



Chemical and biological methods to evaluate the availability of heavy metals in soils of the Siena urban area (Italy)

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Corresponding Author: Dr. Francesco Nannoni, Ph.D.

Corresponding Author's Institution: University of Siena

First Author: Francesco Nannoni, Ph.D.

Order of Authors: Francesco Nannoni, Ph.D.; Giuseppe Protano, Dr

Abstract: A biogeochemistry field study was conducted in the Siena urban area (Italy) with the main objective of establishing the relationship between available amounts of heavy metals in soil assessed by a chemical method (soil fractionation) and bioavailability assessed by a biological method (bioaccumulation in earthworm tissues). The total content of traffic-related (Cd, Cu, Pb, Sb, Zn) and geogenic (Co, Cr, Ni, U) heavy metals in uncontaminated and contaminated soils and their concentrations in soil fractions and earthworms were used for this purpose. The bioavailability of heavy metals assessed by earthworms did not always match the availability defined by soil fractionation. Earthworms were a good indicator to assess the bioavailability of Pb and Sb in soil, while due to physiological mechanisms of regulation and excretion, Cd, Cu and Zn tissue levels in these invertebrates gave misleading estimates of their bioavailable pool. No relationship was identified between chemical and biological availability for the geogenic heavy metals, characterized by a narrow range of total contents in soil. The study highlighted that chemical and biological methods should be combined to provide more complete information about heavy element bioavailability in soils.

Response to Reviewers: Reviewer #1

The manuscript Number: STOTEN-D-16-01282: "Chemical and biological methods to evaluate the bioavailability of heavy metals in soils of the Siena urban area (Italy)" proposed for Research Paper to Science of the Total Environment, concerns bioavailability (earthworms), soil fractionation (chemical extractions on soil) in the case of urban soil impacted by car traffic.

The subject is interesting and persistent metals are widely observed in urban soils with environmental and sometimes health impact.

The study is seriously performed and the manuscript is well organized. My opinion is that the manuscript could be published in STOTEN, however, before acceptance the following changes are needed.

1. The discussion could be improved in order to better highlight the link between compartmentalization of metals in soils and earthworm absorption.

Chemical and biological methods to evaluate the availability of heavy metals in soils of the Siena urban area (Italy)

Francesco Nannoni*, Giuseppe Protano

Department of Environmental, Earth and Physical Sciences, University of Siena, Via del Laterino 8, I-53100, Siena, Italy

*Corresponding author:

Tel: +39 0577 233944

E-mail address: nannoni@unisi.it (F. Nannoni)

Department of Environmental, Earth and Physical Sciences, University of Siena

Via del Laterino 8, I-53100, Siena, Italy

ABSTRACT

A biogeochemistry field study was conducted in the Siena urban area (Italy) with the main objective of establishing the relationship between available amounts of heavy metals in soil assessed by a chemical method (soil fractionation) and bioavailability assessed by a biological method (bioaccumulation in earthworm tissues). The total content of traffic-related (Cd, Cu, Pb, Sb, Zn) and geogenic (Co, Cr, Ni, U) heavy metals in uncontaminated and contaminated soils and their concentrations in soil fractions and earthworms were used for this purpose. The bioavailability of heavy metals assessed by earthworms did not always match the availability defined by soil fractionation. Earthworms were a good indicator to assess the bioavailability of Pb and Sb in soil, while due to physiological mechanisms of regulation and excretion, Cd, Cu and Zn tissue levels in these invertebrates gave misleading estimates of their bioavailable pool. No relationship was identified between chemical and biological availability for the geogenic heavy metals, characterized by a narrow range of total contents in soil. The study highlighted that chemical and biological methods should be combined to provide more complete information about heavy element bioavailability in soils.

Keywords: Chemical availability; Biological availability, Heavy metals; Soil fractionation; Earthworms; Urban area.

1. Introduction

The distribution of heavy metals in soil fractions, so-called chemical fractionation, is an important aspect of soil geochemistry as it governs the behavior of these elements in soil and plays a relevant role in determining their availability for soil biota. Availability is considered an essential parameter for effective uptake and accumulation of heavy metals in soil organisms, and therefore an important tool in assessment of environmental risk. A number of physico-chemical and biological processes influence the bioavailability of heavy metals in soil, indicating that their transfer from soil to biota is as a very complex phenomenon.

According to a general definition, “*The bioavailable fraction of a chemical is the fraction of its total amount present in a specific environmental compartment that, within a given time span, is either available or can be made available for uptake by (micro)organisms from either the direct surrounding of the organism or by ingestion of food*” (Peijnenburg et al., 2007). In this view, the available fraction of a chemical element in soil depends both on soil factors such as element distribution in soil fractions and physico-chemical processes occurring with time (e.g. ionic exchange, acid dissolution and redox reactions), and on biological factors such as the physiological activities of soil organisms, especially affecting ingested soil (Harmsen, 2007; Peijnenburg et al., 2007).

Considering the various factors influencing element availability in soil, more specific available pools may be defined (Harmsen, 2007; Peijnenburg et al., 2007; Maleri et al., 2008; Alvarenga et al., 2012): 1) the “effective available” pool consisting of water-soluble and extractable fractions, which are the most mobile and very active pool of a chemical element, mainly regulated by rapid reactions in soil such as water solubilization, ionic exchange and acid dissolution; 2) the “potentially available” pool, consisting of reducible and oxidable fractions, namely the leachable and partly active pool mainly mobilizable by redox reactions; 3) the “bioaccessible” pool, the amount of a chemical element that can be released through digestion by water and ingested soil; this is the fraction most available for intestinal uptake.

The availability of a chemical element for soil organisms may be assessed by two complementary ways: chemical and biological methods. The chemical methods are single or sequential extraction procedures to determine the amount of a chemical associated with a specific soil fraction, such as water-soluble, extractable, reducible, oxidable and residual. The partitioning of heavy metals in these fractions is mainly

controlled by the physico-chemical properties and composition of soil (e.g. pH, cation exchange capacity and organic matter content) as well as reactions such as sorption, precipitation and coprecipitation. The element fractions determined by chemical extractions can therefore be defined operationally by the procedures used, as well as functionally as effective and potentially available pools (Gupta et al., 1996; Peijnenburg et al., 2007). As bioavailability is not merely a chemical concentration, the extraction procedures may only offer a snapshot of the different fractions of chemicals to which soil species may be exposed, but this does not consider the biological aspects, such as physiology, behavior and exposure time. Therefore, assessment of the available fraction by chemical methods is empirical and highly dependent on the extraction procedure used.

Biological methods identify the aliquot of the element available fraction that a soil organism can absorb and accumulate. These methods evaluate the bioavailability of a chemical element in soil through its bioaccumulation in soil organisms (mainly invertebrates), used as bioindicators exposed to a single or several chemicals. Biological methods also consider the physiological capacity of a soil organism to uptake chemical elements by dermal absorption and digestion in the gut, and to accumulate them in tissues.

An organism frequently used to assess bioavailability of heavy metals in soil is the earthworm (Peijnenburg et al., 1999; Conder et al., 2002; Maddocks et al., 2005; Nahmani et al., 2007). Earthworms are relatively efficient accumulators of essential and non-essential metals such as Cd, Cu, Pb and Zn (Morgan and Morgan, 1999; Lanno et al., 2004; Suthar et al., 2008). Several environmental and ecotoxicological studies have used these invertebrates as bioindicators of heavy metal levels in soil in contaminated areas, providing data for the assessment of environmental risk (e.g. Kennette et al., 2002; Nahmani et al., 2007; Suthar et al., 2008; Goix et al., 2015). Furthermore, chemical analysis of earthworms may provide an indication of bioavailable amounts of heavy metals (Maleri et al., 2008; Ruiz et al., 2011).

Uptake of heavy metals by earthworms is mainly from pore water, food and ingested soil particles (Oste et al., 2001; Lanno et al., 2004; Hobbelen et al., 2006). The storage, accumulation and excretion of these chemicals seem to be controlled by ecological and physiological factors (Spurgeon and Hopkin, 1996; Morgan and Morgan, 1999; Kamitani and Kaneko, 2007; Suthar et al., 2008; Nannoni et al., 2014). Field and laboratory studies have demonstrated that uptake and bioaccumulation of heavy metals

by earthworms are not related to the total content of these contaminants in soil, as these organisms only respond to the biologically available and accessible fractions (Alexander, 2000; Dai et al., 2004; Harmsen, 2007).

An important target of biogeochemistry studies is to define the relationships between the chemical fractionation of heavy metals in soil and their uptake and bioaccumulation by soil organisms, that is, the relationships between the available (effective and potentially) and bioaccessible pools of heavy metals and the amounts accumulating in tissues.

The question is of particular concern in areas contaminated by heavy metals, such as urban areas. Urban areas are affected by several human activities such as vehicle traffic, industries, domestic heating and municipal waste incinerator, which are point and diffuse sources of toxic and potentially toxic heavy metals such as Cd, Cu, Pb, Sb and Zn. In urban settings, soil plays a key role in the environmental pathway of heavy metals, as it controls their accumulation and release to natural waters and soil biota. Soil is therefore a good indicator of human disturbance (contamination) and potential risk for the surface environment, especially the urban ecological system.

A number of studies have dealt with heavy metal contamination in urban areas, assessing the influence of human sources, mainly vehicle traffic, on the total contents of these contaminants in surface soils (e.g. Imperato et al., 2003; Acosta et al., 2011; Sun et al., 2010). To our knowledge, few studies have focused on the fractionation and mobility of heavy metals in contaminated urban soils (Burt et al., 2014; Acosta et al., 2015; Gu et al., 2016), and even less their availability in soil and transfer to earthworms (Pizl and Josens, 1995; Kennette et al., 2002; Nannoni et al., 2011).

In this context, a biogeochemistry field study was conducted in the urban area of Siena (central Italy) with the following aims: i) to determine the influence of contamination, mainly related to vehicle traffic, on the partitioning of heavy metals in soil fractions; ii) to establish the main soil fractions involved in uptake of heavy metals by earthworms; iii) to define the relationships between available and bioaccessible amounts of heavy metals in soil, evaluated by chemical extraction, and bioavailability assessed by bioaccumulation in earthworm tissues.

The present research is a prosecution of a previous study of Nannoni et al. (2014) focused on the relationships between the total soil contents of heavy metals in Siena urban soils and their accumulation in earthworm tissues, as well as the evaluation of the main soil properties as possible factor influencing metal uptake by these invertebrates.

2. Materials and methods

2.1. Study area

The present study was realized in the territory of Siena municipality including both the urban centre and the peri-urban, green-urban and non-urban zones. The resident population in the Siena city is of about 55,000 inhabitants, and the main human sources of heavy metals in the surface environment are the vehicle traffic and domestic heating in wintertime (Nannoni et al., 2014). About 50,000 vehicles move daily mostly along the ring road extending in the peri-urban sector of the Siena city. Since 1965 a limited traffic zone (LTZ) limits the vehicle traffic in the historic centre of Siena.

The study area is located in the northern part of the Siena basin. This basin is a NW-SE graben-type tectonic depression in which clayey-silty-marly to sandy-marly marine sediments of the Neogene-Quaternary succession deposited during Pliocene. These sediments represent the lithological substratum from which soils of the study area were formed.

2.2. Soils and earthworms sampling procedures

A recent study of Nannoni et al. (2014) provided the main physico-chemical properties and the total content of Cd, Co, Cr, Cu, Ni, Pb, Sb, U and Zn in 30 soil samples collected in non-urban, green-urban, peri-urban and urban sites of the Siena municipality. Furthermore, these authors measured the concentrations of these heavy metals in tissue of 150 mature (clitellated) specimens of *Nicodrilus caliginosus* (Savigny) earthworm collected in 15 of 30 soil sampling sites (10 earthworm for each site).

The present study concerned the 15 sampling sites of the territory of Siena municipality where both soil and earthworms were collected. These sites were as follows: (i) urban sites ($n=5$) placed close (<2 m) to the main urban roads outside the limited traffic zone of Siena city; (ii) peri-urban sites ($n=3$) located close (<2 m) to the ring road and its branches; (iii) green-urban sites ($n=2$) placed in green areas within the old town of Siena city; (iv) non-urban sites ($n=5$) from uncultivated terrains in rural zones outside the urban and peri-urban settings.

Each soil sample (20 cm deep) was a composite sample consisting of three sub-samples collected a few metres apart. In the urban and peri-urban sites, soils were sampled near the edge of the roadway within a distance of 2 m.

Mature individuals of the earthworm *N. caliginosus*, the commonest species in the study area, were collected by manual digging and hand-sorting; the sampling period was restricted to one month. These specimens live permanently in horizontal tunnels dug in the upper 20-25 cm of soil and feeds mainly on soil organic matter.

See Nannoni et al. (2014) for more details.

2.3. Soil and earthworm laboratory treatments

In the laboratory soil samples were air dried at +40 °C, sieved to <2 mm, homogenized by quartering and powdered by mechanical pulverizer. The soil samples were solubilised by acid digestion: 1 mL HF, 2 mL HNO₃, 2 mL HCl and 1 mL HClO₄ (ultrapure reagents) were added to 200 mg of powdered soil. The mixture was processed in Teflon bombs using a Milestone Ethos 900 microwave lab station (EPA, 1996).

In the laboratory earthworms were rinsed with deionised water, placed in plastic Petri dishes containing Whatman No.1 filter paper and a few drops of deionised water and kept at 18 °C for 72 h. The specimens were frozen at -80 °C, freeze-dried for 48 h and then digested individually with an acid mixture of 3 mL HNO₃ and 1 mL H₂O₂ (ultrapure reagents) in Teflon bombs placed in the microwave lab station.

See Nannoni et al. (2014) for more details.

2.4. Sequential extraction procedure

A five-step sequential extraction procedure was utilized to determine the partitioning of Cd, Co, Cr, Cu, Ni, Pb, Sb, U and Zn in the water-soluble (F_{sol}), extractable (F_{ext}), reducible (F_{red}), oxidable (F_{oxi}) and residual (F_{res}) fractions of soil samples ($n=15$) collected in the above illustrated sites. The steps 2, 3 and 4 correspond to the steps A, B and C of the protocol proposed by the Standard Measurements and Testing Programme (formerly BCR, Bureau Community of Reference) of the European Commission (Rauret et al., 1999; Pueyo et al., 2001). This protocol consists of the following extraction steps: (i) step A, extraction of extractable fraction accounting for water-soluble, exchangeable and carbonate-bound fractions, with 0.11 M solution of acetic acid; (ii) step B, extraction of reducible fraction with 0.5 M solution of hydroxylamine hydrochloride at pH 1.5; (iii) step C, extraction of oxidable fraction with 8.8 M solution of H₂O₂ and 1 M solution of ammonium acetate at pH 2. The extraction of water-soluble (step 1) and residual (step 5) fractions were added in the procedure adopted in this study. The water-

soluble fraction was extracted using ultrapure water, and the residual fraction by acid digestion following the 3052 EPA method (EPA, 1996).

The five-step sequential extraction procedure was performed using 1 g of powdered soil sample placed in a 50 mL polypropylene centrifuge tube. After reaction with the appropriate reagent of each step, the mixture was centrifuged in order to separate the supernatant liquid and solid residue. The supernatant liquid was used for the analytical determinations, whereas the solid residue was washed with ultrapure water and utilized for the next extraction step.

2.5. Analytical determinations

The total content of Cd, Co, Cr, Cu, Ni, Pb, Sb, U and Zn in soils and earthworm tissues as well as in soil fractions (F_{sol} , F_{ext} , F_{rid} , F_{oxi} and F_{res}) were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using the Perkin Elmer Sciex Elan 6100 spectrometer. The accuracy of heavy metal concentrations in soil samples was evaluated by the NIST 2709 (San Joaquin Soil) and 2710 (Montana Soil) standard reference materials. The heavy metal determinations in these standard materials were in good agreement with the certified values with recoveries from 94% to 100%. The precision estimated as repeatability of the analytical measures ($n=5$) and expressed as percent relative standard deviation (% RSD), was below 4.7% for all the elements.

The NIST 2977 (Mussle tissue) was utilized as standard reference material to check the accuracy of analytical determinations in earthworms. The recoveries were from 92% to 110%. Precision was determined by means of 5 replicate analyses of each earthworm specimen, and was below 4% (% RSD) for all the elements.

To validate the five-step sequential extraction procedure, the concentrations of Cd, Cr, Cu, Ni, Pb and Zn were determined in the extractable, reducible and oxidable fractions of the BCR-701 standard reference material. The measured concentrations of Cd, Cr, Cu, Ni, Pb and Zn in these fractions were in agreement with the certified values for steps A, B and C of the protocol proposed by the Standard Measurements and Testing Programme. The quality of the extraction procedure was also assessed by comparing for each chemical element the sum of concentrations in the five soil fractions and the total soil content. Recoveries were in the range of 82-121% (Cd), 103-120% (Co), 109-120% (Cr), 98-120% (Cu), 106-121% (Ni), 93-121% (Pb), 93-118% (Sb), 92-119% (U) and 98-120% (Zn).

2.6. Geochemical indexes

The enrichment factor (EF) was used as geochemical index to establish the level of soil contamination by heavy metals. EF was calculated as the ratio between the element content in soil sample and the maximum value of its natural variability in soils of the study area (geochemical background). The local geochemical background of Cd, Co, Cr, Cu, Ni, Pb, Sb, U and Zn was evaluated using the range of the element contents in the non-urban soils (Nannoni et al. 2014).

2.7. Statistical analysis

The Shapiro-Wilks W test was applied to verify the normal distribution of analytical data. The homogeneity of variances was verified by means of the Fischer F test. Significant statistical differences between datasets were determined by the parametric Student's t test when data were normally distributed, and the non-parametric Mann-Whitney U test for data not normally distributed, at the 5% significance level.

Spearman's correlation test ($p < 0.05$) was utilized to check the significant correlations between the concentrations of heavy metals in earthworms and in water-soluble, extractable, reducible and oxidable soil fractions.

A simple linear regression analysis was applied to describe the relationships between the heavy metal concentrations in earthworms (dependent variable) and those in effective available, potentially available and bioaccessible pools (explanatory variables).

3. Results and discussion

3.1. Heavy metal total contents in soil

Based on the total contents of Cd, Co, Cr, Cu, Ni, Pb, Sb, U and Zn, and the values of enrichment factor (EF) determined by Nannoni et al. (2014), the soil samples were divided into two groups for each heavy metal: the first group included uncontaminated soils in which heavy metal content was within the respective local natural variability ($EF \leq 1$); the second group consisted of contaminated soils with heavy metal contents exceeding the local background ($EF > 1$).

Our analytical results suggest that soils of the Siena urban area were contaminated by Cd, Cu, Pb, Sb and Zn, and show statistically significant differences ($p < 0.05$) between

the concentrations of these heavy metals in uncontaminated and contaminated soils (Tab. 1). No contamination by Co, Cr, Ni and U was detected in soils of the study area. As indicated by Nannoni et al. (2014), Cd, Cu, Pb, Sb and Zn may be regarded as traffic-related heavy metals, whereas Co, Cr, Ni and U as geogenic heavy metals.

Table 1

Among the traffic-related heavy metals, Sb and Pb were the main soil contaminants. These heavy metals reached their highest levels ($EF > 2$) in soils collected in urban and peri-urban areas, with peaks mainly in urban sites characterized by stop-and-go traffic near traffic lights, crossings and roundabouts. These findings suggest that Sb and Pb contents in contaminated soils were strongly influenced by vehicle traffic, as shown by other studies (Li et al., 2014; Mitchell et al., 2014; Wiseman et al., 2015).

Cd, Cu and Zn levels were also affected by vehicle circulation, though they accumulated slightly to moderately in urban and peri-urban soils (EF usually from 1 to 2). The vehicular origin of Cd, Cu and Zn in the urban environment is well known (Hjortenkrans et al., 2006; Amato et al., 2009; Harrison et al., 2012).

The contents of Co, Cr, Ni and U in soil were rather homogeneous in all sampling sites and within the respective local geochemical background ($EF \leq 1$). Thus, their levels in soils of the study area were mainly defined by the mineralogical and geochemical features of the parent rock, as well as the pedogenetic processes.

3.2. Heavy metal fractionation in soil

Table 2 shows the contents of traffic-related elements in the water-soluble (F_{sol}), extractable (F_{ext}), reducible (F_{rid}), oxidable (F_{oxi}) and residual (F_{res}) fractions of soils. In order to evaluate the influence of contamination on the fractionation of heavy metals, uncontaminated and contaminated soils were divided on the basis of their total contents.

Table 2

Cadmium. In uncontaminated soils, Cd was preferentially associated with F_{ext} . Cd concentrations in F_{ext} varied from 0.04 to 0.09 mg kg⁻¹ (Tab. 2 and Fig. 1), representing 49-74% of Cd_{tot} (median=61%). The Cd contents in F_{oxi} , F_{red} and F_{res} were rather similar ranging from 0.005 to 0.03 mg kg⁻¹, and constituting 18, 11 and 9% (median values),

respectively, of the total element content. Low Cd concentrations ($\leq 0.0002 \text{ mg kg}^{-1}$) were recorded in F_{sol} (0.13% of Cd_{tot}).

The comparison between uncontaminated and contaminated soils highlighted some variations in Cd distribution in soil fractions. In contaminated soils the man-made contribution of Cd was mostly distributed in F_{ext} (median=0.17 mg kg^{-1}), F_{red} (0.09 mg kg^{-1}) and F_{res} (0.02 mg kg^{-1}) that constituted 60, 26 and 6% of Cd_{tot} , respectively.

Analytical data suggest that Cd was mobile in uncontaminated and contaminated soils, since about 2/3 of its total content was in F_{ext} . This finding was in line with the usual behaviour of Cd in soil fractionation (Luo et al., 2012).

Copper. In uncontaminated soil samples, Cu was largely in F_{res} (19.7-22.3 mg kg^{-1} ; Tab. 2 and Fig. 1) accounting for 82% of total Cu content. Cu concentrations were 1.4-3.4 mg kg^{-1} in F_{red} (9% of Cu_{tot}), 1.9-2.5 mg kg^{-1} in F_{oxi} (8%), 0.14-0.2 mg kg^{-1} in F_{ext} (0.6%) and 0.06-0.1 mg kg^{-1} in F_{sol} (0.3%). Cu fractionation in uncontaminated soils of the Siena urban area matched the usual distribution of this element in soil fractions, characterized by a prevalent association with the immobile F_{res} and subordinately with F_{oxi} and F_{red} (Acosta et al., 2015). This observation suggests that Cu has an equivalent affinity for adsorption by organic matter and Fe-Mn oxyhydroxides (Impellitteri et al., 2002; Kabata-Pendias, 2010).

Cu concentrations were significantly higher in all soil fractions of contaminated soils compared to uncontaminated ones, as shown by the median contents in F_{res} (35.3 vs 20.5 mg kg^{-1}), F_{oxi} (8.8 vs 2.1 mg kg^{-1}), F_{red} (5.1 vs 2 mg kg^{-1}), F_{ext} (0.42 vs 0.15 mg kg^{-1}) and F_{sol} (0.15 vs 0.08 mg kg^{-1} ; Tab. 2 and Fig. 1). This data highlighted that Cu contamination was mainly split between F_{res} , F_{oxi} and F_{red} , indicating an important role of organic matter and Fe-Mn oxyhydroxides in copper sorption. In contaminated soils Cu was therefore largely associated with the immobile fraction (70% of Cu_{tot}) and to a lesser extent with the redox-sensitive fractions (18% in F_{oxi} and 11% in F_{red}).

Figure 1

Lead. In uncontaminated soils, Pb was largely in F_{res} (8.1-19.7 mg kg^{-1}) and F_{red} (8-17.9 mg kg^{-1}), constituting 49 and 44% of Pb_{tot} , respectively (Tab. 2 and Fig. 1). Pb concentrations in F_{oxi} (0.73-2.8 mg kg^{-1}) accounted for 5% of total Pb content in soil, whereas those in F_{ext} (0.06-0.29 mg kg^{-1}) and F_{sol} (0.005-0.04 mg kg^{-1}) only accounted for 0.5 and 0.04%, respectively. Pb fractionation in uncontaminated soils of the Siena

urban area as in line with its usual distribution in soil fractions that is characterized by a prevalent presence of the element included in residual and neoformation minerals (e.g. k-feldspars and clay minerals) and adsorbed by Fe-Mn oxyhydroxides (Li et al., 2001; Wong and Li, 2004; Acosta et al., 2015).

In contaminated soils of the study area, Pb enrichment was distributed in all soil fractions. The largest increases in Pb concentrations were recorded in F_{red} (42.2 mg kg⁻¹ as median value), F_{res} (32.8 mg kg⁻¹) and F_{oxi} (9.3 mg kg⁻¹), constituting 50, 39 and 11% of Pb_{tot} , respectively. Pb contents in F_{ext} (<2 mg kg⁻¹) and F_{sol} (<0.2 mg kg⁻¹) of contaminated soils were also rather low, accounting for 0.9 and 0.1% of total Pb in soil, respectively.

Antimony. In uncontaminated and contaminated soils, Sb showed a similar distribution pattern in soil fractions: $F_{\text{res}} \gg F_{\text{ext}} > F_{\text{oxi}} > F_{\text{red}} > F_{\text{sol}}$. Sb was mostly in F_{res} , with median concentrations of 1 mg kg⁻¹ in uncontaminated and 3.8 mg kg⁻¹ in contaminated soils (Tab. 2 and Fig. 1), constituting about 93% of Sb_{tot} in both soil groups. This finding suggests that Sb was mainly immobile in soil of the Siena urban area. The affinity of Sb for the residual fraction of soil has also been reported in other studies (Hammel et al., 2000; Ettler et al., 2010). Other authors observed that Sb has a relatively low mobility in soil, even in highly contaminated terrains of former mining and smelting sites (Flynn et al., 2003; Wilson et al., 2004). Significantly, Sb levels in mobile fractions (F_{sol} and F_{ext}) of contaminated soils together accounted for about 3% of Sb_{tot} compared to 2% for F_{red} and 1% for F_{oxi} .

Zinc. In uncontaminated soil samples, the highest Zn concentrations were recorded in F_{res} (71.1 mg kg⁻¹ as median content; Tab. 2 and Fig. 1) accounting for about 84% of Zn_{tot} . The order of abundance of Zn in the other soil fractions was as follows: 6.8 mg kg⁻¹ in F_{red} (9% of Zn_{tot}), 3.5 mg kg⁻¹ in F_{oxi} (4%), 1.4 mg kg⁻¹ in F_{ext} (2%) and 0.04 mg kg⁻¹ in F_{sol} (0.05%). This fractionation pattern in uncontaminated soils of Siena urban area was in line with the usual distribution of the element in soil fractions (Kaasalainen and Yli-Halla, 2003; Acosta et al., 2015). As the other heavy metals, Zn was enriched in all fractions of contaminated soils. The largest increases were found in F_{res} (97.6 mg kg⁻¹), F_{red} (34.5 mg kg⁻¹) and F_{oxi} (13.9 mg kg⁻¹). The median of Zn contents in the mobile fractions of contaminated soils was 7.5 mg kg⁻¹ in F_{ext} and 0.08 mg kg⁻¹ in F_{sol} .

Statistical analysis indicated that on the whole, the concentrations of traffic-related heavy metals in all soil fractions of contaminated soils were significantly higher than those of uncontaminated soils ($p < 0.05$; Fig. 1), except Cd in F_{oxi} . This result confirms

that heavy metal inputs due to traffic emissions were distributed in all soil fractions, in relation to the behaviour of each metal in soil fractionation.

Cobalt, chromium, nickel and uranium. In soils of the study area, Co, Cr, Ni and U, reported as geogenic heavy metals, were mainly associated with the residual fraction (Tab. 3 and Fig. 2).

Table 3

The relative abundances of Cr, U and Ni in F_{res} were very high, constituting 86, 82 and 76%, respectively, of their total content in soil. About half of the Co in soil (51%) pertained to the immobile fraction. Co, Cr and Ni showed different distribution patterns in non-residual soil fractions: Co was mainly in F_{red} (29%) and F_{ext} (12%), Cr in F_{oxi} (11%), and Ni in F_{oxi} (10%) and F_{red} (9%). The non-residual aliquot of U was largely partitioned in F_{oxi} (13%) in line with the affinity of this element for organic matter.

Figure 2

The analytical data indicated that the concentrations of Cd, Cu, Pb, Sb and Zn in the effective available ($F_{sol} + F_{ext}$), potentially available ($F_{rid} + F_{oxi}$) and bioaccessible ($F_{sol} + F_{ext} + F_{rid} + F_{oxi}$) pools were significantly higher (from 2.5 to 6 times) in contaminated than uncontaminated soils (Tab. 4).

Table 4

Nevertheless, the relationships between the effective available fraction (EAF) and potentially available fraction (PAF) of these heavy metals were similar in uncontaminated and contaminated soils, with $EAF > PAF$ for Cd, $EAF < PAF$ for Cu, Pb and Zn, and $EAF \approx PAF$ for Sb. The percentage aliquots of Cd and Sb in the effective available, potentially available and bioaccessible pools were comparable in uncontaminated and contaminated soils, whereas Cu, Pb and Zn showed higher values in the potentially available pool of contaminated soils (Tab. 4). For Co, Cr, Ni and U, the concentrations in the potentially available pool were constantly higher than in the effective available pool.

Lastly, the percentage aliquots of heavy metals in the effective available pool established the following mobility order: Cd >> Co >> Ni > Sb > U > Zn > Cu > Pb > Cr. Thus, Cd was the heavy metal by far most associated with the most mobile and very active fraction of the studied soils, whereas Cu, Pb and Cr were mainly in the immobile residual fraction.

3.3. Heavy metal concentrations in earthworms

This section summarizes the results of Nannoni et al. (2014) concerning the concentrations of heavy metals in *N. caliginosus* earthworms collected in soils of Siena urban area. Specimens were divided in two groups: the first group included earthworms collected in uncontaminated soils, whereas the second group included individuals from contaminated soils.

The concentrations of traffic-related heavy metals (Cd, Cu, Pb, Sb and Zn) in earthworms of contaminated sites were usually higher than in specimens from uncontaminated sites (Tab. 5). Statistical analysis confirmed significant differences between concentrations of these heavy metals in earthworms from contaminated and uncontaminated soils ($p < 0.05$; Tab. 5).

Table 5

The similar distribution pattern of Cd, Cu, Pb, Sb and Zn contents in earthworms and soil samples suggested that soil contamination likely influenced uptake of these traffic-related heavy metals by these invertebrates. Moreover, statistical analysis showed significant positive relationships ($p < 0.01$) between Cd, Pb and Sb concentrations in earthworms and their total contents in soil (Nannoni et al., 2014).

For Co, Cr, Ni and U, there were no significant correlations between their concentrations in earthworms and soils. The same was found for Cu and Zn, although the concentrations of these elements increased in earthworms sampled in the most contaminated soils. This absence of correlation was probably due to physiological regulation of Cu and Zn by *N. caliginosus*.

The interaction between heavy metals such as Cd, Cu and Zn was documented in several soil invertebrates, including earthworms (Khalil et al., 1996; Lemtiri et al., 2105). In contaminated soils simultaneous exposure to several heavy metals can produce antagonistic or synergistic effects in earthworms. In addition to toxic effects,

interference between heavy metals can influence the uptake mode and internal pathway of regulation and detoxification of earthworms (Rüdiger and Ralf-Rainer, 2010; Vijver et al., 2011).

3.4. Relationship between heavy metal concentrations in earthworms and in soil fractions

Although correlations between the concentrations of some heavy metals in earthworms and total contents in soils were found, several authors have suggested that levels of these elements in earthworms are mainly related to their biologically available fraction in soil (e.g. Dai et al., 2004; Harmsen, 2007; Alvarenga et al., 2013).

In order to determine which soil fractions most affected uptake of heavy metals by *N. caliginosus* in the Siena urban area, the relationships between heavy metal concentrations in earthworms and soil fractions were checked (Tab. 6).

Table 6

Statistical analysis indicated that there were statistically significant correlations between Pb, Sb and Zn concentrations in *N. caliginosus* and those in the extractable, reducible and oxidable fractions (Tab. 6). Zn concentrations in earthworms also showed significant correlation with the soluble fraction. For Cd, statistically significant correlations were only found with the soluble and extractable fractions. This finding is in line with the partitioning of Cd in soils of the Siena urban area, as this element was the most mobile. Cu concentrations in earthworm tissues only showed significant correlation with element contents in the oxidable fraction. No statistically significant correlation was found between the concentrations of Co, Cr, Ni and U in earthworms and soil fractions (Tab. 6). These results were presumably due both to the behaviour of each heavy metal in soil fractionation and to their uptake pathway from each soil fraction by earthworms.

The water-soluble and extractable fractions are the most mobile and very active pools of chemical elements in soil. In particular, the extractable fraction is a good surrogate for defining element bioavailability for earthworms (Streit, 1984; Morgan and Morgan, 1999). The oxidable and reducible fractions are considered the leachable and partly active pool that is mainly mobilizable by redox reactions. The oxidable fraction, which mainly includes the organically bound aliquot of chemical elements, may mostly

influence the uptake of heavy metals by earthworms (e.g. Hobbelen et al., 2006; Suthar et al., 2008). It is known that soil organic matter is a major food source for earthworms. The metals associated within this ingested component therefore pass through the earthworm gut and are absorbed. This supports the hypothesis that after ingestion, soil organic matter is subject to the conditions and chemical/microbial changes (e.g. digestive enzymes, surfactants and difference in pH with respect to bulk soil) in the gastrointestinal tract of earthworms. As a consequence, the organic compounds may undergo modifications and release adsorbed heavy metals. In fact, earthworms extract both exchangeable and non-exchangeable elements sequestered by organic compounds. On the other hand, organic matter is an important factor controlling the retention of heavy metals, such as Cu, Pb and Zn in soil. Humic substances may act as absorbent for heavy metals through sorption reactions such as ionic exchange and organic complexation (Burt et al., 2003; Kabata-Pendias, 2010). In the present study, soil samples were from the upper layer (top 20 cm) where earthworms live and which is usually the soil profile layer richest in organic matter. Moreover, in the urban and peri-urban soils of Siena, the oxidable fraction may also include heavy metals bound to carbon-rich aerosol particles emitted by vehicles in the urban setting.

About the reducible fraction, it is not clear if earthworm gut conditions can determine an increase of bioavailability, releasing heavy metals by partial to total dissolution of reducible mineralogical phases such as Fe-Mn oxyhydroxides. Some authors (Karsten and Drake, 1995; Horn et al., 2003) have mentioned that anaerobic conditions in the earthworm gut may destabilize the mineralogical phases of the reducible fraction, releasing bound elements.

As the analytical data indicated that the concentrations of heavy metals in earthworms do not depend on a single soil fraction but on the contribution of multiple fractions, the concentrations of heavy metals in these invertebrates were compared with: i) the effective available pool represented by the sum of F_{sol} and F_{ext} , ii) the potentially available pool represented by the sum of F_{rid} and F_{oxi} , and iii) the bioaccessible pool represented by the sum of F_{sol} , F_{ext} , F_{rid} and F_{oxi} . A simple linear regression was used to determine the variance of heavy metal concentrations in earthworms (Tab. 7).

Table 7

For earthworms and soils from all sampling sites, the regression models showed that the bioaccessible fraction of Pb and Sb explains more of the variance of their concentrations in earthworms ($R^2=0.9$ and 0.86 , respectively; Tab. 7). This result agrees with the fact that the extractable, reducible and oxidable fractions were those that mostly affected the uptake of Pb and Sb by *N. caliginosus* (Tab. 6). Moreover, for these heavy metals, the values of the regression model coefficient increased ($R^2=0.95$ and 0.92 , respectively; Tab. 7) when only earthworms and soils from contaminated sites were considered. As a result, the concentrations of Pb and Sb in earthworms indicated that the bioavailable pool of these heavy metals included both the effective and potentially available pools. Consequently, the Pb and Sb contents in earthworm tissue may provide a good evaluation of their bioavailable amounts associated with the non-residual soil fractions. The effective available pool of Cd was the most correlated with body levels in earthworms collected in all sampling sites ($R^2=0.82$; Tab. 7). Like for Pb and Sb, the value of the regression model coefficient increased ($R^2=0.86$) selecting only the earthworms and soils from contaminated sites. Thus, Cd concentrations in earthworms could be a predictor of the amount of this heavy metal in the soluble and extractable fractions, by virtue of the fact that uptake of Cd by *N. caliginosus* was mainly influenced by the more mobile and very active pool represented by the effective available pool (Tab. 6). Nevertheless, our analytical data indicates that Cd is bioaccumulated by earthworms as reported by other authors (Marino and Morgan, 1999; Spurgeon and Hopkin, 1999; Conder et al., 2002; Dai et al., 2004). This means that Cd bioavailability assessed by its contents in earthworm tissue underestimates availability obtained the by chemical extraction method.

For Cu and Zn, the bioaccessible fraction explained more of the variance of their concentrations in earthworm tissues. Nevertheless, the low values of the regression model coefficient (data not reported in Table 7) suggested that earthworm body levels of Cu and Zn were not suitable for assessing bioavailability of these elements in soil. This is presumably due to the fact that Cu and Zn are essential elements regulated physiologically by *N. caliginosus* (Pejinenburg et al., 1999; Kennette et al., 2002; Kamitani and Kaneko, 2007).

4. Conclusions

The relationships between the heavy metal concentrations in earthworms and in soil fractions in the Siena urban area lead to the following consideration. The bioavailability of heavy metals assessed by the biological method (using earthworms as bioindicator) did not always agree with availability evaluated by the chemical method (using soil fractionation).

Our results indicated that earthworms were a good indicator of Pb and Sb bioavailability in soil. Conversely, the physiological mechanisms of regulation and excretion by these invertebrates influenced tissue levels of heavy metals such as Cd, Cu and Zn, resulting in misleading estimates of their bioavailable pool in soil. Moreover, it was not possible to define a relationship between chemical and biological availability for the geogenic heavy metals (Co, Cr, Ni and U), the total soil contents of which lay in a narrow range of values.

The results of our study indicated that chemical and biological methods both have their limitations. Chemical methods such as extraction techniques are appropriate for defining the fractionation of heavy metals in soil, but they may not be used as an absolute method to assess the availability of elements for soil organisms such as earthworms. In fact, these methods provide a snapshot of heavy metal amounts in soil fractions to which earthworms may be exposed, but do not consider the biological factors influencing uptake and accumulation of the elements. Biological methods provide an integration of uptake and accumulation of heavy metals by soil organisms over the duration of the exposure period. In this case, evaluation of bioavailability is strongly influenced by physiological and behavioural characteristics of soil organisms that affect body levels of heavy metals.

In conclusion, our study highlighted that estimation of heavy metal availability is useful and important for a better understanding of exposure and the effects of these contaminants on soil fauna, and that chemical and biological measures should be combined to provide more complete information about heavy metal availability in soil.

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Table 1.

Statistics of traffic-related (Cd, Cu, Pb, Sb, Zn) and geogenic (Co, Cr, Ni, U) heavy metal concentrations in uncontaminated and contaminated soils of the Siena urban area (data in mg kg⁻¹ dry weight).

Element	<u>TRAFFIC-RELATED ELEMENTS</u>					<u>GEOGENIC ELEMENTS</u>			
	Cd	Cu	Pb	Sb	Zn	Co	Cr	Ni	U
<u>Uncontaminated soils</u>									
Minimum	0.05	20.4	16.3	0.77	57.2	6.8	47.6	24.8	1.2
Maximum	0.17	24	37.5	1.3	90.1	15.4	91.5	55.6	2.7
Mean	0.12	22.5	26.1	1	75.3	11.1	70.9	40.3	1.7
Median	0.14 *	22.5 *	26.1 *	1 *	74.5 *	10.8	72.7	40.8	1.5
St. Dev.	0.05	1.3	8	0.27	10.7	2.6	11.7	7.3	0.52
<u>Contaminated soils</u>									
Minimum	0.2	32.2	39.7	1.5	95.3				
Maximum	0.48	82.1	178	14.8	207				
Mean	0.31	52.3	104	5.4	142				
Median	0.26 *	49 *	73.7 *	3.8 *	121 *				
St. Dev.	0.12	15.6	64	4.5	44.8				

* Significant differences between the element concentrations in uncontaminated soils compared to contaminated ones ($p < 0.05$).

Table 2.

Statistics of traffic-related heavy metal (Cd, Cu, Pb, Sb, Zn) concentrations in the water-soluble, extractable, reducible, oxidable and residual fractions of uncontaminated and contaminated soils of the Siena urban area (data in mg kg⁻¹ dry weight). *n* = number of soil samples.

Soil fraction	Soluble	Extractable	Reducible	Oxidable	Residual	Soluble	Extractable	Reducible	Oxidable	Residual
<u>UNCONTAMINATED SOILS</u>						<u>CONTAMINATED SOILS</u>				
Cd (<i>n</i> = 9)						Cd (<i>n</i> = 6)				
Minimum	0.0001	0.04	0.005	0.007	0.005	0.0001	0.11	0.03	0.02	0.01
Maximum	0.0002	0.09	0.02	0.03	0.02	0.0007	0.37	0.1	0.04	0.04
Mean	0.0001	0.07	0.01	0.02	0.01	0.0003	0.21	0.08	0.03	0.02
Median	0.0001	0.06	0.02	0.02	0.01	0.0002	0.17	0.09	0.02	0.02
St. Dev.	0.0001	0.02	0.005	0.008	0.005	0.0003	0.1	0.03	0.01	0.01
Cu (<i>n</i> = 6)						Cu (<i>n</i> = 9)				
Minimum	0.06	0.14	1.4	1.9	19.7	0.06	0.14	1.9	3.4	25.6
Maximum	0.1	0.2	3.4	2.5	22.3	0.34	1	21	23.6	60.3
Mean	0.08	0.16	2.2	2.1	20.8	0.15	0.43	6.2	11.1	40
Median	0.08	0.15	2	2.1	20.5	0.15	0.42	5.1	8.8	35.3
St. Dev.	0.01	0.02	0.8	0.2	1.2	0.09	0.26	5.9	7.5	11.2
Pb (<i>n</i> = 8)						Pb (<i>n</i> = 7)				
Minimum	0.005	0.06	8	0.73	8.1	0.001	0.3	19.8	3.1	19.5
Maximum	0.04	0.29	17.9	2.8	19.7	0.19	2	120	35.7	59.3
Mean	0.01	0.15	12.1	1.6	12.8	0.09	1	61.6	14.7	36.3
Median	0.009	0.13	11.1	1.4	11.8	0.06	0.9	42.2	9.3	32.8
St. Dev.	0.01	0.09	3.9	0.79	4.5	0.07	0.7	44.3	13.9	15.9
Sb (<i>n</i> = 6)						Sb (<i>n</i> = 9)				
Minimum	0.004	0.01	0.01	0.009	0.69	0.003	0.03	0.02	0.01	1.4
Maximum	0.02	0.04	0.05	0.02	1.4	0.05	0.32	0.23	0.46	15.2
Mean	0.01	0.03	0.02	0.01	1	0.02	0.14	0.1	0.11	5.5
Median	0.01	0.02	0.02	0.01	0.99	0.02	0.1	0.07	0.04	3.8
St. Dev.	0.006	0.01	0.02	0.006	0.33	0.01	0.11	0.08	0.15	4.8
Zn (<i>n</i> = 8)						Zn (<i>n</i> = 7)				
Minimum	0.02	1	4.9	1.5	46.9	0.03	1.2	10.5	5.9	89.5
Maximum	0.08	2.1	10	7	87.9	0.13	9.1	67.3	27.9	140
Mean	0.04	1.5	7.4	4	70.6	0.08	6.1	31.6	14.9	104
Median	0.04	1.4	6.8	3.5	71.1	0.08	7.4	34.5	13.9	97.6
St. Dev.	0.02	0.5	1.8	2.1	15	0.03	3.2	21.1	8.2	19.3

Table 3.

Statistics of geogenic heavy metal (Co, Cr, Ni, U) concentrations in the water-soluble, extractable, reducible, oxidable and residual fractions of soils of the Siena urban area (data in mg kg⁻¹ dry weight). *n* = number of soil samples.

Soil fraction	Soluble	Extractable	Reducible	Oxidable	Residual	Soluble	Extractable	Reducible	Oxidable	Residual
	Co (<i>n</i> = 15)					Cr (<i>n</i> = 15)				
Minimum	0.009	7	13.7	5	44.5	0.002	0.08	1.1	6	45.7
Maximum	0.03	20.6	33.2	7.9	71.9	0.01	0.19	3.7	11.9	89.3
Mean	0.02	13.4	26.5	6.5	53.6	0.007	0.13	2.2	9.4	70.7
Median	0.01	12.4	28.6	6.5	51.3	0.007	0.13	2.1	9.8	75.7
St. Dev.	0.007	4.1	6	1	6.9	0.002	0.03	0.66	1.6	13.1
	Ni (<i>n</i> = 15)					U (<i>n</i> = 15)				
Minimum	0.01	0.94	2.5	2.5	21.6	0.001	0.004	0.02	0.1	1
Maximum	0.03	3.1	5.7	6.1	49.4	0.002	0.08	0.1	0.46	2.2
Mean	0.02	2.3	4	4.5	35.9	0.001	0.04	0.06	0.24	1.5
Median	0.02	2.1	4.4	5	36.8	0.001	0.04	0.06	0.2	1.3
St. Dev.	0.005	0.55	0.99	1.3	6.8	0.001	0.02	0.02	0.1	0.33

Table 4.

Mean of concentrations (data in mg kg^{-1}) and percentage aliquots (data in %) of heavy metals in the effective available, potentially available and bioaccessible pools of uncontaminated and contaminated soils of the Siena urban area.

Element	<i>Effective available pool</i>		<i>Potentially available pool</i>		<i>Bioaccessible pool</i>		<i>Effective available pool</i>		<i>Potentially available pool</i>		<i>Bioaccessible pool</i>	
	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%
	<u>UNCONTAMINATED SOILS</u>						<u>CONTAMINATED SOILS</u>					
Cd	0.07	61.8	0.03	28.9	0.1	90.6	0.21	60.8	0.1	32.6	0.31	93.3
Cu	0.24	0.95	4.3	17.0	4.6	18	0.58	0.98	17.3	29.1	17.9	30.1
Pb	0.17	0.61	13.7	51.4	13.9	52	1	0.83	76.3	62.4	77.3	63.3
Sb	0.04	3.6	0.04	3.4	0.08	7	0.16	3	0.21	3.3	0.37	6.3
Zn	1.5	1.9	11.4	14.0	12.9	15.9	6.2	3.9	46.5	27.6	52.7	31.5
Co	1.7	13.4	4.3	33.0	5.9	46.4						
Cr	0.14	0.17	11.6	14.1	11.7	14.3						
Ni	2.3	4.8	8.6	18.4	10.9	23.3						
U	0.04	2.1	0.3	16.1	0.34	18.2						

Table 5.

Statistics of traffic-related (Cd, Cu, Pb, Sb, Zn) and geogenic (Co, Cr, Ni, U) heavy metal concentrations in earthworms collected in uncontaminated and contaminated soils of the Siena urban area (data in mg kg⁻¹ dry weight).

Element	TRAFFIC-RELATED ELEMENTS					GEOGENIC ELEMENTS			
	Cd	Cu	Pb	Sb	Zn	Co	Cr	Ni	U
<u>Earthworms from uncontaminated soils</u>									
Minimum	1.5	9.6	0.45	0.1	300	1.7	1.3	2.4	0.05
Maximum	2.9	19.3	1.5	0.21	753	11.7	2.4	8.7	0.18
Mean	2	13.4	1.1	0.13	514	5.4	1.8	5.4	0.08
Median	2 *	11.9 *	1.2 *	0.12 *	471 *	4.5	1.9	4.9	0.08
St. Dev.	0.45	3.9	0.38	0.04	162	3	0.3	2.2	0.04
<u>Earthworms from contaminated soils</u>									
Minimum	1.9	13.2	1.1	0.18	538				
Maximum	4.2	27.6	4	0.53	909				
Mean	3	20.6	2.4	0.3	771				
Median	2.9 *	20.7 *	2.3 *	0.24 *	842 *				
St. Dev.	1	4.4	1.3	0.13	153				

* Significant differences between the element concentrations in earthworms from uncontaminated soils compared to earthworms from contaminated ones ($p < 0.05$).

Table 6.

Spearman's correlation coefficients between heavy metal concentrations in *N. caliginosus* specimens and in water-soluble, extractable, reducible and oxidable soil fractions. Marked correlations are significant at: * $p < 0.05$; ** $p < 0.01$; n.s. not significant.

Element	Soluble	Extractable	Reducible	Oxidable
Cd	0.683 **	0.606 *	n.s.	n.s.
Co	n.s.	n.s.	n.s.	0.614 *
Cr	n.s.	n.s.	n.s.	n.s.
Cu	n.s.	n.s.	n.s.	0.839 **
Ni	n.s.	n.s.	n.s.	n.s.
Pb	n.s.	0.518 *	0.516 *	0.514 *
Sb	n.s.	0.765 **	0.807 **	0.750 **
U	n.s.	n.s.	n.s.	n.s.
Zn	0.614 *	0.736 **	0.761 **	0.796 **

Table 7.

Simple linear regression models and their determination coefficients (R^2) for Cd, Cu, Pb, Sb and Zn. $[X]_{EW}$ = element concentrations in earthworm tissue; $[X]_{\Sigma sol+ext}$, $[X]_{\Sigma red+oxi}$, $[X]_{\Sigma sol+ext+red+oxi}$ = element concentration in effective available, potentially available and bioaccessible pools, respectively.

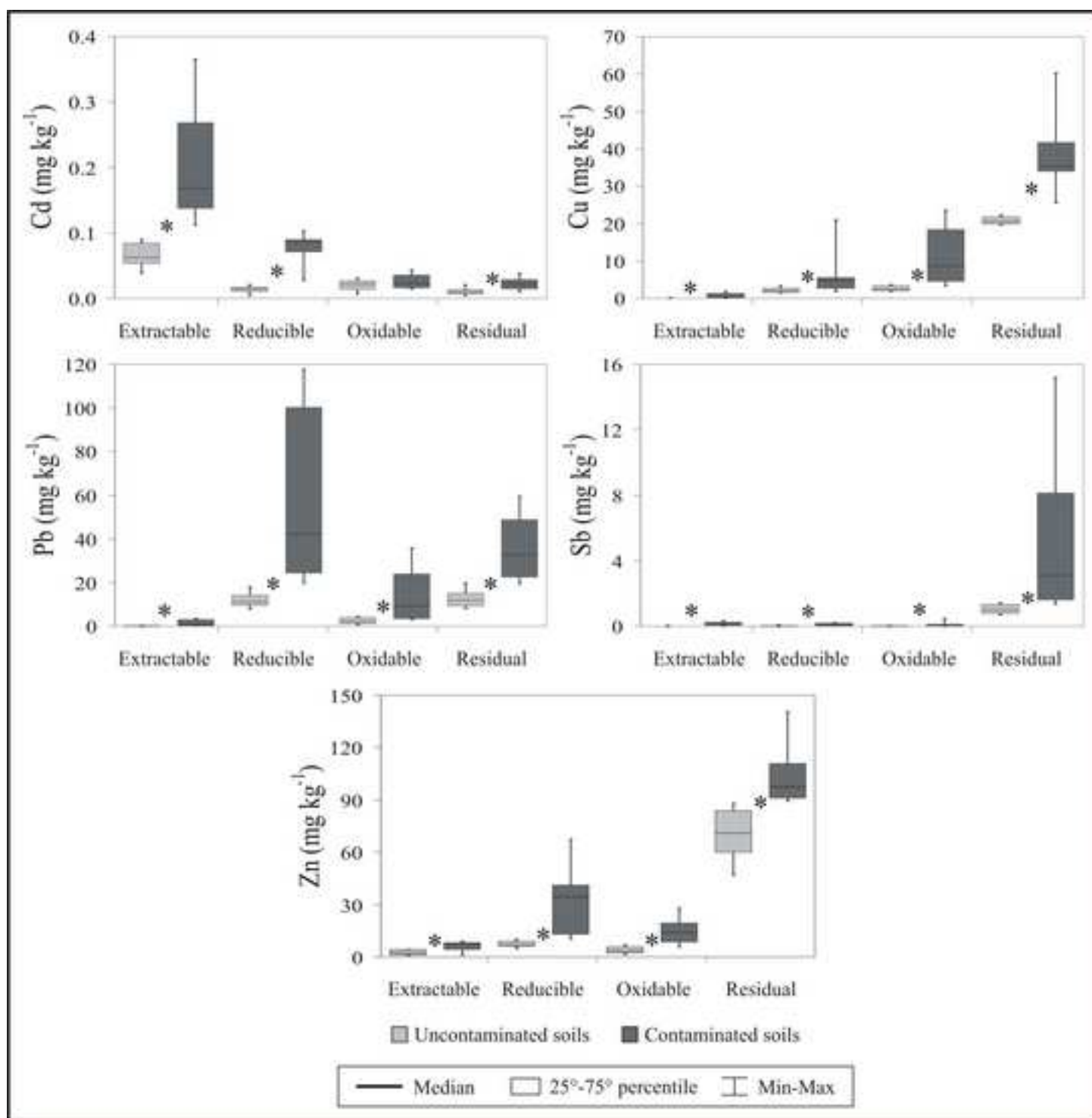
Model	R^2	Model	R^2	Model	R^2
<i>Effective available pool</i>		<i>Potentially available pool</i>		<i>Bioaccessible pool</i>	
<i>Earthworm from all soil sampling sites</i>					
$[Cd]_{EW} = 8.01 [Cd]_{\Sigma sol+ext} + 1.37$	0.82	$[Cd]_{EW} = 14.83 [Cd]_{\Sigma red+oxi} + 1.5$	0.53	$[Cd]_{EW} = 6.21 [Cd]_{\Sigma sol+ext+red+oxi} + 1.36$	0.58
$[Pb]_{EW} = 1.53 [Pb]_{\Sigma sol+ext} + 0.93$	0.64	$[Pb]_{EW} = 0.02 [Pb]_{\Sigma red+oxi} + 0.89$	0.81	$[Pb]_{EW} = 0.02 [Pb]_{\Sigma sol+ext+red+oxi} + 0.77$	0.90
$[Sb]_{EW} = 1.16 [Sb]_{\Sigma sol+ext} + 0.12$	0.61	$[Sb]_{EW} = 0.61 [Sb]_{\Sigma red+oxi} + 0.15$	0.79	$[Sb]_{EW} = 0.37 [Sb]_{\Sigma sol+ext+red+oxi} + 0.13$	0.86
<i>Earthworm from uncontaminated soils</i>					
$[Cd]_{EW} = 5.18 [Cd]_{\Sigma sol+ext} + 1.67$	0.05	$[Cd]_{EW} = 3.82 [Cd]_{\Sigma red+oxi} + 1.89$	0.01	$[Cd]_{EW} = 2.69 [Cd]_{\Sigma sol+ext+red+oxi} + 1.75$	0.03
$[Pb]_{EW} = 0.41 [Pb]_{\Sigma sol+ext} + 1.07$	0.01	$[Pb]_{EW} = 0.02 [Pb]_{\Sigma red+oxi} + 0.89$	0.04	$[Pb]_{EW} = 0.02 [Pb]_{\Sigma sol+ext+red+oxi} + 0.9$	0.04
$[Sb]_{EW} = 1.48 [Sb]_{\Sigma sol+ext} + 0.07$	0.01	$[Sb]_{EW} = -0.98 [Sb]_{\Sigma red+oxi} + 0.16$	0.06	$[Sb]_{EW} = -0.85 [Sb]_{\Sigma sol+ext+red+oxi} + 0.2$	0.22
<i>Earthworm from contaminated soils</i>					
$[Cd]_{EW} = 9.11 [Cd]_{\Sigma sol+ext} + 1.1$	0.86	$[Cd]_{EW} = 20.91 [Cd]_{\Sigma red+oxi} + 0.84$	0.44	$[Cd]_{EW} = 7.54 [Cd]_{\Sigma sol+ext+red+oxi} + 0.65$	0.87
$[Pb]_{EW} = 1.55 [Pb]_{\Sigma sol+ext} + 0.91$	0.57	$[Pb]_{EW} = 0.02 [Pb]_{\Sigma red+oxi} + 1.01$	0.77	$[Pb]_{EW} = 0.02 [Pb]_{\Sigma sol+ext+red+oxi} + 0.46$	0.95
$[Sb]_{EW} = 0.92 [Sb]_{\Sigma sol+ext} + 0.17$	0.47	$[Sb]_{EW} = 0.52 [Sb]_{\Sigma red+oxi} + 0.19$	0.73	$[Sb]_{EW} = 0.34 [Sb]_{\Sigma sol+ext+red+oxi} + 0.17$	0.92

FIGURE CAPTIONS

Figure 1. Box plots of traffic-related heavy metal (Cd, Cu, Pb, Sb and Zn) concentrations in fractions of uncontaminated and contaminated soils of the Siena urban area. * Significant differences of element concentrations in each fraction between uncontaminated and contaminated soils ($p < 0.05$). The concentrations in water-soluble fraction were not reported.

Figure 2. Distribution of the geogenic heavy metals (Co, Cr, Ni and U) in fractions of soils of the Siena urban area.

Figure_1

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Figure_2

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