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Using species-specific enriched stable isotopes to study the effect of fresh mercury inputs in soil-earthworm systems

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ABSTRACT

The fate of mercury (Hg) in the soil-earthworm system is still far from being fully understood, especially regarding recurrent and challenging questions about the importance of the reactivity of exogenous Hg species. Thus, to predict the potential effect of Hg inputs in terrestrial ecosystems, it is necessary to evaluate separately the reactivity of the endogenous and exogenous Hg species and, for this purpose, the use of enriched stable isotope tracers is a promising tool. In the present work, earthworms (*Lumbricus terrestris*) were exposed to historically Hg contaminated soils from the Almadén mining district, Spain. The soils were either non-spiked, which contain only endogenous or native Hg naturally occurring in the soil, or spiked with isotopically enriched inorganic Hg (^{199}IHg), representing exogenous or spiked Hg apart from the native one. The differential reactivity of endogenous and exogenous Hg in the soil conditioned the processes of methylation, mobilization, and assimilation of inorganic Hg by earthworms. Both endogenous and exogenous Hg species also behave distinctly regarding their bioaccumulation in earthworms, as suggested by the bioaccumulation factors, being the endogenous methylmercury (MeHg) the species more readily bioaccumulated by earthworms and in a higher extent. To the best of our knowledge, this work demonstrates for the first time the potential of enriched stable isotopes to study the effects of fresh Hg inputs in soil-earthworm systems. The findings of this work can be taken as a case study on the dynamics of Hg species in complex terrestrial systems and open a new door for future experiments.

1. Introduction

A comprehensive study of the fate of mercury (Hg) in terrestrial ecosystems would require a thorough assessment of the potential effects of fresh Hg inputs in these systems, but recurrent and challenging questions about the importance of the exogenous Hg species reactivity remain open. The traditional approach to these studies has been the addition of natural or radioactive elements to the samples (Suseno et al., 2009; Wang and Wang, 2010). The use of radioisotopes allows to distinguish endogenous from exogenous inputs, but has important limitations associated with the necessary radioactive safety precautions and the requirements of the waste handling procedures (Jackson, 2001). The instrumental advances in mass spectrometry, as well as the concern about exposure to ionizing radiation, led to a general shift away from radioisotopes, thereby increasing the use of enriched stable isotopes in recent times. In this context, stable isotopic tracers have become a powerful tool to follow simultaneously the fate of native (endogenous) and spiked (exogenous) trace elements (Björn et al., 2007; Michener and Lajtha, 2007). This methodology also enables to track coupled and often overlying processes by spiking with different isotopes of the same element (Björn et al., 2007). Therefore, several studies have tracked Hg transformations using enriched stable Hg isotopes, but most of them have been focused on the aquatic environment (Bouchet et al., 2011, 2013; Harris et al., 2007; Monperrus et al., 2007; Rodríguez Martín-Doimeadios et al., 2004; Rodríguez-González et al., 2013). More recently, this tool has also been used in large scale and/or mesocosm experiments investigating terrestrial ecosystems (Graydon et al., 2012; Jonsson et al., 2012, 2014; Oswald et al., 2014; Tjerngren, 2012), but it has never been used to study the soil-earthworm interaction.

Earthworms are key organisms in terrestrial food chains since they are a very important food source for other organisms and account for a major proportion of the animal biomass in soil (Ernst et al., 2008; Ernst and Frey, 2007; Zhang et al., 2009). Earthworms also modify chemical

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and physical properties of the soil that influence the regulation of its structure and, accordingly, its fertility and quality. They also play an important role in metal geochemistry because they contribute to organic matter incorporation and decomposition (Zhang et al., 2009). As a result, these organisms have been proposed as bioindicator species with regard to toxic levels of pollutants such as Hg in soils (Spurgeon et al., 2003). Previous experiments performed to determine the uptake, depuration, and bioaccumulation of Hg species by earthworms exposed to Hg contaminated soils (Burton et al., 2006; Hinton and Veiga, 2008; Kaschak et al., 2014; Rieder et al., 2013; Rodríguez Álvarez et al., 2014) have already demonstrated that MeHg could be generated in the soil and/or within the digestive tract of earthworms and, then, assimilated and bioaccumulated by earthworms. However, knowledge on many aspects of Hg methylation-demethylation in soil and uptake-depuration processes in earthworms is still limited.

Therefore, the aim of this study was to explore by using enriched stable isotopic tracers the potential alterations in the fate of Hg species induced by Hg inputs in the soil-earthworm system. This experiment is based on the consideration that isotopically enriched species are representative of the behaviour of the fresh Hg inputs entering the system. Field soils contaminated with Hg from the Almadén mining district (Spain), with Hg mining activities dating back 2000 years, were used in this study. It allows tracking both native and spiked Hg. This approach provides an estimation for risk assessment that is closer to reality than the ones previously reported (Le Roux et al., 2016; Tang et al., 2016), in which Hg-free soils spiked with Hg were taken as representative of field soils. Species-specific labelling with enriched stable Hg isotopes and subsequent quantification by isotope dilution have been applied to track methylation and demethylation pathways of endogenous and exogenous Hg and also to evaluate the potential alterations in Hg species distribution and/or transformations in the soil-earthworm system induced by new Hg inputs.

2. Materials and methods

2.1. Preparation of natural and isotopically enriched Hg solutions

Stock solutions of inorganic Hg (IHg) and methylmercury (MeHg) of natural isotopic composition were prepared by dissolving Hg(II) chloride (Panreac) in 1% HNO_3 and MeHg chloride (Strem Chemicals) in methanol, respectively. Working standard solutions were prepared daily by appropriate dilution of the stock standard solutions in 1% ultrapure HCl and stored in the dark at 4 °C.

Stock solutions of $^{199}HgCl_2$ were prepared by dissolving 22 mg of ^{199}HgO (Oak Ridge National Laboratory) in 1 mL of concentrated HCl. Stock solutions of $Me^{201}Hg$ were synthesized using ^{201}HgO (Oak Ridge National Laboratory) and methylcobalamin (Sigma-Aldrich) as described elsewhere (Rodríguez Martin-Doimeadios et al., 2002). The solutions were characterized for concentration (calculated by reverse isotope dilution using IHg and MeHg standards with natural isotopic abundances) and for isotopic composition.

Spike solutions of ^{199}IHg and $Me^{201}Hg$ were prepared by diluting the stock solutions in 1% ultrapure HCl.

2.2. Experimental design

The experimental set-up is shown in Fig. S-1. Briefly, test organisms were commercially available clitellate specimens of *Lumbricus terrestris*. Clitellate adults were preferred to juveniles' specimens in order to assure their sexual maturity. A naturally contaminated Hg soil (~250 μg/g of total Hg) from the Almadén mining district (Ciudad Real, Spain) was used in the experiments. The upper 5–10 cm layer was the part of the soil collected for the experiment. Physico-chemical parameters that potentially influence the fate of Hg in soil such as pH, conductivity or organic matter were determined in soils as described elsewhere (Rodríguez Álvarez et al., 2014).

Two experiments were conducted using the Almadén soil. The "Almadén-^{199}Hg" experiment was carried out in a soil spiked with an isotopically enriched solution of $^{199}HgCl_2$. On the other hand, the "Almadén" experiment was carried out in a soil without spiking. Each entire experiment was performed in duplicate.

In order to prepare the soil for the "Almadén-^{199}Hg" experiment, 250 g of soil taken from the Almadén mining district was spiked with Hg chloride solutions isotopically enriched in ^{199}Hg. These solutions were thoroughly mixed with soil to ensure an even distribution. The amount of solution was 30% of the dry weight of the soil. The concentration of the spiked IHg was ~46 mg ^{199}Hg/kg soil, about 20% of the endogenous concentration in the soil. The soil was allowed to stand for 48 h to assure the stabilization of the isotopically enriched species in it.

The soils for the "Almadén" experiment were also moistened with the same volume of a slightly (< 1%) acidic water solution in order to have comparable conditions in both experiments.

Groups of depurated earthworms (n = 12) were exposed to 250 g of each type of soil for 28 days as uptake phase, in containers covered with holed parafilm. Then, the remaining earthworms were moved to a Hg-free soil for a 14 days depuration phase.

Both uptake and depuration phases were conducted under dark conditions at a constant temperature of 4 °C so that the soil moisture was maintained at approximately 30–35%, as recommended for toxicity assays in soil (OECD, 1984), without the need of further water additions.

After the spike stabilization and just before the introduction of earthworms, two replicates of soil samples from each of the two experiments were collected and analysed for total Hg and MeHg. Likewise, five earthworms were randomly selected for total Hg and MeHg analysis prior to exposure to soils. During the uptake phase, two randomly selected earthworms and a soil subsample were taken from each experiment at 2, 8, 14, and 28 days of exposure for analysis. After both uptake and depuration phases, worms were carefully washed, placed into Petri dishes with damp filter paper for 48 h to void the gut contents. The filter paper was changed every 24 h over the 48 h period. Then the earthworms were rinsed with deionized water and sacrificed by deep freezing (−20 °C). Finally, earthworms were ground before the extraction of Hg species.

2.3. Mercury analyses

The methods used for the determination of Hg species in soils and earthworms were satisfactorily validated using adequate certified reference materials as detailed in Supplementary material.

2.3.1. Hg in soils

Total Hg, MeHg and acid-labile IHg, the nitric acid extractable fraction of IHg, were determined in soil aliquots taken at different times according to the experimental design. Inorganic Hg was calculated by difference between total Hg and MeHg concentrations.

Soils were analysed for total Hg by ICP-MS after HCl/HNO_3 ("aqua regia") extraction with closed-vessel microwave heating (irradiation with a Milestone Ethos Plus system for 15 min at 200 °C after a 10 min ramping time to reach the temperature). Under these conditions, the procedural detection limit was 3.2 ng/g of Hg (d.w.).

For Hg speciation analysis, soils were extracted with 6 M HNO_3 in the same closed-vessel microwave system, as described in literature (Berzas Nevado et al., 2008). The concentration of MeHg was then determined in the 6 M HNO_3 extract by ethylation followed by injection into a gas chromatograph coupled to an inductively coupled plasma mass spectrometer (GC-ICP-MS) (Berzas Nevado et al., 2011). When the concentration of IHg was close to 500 ng/g, methylene chloride extraction was used before the ethylation procedure to avoid potential artifact formation of MeHg (Berzas Nevado et al., 2008). In addition, when MeHg was not detectable, the organic extracts were