZnO B, and ionic Zn showed little to no difference in the relative distribution between the observed soil fractions in both forest soil and agricultural soil. Since ionic Zn is more available for plant uptake, BCR sequential extraction procedure may overestimate the easily available Zn when amendment with ionic Zn is compared to particulate Zn. The absence of a first extraction step with mild extractant, such as deionized water, oversimplifies the processes the particulate Zn undergoes in soils.

**Keywords:** zinc; nanoparticle; chemical speciation; BCR Sequential extraction; soil fractions; contamination

## 1. Introduction

The mobility of Zn in soils is strongly affected by its physicochemical forms and by the types of bonds formed with soil constituents. Its bioavailability and toxicity are tied to the Zn forms that are the most mobile rather than to the total Zn concentration in soils [1]. Taking this knowledge into consideration, studies on the speciation of trace elements were conducted for many environmental partitions to evaluate their effects on the environment. Metals, including Zn, are present in soils as simple and complex ions, bound with organic or organo-mineral complexes, adsorbed on or coprecipitates with other metal oxides, carbonates, phosphates, aluminosilicates or other secondary minerals, and are also incorporated in the crystal structure of primary minerals [2]. The size of soil particles that Zn and

other metals are incorporated into also ranges from macroparticles visible to the naked eye all the way down to microscopic particles, such as microparticles and nanoparticles [3,4].

Amendment by ZnO nanoparticles (NP) poses an ecological risk to soils. Most ZnO NP currently enter soil via applied activated sludge, which is used as a fertilizer, and thus the ZnO NP enter the soil transformed, either partially dissolved and/or transformed into Zn phosphates and Zn bound to Fe oxyhydroxides [5,6]. Deficiency of Zn in soils is a common problem [7,8], and ZnO NP can be used as a micronutrient nanofertilizer to alleviate it [9,10]. Nanofertilizers offer new avenues of application to plants with the potential to increase growth yield while at the same time reducing the volume of the used fertilizer; this may result in economic and environmental benefits. Thus, their direct application into soils is very likely in the near future [9]. Even though ZnO NP enhance plant growth at the right concentrations [10–12], their repeated use may result in toxic effects on soil organisms and plants when ZnO NP are transformed or dissolved to more bioavailable or toxic forms [13–15].

Two types of factors affect the distribution and bioavailability of Zn in soils: (1) environmental factors, including soil pH, quantity and types of clay minerals, oxyhydroxides of Al, Fe, Mn, and organic matter affect the behavior and mobility of Zn in soils [13,16–19], and (2) the properties of the physicochemical form of Zn. Therefore, ZnO NP, as well as particles of Zn (bulk Zn), may not be present in smaller pores, nor do they always attach to the same binding sites as ionic Zn. Additionally, while ionic Zn stays dissolved in soil pore water in most cases, the particulate Zn, especially in the form of ZnO NP may dissolve in more acidic soils and behave similarly to ionic Zn. In alkaline soils, ZnO can be stable as particulates [17,19] and these ZnO particles have a lower uptake to plants than the dissolved ionic form [20–23].

Extraction procedures, both one-step and sequential, have been developed to evaluate the mobility of trace elements in various media, including soils [24–28]. Sequential extractions provide details about the mobility and interaction of trace elements with soil matrices and they are assumed to mimic certain environmental conditions that induce surface exchange or mineral dissolution to soil pore water. These extractions are also used instead of direct bioavailability tests, since they are simpler and require less time and resources. A modified BCR sequential extraction procedure, which was created and named by the Community Bureau of Reference of the European Union, is widely used in the analysis of sediments, soils, particulate matter, ferromagnesian clays, mining, mineral processing, industrial wastes, and sewage sludges [29–35]. It solves some inconsistencies detected during trace elements' extractions by BCR [36] and sediment from Lake Orta Piemonte, Italy, and it was used to create a certified reference material (BCR-701) in 2001 [37,38]. Certified concentration values for six trace elements (Cd, Cr, Cu, Ni, Pb, and Zn) in the three extraction steps were made for this reference material. All three steps extract an ion-exchangeable fraction, a fraction of reducible Fe and Mn oxides, as well as a fraction of oxidizable organic matter and sulfides.

In our previous study, we looked at the size distribution of ionic zinc in the form of  $\text{ZnSO}_4$ , as well as the particulate zinc in the form of ZnO NP and ZnO B in soil solutions and their solid-liquid distribution [17]. However, the bioavailability of Zn in soils does not only depend on the solid-liquid distribution, but also on the strength of the bonds with soil constituents. Adsorbed Zn or Zn with weaker bonds is more bioavailable to plants. In addition, ionic Zn is more bioavailable than particulates. The BCR sequential extraction procedure, along with other sequential procedures, are used to evaluate the potential mobility of elements in soils and their accessibility to plants. However, we are not aware of any evaluation that was previously performed on soils amended with both ionic and particulate zinc. Therefore, we (1) evaluated the usefulness of the BCR sequential extraction procedure in differentiating the fractionation of Zn coming from different Zn forms, particulate or ionic, and its ability (2) to meaningfully assess the strength of the Zn bonding of  $\text{ZnSO}_4$ , ZnO NP, and ZnO B to one alkaline and one acidic soil. We expected that particulate and ionic Zn forms would differ significantly in bonding to soil constituents upon their sorption to soils, and that the ionic Zn would be distributed in relatively higher concentrations in the most accessible fraction—the ion-exchangeable fraction—of the BCR sequential extraction procedure.

## 2. Materials and Methods

### 2.1. Selected Soils

Two topsoil samples with different characteristics were collected in the western part of Slovakia. Both soils were collected at a depth ranging between 5 and 15 cm. The soils were classified according to the Morphogenetic Soil Classification System of Slovakia [39] and the World Reference Base for Soil Resources (WRB) [40] as Chernozem kultizemna karbonatova/Calcic Chernozem (CH-cc) (alkSoil) and Kambizem luvizemna pseudoglejova/Dystric Stagnic Cambisol (CM-st.dy) (acidSoil). The methods used to characterize the soil samples can also be found in our previous work [17]. Soil samples were decomposed by digestion of 1 g of soil in acid mixture of HF + HNO<sub>3</sub> + HClO<sub>4</sub> + H<sub>2</sub>O<sub>2</sub> (4:3:4:1, 60 mL) in an open system at 200 °C to determine total concentrations of Al, Fe, Mn, and Zn. Oxalate-extractable forms of Al, Fe, and Mn were determined using extraction with 0.2 mol·L<sup>-1</sup> ammonium oxalate at pH 3 on a soil sample that was ground to a particle size below 0.5 mm [41]. Total organic carbon (TOC) was determined according to the method by Walkley and Black [42]. Soil pH was measured in 1 mol·L<sup>-1</sup> KCl (pH<sub>KCl</sub>) and deionized water (pH<sub>H<sub>2</sub>O</sub>). A 1:2.5 soil to solution ratio was used. The content of CaCO<sub>3</sub> was measured volumetrically using a method with Janko's calcimeter [43]. The size distribution of soil particles was determined by a pipette method [43]. The USDA-FAO soil-texture triangle was used to classify soil samples into textural classes [44]. Concentrations of humic substances, humic acids, and fulvic acids in soil samples were determined by the method developed by Kononova and Belchikova [45]. The soil cation exchange capacity (CEC) was measured by the Kappen method [46]. The characteristics of the two soils are shown in Table 1.

**Table 1.** Characteristics of soil samples used in sorption experiments.

Soil Samples	alkSoil	acidSoil	Soil Samples	alkSoil	acidSoil
Soil code [40]	CH-cc	CM-st.dy	pH <sub>H<sub>2</sub>O</sub>	7.98	4.10
Location	Senec	Stara Tura	pH <sub>KCl</sub>	7.45	3.40
Land use	Crop	Forest	CaCO <sub>3</sub> (%)	3.3	0.4
Texture	loam	loamy sand	Tot Al (mg·kg <sup>-1</sup> ) <sup>e</sup>	51,800	44,600
Sand (%)	34.3	79.2	Tot Fe (mg·kg <sup>-1</sup> ) <sup>e</sup>	25,700	16,200
Silt (%)	45.8	16.4	Tot Mn (mg·kg <sup>-1</sup> ) <sup>e</sup>	604	318
Clay (%)	19.9	4.5	Tot Zn (mg·kg <sup>-1</sup> ) <sup>e</sup>	82.4	142
TOC (%) <sup>a</sup>	2.82	4.73	Ox Al (mg·kg <sup>-1</sup> ) <sup>f</sup>	920	1560
HS (%) <sup>b</sup>	1.12	2.48	Ox Fe (mg·kg <sup>-1</sup> ) <sup>f</sup>	1270	2970
HA (%) <sup>c</sup>	0.53	0.79	Ox Mn (mg·kg <sup>-1</sup> ) <sup>f</sup>	390	320
FA (%) <sup>d</sup>	0.59	1.69	CEC (mmol·kg <sup>-1</sup> ) <sup>g</sup>	484	291

CH-cc—Calcic Chernozem, CM-st.dy—Dystric Stagnic Cambisol, <sup>a</sup> Total organic carbon, <sup>b</sup> Humic substances, <sup>c</sup> Humic acids, <sup>d</sup> Fulvic acids, <sup>e</sup> Total concentration in the soil sample, <sup>f</sup> Oxalate-extractable phase of the element, <sup>g</sup> Cation exchange capacity.

To verify the accuracy of the procedure and determination of Zn contents, lake sediment BCR-701 (EUR 19,775), with certified extractable contents of Zn for the modified BCR three-step sequential extraction procedure, was used.

### 2.2. Amendment of Selected Soils and BCR Extraction

Batch sorption was used for the amendment of alkSoil, acidSoil and BCR-701 by ionic Zn as ZnSO<sub>4</sub> (p.a., Centralchem), a colloidal solution of ZnO nanoparticles (ZnO NP) (dispersion, <100 nm particle size (TEM), ≤40 nm avg. part. size (APS), 20 wt.% in H<sub>2</sub>O, 721077, Sigma Aldrich) and microsized conventional ZnO powder (ZnO B) (p.a., Chemapol). A solution containing 196 mg·L<sup>-1</sup> Zn (3 mmol·L<sup>-1</sup>) of either ZnSO<sub>4</sub> or ZnO NP was obtained by the addition of an appropriate amount of 0.1 mol·L<sup>-1</sup> ZnSO<sub>4</sub> or dispersion of ZnO NP and 0.1 mL of artificial rainwater concentrate (see Supplementary Materials) to a 100 mL volumetric flask filled to the mark with distilled water. In the case of ZnO NP, the colloidal solution was created right before the experiment and sonicated for

15 min in an ultrasonic bath. One gram of either alkSoil, acidSoil, or BCR-701 was placed into a 50 mL centrifuge tube (metal-free, sterile, VWR) and 20 mL of either 196 mg·L<sup>-1</sup> Zn of ZnSO<sub>4</sub> or ZnO NP or 4.9 mg of ZnO B and 20 mL of artificial rainwater was added (see Supplementary Materials). A liquid–solid ratio of 20:1 was created. For each of the solid samples, experiments for all three Zn forms were undertaken as well as controls with no added Zn form. All experiments were done in triplicate. Centrifuge tubes were mixed on an over-head rotator at 5 rpm for 24 h.

After 24 h, centrifuge tubes were removed and centrifuged for 1 min at 700 g. Particles of ZnO smaller than 1000 nm stayed suspended in the supernatant [17]. The supernatant was removed from the centrifuge tubes and analyzed for the Zn content by flame atomic absorption spectrometry (FAAS) (see Section 2.3 of Materials and Methods).

The remaining contaminated sedimented solids were subjected to the modified BCR 3-step sequential extraction procedure [29]. The full details on the procedure can also be found elsewhere [37,38] and in the Supplementary Material.

The relative concentrations of Zn acquired during the three BCR extraction steps (ion exchangeable + reducible + oxidizable = 100%) were compared among the three Zn forms in one soil using single factor ANOVA. An extension Analysis ToolPak in Microsoft Excel (Redmond, WA, USA) was used to find the differences between the means. If a difference was found, ionic Zn, ZnO NP, and ZnO B were compared in pairs to find the source of difference by *t*-Test: Two-Sample Assuming Equal Variances. All tests were done on significance level  $\alpha = 0.05$ .

### 2.3. Analysis of Zn Content in Solids and Liquids

A flame atomic absorption spectrometer (Perkin-Elmer 1100, Perkin-Elmer, Waltham, MA, USA) with an air–acetylene flame (air flow rate 8.0 L min<sup>-1</sup>, acetylene flow rate 2.5 L min<sup>-1</sup>) was used to determine zinc content in digests and extracts. The limit of quantification for zinc in the conducted experiments was 0.005 mg L<sup>-1</sup>. The operational instrumental parameters are shown in Supplementary Material Table S1.

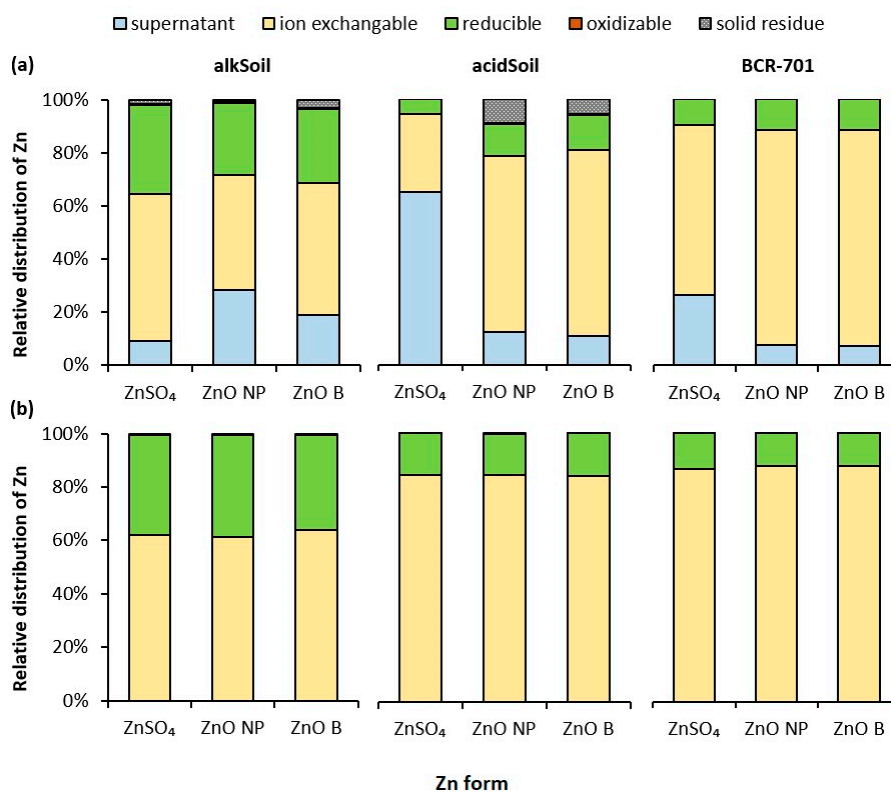
## 3. Results

### 3.1. Amendment of the Soils

The two soils (and BCR-701 for reference) were contaminated by batch sorption with three forms of Zn, ionic Zn added as ZnSO<sub>4</sub>, ZnO NP, and ZnO B. The sorption pattern significantly differed between alkSoil and acidSoil, and between applied Zn forms as well. In the latter case, the contrast was most apparent in the comparison between the ZnSO<sub>4</sub> and particulate ZnO forms (Figure 1 and Supplementary Material Tables S4–S7).

The distribution between liquid- and solid-state was similar for ZnO NP and ZnO B in acidSoil, while the ionic Zn (dissociated from ZnSO<sub>4</sub>) was sorbed onto the soil to a much lesser extent (32.0%) than both particulate forms of Zn—ZnO NP and ZnO B (87.7% and 89.0%, respectively).

In alkSoil, a different pattern of solid-liquid distribution was observed. The nanoparticulate form of Zn—ZnO NP—was the form that attached the least onto the soil-particles' surfaces (71.8%). Retention of larger ZnO B followed with 80.9% sorption, and ionic Zn had the highest retention efficiency at 91.1%.



**Figure 1.** Zn distribution obtained for the contaminated solid materials alkSoil, acidSoil and BCR-701 using the modified BCR sequential extraction procedure. (a) presents relative content of Zn contained in all fractions of the experiment and (b) only in the three fractions of modified BCR sequential extraction procedure: ion exchangeable, reducible and oxidizable fractions.

### 3.2. Fractionation of Sorbed Zn in the Soils

After the amendment by batch sorption, the BCR extraction procedure was used on both soils (and BCR-701 for reference) to extract the Zn from the three soil fractions.

Relative contents of Zn in ion-exchangeable, reducible and oxidizable fractions were very similar for all three Zn forms (Figure 1b and Supplementary Material Tables S4–S7). Only ZnO B in alkSoil had a relatively higher content of Zn in the ion-exchangeable fraction, by ca. 2% compared to both ZnO NP and ZnSO<sub>4</sub>. Particulate and ionic Zn have different retentions in the same soil. Hence, depending on whether Zn is in the particulate form or ionic, the dissolved form affects the quantitative nature of the distribution between solids and liquid in alkSoil and acidSoil as defined under the operational conditions of the experiment (Figure 1a). However, the general affinity towards the three studied soil fractions—ion-exchangeable, reducible and oxidizable—was very similar for all three Zn forms (Figure 1b and Table S7).

The 24 h batch sorption of three Zn forms onto alkSoil and acidSoil resulted in the retention of Zn that was bound mainly to soil fraction forming weaker bonds with soil, which is easily extractable by the first step of sequential extraction with a more benign extraction reagent used. More specifically, the highest amount of Zn was measured in ion-exchangeable soil fraction (Figure 1, Tables S4–S7). The measured relative distribution of Zn in ion-exchangeable fraction differed between the two soils and was higher in acidSoil (ca 85.0%) compared to alkSoil (ca 60.0%).

The fraction of reducible Fe and Mn oxides held the second-highest amount of Zn (Figure 1). Its relative amount in reducible fraction differed between alkSoil and acidSoil. While the reducible fraction from acidSoil amounted to around 15.0% of Zn, it was approximately 40.0% for alkSoil. A lower amount of Zn was bound to the reducible fraction of acidSoil even though the soil contains a

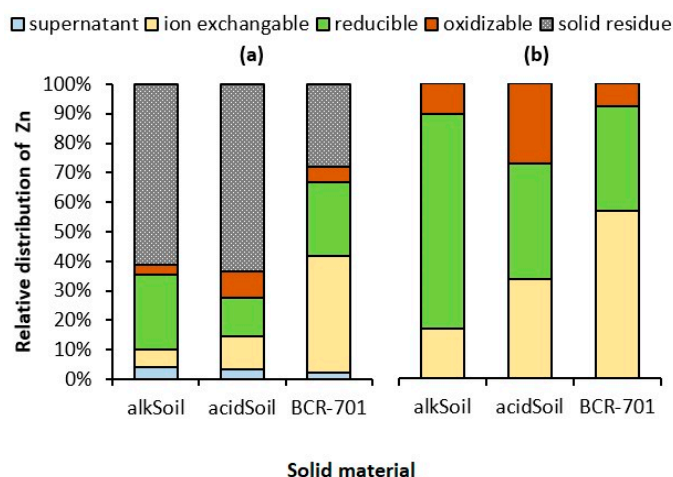


higher content of oxalate extractable Al, Fe and Mn, which approximate the concentrations of reducible Fe and Mn oxides in soils (Table 1).

Ion-exchangeable and reducible fractions combined contained nearly all Zn, while oxidizable fraction and solid residue contained only negligible amounts (Figure 1b and Tables S4–S7).

### 3.3. Comparison between Amended and Unamended Soils

Control experiments with no addition of Zn and extraction validation with certified reference material BCR-701 were also undertaken. The distribution of Zn in the entire experimental setup is highlighted in Figure 2 (and in Supplementary Material Table S2). The artificial rainwater extracted between 2.2% and 4.3% of the natural Zn found in the solid materials. The first step of the BCR extraction procedure—the ion-exchangeable fraction—contained different relative amounts of natural Zn bound in the solid materials—5.9, 11.3, and 39.7% for alkSoil, acidSoil and BCR-701, respectively. The reducible fraction amounted to 25.3, 13.0, and 24.8% of Zn bound in the solid materials alkSoil, acidSoil, and BCR-701, respectively; and 3.6, 9.1, and 5.4% of Zn was extracted from the oxidizable fraction of alkSoil, acidSoil, and BCR-701, respectively. For solid residue, a relative amount of 61.0, 63.3, and 28.0% Zn bound in the solid material alkSoil, acidSoil, and BCR-701 was found, respectively. The two soils contain relatively more Zn in less accessible, reducible and oxidizable fractions and in the solid residue than BCR-701. The total concentration of Zn in the two soils is lower than in BCR-701 (see Supplementary Material Table S2). The concentrations of Zn in both alkSoil and acidSoil were below the threshold of amendment by Zn, according to the decree of the Slovak Ministry of Agriculture [47], and they are typical for the region where the soil samples were collected [48]. The concentrations in both soils are above the average value for non-contaminated soils, which is around  $64 \text{ mg kg}^{-1} \text{ Zn}$  [7].



**Figure 2.** Zn distribution obtained for the uncontaminated solid materials alkSoil, acidSoil and BCR-701 using the modified BCR sequential extraction procedure. (a) presents the relative content of Zn contained in all fractions of the experiment and (b) only in the three fractions of modified BCR sequential extraction procedure: ion exchangeable, reducible and oxidizable fractions.

The results of the control experiment with certified referential material BCR-701 were compared with certified values provided for the referential material, and are summarized in Supplementary Material Table S3. The whole procedure, including aqua regia digestion of the residue, extracted an amount of Zn from BCR-701 that is within the confidential limit (95% confidence interval) stated in the certified values [37,38]. However, the ion exchangeable and oxidizable fraction had lower extraction efficiency and their recovery rates were 93.6% and 54.4% of Zn, respectively. The unreleased Zn from the previous steps was later measured in the solid residue with a Zn recovery rate of 135.4% compared to the certified value (Supplementary Material Table S3).

The amendment of alkSoil, acidSoil, and BCR-701 by sorption of three Zn forms (Figure 1b) changed the relative distribution of Zn in the different fractions. The relative concentration of Zn in the ion-exchangeable fraction increased. Therefore, relatively less Zn was extracted from the fraction of reducible Fe and Mn oxides. The amount of Zn extracted from the fraction of oxidizable organic material and sulfides was negligible. Less than 0.5% of added Zn was extracted from the oxidizable fraction. Only a small fraction of Zn created strong bonds with oxidizable organic matter or sulfides during the experiment. In total, reducible and oxidizable fractions did not play a substantial role in the sorptive binding of Zn in soils amended with the three Zn forms compared to the unamended alkSoil, acidSoil, and BCR-701 (Figures 1 and 2).

#### 4. Discussion

Chemical properties of Zn, in both ionic or particulate forms, play an important role in their association with soil particles, their distribution pattern in various soil fractions, and in soil solutions as well [17,49]. The BCR sequential extraction procedure was used to acquire three soil fractions, which are important when the association of elements with soil is taken into account, and it is considered to provide enough information to assess the potential bioaccessibility and mobility of Zn in soil environments [24,34]. Elements from different soil fractions are usually separated via one-step or sequential extraction with precisely defined reagents that mimic various natural weathering conditions. However, the first step of the BCR extraction uses a reagent that is strong enough to dissolve particulate forms of ZnO NP and ZnO B. This may hinder the usefulness of the BCR extraction when the contamination comes from particulate Zn forms, and thus bioaccessibility of Zn from such contamination may be overestimated. In the following subsections, the inability to discern ionic and particulate forms is described and discussed in more detail.

In addition, when the results in Figure 1 are compared to Figure 2, it can be assumed that the experiment was too short for Zn to create stronger bonds with Fe and Mn reducible oxides or soil organic matter. This corresponds to the existing literature, thus suggesting that soil aging is necessary to increase the concentrations of Zn in fractions of reducible oxides, as well as in oxidizable carbon represented by soil organic matter [50].

##### 4.1. Amendment of the Soils

During the batch sorption a higher amount of particulate Zn, both in the form of ZnO NP and ZnO B, was retained in acidSoil compared to the ionic Zn form (Figure 1). Aggregation of particulate ZnO has been reported to be more favorable under acidic conditions [17,51,52]. The pH-dependent surface-charge aggregation process forms large aggregates that are more easily retained in the solid phase of acidSoil. Thus, the apparent higher retention of particulate Zn, in comparison to dissolved Zn ions, most likely relates to higher sedimentation and attachment rates of aggregates with a size over 1000 nm [17,51,52]. It is important to note that in this case, the apparent sorption does not reflect the 'chemical' characteristics of the sorption process. The dissolution of particulate ZnO, which is expected under acidic conditions, was only partial since the full dissolution would have resulted in a similar distribution of ZnSO<sub>4</sub> and particulate ZnO [53]. If the dissolution in soils is only partial, only a fraction of the particulate form will be easily available for a plant root system to uptake without the plant actively increasing the production of exudates to promote the uptake of elements from the soil environment. So even in more acidic soils, BCR extraction may not be able to capture the differences between the uptake of Zn from different sources of contamination.

The results from acidSoil contrast considerably with the results from AlkSoil and the difference is most likely related to the processes connected to the stabilization of solid ZnO particles by soil organic matter in a soil solution. Other differences in mechanisms governing the retention of ionic Zn compared to particulate ZnO, such as the different availability of binding sites for the particulate vs. ionic form, along with the inability of particulate forms to enter certain small soil pores, also play a role [17,50,52,54]. Compared to the control, some of the added Zn remained in the solid residue

of alkSoil. This may suggest that new insoluble minerals formed from the ionic Zn or dissolved Zn released from ZnO NP or ZnO B. Wan et al. [55] found that above pH 7, Zn forms Zn-Al layered double hydroxides that can form within 24 h. Newly formed minerals of this kind may not fully dissolve during extraction and, thus, the Zn may not have been extracted during the BCR extraction procedure.

The particulate ZnO was stabilized by the presence of soil organic matter, decreasing the efficiency of sorption to the soil particles [52,54]. Furthermore, the particulate ZnO forms may not be able to attach to (i) some sorption sites that are in pores smaller than the size of a Zn-containing particle, or (ii) to the sorption sites that are inside inaccessible surface depressions. However, these sites are still easily accessible to ionic Zn [17,56–59]. Therefore, a lower attachment of ZnO NP and ZnO B compared to ZnSO<sub>4</sub> was observed in alkSoil. In our previous experiment, where size fractions of 1 to 100 nm, 100 to 450 nm, and 450 to 1000 nm were separated by sequential sedimentation [17], most of the Zn in alkSoil supernatant was in the form of particulate aggregates of ZnO NP or ZnO B. Therefore, we presume that most of the retained Zn was also in the form of solid particles attached to the soil that is less easily available to plants.

#### 4.2. Fractionation of Zn Retained in the Soils

In both acidSoil and alkSoil, ZnO NP and ZnO B may have retained a positive surface charge [52,53] and, thus, their sorption sites resembled those of the ionic Zn. Even though we expected that the availability of suitable bonding sites might differ, the general type of these sites may be very similar. Similarities in interactions of all Zn forms with organic matter and soil mineral surfaces have been reported for both ionic [60,61] and particulate Zn [52,59,62,63]. Our observation that the Zn forms have the same relative distribution of Zn between the fractions is in line with the existing literature (Figure 1b). However, if the Zn is bound to this fraction in the form of solid particles, Zn from these particles has a lower probability of being taken up by plants, since it firstly needs to dissolve. While the first step of the BCR extraction procedure is suitable for the extraction of the Zn in all ion-exchangeable types of bonds, it also dissolves the particulate Zn associated with the soil particles. Since there is a difference between the uptake of particulate and ionic Zn by plants, an extraction step with a more benign reagent, such as deionized water, is required. Ionic Zn was shown to be absorbed to a greater degree compared to particulate forms of Zn in soils [20–23].

The relative amount of Zn in the measured fractions differed between acidSoil and alkSoil. A higher amount of Zn in reducible fraction reflects the amphoteric character of surface-reactive groups of mineral phases. More specifically, hydrated oxides and oxyhydroxides of Fe and Mn contain groups that have a variable charge that changes to positive in acidic condition. Although acidSoil has higher contents of oxalate-extractable Fe and Mn, the positive charge of most binding sites in this fraction would not be suitable for the sorption of particulate and ionic Zn forms with the same charge. Therefore, as reported here and by others, the higher sorption of Zn in reducible oxides was observed in alkSoil [64,65].

These findings may also play a role in the reported similarities in uptake, toxicity, and growth stimulation between all Zn forms [20], while they still fail to explain the higher uptake and increased toxicity of ionic Zn in other papers [21]. Therefore, our observation has some restrictions regarding the evaluation of actual Zn bioaccessibility: (1) the ion-exchangeable step of the BCR extraction procedure uses a strong enough extractant to dissolve ZnO NP and ZnO B. Thus, it cannot fully mimic the differences between the plant uptake of Zn in ionic forms or particulate forms, especially in alkaline soils where plant roots' exudates cannot fully dissolve particulate ZnO. (2) When treatment of soils comes from one Zn form, the BCR extraction procedure can safely estimate the difference in potential bioaccessibility between different soils. However, a comparison of bioaccessibility in soils amended by various Zn forms, especially when comparing soil amended with particulate Zn with soil amended with ionic Zn, is not recommended, as it might lead to overestimating the bioaccessibility of particulate Zn form. To alleviate the problem with differentiating the bioavailability between ionic Zn, ZnO NP and ZnO B, we propose using an additional step—step 0—which comes before the BCR extraction.



Deionized H<sub>2</sub>O should be a mild enough extractant to differentiate between the ionic and particulate forms. The dissolved, ionic forms of Zn are the most bioavailable to plants. The dissolution of ZnO NP and ZnO B in distilled water is comparable to the dissolution under the environmental conditions of rainwater, and this step should provide the missing information that can differentiate between the bioavailability of ionic and particulate Zn forms in the soil environment [66].

## 5. Conclusions

Three physicochemical forms of Zn were used to contaminate the soils. Upon sorption, Zn forms were primarily immobilized in ion exchangeable soil fraction, which is easily accessible for plants and microorganisms as well. When the relative amount of Zn in a different fraction between contaminated and uncontaminated samples was compared, there was a larger percentage of zinc in the ion exchangeable fraction of the contaminated soils. The results suggest that the formation of stronger bonds with Fe and Mn oxyhydroxides and oxidizable organic matter is a slower process and amended soils did not form the same quantity of the stronger bonds throughout the duration of the experiment when compared to the Zn fractionation in the same pristine soils. As defined by BCR, there was no difference between the relative distribution of Zn in soil fractions after being applied as either dissolved ZnSO<sub>4</sub> or particulate ZnO including nanoforms, which contradicts the results of Zn uptake from particulate and ionic Zn form in other studies. Therefore, the used BCR 3-step sequential extraction procedure may not be ideal for assessing Zn distribution in soils amended by particulate and ionic forms of Zn. For future studies that compare the partitioning of Zn in soils from both particulate and ionic forms, we propose that bioaccessibility should be studied with an extraction step that uses a benign reagent that will not dissolve all of the applied particulate ZnO in a soil. Extraction procedures that are more benign towards the dissolution of nanoparticles are necessary to simulate the bioavailable fractions of an element for plants or other soil microorganisms.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1999-4907/11/10/1077/s1>, 1. Artificial rainwater; 2. Modified BCR 3-step sequential extraction procedure; 3. Aqua regia digestion; 4. Solids and liquids analyses; 5. Results of control experiments; 6. Results of the batch experiment and Zn extraction from contaminated solid materials; 7. Statistical evaluation of relative Zn concentrations of different Zn forms extracted from the same soil; Table S1: Instrumental parameters for determination of analytes by FAAS, Table S2: Distribution of Zn in the experimental setup of the control experiments with standard deviations, Table S3: Comparison of results of the control experiment with certified referential material BCR-701 with the certified values for the BCR-701 Table S4: Distribution of Zn in the system of solid material alkSoil—CH-cc, an alkaline soil, Table S5: Distribution of Zn in the system of solid material acidSoil—CM-st.dy, an acidic soil, Table S6: Distribution of Zn in the system of solid material BCR-701, lake sediment certified reference material, Table S7: Relative concentrations of Zn extracted by the BCR extraction.

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