Article

Effect of the Long-Term Application of Sewage Sludge to A Calcareous Soil on Its Total and Bioavailable Content in Trace Elements, and Their Transfer to the Crop

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Abstract: Sewage sludge (SS) can be used as an organic amendment in agricultural soils, provided they comply with the relevant legislation. This use can incorporate traces of metals into the soil, which can cause environmental or human health problems. In the study period between 1992 and 2018 (26 years), it was observed that the use of SS as an organic fertilizer significantly increased the total concentration of Zn, Cu, Cr, Ni and Hg of this study between 55.6% (Hg) and 7.0% (Ni). The concentration of Zn, Cu, Pb, Ni and Cd extracted with DTPA, also increased between 122.2% (Zn) and 11.3% (Cd). In contrast, the Mn concentrations extracted with Diethylene Triamine Pentaacetic Acid (DTPA)were 6.5% higher in the treatments without SS. These changes in the soil had an impact on the crop, which showed a significant increase in the concentration of Zn, Cu and Cr in the grain, between 15.0% (Cr) and 4.4% (Cu), and a decrease in the concentration of Mn, Cr and Ni in the barley straw when SS was added to the soil between 32.2% (Mn) and 29.6% (Ni). However, the limits established by current legislation on soil protection and food were not exceeded. This limited transfer to the crop, is likely due to the high content of carbonates and organic matter in the soil, which limit the bioavailability of most of the trace metals (TM) in the soil. As a conclusion, we observe that the use of SS as an organic amendment increased the concentration of some TM in the soil, in its bioavailable forms, and in the crop.

Keywords: trace metals; sewage sludge; calcareous soil; extraction DTPA; crop; transfer; long time

1. Introduction

As a consequence of the urbanization and industrialization of urban environments, there is a growing generation of wastewater of domestic and industrial origin. This water must be treated to ensure an environmentally safe and economically viable final destination. This wastewater treatment is generally carried out in treatment plants, and involves a generation of sludge (sewage sludge, SS). As in many other countries, in Spain, SS is considered waste, in accordance with the provisions of Law 22/2011 [1] on waste and contaminated soils, and must be managed in accordance. Depending on its final composition, SS can have three possible destinations within the Spanish legislative framework. By order of priority, these destinations are: (i) the application on agricultural soils, (ii) incineration or co-incineration, and (iii) deposition in landfills. In Spain, the annual generation of SS accounts for approximately 1,000,000 tons of dry matter, remaining stable in recent years, approximately 80% of which is used in agricultural soils [2].

The agricultural use of SS as an organic amendment has been reported to provide several benefits to agrosystems [3–6]. They include an increase in macro and micro nutrients

in soil [7,8] and an increase in the content of organic matter [9–12]. These inputs normally result in higher crop yields [10,13,14].

However, this application involves a series of environmental and human health risks. Some of these risks are the possible transfer of genes encoding resistance to antibiotics [11], and the accumulation of persistent organic pollutants [4] or of trace metals (TM) in soil. Trace metals can accumulate in the surface layers [12,15–17] and/or leach out of the agrosystem [18–20], causing environmental contamination [4,21], ecotoxicity problems [14,22,23] or becoming part of the trophic chain due to their translocation in crops [21,24].

To control these risks, different strategies have been developed in order to minimize the impact of TM on soils where SS is applied [25]. On the one hand, there are legislative tools addressing soil protection [25–27] and animal and human food protection [28,29]. On the other hand, SS purification and stabilization techniques have been improved to obtain higher quality biosolids with a lower concentration in TM [30–32].

Along with these strategies, long-term studies are of great importance, as they allow observing the actual evolution of TM in soil, and therefore, the assessment of their true risk of accumulation and possible transfer to food, in the particular conditions of each soil and crop [16,17,32–34]. In this sense, the literature shows variable results, with some studies showing enrichments of TM in crops [16,33], and other studies reporting no accumulation [24,35]. This reflects the possible influence of edaphoclimatic conditions and SS composition on the consequences of their application, and highlights the need to consider these conditions in its study.

In this framework, a relevant factor in the assessment of the consequences of SS application to soil is to determine the proportion of TM present in the soil that are available to be adsorbed by crops. This proportion constitutes the so-called bioavailable fraction, and is influenced by the nature of the element, their interaction with soil components, soil properties, and the contact time of soil with the metal [36]. Some studies have shown that this concentration increases faster than the total concentration in soil as a consequence of the application of SS [37]. A series of methodologies have been developed to determine the concentration of bioavailable TMs in soils. A widely extended and accepted one is the use of the chelating agent DTPA (Diethylene Triamine Pentaacetic Acid) as an extractant [16,37–41].

Among the aspects related to the composition of soil that can modulate the bioavailability of TM, the amount and type of organic matter received with SS is determinant, as it can determine the formation of chelates, exchange complexes or organometallic complexes [12,42,43]. These phenomena can lengthen the retention time of trace metals in soil [44], limiting their bioavailability and their introduction into the trophic chain. On the other hand, in calcareous soils, the bioavailability of TMs in relation to their total content is assumed to be lower than in other types of soils due to the presence of carbonates. Carbonates maintain a high soil pH and are associated with sorption and precipitation processes of TM [15,45].

The general objective of this study was to assess the effect of long-term application of SS on the content of TM and their analytical bioavailability, and actual transfer to the most frequent crop in the rotation used in the area of study, in a cultivated calcareous soil. To this end, we studied the effect of the accumulated dose of SS on the total and DTPA-extracted content of TM in the soil after 26 years of SS application at different doses and frequencies in an experimental field. Then, the actual absorption by the crop was assessed by studying the concentration in TM of grains and leaves of barley grown after 26 years of experimentation.

2. Materials and Methods

2.1. Site Description

The study was carried out in an experimental field located in the municipality of Arazuri, in the region of Navarre, in Northern Spain. The field belongs to the local municipal wastewater treatment company (Commonwealth of Pamplona, Navarre, Spain).

The experimental field was setup in 1992, in order to assess the long-term effect of the direct application of SS as an agricultural amendment both in the soil and crops.

The experimental field is located on a Calcaric Cambisol [46], with a loam-clay-silty texture and more than 16% carbonates in the upper horizon (0–30 cm). The main soil characteristics at this depth are shown in Table 1. It is a well-drained soil, without problems of effective depth and under rainfed agricultural use for decades. Climate in this region is temperate sub-humid Mediterranean, with an average annual temperature of 12.9 °C, an average annual rainfall of 771 mm, and an average potential evapotranspiration according to Thornthwaite [47] of 696.7 mm between 1981 and 2010. [48].

Table 1. Average values of the physicochemical properties of the tilled horizon (0–30 cm) of the study soil and analytical methods used. Control soil and soil amended with sewage sludge for 26 years. Values are given as the mean $\pm$ standard deviation (n = 4).

Soil Physical-Chemical Properties	Control	After 26 Years	Analysis Methods
pH	8.67 ± 0.03	8.45 ± 0.11	Soil pH in water 1:2 [49]
Electric Conductivity ($\mu s \, cm^{-3}$)	169 ± 10	233 ± 11	Diluted soil:water Extract 1:2.5 [50]
Bulk density ($g \, cm^{-3}$)	1.59 ± 0.08	1.54 ± 0.01	Core Method [51]
Carbonates (%)	16.0 ± 2.1	16.7 ± 1.4	Bernard calcimeter [52]
Organic Carbon (%)	1.35 ± 0.02	1.56 ± 0.03	Wet Oxidation-Redox Titration Method [53]
Total Phosphorus ($mg \, kg^{-1}$)	603 ± 52	945 ± 38	Microwave digestion + ICP-MS [54,55]
Available Phosphorus ($mg \, kg^{-1}$)	32.2 ± 1.5	99.4 ± 8.4	Sodium Bicarbonate-Extractable P at pH 8.5 [56]
Total Potassiun ($mg \, kg^{-1}$)	9294 ± 550	9709 ± 185	Microwave digestion + ICP-MS [54,55]
Available Potassiun ($mg \, kg^{-1}$)	109 ± 15.0	110 ± 9.00	Ammonium Acetate Method at pH 7.0 [57]

2.2. Experimental Design

The experimental field was setup to study the effect of SS in the soil and crops, by verifying which doses were the most adequate ones to avoid risks of contamination by TM, on a conventional crop rotation in this area. The design is block factorial, with four repetitions (n = 4), and with combinations of two application rates (40 Mg ha^{-1} and 80 Mg ha^{-1}) of SS, and three frequencies (1, 2 and 4 years). Therefore, the treatments applied are 40 Mg ha^{-1} annually, 40 Mg ha^{-1} every two years, 40 Mg ha^{-1} every four years, 80 Mg ha^{-1} annually, 80 Mg ha^{-1} every two years and 80 Mg ha^{-1} every four years. There is also a control treatment in which SS or other fertilizers are not applied denominated "Control", and a treatment that receives mineral fertilization (46% urea and Ammonium Sulfate) at the commercially recommended dose denominated "MinFer". The individual plots per replicate, corresponding to the different treatments, had a surface area of 35 m^2 (10 m $\times$ 3.5 m).

The doses were calculated in 1992 based on the restrictive regulation of 250 N fertilizer units (NFU) per hectare and year, without taking into account the N yield for the year of application. As such, the treatment using 40 Mg ha^{-1} per year was set as the one corresponding to this dose, and those receiving less or more SS were used to follow the evolution of soil and crops below and above the recommended dose at the time. The frequencies of two and four years were established according to the common practices of farmers in the area, and the recommendations of the entity that supplied the SS. The N equivalence of the added doses has varied (Table 2) due to the SS composition changes over time (Table 3).

Table 2. N fertilizer units (NFU) provided by the study doses according to the N concentration in SS as used at the beginning of the study (1992), and at the end of the study (2018), without taking into account the annual yield of N for the dose provided.

Doses Sewage Sludge	NFU 1992	NFU 2018
40 Mg ha^{-1} every year	250	480
80 Mg ha^{-1} every year	500	960

Due to the interest of the cumulative study of TM, from the total doses provided in each treatment during the 26 years of the study, 5 ranges of accumulated SS were established: (i) 0 Mg ha^{-1} of SS (MinFer and Control), (ii) 250 Mg ha^{-1} of SS (40 Mg ha^{-1} every 4 years), called 250MgSS, (iii) correspondent 520 Mg ha^{-1} of SS and 560 Mg ha^{-1} of SS (40 Mg ha^{-1} every 2 years and 80 Mg ha^{-1} every 4 years) called 500MgSS, (iv) 1040 Mg ha^{-1} of SS (40 Mg ha^{-1} every year and 80 Mg ha^{-1} every 2 years) called 1000MgSS and (v) 2080 Mg ha^{-1} of SS (80 Mg ha^{-1} every year) called 2000MgSS. The crops used corresponded to a rainfed conditions in a rotation of 3 years (barley–barley–sunflower), managed in a conventional way (annual tillage with a 30 cm deep moldboard plough, and application of phytosanitary products according to the needs of the crops each year). The 2018 yield values expressed in percent 12% moisture were provided as supplementary material, firstly, for each fertilization treatment (Table S1) and, secondly, an average value was provided for treatments that had received SS and those that had not received SS (Table S2).

2.3. Sewage Sludge Characteristics and Application

The application of SS in each campaign was carried out in September, coinciding with the work of preparing the soil for cultivation. This application was made using a 3.5-m wide spreader trailer. Once the amount of SS corresponding to each treatment was spread, it was mixed with a moldboard plough down to 30 cm. This was conducted annually between three and four weeks before seeding.

The SS used in this study comes from the wastewater treatment plant corresponding to the city of the Pamplona region and its metropolitan area, with approximately 335,000 inhabitants [58]. Wastewater undergoes a primary treatment, followed by a biological treatment and a nitrification and denitrification treatment. The resulting sludge undergoes a mesophilic and dehydration treatment, obtaining biologically stable SS, with the physical-chemical characteristics detailed in Table 3. These characteristics comply with the Spanish national regulations on the maximum concentration of TM in SS used as organic amendments in Agriculture [26].

Table 3. Physical-chemical properties of the sewage sludge (SS) used in this study in 1992 (start of the experiment) and 2018 (sampling) together with the analytical methods used. Mean annual values ± standard deviation. The maximum legal limits of TM allowed in SS intended for agricultural use are indicated for each TM.

Sewage Sludge Physical-Chemical Properties	1992	2018	Legal Limit	Analysis Method
		General parameters		
pH	8.01	8.16 ± 0.03	NA	Soil pH in water 1:5 [49]
Electric Conductivity (µs cm^{-3})	NA	1795 ± 28	NA	Diluted Extracts 1:5 [50]
Dry material (%)	28.8	18.1 ± 0.4	NA	Direct calcination at 540 °C [59]
Volatile matter (% *of dry substance*)	NA	62.8 ± 1.9	NA	Direct calcination at 540 °C [59]
C/N	10	5.35 ± 0.08	NA	
		Fertilizing elements (% of dry substance)		
N total	2.18	5.85 ± 0.13	NA	Kjeldahl digestion and distillation [60]
N ammonium	0.18	0.75 ± 0.02	NA	Kjeldahl digestion and distillation [60]
Phosphorus (P$_2$O$_5$)	3.62	5.59 ± 0.22	NA	Microwave digestion + ICP-MS [54,55]
Potassium (K$_2$O)	0.51	0.62 ± 0.05	NA	Microwave digestion + ICP-MS [54,55]
Iron (Fe)	1.48	1.68 ± 0.04	NA	Microwave digestion + ICP-MS [54,55]
Calcium (CaO)	NA	7.98 ± 0.29	NA	Microwave digestion + ICP-MS [54,55]
Magnesium (MgO)	NA	1.10 ± 0.05	NA	Microwave digestion + ICP-MS [54,55]
		Trace metals (mg Kg^{-1} dry weight)		
Cadmium (Cd)	<10	0.88 ± 0.09	40	Microwave digestion + ICP-MS [54,55]
Copper (Cu)	302	187 ± 11	1750	Microwave digestion + ICP-MS [54,55]
Nickel (Ni)	75	32.1 ± 0.77	400	Microwave digestion + ICP-MS [54,55]
Lead (Pb)	191	39.0 ± 1.2	1200	Microwave digestion + ICP-MS [54,55]
Zinc (Zn)	1230	874 ± 38	4000	Microwave digestion + ICP-MS [54,55]
Mercury (Hg)	NA	0.003 ± 0.003	25	Microwave digestion + ICP-MS [54,55]
Chromium (Cr)	112	58.3 ± 3.2	1500	Microwave digestion + ICP-MS [54,55]
Manganese (Mn)	NA	NA	NA	Microwave digestion + ICP-MS [54,55]

NA: Not analyzed.

2.4. Soil and Crops Sampling

After 26 years from the beginning of the trial, a specific sampling was carried out to evaluate the concentration and availability of trace metals in the soil, as well as in the aerial parts (grain and straw) of barley.

Soil sampling was carried out in September, after the previous crop cycle ended, and before the application of SS for the following campaign. Samples of each treatment and replicate were collected at 0–30 cm depth, with an auger. A sample composed of three subsamples was collected in each plot. Samples were immediately transferred to the laboratory in polyethylene bags protected from sunlight, were dried at room temperature and ground to 2 mm.

Crop sampling was carried out at complete physiological maturity, 5 days before harvest. A sample of the aerial part of the crop was taken from each treatment and repetition, avoiding the edges of each plot. Crop samples were transferred to the laboratory in paper bags, and oven-dried at 50 °C for 7 days. Once dry, they were shelled with a 6mm sieve to separate the grain from the straw, and each part was ground separately with an agate ball mill.

2.5. Analytical Methods

2.5.1. Total Concentration in TM in Soil and Crop Samples

Soil, grain and straw samples for the determination of Cr, Cu, Mn, Ni, Zn, Cd, Pb and Hg were microwave-digested. Following the methodology described in EPA 3051 [55], 0.3 g of soil, 6 mL of 67–69% TraceMetal™ HNO_3, 2 mL 34–37% TraceMetal™ HCl and 2 mL of Milli-Q water were used for total Soil TM determinations. For the analysis of both parts of the crop (straw and grain), the methodology described in EPA 3052 [61] was followed; 0.25 g of sample, 4 mL of 67–69% TraceMetal™ HNO_3, 2 mL of H_2O_2 at 30% and 4 mL of Milli-Q water were used.

The mixing of the samples with the reagents was carried out directly in teflon tubes (PTFE) and then closed. They were treated in an ETHOS UP, Microwave digestion system (Milestone, Sorisole, Italy). For soil and crop samples, microwaves were emitted for 50 min and 1 h, respectively. To obtain a complete digestion, soil samples reached 200 °C for 20 min, and crop samples, 220 °C for 30 min. The temperature was controlled by a system of probes that constantly measure the process temperature. Once the digested samples had cooled within the system, each sample was filtered and transferred pre-tared 50 mL polypropylene containers, which were made up to their capacity with Milli-Q water. Between samples, the PTFE was rinsed with 5% HNO_3, five times and subsequently washed with Milli-Q water. Between different matrices, to avoid contamination, a blank digestion was carried out at 170 °C and, subsequently rinsed with Milli-Q water.

External calibration standards, and internal standards, were included in the sample set by means of a T system in the equipment's sample introduction system.

2.5.2. Extraction with DTPA of Soil Samples

To evaluate the bioavailability of TM in the soil, the DTPA-extractable concentrations of Cr, Cu, Mn, Ni, Zn, Cd and Pb were determined, using the extraction procedure described in the UNE 77315:2001 standard [62], equivalent to the international standard ISO 14870:2001 [63]. This extraction procedure aims to detect the TM concentrations in the most labile fractions of soil, and therefore potentially absorbable by plants [64].

The extraction solution was prepared by mixing, first, 0.735 g of $CaCl_2 \cdot 2H_2O$, 0.984 g of diethylene triamine pentaacetic acid—DTPA ($C_{14}H_{23}N_3O_{10}$) and 7.46 g of Triethanolamine—TEA ($C_6H_{15}NO_3$) in a beaker. The mixture was then diluted with 800 mL of deionized water and the pH was adjusted to 7.3 with HCl. The solution was finally transferred to a 1000 mL flask, made up to the mark and homogenized. The solution was stored at 20 °C until used. Subsequently, in a 100 mL wide-mouth polypropylene container, 20 g of soil sample and 40 mL of the solution were mixed. The recipient was hermetically closed and stirred for 2 h at 20 °C on a reciprocating shaker at 30 rpm. Then a fraction of the extract

was decanted, placed in a centrifuge tube, and centrifuged for 10 min at 6000 rpm. The supernatant was filtered with a membrane filter with a pore size of 0.45 μm, collected in a polyethylene bottle, and stored in the refrigerator. The blank extractions for each batch of analysis were carried out in the same way but without soil samples.

All glass and plastic material used in this procedure were previously washed in 5% HNO_3, and in deionized water, 3 times. Then they were rinsed with distilled water and with the extracting solution, 3 and 1 times, respectively.

2.5.3. Trace Metals Determination

The determination of TM concentrations in digested soil and crop samples was carried out by ICP-MS in a 7700x analyzer (Agilent Technologies, Santa Clara, CA, USA), following the UNE-EN 17053 standard [54]. The DTPA extracts were analyzed before 48 h from their preparation, using the same analysis method.

2.6. Statistical Analysis

A statistical analysis was carried out for each MT, with a significance level of 5%, to contrast the difference in means depending on the fertilizer dose received, understand this factor in six different levels associated with the ranges of accumulated contribution of SS, and distinguish the control of mineral fertilization. Thus, it was analyzed by one-factor ANOVA, carried out with R (R Core Team 2019, Vienna, Austria), with six levels: control (Control), mineral fertilizer (MinFer), 250 Mg per ha of SS (250MgSS), 520–560 Mg per ha of SS (500MgSS), 1040 Mg per ha of SS (1000MgSS) and 2080 Mg per ha of SS (2000MgSS). We will refer to the factor as "Fertilizer Treatment".

The ANOVA results are presented accompanied by BoxPlot graphs with whiskers where indicated, when the existence of any difference was confirmed, the groupings of factor levels (a, b, c...) after a study of multiple comparison of means two to two using the Tukey test.

In a complementary way, in order to contrast the effect of the application of SS per se, a study was carried out for each TM for the difference in means depending on whether the treatment had received SS (regardless of the quantity) called "With SS" or had not received SS (control and MinFer) called "Without SS", at 5% significance. This statistical analyses were carried out with R (R Core Team 2019, Vienna, Austria).

3. Results

3.1. Total Concentration of Trace Metals in Soil

The ANOVA showed significant differences in the total concentration of Zn, Cu, Cr, Ni and Hg in soil between the different Fertilizer Treatments (Figure 1).

The total concentrations of Cu, Cr and Hg showed significant differences between the control treatment (either SS or mineral fertilization) and the treatment with the highest accumulated SS dose (2000MgSS), which displayed the lowest and highest concentrations, respectively.

The total concentration of Ni in soil was significantly different between the treatments that had not received SS (control and MinFer) and those that had received SS.

In the case of Zn, significant differences were found between the treatments that had not received SS and those that had received SS. Moreover, in treatment 2000MgSS, the concentration increased.

No significant differences were found in the total concentration in Pb, Cd, and Mn as a function of the different fertilizer treatments studied.

The complementary study based on the contrast between treatments with SS and without SS (Table 4) confirmed the results obtained from ANOVA. This analysis, revealed higher values overall for the total concentration of Zn, Cu, Cr, Ni and Hg in the treatments that had received SS than in those that had not received SS. The average increase with SS was of 55.6% in Hg, 29.3% in Zn, 21.5% in Cu, 7.7% in Cr and 7.0% in Ni.
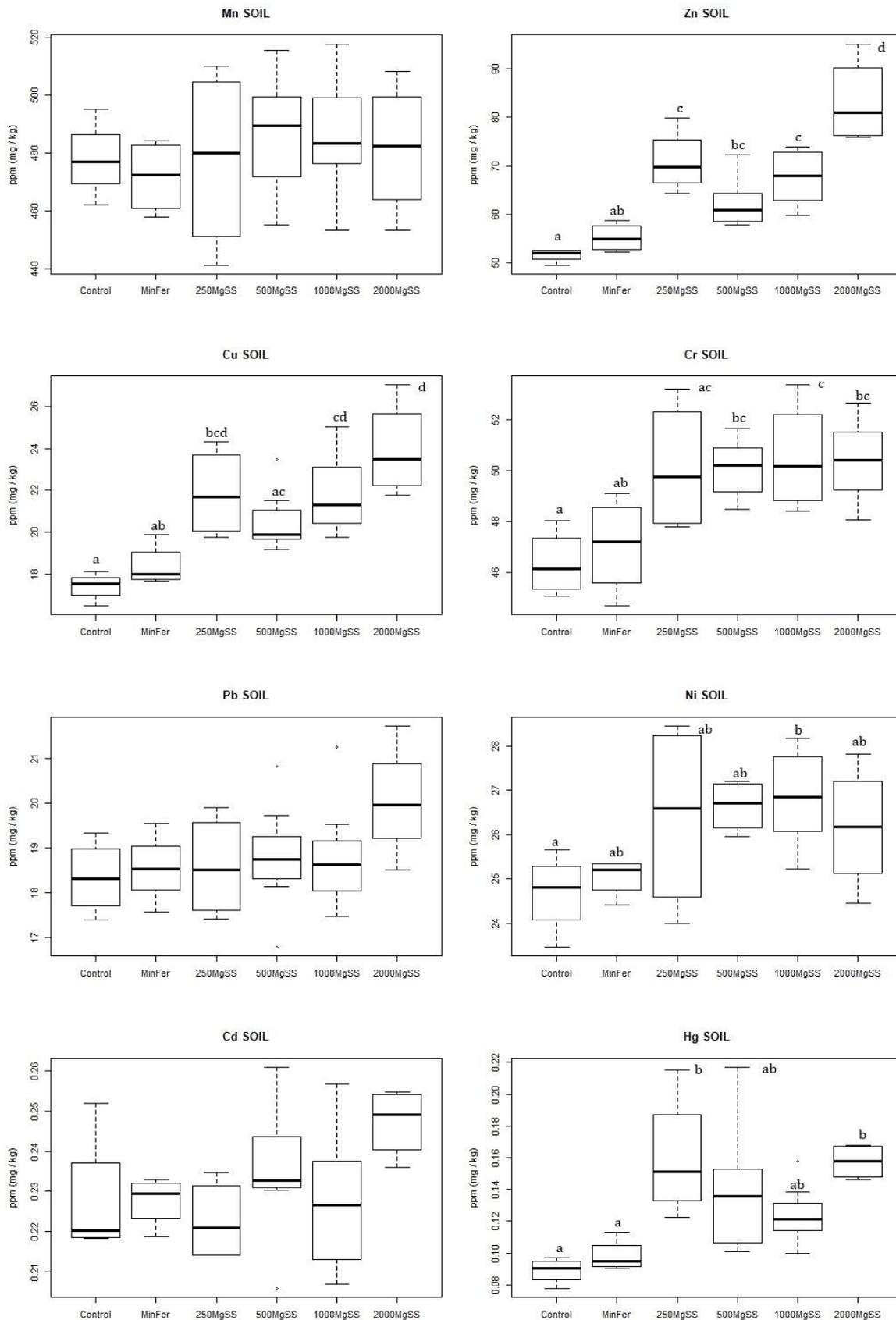
Figure 1. Box-and-whisker plot indicating different homogeneous groups in the multiple comparison Scheme (0–30 cm). Values showing the same letter belong to the same homogeneous group according to Tukey's test ($p < 0.05$).

Table 4. Mean ± standard deviation of the total concentration of Mn, Zn, Cu, Cr, Pb, Ni, Cd and Hg in soil, depending on whether they have received SS or not, together with their *p*-value.

Treatment	$TM_{SOIL\ TOTAL}$ (mg kg^{-1})							
	Mn	**Zn**	**Cu**	**Cr**	**Pb**	**Ni**	**Cd**	**Hg**
With SS	484 ± 21	69.0 ± 9.2	21.7 ± 2.1	50.3 ± 1.7	19.0 ± 1.3	26.6 ± 1.2	0.23 ± 0.02	0.14 ± 0.03
Without SS	475 ± 13	53.4 ± 2.3	17.9 ± 1.0	46.7 ± 1.6	18.4 ± 0.8	24.9 ± 0.7	0.23 ± 0.01	0.09 ± 0.01
p-value	0.13	0.00 *	0.00 *	0.00 *	0.14	0.00 *	0.22	0.00 *

* Significant difference.

3.2. Concentration of DTPA-Extracted Trace Metals in Soil

The results showed that the concentration in DTPA-extracted Zn, Cu, Pb, Ni and Cd, increased with the use and dose of SS (Figure 2).

In all TM except for Mn, DTPA-extracted concentrations were found to be significantly lower in the control than in the treatment 2000MgSS. Furthermore for Pb, a significant difference was observed between the treatments that did not receive SS and those that received SS. For these TMs, the minimum amount of SS applied represented a significant increase in the extractable concentration by DTPA.

In the case of Zn extracted with DTPA (Figure 2), a significant difference was observed between the treatments that did not receive SS and those that received SS. At treatment 2000MgSS, the bioavailable concentration in soil increased.

Cu showed three statistically different groups, with the treatments corresponding to 0 Mg per ha of SS accumulated (control and MinFer) grouped together with the lowest concentrations, the treatments comprised between 250MgSS to 1000MgSS in another homogeneous group with intermediate values, and the treatment 2000MgSS with the highest observed value.

Finally, it was not possible to quantify the concentrations of Cr extracted by DTPA, as it was below the detection limit of 0.5939 ppb.

The complementary statistical study found that the average concentration in Mn extracted with DTPA was 6.5% higher in the plots that had not received any dose of SS, compared to those that had received SS (Table 5). This same analysis confirmed the results of the ANOVA analysis, revealing higher values in the treatments that had received SS applications compared to the treatments without SS for DTPA-extractable Zn, Cu, Pb, Ni and Cd. The average gains were of 122.2% in Zn, 61.1% in Cu, 27.3% in Ni, 14.9% in Pb and 11.3% in Cd. Finally, only Cu and Zn showed a clear correlation in their DTPA-extracted concentrations and those found in bulk soil (Figure S1).

Table 5. Mean ± standard deviation of the DTPA extractable concentration of Mn, Zn, Cu, Pb, Ni and Cd in soil, depending on whether they have received SS or not, together with their *p*-value.

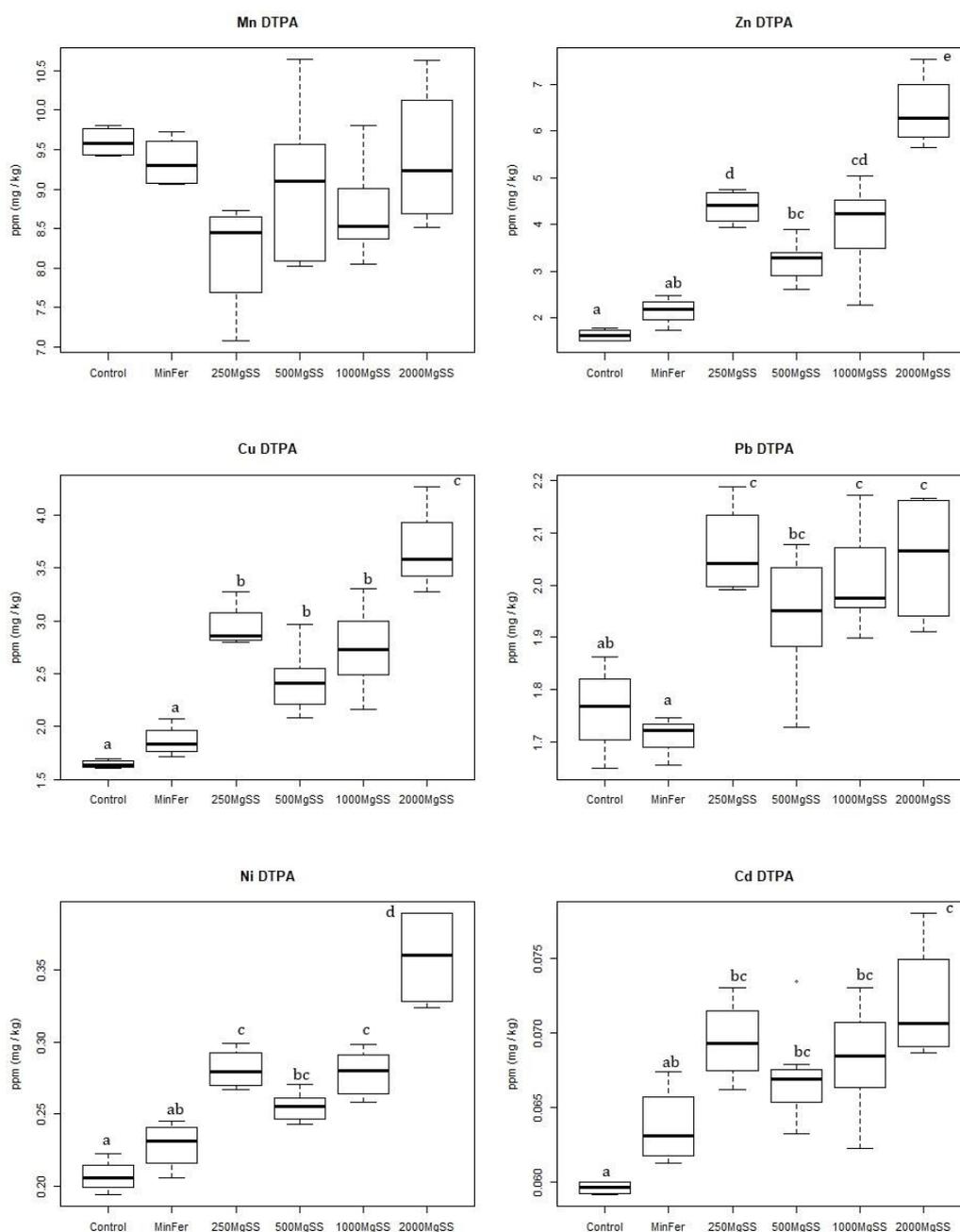| Treatment	$TM_{SOIL\ DTPA}$ (mg kg^{-1})					
	Mn	**Zn**	**Cu**	**Pb**	**Ni**	**Cd**
With SS	8.85 ± 0.86	4.20 ± 1.27	2.82 ± 0.53	2.00 ± 0.11	0.28 ± 0.04	0.07 ± 0.00
Without SS	9.47 ± 0.28	1.89 ± 0.36	1.75 ± 0.16	1.74 ± 0.07	0.22 ± 0.02	0.06 ± 0.00
p-value	0.01 *	0.00 *	0.00 *	0.00 *	0.00 *	0.00 *

* Significant difference.

Figure 2. Box-and-whisker plots that indicate different homogeneous groups in the multiple comparison of means two to two of the concentration extracted by DTPA of the trace metal Mn, Zn, Cu, Pb, Ni and Cd existing in the soil according to the fertilizer treatment received in the study horizon (0–30 cm). Values showing the same letter belong to the same homogeneous group according to Tukey's test ($p < 0.05$).

3.3. Concentration of Trace Metals in Barley Grain and Straw

In relation to grain, no significant differences were detected in the concentration of TM in grain as a function of the different treatments, with the exception of Zn (Table 6). Zn concentration showed the highest concentration in grain in the fertilizer treatment corresponding to 2000MgSS. The result of the complementary analysis, confirmed the significant difference in Zn, and revealed differences in Cu and Cr (Table 7). For these TM, the concentrations in barley grains were significantly higher in the treatments that had received SS than in those without SS application. The average increases were of 15.0% in Cr, 8.3% in Zn and 4.4% in Cu.

Table 6. Different homogeneous groups in the multiple comparison of two to two means of the concentration of the trace metal Mn, Zn, Cu, Cr, Pb, Ni, Cd and Hg in grain and straw according to the fertilizer treatment received in the study horizon (0–30 cm). Mean ± standard deviations. Values in the same column showing the same letter belong to the same homogeneous group according to Tukey's test ($p < 0.05$).

Fertilizer Treatment	TM Crop (mg kg^{-1})															
	Mn		Zn		Cu		Cr		Pb		Ni		Cd		Hg	
	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw	Grain	Straw
250MgSS	17.3 ± 0.6	16.8 [ab] ± 2.4	34.3 [ab] ± 2.4	8.16 ± 1.00	5.94 ± 0.39	2.88 ± 0.28	1.35 ± 0.23	1.71 ± 0.44	0.10 ± 0.00	0.24 ± 0.01	0.83 ± 0.11	0.95 ± 0.25	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
500MgSS	16.8 ± 1.6	17.4 [a] ± 2.2	31.0 [a] ± 2.6	7.31 ± 0.96	5.69 ± 0.32	2.50 ± 0.41	1.23 ± 0.18	1.58 ± 0.19	0.10 ± 0.01	0.25 ± 0.07	0.81 ± 0.09	1.01 ± 0.21	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
1000MgSS	17.1 ± 1.8	15.6 [a] ± 4.6	35.2 [ab] ± 3.2	6.98 ± 1.68	5.67 ± 0.32	2.67 ± 0.63	1.31 ± 0.3	1.84 ± 0.71	0.10 ± 0.00	0.22 ± 0.09	0.83 ± 0.13	1.02 ± 0.32	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
2000MgSS	17.8 ± 1.2	17.7 [ac] ± 3.1	37.1 [b] ± 4.1	7.00 ± 0.37	5.63 ± 0.44	2.78 ± 0.53	1.41 ± 0.08	1.54 ± 0.82	0.11 ± 0.01	0.27 ± 0.14	0.90 ± 0.02	0.96 ± 0.48	0.01 ± 0.00	0.03 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
MinFer	18.4 ± 1.1	24.4 [bc] ± 3.4	30.4 [ab] ± 2.6	6.91 ± 1.76	5.66 ± 0.21	2.82 ± 0.55	1.03 ± 0.06	2.26 ± 1.01	0.10 ± 0.01	0.31 ± 0.10	0.73 ± 0.05	1.51 ± 0.57	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Control	17.3 ± 1.8	25.1 [c] ± 5.3	32.1 [ab] ± 2.6	9.26 ± 1.60	5.27 ± 0.17	2.25 ± 0.39	1.26 ± 0.47	2.55 ± 0.45	0.12 ± 0.03	0.23 ± 0.04	0.78 ± 0.25	1.34 ± 0.40	0.01 ± 0.00	0.03 ± 0.01	0.00 ± 0.00	0.01 ± 0.00

Table 7. Mean ± standard deviation of the concentration of Mn, Zn, Cu, Cr, Pb, Ni, Cd and Hg in the crop (grain and straw), depending on whether or not they have received SS, together with their *p*-value.

Treatment	TM$_{CROP}$ (mg kg^{-1})							
	Mn	**Zn**	**Cu**	**Cr**	**Pb**	**Ni**	**Cd**	**Hg**
GRAIN With SS	17.1 ± 1.4	34.0 ± 3.7	5.71 ± 0.35	1.30 ± 0.22	0.10 ± 0.01	0.83 ± 0.10	0.01 ± 0.00	0.01 ± 0.00
GRAIN Without SS	17.8 ± 1.6	31.4 ± 2.6	5.47 ± 0.27	1.13 ± 0.30	0.11 ± 0.03	0.75 ± 0.15	0.01 ± 0.00	0.01 ± 0.00
p-value	0.32	0.045 *	0.038 *	0.047 *	0.64	0.062	0.25	0.59
STRAW With SS	16.8 ± 3.3	7.29 ± 1.21	2.66 ± 0.48	1.68 ± 0.53	0.24 ± 0.09	1.00 ± 0.29	0.03 ± 0.01	0.01 ± 0.00
STRAW Without SS	24.8 ± 4.1	8.08 ± 2.00	2.53 ± 0.54	2.40 ± 0.74	0.27 ± 0.08	1.42 ± 0.47	0.03 ± 0.01	0.01 ± 0.00
p-value	0.00 *	0.19	0.26	0.01 *	0.40	0.00 *	0.71	0.46

* Significant difference.

In straw, no significant differences were found in the concentration of TM analyzed as a function of the fertilizer treatment, with the exception of Mn (Table 6). The Mn concentration in straw in the treatments without SS was significantly higher than in those that had received SS. The result of complementary analysis confirmed the significant difference in Mn and revealed differences in Cr and Ni (Table 7). In the three TM, the concentrations in the straw of the treatments that had not received SS were higher than those that had received SS. The average decrease was 32.2% in Mn, 30.0% in Cr and 29.6% in Ni.

4. Discussion

4.1. Influence of Sewage Sludge in Total and DTPA-Extracted Concentrations of Trace Metals in Soil

4.1.1. Total TM Concentrations in Soil

In general, the TM concentration found in soil, in descending order was Mn> Zn> Cr> Ni> Cu> Pb> Cd> Hg. The application of SS significantly increased the total concentration of Zn, Cu, Cr, Ni and Hg in the soil. These results are in line with those found in other locations and experimental sites. For instance, in a study carried out on a Mediterranean agricultural soil, Reference [16] obtained similar results, where the concentrations of Cu, Zn, Ag, Sb, Hg and Pb in the soil increased with the application of SS over 15 years, compared to the control and to mineral fertilization. In studies conducted on other soil types such as Cumulic Haplustolls and an Udic Calciustoll [65], it was also concluded that Cr, Cu, Cd, Pb and Zn accumulated in the most superficial layers of the soil after the application of SS over several years.

The different behavior of the TM analyzed can be explained by both their concentration in SS, and their particular behavior in soil. As it can be seen in Figure 1, the concentrations of Zn, Cu, Cr and Hg were significantly higher in the treatments receiving the highest dose of SS, 2000MgSS (corresponding 80 Mg ha^{-1} of SS every year for 26 years), compared to the control plots. This dose is possible only within the framework of research. Although the annual amounts of TM that can be applied to agricultural soils comply with the current legislation RD 1310/1190 [26], the regional regulations on the use of SS in agriculture in Navarra (Orden Foral 359/2010 [66]) is more strict. This regulation prohibits exceeding the dose of 250 NFU annually in general, and of 170 NFU in areas designated as vulnerable to nitrate contamination according to EU Directive 91/676/CEE [67]. The dose of 80 Mg ha^{-1} of SS every year is therefore well above the one allowed at the local level (Table 2), which corresponds to 20 Mg ha^{-1} every four years in rainfed crops. To this, it has to be added that current regulations on organic fertilization also impose limits that make this dose above those allowed [68].

In the particular case of Zn, the significant enrichment observed in the soil with increasing SS application can be related to the high concentration of Zn in SS (874 mg Kg^{-1}, Table 3). These results were also observed in a previous study carried out in this experimental field [11].

In Ni, a significant difference was observed between the treatments that had received SS compared to those that had not received SS (Figure 1). This was not observed in studies previously conducted under similar conditions [39], and in the same experimental field [11]. This suggests, that although the concentration of Ni in SS was low (32.1 mg Kg^{-1}, Table 3) it was sufficient to generate an increase in concentration in the plots that received SS at any dose after 26 years of continuous application. The application time and the accumulated dose seem therefore to have consequences in the accumulation of this element in soils like the one studied here. A significant increase in total Ni was also observed in a study carried out in two soils in India, with neutral pH, warm steppe climate, sunflower and wheat cultivation, fewer applications and lower doses of SS than those used in this study [41].

In Mn, Pb and Cd there were no significant differences in their total concentration in the soil between any fertilizer treatment, whether they received SS or not, as previously found in this experimental site [11], and in other studies with similar conditions but

lower doses of SS, for Mn and Cd [16]. Despite amending with lower doses, Iglesias [16] found a significant increase in Pb, probably due to the concentration of this element in the SS used (112 ± 21 mg ka^{-1}), while in our study, the concentration of Pb was lower (39 ± 1.2 mg ka^{-1}, Table 3). In another study in Sweden, since 1997, in a silty clay soil (42% clay), cereal crop but acid pH, Pb did not suffer significant increases and Cd increased only when SS with metal salts was added [10]. It is important to note that the doses applied were lower than in our case study.

It has to be noted that, despite the observed cumulative effect of SS application on total Zn, Cu, Cr, Ni and Hg concentrations, these concentrations were always below the legal limit for soils with a pH greater than 7, as indicated by the Spanish national legislation RD 1310/1190 [26], and the European Directive 86/278/EEC [27]. Previous studies carried out under similar conditions coincide with this observation [12,16,17]. Our results indicate, however, that the use of SS for fertilization purposes, in the conditions used here can have a relevant and cumulative effect on the total concentration of TM in the soil, which should be monitored in the long term.

4.1.2. DTPA-Extractable TM Concentrations in Soil

In general, the order of concentration of TM in the DTPA extractable fraction was Mn > Zn > Cu > Pb > Ni > Cd. It was observed that for Zn, Cu, Pb, Ni and Cd, the concentration extracted with DTPA was significantly higher in the treatments that received SS than in those without SS, as already observed in many other studies [22,35]. Assuming that these concentrations represent the most readily available forms of TMs in soil, they would be affected by three factors: the source or origin of these TM (in the case, the SS), the characteristics of the soil and soil conditions associated with their application, and the particular behavior of each TM. In relation to the source, anthropogenic TMs are known to be more bioavailable than pedogenic metals [42,69,70].

In relation to soil parameters that might influence TMs bioavailability in the experimental site of this study, the most relevant ones are pH, the presence of carbonates, and the possible modifications in the retention of cations associated with the presence of organic matter. The high pH (8.5) associated with a high content of carbonates in the depth of study, hinders the mobility of TM in the soil to phases easily absorbed by the crops [15,45]. To this fact we must add that increasing the content of organic matter by continuous addition of SS can favor the formation of organo-metallic complexes [12,42,43], and increase the cation-exchange capacity of soil [42,71,72], which would also reduce the mobility and availability of TMs. In addition, the use of phosphate compounds has been proven to be efficient for the chemical immobilization of heavy metals in soils [73,74]. In this study, SS contributed a high concentration of phosphorus (P) to the soil, due to the affinity of this element with the solid fraction of SS during the wastewater treatment process [6]. It has been indeed observed in this experimental field that the application of SS increased the total and available content of P in soil [11], which could increase the immobilization of different TMs [71]. The behavior of the different TM analyzed was however not homogeneous. In Ni and Cd extracted by DTPA, only the treatment 2000MgSS caused a significant increase in the concentration with respect to the control (Figure 2). Both TMs have similar mobility in SS [39]. Cd tends to be found in a greater proportion strongly absorbed to organic matter and/or easily reducible Mn oxides and amorphous iron oxides, while Ni can also be strongly bound or incorporated into organic matter or into other oxidizable species [39]. When SS is incorporated into the soil, these TM undergo a minimum redistribution, remaining above all strongly adsorbed to organic matter and carbonates [39,75,76]. This mobility in SS and soil, together with the low concentrations of both TMs in SS (Table 3), can explain the observed increase in bioavailability only in the treatments corresponding to the highest SS doses, as well as the lack of a clear relationship between total and DTPA-extractable concentrations (Figure S1). These results coincide with those described by Navarro [77], who determined, by means of a column experiment, that the Cd and Ni extracted by DTPA increased only after the use of large doses of SS in calcareous soils. In the case of Cd, it

has however been observed that in soils modified with biosolids greater mobility can be observed as exchangeable Cd associated to the greater exchange capacity of some organic components added to the soils [76]. It is important to take this into account since Cd is a non-essential TM.

For Pb extracted by DTPA, a significantly higher concentration was observed in the treatments with SS compared to those that had not received SS. In this case, the contributions through SS were low (39.0 mg Kg^{-1}, Table 3), and considering its affinity with organic ligands [78], it can be thought that a relevant part of its concentration was associated to organic matter in SS. The mineralization of organic matter would thus imply a gradual release of the Pb attached to it. In the case of calcareous soils, this release would be associated with the formation of forms that are not readily bioavailable for crops, mostly in the form of carbonates [76,79,80]. As such, the increasing concentration of Pb with increasing accumulation of SS-derived organic matter would be outbalanced by this behavior of Pb in calcareous soils that would imply its progressive incorporation into stable forms as it is released by organic matter mineralization, explaining also the low correlation between total and DTPA-extractable Pb (Figure S1).

In relation to Zn extracted by DTPA (Figure 2), the response was similar to that observed for total concentration in soil (Figure 1 and Figure S1). This is probably due to the high concentration of Zn in SS (874 mg Kg^{-1}, Table 3) and the mobility of this TM. According to Morera [39], in SS such as the one used, Zn is mainly found together to organic matter and Fe and Mn oxides. However, about 10% can be in easily mobile forms. Some studies have determined that Zn may be biologically available and mobile in soils that have been treated with SS for long periods [81]. Kabata Pendias [82] already mentioned that the long-term addition of biosolids to the soil can increase the fraction of bioavailable Zn. This mobility has been observed to increase with time of SS addition to soil since, as it can form soluble chelates with humified and non-humidified forms of organic matter, which in turn can be easily degraded by soil microorganisms [83,84].

DTPA-extractable Cu showed significant differences between the different doses of accumulated SS, with an increasing concentration with the increase of cumulative SS dose (Figure 2 and Figure S1). This behavior can be related to the great affinity that Cu has with organic matter [78,85]. It is likely that the Cu present in SS was mostly bound to organic matter. When added to the soil would hardly undergo any redistribution, as it can be retained to the organic fraction of the soil through stable organic-mineral complexes [40]. At the same time, the increment in the density and activity of the soil microbial biomass associated to the addition of SS with a low concentration in TM to the soil [86], can contribute to gradually incorporate this Cu into more available forms. Some studies have shown that the amount of SS that actually affects the increase in Cu availability is that accumulated mainly on the last 4 years of application [87,88]. This seems to support the observation that the fertilizer treatment with 2000MgSS, corresponding to an annual application of 80 Mg ha^{-1} of SS, was the treatment with the highest concentration of Cu extracted with DTPA. This observed correlation of DTPA-extracted Zn and Cu and their total concentration in soil has been observed in fact in other studies including SS additions to calcareous soils [38].

In the Mn extracted with DTPA, a higher concentration was observed in the treatments that had not received SS. This may be due to the affinity that Mn has with carbonates in SS and in soil, coupled with the low Mn contribution normally associated with SS [33]. Mn in SS is usually bound to carbonates, Fe and Mn oxides, and/or bound to the residual fraction [89,90]. When incorporated into calcareous soils, Mn hardly undergoes a redistribution [80], remaining bound to carbonates. This could explain the low efficiency of SS in incorporating Mn into the soil and a higher efficiency of mineral amendments. Previous results where the highest concentration of Mn extractable by DTPA was obtained in the control amended with mineral fertilization, support this hypothesis [40].

4.2. Concentration of TM in Barley

The study of TM concentrations in crops, as a reliable method to assess the actual bioavailability of TMs in the studied experimental field, detected some differences and trends between TMs, and for the different parts of the crop (grains and straw).

As a general trend, it was observed how the essential TM were adsorbed in higher concentrations than the non-essential ones, being the order of concentration from highest to lowest Zn > Mn > Cu > Cr > Ni > Pb > Cd > Hg, and Mn > Zn > Cu > Cr > Ni > Pb > Cd = Hg, for grain and straw respectively. However, significant differences were observed for Mn, Zn, Cu, Cr and Ni accumulation in barley grains and straw. In grain, Zn, Cu and Cr showed higher concentrations in the treatments that had received SS than in those without SS (Table 7). On the contrary, in straw, Mn, Cr and Ni showed higher concentrations in the treatments that had not received SS (Table 7).

In the case of Zn, the highest concentration in grain coincided with the highest concentration extracted with DTPA, corresponding to 2000MgSS, in line with the general correspondence between the total concentration of this TM and of Cu in the soil and that found in plants in soils amended with SS [3,38]. However, the differences observed for soil Cu concentrations between doses were not observed in grain (Table 6). This could be explained by what was observed by Montaghian [91], who found that in calcareous soils amended with SS, the concentration of bioavailable Cu was lower in the soil of the rhizosphere than in the rest of the soil. They observed a redistribution of Cu between the different soil fractions, increasing the proportion linked to organic matter as a consequence of an increase in dissolved organic carbon released by the roots. This suggests a possible antagonistic effect, hindering the accumulation of Cu in grain (the organ that normally accumulates the most Cu in mature plants [92]) beyond a certain level of accumulation of Cu in the soil of this study. In the case of Mn, a tendency to a greater accumulation was observed in the grains of barley plants from the treatment MinFer. This highlights the low efficiency of SS to supply Mn to crops.

In straw, the treatments without SS contained higher concentrations of Mn, Ni and Cr than the treatments that had received any dose of SS. This can be related to the "dilution effect" for these elements caused differences in yield between treatments. As SS produced an increase in yields (Supplementary Tables S1 and S2) also observed in other studies [14,38,93] and the applied and available concentrations of these TM were low, a dilution of these TM in the crop can be expected. Similar results have been reported before for Mn in long-term field [94] and pot experiments for Mn and Ni [71].

Finally, in the case of Cr, for which the DTPA-extracted concentration was below the method's detection limit, it was not possible to relate this concentration and that observed in the crop aerial parts. However, our results seem to indicate a different efficiency in the use of Cr depending on the plant organ and SS dose received. In a study carried out in pots, a high correlation was observed between the Cr concentration in the grain of different varieties of wheat and that extracted from soil with EDTA [35].

The study of TM concentrations in aerial crop parts allowed to contrast them with the limits and thresholds defined in the legislation. The European regulation referring to food products intended for human [29] and animal consumption [28] only contemplates Pb, Cd and Hg as risk elements. In our study, in general terms, it was found that the concentrations found in barley that complied with current regulations, coinciding with other studies [16,94]. In the case of grain, the concentrations were below the limit allowed for both norms. In the case of straw, however Pb concentrations were above that allowed by the legislation for human consumption. Despite the fact that straw is not intended for this type of use, it should be noted that even the control was found above this limit, which indicates that SS was not the main reason for Pb exceeding the value allowed by the legislation.

5. Conclusions

The application of SS as a long-term organic amendment in the conditions of soil, climate, management and type of sludge studied here, caused an accumulation of total Zn, Cu, Cr, Ni and Hg in soil, of Zn, Cu, Pb, Ni and Cd in their bioavailable form extracted by DTPA and of Zn, Cu and Cr in grain. This suggests the existence of potential environmental risks and supports the need for long-term monitoring of this type of practice. On the other hand, a higher concentration of Mn extracted with DTPA, as well as a higher concentration of Mn, Ni and Cr in the straw was observed, in the treatments without SS amendment.

Therefore, it was observed that Zn, Cu and probably Cr, suffered an increase in their concentration in soil and extractable by DTPA, that was visualized in grain. Other TM, such as Hg, increased their total concentration in soil and others, such as Cd and Pb, increase their concentration extracted by DTPA. In the case of Ni, both the total and DTPA-extracted concentration increased without any influence in the grain content.

Despite the existence of an increase in the total and bioavailable concentration of some TMs, the resulting crop complied with the current regulations imposed by the European Union for human and animal nutrition.

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