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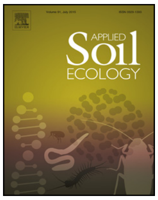

# Earthworms *Eisenia fetida* affect the uptake of heavy metals by plants *Vicia faba* and *Zea mays* in metal-contaminated soils

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ABSTRACT

Earthworms increase the availability of heavy metals in some situations and aid in maintaining the structure and quality of soil. The introduction of earthworms into metal-contaminated soils has been suggested as an aid for phytoremediation processes. In Wallonia, Belgium, a century of industrial metallurgic activities has led to the substantial pollution of soils by heavy metals, including copper (Cu), zinc (Zn), lead (Pb) and cadmium (Cd), due to atmospheric dusts. Two plant species, *Vicia faba* and *Zea mays*, and earthworms (*Eisenia fetida*) (Savigny, 1826) were exposed to different concentrations of long-term-contaminated soils for 42 days. The soil samples, which were collected from the land surrounding a former Zn-Pb ore-treatment plant, exhibited different levels of heavy metals. Our aim was to evaluate the role of earthworms *E. fetida* on the availability of metals in soils and their effects on metal uptake by *V. faba* and *Z. mays* plants at different soil concentrations.

The results suggest that earthworms and plants modified the availability of metals in contaminated soils after 42 days of exposure. Earthworm life-cycle parameters were affected by metal contamination and/or the addition of plants; cocoon production and weight were more responsive to adverse conditions than earthworm survival or weight change. The concentrations of Pb and Cd in earthworm tissues decreased in the presence of plants. Results showed that metal accumulation in plants depended on the metal element considered and the presence of earthworms. However, the presence of earthworms did not change the concentrations of metals in plants, except for Cd. In the presence or absence of earthworms, *V. faba* accumulated higher concentrations of Cu and Zn compared with *Z. mays*, which accumulated higher concentrations of Cd. These findings have revealed that earthworm activities can modify the availability of heavy metals for uptake by plants in contaminated soils. Moreover, the study results show that the ecological context of phytoremediation should be broadened by considering earthworm-plant-soil interaction, which influence both the health of the plant and the uptake of heavy metals by plants.

## 1. Introduction

Heavy metals are continuously being added to soils through anthropogenic activities such as industrialization, mining, smelting, and land application of sewage sludge (Harlavan et al., 2010). In Wallonia (South region of Belgium) during the two last centuries, processing of metal-bearing ore has emitted metal-bearing particulates laden with Cd, Pb and Zn (Graitson, 2005). The fallouts in the vicinity of plants have led to significant contamination of topsoil (Liénard et al., 2014). Among the heavy metals, copper (Cu), zinc (Zn), lead (Pb), and cadmium (Cd) levels in soil are receiving extensive attention because of their acute and chronic toxicological effects on plants and animals (Cheng and Wong, 2002; Li, M. et al., 2009; Li, N.Y. et al., 2009). When these four elements co-occur in the soil, their combined effects are very complex. Mixture toxicity is difficult to study because metals can interact at various levels (Dickson et al., 1994) such as the exposure level, the uptake level, the target level (Rüdiger and Ralf-Rainer, 2010), and the internal pathway of detoxification (Vijver et al., 2011). Cleaning up soils contaminated with heavy metals using traditional techniques can be

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destructive to the soil. Remediation of contaminated soils using earthworms and plants appears to be cost-effective and environmentally friendly technology. Most of metal-accumulating plants are characterized by slow growth, low biomass yield and broad growth requirements. Moreover, they are often incapable of accumulating high concentrations of potentially phytotoxic metals, such as Cu, Zn, Pb, and Cd, in their above ground biomass (Kumar et al., 1995; Komarek et al., 2007). Plants vary in response to metals, in mechanism of uptake and of avoiding damage. Metal tolerant plants such as *Thlaspi caerulesens* (Papoyan et al., 2007; Salt et al., 1998) and *Brassica juncea* (Brunet et al., 2009) have been used for phytoextraction tests of lead from contaminated sites. A plant, which can be used for phytoextraction, should be able to grow rapidly, to produce large biomass and to accumulate high concentrations of metals. Availability of metals in soil has been reported to be dependent on soil type, metal species, metal kinetics and age of metal contamination in the soil (Beeby, 1993; van Gestel et al., 1995). The availability of heavy metals in soil is determined by their chemical speciation (Babich and Stotzky, 1980) and their interactions with organic matter and mineral soil particles (Li and Li, 2000), which can be influenced by soil organisms. There have been many studies concerned with the combined effects of heavy metals on earthworms and plants (Morgan, 1988; Brokbartold et al., 2012; Qiu et al., 2011; Israr et al., 2011).

Earthworms improve soil structure, contribute to organic matter decomposition and nutrient cycling (Lemtiri et al., 2014) and play a key role in terrestrial ecotoxicological risk assessment (Sheppard et al., 1997; Weeks et al., 2004). The beneficial role of earthworms in soil, influencing a range of chemical, physical and biological processes, has been reported (Scheu, 1987; Edwards and Bohlen, 1996; McCredie and Parker, 1992; Curry and Baker, 1998). Some earthworm species are easy to culture and handle for experiments, and thus have become standard test organisms in ecotoxicology (Organisation for Economic Co-operation and Development (OECD), 1984, 2004). On the other hand, it is recognized that the accumulation of heavy metals in earthworms depends more strongly on the fraction of metal that is available for uptake rather than the total metal concentration. Dai et al. (2004), Spurgeon and Hopkin, (1996) and Hobbelen et al. (2006) observed significant correlations between the concentrations of heavy metal accumulated in earthworms and available metal concentrations of field soils. Because of their capacity to accumulate and concentrate large quantities of organic and inorganic pollutants, earthworm species are widely recognized as suitable organisms for biomonitoring the effects of heavy metals in contaminated soils (Reddy and Rao, 2008; Peijnenburg and Vijver, 2009). Numerous studies investigated the effects of metals on earthworms in terms of mortality (Neuhauser et al., 1985; Fitzpatrick et al., 1996; Spurgeon et al., 1994, 2000), loss of weight (e.g. Khalil et al., 1996; Spurgeon and Hopkin, 1996; Maboeta et al., 2004), cocoon production (e.g. Ma, 1988; Spurgeon and Hopkin, 1996), cocoon viability (e.g. van Gestel et al., 1992; Spurgeon and Hopkin, 1996) and growth (e.g. van Gestel et al., 1991; Khalil et al., 1996). Most of these studies have been short-term experiments (14 or 21 days), performed in artificial soils or soil artificially contaminated by the addition of metal in solution (Nahmani et al., 2007). Few studies have investigated field-contaminated soils with multiple metals (Weltje, 1998; Conder and Lanno, 2000; Feisthauer et al., 2006). Despite a large body of literature on the impact of earthworms on the availability of metals in soils, only a few studies have been carried out addressing earthworm-facilitated metal extraction by plants.

In order to gain better understanding of the metal uptake by plants in contaminated soils, in the presence of earthworms, the aims of the present study were to: (1) investigate the change in metal availability in the soil given the presence of earthworms and plants; (2) evaluate the effects of metal concentrations on survival, body weight, cocoon production and cocoon weight of earthworms (*Eisenia fetida*) and (3) assess the effect of earthworms on metal uptake by plants. Earthworm and plant metal bioaccumulation studies were performed to better understand the relationships between availability of metals in soil and their bioaccumulation in organisms.

## 2. Materials and methods

### 2.1. Study area and characterization of experimental soils

The study area consists of a 3 km radius circle surrounding the Sclaigneaux calaminary site. The term “calaminary” originates from “Calamine” which is a general description of zinc-ores such as zinc-silicate, zinc–carbonate or the assemblage of hemimorphite, smithsonite, hydrozincite and willemite (Dejonghe, 1998). This area is known for its historical soil contamination by a former Zn–Pb ore treatment plant (Liénard et al., 2014). The Cu, Cd, Pb and Zn metals originate from historic atmospheric fallout of dusts enriched in these four metals. The soils used in the experiment represent one of the three major soils type present on the study area (Liénard et al., 2011), and are characterized as loamy-stony soils with gravel (Cambisols (Siltic) (WRB, 2014)) developed on ancient alluvial gravely terraces of Meuse river. Three samples were collected from contaminated fields and a control sample was collected from an uncontaminated field. Sampling points were located according to distance from the metal source. Four plots of about 20–25 $m^2$ in size were selected for the study. The study sites were as follows: C3 (most polluted), 1 km from the former Zn–Pb ore treatment plant (source); C2, 2.3 km from the source; C1, 3.5 km from the source; and C0 (the control), a site that has soil properties comparable to the contaminated sites, but with no history of contamination. For analysis of the physicochemical characteristics of the soil (including heavy metal concentrations), soil samples of approximately 1 kg each, were taken in April 2014 from four places in each plot and mixed together. Four core samples randomly taken from each plot to a depth of 20 cm were combined to form a composite sample for each plot. The soil samples were dried under shade conditions for two weeks and sieved at 8 mm to remove gravel. This sieved soil was used for the pot experiment. For physicochemical analyses, the soil samples were sieved at 2.0 mm and a subsample was crushed to 200 μm. Particle size distribution (clay, silt and sand fractions) was determined by sedimentation using the pipette method (van Ranst et al., 1999). Soil pH was measured by creating a slurry in distilled water ($pH_{water}$) and 1N KCl ($pH_{KCl}$) (w:v 2:5 ratio). Total organic carbon (TOC) was determined following the Walkley and Black method (Walkley and Black, 1934) and total nitrogen (N) was estimated by modified Kjeldahl method (Nelson and Sommers, 1996). Effective cation exchange capacity (CEC) was determined by using a hexamminecobalt trichloride solution following ISO 23470. Pseudo-total trace element (Cu, Cd, Pb, Zn) concentrations were determined after aqua regia digestion following ISO 11466 (referred to as ARmetal concentration). Available major (Ca, Mg, K, P) and trace (Cu, Cd, Pb, Zn) elements were determined after extraction with $CH_3COONH_4$ (0.5 M) and EDTA (0.02 M) at pH 4.65 (w:v 1:5 ratio) and agitation for 30 min (referred to as available metal concentration) (Lakanen and Erviö, 1971). The concentration of P in the resulting extract was determined by colorimetry at 430 nm. The concentrations of the other elements in the solution were measured by flame atomic absorption spectrometry (VARIAN 220, Agilent Technologies, Santa Clara, CA, USA). All concentrations were calculated on the basis of dry weight (DW) of samples (40 °C, 24 h). The detection limits for AR/available metals were, respectively, 0.66/0.10 mg $kg^{-1}$ (Cd), 0.99/0.15 mg $kg^{-1}$ (Cu), 3.33/0.50 mg kg$^{-1}$ (Pb) and 0.33/0.05 mg kg$^{-1}$ (Zn). To assess the available (soluble) fraction of metals in the soil, $CaCl_2$ extraction was used (at both the start and the end of the experiment) (McGrath and Cegarra, 1992). Extractions with 0.01 M $CaCl_2$ have been widely used for assessing the mobility and availability of heavy metals in soils. As part of the quality control program for the study, a standard reference material was used and analyzed with each set of samples. Selected physicochemical soil characteristics and metal concentrations in the soils are presented in Table 1.

### 2.2. Experimental procedure

The experimental design involved four factors: (i) metal concentration represented by four different soils with an increasing level of contamination ($C_0$, $C_1$, $C_2$ and $C_3$), (ii) plants (*Zea mays*, *Vicia faba*), (iii) earthworms *E. fetida*, and (iv) food for earthworms (Table 2a). Ten treatments were prepared from the soil samples to create a set of samples with different combinations of (the two) plants, earthworms and food presence and each treatment was replicated four times, except for the control sample with food only ($CP_0E_0F_1$) which was replicated once (Table 2b). The experiment was conducted under controlled conditions (i.e. 16 h light and 8 h dark at 20 ± 1 °C) (Reneicke and Kriel, 1981) during 42 days. Pots (16 cm diameter × 20 cm high) were prepared with 2.25 kg of dry soil. At the beginning of the experiment, soil moisture content was adjusted to 18%, corresponding to 65% of the soil's water holding capacity, checked regularly and adjusted to the desired value by adding deionized water.

**Table 2**
Experimental design for ecotoxicity test: (a) presentation of different factors and treatments, (b) description of treatments with different combinations of factors.

| (a) | Factors | Treatments |
|---|---|---|
| | Concentration | $C_0$, $C_1$, $C_2$, $C_3$ |
| | Plants | No plant ($P_0$)<br>*Vicia faba* ($P_1$)<br>*Zea mays* ($P_2$) |
| | Earthworms | Presence ($E_1$)<br>Absence ($E_0$) |
| | Food | Presence ($F_1$)<br>Absence ($F_0$) |

| (b) | Treatment | Description | n |
|---|---|---|---|
| | 1 | $C_{0,1,2\&3}$ $P_0$ $E_0$ $F_1$ | 4 |
| | 2 | $C_{0,1,2\&3}$ $P_0$ $E_0$ $F_0$ | 16 |
| | 3 | $C_{0,1,2\&3}$ $P_0$ $E_1$ $F_0$ | 16 |
| | 4 | $C_{0,1,2\&3}$ $P_0$ $E_1$ $F_1$ | 16 |
| | 5 | $C_{0,1,2\&3}$ $P_1$ $E_0$ $F_0$ | 16 |
| | 6 | $C_{0,1,2\&3}$ $P_1$ $E_0$ $F_1$ | 16 |
| | 7 | $C_{0,1,2\&3}$ $P_1$ $E_1$ $F_1$ | 16 |
| | 8 | $C_{0,1,2\&3}$ $P_2$ $E_0$ $F_0$ | 16 |
| | 9 | $C_{0,1,2\&3}$ $P_2$ $E_0$ $F_1$ | 16 |
| | 10 | $C_{0,1,2\&3}$ $P_2$ $E_1$ $F_1$ | 16 |

**Table 1**
Aqua regia, available and soluble metal concentrations (mg kg$^{-1}$ DW), available major elements (mg 100 g$^{-1}$ DW), and other chemical parameters measured in the four experimental soils ($C_0$: control soil, $C_1$, $C_2$ and $C_3$: contaminated soils).

| Parameters | $C_0$ | $C_1$ | $C_2$ | $C_3$ |
|---|---|---|---|---|
| pH$_{water}$[a] | 8.20 | 7.45 | 7.11 | 8.08 |
| pH$_{KCl}$[a] | 7.64 | 6.78 | 6.61 | 7.75 |
| TOC (%)[b] | 2.04 | 1.74 | 1.67 | 1.50 |
| N (%)[c] | 0.190 | 0.160 | 0.180 | 0.150 |
| CEC (meq 100 g$^{-1}$)[d] | 15.2 | 11.8 | 10.7 | 10.5 |
| Clay (%)[e] | 21.0 | 12.3 | 13.3 | 14.6 |
| Silt (%)[e] | 62.5 | 72.4 | 59.4 | 46.1 |
| Sand (%)[e] | 16.5 | 15.3 | 27.2 | 39.3 |
| Aqua Regia[f] | | | | |
| Cd (mg kg$^{-1}$, DW) | 0.710 | 2.48 | 4.27 | 9.28 |
| Cu (mg kg$^{-1}$, DW) | 15.0 | 11.7 | 21.7 | 19.3 |
| Pb (mg kg$^{-1}$, DW) | 29.0 | 69.0 | 129 | 255 |
| Zn (mg kg$^{-1}$, DW) | 103 | 208 | 384 | 743 |
| Available[g] | | | | |
| Cd (mg kg$^{-1}$, DW) | 0.550 | 1.95 | 2.89 | 6.07 |
| Cu (mg kg$^{-1}$, DW) | 3.59 | 13.34 | 4.89 | 6.97 |
| Pb (mg kg$^{-1}$, DW) | 12.4 | 41.1 | 71.2 | 169 |
| Zn (mg kg$^{-1}$, DW) | 15.8 | 61.9 | 65.6 | 172 |
| Ca (mg 100 g$^{-1}$, DW) | 479 | 191 | 221 | 554 |
| Mg (mg 100 g$^{-1}$, DW) | 23.2 | 22.2 | 11.2 | 10.6 |
| K (mg 100 g$^{-1}$, DW) | 23.4 | 37.8 | 20.4 | 27.7 |
| P (mg 100 g$^{-1}$, DW) | 13.5 | 16.1 | 14.7 | 16.2 |
| Soluble[h] | | | | |
| Cd (mg kg$^{-1}$, DW) | 0.010 | 0.050 | 0.100 | 0.060 |
| Cu (mg kg$^{-1}$, DW) | 0.140 | 0.12 | 0.11 | 0.14 |
| Pb (mg kg$^{-1}$, DW) | <DL | <DL | <DL | <DL |
| Zn (mg kg$^{-1}$, DW) | 0.020 | 0.370 | 0.690 | 0.240 |

(DL: detection limit).

[a] pH$_{water}$ with distilled water (w:v 2:5 ratio) and pH$_{KCl}$ with 1N KCl (w:v 2:5 ratio).

[b] Walkley and Black method (1934).

[c] Modified Kjeldahl method (Nelson and Sommers, 1996).

[d] Hexamminecobalt trichloride solution following ISO 23470.

[e] Size particles by sedimentation using the pipette method (van Ranst et al., 1999).

[f] Aqua regia digestion following ISO 11466.

[g] Extraction with $CH_3COONH_4$ (0.5 M) and EDTA (0.02 M) at pH 4.65 (w:v 1:5 ratio) (Lakanen and Erviö, 1971).

[h] Extraction with 0.01 M $CaCl_2$.

For each treatment with earthworms, 20 specimens *E. fetida* were deposited on the surface of the soil in pots. According to the OECD methods (OECD, 2004), earthworms were acclimatized for at least 48 h prior to exposure, in the experiment conditions (temperature, light, soil moisture, etc.). Healthy earthworms weighing between 200 and 600 mg each, and well-developed clitellum were introduced seven days after plant seeding. To ensure the earthworms survival during the experimentation period, they were fed weekly with a dried mixture of horse manure (75%) and oat flakes (25%) to provide 0.5 g per earthworm. The test containers were covered with a perforated lid to limit water loss due to evaporation, to allow aeration and prevent the earthworms from escaping.

*Z. mays* and *V. faba* were the plant species used during this experiment. Ten seeds were seeded in pots according to experimental design. Plant growth was held under controlled conditions: 20 ± 1 °C and 18 ± 1 °C day and night temperatures respectively, with 60% ± 5% relative humidity. Germination was determined by visual seedling emergence (Gong et al., 2001). The plants were watered with deionized water. The distribution of the microcosms in the chamber was randomized and changed biweekly.

### 2.3. Acquisition of organismal data

After 42 days of exposure, earthworm mortality was checked, and the living adults were hand-collected. The earthworms and cocoons in each pot were counted using the procedure described in the OECD method (OECD, 2004). After 48 h cleaning out of gut content on moist filter paper, the earthworms were weighed and checked for any morphological symptoms. The filter paper was changed three times per day to prevent coprophagy. All earthworms in each container were weighed as a group and recorded as a datum. The relative growth rate of earthworms was calculated according to the following equation: relative growth rate = $(W_t–W_0)/(W_0) \times 100\%$, where $W_0$ is the initial average weight of earthworms, and $W_t$ is the average weight after 42 days.

With regard to the plants, after 42 days of exposure, the shoots were removed by cutting the *V. faba* and *Z. mays* plants close to the soil surface and weighed. Root mass was determined following washing with DI water and drying in paper bags (40 °C) for one week. Similarly, and following 48 h for gut clearing, earthworms were placed into an oven at 40 °C over night; the dry earthworms were weighed (DW).

Samples of dried plant and earthworm tissues were digested in a mixture of 15 ml of nitric acid ($HNO_3$, 65%) and 15 ml of perchloric acid ($HClO_4$, 70%) during 16 h. After complete evaporation, 5 ml of HCl (10%) was added and the samples were brought to a 25 ml volume with distilled water. These solutions were stored at 4 °C in polyethylene tubes before analysis. Metal concentrations in the samples were measured by flame atomic absorption spectrometry (VARIAN 220, Agilent Technologies, Santa Clara, CA, USA). The detection limits for Cu, Zn, Pb and Cd were 0.6, 0.2, 2 and 0.4 mg kg$^{-1}$, respectively.

### 2.4. Statistical analysis

Data normality and homoscedasticity were verified with Shapiro-Wilk's and Bartlett's tests. In most cases, parametric tests were relevant, but in others equivalent non-parametric tests were more appropriate.

Firstly, the effects of soil metal concentration ($C_{0,1,2\&3}$), plants ($P_0$, $P_1$, $P_2$), earthworms ($E_0$,$E_1$), food ($F_0$, $F_1$), time ($T_0$, $T_1$) and sampling block (random factor) on metal availability in soil were evaluated through analysis of variance (ANOVA) within a general linear model in Minitab 16 software (Minitab Inc., State College, PA, USA). Secondly, the effects of soils concentration ($C_{0,1,2\&3}$), plants ($P_0$, $P_1$, $P_2$), food ($F_0$, $F_1$) and sampling block (random factor) on earthworm mortality, earthworm weight, earthworm reproduction (number and weight of cocoons) and the concentration of metals in earthworm tissues were evaluated. Finally, the effects of soil metal concentration ($C_{0,1,2\&3}$), earthworms ($E_0$,$E_1$), food ($F_0$, $F_1$) and sampling block (random factor) on the uptake of metals by plants ($P_1$, $P_2$) were investigated. Differences were considered significant at $P \leq 0.05$.

When the difference was significant, a comparison of means was conducted by a post-hoc Tukey test. If the hypothesis of parameters of that model was not satisfied, a Kruskal–Wallis test was used instead to detect the effects of the various factors.

## 3. Results

### 3.1. Effect of earthworm and plant activities on the availability of metals in soil

Soil samples taken from the sample treatments (control, soil with earthworms, soil with plants, and soil with both earthworms and plants) were analyzed to compare pH water and the available metal fraction. Table 3 gives pH values and metal fraction (0.01 M $CaCl_2$) remaining in the soil after 42 days of exposure in the presence or absence of organisms (plants or earthworms).

Soil pH values varied from 6.3 to 7.2. No significant difference was observed among the six treatments ($P > 0.05$). The introduction of earthworms, or plants, or the combination between earthworms and plants did not affect soil pH values.

Presence of *V. faba* decreased the availability of Cd in soil ($P = 0.02$), but no significant differences on the availability of metals were observed for the other treatments.

### 3.2. Earthworm traits

The mean concentrations of Cu, Zn, Pb and Cd (expressed as mg kg$^{-1}$ DW) in *E. fetida* tissues after 42 days of exposure are reported in Fig. 1. Significant differences were observed between

**Table 3**

Mean (±s.e) $pH_{water}$ and metal fraction extractable using 0.01 M $CaCl_2$ procedure in soil (mg kg$^{-1}$ DW) before experiment (Soil) and after 42 days exposure (Treatments) in the absence/presence of plants (*Vicia faba*/*Zea mays*) and/or absence/presence of earthworms (*Eisenia fetida*). Different letters indicate a significant difference according to Tukey's test at $P \leq 0.05$.

| Soil parameters | Soil | | Treatments | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $P_0E_0F_0$ | $P_0E_1F_1$ | $P_1E_0F_0$ | $P_2E_0F_0$ | $P_1E_1F_1$ | $P_2E_1F_1$ |
| pH | $C_0$ | 7.25 | 7.2 ± 0.17 | 7.2 ± 0.16 | 7.2 ± 0.18 | 7.1 ± 0.18 | 7.1 ± 0.14 | 7.1 ± 0.21 |
| | $C_1$ | 6.40 | 6.4 ± 0.23 | 6.3 ± 0.19 | 6.4 ± 0.12 | 6.4 ± 0.11 | 4.9 ± 0.15 | 6.6 ± 0.04 |
| | $C_2$ | 6.28 | 6.4 ± 0.05 | 6.4 ± 0.16 | 6.3 ± 0.18 | 6.4 ± 0.12 | 6.4 ± 0.11 | 6.6 ± 0.19 |
| | $C_3$ | 6.67 | 7.1 ± 0.13 | 7.1 ± 0.13 | 7.1 ± 0.18 | 7.1 ± 0.15 | 7.0 ± 0.15 | 7.0 ± 0.15 |
| Cd | $C_0$ | 0.01 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 |
| | $C_1$ | 0.06 | 0.04 | 0.04 | 0.03 | 0.03 | 0.03 | 0.08 |
| | $C_2$ | 0.10 | 0.04 a | 0.07 ab | 0.07 b | 0.05 ab | 0.07 ab | 0.06 ab |
| | $C_3$ | 0.10 | 0.04 | 0.04 | 0.05 | 0.03 | 0.06 | 0.06 |
| Cu | $C_0$ | 0.14 | 0.01 | 0.00 | 0.02 | 0.04 | 0.00 | 0.15 |
| | $C_1$ | 0.12 | 0.02 | 0.02 | 0.01 | 0.00 | 0.00 | 0.02 |
| | $C_2$ | 0.11 | 0.02 | 0.00 | 0.02 | 0.00 | 0.08 | 0.23 |
| | $C_3$ | 0.14 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.09 |
| Pb | $C_0$ | 0.00 | <DL | <DL | <DL | <DL | <DL | <DL |
| | $C_1$ | 0.00 | <DL | <DL | <DL | <DL | <DL | <DL |
| | $C_2$ | 0.00 | <DL | <DL | <DL | <DL | <DL | <DL |
| | $C_3$ | 0.00 | <DL | <DL | <DL | <DL | <DL | <DL |
| Zn | $C_0$ | 0.00 | <DL | <DL | <DL | <DL | <DL | <DL |
| | $C_1$ | 0.36 | 0.05 | 0.46 | 0.43 | 0.38 | 0.31 | 0.30 |
| | $C_2$ | 0.69 | 0.57 | 0.74 | 1.05 | 0.64 | 0.62 | 0.65 |
| | $C_3$ | 0.24 | 0.39 | 0.22 | 0.38 | 0.27 | 0.40 | 0.57 |

$C_0$: control soil, $C_1$, $C_2$ and $C_3$: contaminated soils, $P_0$: no plant, $P_1$: *Vicia faba*, $P_2$: *Zea mays*, $E_0$: no earthworms, $E_1$: earthworms, $F_0$: no food, $F_1$: food, DL: detection limit.

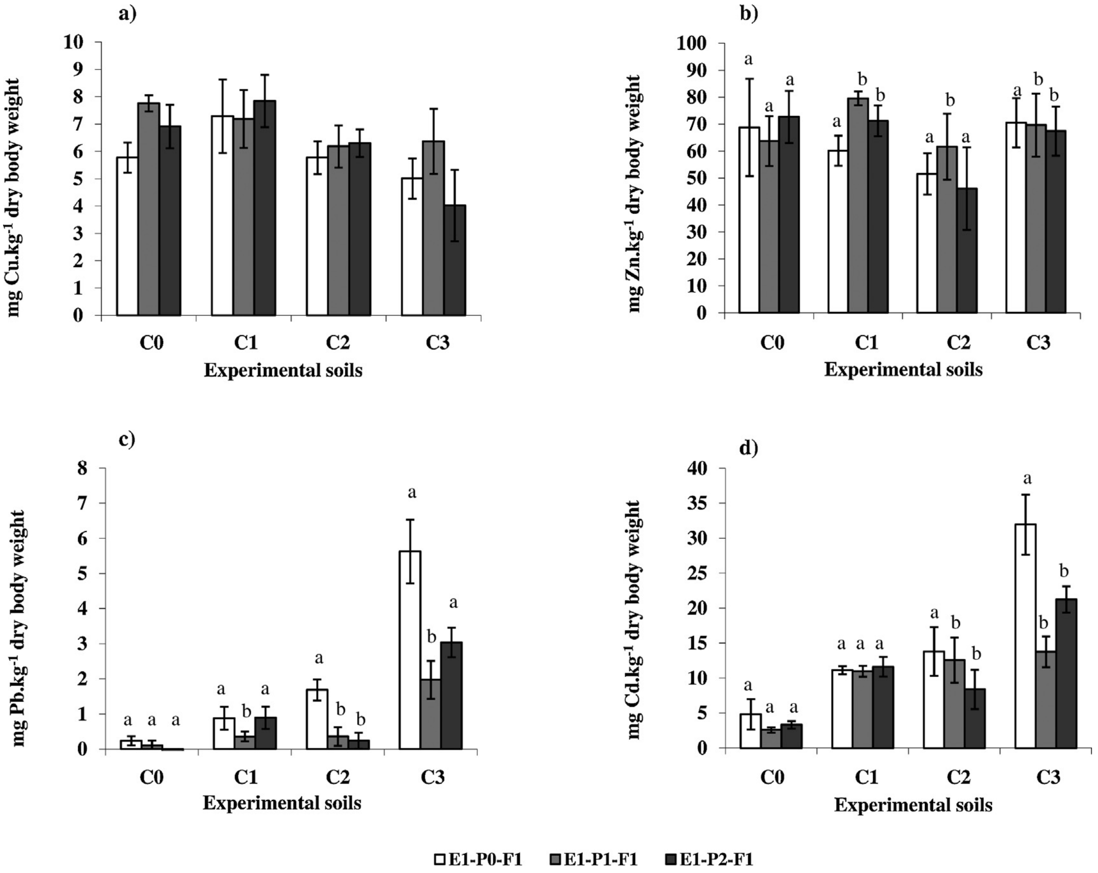

**Fig. 1.** Mean (±s.e) tissue concentrations (mg $kg^{-1}$ dry body weight) of Cu (a), Zn (b), Pb (c) and Cd (d) in *Eisenia fetida* ($E_1$) specimens in the presence of food ($F_1$), after 42 days exposure to control ($C_0$) and contaminated ($C_1$,$C_2$ and $C_3$) soils, in the absence ($P_0$) or presence of *Vicia faba* ($P_1$) and *Zea mays* ($P_2$) plants. Different letters indicate a significant difference according to Tukey's-test at $P \leq 0.05$.

the concentrations of elements found in *E. fetida* for all the contaminated soils, except for copper. The concentration of Pb metal in earthworm tissues clearly increased with exposure to raised soil Pb concentration ($P < 0.001$).

Our results showed that addition of plants affected the metal concentrations in earthworm tissues except for Cu (Fig. 1a). Tissue concentration of Zn in *E. fetida* was significantly higher when plants were added. *V. faba* plant affected significantly the accumulation of Zn by earthworms (Fig. 1b). Pb and Cd metals concentration in *E. fetida* tissues decreased in the presence of *V. faba* or *Z. mays* plants for $C_2$ and $C_3$ soils (Fig. 1c and d)

### 3.2.1. Earthworm mortality

An analysis of variance was performed on the results of earthworm life-cycle parameters in order to evaluate whether there was a change in mortality after 42 days of exposure. Throughout the experimental period, no earthworms died in the control soils ($C_0$). Thus, this observation suggested that the experimental conditions were valid in terms of providing suitable media for earthworm's survival. After 42 days of exposure, mortality rates in the contaminated soils were not different from the controls (data not shown). In addition, regardless of the concentration of polluted soil in any sample, earthworm mortality was not affected by the addition of plants *V. faba* or *Z. mays*. Only the "food" factor significantly affected the mortality of *E. fetida* ($P = 0.036$).

### 3.2.2. Earthworm body weight

Twenty worms were weighed at the beginning of the experiment and the remaining number of earthworms after 42 days was >17 for all samples. The questions investigated were: was there a general change in earthworm weight or did the change vary according to worm feeding, soil contamination or the presence of plants?

Significant difference in body weight was found between earthworms in pots with food and earthworms in pots without food (Table 4) so we performed a separate analysis for pots with and without a food supply. In the pots without food, no plants were cropped. No significant interaction was found with time, while the factors "Soil" and "Time" were significant ($P = 0.021$) and highly significant ($P = 0.0001$), respectively. Regarding the effect of soil contamination, it seems that the differences were linked to the initial weight of the earthworms. The change in their body weight over time can be considered as equivalent for all the soil types, that is a mean decrease of 195 mg per earthworm after 6 weeks.

For pots which received food, significant interactions were found between "Plant and Time", "Bloc and Time" and "Plant and Soil". We found that the mean weight of earthworms at the beginning of the experiment was higher (40 mg) in Bloc 2 than in the other three. Separate analyses of variance were performed for the treatments without plants, with *Z. mays* or with *V. faba*. The first point to be noted is that there was an increase of worm weight for every pot without a plant, and a smaller increase for soil $C_0$, $C_2$

**Table 4**
Mean (±s.e) weight of earthworms *Eisenia fetida* (g) at the beginning ($T_0$) and after 42 days exposure ($T_1$) with different combinations, in the presence ($F_1$) and absence ($F_0$) of food. ($C_0$: control soil, $C_1$–$C_2$–$C_3$: contaminated soils, $P_0$: no plant, $P_1$: *Vicia faba*, $P_2$: *Zea mays*, ↘: decrease between $T_0$ and $T_1$, ↗: increase between $T_0$ and $T_1$, ↗↗: high increase between $T_0$ and $T_1$). Different letters indicate a significant difference according to Tukey's-test at $P \leq 0.05$.

| | $F_0$ | | | $F_1$ | | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_1$ | | $T_0$ | $T_1$ | |
| $C_0$ $P_0$ | 22.10 ± 4.65 ab | 13.10 ± 3.20 ab | ↘ | 21.85 ± 4.20 ab | 25.45 ± 5.15 bc | ↗↗ |
| $C_0$ $P_1$ | | | | 22.05 ± 4.30 ab | 23.40 ± 4.95 ab | ↗ |
| $C_0$ $P_2$ | | | | 21.55 ± 3.95 ab | 20.60 ± 4.45 a | |
| $C_1$ $P_0$ | 21.60 ± 4.10 ab | 12.45 ± 2.95 ab | ↘ | 22.75 ± 4.45 ab | 24.70 ± 6.35 bc | ↗ |
| $C_1$ $P_1$ | | | | 21.95 ± 4.35 ab | 22.00 ± 5.15 ab | |
| $C_1$ $P_2$ | | | | 22.45 ± 4.50 ab | 24.75 ± 5.40 bc | ↗ |
| $C_2$ $P_0$ | 21.35 ± 4.25 a | 11.35 ± 3.00 a | ↘ | 21.45 ± 4.15 ab | 25.80 ± 6.45 bc | ↗↗ |
| $C_2$ $P_1$ | | | | 21.50 ± 4.20 ab | 22.45 ± 4.35 ab | ↗ |
| $C_2$ $P_2$ | | | | 22.95 ± 4.15 ab | 22.60 ± 5.95 ab | |
| $C_3$ $P_0$ | 23.30 ± 4.00 b | 12.65 ± 3.05 b | ↘ | 21.05 ± 3.85 a | 27.15 ± 4.90 c | ↗↗ |
| $C_3$ $P_1$ | | | | 23.10 ± 4.40 b | 24.30 ± 5.10 bc | ↗ |
| $C_3$ $P_2$ | | | | 22.75 ± 4.00 ab | 21.95 ± 5.30 ab | |

and $C_3$ with *V. faba*. Regarding *Z. mays*, only the soil $C_1$ showed an increase of the mean mass of the worms. We can conclude that the action of giving food to worms had a significant impact when no plant was cultivated, while the presence of a plant did facilitate the gain of mass.

### 3.2.3. Earthworm reproduction

Cocoon production was calculated per surviving earthworm. Table 5 shows the effects of metals and the addition of plants on reproductive parameters of *E. fetida*, including cocoon production per earthworms and weight per cocoon in soils after 42 days of exposure. A significant ($P < 0.001$) reduction in cocoon production and cocoon weight was seen for the control and contaminated soils, when food was unavailable. With the addition of food, earthworms produced more cocoons (6.65 per worm) and with a greater mean weight (19 mg per cocoon) than in the absence of food (1.75 per worm; 12 mg per cocoon). However, no significant difference in earthworm reproduction was observed between earthworms kept in control soil and contaminated soils.

The presence of plants also enhanced the reproduction of *E. fetida*. Cocoon production in the treatments with plants was significantly higher than in the treatments without plants, suggesting that the reproduction response was influenced by the presence of plants in the soil. The presence of *V. faba* or *Z. mays* significantly increased the reproduction activity of *E. fetida* in control and contaminated soils. This suggests that reproduction and cocoon weight were sensitive to environmental changes such as the introduction of plants.

### 3.3. Earthworm metal concentrations

The mean concentrations of Cu, Zn, Pb and Cd (expressed as mg kg$^{-1}$ DW) in *E. fetida* after 42 days of exposure are reported in Fig. 1. Differences were observed between the concentrations of elements found in the tissues of *E. fetida* for all the contaminated soils. The concentration of Pb in earthworm tissues clearly increased with exposure to raised soil Pb concentration ($P < 0.001$).

The addition of plants also affected earthworm metal concentrations. The concentration of Pb and Cd in earthworm tissues decreased in the presence of *V. faba* or *Z. mays* for $C_2$ and $C_3$ samples. The concentration of Zn in *E. fetida* tissues was significantly higher in the presence of plants. The presence of *V. faba* affected significantly the concentration of Zn in earthworm tissues.

**Table 5**
Total number of cocoons, cocoon production rates (number of cocoons/surviving adult earthworms/month), and mean weight of cocoons (±s.e) collected after 42 days exposure and produced by *Eisenia fetida* exposed to soils collected from the three metal-contaminated sites ($C_1$, $C_2$ and $C_3$) and the control soil ($C_0$).

| Treatments | Cocoons number | Cocoons production rate (Nb/adult/month) | Mean cocoon weight (mg) |
|---|---|---|---|
| $C_0$ $P_0$ $E_1$ $F_0$ | 151 | 1.35 ± 0.45 | 11.56 ± 1.84 |
| $C_1$ $P_0$ $E_1$ $F_0$ | 187 | 1.67 ± 0.43 | 10.43 ± 2.45 |
| $C_2$ $P_0$ $E_1$ $F_0$ | 97 | 0.87 ± 0.32 | 12.48 ± 3.38 |
| $C_3$ $P_0$ $E_1$ $F_0$ | 123 | 1.10 ± 1.32 | 13.43 ± 3.00 |
| $C_0$ $P_0$ $E_1$ $F_1$ | 451 | 4.03 ± 1.14 | 17.81 ± 2.78 |
| $C_1$ $P_0$ $E_1$ $F_1$ | 436 | 3.89 ± 0.81 | 18.95 ± 1.92 |
| $C_2$ $P_0$ $E_1$ $F_1$ | 457 | 4.08 ± 2.23 | 20.16 ± 1.53 |
| $C_3$ $P_0$ $E_1$ $F_1$ | 536 | 4.78 ± 0.33 | 18.68 ± 1.91 |
| $C_0$ $P_1$ $E_1$ $F_1$ | 539 | 4.81 ± 1.13 | 15.28 ± 2.70 |
| $C_1$ $P_1$ $E_1$ $F_1$ | 560 | 5.00 ± 0.38 | 14.80 ± 1.11 |
| $C_2$ $P_1$ $E_1$ $F_1$ | 571 | 5.10 ± 1.00 | 17.25 ± 4.55 |
| $C_3$ $P_1$ $E_1$ $F_1$ | 608 | 5.43 ± 0.97 | 17.87 ± 4.25 |
| $C_0$ $P_2$ $E_1$ $F_1$ | 500 | 4.46 ± 1.14 | 16.95 ± 3.95 |
| $C_1$ $P_2$ $E_1$ $F_1$ | 547 | 4.88 ± 0.76 | 18.88 ± 2.88 |
| $C_2$ $P_2$ $E_1$ $F_1$ | 505 | 4.51 ± 1.25 | 16.29 ± 1.33 |
| $C_3$ $P_2$ $E_1$ $F_1$ | 594 | 5.30 ± 0.96 | 15.77 ± 1.27 |

$P_0$: no plant, $P_1$: *Vicia faba*, $P_2$: *Zea mays*, $E_0$: no earthworm, $E_1$: earthworms, $F_0$: no food, $F_1$: food.

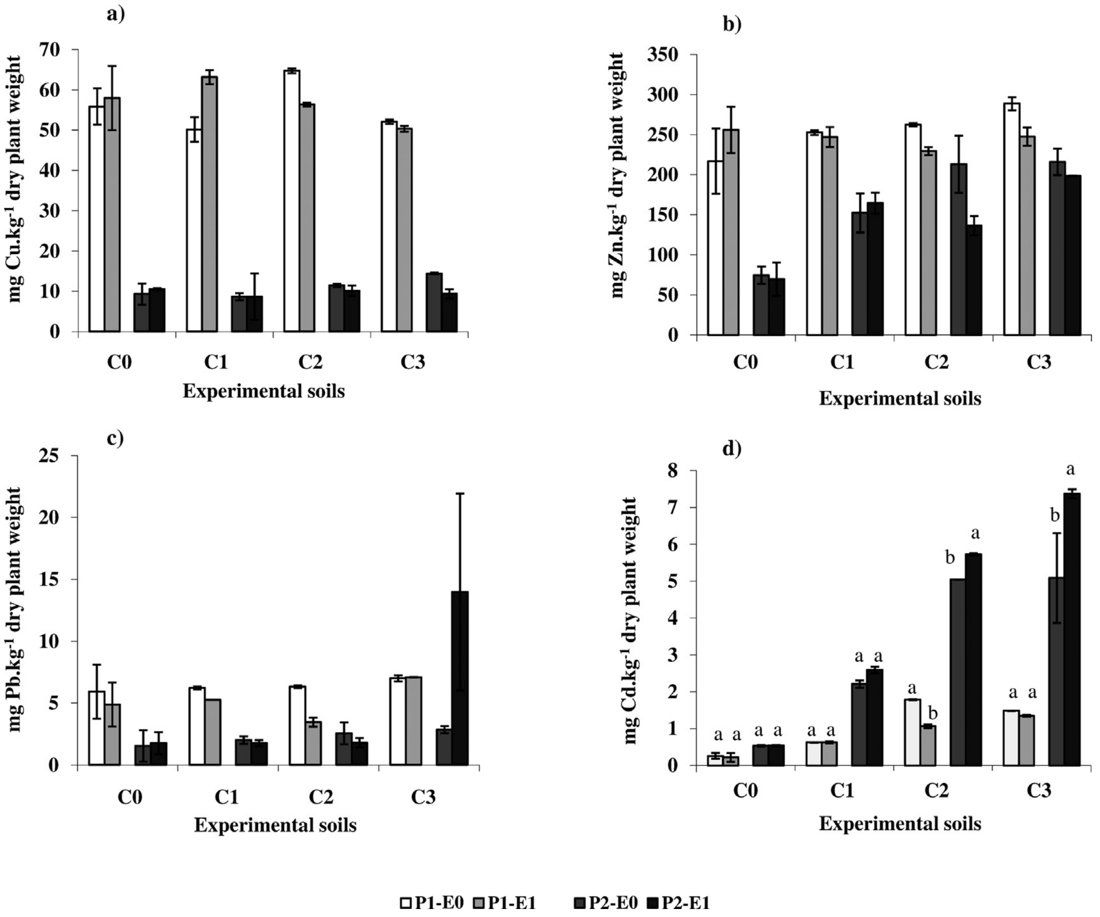

**Fig. 2.** Effects of *Eisenia fetida* ($E_1$) on mean concentrations (±s.e) (mg kg$^{-1}$ dry plant weight) of Cu (a), Zn (b), Pb (c) and Cd (d) in *Vicia faba* ($P_1$) and *Zea mays* ($P_2$), after 42 days exposure to control ($C_0$) and contaminated ($C_1$, $C_2$ and $C_3$) soils. Different letters indicate a significant difference according to Tukey's-test at $P \leq 0.05$.

### 3.4. Plant metal concentrations

The mean of the concentrations of heavy metals within *V. faba* and *Z. mays* plants, with and without the presence of *E. fetida*, are given in Fig. 2. In contaminated soils, metal concentrations were significantly different between the two plants. Cu and Zn concentrations were lower in *Z. mays* compared with *V. faba* (Fig. 2a and b). Pb concentration of *Z. mays* and *V. faba* as compared to the control (Fig. 2c). Cd concentration was higher in *Z. mays* in comparison with *V. faba* (Fig. 2d). The data showed a complex interaction between the metals in the soil solution and their accumulation in plants (both species and metal dependant). When the plant tissue concentration data was compared to the available concentration, no correlation was observed for *V. faba* and *Z. mays*. The addition of earthworms to soils contaminated by Cu, Zn and Pb did not affect metal concentrations in plants; only for Cd did we observed an increased concentration in *Z. mays*. As the concentration of Cu, Zn and Cd in soil increased, the concentrations of Cu, Zn and Cd in *Z. mays* plants also increased, while for *V. faba*, the plants' metal concentrations remained unchanged.

## 4. Discussion

### 4.1. Effects of earthworms and plants on the pH and the availability of metals in soils

It is well established that soil pH is a key factor affecting the adsorption–desorption behaviors and hence availability of heavy metals in soil. In this study, it was found that the presence or absence of organisms (earthworms and plants) did not change the pH values. Our results are however inconsistent with most previous studies, which have shown that, in contaminated soils earthworm activity increased soil pH (Wen et al., 2006; Udovic and Lesta, 2007). The mechanisms by which *E. fetida* should change pH value are still unclear (Sizmur and Hodson, 2009).

When the soil is contaminated with a mixture of metals, their combined effects have proven to be very complex. After 42 days of exposure, following the addition of *E. fetida*, it can be seen that Cd and Zn fractions were less available than other metals. The significant decrease in the available fraction of Cd in soils suggested that *E. fetida* either accumulated those metals in their tissues or that earthworm activity may change the chemical form and availability of Cd fraction in soils. The significant difference was observed for the $C_2$ concentration, which represents the highest $CaCl_2$ extractable Cd soil concentration. These findings are inconsistent with previous studies, which suggested an increase in the availability of metal fractions as a result of earthworm activities (Wen et al., 2004; Coeurdassier et al., 2007; Udovic and Lesta, 2007). Lukkari et al. (2006) however reported an increase of Zn fractions in the presence of earthworms, concluding that earthworms tended to decrease the mobility of Zn in the soil. In some studies, the addition of earthworms has been shown to decrease or not affect the fraction of metals bound to either organic matter or carbonates (Wen et al., 2004; Ruiz et al., 2011). The lack of observed differences in the availability of other metals (Cu, Pb, and Zn) between the control and the earthworm-inhabited soils may indicate that although passage through the earthworm gut has an impact on metal mobility, this is only a temporary effect (Lukkari et al., 2006). These conflicting results could be mainly due to differences in (i) soil characteristics, (ii) pollution mode, (iii) metal type, and (iv) even physiological and ecological differences among earthworm species. The effect of earthworms on metal availability in soils has not been extensively documented (Sizmur and Hodson, 2009).

Our findings showed a significant correlation between extractable Zn and pH level. Changes in pH affect the availability of Zn. The pH level, organic matter content and availability of heavy metals in soil are critical factors for the heavy metal accumulation by both plants and animals (Spurgeon and Hopkin, 1996; Oste et al., 2001). The addition of *V. faba* increased the available fractions of Cd and Zn. This result can be explained by the vital role of plant roots, which played in altering metal speciation in soils through rhizosphere processes. Plant roots can modify the speciation and availability of metal (Hashimoto et al., 2010). Another possibility for enhancing metal availability is the involvement of soil microorganisms and plant root-associated bacteria (Kamnev and van der Leile, 2000; van der Lelie, 1998).

### 4.2. Earthworm traits

#### 4.2.1. Earthworm adult survival and growth

The high survival rate observed for the earthworms in the metal contaminated soils after 42 days of exposure and the absence of visible damage on the plants may be explained by the low range of metal contamination. The stability and lack of mortality for biological organisms observed in old contaminated soils were reported by Schreck et al. (2011). The availability of metal in field soils often decreases over time (Lock and Janssen, 2003a,b).

The body weight of *E. fetida* was significantly affected by food. In the treatments without food, the loss of body weight was most likely due to the fact that organic carbon and food supply available in these soils were insufficient to maintain the initial body weight of earthworms. Body weight of the earthworms was not significantly affected by metal contamination. This could be explained by the fairly low of metal concentrations or by the competition of metals with other essential elements in the soils. These effects suggested that soil characteristics can decrease the availability of metals and modify their toxicity. As reported by Ernst et al. (2008), soil properties can affect metal bioavailability and their subsequent impacts on earthworm physiology and behavior. Some reports suggested that in some cases, organisms primarily responded to certain attributes rather than to the metal concentration (Chang et al., 1997). Soil pH and organic carbon have been claimed to be important factors for affecting metal bioavailability to ecological receptors (Spurgeon and Hopkin, 1996; Peijnenburg and Jager, 2003; Basta et al., 2005; Dayton et al., 2006). In our study, soil pH remained in the neutral to slightly alkaline range. Many other studies have reported the metal's toxicity to earthworms, tested both in artificial and field soil (Neuhauser et al., 1985; Spurgeon et al., 1994; Spurgeon and Hopkin 1995). They concluded that the toxic effects of metals were less severe in field soils (Spurgeon and Hopkin, 1995). It should be noted that simultaneous exposure to several metals can also lead to antagonistic, not necessarily to additive or synergistic effects, as observed by Khalil et al. (1996).

The addition of *V. faba* and *Z. mays* did not affect the earthworm weight after 42 days in all concentrations. We can suppose that metallic elements adhered quickly on the root surface and penetrated into plant tissues, which explained the inhibited development seen after 28 days for both plants, which in turn would make metals less available (data not shown). A consequence of the presence of earthworms and plants in the same pots could have changed resource allocation and create a competition between the plants and the earthworms.

#### 4.2.2. Cocoon production and cocoon weight

Several studies indicated that cocoon production is one of the most sensitive biological responses in toxicity tests (Ma, 1984; Spurgeon et al., 1994; Spurgeon and Hopkin, 1996, 1999; Kula and Larink, 1997; Reinecke et al., 2001; Homa et al., 2003), with a strong decrease in cocoon production with increased metal concentrations.

The introduction of plants (*V. faba* or *Z. mays*) affected cocoon production. This suggested that an important amount of energy has been allocated to the production of earthworm cocoons and the rest was allocated to cocoon development. The opposite response of the two endpoints for *E. fetida* suggested a trade-off of energy between cocoon production and cocoon weight. This pattern were very difficult to explain: it is possible that *E. fetida* living in a stressed environment is “forced to choose” on which process to spend its energy. Indeed, it was the availability of sufficient food that was of prime importance for maintaining a high cocoon production and weight (Reinecke and Viljoen, 1990) and this could have explained the decrease in cocoon weight we observed. Spurgeon and Hopkin (1996) observed that *E. fetida* produced cocoons that increased in weight with increased soil concentrations of a metal mixture, mainly Cu, Zn, Pb and Cd. In our study, the cocoon weight was independent of the metal concentrations. Our findings disagreed with several authors (Spurgeon et al., 2000; Ávila et al., 2009) who found negative effects on earthworm reproduction in metal contaminated soils. The direct relationship between *E. fetida* reproduction and soil metal concentration must be discussed with caution since earthworm reproduction depends on many different soil factors. In particular soil organic matter content played an important role in mitigating the effects of metals (Ávila et al., 2009van Gestel et al., 2011), while earthworm reproduction may also be reduced at high soil pH (van Gestel et al., 1992).

### 4.3. Metal accumulation in earthworms

Earthworms are considered to have two uptake pathways for heavy metal: dermal and intestinal. According to Lanno et al. (2004), earthworms can take up metals from soil either through direct dermal contact with them in soil solution or by ingestion of bulk soil or specific soil fractions. Distribution of metals among soil fractions is also considered to be important for their toxicity and bioavailability to earthworms (Becquer et al., 2005). The results we obtained showed that Zn and Cu were accumulated in *E. fetida* tissues without exceeding 80 mg kg$^{-1}$ and 8 mg kg$^{-1}$ DW, respectively. Numerous authors have suggested that *E. fetida* is able to regulate Zn by binding Zn in their chloragogenous tissue (Morgan, 1981; Morgan and Morris, 1982; Morgan and Winters, 1982; Cotter-Howells et al., 2005). Since the binding of Zn to metallothioneins is reversible, it was likely that Zn-thioneins may regulate the concentrations of metal in the body tissue by allowing rapid elimination of Zn (van Gestel et al., 1993; Marinussen et al., 1997). Indeed, Zn is an essential element and its internal level is regulated by earthworms; the efficiency of Zn accumulation probably relates to a necessity for a stored pool of available Zn in anticipation of future physiological demand (Nannoni et al., 2011). Our findings showed that Cu concentrations were relatively low. This essential metal may also be regulated by earthworms. In addition, in the present experiment, the metal concentration was measured in earthworm tissues following 48 h of gut depuration, which may have resulted in a loss of metals from the tissues of the earthworms (Spurgeon and Hopkin, 1999).

The accumulation of Cd and Pb by *E. fetida* increased with the increasing concentrations of metals in the soil. Cd was highly accumulated by *E. fetida*. This phenomenon could be explained by its high mobility in soil and its chemical analogy with Zn (Li and Thornton, 2001; Nannoni et al., 2011). Less Pb uptake and accumulation by *E. fetida* was observed which should be linked with lower availability of Pb.

Metal toxicity involves three steps: exposure, uptake, and reaction with the biological target. In *E. fetida*, the Pb and Cd accumulation increased in soil with greater soil metal concentrations. By contrast, Zn and Cu have a steady level of accumulation. It is likely that Cd and Pb did not use the same uptake pathways as Zn and Cu. Li et al. (2010) provided evidence that the uptake of Cd by *E. fetida* precedes through calcium channels, whereas Zn uptake is carrier-mediated by proteins or other sulfhydryl-containing compounds, implying that the mechanisms of Cd and Zn uptake in *E. fetida* are essentially different. However, interaction between metals should also be considered. Weltje et al. (1998) compiled data on sublethal toxicity and tissue concentrations of Cu, Zn, Pb and Cd mixtures in earthworms. Mixture toxicity shifted from mainly antagonism towards nearly concentration-addition when the endpoints were based on extractable metal concentrations instead of total soil concentrations.

### 4.4. Earthworm and plants interaction

This part of the study investigated the effect of *E. fetida* on the ability of *V. faba* and *Z. mays* to extract metals in contaminated soils. Plants accumulated elements to varying degrees depending on the soils and metals. *V. faba* accumulated higher concentrations of Cu and Zn than *Z. mays* which accumulated higher concentrations of Cd. These results suggested that *Z. mays* and *V. faba* can facilitate Cd and Cu extraction from contaminated soils, respectively. *V. faba* is not known as a metal tolerant plant. To the contrary, its sensitivity to metals justified its use in ecotoxicity tests (Cordova Rosa et al., 2003; Ünyayar et al., 2006) as it seems to be one of the most metal sensitive plant species (Rahoui et al., 2008). *Z. mays* plant appeared to be a good candidate for phytoextraction. Previous reports have classified *Z. mays* as a root accumulator (Li, N.Y. et al., 2009; Mench and Martin, 1991). The difference between the two plants could be explained by the differences in metal absorption mechanisms. Looking at plant accumulation of elements, Pignattelli et al. (2012) also found differences in the influence of the available and total concentrations between arsenic and other elements (e.g., Zn and Cd), which were attributed to the difference in the uptake mechanisms of metals. We can hypothesize that the metal uptake by plants is not only controlled by the element distribution in the soil but also by the exposure pathways. The concentration of Cu in *V. faba* and *Z. mays* follow the same trend whatever the metal concentrations in soil. These findings may be explained because Cu is an essential micronutrient for plant nutrition and deficiency effects could be mistaken for toxic responses. Concentrations between 5 and 20 $mg\,kg^{-1}$ DW plant are considered adequate for normal growth, whereas concentrations higher than 20 $mg\,kg^{-1}$ DW are considered toxic (Adriano, 2001). The analysis of Pb metal concentrations in plants did not reveal significant differences. The extent of assimilation of heavy metals from soil depends on whether they are present in a form that can be absorbed by plants. Two mechanisms can explain this result: (i) Pb could be strongly adsorbed by soil particles (Smical et al., 2008) and (ii) the low affinity of plants for this element, which has no role in their metabolism; while Cd element is relatively mobile in soil and can be more easily absorbed by *Z. mays.*

The metal uptake of the two types of plant grown in contaminated soil under presence of earthworms varied according to the plant species and the metal. This suggested that these plants have different capacities to absorb and eliminate toxic elements, but the detailed mechanism needs to be further investigated. As suggested by Wang and Li (2006), the higher uptake of heavy metals by plants under earthworm inoculation was probably due to the increase in dry matter production stimulated by earthworms. The presence of earthworm increased Cd concentration in *Z. mays*, except for the control, probably because the amount of available Cd was too low. An increase in metal concentration for plants in the presence of earthworms was also observed by Wen et al. (2004). Yu et al. (2005) found that earthworm activities increased Cd uptake and plant growth, thereby improving the phytoextraction efficiency of metal hyperaccumulators in low to medium level metal-contaminated soils. As earthworms are known to affect the distribution of microorganisms (Brown, 1995), we hypothesized that the effects of earthworms on metal uptake by plants resulted in part from their impact on soil microorganisms. Microorganisms associated with plant-roots are known to be major drivers of metal speciation in soils and could promote a better efficiency in phytoremediation (Abou-Shanab et al., 2003). Dandan et al. (2007) found that the earthworm bodies and earthworm casts are rich in amino acids and proteins, and soil-available carbon. These organic materials may form chelates with heavy metals, thus contributing to enhance the transport of heavy metals from soil to plant.

In our experiment, when plants were inoculated with earthworms, Cd and Pb accumulation in *E. fetida* decreased. The presence of *Z. mays* and *V. faba* can prevent and reduce the accumulation of Cd and Pb in earthworm tissues. Probably, plants can accumulate part of the Cd and Pb, which may create competition between the two organisms. Pb and Cd uptake by *E. fetida* was higher in the presence of *Z. mays* compared with *V. faba* for the treatment with the highest metal concentration ($C_3$). The effect of the two plants on earthworm metal uptake was different. Actually, by producing exudates, plants can modify metal speciation and their behaviour in soil, especially in the rhizosphere (Chaignon and Hinsinger, 2003; Uzu et al., 2009) and hence, as results showed, plants can modify metal accumulation in earthworms.

## 5. Conclusion

The present study showed that survival and growth of *E. fetida* is insensitive to Cu, Zn, Pb and Cd mixtures, and regardless of the presence of *V. faba* or *Z. mays*. However, negative effects of contaminated soils on cocoon production were observed. The introduction of plants did not affect significantly cocoon production or weight per cocoon. These results suggested that the adult earthworms used had a tolerance for the tested concentrations of metals.

This study showed that inoculating metal-contaminated soils with *E. fetida* decreased Cd and Zn chemical availability. The only contrary result was observed for concentrations of Cd and Zn in the presence of *V. faba*. Zn and Cu did accumulate in *E. fetida* tissues. The concentrations of Cd and Pb in the earthworms increased with increasing concentration in the soil. These findings suggest that earthworms are likely to employ different pathways for the uptake of different metals. Furthermore, the addition of *Z. mays* or *V. faba* reduced the accumulation of Cd and Pb in earthworm tissues. This is probably because plants can accumulate part of the available Cd and Pb concentrations, effectively creating an uptake competition between the different organisms. In plants, the accumulation of metals varied between the two species: *V. faba* plants accumulated higher Cu and Zn concentrations, whereas *Z. mays* accumulated higher Cu concentrations. After the addition of *E. fetida*, higher uptake of Cd by *Z. mays* was observed, indicating that *E. fetida* earthworm activity enhances Cd uptake for this plant species.

Our study showed that metal accumulation is a complex process that cannot be predicted by measuring the available fraction of metals alone. The final tissue concentrations after exposure to the metals depended on the physiological characteristics of the organisms (earthworms or plants), not only on its regulation pathways but also on its exposure routes. Nevertheless, while further research to establish the optimum species and combinations of them to use is needed, this study suggests that improving phytoextraction treatment of industrial sites polluted with a mixture of metals by use of earthworms and plants is possible.

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