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1 *In situ* assessment of phyto and zooavailability of
2 trace elements: a complementary approach to
3 chemical extraction procedures

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Abstract

For an accurate risk assessment of sites contaminated by trace elements (TE), measurements of bioavailability must be performed. This is routinely achieved using the standardized 0.01 M CaCl₂ method. However, the suitability of chemical extractions as proxies of bioavailability is questionable. We analyzed the correlations between chemically estimated TE bioavailability and TE actually accumulated by coupling plant and snails bioindicators. Results showed a better correlation between plant TE contents and CaCl₂ fraction while total soil concentration better explained snail TE contents. However in both cases chemical measures were not suitable to predict TE accumulation and bioavailability. Considering the soil properties only improve the estimation of Cr, Ni and Pb accumulation by plants while for snails, TE contents in viscera were dependent both on soil and plant contents and soil properties. It highlights the complementarities of biomonitoring methods to assess bioavailability. This dual approach allows a “physiologically defined” evaluation of bioavailability.

1. Introduction

Since a few decades, soil degradation (*e.g.*, erosion, loss of organic matter, sealing, pollution, ...) is an increasing problem worldwide. Contamination by trace elements (TE) is considered as one of the main threats (Jeffery *et al.*, 2010) because of human health issues (Qingdong *et al.*, 2007 and Science Communication Unit, 2013), and also as they may cause severe ecological disturbances to both organisms and their habitats (Moriarty, 1999). For these reasons, various protective thresholds for total contents in soils have been proposed for different TE (Carlon, 2007). However it is largely acknowledge today that the toxicity of TE rather depends on their bioavailability than on their total contents in soils (Van Gestel *et al.*, 2009). Consequently, protective thresholds based on total TE contents in soils are only coarse indications of the potential hazard, and should be completed by methods allowing to assess TE's bioavailability (ISO, 17402, 2008).

However, although the term “bioavailability” can be easily understood as how much of a coowever, although the term “bioavailability” can be easily understood as how much of a contaminant is available for living organisms, the underlying concept is much more complex and a great number of definitions, and assessment methods, have been proposed so far (Harmsen, 2007, Naidu *et al.*, 2008 and Semple *et al.*, 2004). In an effort to offer a clear working definition of this concept, Semple *et al.* (2004) proposed to distinguish the part of the contaminant which is “bioaccessible” (*i.e.*, “which is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical”), from that which is actually “bioavailable” (*i.e.*, “which is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time”). However this distinction has not been retained in the definition adopted in ISO 17402 (ISO, 17402, 2008) which simply states that “bioavailability is the degree to which chemicals present in the soil may be absorbed or metabolized by human or ecological receptors or are available for interaction with biological systems”. Whatever the terms and definitions considered, there is however a consensus today to regard bioavailability as a multi-level concept involving three distinct notions: the environmental availability, the environmental bioavailability and the toxicological bioavailability (Gimbert *et al.*, 2006, ISO 17402, 2008, Lanno *et al.*, 2004 and Peijnenburg *et al.*, 1997).

The environmental availability depends on multiple physico-chemical processes governing metal partition between the solid and liquid phases of the soil. Environmental availability is generally assessed as the so-called “available” or “easily exchangeable” TE fractions, using more or less weak chemical extractants, such as neutral salt solutions at low concentration or diluted weak acids. Because these chemical extraction methods are easy to use, reproducible and based on an easily understandable concept (the more the TE are easy to extract, the more they can interfere with living organisms), they are routinely used for risk assessment of contaminated sites. However numerous studies (Meers *et al.*, 2007, Pauget *et al.*, 2012, Peakall and Burger, 2003 and Van Gestel, 2008) have shown that the level of “available” elements, as determined by chemical extraction methods, is often a poor proxy of the fraction of TE that actually interacts with living organisms. By contrast, the environmental

bioavailability refers to the fraction of contaminant that is actually taken up by biological receptors. It depends on complex species-specific physiological processes, controlling desorption of contaminants from the solid matrix and their assimilation (absorption and excretion) by the organism. To assess the environmental bioavailability of TE in soils, the use of accumulation bioindicators is obviously highly relevant (Peakall and Burger, 2003). Indeed, during the last two decades, plants (Le Guédard *et al.*, 2012, Remon *et al.*, 2013 and Vergé *et al.*, 2002) or soil organisms, such as snails or earthworms (Dallinger and Berger, 1992, Gimbert *et al.*, 2008a, Pauget *et al.*, 2013, Rabitsch, 1996 and Scheifler *et al.*, 2003), have been proposed as accumulation bioindicators. However, the transfer of TE and their accumulation by living organisms and in food webs depends both on the species, its trophic level and exposure pathways. It is thus questionable to use a single species or to consider a single trophic level for assessing bioavailability, as this may lead to misinterpretations.

In this work we hypothesized that considering different organisms, representative of different trophic levels (primary producers and primary consumers), could be a relevant approach to get information on the environmental bioavailability of TE in soils. Transfer to primary producers was estimated by analyzing TE content in composite plant samples, as recently proposed by Remon *et al.* (2013). This passive biomonitoring approach informs on the phytoavailability of TE for a plant community, considered as a whole. Transfer to primary consumers was assessed by active biomonitoring with garden snails, informing on the zooavailability of TE (Fritsch *et al.*, 2011 and Gimbert *et al.*, 2008a). We also assumed that the transfer of TE from soil to organisms at various spatial scales, could be partly conditioned by their environmental availability or/and by the soil properties. To address these issues, we performed an extensive study on 25 experimental plots (7 geographical sites) exemplifying different land use and contamination levels. We analyzed (i) the correlations between environmental availability and environmental bioavailability, as determined by chemical and biological methods, (ii) the influence of soil properties on TE accumulation by snails and plants and (iii) the relationship between TE concentrations in plants and TE accumulation by snails.

2. Materials and Methods

2.1. Selected sites and studied soils

Seven sites (hereafter named Andra, Auzon, GISFI, Metaleurop, RENECOFOR, SHSE and Yvetot, S1) were selected throughout France (Fig. 1), among those studied in the French national research program “Bioindicators 2”; <http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur>. Each site was subdivided into two to six 100 m² (10 m x 10 m) experimental plots, according to the local typology and/or land use. The Auzon (6 plots), Metaleurop (4 plots), SHSE (3 plots) and GISFI (2 plots) sites were industrial landfills or peri-industrial woodlands and grasslands more or less impacted by industrial activities; the RENECOFOR site (4 plots) belonged to a network of French forests (<http://www.onf.fr/renecofor>); the Yvetot site (4 plots) was a cultivated grassland (Plassart *et al.*, 2008) and the Andra site presented two plots, one in a forest and one in a grassland. All these sites and experimental plots have been previously described and located in Pérès *et al.* (2011) and in Pauget *et al.* (2013).

Samples of each plot were taken on a grid (10 m × 10 m) subdivided into 4 sampling-zones (5 m × 5 m). In each 25 m² sampling zone, 12 randomized soil samples were taken (0–15 cm depth after removal of the humus) and pooled to characterize soil physico-chemical parameters.

Soils from the 25 plots studied were analyzed for their pedological characteristics, as well as for their total and “available” TE contents. All analyses were performed by the Laboratory for Soil Analyses of the National Institute for Agronomic Research (INRA Arras, France), which benefits from the COFRAC (French Accreditation Committee) accreditation n°1-1380 (available at www.cofrac.fr) for its analytical insurance in soil metal(loid) measurements. Briefly, total metals (Cd, Cr, Cu, Ni, Pb and Zn) in soil samples (routinely 250 mg dry soil, ground at < 250 µm) were extracted using hydrofluoric (HF) and perchloric (HClO₄) acids, according to the NF X 31-147 procedure (AFNOR, 1996). For the determination of total As, soil samples were extracted with a mixture of sulfuric acid (H₂SO₄)/nitric acid (HNO₃) (2/1, V/V) in the presence of vanadium pentoxide (V₂O₅) at 100 °C for 3

h. For the determination of “available” metal(oids), extractions were performed with 0.01 M calcium chloride (CaCl₂), according to the NEN 5704 procedure (NEN 5704, 1996). Metal concentrations in the various extracts were measured using inductively coupled plasma atomic emission spectrometry (ICP-OES). Selected pedological characteristics of the 25 plots studied and their total and CaCl₂ extractable TE contents are given in Supplementary Material S2 and S3.

2.2. Biological material and sampling protocol

2.2.1. Plants

To get a general insight into TE transfer toward plants, and to avoid species-specific responses, metal analyses were performed at a plant community level, *i.e.*, using composite plant samples (Remon *et al.*, 2013). The basic assumption of this approach is that TE concentrations in leaves of an ensemble of species colonizing a site, is a relevant indicator of TE phytoavailability in its whole.

Composite plant samples were prepared by collecting green leaves (during the months of June to August) from the most abundant species identified at each site, *i.e.*, those species which were representative of the plant community in place. Ten to 15 g fresh weight (FW) of leaves was taken from four to five different species collected in the same area (approx. 5 m²). This elementary sampling was repeated five times in each plot (at each of the corners and in the center), with different species for each replica, when possible.

For each elementary sampling, specimens of each species were washed thoroughly with tap water and rinsed with distilled water. They were then dried at 40 °C to constant weight and individually ground up to pass through a 2-mm sieve. A composite sample (“pool”) was then prepared by mixing the same quantity (routinely 100 mg DW) of each species constituting the pool. Five pools of plants were prepared in this way, for each plot studied.

2.2.2. Snails

Garden snails (*Cantareus aspersus*), were obtained from our laboratory breeding. They were fed with commercial snail meal (Helixal®, Antigny Nutrition S.A., France) and reared under controlled

conditions until the age of 7–9 weeks, as described by Gomot-de Vaufleury (Gomot-de Vaufleury, 2000). To avoid strong modification of mass during the exposure time that could lead to misinterpretation of internal concentration variations (Gimbert *et al.*, 2008b), only sub-adults weighing 5.0 ± 0.6 g ($n = 1230$) were used. At the beginning of the exposure, TE concentrations in the viscera were 0.33 ± 0.11 kg⁻¹ for As DW, 0.73 ± 0.10 mg kg⁻¹ Cd DW, 2.19 ± 0.48 mg kg⁻¹ Cr DW, 139 ± 40.1 mg kg⁻¹ Cu DW, 0.59 ± 0.26 mg kg⁻¹ Pb DW, and 881 ± 182 mg kg⁻¹ Zn DW (mean \pm SD, $n = 10$).

For in situ exposure, snails were caged in microcosms (25×25 cm stainless steel cylinders), during a 28-days period (during the months of June to August), as described by Fritsch *et al.* (2011). They were exposed to the soil and vegetation of each plot, under natural climatic conditions, from June to August, in 2009 (for Metaleurop, GISFI, F08 and F57), 2010 (for Auzon, SHSE, Yvetot, F63 and F76) and 2011 (for ANDRA). Fifteen snails were placed in each microcosm. On forest and grassland sites, one microcosm per plot was used and 6 individuals were sampled for TE analysis after 28 days. On sites contaminated by industrial activities, 3 microcosms per plot were used to account for the heterogeneity in soil contamination, and two snails per microcosm (= 6 snails) were sampled for TE analysis after 28 days.

2.3. Analyses of trace elements

2.3.1. Plants

Ground composite samples (“pools”) were dried overnight at 40 °C, before processing. Samples were then weighted (500 mg DW) into clean, dry PTFE (Teflon®) screw cap digestion tubes, and 4 ml concentrated nitric acid (65% HNO₃, analytical grade) were added. Open tubes were heated up to 50 °C for 1 h, and kept overnight at room temperature. Three ml hydrogen peroxide (36.5% H₂O₂, analytical grade) were then added and tubes were stand for 30 min at room temperature; they were then heated to 70 °C and allowed to evaporate until about 1 ml. A final aquae-regia digestion was then performed, by adding 2 ml concentrated HNO₃ and 4 ml concentrated hydrogen chloride (37% HCl,

analytical grade); the tubes were hermetically closed and heated up to 150 °C for 2 h. Samples were then cooled, tubes were opened, and kept at 70 °C until complete evaporation. Dry samples were finally solubilized in 10 ml HCl (2 M) by heating for 1 h at 100 °C in closed tubes. Solutions were cooled to room temperature and metal concentrations in the extracts were determined by ICP-OES (Jobin-Yvon, Activa).

The detection limits for metal analysis by ICP-OES were 0.11, 0.060, 0.013, 0.080, 0.064, 0.341 and 0.028 mg/kg DW, for As, Cd, Cr, Cu, Ni, Pb and Zn respectively. The analytical precision was checked by measuring in triplicate about 20% of the samples. The relative standard deviation routinely was between 1 and 8%, and never higher than 10%. For the quality assurance of plant analysis, the certified reference CTA-OTL-1 (Oriental Tobacco Leaves from the Bulgarian Institute for Plant Protection) was employed. Average recoveries (n = 6) were 91, 87, 66, 98, 85, 58, and 79% for As, Cd, Cr, Cu, Ni, Pb and Zn, respectively.

2.3.2. Snails

The snails sampled were fasted for 48 h (the feces were removed after 24 h) and then weighed and sacrificed by freezing at – 80 °C. After thawing, the whole soft body was removed from the shell and the foot was separated from the viscera. The viscera were studied because they are the main site of metal accumulation in snails (Hopkin, 1989). The viscera were oven-dried at 60 °C until they reached a constant weight (~ 0.2/0.3 g DW), digested in HNO₃ (65% HNO₃, Carlo-Erba analytical quality) as previously described (Pauget *et al.*, 2011) and analyzed by ICP-MS. The validity of the analytical methods was checked by analyzing standard biological reference material (TORT-2, lobster hepatopancreas; National Research Council of Canada-Institute for National Measurement Standard, Ottawa, ON, Canada). The quantification limits for metal analysis by ICP-MS were 0.081, 0.003, 0.104, 0.391, 0.065, 0.009 and 0.853 µg/L, for As, Cd, Cr, Cu, Ni, Pb and Zn respectively.

2.4. Statistical analyses

In a first approach, simple linear regressions were performed to assess the ability of soil chemical extraction methods (*i.e.*, total concentrations and CaCl₂ extraction for “available” concentrations) to predict TE accumulation by plants and snails.

Then multiple linear regression (MLR) models were established to better estimate the influence of the soil's characteristics on TE accumulation in plant and in snails (Eq. (1)):

$$Y = x*A + y*B + , \dots z \quad (1)$$

The dependent variables (Y) were the medians of concentrations (C) of each metal(loid) in the organisms studied, after log (C + 1) transformation. The explanatory variables (A, B, ...) were the main soil's related-parameters, *i.e.*, pH, cation exchange capacity (CEC), organic carbon (C_{org}) content, clay content, sum of exchangeable cations (SEC : Al, Ca, Fe, Mg, Mn, Na.), sands, silts, and total metal concentrations (some parameters are compositional in nature, but some were not (*e.g.*, pH, CEC); thus MLR were used because they allow the specific influence of each soil parameter on the biological response to be taken into account). All values, except pH, were log (a + 1) transformed before data processing and x, y, ... represent the coefficients of the explanatory variables.

Lastly, another set of MLR analyses was performed, by only considering the internal metal contents in snails as the dependent variables, and by adding the metal contents in plants as supplementary independent variables. The goal of this last approach was to integrate the trophic level of snails (herbivorous) to better assess metal accumulation in this species.

For each set of regressions, the best model (*i.e.*, the one providing the best adjusted coefficient of determination, with the lowest number of independent variables) was chosen using corrected Akaike criterion –AICc– (Burnham and Anderson, 2004). All calculations were performed using the R program (version 2.15.2) (R Development Core Team, 2011).

3. Results

3.1. Accumulation of TE in plants and snails

The main distribution parameters of TE contents in plants and snails sampled on the 25 plots studied are given in Table 1. The median concentrations in Cd, Cu, Pb and Zn were 7- to 21-fold higher in snail viscera than in plant leaves. For Cr and Ni the median concentrations in snails and plants were about the same, while for As the measured concentrations were about 2-fold higher in plants than in snails. However there was a great variability in TE contents, both in plants and snails, as shown by the wide ranges of the data, and the median absolute deviation (MAD) values which were often close to the medians. This was obviously due to the large diversity of the studied sites, in terms of pedological characteristics and contamination levels (see S2 and S3). Consequently, we further examined the data in more details by separating values from the different sites and plots studied.

TE contents in plants and snails for each individual plot are given in Fig. 2. Comparison of accumulation profiles allowed to easily distinguishing some plots, where both plant and snail TE contents were distinctly above those measured at the other plots. For instance, at the four plots from the Metaleurop site (RW, LW, IW and IW), the Cd content in plants and snails was clearly higher. On the same site, the Pb content in both types of organisms was also abnormally high for plots LW, IW and HW. Likewise, the three plots (LCV, ICV and HCV) from the industrial SHSE site were associated with higher Cr contents in plants and snails. Lastly at the LCV (SHSE site) and CoWH (Auzon site) plots, the Ni contents were particularly high, both in plants and snails. It must be noticed that the above mentioned sites (*i.e.*, Metaleurop, Auzon and SHSE) were all considered as highly contaminated (see S2); thus high levels of Cd, Pb, Cr and/or Ni in plants and snails reflected the soil contamination. Nevertheless, soil contamination was not systematically associated with high accumulation of TE in living organisms. Indeed at the plots GHM and GHF (GISFI site) both plants and snails showed low TE contents, despite a quite high soil contamination. Taken together these results highlighted that measuring TE contents in plants and snails, could clearly discriminate the plots studied, with respect to phyto- and zoo-availability.

However in several cases there were some discrepancies between TE contents in plants and snails. For example, the As content in snails exposed on the CoWW plot (Auzon) was very high, but no anomaly was observed in plant As content on the same plot. Likewise at the two Pa and Fo plots (ANDRA) the Cr content in snails was particularly high, while concentrations measured in plants were quite low. At the opposite plant contents in Cd and Pb were very high at the CoWH (Auzon) and F57 (RENECOFOR) plots respectively, while no anomaly in snail contents were evidenced. Thus, on these plots, there was no clear cut relationship between metal contents in plants and snails. This suggested that the levels of metals accumulated in both type of organisms probably depend on complex environmental factors. Consequently, to go a step further in the understanding of metal accumulation in plants and snails, we performed regression analyses taking into account the soil's parameters.

3.2. Relationships between TE contents in soil and TE accumulation in plants and snails.

A synthetic representation of TE contents in soil (total fractions extracted with HF + HClO₄ and “available” fractions extracted with CaCl₂), in plants and in snails on the 25 plots studied is given in Fig. 3. This representation illustrated a complex pattern where no obvious relationship between TE in soil and in living organisms was distinguished. Consequently in order to better explain the observed TE levels in plants and snails, we first performed simple linear regression analyses and correlation studies using total and “available” TE contents in soil as explanatory variables, respectively. Results are shown in Table 2 and Table 3.

When total TE contents in soil were considered as the explanatory variables (Table 2) for accumulation in plants, significant correlations were only evidenced for As, Cr, Ni and Pb. However, the coefficients of correlation were relatively low. The highest value was observed for Cr ($r^2_{\text{adj}} = 0.49$) while for As, Ni and Pb the r^2_{adj} values were comprised between 0.15 and 0.35. For Cd, Cu and Zn no significant correlations between total TE in soil and plant contents were found. In contrast for snails, internal TE concentrations were significantly correlated with total contents in soil for all elements, excepted for Cu (Table 2). The highest coefficient of correlation was observed for As ($r^2_{\text{adj}} = 0.63$), the r^2 values for the other elements ranging between 0.14 and 0.45.

When “available” TE contents in soil were considered as the explanatory variables for accumulation in plants and snails (Table 3), quite different results were obtained. For plants, significant, albeit relatively low ($0.23 < r^2_{\text{adj}} < 0.54$), correlations were observed for As, Cd, Ni and Pb, while no significant correlation was evidenced for Cu and Zn. For snails, significant correlations between “available” TE in soil and concentrations in viscera were only found for As and Cd ($r^2_{\text{adj}} = 0.60$ and 0.20 , respectively); for the other elements studied (Cr, Cu, Ni, Pb and Zn) no significant correlations were observed.

3.3. Influence of soil properties on TE accumulation by plants and snails

Because simple regression models solely based on TE contents in soil poorly predicted the observed TE contents in plants and snails, we further performed MLR by adding the main soil characteristics as supplementary explanatory variables. Results are given in Table 4.

For plants, the addition of the soil parameters in the regression models greatly improved the estimation of Cr and Ni accumulation. The main variables influencing the accumulation of these metals were the SEC for Cr, the pH for Pb and pH coupled with silt content for Ni. On the other hand for As multiple regression model did not improve the estimation of accumulation in plants, as compared with simple models (using either total or “available” contents in soil). Lastly, for Cd, Cu and Zn no statistically significant regression was found, suggesting that accumulation of these metals in plants was not dependent on the soil parameters for the set of plot studied.

For snails, adding the soil characteristics in the regression models improved the assessment of metal accumulation in viscera for Cd, Cr, Ni and Zn. This increased the adjusted coefficients of correlation of approx. 10%, 22%, 12% and 10% for Cd, Cr, Ni, Zn respectively. Beside total element contents in soils, the main parameters that modulated metal accumulation were the SEC (for Ni and Zn), the organic carbon content (for Cd) and the pH and CaCO_3 content (for Cr). On the other hand, for As and Pb no significant influence of the soil parameters was observed. Lastly for Cu, no significant model was found.

3.4. Influence of plants on TE accumulation by snails

In order to assess the impact of the different potential sources of contamination (*i.e.*, plants and soils) on snails' TE contents, we performed a last set of simple and multiple linear regressions. Firstly, TE contents in snails were assessed with simple regression models, using plants contents as the sole source of contamination. Secondly, we performed multiple regressions using TE concentrations in plants and in soils, as explanatory variables. Lastly, TE contents in snails were estimated taking into accounts both contamination sources and soil parameters. Results are shown in Table 5.

Simple regression analyses using plant TE contents as explanatory variables showed significant correlations with TE in snails' viscera for all the elements but Cu, with adjusted correlation coefficients ranging from 0.21 for Cr to 0.56 for Pb. Considering both sources of contamination (TE contents in soil and in plants) did not improve the simple regression models, except for Cd where both soil and plant TE content were significantly correlated with internal contents in snails. For Ni, Pb and Zn, the plant contents were the best predictors of snail's contents, while for As it was the soil total content that gave the best r^2_{adj} . For Cr, considering either the soil or the plants as explanatory variable gave comparable r^2_{adj} .

Lastly using more complex multiple regression models, taking into account the two sources of contamination plus the soil parameters (Table 5), only improved the assessment of Cd, Cr and Pb accumulation. For Cd, both soil content, plant content and soil organic carbon significantly influenced the concentration in snail's viscera. For Cr, the observed increased in the r^2_{adj} was only due to the soil's parameters (pH and CaCO_3). For Pb, the concentration in snail's viscera was significantly influenced by plant content and soil parameters (*i.e.*, pH) but not by total content in soil. For the other TE (As, Cu, Ni and Zn), complex multiple regression models did not improve the r^2_{adj} values, as compared with those using only soil or plant TE contents as explanatory variables.

4. Discussion

Although separating bioavailability into three operational insights (*i.e.*, environmental availability, environmental bioavailability and toxicological availability) allows to clarify the concept and to propose various measurement methods, the underlying physical, chemical and biological factors that ultimately govern the transfer of TE from a contaminated habitat to living organisms are still poorly understood. In particular the question remains to know the relationships between environmental availability and environmental bioavailability and, ultimately, how these descriptors should be handled in a view of environmental risk assessment. In this work, we studied the correlations between TE extracted by standardized chemical methods and those actually accumulated in two model organisms (plants and snails). We also investigated the influence of soil properties and contamination source on TE bioaccumulation.

4.1. Standardized chemical methods for TE extraction from soils poorly predict TE accumulation in plants and snails

Classically, two different points of view can be considered for TE analysis in soil, with the aim of risk assessment for ecological receptors. The default assumption is that the totality of TE present in the soil could pose a risk at a short-, medium- or long-term scale, *i.e.*, that availability could be 100%. A less conservative assumption is that only part of total TE is actually bioavailable and thus poses a risk to living organisms. From these points of view, a number of total and partial extraction schemes have been designed for soil analysis in risk assessment procedures. Among the most commonly used methods, are the standardized HF-HClO₄ mineralization for total TE (AFNOR, 1996), and the 0.01 M CaCl₂ extraction, for “available” TE (NEN 5704, 1996). These are the methods we retained in this work.

Our correlation studies showed that the total TE contents in soils (HF-HClO₄ extraction) were poorly correlated to the concentrations of TE measured in plants. Indeed on average, only 18% of the variability in plant TE contents was explained by the total contents in soils. There were however noticeable differences between elements, with correlation coefficients ranging from 0 for Cd, Cu and

Zn, to > 0.3 for Cr and Pb. To consider “available” TE contents in soils (CaCl_2 extraction) was clearly a better proxy for Cd ($r^2_{\text{adj}} = 0.3$) and, in a lesser extent, for Ni and Pb. This is in accordance with the basic assumption that plants take up TE from the soil solution (Degryse *et al.*, 2009 and Smethurst, 2000), and that CaCl_2 extractions better reflect this soil compartment than do total extractions (Kabata-Pendias, 2004, Meers *et al.*, 2007 and Römken *et al.*, 2009). However CaCl_2 extractions still did not detect any correlation between soil and plant contents for Cu and Zn, while for As, plant contents remained better explained by total than by “available” TE in soils.

For snails, about 29% of the variability of TE contents in viscera was explained by total concentrations in soil; at the opposite, “available” TE explained only 11% of the variability of viscera's contents. The greater influence of soil total TE content, as compared to “available” concentrations, on snail's internal TE levels could be partly due to its foraging mode which multiplies the sources of exposure. Indeed, in addition to their plant-based diet, snails also ingest significant amounts of soil particles to satisfy their physiological needs (Gomot *et al.*, 1989). This could allow snails to pick up quite strongly bounded TE on soil constituents (Pauget *et al.*, 2012). Thus, as previously shown for earthworms (van Gestel, 2008), total soil concentrations are a better indicator of the amounts of TE that are available for snails, than the CaCl_2 extractable pools. However, as observed for plants, there were clear cut differences between elements, and Cu, Ni and Zn concentrations in viscera were not, or slightly ($r^2 < 0.15$), correlated with soil contents.

It is interesting to note that, whatever the extraction method used and the organism considered, there was no, or very low, correlations between the content in soils and the content in living organisms, for those elements that are essential for nutrition (*i.e.*, Cu, Ni and Zn). This is likely due to the complex biological processes regulating homeostasis of essential elements, allowing living organisms to maintain almost stable internal levels under a certain range of external concentrations (Bargagli, 1998 and Menta and Parisi, 2001). Thus, it is only when homeostasis can no be longer controlled that the internal levels of essential elements will rise significantly. It is obvious that chemical extraction methods cannot take into account such biological response, making these methods poor proxies for

assessing environmental bioavailability of essential elements. At the opposite, for non essential TE (As, Cd, Cr, Pb) whose concentrations in living organisms are not under strict homeostatic control, correlations between contents in soil and in organisms were better, but were clearly dependent on the fraction of TE (total vs “available” fraction) and the organism considered (plants vs snails).

Thus, as already emphasized by a number of authors for different types of soil, contamination levels and living organisms (Lopes *et al.*, 2012, McLaughlin *et al.*, 2000, Mourier *et al.*, 2011, Murphy *et al.*, 2000, Remon *et al.*, 2005 and Van Gestel, 2008), these results confirm that neither total soil concentrations nor “available” concentrations give enough information to foresee the actual environmental bioavailability of TE on contaminated sites.

4.2. Factors modulating TE accumulation are different for plant and snails

It is well known that the speciation of TE in soils depends on the soil's characteristics such as pH, redox potential, CEC or organic matter content and quality (Park *et al.*, 2011, Unamuno *et al.*, 2009 and Zeng *et al.*, 2011). Thus, we hypothesized that beside total or “available” TE contents in soils, as determined by chemical extraction methods, the accumulation of TE in plants and snails could be partly correlated to some soil parameters. Moreover, because snails are mostly herbivorous, we postulated that TE accumulation in viscera could be closely linked to plant contamination.

When plants were considered, MLR models indeed improved the assessment of Cr and Ni accumulation, and highlighted that soil factors such as pH, carbonates or total content of exchangeable cations may play a significant role in the phytoavailability of TE. However for the other TE studied (As, Cd, Cu, Pb and Zn) the MLR did not give better correlation coefficients than simpler models using only “available” soil contents as explanatory variable. At the opposite for snails, considering the soil parameters and/or plant contents in the regression models improved the assessment of all TE, but As and Cu. As a rule, TE contents in snail viscera were mostly correlated to plant contents, except for Cu. This is in agreement with the results of Scheifler *et al.* (2006) who showed that up to 90% of Cd accumulated by snails came from plants. Internal TE contents in snails were also correlated to total contents in soil (As, Cd, Cr) and/or to organic carbon (Cd), pH (Cr, Pb) and carbonates (Cr).

The aim of this work was not to elucidate the physicochemical drivers of TE accumulation in plants and snails, but rather to compare the main parameters explaining variations in TE contents in both organisms. Our results showed that TE contents in plants and snails were depending on very different parameters. These differences are obviously due to the different exposition pathways, and finally to the specific physiology of each type of organism. In fact, because of the development of their root system, plants can accumulate TE from deeper soil layers than do snails, which are only exposed to the topsoil (Berger and Dallinger, 1993). Moreover, the activity of plant roots and associated microorganisms may trigger localized soil modifications, leading to a significant increase or decrease of pH, redox potential and/or organic matter content (Ehrenfeld, 2013). Thus, these so-called rhizosphere processes, can considerably modify the speciation of TE (Kabata-Pendias, 2004) and their availability for plants; at the opposite, snails do not significantly modify soil properties and have very likely no effect on TE speciation (Coeurdassier *et al.*, 2007). Also, the fate of TE in plants and snails is very different, in that snails are able to excrete some accumulated TE (Gimbert *et al.*, 2008b), while in plants they are stored in the roots or above ground tissues but are rarely excreted (Weis and Weis, 2004). Lastly a major difference between these biological receptors lies in their source of contamination. Indeed plants are mainly exposed to TE via the soil, whereas snails are exposed to both soil and contaminated plants. Our results indeed confirmed that both sources of contamination had a significant influence on snail's TE content.

4.3. Implications in risk assessment

It is widely admitted today that assessing and managing polluted soils on the sole basis of their total contaminant concentrations (IEM, 2007, ISO 17402, 2008 and US EPA, 2007) may lead to inaccurate conclusions and decisions (Brand *et al.*, 2013, Mourier *et al.*, 2011 and Van Gestel, 2008), resulting in misestimating the actual risk for biological receptors. Thus considering the bioavailability of contaminants could be a more relevant approach, as this integrates all aspects modulating their transfer to living organisms (Fig. 4) (van Gestel, 2008). Given this, it is now argued that integrating the concept of bioavailability in risk assessment methodologies is a necessity (ISO, 17402, 2008). In this

objective the use of CaCl_2 extraction is usually presented as a good chemical proxy for bioavailability. However in this study we showed that such a chemical extraction poorly estimated TE accumulation in plants and snails in large scale in situ experiments, *i.e.*, considering numerous elements, contamination levels and soil types. This confirms the conclusions of several authors (Feng *et al.*, 2005 and Van Gestel, 2008) who emphasized the limitation of the CaCl_2 method for the prediction of TE bioavailability in some field situations. To fill this gap a number of alternative weak extraction procedures have been proposed so far, each focusing on particular elements, soil types or biological receptors (Mourier *et al.*, 2011 and Smith *et al.*, 2010). This underlines the difficulty to find a universal chemical extractant that could predict TE bioavailability. In fact, whatever the extractant used, one of the major limitations of the “chemical extraction” approach to assess bioavailability, is that it intrinsically considers the soil as the sole source of exposure. This may be a correct assumption for plants, but this is obviously not true for numerous other organisms whose nutrition does not exclusively depend on soil. For instance in snails, we showed that considering various exposition pathways according to the trophic level of the target organism, strongly improved the prediction of accumulation of several TE in viscera. This result highlights the importance of taking into account all contamination sources, and not only the total or “available” TE contents in soil, when assessing the bioavailability of contaminants on a given site. Consequently, although chemical extraction methods are routinely used with the underlying idea that they can give a general insight into metal bioavailability, they actually only inform about the easiness of metal leaching from soil by chemical weak extractions, which is a measure of the environmental availability and not of the actual bioavailability. So, owing that the accumulation of TE in living organisms depends on both the metal speciation in soil and the physiological traits of the target organisms, the study of bioindicators could be a relevant complement to chemical procedures, to get insights into TE bioavailability on contaminated sites (Reeves and Chaney, 2008).

In this study we considered two types of bioindicators (plants and snails), representative of two trophic levels. Indeed we assumed that coupling biological measures, by permitting the differences

between organisms to be taken into account, should allow a more accurate assessment of TE bioavailability and a better site characterization. For bioindication using plants, the “composite sample” strategy (Remon *et al.*, 2013) makes easy the prospection of large areas and allows to overcome interspecific variations. Consequently, such a passive bioindication approach informs on the global phytoavailability of TE and their potential transfer toward primary consumers. On the other hand, bioindication using snails gives information about TE transfer to a specific link of the food chain integrating multiple contamination sources (*i.e.*, soil and plants) and being a prey for a number of carnivorous species (Barker, 2004 and Scheifler *et al.*, 2006). The snail-watch approach permits accurate between-sites comparisons of TE bioavailability (Beeby and Richmond, 2003) as it uses laboratory-born and calibrated animals. This active bioindication strategy informs on a species-specific zooavailability of TE, allowing their potential transfer toward higher levels of the trophic webs to be partly anticipated.

However, even though both bioindication strategies have many advantages, they also pose some questions or present some limits (Varrault and Bermond, 2011). For instance, because of the potential adaptation of plants to contaminated soils (Schipper *et al.*, 2011), TE phytoavailability on a given site may change if plant communities are modified. Likewise, because snails only inform on the bioavailability of TE inside the small area of the microcosms, the study of large and heterogeneous sites may be relatively time consuming. Lastly it must be kept in mind that bioavailability of TE cannot be quantified by a single value, as it closely depends on the organism of interest. Nevertheless, accumulation bioindicators provide specific information about the extent of actual TE transfer toward living organisms; consequently they allow a “physiologically defined” approach (Peijnenburg *et al.*, 2007) for measuring bioavailability. Thus, together with the classical chemical methods for assessing environmental availability, actual uptake studies could be used as a guideline to assess the environmental bioavailability of TE. These dual chemical and biological approaches could constitute a more accurate way for raising (or decreasing) the alarm on soil quality for environmental risk assessment.

475 ASSOCIATED CONTENT

476 **Supporting Information:**

477 S 1: Main pedological characteristics of the 25 plots from the 7 sites studied (median values, n=5)

478 S 2: Total (HF + HClO₄ extraction) and “available” (CaCl₂ extraction) TE contents at the 25 plots
479 from the 7 sites studied (median values, n=5). NA: no available data. *Italic values correspond to value*
480 *below the detection limit*

481 S 3: Principal plant species on the studied plots.

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489 ABBREVIATIONS

490 CEC, cation exchange capacity; MAD, median absolute deviation; MLR, multiple linear regression;
491 OM, organic matter; TE, trace element; SEC, sum of exchangeable cation

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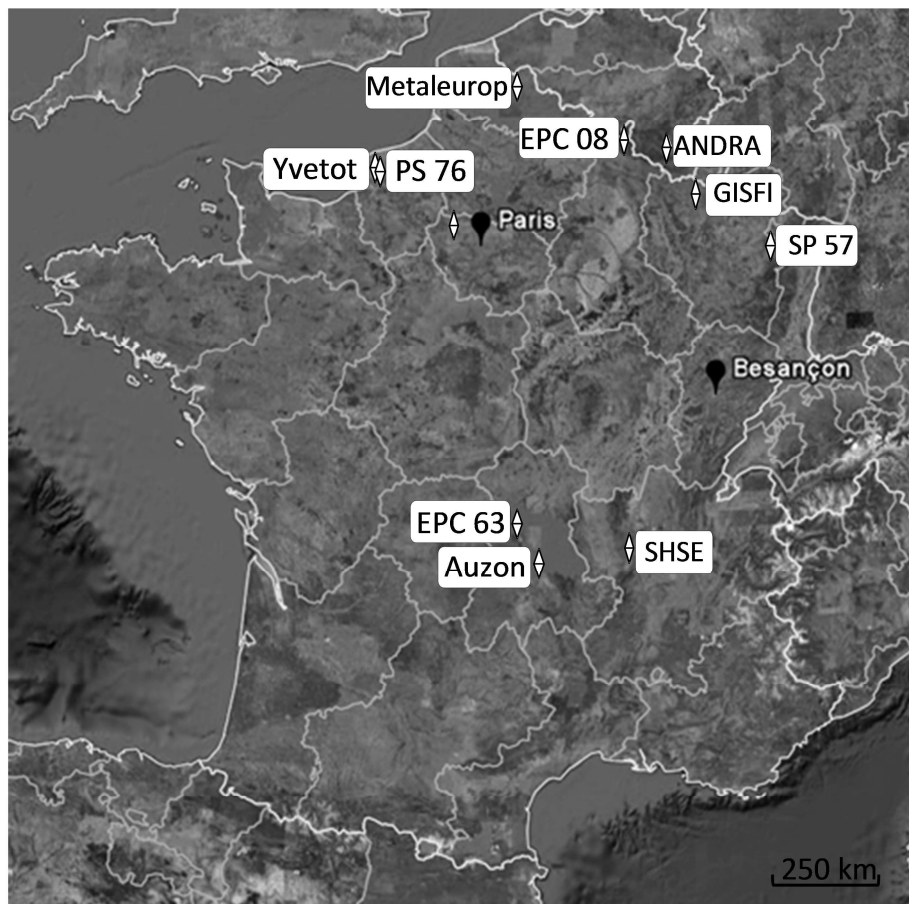
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670

672 FIGURES
673



674

675 Figure 1: Sites localization. <http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/>
676

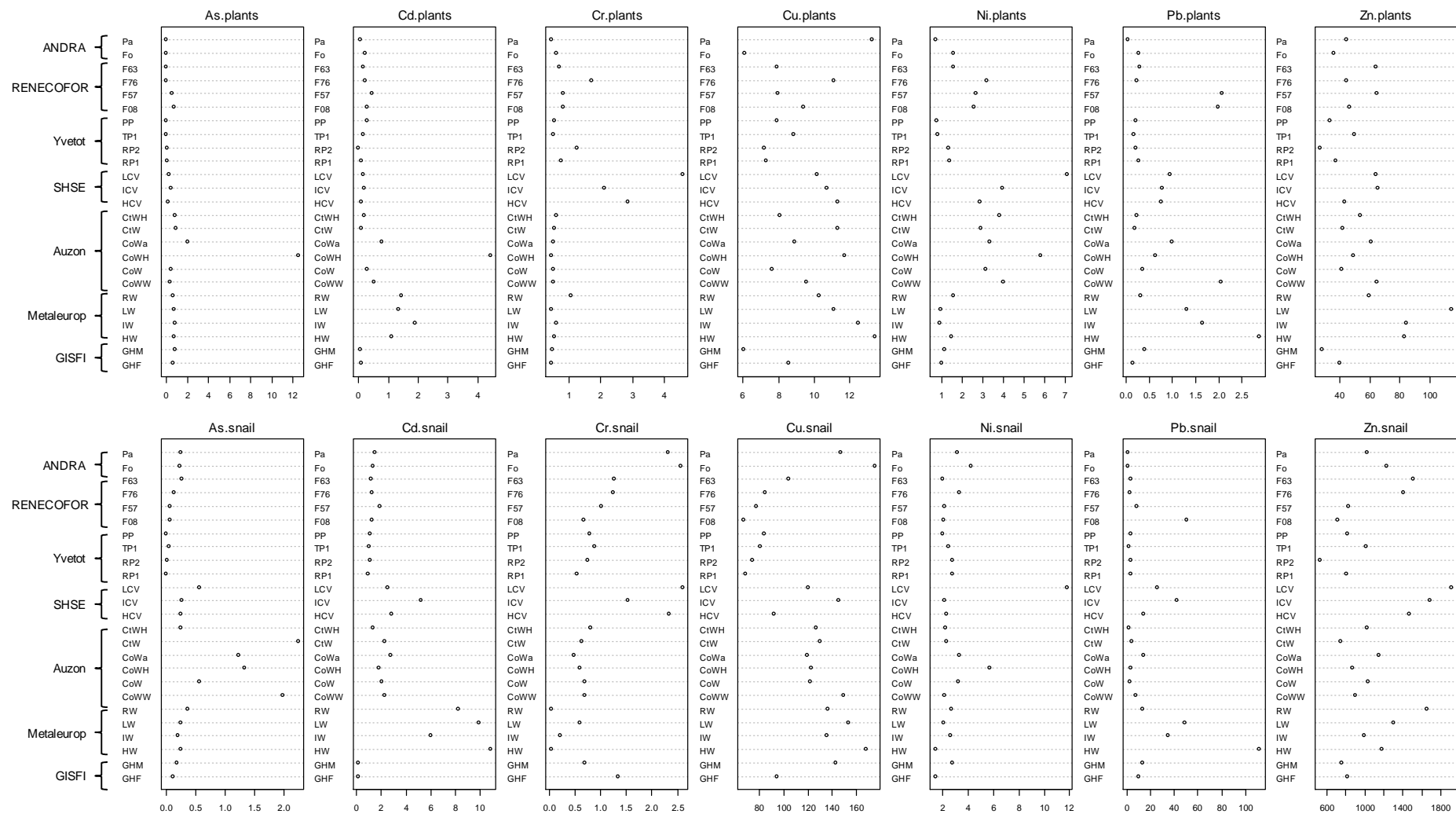


Figure 2: TE contents (mg kg^{-1}) in plants (up) and snails (down) sampled on 25 plots from 7 sites (median values, $n=5$ for plants, $n=6$ for snails).

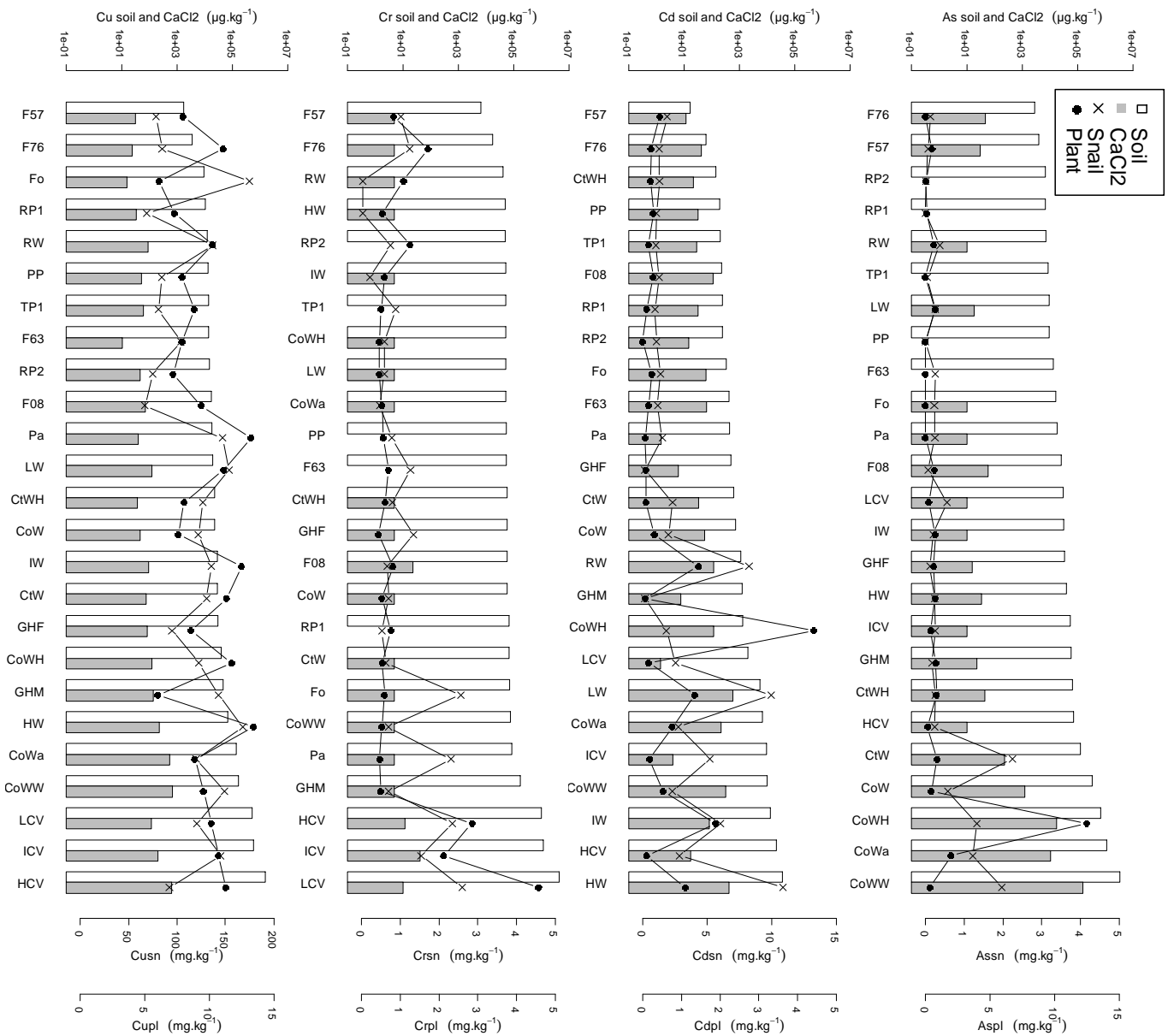


Figure 3 : TE contents in soil (total and CaCl₂ extractable fractions), in plants and in snails from 25 experimental plots (n= 12 for soils, n= 5 for plants, n= 6 for snails).

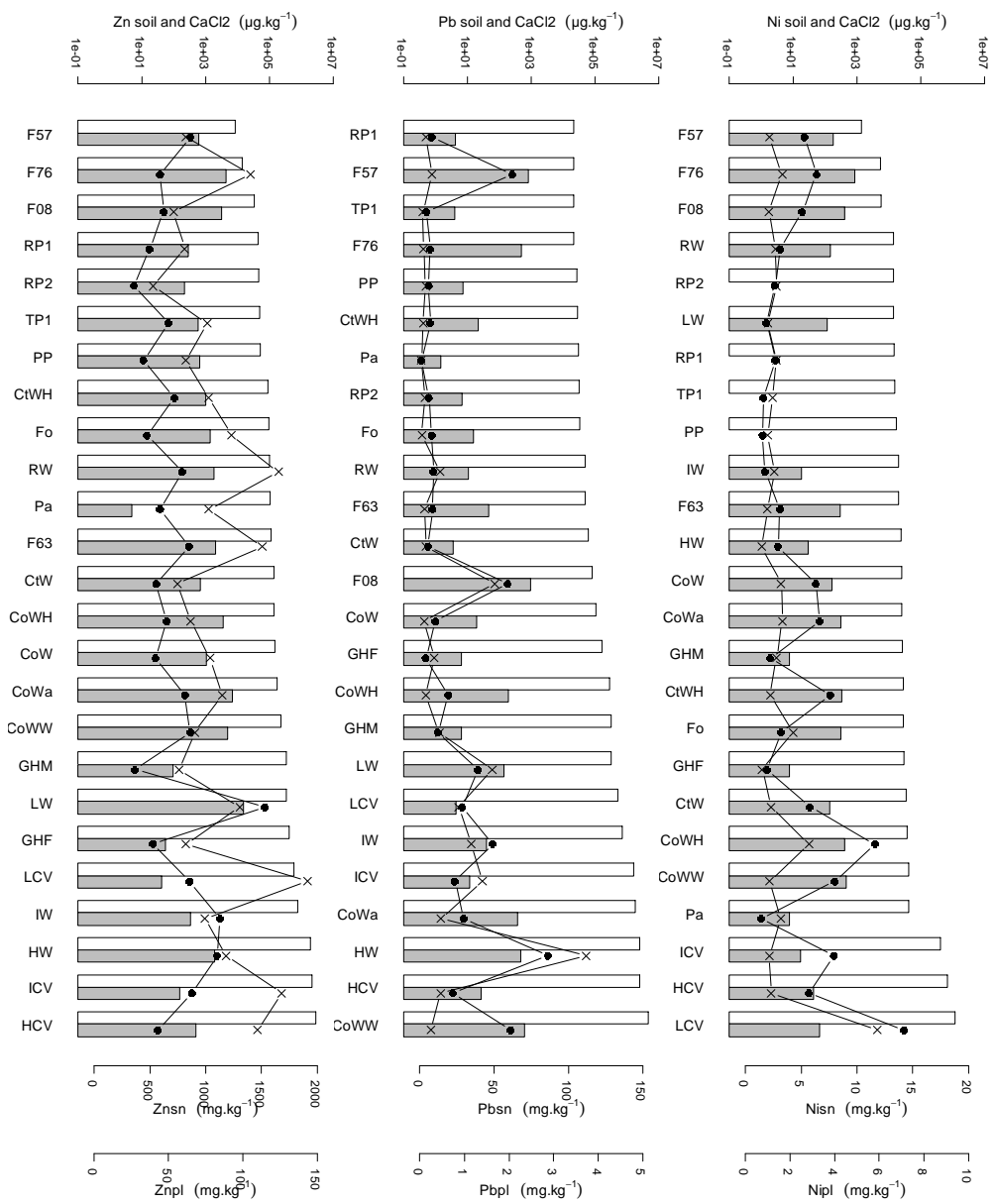
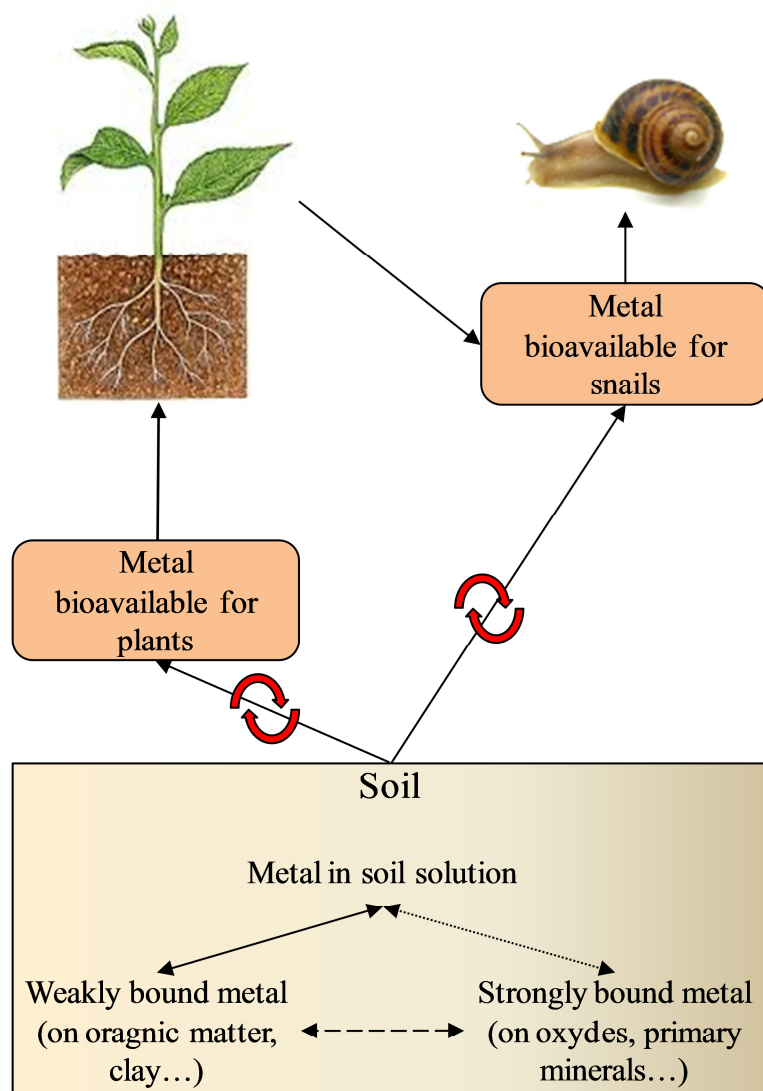


Figure 3: (continued)




 Influence of soil properties

Figure 4: Synthetic schema of metal mobility and transfer from soil to organisms

TABLES

Table 1: Main distribution parameters of TE contents (mg kg^{-1} DW) in plants, snails and soil (total and CaCl_2) sampled on 25 plots from 7 sites

Metal		As	Cd	Cr	Cu	Ni	Pb	Zn
Plants (n=125)	Median	0.519	0.233	0.644	9.20	1.75	0.395	50.4
	Min	0.000	0.000	0.250	4.98	0.359	0.000	17.9
	Max	28.6	7.42	165	25.6	10.4	6.24	193
	MAD	0.609	0.233	0.264	2.78	1.22	0.374	22.8
Snails (n=150)	Median	0.227	1.65	0.839	121	2.57	5.26	1072
	Min	0.001	0.142	0.030	22.9	0.033	0.689	391
	Max	11.1	33.0	20.0	222	33.4	313	2422
	MAD	0.237	1.01	0.532	44.4	1.11	4.91	381
Soil concentration (Total, n=25)	Median	30.35	0.612	56.8	22.8	25.7	78.9	138
	Min	2.905	0.0165	6.55	1.73	1.4	21.045	8.61
	Max	3285	34.4	4345	1555	1180	4575	2830
	MAD	34.7	0.709	12.2	15.6	15.8	85.8	135
Soil concentration (CaCl_2 , n=25)	Median	26.925	33.6	5	76	150.5	19	689
	Min	10	1.415	5	10.65	7.5	1.5	5
	Max	155050	578	41.9	681	847	924	15100
	MAD	25.1	43.1	0	61.8	212	22.2	940

Table 2: Simple linear regression analysis and correlation study between TE contents in plants or in snails, and total TE in soil (extracted with HF + HClO₄). (n= 25)

Element	Organism	Regression equation	r^2_{adj}	<i>p-value</i>
As	Plants	$As_{pl} = -0.081^{ns} + 0.170 As_{tot}^{**}$	0.27	0.005
	Snails	$As_{sn} = -0.108^* + 0.153 As_{tot}^{***}$	0.63	<0.001
Cd	Plants	$Cd_{pl} = ns$	--	--
	Snails	$Cd_{sn} = 0.324^{***} + 0.395 Cd_{tot}^{***}$	0.45	<0.001
Cr	Plants	$Cr_{pl} = -0.095^{ns} + 0.190 Cr_{tot}^{***}$	0.49	<0.001
	Snails	$Cr_{sn} = 0.019^{ns} + 0.135 Cr_{tot}^{**}$	0.23	0.009
Cu	Plants	$Cu_{pl} = ns$	--	--
	Snails	$Cu_{sn} = ns$	--	--
Ni	Plants	$Ni_{pl} = 0.285^{**} + 0.140 Ni_{tot}^*$	0.15	0.032
	Snails	$Ni_{sn} = 0.417^{***} + 0.106 Ni_{tot}^*$	0.15	0.031
Pb	Plants	$Pb_{pl} = -0.070^{ns} + 0.132 Pb_{tot}^{**}$	0.35	0.001
	Snails	$Pb_{sn} = 0.104^{ns} + 0.408 Pb_{tot}^{***}$	0.42	<0.001
Zn	Plants	$Zn_{pl} = ns$	--	--
	Snails	$Zn_{sn} = 2.821^{***} + 0.091 Zn_{tot}^*$	0.14	0.037

Symbols for *p-values* in the regression equations are: °<0.1, *<0.05, **<0.01, ***<0.001

Table 3: Simple linear regression analysis and correlation study between TE contents in plants or in snails, and “available” TE in soil (extracted with 0.01 M CaCl₂). (n= 25)

Element	Organism	Regression equation	r^2_{adj}	<i>p-value</i>
As	Plants	$As_{pl} = 0.034^{ns} + 0.103 As_{CaCl_2}$	0.24	0.016
	Snails	$As_{sn} = -0.017^{ns} + 0.093 As_{CaCl_2}^{***}$	0.60	<0.001
Cd	Plants	$Cd_{pl} = -0.097^{ns} + 0.160 Cd_{CaCl_2}$	0.32	0.001
	Snails	$Cd_{sn} = 0.178^{ns} + 0.201 Cd_{CaCl_2}^*$	0.20	0.013
Cr	Plants	$Cr_{pl} = ns$	--	--
	Snails	$Cr_{sn} = ns$	--	--
Cu	Plants	$Cu_{pl} = ns$	--	--
	Snails	$Cu_{sn} = ns$	--	--
Ni	Plants	$Ni_{pl} = 0.220^{\circ} + 0.153 Ni_{CaCl_2}^*$	0.23	0.017
	Snails	$Ni_{sn} = ns$	--	--
Pb	Plants	$Pb_{pl} = -0.014^{ns} + 0.149 Pb_{CaCl_2}^{***}$	0.54	<0.001
	Snails	$Pb_{sn} = ns$	--	--
Zn	Plants	$Zn_{pl} = ns$	--	--
	Snails	$Zn_{sn} = ns$	--	--

Symbols for *p-values* in the regression equations are: [°]<0.1, *<0.05, **<0.01, ***<0.001

Table 4: Multiple linear regression analysis and correlation study between TE content in plants or in snails, and the main soil parameters as independent variables. Selected soil parameter were pH, cation exchange capacity (CEC), sum of exchangeable cations (SEC), organic carbon content (C_{org}), clay and silts contents and total soil concentration (extracted with HF + HClO₄) of each studied element. (n= 25)

Element	Organism	Regression equation	r^2_{adj}	<i>p-value</i>
As	Plants	$As_{pl} = -0.081^{ns} + 0.170 As_{tot}^{**}$	0.27	0.005
	Snails	$As_{sn} = -0.108^* + 0.153 As_{tot}^{***}$	0.63	<0.001
Cd	Plants	$Cd_{pl} = ns$	--	--
	Snails	$Cd_{sn} = 0.750^{***} + 0.403 Cd_{tot}^{***} - 0.271 C_{org}^*$	0.55	<0.001
Cr	Plants	$Cr_{pl} = 0.178^{\circ} + 0.205 Cr_{tot}^{***} - 0.277 SEC^{***}$	0.69	<0.001
	Snails	$Cr_{sn} = 0.464^{**} + 0.230 Cr_{tot}^{***} - 0.117 pH^{**} + 0.153 CaCO_3^*$	0.45	0.001
Cu	Plants	$Cu_{pl} = ns$	--	--
	Snails	$Cu_{sn} = ns$	--	--
Ni	Plants	$Ni_{pl} = 1.484^{***} + 0.226 Ni_{tot}^{**} - 0.089 pH^{***} - 0.310 Silts^{**}$	0.62	<0.001
	Snails	$Ni_{sn} = 0.641^{***} + 0.128 Ni_{tot}^{**} - 0.235 SEC^*$	0.27	0.012
Pb	Plants	$Pb_{pl} = 0.106^{ns} + 0.184 Pb_{tot}^{***} - 0.046 pH^*$	0.45	<0.001
	Snails	$Pb_{sn} = 0.104^{ns} + 0.408 Pb_{tot}^{***}$	0.42	<0.001
Zn	Plants	$Zn_{pl} = ns$	--	--
	Snails	$Zn_{sn} = 2.960^{***} + 0.150 Zn_{tot}^{**} - 0.245 SEC$	0.24	0.02

Symbols for *p-values* in the regression equations are: $^{\circ}$ <0.1, *<0.05, **<0.01, ***<0.001

Table 5: Regression analysis and correlation study between TE content in snails, and various sets of explanatory variables (n=25). PC: plant TE content; STC: soil TE total content; SSP: selected soil properties (pH, CEC, SEC, C_{org}, clay and silts contents).

Element	Explanatory variables	Regression equation	r ² _{adj}	p-value
As	PC	As _{sn} = 0.075* + 0.310 As _{pl} **	0.22	0.01
	STC + PC	As _{sn} = -0.108* + 0.153 As _{tot} ***	0.63	<0.001
	STC + PC + SSP	As _{sn} = -0.108* + 0.153 As _{tot} ***	0.63	<0.001
Cd	PC	Cd _{sn} = 0.365*** + 0.838 Cd _{pl} **	0.27	0.004
	STC + PC	Cd _{sn} = 0.272*** + 0.324 Cd _{tot} *** + 0.530 Cd _{pl} *	0.54	<0.001
	STC + PC + SSP	Cd _{sn} = 0.660*** + 0.339 Cd _{tot} *** + 0.468 Cd _{pl} * - 0.241 C _{org} *	0.62	<0.001
Cr	PC	Cr _{sn} = 0.146* + 0.496 Cr _{pl} *	0.21	0.012
	STC + PC	Cr _{sn} = 0.019 ^{ns} + 0.135 Cr _{tot} **	0.23	0.009
	STC + PC + SSP	Cr _{sn} = 0.464** + 0.230 Cr _{tot} *** - 0.117 pH** + 0.153 CaCO ₃ *	0.45	0.001
Cu	PC	Cu _{sn} = ns	--	--
	STC + PC	Cu _{sn} = ns	--	--
	STC + PC + SSP	Cu _{sn} = ns	--	--
Ni	PC	Ni _{sn} = 0.376*** + 0.402 Ni _{pl} **	0.25	0.006
	STC + PC	Ni _{sn} = 0.376*** + 0.402 Ni _{pl} **	0.25	0.006
	STC + PC + SSP	Ni _{sn} = 0.376*** + 0.402 Ni _{pl} **	0.25	0.006
Pb	PC	Pb _{sn} = 0.519*** + 2.171 Pb _{pl} ***	0.56	<0.001
	STC + PC	Pb _{sn} = 0.519*** + 2.171 Pb _{pl} ***	0.56	<0.002
	STC + PC + SSP	Pb _{sn} = -0.333 ^{ns} + 2.085 Pb _{pl} *** + 0.139 pH***	0.75	<0.001
Zn	PC	Zn _{sn} = 2.196*** + 0.481 Zn _{pl} **	0.24	0.007
	STC + PC	Zn _{sn} = 2.196*** + 0.481 Zn _{pl} **	0.24	0.007
	STC + PC + SSP	Zn _{sn} = 2.196*** + 0.481 Zn _{pl} **	0.24	0.007

Symbols for p-values in the regression equations are: °<0.1, *<0.05, **<0.01, ***<0.001

ASSOCIATED CONTENT : **Supporting Information**

S 1: Studied sites: land use, soil occupation, constraints and number of plots.

Site	Land uses	Soil occupation	constraints	No. of plots
GISFI (French Scientific Interest Group - Industrial wasteland)	Contaminated	Wastelands	Contamination in PAH	2
Metaleurop	Contaminated	Arable, woodlands	Multi-metals contamination (Cd, Pb, As)	7
Auzon	Contaminated	Arable, woodlands	Located contamination (As)	6
SHSE (Slag Heap of Saint-Etienne)	Contaminated	Wastelands	Metal diffused contamination (Pb, Cd, Zn...)	3
Yvetot	Cultivated	Arable, pastures	Age of pastures	6
RENECOFOR (national Network of long-term follow-up of the forest ecosystems)	Forest	Forest	Scots pines, spruces, fir tree	4
Andra (French national radioactive waste management agency)	Cultivated / Forest	Arable, forest	Different soil occupations	2

S 2: Main pedological characteristics of the 25 plots from the 7 sites studied (median values, n=5)

Site	Plot	Clay (g kg ⁻¹)	Silts (g kg ⁻¹)	Sands (g kg ⁻¹)	C _{org} (g kg ⁻¹)	pH _w	CEC (cmol kg ⁻¹)	SEC cmol+.kg ⁻¹	CaCO ₃ (g kg ⁻¹)	N _{tot} (g kg ⁻¹)
GISFI	GHF	143	225	611	121	8.3	15.0	17.8	249	2.99
	GHM	94.0	172	736	210	8.3	11.0	14.6	137	4.17
Metaleurop	HW	298	601	101	48.3	8.0	29.3	31.4	50.6	3.40
	IW	294	609	96.0	26.6	8.2	24.3	27.0	66.4	2.05
	LW	203	283	517	31.8	6.5	17.2	17.1	0.500	2.27
	RW	163	525	313	20.2	6.5	12.2	12.4	0.500	1.58
Auzon	CoWW	238	427	346	59.6	5.4	19.1	19.5	0.500	3.96
	CoW	148	279	574	40.8	5.7	13.0	13.6	0.500	2.90
	CoWH	89.5	167	757	44.2	5.2	11.0	11.5	0.500	3.33
	CoWa	98.5	167	713	34.9	5.8	9.46	9.90	0.500	2.48
	CtW	138	299	556	40.8	6.2	14.9	15.6	0.500	2.90
	CtWH	148	225	628	26.2	5.1	8.90	9.29	0.500	2.03
SHSE	HCV	48.5	142	803	46.9	8.1	10.5	13.0	48.6	2.26
	ICV	44.5	131	820	30.4	8.7	6.60	10.8	74.5	1.36
	LCV	33.5	77.0	886	14.8	8.6	3.47	5.72	39.9	0.905
Yvetot	RP1	140	665	196	11.1	5.6	5.43	6.40	0.500	1.14
	RP2	128	665	210	14.4	6.0	6.97	7.71	0.500	1.41
	TP1	149	652	202	18.9	5.5	7.25	8.09	0.500	1.83
	PP	163	634	203	25.8	5.5	8.06	8.59	0.500	2.45
RENECOFOR	F08	262	630	106	87.4	4.0	10.4	9.76	0.500	4.72
	F57	44.5	73.0	886	13.0	4.1	2.14	1.82	0.500	0.689
	F76	78.0	354	566	104	3.8	7.10	4.55	0.500	3.07
	F63	257	411	326	156	4.9	9.42	8.94	1.40	9.17
Andra	Fo	255	425	299	27.5	4.9	6.81	6.86	0.500	1.88
	Pa	499	360	134	59.1	7.3	33.5	34.7	21.3	5.61

SEC : sum of exchangeable cations (Al, Ca, Fe, Mg, Mn, Na,)

S 3: Total (HF + HClO₄ extraction) and “available” (CaCl₂ extraction) TE contents at the 25 plots from the 7 sites studied (median values, n=5). NA: no available data. *italic values correspond to value below the detection limit*

Site	Plot	Total fraction (mg kg ⁻¹)							Available fraction (µg kg ⁻¹)						
		[As] _{tot}	[Cd] _{tot}	[Cr] _{tot}	[Cu] _{tot}	[Ni] _{tot}	[Pb] _{tot}	[Zn] _{tot}	[As] _{CaCl2}	[Cd] _{CaCl2}	[Cr] _{CaCl2}	[Cu] _{CaCl2}	[Ni] _{CaCl2}	[Pb] _{CaCl2}	[Zn] _{CaCl2}
GISFI	GHF	32.8	0.499	57.1	30.3	29.0	165	408	15.5	6.12	<i>5.00</i>	84.6	<i>7.50</i>	6.39	55.1
	GHM	58.5	1.23	172	45.3	26.9	309	323	23.4	7.56	<i>5.00</i>	143	<i>7.50</i>	6.52	94.8
Metaleurop	HW	39.3	34.4	48.6	68.4	23.5	2485	1885	34.2	415	<i>5.00</i>	234	30.4	465	1965
	IW	30.9	13.3	50.3	27.6	19.8	731	745	<i>10.0</i>	79.3	<i>5.00</i>	93.3	18.3	39.1	340
	LW	9.42	5.44	52.0	19.4	14.2	319	332	18.6	578	<i>5.00</i>	123	120	142	15100
	RW	7.11	1.09	41.5	12.3	13.6	48.8	102	<i>10.0</i>	122	<i>5.00</i>	87.8	151	10.7	1835
Auzon	CoWW	3285	9.97	76.5	159	41.4	4575	225	155050	326	<i>5.00</i>	681	466	617	5020
	CoW	339	0.722	57.9	22.9	25.7	104	148	1238	53.8	<i>5.00</i>	47.7	163	19.0	1080
	CoWH	661	1.32	51.8	38.6	36.3	282	140	16895	120	<i>5.00</i>	126	418	194	3500
	CoWa	1087	6.73	52.3	140	25.7	1834	173	10735	219	<i>5.00</i>	538	318	377	6660
	CtW	123	0.612	67.3	27.7	35.0	60.1	138	233	33.6	<i>5.00</i>	76.0	136	3.54	689
	CtWH	62.5	0.143	56.8	22.8	27.6	28.1	88.1	44.6	21.2	<i>5.00</i>	38.0	335	21.4	1006
SHSE	HCV	73.3	21.0	982	1555	685	2525	2830	<i>10.0</i>	17.6	11.6	653	43.5	26.5	492
	ICV	54.8	9.37	1158	570	405	1616	2180	<i>10.0</i>	3.98	41.9	200	17.4	11.8	153
	LCV	30.3	1.99	4345	525	1180	513	577	<i>10.0</i>	1.42	10.2	118	69.5	4.35	42.2
Yvetot	RP1	6.93	0.236	66.3	10.3	14.6	21.0	42.7	NA	32.6	NA	34.3	NA	4.21	284
	RP2	6.76	0.243	49.0	14.4	13.9	30.6	47.0	NA	14.7	NA	46.1	NA	6.80	215
	TP1	8.52	0.205	51.2	13.7	15.6	21.4	48.4	NA	29.3	NA	61.4	NA	4.04	587
	PP	9.48	0.198	53.8	13.4	16.9	26.3	51.5	NA	32.3	NA	51.2	NA	7.24	640
RENECOFOR	F08	25.4	0.235	57.9	17.1	5.68	78.9	34.0	58.9	111	22.5	71.5	427	924	3175
	F57	3.83	0.017	6.55	1.73	1.40	21.1	8.61	30.5	11.7	<i>5.00</i>	32.5	179	826	604
	F76	2.91	0.063	17.7	3.48	5.53	21.9	13.5	46.4	41.5	<i>5.00</i>	24.0	847	485	4290
	F63	12.8	0.414	55.0	14.1	20.2	49.7	113	NA	64.0	NA	10.7	300	46.7	2035
Andra	Fo	16.6	0.328	71.1	9.57	27.9	32.5	93.3	<i>10.0</i>	62.7	<i>5.00</i>	15.9	316	16.0	1410
	Pa	18.4	0.447	82.7	18.5	42.3	29.9	104	<i>10.0</i>	1.45	<i>5.00</i>	38.9	<i>7.50</i>	<i>1.50</i>	<i>5.00</i>

S 3: Principal plant species on the studied plots.

Site	Plot	Principal species on the plots
GISFI	GHF	<i>Achillea millefolium</i> , <i>Agrostis stolonifera</i> , <i>Arrhenatherum elatius</i> , <i>Artemisia vulgaris</i> , <i>Artemisia campestris</i> , <i>Bromus hordeaceus</i> , <i>Bromus sterilis</i> , <i>Cerastium</i> sp., <i>Chenopodium album</i> , <i>Dactylis glomerata</i> , <i>Echium ulgare</i> , <i>Epilobium parviflorum</i> , <i>Erigeron annuus</i> , <i>Euphorbia cyparissias</i> , <i>Hypericum perforatum</i> , <i>Medicago lupulina</i> , <i>Melilotus albus</i> , <i>Malva moschata</i> , <i>Myosotis</i> sp., <i>Oenothera biennis</i> , <i>Petrorhagia rolifera</i> , <i>Picris hieracioides</i> , <i>Plantago lanceolata</i> , <i>Prunus spinosa</i> , <i>Robinia pseudoacacia</i> , <i>Rubus</i> sp., <i>Scrophularia</i> sp., <i>Sedum acre</i> , <i>Senecio jacobaea</i> , <i>Silene latifolia</i> , <i>Tanacetum vulgare</i> , <i>Tragopogon ubius</i> , <i>Veronica chamaedrys</i> , <i>Vicia hirsuta</i> , <i>Vulpia ciliata</i>
	GHM	<i>Acer pseudoplatanus</i> , <i>Arrhenatherum elatius</i> , <i>Artemisia vulgaris</i> , <i>Astragalus glycyphyllos</i> , <i>Centaurea jacea</i> , <i>Chenopodium album</i> , <i>Cirsium arvense</i> , <i>Clematis vitalba</i> , <i>Dactylis glomerata</i> , <i>Dipsacus fullonum</i> , <i>Epilobium</i> sp, <i>Erigeron annuus</i> , <i>Euphorbia cyparissias</i> , <i>Geranium robertianum</i> , <i>Hypericum perforatum</i> , <i>Medicago sativa</i> , <i>Oenothera biennis</i> , <i>Papaver rhoeas</i> , <i>Plantago lanceolata</i> , <i>Reseda lutea</i> , <i>Rosa</i> sp, <i>Rubus</i> sp, <i>Rumex acetosella</i> , <i>Sanguisorba minor</i> , <i>Silene latifolia</i> , <i>Verbascum</i> sp., <i>Vulpia ciliata</i>
Metaleurop	HW	<i>Acer pseudoplatanus</i> , <i>Alnus glutinosa</i> , <i>Arrhenatherum elatius</i> , <i>Betula pubescens</i> , <i>Calystegia sepium</i> , <i>Carex palustris</i> , <i>Cornus sanguinea</i> , <i>Crataegus monogyna</i> , <i>Elytrigia repens</i> , <i>Epilobium</i> sp, <i>Fraxinus excelsior</i> , <i>Galium aparine</i> , <i>Glechoma hederacea</i> , <i>Myosotis arvensis</i> , <i>Quercus</i> sp., <i>Ranunculus repens</i> , <i>Robinia pseudoacacia</i> , <i>Rubus</i> sp, <i>Salix</i> sp., <i>Sambucus nigra</i> , <i>Symphytum officinale</i> , <i>Urtica dioica</i>
	IW	<i>Acer pseudoplatanus</i> , <i>Arrhenatherum elatius</i> , <i>Cornus sanguinea</i> , <i>Crataegus monogyna</i> , <i>Dipsacus fullonum</i> , <i>Elytrigia repens</i> , <i>Epilobium</i> sp., <i>Fragaria vesca</i> , <i>Galium aparine</i> , <i>Glechoma hederacea</i> , <i>Myosotis arvensis</i> , <i>Populus</i> sp., <i>Ranunculus repens</i> , <i>Rubus</i> sp, <i>Sambucus nigra</i> , <i>Symphytum officinale</i> , <i>Urtica dioica</i>
	LW	<i>Acer pseudoplatanus</i> , <i>Alnus glutinosa</i> , <i>Betula pubescens</i> , <i>Carpinus betulus</i> , <i>Castanea sativa</i> , <i>Corylus avellana</i> , <i>Crataegus monogyna</i> , <i>Epilobium</i> sp., <i>Fraxinus excelsior</i> , <i>Galium aparine</i> , <i>Geranium robertianum</i> , <i>Glechoma hederacea</i> , <i>Hedera helix</i> , <i>Ilex aquifolium</i> , <i>Lamium album</i> , <i>Milium effusum</i> , <i>Myosotis arvensis</i> , <i>Parthenocissus inserta</i> , <i>Poa</i> sp., <i>Populus</i> sp., <i>Prunus avium</i> , <i>Quercus</i> sp., <i>Rubus</i> sp., <i>Sambucus nigra</i> , <i>Stellaria holostea</i> , <i>Symphytum officinale</i> , <i>Urtica dioica</i>
	RW	<i>Acer pseudoplatanus</i> , <i>Clematis vitalba</i> , <i>Crataegus monogyna</i> , <i>Epilobium</i> sp., <i>Equisetum arvense</i> , <i>Euonymus europaeus</i> , <i>Fraxinus excelsior</i> , <i>Galium aparine</i> , <i>Geranium robertianum</i> , <i>Glechoma hederacea</i> , <i>Hedera helix</i> , <i>Lamium album</i> , <i>Myosotis arvensis</i> , <i>Populus</i> sp., <i>Prunus avium</i> , <i>Quercus</i> sp., <i>Ranunculus repens</i> , <i>Rubus</i> sp., <i>Salix</i> sp., <i>Urtica dioica</i> , <i>Vicia hirsuta</i>
Auzon	CoWW	<i>Agrostis capillaris</i> , <i>Arum maculatum</i> , <i>Cornus sanguinea</i> , <i>Crataegus monogyna</i> , <i>Equisetum arvense</i> , <i>Euphorbia dulcis</i> subsp <i>incompta</i> , <i>Hedera helix</i> , <i>Lonicera periclymenum</i> , <i>Quercus</i> sp., <i>Rubus</i> sp., <i>Sambucus nigra</i> , <i>Urtica dioica</i> ,
	CoW	<i>Apiaceae</i> sp., <i>Cornus sanguinea</i> , <i>Corylus avellana</i> , <i>Crataegus monogyna</i> , <i>Euonymus europaeus</i> , <i>Hedera helix</i> , <i>Ligustrum vulgare</i> , <i>Lonicera periclymenum</i> , <i>Quercus</i> sp., <i>Rubus</i> sp.
	CoWH	<i>Agrostis capillaris</i> , <i>Alliaria petiolata</i> , <i>Apiaceae</i> sp., <i>Cirsium arvense</i> , <i>Cornus sanguinea</i> , <i>Crataegus monogyna</i> , <i>Epilobium</i> sp.1, <i>Epilobium</i> sp. 2, <i>Equisetum arvense</i> , <i>Euonymus europaeus</i> , <i>Humulus lupulus</i> , <i>Rubus</i> sp., <i>Urtica dioica</i> ,
	CoWa	<i>Achillea millefolium</i> , <i>Agrostis capillaris</i> , <i>Apiaceae</i> sp., <i>Cytisus scoparius</i> , <i>Epilobium</i> sp., <i>Euphorbia cyparissias</i> , <i>Galium aparine</i> , <i>Populus tremula</i> , <i>Quercus</i> sp., <i>Rubus</i> sp., <i>Rumex acetosella</i> , <i>Urtica dioica</i> ,
	CtW	<i>Alliaria petiolata</i> , <i>Anthriscus sylvestris</i> , <i>Arum maculatum</i> , <i>Chelidonium majus</i> , <i>Crataegus monogyna</i> , <i>Euonymus europaeus</i> , <i>Galium aparine</i> , <i>Hedera helix</i> , <i>Lamium galeobdolon</i> , <i>Ligustrum vulgare</i> , <i>Lunaria annua</i> , <i>Prunus spinosa</i> , <i>Sambucus nigra</i> , <i>Tillia platyphyllos</i> , <i>Urtica dioica</i> ,
	CtWH	<i>Euonymus europaeus</i> , <i>Galium aparine</i> , <i>Glechoma hederacea</i> , <i>Hedera helix</i> , <i>Lamiaceae</i> sp., <i>Prunus spinosa</i> , <i>Quercus</i> sp., <i>Rubus</i> sp., <i>Tillia platyphyllos</i>
	HCV	<i>Ailanthus altissima</i> , <i>Calamintha nepeta</i> , <i>Clematis vitalba</i> , <i>Cornus sanguinea</i> , <i>Echium vulgare</i> , <i>Euonymus europaeus</i> , <i>Hypericum perforatum</i> , <i>Lactuca serriola</i> , <i>Melilotus albus</i> , <i>Oenothera biennis</i> , <i>Urtica dioica</i> , <i>Verbascum pulverulentum</i>
SHSE	ICV	<i>Ailanthus altissima</i> , <i>Calamintha nepeta</i> , <i>Fraxinus excelsior</i> , <i>Hypericum perforatum</i> , <i>Melilotus albus</i> , <i>Oenothera biennis</i> , <i>Plantago sacbra</i> Moench subsp <i>scabra</i> , <i>Populus nigra</i> , <i>Reseda lutea</i> , <i>Rosa</i> sp., <i>Sambucus nigra</i> , <i>Urtica dioica</i> , <i>Verbascum pulverulentum</i>
	LCV	<i>Echium vulgare</i> , <i>Lactuca serriola</i> , <i>Melilotus albus</i> , <i>Hypericum perforatum</i> , <i>Oenothera biennis</i> , <i>Plantago sacbra</i> Moench subsp <i>scabra</i> , <i>Populus nigra</i> , <i>Reseda lutea</i> , <i>Scrophularia canina</i> , <i>Verbascum pulverulentum</i>
Yvetot	RP1	<i>Carpinus betulus</i> , <i>Fumaria officinalis</i> , <i>Lamium purpureum</i> , <i>Lolium perenne</i> , <i>Poa annua</i> , <i>Rumex acetosa</i> , <i>Stellaria media</i> , <i>Taraxacum</i> sp., <i>Trifolium repens</i> , <i>Urtica dioica</i> , <i>Veronica chamaedrys</i> ,

	RP2	<i>Lolium perenne</i> , <i>Poa annua</i> , <i>Stellaria media</i> , <i>Trifolium repens</i> ,
	TP1	<i>Cirsium sp.</i> , <i>Holcus lanatus</i> , <i>Lamium album</i> , <i>Lamium purpureum</i> , <i>Lolium perenne</i> , <i>Ranunculus repens</i> , <i>Rumex acetosa</i> , <i>Rumex acetosella</i> , <i>Stellaria holostea</i> , <i>Stellaria media</i> , <i>Taraxacum sp.</i> , <i>Trifolium repens</i> , <i>Urtica dioica</i>
	PP	<i>Cirsium sp.</i> , <i>Crataegus monogyna</i> , <i>Fagus sylvatica</i> , <i>Lamium album</i> , <i>Lamium purpureum</i> , <i>Plantago major</i> , <i>Ranunculus repens</i> , <i>Rumex acetosella</i> , <i>Stellaria holostea</i> , <i>Symphytum officinale</i> , <i>Taraxacum sp.</i> , <i>Urtica dioica</i>
RENECOFOR	F08	<i>Picea abies</i> , <i>Calluna vulgaris</i> , <i>Carex pilulifera</i> , <i>Deschampsia flexuosa</i> , <i>Galium mollugo</i> , <i>Rubus fruticosus</i> , <i>Sorbus aucuparia</i> , <i>Vaccinium myrtillus</i>
	F57	<i>Abies alba</i> , <i>Deschampsia flexuosa</i> , <i>Fagus sylvatica</i> , <i>Festuca altissima</i> , <i>Luzula luzuloides</i> , <i>Oxalis acetosella</i> , <i>Picea abies</i> , <i>Rubus fruticosus</i> , <i>Rubus idaeus</i> , <i>Sorbus aucuparia</i> , <i>Vaccinium myrtillus</i>
	F76	<i>Pinus sylvestris</i> , <i>Calluna vulgaris</i> , <i>Deschampsia flexuosa</i> , <i>Fagus sylvatica</i> , <i>Molinia caerulea</i> , <i>Rubus fruticosus</i> , <i>Vaccinium myrtillus</i>
	F63	<i>Abies alba</i> , <i>Agrostis capillaries</i> , <i>Campanula rotundifolia</i> , <i>Carex pilulifera</i> , <i>Cytisus scoparius</i> , <i>Digitalis purpurea</i> , <i>Epilobium montanum</i> , <i>Fagus sylvatica</i> , <i>Gallium rotundifolium</i> , <i>Hypericum humifusum</i> , <i>Picea abies</i> , <i>Rubus idaeus</i> , <i>Rumex acetosella</i>
ANDRA	Fo	<i>Fagus sylvatica</i> , <i>Galium aparine</i> , <i>Glechoma hederacea</i> , <i>Hedera helix</i> , <i>Lamium album</i> , <i>Lamium galeobdolon</i> , <i>Rubus fruticosus</i>
	Pa	<i>Lolium perenne</i> , <i>Ranunculus repens</i> , <i>Rumex acetosella</i> , <i>Taraxacum sp.</i> , <i>Trifolium repens</i>