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The Effect of Complexed, Nanosized, and Conventional Zinc Sources Applied at Varying Rates to an Acidic Mediterranean Soil on Two Successive Lettuce Crops

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Abstract: This study investigates the current application and ageing effects of various Zn sources on acidic Mediterranean soil. Two successive lettuce crops were grown in soil fertilised with 0, 15, 30, 60, and 140 mg Zn kg^{-1} using commercial ZnO nanoparticles, Zn complex, and Zn sulphate. Plant growth, Zn biofortification, dietary implications, human health, and the soil Zn status were evaluated. Zinc bioavailability was influenced by the source, application rate, and chemical ageing. The bioavailability of Zn in the soil increased from 4.60 to 66.7 compared to the control treatment. Zinc applied in the form of $ZnSO_4$ was the most bioavailable form in the first year of cultivation. Advanced specialty fertilisers such as ZnO nanoparticles and Zn-lignosulfonate, along with the conventional fertiliser $ZnSO_4$, demonstrated a residual effect allowing effective Zn uptake by plants in the second crop. Zn concentrations in lettuce leaves were 3.33–34.6 times higher than the control treatment. Application of 30 mg Zn kg^{-1} and higher of commercial ZnO nanoparticles, Zn complex, and Zn sulphate heptahydrate resulted in some toxicity. Higher application rates of these sources may pose a potential risk to the population, as indicated by the health risk index. These Zn sources represent a promising alternative for enhancing plant growth and providing a sustained release of Zn in several successive crops, making them a potential alternative to conventional fertilisers. Their unique properties can optimise nutrient management strategies and promote sustainable crop production.

Keywords: ZnO nanoparticles; Zn complex; biofortification; dietary; aging

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1. Introduction

Zinc (Zn) deficiency is a widespread issue among the human population, with inadequate levels of Zn in food crops being a major contributing factor [1,2]. As an essential micronutrient, zinc plays a crucial role in numerous physiological functions. Its deficiency can lead to impaired immune function, stunted growth in children, and adverse pregnancy outcomes in women [3]. Additionally, Zn is crucial for male fertility and sperm quality [4].

Traditionally, crop production has primarily focused on increasing yields and agricultural productivity, with comparatively less attention given to the nutritional quality of these agricultural products. This approach has led to a progressive global rise in micronutrient malnutrition [5]. Micronutrient deficiencies for humans, particularly Zn and iron (Fe), have

emerged as significant contributors to the phenomenon of "hidden hunger". Improving the nutritional composition of agricultural crops provides a viable solution to this issue. Enhancing the mineral content in the consumable portions of crops through agronomic approaches is commonly referred to as agronomic biofortification. Zinc biofortification has emerged as a promising strategy to increase the accumulation of high concentrations of Zn in the edible parts of plants, aiming to address human health concerns related to the inadequate intake of this micronutrient [6].

The total adequate amount of Zn in the human body is approximately 1.5 g in women and 2.5 g in men [7]. The average dietary Zn requirements to meet physiological needs range from 6.2 to 10.2 mg day^{-1} for women (with a reference weight of 58.5 kg), from 7.5 to 12.7 mg/day^{-1} for men (with a reference weight of 68.1 kg), and from 2.4 to 11.8 mg/day^{-1} for infants and children [8]. Approximately 17.3% of the world's population is affected by zinc deficiency [9].

Conversely, most studies suggest humans have a high tolerance for elevated Zn intake [10]. However, toxic levels of Zn can damage plants and soil quality. These include reduced crop yields, impaired nutrient uptake and essential nutrient utilisation, and detrimental biochemical effects. Such effects may involve a decline in photosynthetic pigments, increased oxidative stress, changes in membrane structure and function, and inhibition of protein function. Excessive Zn concentrations negatively impact soil health and microbiological processes [11], contributing to increased groundwater contamination and posing an ecotoxicological risk factor. Ensuring soil quality is vital for sustainable soil management and food security, which aligns with the United Nations Sustainable Development Goals (UN SDGs).

Plant growth and development primarily rely on nutrients obtained from the soil [12]. Plants absorb these nutrients from the soil in varying amounts based on their specific requirements [13]. The bioavailability of Zn in soil depends on its micronutrient status, i.e., the concentration of Zn in different soil forms. This is influenced by the chemical form in which zinc is applied as a fertiliser.

Traditionally, various sources of Zn have been used to treat Zn deficiencies and facilitate biofortification in other crops. Advanced specialty fertilisers, such as nanofertilizers, chelated fertilisers, and water-soluble fertilisers, have become increasingly important. These fertilisers are characterised by their high nutrient uptake efficiency and ability to enhance nutrient translocation to the edible parts of the crop [14].

Emerging research has highlighted the positive impact of ZnO nanoparticles (ZnO NPs) on plant growth when applied at appropriate concentrations, indicating their potential as a viable alternative to conventional fertilisers. The long-term solubility of ZnO NPs has garnered increased attention due to its ability to provide a sustained release of Zn ions, ensuring a continuous nutrient supply to plants [15]. The unique properties of ZnO NPs make them a promising candidate for optimising nutrient management strategies in agriculture and advancing sustainable crop production [16].

The physicochemical properties of different Zn fertilisers influence processes such as adsorption, aggregation, dispersion, and particle solubility within the soil [17]. Over time, the availability of Zn sources in the soil undergoes significant changes. The more soluble forms of Zn lose activity and extractability, transforming into more stable and less available chemical forms. This shift results in reduced efficacy of the initial Zn application, leading to diminished plant uptake of the micronutrient. While this phenomenon has been extensively studied with traditional and advanced speciality fertilisers, to the best of our knowledge, there are no comparative studies evaluating the behaviour of these zinc sources and their residual effects on high-value and widely consumed crops such as lettuce (*Lactuca sativa* L).

Lettuce is a zinc-sensitive crop with a substantial global production, reaching 1213M ha in 2021 [18,19]. Spain ranks as the fourth largest producer, behind China, the United States, and India. Lettuce is recommended by USEPA [20] for assessing ecological effects, as its seeds are recognised as excellent bioindicators of toxicity due to their heightened sensitivity to chemical stressors [21].

The study aims to explore the role of zinc in lettuce crop biofortification and yield, focusing on zinc oxide nanoparticles (ZnO NPs) as a source of fertilisation. It is proposed that the application of ZnO NPs could enhance both crop yield and Zn bioaccumulation in plants. Furthermore, the study investigates whether ZnO NPs offer a beneficial residual effect, potentially reducing the need for successive fertiliser applications. Finally, its efficiency is compared with other Zn sources, such as natural complexes and $ZnSO_4$. To achieve these objectives, the research was guided by the following questions: (i) Can an adequate concentration of Zn in the form of ZnO NPs improve the yield and biofortification of lettuce crops? (ii) Can the residual availability of ZnO NPs in the soil eliminate the need for successive Zn applications? (iii) How does the effectiveness of ZnO NPs compare with traditional Zn sources like $ZnSO_4$ and natural Zn complexes?

2. Materials and Methods

2.1. Original Soil Characterisation

The original soil was located in Castilla y Leon, Spain (41°13′ N, 5°17′ W). This soil was obtained from the Ap horizon (0–20 cm) and was air-dried. The fractions smaller than 2 mm were selected for the experiment. The soil was classified as Cambisols [22], and its main characteristics were as follows: pH, acidic 5.9; sand, 803 g kg^{-1}; silt, 116 g kg^{-1}; clay, 81 g kg^{-1}; texture, sandy loam; electrical conductivity, 67.2 μS cm^{-1}; extractable P, 18.3 mg kg^{-1}; oxidisable OM, 4.1 g kg^{-1}; total N, 0.6 g kg^{-1}; C:N ratio, 3.7; exchange Na, 58 mg kg^{-1}; exchange K, 115 mg kg^{-1}; exchange Ca, 805 mg kg^{-1}; and exchange Mg, 263 mg kg^{-1}. The soil was found to be Zn-deficient, with an available Zn concentration (DTPA–triethanolamine (TEA)-extractable Zn) (0.38 mg kg^{-1}). This soil is mainly characterised by its acidic pH and low organic matter content.

2.2. Zinc Fertiliser Application in Soil and First Lettuce Crop

According to the primary macronutrient extractions of the lettuce crop [23], the soil pots (350 g soil dry weight) were fertilised with NPK: 100 mg N kg^{-1} as urea, 66.41 mg P kg^{-1} (50 mg P_2O_5 kg^{-1}) as KH_2PO_4, and 5.46 mg K kg^{-1} (160 mg K_2O kg^{-1}) as KH_2PO_4 and K_2SO_4.

The different Zn treatments were then applied to the NPK-fertilised soils at varying Zn application rates: D0 (control, without Zn application; the total Zn concentration in this treatment corresponds to the concentration in the original soil, 16.3 mg Zn kg^{-1}), D1 (15 mg Zn kg^{-1}; total Zn concentration, 31.3 mg Zn kg^{-1}), D2 (30 mg Zn kg^{-1}; total Zn concentration, 46.3 mg Zn kg^{-1}), D3 (60 mg Zn kg^{-1}; total Zn concentration, 76.3 mg Zn kg^{-1}), and D4 (140 mg Zn kg^{-1}; total Zn concentration, 156.3 mg Zn kg^{-1}). These doses were selected to examine the agro-environmental effects of different concentrations (agricultural, medium, and high) on an agricultural crop's biofortification and determine the application limits to avoid agro-environmental damage. Furthermore, fertilisation was carried out by adding the solid Zn product and homogenising it with the entire potting soil to prevent any potential aggregation of Zn particles.

The NPK-fertilised soils were treated with different forms of Zn: (i) zinc sulphate heptahydrate (Merck) [SULP]; (ii) a Zn complex called Zn lignosulphonate (complex derived from an organic source, a byproduct of the paper pulp production process from wood pulp) with sulfonated lignin wastes produced by the paper (Zn concentration: 12% *p*/*p*)

(commercial product Rayplex Zinc®, obtained from CQ Massó, Barcelona, Spain) [LIG]; and (iii) commercial ZnO NPs (Aldrich Chemistry, Stenheim, Germany) [NANO]. The ZnO NPs employed in this research were commercially sourced, with a primary particle size of $\leq$50 nm and a specific surface area ranging from 15 to 25 m^2 g^{-1}. The authors previously determined the nanoparticles' size and morphology using a transmission electron microscope (TEM). The ZnO-NPs exhibited a rod-like, elongated shape with a mean length (longest dimension) of 55 nm. The zeta potential of the Zn nanoparticles in solution was measured at -7.2 (indicating instability in the aqueous solution since the zeta potential value fell within the range of -30 to 30 mV). The average hydrodynamic diameters of the nanoparticle aggregates were 503 ± 142 nm (10% intensity) and 1486 ± 244 nm (90% intensity).

Four replicates were used for each treatment, with a total of 52 pots in a randomised complete block design. Three days after soil treatments with Zn, 8 lettuce seeds (long, light-green romaine lettuce, Verdecora) were sown in each pot. This 3-day period is intended to emulate actual fertilisation and planting conditions. Following sowing, the seeds were subjected to controlled germination conditions in a growth chamber. The growth chambers provided a controlled environment with a constant temperature of 25 °C, a relative humidity of 70%, and a 12 h light/dark cycle. After five days, the number of germinated seeds in each pot was counted. Subsequently, one plant was left in each pot. In March 2022, the pots were transferred to an uncontrolled greenhouse environment on the Universidad Politécnica de Madrid campus, where they were exposed to environmentally realistic conditions. Temperatures ranged from 4 °C (night) to 38 °C (day), with relative humidity varying between 20% and 85%. Soils were irrigated with tap water during the experiment to maintain a soil moisture of 60% water-holding capacity. Soil moisture was controlled through weighing.

Thirty-nine days after planting (DAP), the plants were collected and weighed. To desorb Zn from the root surfaces, they were first washed in deionised water using an ultrasound-assisted bath, followed by washing in Na_4EDTA in the ultrasound-assisted bath. The aerial parts of the plants were washed with deionised water. Subsequently, the samples were dried in an oven at a constant temperature of 60 °C until they reached a stable weight, after which they were stored in sealed containers for analysis. After the plants were harvested, the soil from each container was naturally dried and manually homogenised. A subsample of the soil was taken for laboratory analysis, and the remaining soil was returned to the pots for continued experimentation. The soil in the pots was then incubated at room temperature until the second seeding.

2.3. Second Lettuce Crop and Effect of Ageing Zn

The soils used in this second year were those from the previous crop. No additional Zn was applied to evaluate the residual effect of the previously applied Zn. All other cultivation procedures were similar to those of the prior year's crop. Additional N, P, and K were applied at the same doses as in the previous crop, and 8 lettuce seeds were sown in each pot (March 2023). The seeds underwent controlled germination conditions in a growth chamber, after which one plant was left in each pot. The pots were then placed in an uncontrolled greenhouse environment, where soil moisture was maintained at 60% of the water-holding capacity through regular weighing.

At 39 DAP, the plants were collected for analysis following the same procedure as the previous year. After harvesting, the soil from each container was naturally dried, manually mixed, and sieved (<2 mm), with a subsample retained for laboratory analysis.

2.4. Plant Analyses

The total Zn concentration in lettuce samples (root and aerial parts) was determined through wet digestion in Teflon vessels using a sample preparation block system (SPB Probe, PerkinElmer, Waltham, MA, USA). For this procedure, 0.5 g of dry matter samples were utilised along with 10 mL of HNO_3 (65%) and 5 mL of HCl (48%)]. The Zn concentration in the resulting extracts was then quantified using atomic absorption spectrometry (Analyst 700, PerkinElmer).

The root-to-shoot translocation factor (TF) was determined based on the nutrient concentrations in different plant components, according to the equation:

$$TF = \frac{Zn\ concentration\ in\ shoot}{Zn\ concentration\ in\ root} \quad (1)$$

Additionally, the bioconcentration factor (BF), also known as the transfer factor from soil to plant, serves as an indicator of the element's enhanced mobility from the soil to the plant. This factor was calculated as follows:

$$BF = \frac{Zn\ total\ concentration\ in\ plant\ (aerial\ part)}{Zn\ total\ concentration\ in\ soil} \quad (2)$$

The suitability of Zn concentrations in edible lettuce tissue for human consumption following biofortification with different sources and doses was evaluated. For this purpose, the values of dietary intake of the micronutrient (DIM) and the recommended intake (mg d^{-1}) of Zn were calculated according to Khan et al. [24]:

$$DIM = \frac{fresh\ leaf\ Zn\ concentration \times daily\ dietary\ intake}{body\ average\ weight} \quad (3)$$

The health risk index (HRI) of inhabitants associated with the consumption of biofortified lettuce was assessed using the oral reference dose (RfD) for Zn [25]:

$$HRI = \frac{DIM}{RfD} \quad (4)$$

The oral reference dose is defined as an estimate of the daily exposure for the human population (including sensitive subgroups) that is unlikely to pose a significant risk of adverse effects over a lifetime [26]. The recommended nutrient intake (RDA, mg d^{-1}) includes the following: (children) 3 mg d^{-1} from 7–12 months; 3–5 mg d^{-1} from 1–3 years; 5–8 mg d^{-1} from 4–8 years; 8–9 mg d^{-1} from 9–13 years; 5–8 mg d^{-1} for adolescents (14–18 y) and adults, all of which indicate the recommended values for males and females, respectively; and 11 and 12 mg d^{-1} when pregnant or lactating, respectively.

2.5. Soil Analysis

The total Zn content in the soil was determined through wet digestion in Teflon vessels using a sample preparation block system (SPB Probe, PerkinElmer). The digestion procedure involved the utilisation of 0.5 g of soil combined with a mixture of 10 mL of HNO_3 (65%) and 5 mL of HF (48%). Two certified reference soils, ERM-CC141 and BCR-143R from the Institute for Reference Materials and Measurements of the European Commission, were used to validate the analytical method.

The distribution of Zn across different fractions was determined using a four-step sequential extraction method. A soil-to-extractant solution ratio of 1:30 (g: mL) was utilised. The extractants and conditions employed for each step were as follows: (i) hydrosoluble Zn [27]—milli-Q® H_2O, shaking for 16 h; (ii) mobile fraction—exchangeable Zn and easily soluble organic complexes [28] with NH_4NO_3 (1M), under shaking for 24 h; and

(iii) exchangeable fraction—specifically adsorbed at mineral surfaces and bound to $CaCO_3$, metal-organic complexes with lesser bond strength [28], with NH_4Ac (1M, adjusted to pH 6), shaking for 24 h. After each step, the samples were centrifuged (4000 g for 600 s), and the supernatant solution was filtered. (iv) The residual Zn fraction was calculated by subtracting the sum of the other fractions from the total Zn concentration. The mobile Zn concentration was calculated as the sum of the hydrosoluble Zn concentration (step 1) and the Zn concentration in the mobile fraction (step 2). The exchangeable Zn concentration was calculated as the sum of the hydrosoluble Zn concentration (step 1), the Zn concentration in the mobile fraction (step 2), and the Zn concentration in the exchangeable fraction (step 3).

The concentration of potentially bioavailable Zn in the soil for lettuce was determined using an extractant solution composed of a mixture of low-molecular-weight organic acids (LMWOAs, employing a rhizosphere-based extraction method) [29]. For this analysis, 2 g of soil was weighed and mixed with 20 mL of a LMWOAs (10 mM) solution. This combination of the organic acid solution contained acetic, lactic, citric, malic, and formic acids in a molar ratio of 4:2:1:1:1, respectively.

The Zn concentration in all extracts was quantified using atomic absorption spectrometry (AAnalyst 900, PerkinElmer, Waltham, MA, USA).

2.6. Statistical Analysis

Correlation and statistical analyses were performed using Statgraphics Centurion 19 software (Manugistic, Rockville, MD, USA). Analysis of variance was conducted using the optimised Box-Cox General Linear Model. The main effects were differentiated using a protected Fisher's LSD test at a probability level of $p \leq 5\%$. In cases where interactions between factors were significant, we conducted a new ANOVA to determine the primary effects of the combined factor.

The EC50 values corresponding to 95% confidence intervals were calculated to evaluate the effect of Zn treatments on fresh weight using the 3-parameter logistic model for the first crop and the 4-parameter hormetic, dose-response model [30] for the second crop (ageing Zn).

The 3-parameter logistic model considers Ymax the maximum response (highest fresh weight), EC50, and b (maximum slope of the model) as main variables:

$$Y(c) = \frac{Y_{max}}{1 + \frac{x}{100-x}\left(\frac{c}{EC_{50}}\right)^{b}} \quad (5)$$

The 4-parameter hermetic model considers f (hermetic parameter), Ymax the maximum response (highest fresh weight), EC50, and b (maximum slope of the model) as main variables:

$$Y(c) = \frac{Y_{max}\ (1 + f \times c)}{1 + (2 \times f \times EC_{50}\ + 1)\left(\frac{x}{100-x}\right)\left(\frac{c}{EC_{50}}\right)^{b}} \quad (6)$$

3. Results

3.1. Effect of Treatments on the Lettuce Plant

Fresh matter (FM) yields, expressed as a percentage of the control, are presented in Figure 1. Increasing Zn doses resulted in average yield reductions of 15% (D1), 21% (D2), 29% (D3), and 34% (D4).

The application of D2 and higher doses with the SULP source exhibited a phytotoxic effect since a significantly lower fresh weight was obtained than in the control treatment. EC50 values for the first crop, determined using the three-parameter logistic model [31], indicated a slightly higher toxicity for the LIG (210.9 mg Zn kg^{-1} soil) compared to SULP

(244.7 mg Zn kg^{-1} soil) and NANO (376.9 mg Zn kg^{-1} soil) sources. In the second year, with the ageing of Zn sources, the hormetic dose-response model [30] showed varying trends across the sources. Doses D1 and D2 of all sources and D3 of SULP and NANO led to higher percentage yields compared to the control (D0). The highest percentage increase (35%) was observed with NANO-D1. The EC50 values for the second crop indicated a slightly higher toxicity for LIG, followed by SULP and NANO (192, 231, and 265 mg Zn kg^{-1} soil, respectively).

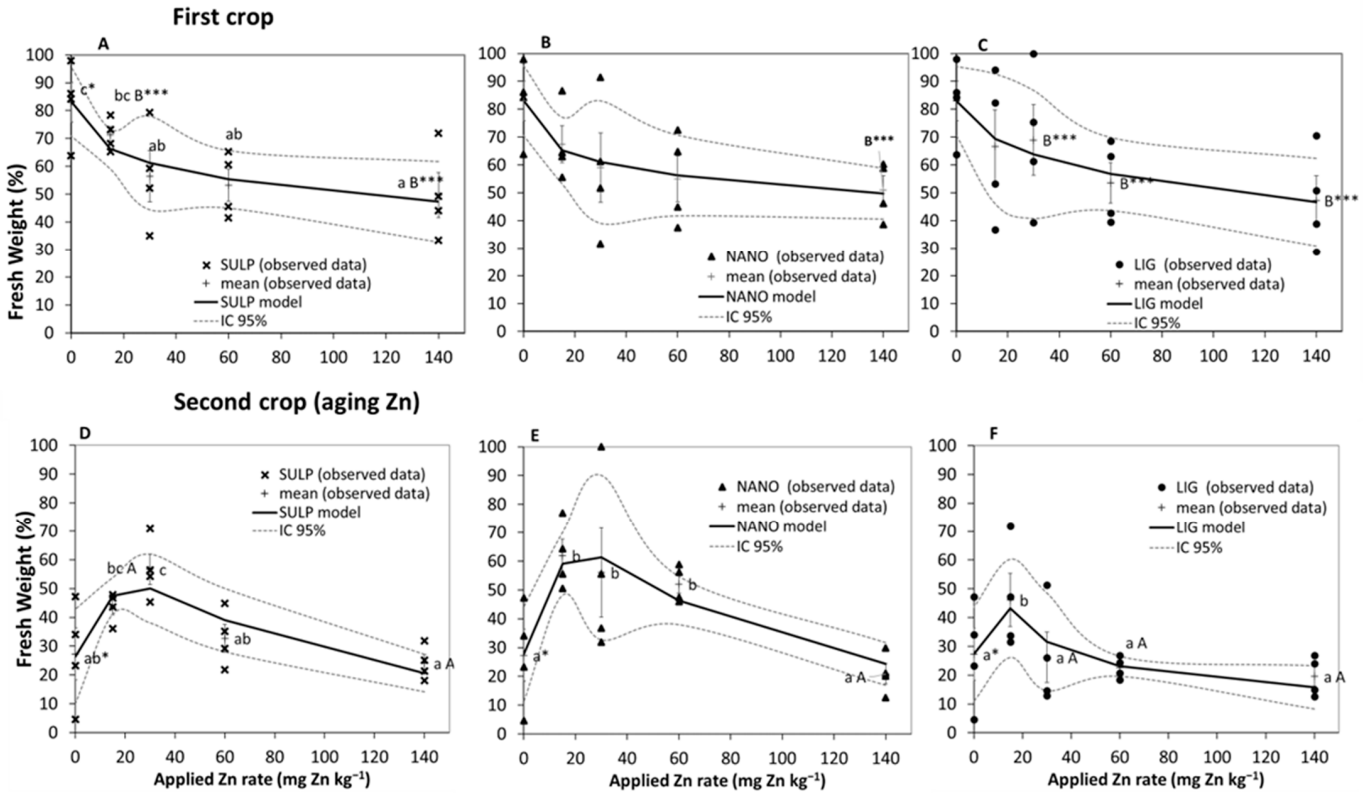

Figure 1. Effect of different Zn forms (SULP, Zn sulphate heptahydrate (**A**,**D**); NANO, commercial ZnO nanoparticle (**B**,**E**), and LIG, Zn lignosulphonate (**C**,**F**)) and Zn application rates (D0, control; D1, 15 mg Zn kg^{-1} soil; D2, 30 mg Zn kg^{-1} soil; D3, 60 mg Zn kg^{-1} soil; and D4, 140 mg Zn kg^{-1} soil) on fresh weight as a percentage of lettuce (*Lactuca sativa* L). The (+) symbol represents the mean of the observed data. The vertical line represents the standard deviation. The values were fitted to the 3-parameter logistic model for the first crop and the 4-parameter hormetic, dose-response model for the ageing Zn crop. The dotted line represents the IC 95% of the model. Statistical differences at $p < 0.05$ (LSD test) are indicated by different letters. *** and * corresponds to significance at 0.01 and 5% levels. Roman letters represent differences between doses for the same source and crop year. Capital letters indicate differences between the time elapsed since Zn application for the same treatment. Columns without letters within each group indicate no significant differences. No significant differences were detected between sources for the same dose and crop year. The statistical analysis considered the combined factor "forms of Zn × Zn application rate × time since Zn application".

Figure 2 presents the Zn concentrations in the edible part (leaves) of lettuces for each treatment and crop. No significant differences were found between Zn sources for each dose in both crop years. In the first year, increasing doses resulted in statistically significant increases in Zn concentration, except between D3 and D4 of NANO and LIG sources. In the second crop, increasing the application rate in the previous year led to increased Zn concentration in lettuce for all sources. Significant differences in Zn concentration between

the first and second crops were observed for SULP-D1, SULP-D2, and LIG-D1, indicating a substantial decline in Zn concentration.

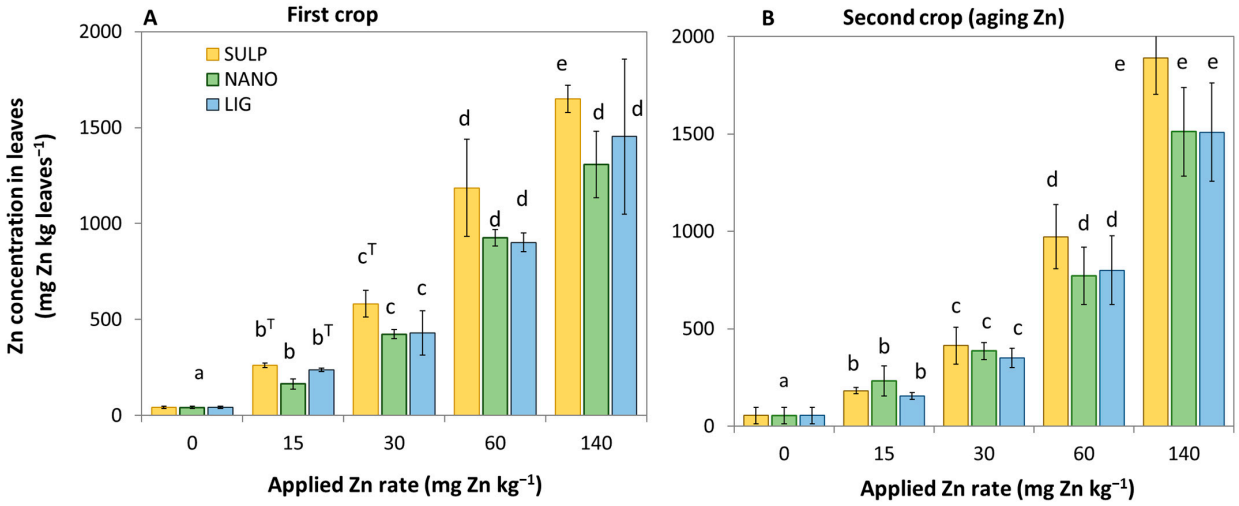

Figure 2. Zinc concentration in leaf obtained with each treatment (SULP, Zn sulphate heptahydrate; NANO, commercial ZnO nanoparticle, and LIG, Zn lignosulphonate) in the first crop (**A**) and in the second crop (**B**). Vertical lines on each data point represent the standard deviation from the mean. Statistical differences at $p < 0.05$ (LSD test) are indicated by different letters (roman letters indicate differences between doses for the same source and crop year). ^{T} highlights the differences between time since Zn application (first crop vs. ageing Zn). Within each group, columns without letters indicate no significant differences. The statistical analysis used a combined factor, "forms of Zn × Zn application rate × time since Zn application". The root–shoot TF values ranged between 0.44 and 1.35 in the first crop (for D0 and LIG-D3, respectively) and between 0.44 and 1.29 in the second crop (for D0 and NANO-D4, respectively) (Figure 3). TF values higher than unity were observed in specific treatments. In the first crop, SULP-D1, SULP-D2, SULP-D3, SULP-D4, LIG-D3, and LIG-D4 exceeded unity. In the ageing Zn crop, the TF values for SULP-D4, NANO-D3, NANO-D4, LIG-D1, LIG-D2, LIG-D3, and LIG-D4 also reached unity.

3.2. Dietary and Health Risks in Humans

The Zn intake from consuming the cultivated lettuce was calculated using the dietary intake of micronutrients (DIM) (Table 1). As expected, DIM increased with Zn treatments compared to the control treatment. The recent application of Zn at the lowest doses significantly increased Zn intake, with DIM values 6.17, 3.89, and 5.64 times higher than the control for SULP, NANO, and LIG at the D1 rate, respectively. Residual Zn (second crop) resulted in relatively smaller increases in DIM with 3.34, 4.25, and 2.84 times higher than control for SULP, NANO, and LIG at D1, respectively. A general trend of increasing Zn dosages was observed across all sources. Among the treatments, SULF-D4 exhibited the highest DIM values in both crops. Although this treatment provides a small percentage of the recommended Zn intake in both crops, the HRI exceeded unity, indicating a potential risk to the population [25,32] (Table 1). Zinc toxicity can adversely affect humans' nervous, cardiovascular, respiratory, renal, gastrointestinal, and reproductive systems [33]. These values exceeding unity were obtained with SULP-D3 for children, male and female adolescents, and female adults in the first crop (1.91, 1.11, 1.25, and 1.16 HRI values, respectively) and children and female adolescents in the second crop of lettuce (1.56 and 1.02, respectively) (the data do not show this). A similar pattern is also observed in lettuce fertilised with NANO or LIG treatments at the highest rate (D4), where children consuming the crop fertilised with these products at D3 (1.49, 1.24, 1.45, and 1.29 HRI with NANO-D3 first and second crop and LIG-D3 first and second crop, respectively). In addition, infants fed lettuce at the D3 rate also exhibited potential health risks (Table 1).

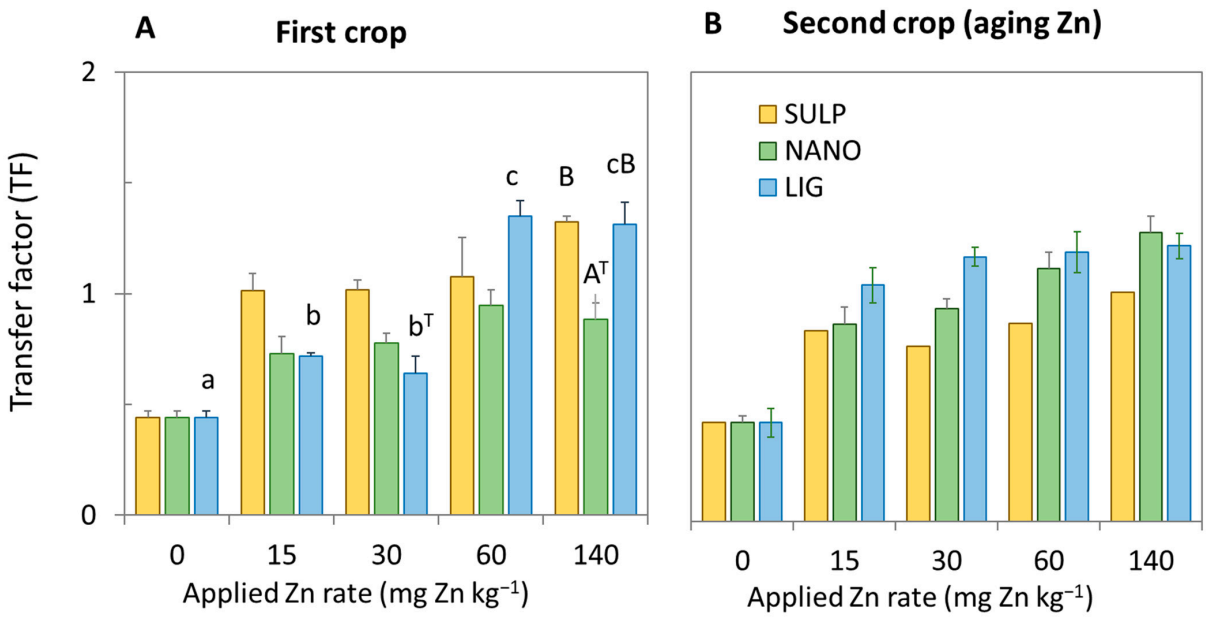

Figure 3. Transfer factor (TF) from root to shoot obtained for each treatment (SULP, Zn sulphate heptahydrate; NANO, commercial ZnO nanoparticle, and LIG, Zn lignosulphonate) in the first crop (**A**) and in the second crop (**B**). Vertical lines on each data point represent the standard deviation from the mean. Statistical differences at $p < 0.05$ (LSD test) are indicated by different letters ($p = 0.0000$). Roman letters indicate differences between doses for the same source and crop year. Capital letters show differences between sources for the same dose and crop year. ^{T} show differences between the time elapsed since Zn application for the same treatment. Columns without letters within each group indicate no significant differences. The statistical analysis considered the combined factors "Zn forms × Zn application rate × time since Zn application".

Table 1. Ranges of daily dietary intake values (DIM) of Zn and the health risk index (HRI) were obtained for the applied treatments. SULP, Zn sulphate heptahydrate; NANO, commercial ZnO nanoparticle; and LIG, Zn lignosulphonate. D0, D1, D2, D3, and D4 corresponds to 0, 15, 30, 60 and 140 mg Zn kg^{-1} soil, respectively.

		Daily Dietary Intake Values (mg d^{-1})				**Health Risk Index (HRI)**			
		First Crop		**Second Crop [1]**		**First Crop**		**Second Crop [1]**	
		MIN [2]	MAX [2]	MIN [2]	MAX [2]	MIN [2]	MAX [2]	MIN [2]	MAX [2]
Control	D0	0.010	0.020	0.013	0.026	0.033	0.068	0.043	0.088
SULP	D1	0.062	0.126	0.043	0.088	0.205	0.419	0.143	0.293
SULP	D2	0.137	0.281	0.098	0.200	0.458	0.935	0.326	0.665
SULP	D3	0.280	0.572	0.230	0.469	0.933	1.908	0.765	1.565
SULP	D4	0.390	0.796	0.446	0.912	1.298	2.654	1.488	3.041
NANO	D1	0.039	0.079	0.055	0.112	0.129	0.264	0.183	0.374
NANO	D2	0.100	0.205	0.091	0.186	0.334	0.682	0.304	0.621
NANO	D3	0.218	0.446	0.182	0.372	0.728	1.488	0.606	1.240
NANO	D4	0.309	0.631	0.357	0.729	1.029	2.103	1.189	2.431
LIG	D1	0.056	0.115	0.037	0.075	0.187	0.383	0.122	0.249
LIG	D2	0.102	0.208	0.083	0.169	0.338	0.692	0.276	0.564
LIG	D3	0.213	0.435	0.189	0.386	0.709	1.450	0.630	1.288
LIG	D4	0.343	0.701	0.356	0.728	1.143	2.337	1.188	2.428

[1] Aging Zn; [2] The lowest values (MIN) of daily dietary intake (mg d^{-1}) and health risk index (HRI) correspond to male adults, and the highest values (MAX) correspond to children.

3.3. Zinc Status in Soil

The application of different Zn sources resulted in variations in all estimated soil Zn concentrations. Multifactor analyses of variance showed significant ($p < 0.0001$) differences for the main effect of the chemical forms of Zn (SULP, CHE, LIG, and NANO). LIG and NANO exhibited the lowest hydrosoluble Zn and bioavailable Zn concentrations (LIG ≈ NANO < SULP) yet contributed a higher percentage of Zn in the mobile fraction (SULP < NANO ≈ LIG). NANO's contribution to the Zn exchangeable fraction was comparable to that of SULP, while LIG had a lower percentage (LIG < SULP ≈ NANO). Zinc application rates also affected soil Zn concentrations. The results also showed variations in the percentage of hydrosoluble Zn ($p < 0.001$) and bioavailable Zn ($p < 0.05$) as a function of the time elapsed since Zn application. While higher percentages of hydrosoluble Zn were observed in the first crop, the percentages of bioavailable Zn were higher in the second crop (with ageing Zn).

An interaction between Zn forms and application rates was observed ($p < 0.0001$) for all variables studied. Figure 4 illustrates the concentration of Zn associated with different soil fractions in the first and second crops. In general, the concentrations of water-soluble Zn for both crop years increased with the application rate. Significant differences between sources (at the same dose and year) were only observed with SULP D3 and D4 in the first year; however, no such differences were detected in the second year of cultivation. The concentration of hydrosoluble Zn decreased from the first to the second year for SULP at D4, while it increased for SULP and NANO at D1, LIG at D3, or NANO and LIG at D4.

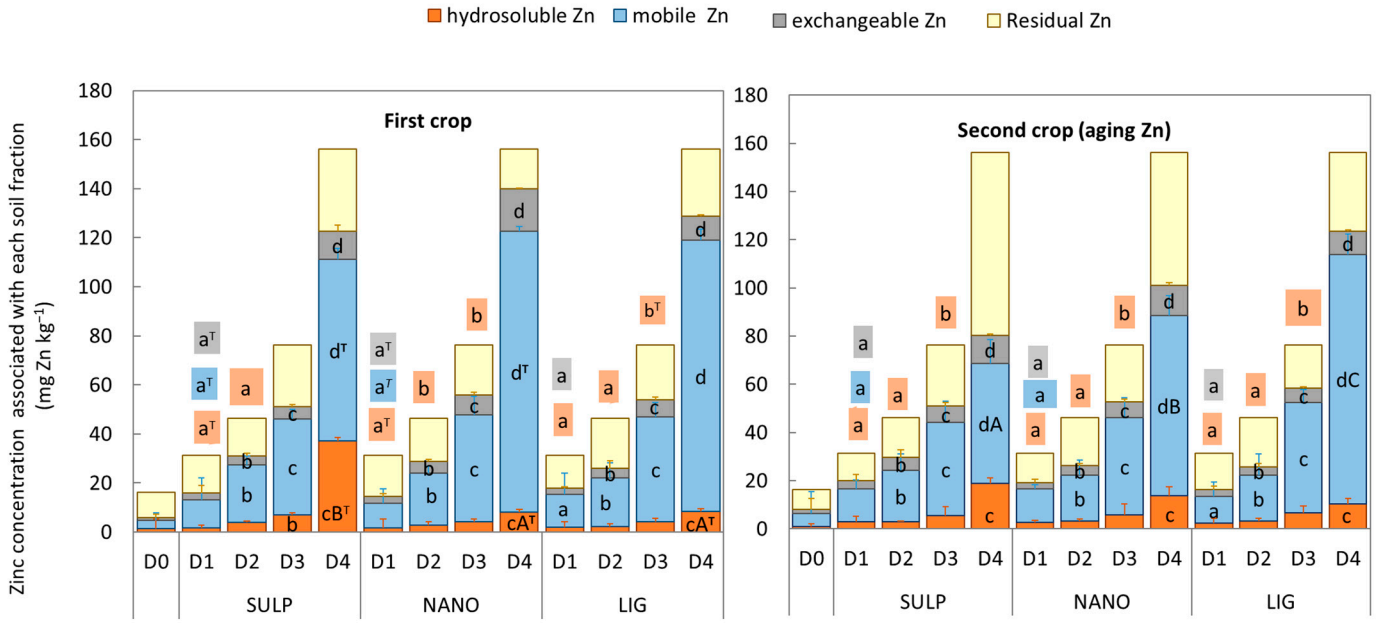

Figure 4. Effect of different sources (control; SULP—zinc sulphate heptahydrate; NANO—commercial ZnO nanoparticle; and LIG—Zn lignosulphonate) and rates (D0, D1, D2, D3, and D4 corresponds to 0, 15, 30, 60, and 140 mg Zn kg^{-1}, respectively) on the cumulative percentage of Zn (hydrosoluble Zn, mobile Zn, exchangeable Zn, and residual Zn). Statistical differences at $p < 0.05$ (LSD test) are indicated by different letters ($p = 0.0000$). Roman letters indicate differences between doses for the same source and crop year. Capital letters show differences between sources for the same dose and crop year. ^{T} show differences between the time elapsed since Zn application for the same treatment. Columns without letters within each group indicate no significant differences. The background colour of the letters corresponds to the colour of the fraction represented. The statistical analysis considered the combined factors "forms of Zn × Zn application rate × time since Zn application".

No significant differences were found between Zn sources at the same rate and crop year for the Zn concentration in the mobile fraction, except in the second year for sources applied at D4. The residual effect showed higher mobile Zn for the LIG source, followed by NANO and finally SULP. In both crop years, the concentrations of water-soluble Zn increased with the application rate. The concentration of mobile Zn decreased from the first to the second year for SULP and NANO at D4, while it increased for SULP and NANO at D1. The trend observed with exchangeable Zn was similar to that of the mobile Zn.

Figure 5 shows the potential bioavailable Zn concentration for the different treatments and times since Zn application. As expected, the results indicated that the concentration of bioavailable Zn in the soil increased with increasing application rates for each source. In both crop years, bioavailable Zn concentrations were similar across sources at each application rate, except for D1 and D4 in the second crop (SULP-D1 ≈ NANO-D1 > LIGNO-D1; SULP-D4 > NANO-D4 ≈ LIGNO-D4). Increases in the bioavailable Zn concentrations were shown between the two years for SULP-D1, NANO-D1, and NANO-D3. As shown in Figure 6, the bioconcentration factor (BF) or soil-to-plant transfer factor showed that SULP is the most efficient source for recent applications. However, in the second crop, a reduction in BF is observed.

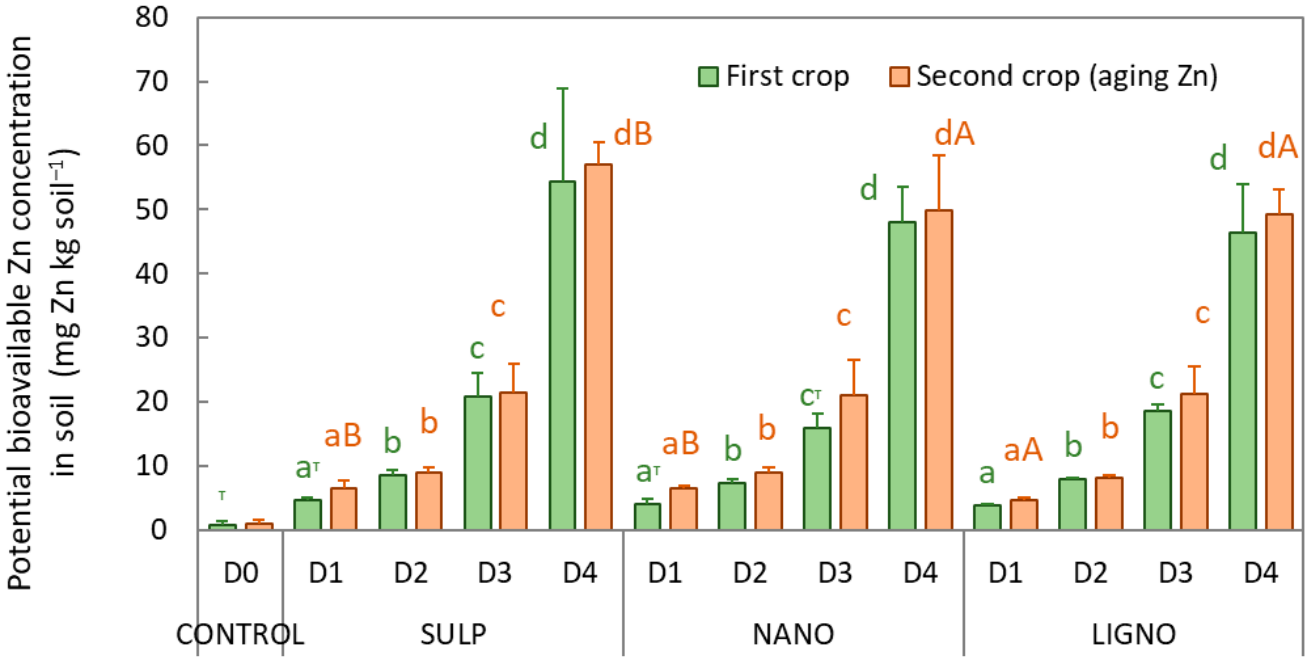

Figure 5. Effect of different sources (control; SULP—zinc sulphate heptahydrate; NANO—commercial ZnO nanoparticle; and LIG—Zn lignosulphonate) and rates of Zn (D0, D1, D2, D3, and D4 corresponds to 0, 15, 30, 60, and 140 mg Zn kg^{-1}, respectively) on potential bioavailable Zn (rhizosphere-based extraction method) in soil at two times (first crop and second crop-aging Zn crop). Statistical differences at $p < 0.05$ (LSD test) are indicated by different letters ($p = 0.0000$). Roman letters indicate differences between doses for the same source and crop year. Capital letters show differences between sources for the same dose and crop year. ^{T} show differences between the time elapsed since Zn application for the same treatment. Columns without letters within each group indicate no significant differences. The statistical analysis considered the combined factor "forms of Zn × Zn application rate × time since Zn application".

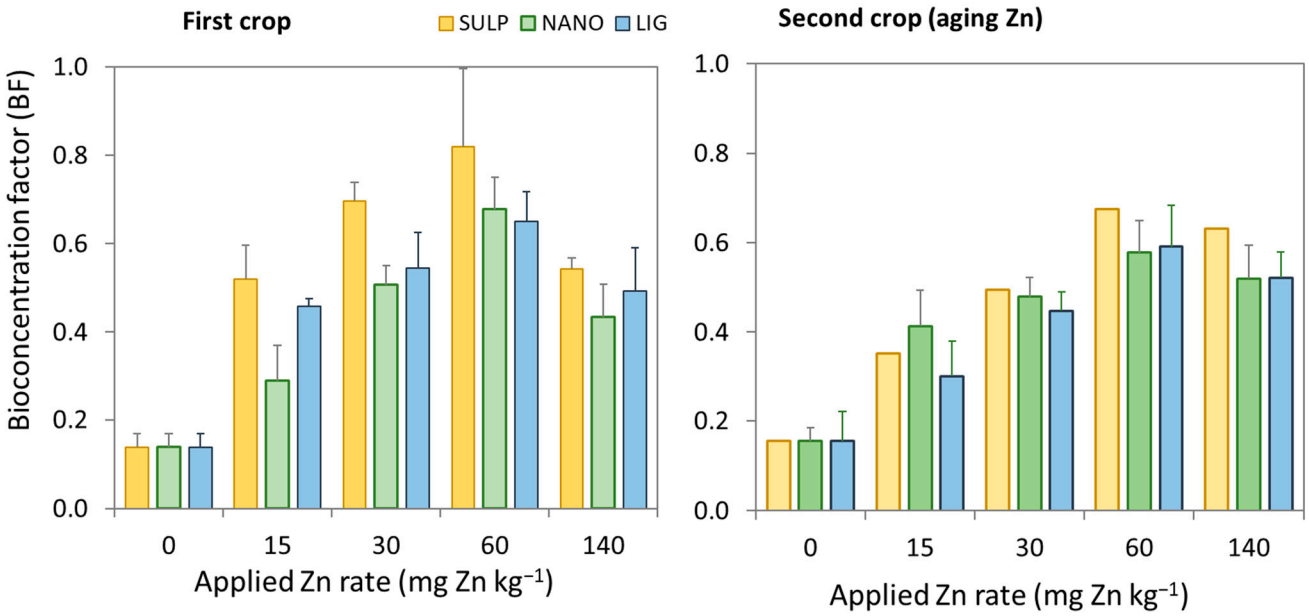

Figure 6. Bioconcentration factor (BF) or transfer factor from soil to plant obtained with each treatment (SULP, Zn sulphate heptahydrate; NANO, commercial ZnO nanoparticle; and LIG, Zn lignosulphonate). The vertical line at each data point represents the standard deviation from the mean.

4. Discussion

The findings indicate that the three Zn sources tested differed in their influence on soil Zn status, leading to variations in their effectiveness as fertilisers and their impact on the plant parameters studied.

Zinc sulphate is widely used, with soil application rates of Zn ranging from 10 to 100 kg $ZnSO_4.7H_20$ per hectare [34]. This Zn source releases significant Zn^{2+} ions due to its high solubility (965 g L^{-1} water at 20 °C). In our study, the application of SULP resulted in a high percentage of water-soluble Zn, which may have influenced the Zn concentration in the leaves. The calculated root-to-shoot transfer factors indicated values above unity when this Zn source was applied at any dose, indicating highly effective internal movement of Zn within the plant. This high root-to-stem transfer when this Zn source is applied can be explained because long-distance Zn transport occurs mainly through the flow of Zn^{2+} ions in the xylem stream [35]. The bioconcentration factor (BF) further supported this, showing that SULP is the most efficient source for recent applications. These findings were also reflected in the percentage of the DIM value obtained with this Zn source, which increased from 517% to 3880% of the control values (D1 and D4, respectively). However, the higher application doses, D3 and D4, may pose a potential risk to the population, as indicated by the health risk index (HRI) values exceeding unity since they are considered unsafe for human health [25,32]. Zinc uptake in plants primarily occurs through the uptake of Zn^{2+} ions from the soil solution, facilitated by a dynamic and complex process. In our study, limitations in root development due to the small pot size may have influenced nutrient uptake by the root, the distribution of Zn in the soil, and the bioconcentration factor [36]. Uptake is influenced by the ion concentrations at the root surface, the plant's demand, and the roots' ability to absorb Zn [37].

For the other two sources studied, NANO and LIG, our results reveal a similar behaviour in soil during the first year of cultivation. The concentrations of hydrosoluble Zn, mobile Zn, and bioavailable Zn in the soil were comparable for both sources. In general, the BF values showed a similar trend for both sources, with an increase up to D3 indicated a very intense bioaccumulation. The differences in behaviour between these sources and

the SULP source may be due to the Zn^{2+} uptake by roots, that is to the difference in the concentration-dependent Zn^{2+} uptake kinetics which is dependent on the state of Zn in the soil [35]. In addition, agents that effectively complex Zn^{2+} (as lignosulphonate) reduce the activity of free Zn^{2+} in solution [38]. Therefore, the actual Zn^{2+} activity at the root surface may be considerably lower than the activity in total solution [35]. On the other hand, different studies using ZnO in nanoparticle sizes have reported that the key factor controlling Zn uptake by the plant is the concentration of dissolved Zn, rather than the concentration of the nanoparticle itself [39,40]. This concentration of dissolved Zn is influenced by the size of the applied nanoparticle. Da-Cruz et al. [39] reported that Zn uptake by the plant with ZnO nanoparticles larger than 300 nm was lower due to their lower solubility.

Furthermore, a high Zn concentration in lettuce leaves indicated a significant enrichment in this micronutrient, although the biofortification capacity was limited by phytotoxicity. Zinc concentration higher than 400 mg kg^{-1} in the dry mass of plant tissue is toxic to plants [41]. This phytotoxicity was evident in crop yield reduction (fresh weight). Some authors have suggested that heavy metal toxicity reduces stem and root hydraulic conductivity and decreases the specific hydraulic conductivity of xylem and leaves [39]. Furthermore, when plants are exposed to very high doses of Zn, they prioritize Zn retention to mitigate toxicity in their aerial tissues [42]. This may explain the decrease in TF and BF values at toxic doses. These high-dose treatments (D3 and D4) may represent a health risk index according to HRI values above unity. On the other hand, although the Zn concentrations in the edible part of lettuce biofortified at the lowest doses (D1 and D2) did not cover the full nutritional needs for Zn intake of the individual, they may represent a significant contribution to the daily Zn intake of the population, since lettuce is the most widely grown and consumed leafy vegetable worldwide. The high-dose treatments (D3 and D4) may pose a health risk index, as indicated by HRI values exceeding unity. On the other hand, while the Zn concentrations in the edible part of lettuce biofortified at the lowest doses (D1 and D2) did not fully meet nutritional needs for Zn intake for individuals, they may still contribute significantly to the daily Zn intake of the population, since lettuce is the most widely grown and consumed leafy vegetable worldwide.

Soil-applied Zn is involved in various physical, chemical, and biological reactions that determine its concentration in the soil solution. Zinc in the soil may become less available for plant uptake due to processes such as precipitation, adsorption, or the formation of complexes with other compounds [18]. Our results did not align with the expected outcomes. No decrease in water-soluble, mobile, or potentially bioavailable Zn in the soil was observed in the second crop compared to the first crop, except for the SULP-D4 treatment.

On the contrary, the opposite effect was observed with the NANO-D4 and LIGNO D4 treatments, where an increase in Zn was found in these soil fractions. The quantity of metals in these soil fractions represents the most accessible fraction, specifically the water-soluble form of Zn. This suggests that these treatments were able to maintain or even enhance Zn availability in forms that the plant more readily absorbs. This effect could be attributed to the incubation conditions between the two crops. Additionally, plants influence the release of certain weakly bound trace metals from the soil rhizosphere. The concentration of bioavailable Zn, estimated by the LMWOAs chemical extraction method, depends on the impact of low-molecular-weight organic acids present in the soil rhizosphere. These organic acids can dissolve Zn and facilitate its uptake by plant roots. The metals extracted by this rhizosphere-based technique correspond to the mobile fractions within the soil solution, contributing to the potential bioavailable Zn. These findings align with those of Obrador et al. [43] who observed higher extractability of bulk ZnO and ZnO NPs compared to $ZnSO_4$ during an experiment with agricultural soils with varying pH. The authors suggested that

this effect could be partially attributed to root exudates, which may have influenced the dissolution of metal oxides from both nanoparticles and bulk materials. Consequently, soluble Zn levels increased, potentially helping to mitigate the effects of Zn adsorption on soil components. Obrador et al. [43] also noted that nanoparticles could function as reservoirs for sustained ion release.

The reduction in BF in the second year suggests a decreased efficiency in the Zn uptake process by the plant. However, in the second year, an increase in root-to-leaf Zn transfer (TF) was observed with LIG-D2 and NANO-D4 compared to the first year. This increase suggests that the plant became more efficient at transporting Zn from the roots to the leaves. Root-to-leaf Zn transfer may be influenced by factors such as changes in plant physiology, nutrient competition, or limitations in the root uptake process [44].

The residual effect of Zn applied to the soil in the first year positively affected plant fresh weight in the second year. Specifically, an increase in FM was observed with all sources (SULF, LIG, and NANO) at doses D1 and D2, compared to the control; the results also indicated a significant decrease in plant Zn concentration in the second crop for the SULP D1 and D2 and LIG D1 treatments. Nevertheless, all treatments resulted in leaf Zn concentrations above the critical concentration, typically between 15 and 20 ppm [34]. Notably, this decrease in Zn concentration only slightly affected the percentage of dietary Zn intake.

5. Conclusions

Zinc bioaccumulation in the first crop plants was high at 15 and 30 mg Zn kg^{-1} soil doses. However, doses of 60 and 140 mg Zn kg^{-1} resulted in toxicity effects on plants by limiting Zn uptake and translocation to aerial tissues. The use of ZnO nanoparticles may be an efficient alternative to natural Zn complexes, such as Zn-lignosulphonate, up to a dose of 30 mg Zn kg^{-1}. The $ZnSO_4$ source demonstrated high availability and effectiveness in the first crop. Residual Zn in the soil can benefit subsequent crops, eliminating the need for the reapplication of high doses. Zinc application in the first year also positively influenced the residual effect on lettuce yield in the second year, especially at low doses. ZnO NP applications can fulfil the need for successive applications by providing available Zn for subsequent crops.

There is a potential health risk for specific populations (children and adolescents) when consuming lettuce grown in soils treated with 60 and 140 mg Zn doses. These findings enhance our understanding of the agronomic and nutritional aspects of Zn supplementation in lettuce cultivation, offering valuable insights into agricultural practices and human health. Further research is needed to explore additional factors influencing the efficiency of Zn uptake and its impact on plant growth and the nutritional quality of edible parts of crops.

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Abbreviations

The following abbreviations are used in this manuscript:

SULP	Zn sulphate heptahydrate
LIG	Zn complex: Zn lignosulphonate
NANO	commercial ZnO nanoparticle
TF	root–shoot translocation factor
BF	bioconcentration factor or transfer factor from soil to plant
DDI	human daily dietary intake
RDA	recommended dietary allowance
FM	fresh matter

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