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Is there a relationship between earthworm energy reserves and metal availability after exposure to field-contaminated soils?

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ABSTRACT

Generic biomarkers are needed to assess environmental risks in metal polluted soils. We assessed the strength of the relationship between earthworm energy reserves and metal availability under conditions of cocktail of metals at low doses and large range of soil parameters. *Aporrectodea caliginosa* was exposed in laboratory to a panel of soils differing in Cd, Pb and Zn total and available ($CaCl_2$ and EDTA-extractable) concentrations, and in soil texture, pH, CEC and organic-C. Glycogen, protein and lipid contents were recorded in exposed worms. Glycogen contents were not linked to the explaining variables considered. Variable selection identified $CaCl_2$ extractable metals concentrations and soil texture as the main factors affecting protein and lipid contents. The results showed opposite effects of Pb and Zn, high inter-individual variability of biomarkers and weak relationships with easily extractable metals. Our results support the lack of genericity of energy reserves in earthworms exposed to field-contaminated soils.

1. Introduction

In soil, assessing environmental risks associated with metallic trace elements is still challenging. Both sensitive and generic indicators of metal bioavailability are needed and despite the great number and variety of candidates, their potential as risk assessment tools has to be questioned. This is especially the case for soil organisms like earthworms, even though metal bioavailability was extensively studied (Nahmani et al., 2007b).

Biomarkers are assumed to be direct measurements of metal bioavailability (Lanno et al., 2004). The decrease of energy reserves (carbohydrates, proteins, lipids) has been proposed as a biomarker of metal stress (Scott-Fordsmand and Weeks, 2000), but the subject was rarely addressed in the case of earthworms and metal contamination. For other soil organisms, several works reported that energy reserves were affected by metal contamination (Weeks et al., 2004; Amorim et al., 2012) while other studies have shown the opposite (Schill and Köhler, 2004; Bindesbøl et al., 2005; Bednarska et al., 2013). These previous surveys considered total metal concentrations and not metal availability.

Weak and stronger metal extractions have been used to describe internal metal concentrations in earthworms, but with variable results (e.g. Vijver et al., 2005; Ernst et al., 2008). No particular chemical extraction was proven to be generic to mimic metal bioavailability for earthworms: neither soil solution concentrations (Nahmani et al., 2009; Veltman et al., 2007), nor weak salt extractions (Bernard et al., 2010; Gaw et al., 2012), nor extractions using chelating agents (Ernst et al., 2008; Daoust et al., 2006). Yet $CaCl_2$ and ethylene diamine tetraacetic acid (EDTA) are extensively used to measure soil metal availability (e.g. Fritsch et al., 2011). Their pertinence to indicate metal bioavailability for earthworms must be confirmed. In order to determine if chemical extraction can mimic bioavailability, a mathematical relationship between the extractable metal content and a biological measurement of bioavailability is needed (Harmsen, 2007; ISO 17402, 2008). If a number of studies correlated chemical extractions with internal concentrations in earthworms, few attempted to link metal availability measurements with earthworm biomarkers.

In complex systems such as soils, several factors are expected to modulate biomarkers responses to metal availability. Although soils are often moderately contaminated, soil organisms energy reserves have been mainly studied in the case of high levels of metal contamination and their response to low doses of metals is not known yet. In aquatic organisms, significant increase of energy reserves at low doses of xenobiotics and decrease at higher doses have been reported (De Coen and Janssen, 2003; Smolders et al., 2003). Low doses of available metals could thus exert hormetic-like effects on earthworm energy reserves. Besides, *in situ*, soils are very often

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contaminated with cocktails of metals. Earthworms regulate metals either by sequestration (Cd, Pb) or excretion (Zn, Cu) (Spurgeon and Hopkin, 1999). Holmstrup et al. (2011) have recently suggested that the ways earthworms coped with internal metals are associated to different energy demands. A complex response of energy reserves in multi-contaminated soils can therefore be expected. Finally, it is likely that the energy status of soil organisms is influenced by other factors in the environment, notably soil characteristics (Amorim et al., 2012). Soil texture and organic carbon content have been shown to influence earthworm biomass (Nahmani et al., 2007a), which could be associated with a change in energy allocation.

In the end, the effects of low doses, multiple contamination and soil characteristics could prevent the use of earthworm energy reserves as generic indicators in risk assessment. In soil, these factors intervene concomitantly, and the response of the entire system can be different from what is observed when considering separately its different parts (Beketov and Liess, 2012). Therefore, it is important to study the system as a whole. In this work, we determined whether the relationship between metal availability and earthworm energy reserves was strong enough to be found after exposure to a wide panel of field soils. The studied soils were chosen to exhibit jointly low doses of metals present in cocktails and various soil parameters.

2. Materials and methods

2.1. Sites description

The design of the experiment included 31 soil samples chosen to achieve a wide array of soil characteristics and levels of metal contamination. They were sampled in four contaminated and four uncontaminated sites located in France. In each site, several soil samples were taken from different plots. All the plots were arable (crops or pastures), had comparable geological origins (mainly wind-deposited silt, and alluvium) and were noncalcareous.

Two of the contaminated sites (Metaleurop (Me) and Mortagne-du-Nord (Mo)) are located in the North of France near former lead and zinc smelters respectively. They were contaminated by atmospheric deposition of dusts (van Oort et al., 2002; Bernard et al., 2010). At the Metaleurop site, five soil samples were taken from five arable cropping plots, at different distances from the smelter. At the site of Mortagne-du-Nord, nine soil samples were collected from nine different plots presenting various soil texture (from sandy to loamy), and different land use (crops (Mo3, Mo8 and Mo9) or pastures (Mo1, Mo2, Mo4, Mo5, Mo6, Mo7)).

The two other contaminated sites (Pierrelaye (Pi) and Triel (Tr)) are located in Paris suburbs and were subjected to raw wastewater spreading for 100 years, leading to the accumulation of large amounts of organic matters and metal pollutants (Lamy et al., 2006). At the Pierrelaye site, soil samples were collected from two arable cropping plots: an unpolluted plot located outside the wastewater irrigated area for which one sample was carried out (Pi1), and a contaminated plot within which four soil samples were collected along a gradient of contamination (Pi2, Pi3, Pi4, Pi5). At the site of Triel, three soil samples were taken from three plots with varying total metal concentrations and land use: one arable cropping (Tr1), one fallow (Tr2), and one grassland (Tr3).

The four uncontaminated sites had no history of metal contamination. One site named Qualiagro-Feucherolles (Fe) is located 50 km west from Paris on a loamy soil cultivated with a maize/wheat rotation (Houot et al., 2002). The Yvetot site (Yv) is located in Haute Normandie region on a loamy plateau (Hedde et al., 2013). The Bannost site (Ba) is located 30 km south east from Paris on clay loamy soils (Pelosi et al., 2013). The Closeaux site (Cl) is in the south west Paris suburbs (Versailles) on a silty loam soil. From these sites, a total of nine uncontaminated soil samples was carried out. Three plots receiving different exogenous organic matters (co-compost of green waste and sludge (Fe2), farmyard manure (Fe3) and no amendment (Fe1)) were sampled from Fe site. At the Yvetot site, three soil samples presenting different land use were selected: one arable cropping (Yv1), one permanent pasture established in 1968 (Yv2), and one crop/pasture rotation (Yv3) under crop at the time of the sampling. Two soil samples were collected from two different organic farming plots at the Bannost site. They presented different clay and organic C contents. One sample was carried out from a meadow plot in the Closeaux site. It was considered as our control since the earthworms used in our study were sampled from this plot.

2.2. Soil sampling and chemical extractions

Soil samples were collected from March to May 2012. Each sample was a composite of five subsamples of the top-soil (0–20 cm depth), taken from an area of about 1 m^2. Soil samples were hand sorted to remove soil fauna, plant material and debris, air-dried, homogenized and sieved at 2 mm. Afterward, samples were quartered and five representative subsamples were isolated. Soil analyses were conducted on each subsample. Soil particle size determination was carried out according to NF X31-107, soil pH determination according to NF ISO 10390 and soil cation exchange capacity (CEC) according to NF X31-130. Total organic C and N assessments were performed according to NF ISO 10694 and NF ISO 13878. Total Zn, Pb, Cu, and Cd according to NF X31-147 (tri-acid HF + HCl + HNO_3 digestion). Soil metal availability was assessed using two extracting reagents: a neutral salt, calcium chloride ($CaCl_2$) and an organic chelating agent, the ethylene diamine tetraacetic acid (EDTA). 0.01 M $CaCl_2$ extraction was carried out according to Houba et al. (1990), with a ratio mass:volume of 1:10. 0.05 M EDTA extraction was performed according to Quevauviller (1997) (BCR method) at pH 7, with a ratio mass:volume of 1:10. Total, $CaCl_2$ and EDTA-extractable metal concentrations in solution were obtained from inductively coupled plasma mass spectroscopy. The quantification limits were 1 $\mu g\ kg^{-1}$ for Cd, 3 $\mu g\ kg^{-1}$ for Pb, 10 $\mu g\ kg^{-1}$ for Zn. Quantification limits (LQ) were determined according to NF T90-210 slightly adapted, taken into account first the analysis of ten blanks and then the analysis of solutions whose metal contents were close to the first approximation of LQ, in order to make adjustment. All analyses were made by the Laboratoire d'Analyse des Sols (INRA, Arras, France) applying standardized methods and quality assurance procedures.

2.3. Exposure of earthworms

Adult earthworms of the species *Aporrectodea caliginosa* (Savigny 1826) were hand sorted during spring 2012 from an uncontaminated meadow of the Closeaux site. They were maintained in the laboratory in their soil of origin that had been subjected to the same experimental procedure than the other soil samples (air dried, sieved to <2 mm). Earthworm biomass was 0.334 ± 0.085 g fresh weight (mean ± standard deviation, $n = 155$).

For each of the 31 soil samples, five microcosms were conducted using the five soil subsamples previously separated for soil analyses. Water holding capacity (WHC) was measured as the maximum quantity of deionized water retained by the soil sample before observing percolation on a cotton placed underneath in a funnel. One week before the onset of the experiment, 600 g of air-dried sieved soil were placed in 1 L glass jars, moistened to 60% of their WHC using deionized water and placed in the dark at 12 °C. 48 h prior to exposure, earthworms were gently washed in tap water and placed in Petri dishes containing moistened filter paper in order to void their gut. Petri dishes were placed in the dark at 12 °C. Filter paper was cleaned and re-moistened every 12 h. Once voided, earthworms were weighted before being randomly assigned to each microcosm. One microcosm contained 6 individuals. Microcosms were placed in the dark at 12 °C for 21 days. No food was added to the soil in order to not disturb initial metal availability. An exposure duration of 21 days was chosen as a compromise between what is recommended to study metal accumulation in earthworms (28 days, Nahmani et al., 2007b) and to avoid that earthworms lack of food (14 days, Rault et al., 2008). Soil moisture was checked regularly by weighting the microcosms. At the end of exposure, earthworms were removed from the microcosms and were gently washed in tap water before being weighted and placed at −80 °C until further analysis.

2.4. Biochemical measurements

Glycogen, protein and lipid contents were measured on individual earthworms. For a given soil sample, five replicates were carried out; one individual was randomly selected among the 6 earthworms contained in each microcosm. Frozen earthworms were individually homogenized using an ultra-turrax (IKA T10, at 15,000 rpm) in 5 mL of ice-cold phosphate buffer 100 mM, pH 7.2, 1 mM EDTA. All biochemical measurements were carried out in duplicate for each individual.

Glycogen was quantified in the tissue homogenates according to Holmstrup et al. (2011). 250 μL of tissue homogenates were placed in 0.5 M NaOH at 80 °C for 3 h and 100 μL of the extraction mixtures were incubated for 2 h at 37 °C with 50 μL of 10 mg mL^{-1} amyloglucosidase (*Aspergillus niger*, Sigma–Aldrich) in 850 μL of 0.25 M acetate buffer pH 4.4. Glucose was quantified using hexokinase reagent (Sigma–Aldrich) by measuring absorbance at 340 nm. Glycogen contents were calculated relative to a glycogen standard curve (Rabbit liver glycogen, Sigma–Aldrich).

Total soluble protein contents were determined using a bicinchoninic assay kit (Smith et al., 1985) and bovine serum albumine as a standard (Sigma–Aldrich).

Lipids were extracted following Folch et al. (1957). Tissue homogenates (500 μL) were stored at −20 °C in 1.25 mL methanol until analysis. Chloroform was added to achieve a methanol/chloroform ratio of 2/1 (v/v). After centrifugation (5 min, 1700 *g*), lipids were separated from the water-soluble material by adding 1 volume chloroform followed by 1 volume of miliQ water (Milipore, 18 MΩ) to achieve a volume ratio of 1/1/0.3. After a second centrifugation (5 min, 1700 *g*), the chloroform layer was evaporated using nitrogen flux. The extraction mixtures were incubated with 5 mL of sulfuric acid in boiling water for 10 min, cooled down on ice for 5 min, and 200 μL of each sample were mixed with 3 mL of phosphoric acid-vanillin reagent freshly prepared following Knight et al. (1972). After an incubation of 15 min at 37 °C, optical density at 525 nm was read and lipid contents were calculated relative to olive oil standards (Sigma–Aldrich).