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Effect of Soil Aging on Cadmium Bioavailability and Bioaccessibility at a Contaminated Site

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Abstract: The effect of aging on cadmium (Cd) bioavailability and bioaccessibility was investigated in naturally aged field soil within a contaminated site. The results, which are based on a comparison of investigations carried out in 2018 and 2022 on the same soil samples, provide a realistic evaluation of the variation in Cd chemical forms due to long-term aging. The data obtained show a significant reduction (from approximately 30% to 60%) in the mobile and bioavailable forms of cadmium, while the total quantity in soil did not change significantly. The effect of aging on the bioavailable fractions is also reflected in the reduction in the amount of the metal absorbed by plants. On the one hand, this indicates a reduction in the potential contamination of the food chain, while on the other, it highlights the limitations of the use of phytoextraction as a clean-up technology in this specific site. In the case under study, it should also be noted that there was no decrease in cadmium bioaccessibility over time, which remained very high even after four years of cadmium aging in the soil, which was about 60% of the total content in the most contaminated soil samples. This highlights the potential health risks related to the incidental ingestion of Cd-contaminated soil, which could become the main exposure route in the case of the final use of the site as a park or public green area.

Keywords: cadmium; soil contamination; bioavailability; bioaccessibility; soil ageing

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1. Introduction

The protection of the soil environment is one of the most critical concerns linked to economic development in all countries. Nowadays, most activities that are part of modern human society can result in direct or indirect release of organic and inorganic pollutants into the environment, from process industry to construction to agricultural/green practices, to name a few [1–3]. These practices, too often accompanied by improper waste disposal policies without adequate resource recovery [4,5], end up having increasingly negative effects on the ecosystem and human health [6].

To face these problems, a wide range of physicochemical and biological approaches have been developed for the recovery of contaminated water [7–10] and soils [11–13]. Indeed, the remediation of contaminated sites is a crucial activity that requires effective, environmental sound and cost-effective technological approaches to restore and preserve natural resources such as soils, which would otherwise be permanently lost [14–16].

Heavy metals are among the most common pollutants in contaminated sites [11,13,17] because of their non-biodegradability and high persistence in soil. Therefore, there is an urgent need to eliminate or at least reduce the hazards from the presence of heavy metals in soil to prevent uncontrolled translocation of pollutants [18], protecting groundwater and the food chain [19,20].

Of all heavy metals, cadmium (Cd) has long attracted particular interest in soil research, because its high toxicity can cause very serious damage to human health and the environment [21]. Since 1993 the International Agency for Research on Cancer has classified cadmium as a category 1 human carcinogen. Many adverse health effects have been linked to cadmium, the most deleterious effects were found in the bone and kidneys [22–26].

Due to cadmium's persistence in the environment and bioaccumulation in the food chain, soil represents a primary matrix for studying the dangers of cadmium. The metal in soil derives from both natural sources (e.g., geological substrate) and anthropogenic activities (e.g., industrial activities, waste incinerators, sewage sludge and phosphatic fertilizers). Human exposure to Cd derives primarily by vegetables grown in soil, which account for about 90% of the total intake of Cd in non-smoking people [27]. The consumption of contaminated vegetables through the diet could thus have significant consequences for human health [22,28,29].

It is known that the total concentration of the metal in soil is inadequate to define its dangerousness; bioavailability is the key to understand the probability of adverse effects of metals on the human health. The bioavailability is described by the fraction of the total amount of a metal in the soil that, within a given time span, is available for plant uptake, and thus potentially transferred into the food chain [30,31].

In addition to the soil-plant-food chain route, further exposure pathways include incidental ingestion of soil, largely dependent on hand-to-mouth contact, dermal contact, and the inhalation of soil particulate. The potential negative effects of these specific pathways can be evaluated in terms of bioaccessibility. Bioaccessibility is defined as the fraction of the total amount of a contaminant present in soil that, after ingestion, becomes soluble in the stomach and is thus available for intestinal absorption [32,33].

Bioavailability and bioaccessibility are related both to the characteristics of soil and contaminants but identify different routes of exposure [34].

Aging is one of the factors affecting the Cd fate in soil which has gained interest, because over time it can significantly change the chemical behavior of the element in the soil environment. The concept of soil aging is closely connected with the variation in bioavailability which generally tends to decrease over time [35–37]. Numerous studies have been carried out on the aging of heavy metals in the soil [38–42], and it has been recognized that aging has a crucial influence on the bioavailability of metals in soil [43,44]. Most metal aging studies are conducted under controlled laboratory conditions, and even long-term experiments fail to fully simulate field aging conditions which include a number of variables that cannot be replicated in the laboratory [45]. For example, compared to controlled laboratory conditions, the temperature variations that occur in the field modify the diffusion of heavy metal ions in the micropores and the consequent modifications of the chemical forms, especially those characterized by weaker bonds with the soil surfaces [46,47]. Furthermore, the seasonal cycles of wetting and drying can modify the structure of soil aggregates and significantly influence the speciation of Cd in soil [48]. Naturally, variations in light intensity, biological reactions and microbial activities can also affect the bioavailability of metals differently between the laboratory and the field. Finally, unlike laboratory tests, in full-scale tests it is possible to take into account the long-term reactions that can redistribute the metals in the various more or less available chemical forms. The contribution derived from field results may thus be of great importance to establish the implications of aging in risk assessment management.

This work reports the results obtained on soil samples taken from a site contaminated by industrial waste after a period of four years ageing. This was possible because, due to bureaucratic delays, initial remediation activity was suspended, and it started again four years later. Thus, two soil sampling campaigns were conducted four years apart from each other at the same sampling points. These two sampling campaigns were exploited to study the aging processes in the open field conditions.

The aim of this study was to detect the variations in the bioavailability and bioaccessibility of cadmium in a soil subject to field aging, in order to provide information that can be used for the management of soil Cd contamination both for overall risk assessment and the selection of possible remediation technologies.

2. Materials and Methods

2.1. Site Description and Soil Sampling

In 2018 contamination from various metals including cadmium was discovered within an abandoned area (5000 m^2) in Tuscany, Central Italy. This peri-urban area, which was originally agricultural, had been used for some years as a shelter for sheep and then became completely abandoned. This area is characterized by a Mediterranean climate with a mean annual rainfall of 1080 mm and a mean annual temperature of 15.2 °C. Preliminary chemical analyses performed by control authorities on the soil in the area revealed significant Cd contamination, with values exceeding threshold concentrations established by the Italian legislation [49]. The contamination was ascribed to the illegal uncontrolled release of waste probably derived from various industrial productions, including tannery residue mixed with sewage sludge which could contain Cd soluble salts. Many activities including soil sampling and characterization were undertaken by the local environmental authorities to obtain a complete picture of the contamination aimed at remediation actions.

The results indicated that the highest levels of cadmium concentrations were in the top layer of the soil (20–30 cm). Below 30 cm the Cd concentration was the same as the neighboring unpolluted soils outside of the contaminated site. The judiciary intervened and closed off the area, but no remediation activity was initiated due to legal disputes and bureaucratic delays.

In 2022, remediation operations started again, and a new characterization campaign was carried out. Within the framework of this new sampling campaign, 15 soil samples were collected in exactly the same points as in 2018. A further sample was collected both in 2018 and 2022 from the neighboring soil with identical characteristics but which had not been affected by the contamination. It was thus possible to study the effects of four years aging processes in the field.

Approximately 2 kg of soil were collected from each sampling point to a maximum depth of 30 cm. The soil samples were preliminary sieved to 2 cm on-site and then transported to the laboratory for the soil characterization analysis. The soil of each sampling point was used both in 2018 and 2022 for bioavailability, bioaccessibility and microcosm bioassay tests.

2.2. Soil Characterization and Cd Bioavailability and Bioaccessibility Evaluation

Soil analyses were performed on the selected samples from both 2018 and in 2022. Chemical analyses were carried out on 2 mm air-dried soil fractions by standard methods [50]. To evaluate the potential Cd bioavailability a Sequential Extraction Procedure (SEP) was used, (H_2O, KNO_3 1M, EDTA 0.01 M pH = 4.65) which was carried out with a soil: extracting agent ratio of 1:5 and 3 h time for each extracting step [51–55]. The bioaccessibility evaluation was operationally described by the amount of metal extractable according to the USEPA method 1340 [56] with 0.4 M glycine (in deionized water) as extractant, adjusted to a pH of 1.50 ± 0.05 at 37 ± 2 °C with trace-metal grade concentrated HCl. The method specific for lead, can be also used for other heavy metals [34,57–59].

Soil contamination was evaluated by the Geo–accumulation Index was originally defined [60] as

$$\mathrm{Igeo} = \log_2\left(\frac{C_m}{1.5 \times R_m}\right) \tag{1}$$

where C_m represents the concentrations of Cd in soil samples; R_m represents the reference value for Cd in the geographical area where the polluted site is located and the constant 1.5 is applied to eliminate the lithological fluctuations [61].

2.3. Microcosm Bioassay Experimental Design

The bioassay trials, prepared as phytoextraction feasibility tests, were carried out at the microcosm scale using 500 g for each pot in a climatic chamber (CCL300BH-AS S.p.A., Perugia, Italy) with a photoperiod of 14 h light, at 24 °C and 10 h dark at 19 °C, and humidity at 70%.

Brassica juncea var. scala was selected as the plant for the bioassay due to its ability to grow in contaminated soils [52,53]. Each microcosm was prepared using 0.3 g *B. juncea* seeds, with a growth of about 30/40 seedlings. For each sampling point, trials were carried out in triplicate, and the experiments lasted 30 days. During the growing period, plants were watered daily with tap water.

After plant harvest, the aerial parts were separated from the roots, and all samples were washed with deionized water. The roots were also washed in an ultrasound bath (Branson Sonifier 250 ultrasonic processor) for 10 min to eliminate soil particles that could have remained on root surfaces. Vegetal samples were left in a ventilated oven at a temperature of 40 °C until constant weight. The dry mass of shoots and roots was gravimetrically determined. Bioassay tests were carried out in 2018 on soil samples collected as a part of a phytoremediation feasibility test. In 2022, the feasibility test was repeated on the new samples collected.

2.4. Cadmium Analysis

Soil and plant samples were digested in aqua regia in a microwave digester according to the USEPA method 3051A [62]. Concentrations of Cd in soil, plant samples and supernatants from sequential extraction procedures were determined by ICP-OES with a Liberty AX Varian. All the analyses were performed in triplicate including certified reference materials and blanks.

2.5. Quality Assurance and Quality Control

Quality assurance and quality control were performed by testing two standard solutions (0.5 and 2 mg L^{-1}) for every 10 samples. CRM ERM—CC141 for soil and CRM ERM—CD281 for plants were used as certified reference materials. The values obtained for Cd were 0.32 ± 0.05 mg kg^{-1} for CRM ERM—CC 141 and 0.121 ± 0.006 mg kg^{-1} for CRM ERM—CD281, in agreement with the certified values of 0.35 ± 0.05 mg kg^{-1} and 0.120 ± 0.003 mg kg^{-1}, respectively.

The detection limit for Cd was 0.003 mg L^{-1}. The recovery from the plant standard ranged from 98.3 to 102.7% and from the soil standard ranged from 97.4 to 101.5% with a relative standard deviation (RSD) of 1.84% from the mean.

2.6. Statistical Analysis

Bioassay test data are reported as the mean of three replicate microcosms, and SEPs were performed in triplicate, recording the mean value ± standard deviation (±SD). Data statistical analyses were performed using Statistica v. 6.0 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Soil Analysis

The soil from the contaminated site was quite homogeneous, and the characterization analysis carried out in 2018 clearly showed that the chemical physical characteristics of the 15 soil samples were essentially the same, including those from the uncontaminated soil except for Cd contamination level. The characterization analysis repeated in 2022 showed that all values remained unchanged. The soil was classified as sandy loam and its characteristics, expressed as mean values (for sake of simplicity the individual values of each sample are not reported), are described in Table 1.

Table 1. Mean values of soil characteristics of the 15 collected samples. Values are reported as mean ± Standard Deviation (SD). EC indicates Electrical conductivity, CEC indicates Cation Exchange Capacity.

Soil Characteristics	Mean Value ± SD
pH	6.3 ± 0.4
EC (mS cm^{-1})	14.4 ± 1.3
Clay (%)	13.8 ± 1.6
Silt (%)	29.2 ± 2.1
Sand (%)	55.5 ± 3.5
CEC (cmol(+)kg^{-1})	14.4 ± 1.3
Organic matter (%)	0.9 ± 0.05

3.2. Soil Contamination

Total content of cadmium in individual samples collected at the site under investigation in the years 2018 and 2022 is reported in Table 2.

Table 2. Total content of cadmium in individual samples collected at the site under investigation (S1–S15). US is the uncontaminated soil Cd concentration. Values are reported as mean ± SD. Different letters indicate significant differences ($p < 0.05$) in total concentrations between 2018 and 2022 in each soil sample.

Sampling Point	Total Cd (2018)	Total Cd (2022)
S1	52.1 ± 3.4 a	50.6 ± 4.2 a
S4	40.7 ± 3.0 a	41.8 ± 3.7 a
S2	30.3 ± 2.5 a	31.6 ± 2.9 a
S5	21.7 ± 2.1 a	22.1 ± 2.5 a
S7	18.6 ± 1.8 a	17.7 ± 1.9 a
S11	15.4 ± 1.2 a	17.1 ± 2.3 a
S12	13.2 ± 1.1 a	11.9 ± 2.7 a
S8	9.4 ± 1.0 a	10.2 ± 1.7 a
S3	7.2 ± 0.7 a	6.9 ± 0.82 a
S9	5.3 ± 0.5 a	5.9 ± 0.9 a
S15	3.6 ± 0.3 a	4.1 ± 0.4 a
S6	1.81 ± 0.2 a	1.63 ± 0.2 a
S13	0.66 ± 0.04 a	0.60 ± 0.07 a
S14	0.54 ± 0.04 a	0.56 ± 0.05 a
S10	0.38 ± 0.03 a	0.40 ± 0.06 a
US	0.5 ± 0.001 a	0.5 ± 0.003 a

Since there were no statistically significant differences in the total concentration of Cd in the selected sampling points after four years, the total Cd can be considered unchanged. The results differ from other studies which reported a reduction in total concentrations due to downward leaching of cadmium with aging [45,63]. In the studied soil, metal leaching probably occurred shortly after contamination, estimated by local authorities at about 15/30 days before the first sampling, and the process had essentially finished by the time of the first sampling in 2018. In fact, leaching tends to be particularly significant in the early days after a contamination event [45].

Of the various approaches to evaluate the degree of heavy metal contamination, the geoaccumulation index (Igeo), was selected because it refers to the local background concentration, which is crucial to evaluate the pollution level of soil [64].

The Igeo index defines seven classes of soil contamination [60,65–67] from non-polluted (Igeo < 0) to extremely polluted (Igeo ≥ 5).

Based on the Cd concentration values of the soil samples in the contaminated site, five different degrees of contamination were found: extremely polluted (Igeo > 5), heavily to extremely polluted (4 < Igeo < 5), "severe" (3 < Igeo < 4), "moderate to severe" (2 < Igeo < 3) and "moderate" (1 < Igeo < 2). The class defined as unpolluted to moderately polluted

(0 < Igeo < 1) in the specific case can be considered unpolluted as the values are identical to the background value (Table 2).

The Igeo index values for each sampling point are reported in Figure 1.

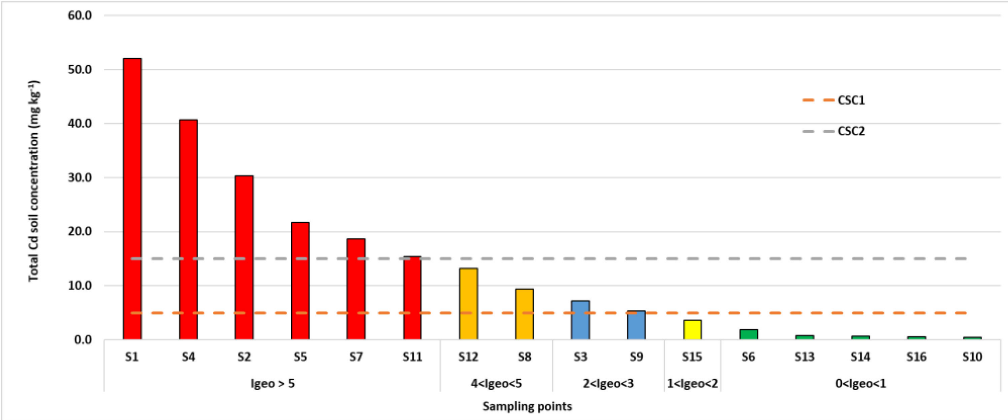

Figure 1. Igeo index values for each sampling site. CSC1 and CSC2 are the Italian legislation threshold limits for soil in green areas and industrial areas, respectively. The total concentration reported is that of the first sampling campaign (2018) as the values remained significantly unchanged after four years.

Although this index is widely used in the scientific literature, it is not used in the European regulations on contaminated soils. In the case of Italy in particular, the level of cadmium concentration (defined by the acronym CSC in Italian legislation) that would trigger an investigation into potential contamination is 5 mg kg^{-1} for public and residential green areas and 15 mg kg^{-1} for industrial soils (CSC1 and CSC2 respectively in Figure 1). However, the Igeo index provides an interesting evaluation of the pollution since the concentration in the contaminated site is compared with the background value of the affected area in order to identify the anthropic contribution to the total concentration of the metal. Anthropic inputs are usually also characterized by greater mobility of the metal and therefore greater environmental danger [35,68]. In the case study it can be noted that the samples with a total metal concentration lower than 1 are substantially identical to the US background reference soil. furthermore, according to Italian legislation, the samples S15, S6, S13, S14, and S10 are considered uncontaminated.

3.3. Changes in Cd Extractability with Aging

The Igeo index helps to define the extent of contamination but fails to provide a picture of the evolution of contamination in the case of heavy metals that remain in place, as in this case. Different tools therefore need to be used to evaluate the effects of soil aging.

Cadmium mobility and bioavailability in soils are determined by chemical forms of the metal, therefore, metal fractionation by sequential extraction procedures (SEPs) is considered as the basis to evaluate Cd aging in soil [69]. There are many varieties of sequential extractions used and all the SEPs are subject to possible criticism [70]. However, although the different fractions of metals are only operationally defined, SEPs can provide valuable data concerning the distribution of metals in different pools of bioavailability in soil [71].

The SEP used (H_2O, KNO_3, EDTA) separates Cd according to the strength of the linkages with soil solid phases in soluble (H_2O), exchangeable fractions (KNO_3) characterized by non-specific adsorption reactions and specific sorbed and complexed fractions (EDTA) [51–55]. Cd extractability is reported in Table 3.

The results obtained showed Cd soluble forms only in the first sampling (2018) and only in some of the selected sampling points belonging to the most contaminated zones. The water extractable species include free ions and soluble Cd complexes which are the most mobile and potentially bioavailable species [30]. The results obtained in 2018 showed that where the contamination was highest (Igeo > 5), the concentration of extractable Cd was rather high at around 1.5 mg kg^{-1} which accounts for about 3% to 6% of the total Cd. However, after four years, also in these sampling points, the concentration of Cd in this fraction was below the detection limit.

Table 3. Cd concentration in the different fractions extracted by the SEP used. Data (mg kg^{-1}) are reported as mean ± SD. US = uncontaminated soil Cd concentration. Nd (not detectable) = below the detection limit. Different letters indicate significant differences ($p < 0.05$) in concentrations between 2018 and 2022 in each fraction of the SEP for each soil sample.

Sample	H_2O		KNO_3		EDTA	
	2018	2022	2018	2022	2018	2022
S1	1.5 ± 0.02	nd	7.2 ± 0.11 b	4.2 ± 0.03 a	34.7 ± 1.48 a	38.1 ± 1.61 b
S4	1.8 ± 0.03	nd	6.6 ± 0.09 b	3.1 ± 0.02 a	23.9 ± 1.30 a	25.4 ± 1.23 b
S2	1.5 ± 0.03	nd	4.2 ± 0.07 b	2.8 ± 0.03 a	20.6 ± 1.15 a	22.4 ± 1.11 b
S5	1.3 ± 0.02	nd	3.1 ± 0.03 b	1.5 ± 0.01 a	13.4 ± 0.72 a	14.5 ± 0.09 b
S7	0.80 ± 0.01	nd	2.6 ± 0.03 b	1.1 ± 0.01 a	12.1 ± 0.68 a	13.0 ± 0.08 b
S11	0.60 ± 0.01	nd	2.1 ± 0.02 b	0.96 ± 0.01 a	7.6 ± 0.10 a	8.8 ± 0.09 b
S12	0.65 ± 0.02	nd	1.5 ± 0.02 b	0.90 ± 0.02 a	6.2 ± 0.09 a	6.9 ± 0.07 b
S8	nd	nd	1.2 ± 0.01 b	0.85 ± 0.01 a	5.2 ± 0.10 a	5.7 ± 0.05 b
S3	nd	nd	1.1 ± 0.02 b	0.80 ± 0.01 a	3.9 ± 0.06 a	4.0 ± 0.05 b
S9	nd	nd	0.82 ± 0.01 b	0.76 ± 0.01 a	3.1 ± 0.06 a	3.2 ± 0.03 b
S15	nd	nd	0.68 ± 0.01 b	0.63 ± 0.02 a	1.8 ± 0.05 a	2.0 ± 0.03 b
S6	nd	nd	0.55 ± 0.02 b	0.28 ± 0.02 a	0.92 ± 0.01 a	1.1 ± 0.01 b
S13	nd	nd	0.14 ± 0.01 b	0.10 ± 0.01 a	0.24 ± 0.01 a	0.26 ± 0.02 b
S14	nd	nd	0.11 ± 0.01 b	0.060 ± 0.002 a	0.28 ± 0.02 a	0.29 ± 0.02 b
S10	nd	nd	0.025 ± 0.02 b	0.010 ± 0.001 a	0.18 ± 0.02 a	0.18 ± 0.01 b
US	nd	nd	0.010 ± 0.01 a	0.010 ± 0.001 a	0.014 ± 0.001 a	0.014 ± 0.001 a

As regards the extractability by KNO_3, the extractable amount was quite high at around 15% of the total in 2018. After four years, Cd extractability in KNO_3 decreased in all sampling points from about 30% to about 60% compared to the 2018 data. However, in the most contaminated samples, the extractability was still quite high: around 8% of the total concentration. Cd chemical forms extracted by KNO_3 include weakly-sorbed metal species, particularly those retained on the soil surface by relatively weak electrostatic interactions and those that can be released by ion-exchange processes [53], thus can be considered potentially bioavailable to plant uptake.

EDTA was used because this complexing agent is able to extract Cd ions that are bound to organic matter [70] (Gleyzes et al., 2002), and to oxide in soils [72]. The EDTA-extractable forms are generally considered to be inner-sphere surface complexed forms [73].

The Cd extractable quantity in EDTA was very high. In 2018 in the most contaminated sampling points, it was around 65% of the total. The extractability in EDTA has an inverse trend compared to the other two extractants with the passage of time. In fact, after four years the Cd extractable quantity increased at all sampling points, reaching approximately 70% of the total. In the absence of leaching, this trend is not surprising and indicates a modification in the chemical forms of the Cd due to aging which induces the passage of the metal from the more mobile forms to the less soluble and less exchangeable ones.

The results from SEP are in accordance with previous studies regarding the change of chemical forms over time [73]. However the results obtained in this long-term experiment differ considerably from those obtained in the laboratory. In fact in laboratory tests most of Cd added to soils is found in the soluble and exchangeable fractions. In our case instead the highest extractability appeared in the EDTA fraction. Even when laboratory tests are conducted for longer times the exchangeable fractions remained the most dominant in all soils [73], while in the field, after 4 years very strong bonds are formed between Cd and soil surfaces, as shown by the increase in amount extractable using EDTA. In the initial period after the contamination event, the metal is assumed to be subject to adsorption reactions on the negatively charged surfaces of soil minerals and organic matter through the formation of outer-sphere complexes. With aging, chemical bonds with soil surfaces can become more stable due to the gradual diffusion of the metal within the micropores and mesopores of the solid phase which can lead to formation of inner-sphere complexes, entrapment within the solid phases as well as precipitation reactions [47,73,74].

The data obtained showed that the sum of the extractable fractions tends to remain constant in soil samples with a low Igeo index, and tends to decrease, albeit with some exceptions, in soils with a higher Igeo index.

3.4. Effects of Aging on Cd Plant Uptake

The uptake of Cd by plants requires the release of the metal from solid soil surfaces and its transfer in the soil solution. Results from the SEP provide useful indications of this process, however the soil extractants measure only the potential Cd bioavailability [75]. The plant physiology and related rhizosphere can modify the relationship between Cd and its plant uptake [76]. Plants can be used to better understand the metal bioavailability and toxicity in soil during the aging process.

The bioassay experiments carried out on the soil sampled at the two different times showed a definite aging effect on the biomass production. In both 2018 and 2022 the plants grown in the contaminated soils showed no visual signs of stress or phytotoxicity, however the biomass yields were somewhat lower than those grown in uncontaminated soil. The results of the shoot biomass production are reported in Figure 2.

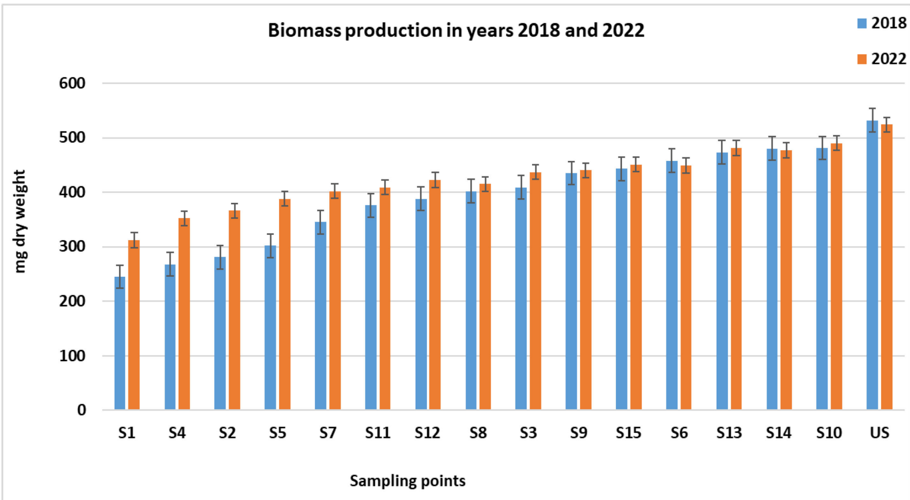

Figure 2. Total shoot biomass (mg dw) of *B. Juncea* obtained from bioassay tests carried out on soils sampled in 2018 and 2022. Data are reported as means ± SD (indicated by the bars).

There was a significant reduction in biomass production compared to the non-contaminated soil. The plants grown in the soil sampled in 2018 show a reduction in yields up to around 53% (sample S1) compared to the yield in US soil. In the tests carried out on the soil sampled in 2022, the biomass production decreased up to about 40% compared to the yield in the US soil. The results obtained are in accordance with other studies which show the direct effect of cadmium on the yield of different plant species [77,78]. After four years the reduction of the mobile and bioavailable chemical forms of Cd, due to aging, promoted an increase of plants biomass with respect to those of 2018.

The Cd concentration in the above ground part of *B. Juncea* grown in the control soil was below the detection limit. In contrast in the microcosms prepared with the soils collected in contaminated areas in 2018, the Cd in shoots ranged from about 0.24 mg kg^{-1} (S10) to about 15.96 mg kg^{-1} (S1). Plants grown in the soils collected after four years showed a trend of reduction in the uptake of Cd and the concentration in the shoots ranged from 0.18 (S10) to 11.74 mg kg^{-1} (S1). The plant uptake in the two years is reported in

Table 4 together with the translocation factor (TF) calculated as the Cd concentration in shoots/Cd concentration in roots [79,80].

Table 4. Mean values of Cd concentration in the shoots and roots of *B. Juncea* and corresponding TF. Data reported are mean values ± SD, obtained in 2018 and 2022 expressed as mg kg^{-1} on a dry weight basis. Different letters indicate significant differences ($p < 0.05$) in concentrations between roots 2018 and 2022 and shoots 2018 and 2022 for each soil sample.

Sample	2018			2022		
	Roots	Shoots	TF	Roots	Shoots	TF
S1	19.50 ± 1.64 b	15.96 ± 1.33 b	0.82	15.40 ± 1.30 a	11.74 ± 1.6 a	0.76
S4	18.01 ± 1.22 b	15.03 ± 1.08 b	0.83	14.10 ± 1.14 a	11.05 ± 0.95 a	0.78
S2	16.93 ± 1.56 b	13.39 ± 1.15 b	0.79	12.60 ± 1.04 a	9.85 ± 0.82 a	0.78
S5	12.10 ± 0.84 b	9.57 ± 0.59 b	0.79	8.72 ± 0.74 a	7.04 ± 0.70 a	0.81
S7	10.08 ± 0.65 b	8.21 ± 0.51 b	0.81	7.68 ± 0.86 a	6.03 ± 0.53 a	0.79
S11	8.51 ± 0.69 b	5.74 ± 0.34 b	0.67	6.35 ± 0.46 a	4.22 ± 0.50 a	0.66
S12	8.07 ± 0.51 b	5.55 ± 0.45 b	0.69	6.00 ± 0.46 a	4.08 ± 0.31 a	0.68
S8	7.09 ± 0.40 b	4.89 ± 0.29 b	0.69	5.48 ± 0.33 a	3.60 ± 0.22 a	0.66
S3	5.41 ± 0.36 b	3.60 ± 0.24 b	0.67	3.93 ± 0.26 a	2.65 ± 0.16 a	0.67
S9	4.72 ± 0.38 b	2.94 ± 0.11 b	0.62	3.25 ± 0.25 a	2.16 ± 0.22 a	0.67
S15	4.50 ± 0.21 b	1.91 ± 0.09 b	0.42	3.15 ± 0.18 a	1.41 ± 0.07 a	0.45
S6	1.98 ± 0.06 b	0.81 ± 0.01 b	0.41	1.45 ± 0.04 a	0.59 ± 0.03 a	0.41
S13	0.75 ± 0.05 b	0.35 ± 0.02 b	0.47	0.63 ± 0.02 a	0.26 ± 0.01 a	0.41
S14	0.70 ± 0.04 b	0.30 ± 0.02 b	0.43	0.58 ± 0.03 a	0.22 ± 0.01 a	0.38
S10	0.61 ± 0.04 b	0.24 ± 0.03 b	0.40	0.49 ± 0.02 a	0.18 ± 0.02 a	0.36
US	0.08 ± 0.005 a	0.03 ± 0.005 a	0.38	0.07 ± 0.005 a	0.024 ± 0.002 a	0.40

When evaluating the contamination of the site under examination, the persistence of cadmium in the soil needs to be differentiated from the persistence of the mobile, soluble or easily exchangeable forms. The results of the sequential extraction provide an image of the aging processes that reduced the mobility and bioavailability of cadmium. Further confirmation is also provided from the results of the biological tests. The bioassay also shows that the bioavailability of cadmium decreased with aging. In fact, the production of biomass increased with time and the Cd concentration in the plant decreased after four years.

Comparing the results of the chemical and biological tests, there is an agreement between the extractability in KNO_3 and the concentration of the metal in the plants. In fact, the reduced extractable quantity corresponds to a decrease in concentration in the plants (Figure 3).

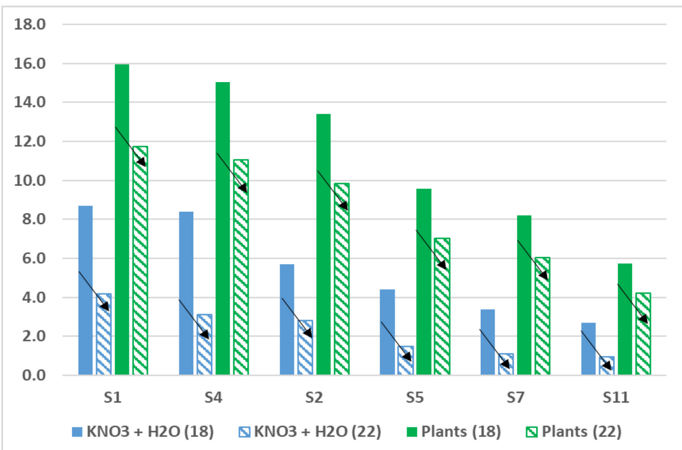

Figure 3. Trend of the concentration of Cd in plants in relation to the extractability of Cd with KNO_3. The arrows indicate the accordance (same direction) between chemical and biological tests.

On the contrary, while the extractable fraction in EDTA increased over time, there was no increase in plant uptake, thus the extraction in EDTA often used to define the bioavailable forms of metals [81] resulted in contrast with the data of the biological test.

The data are reported in Figure 4.

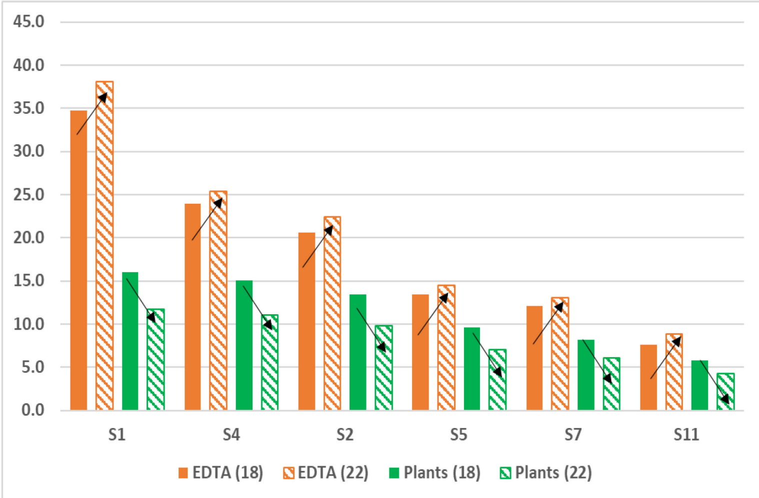

Figure 4. Trend of the concentration of Cd in plants in relation to the extractability of Cd with EDTA. The arrows indicate the discordance (contrasting direction) between chemical and biological tests.

Since the extractable quantity of EDTA increased over time, while the uptake by the plants was reduced after four years, the extraction with EDTA, in this case, does not provide reliable information regarding the actual uptake by plants. Therefore, the evaluation of bioavailability needs an understanding of the chemical processes involved not only in the soil, but also of the complex interactions at the soil-plant interface. The results obtained in this case study indicate that the extractability in EDTA tends to overestimate the bioavailable amount of cadmium, which is best represented by the extractable amount in KNO_3. However, EDTA extraction provides interesting data that can be used to evaluate the possibility of using this complexing agent as a Cd mobilizer in the case of assisted phytoextraction.

This result is important in terms of soil remediation. In this study it is clear that Cd concentration in soil affected the metal uptake. However, it should be noted that even in the most contaminated soil samples, the effect of the soil-plant barrier [82] limited the absorption of Cd by *B. juncea*. Moreover, the translocation of the metal in the aerial part of the plants was low. In both 2018 and 2022, for plants growing in all the 15 soil samples, the translocation factor (TF) defined by the ratio between the Cd concentration in shoots and Cd concentration in roots was always smaller than 1, ranging from 0.36 to 0.82. These results are in accordance with previous studies [83,84], and confirm that roots act as a barrier against the translocation of the metal to the aerial parts of *B. juncea*.

Evaluation of the bioassays results as a feasibility test for the possible use of phytoextraction, must be based on the total removal of the metal from the soil by the plants. The efficiency of Cd phytoextraction was thus calculated according to the following relation [80,85,86]:

$$\text{Cd Total uptake} = \text{shoot dry biomass of plants in a microcosm} \times \text{shoot Cd concentration}$$

$$\text{Phytoextraction efficiency (\%)} = (\text{total uptake}/\text{soil Cd content in a microcosm}) \times 100$$

The data reported in Table 5 show that the brassica plants remove only a very low fraction of the Cd present in the soil, less than 0.1% even in the most contaminated samples.

Table 5. Total uptake (µg) and removal percentage (%) by *B. juncea* in 2018 and 2022.

Sample	Year 2018		Year 2022	
	Total Uptake	% Removal	Total Uptake	% Removal
S1	5.32	0.020	4.97	0.019
S4	5.48	0.027	5.21	0.026
S2	5.12	0.034	5.16	0.034
S5	3.93	0.036	3.81	0.035
S7	3.85	0.041	3.10	0.033
S11	2.93	0.038	2.23	0.029
S12	2.93	0.044	2.24	0.034
S8	2.67	0.057	2.08	0.044
S3	2.00	0.056	1.68	0.047
S9	1.74	0.066	1.43	0.054
S15	1.15	0.064	0.73	0.041
S6	0.50	0.056	0.33	0.037
S13	0.23	0.061	0.0051	0.0014
S14	0.20	0.060	0.0050	0.0015
S10	0.16	0.059	0.0050	0.0018
US	0.024	0.013	0.0094	0.0050

Even considering the total removal in relation to only the bioavailable fraction, as is often recommended in the evaluation of phytoextraction [87,88], the amount removed from the plants, would not reach, on average, more than 5% (data not reported). Clearly, no conclusions can be drawn from the evaluation of a single plant species, and different species should be tested. *Brassica juncea* which is considered a plant with great potential for phytoremediation [89] is a good indicator for Cd uptake even if it is not an accumulator species [90–93]. However, the data obtained strongly suggest that the technology could only be applied in the form of "assisted phytoremediation" by applying, for example, a complexing agent such as EDTA [94]. However, the use of complexing agents can also cause further environmental risks, in fact the quantity of cadmium mobilized may be higher than the quantity absorbed by the plants, and being in soluble form, it could percolate along the soil profile [95].

3.5. Effect of Ageing on Cd Bioaccessibility

In addition to the soil-plant-food chain route, incidental ingestion of soil is an important exposure pathway. Oral ingestion is often the critical exposure pathway for children [96]. The negative effects of this pathway can be assessed in terms of bioaccessibility, which is defined as the fraction of a metal in soil which, after ingestion, becomes soluble in the stomach and is thus available for intestinal absorption [32,33,97,98]. An estimation of Cd bioaccessibility is therefore useful to better assess the human health risks from soil ingestion exposure. The data on bioaccessible Cd in the two sampling campaigns are reported in Table 6.

In natural soils the bioaccessible fraction is generally very low [99,100] and also in this study in the unpolluted soil, the Cd bioaccessible concentration was very low (0.02 mg kg^{-1}). In contrast in the contaminated samples, the Cd concentration values in the bioaccessible extracts were often rather high in both sampling campaigns with values ranging from 40 to 60% of the total content similarly to what was found for this metal in previous studies [99,101]. The differences between the two concentrations were not statistically significant (Table 6).

The amount of bioaccessible Cd was significantly related to the total metal content (R^2 = 0.988) in soil and less to the most available fractions such as water-soluble and exchangeable fractions. This is in agreement with a previous study where different levels of bioaccessible Cd in soils were mostly related to the total metal content [33], and it is consistent with the fact that both total and bioaccessible quantities are based on acid extraction.

Table 6. Bioaccessibility values (mg kg^{-1}), determined in the two years of soil sampling. Data are reported as mean ± SD. Different letters indicate significant differences ($p < 0.05$) in concentrations between bioaccessibility in 2018 and in 2022 for each soil sample.

Sample	Bioaccessibility	
	2018	2022
S1	31.4 ± 2.30 a	30.4 ± 2.10 a
S4	21.8 ± 1.50 a	20.9 ± 1.10 a
S2	19.9 ± 1.15 a	18.6 ± 1.11 a
S5	11.6 ± 0.88 a	10.4 ± 0.91 a
S7	11.5 ± 0.86 a	10.1 ± 0.79 a
S11	7.32 ± 0.42 a	7.82 ± 0.35 a
S12	6.31 ± 0.38 a	5.73 ± 0.33 a
S8	5.29 ± 0.35 a	4.80 ± 0.37 a
S3	3.54 ± 0.29 a	3.21 ± 0.20 a
S9	2.91 ± 0.24 a	2.66 ± 0.26 a
S15	2.26 ± 0.27 a	1.97 ± 0.25 a
S6	0.88 ± 0.12 a	0.68 ± 0.13 a
S13	0.024 ± 0.08 a	0.021 ± 0.07 a
S14	0.025 ± 0.06 a	0.023 ± 0.05 a
S10	0.025 ± 0.01 a	0.024 ± 0.02 a
US	0.022 ± 0.01 a	0.022 ± 0.01 a

In other cases, bioaccessible metals were found to be correlated with the exchangeable fractions [44,102]. However, in the latter studies, the soils were spiked with heavy metals, which could be important in explaining the diversity of the results obtained in our study where the effects of aging may have changed the original chemical forms of the metal.

Soil oral ingestion together with inhalation of soil dust and dermal contact have been recognized as a critical exposure pathway [103,104]. Including metal bioaccessibility in soil risk assessment and monitoring strategies has increased in the last few years in relation to human health protection [101]. The influence of aging on bioaccessibility has been well documented for organic contaminants [105], while several aspects of the aging trend of the bioaccessible fractions of metals in soil over time still needs to be still clarified. The speciation of cadmium in soil largely regulates its dissolution in the gastrointestinal system which is also promoted by the low pH in the stomach. Evaluating the change with time of the bioaccessible fraction of the metal may thus be of great help for the risk assessment of soil in relation to the potential negative health effects [37,106]. Even when long incubation times are used in the laboratory, the conditions are not fully comparable with those of naturally aged field soils [42,45,107]. To explain the changes in bioavailability and bioaccessibility of Cd it must be taken into account not only the fast distribution of the metals among the soil phases after the first days of contamination, but also the slow sorption and desorption processes in the long time. In addition to the fast reactions of adsorption of the first phase which are studied in laboratory experiments with spiked soils, in this field experiment also the very much slower continuing sorption reactions impacted Cd speciation is soil. Thus bioavailability and bioaccessibility were influenced by the Cd diffusive penetration into soil constituents, precipitation and slow chemisorption on reactive soil surfaces where the metal strongly bound may not be likely to be easily released. The study of the aging of cadmium under real field conditions is therefore an important step for the accurate assessment of soil contamination.

4. Conclusions

It is recognized that high levels of contaminants do not necessarily imply an increased risk. Assessing the bioavailability and bioaccessibility is thus essential to determine whether the concentration of a contaminant in the soil has negative effects on humans and the environment. Few studies have dealt with aging in real field conditions. In this case study, some aspects found in other soils and with different types of contamination were confirmed, such as the modification of the distribution of Cd in the fractions of a sequential extraction, with a clear decrease in the more mobile and soluble fractions. Thus, phytoextraction treatment could be less effective over time, and to counterbalance the reduced bioavailability the addition of Plant Growth Promoting Rhizobacteria (PGPR) or biodegradable complexing agents should be necessary. For a complete assessment of the risks for human health, the results of the bioaccessibility of Cd should not be overlooked. In contrast to bioavailability, in our study bioaccessibility did not decrease after four years. It is crucial that oral ingestion is therefore closely monitored depending on the final destination of the site.

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