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Fate and *O*-methylating detoxification of Tetrabromobisphenol A (TBBPA) in two earthworms (*Metaphire guillelmi* and *Eisenia fetida*)☆

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ABSTRACT

Tetrabromobisphenol A (TBBPA) is the world's most widely used brominated flame retardant but there is growing concern about its fate and toxicity in terrestrial organisms. In this study, two ecologically different earthworms, *Metaphire guillelmi* and *Eisenia fetida*, were exposed to soil spiked with ^{14}C-labeled TBBPA for 21 days. *M. guillelmi* accumulated more TBBPA than *E. fetida*, evidenced by a 2.7-fold higher ^{14}C-uptake rate and a 1.3-fold higher biota-soil accumulation factor. Considerable amounts of bound residues (up to 40% for *M. guillelmi* and 18% for *E. fetida*) formed rapidly in the bodies of both earthworms. ^{14}C accumulated mostly in the gut of *M. guillemi* and in the skin of *E. fetida*, suggesting that its uptake by *M. guillelmi* was mainly via gut processes whereas in *E. fetida* epidermal adsorption predominated. The TBBPA transformation potential was greater in *M. guillelmi* than in *E. fetida*, since only 5% vs. 34% of extractable ^{14}C remained as the parent compound after 21 days of exposure. Besides polar metabolites, the major metabolites in both earthworms were TBBPA mono- and dimethyl ethers (*O*-methylation products of TBBPA). Acute toxicity assessments using filter paper and natural soil tests showed that the methylation metabolites were much less toxic than the parent TBBPA to both earthworms. It indicated that earthworms used *O*-methylation to detoxify TBBPA, and *M. guillelmi* exhibited the higher detoxification ability than *E. fetida*. These results imply that if only the free parent compound TBBPA is measured, not only bioaccumulation may be underestimated but also its difference between earthworm species may be misestimated. The species-dependent fate of TBBPA may provide a better indicator of the differing sensitivities of earthworms to this environmental contaminant.

1. Introduction

Tetrabromobisphenol A (TBBPA) is widely used as an additive or reactive component in polymers and electronics and accounts for ~60% of the total brominated flame retardant (BFR) market (Cruz et al., 2015). TBBPA has been frequently detected in the environment, including its bioaccumulation in aquatic organisms, plants, and humans (Cariou et al., 2008; Covaci et al., 2009; Cruz et al., 2015; Li et al., 2011; Liu et al., 2016; Sun et al., 2014). However, there is little information on its occurrence in terrestrial animals. Despite its more extensive application, relatively low concentrations of TBBPA generally at ppb level have been measured in soils, sediments and biota (EFSA, 2011; Luigi et al., 2015; Morris et al., 2004), and occasionally the concentrations at ppm level were observed in contaminated sites (Covaci et al., 2009; Liu et al., 2016), such that there has been less concern about TBBPA than other major BFRs (Cruz et al., 2015).

Conventional assessment methods based on solvent extraction may have greatly underestimated the real accumulation of TBBPA as they consider only the extractable fraction of the parent compound and ignore the fact that TBBPA can form non-extractable (bound) residues in soil/sediment and biota. Li et al. (2015a,b) reported that bound ^{14}C accounted for >50% of the initially applied ^{14}C-TBBPA in soil and sludge. Bound residues of organic compounds can also be formed by covalent binding to the cellular components of organisms (Shan et al., 2010). For example, many organic pollutants rapidly formed large amounts of bound residues in

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earthworms (Belden et al., 2011; Huang et al., 2017; Liu et al., 2015; Sarrazin et al., 2009; Shan et al., 2010). In addition, because TBBPA is chemically reactive it undergoes biotransformation, and several biotransformation pathways have been described (An et al., 2011; George and Häggblom, 2008; Li et al., 2014, 2015a,b; Liu et al., 2013; Sun et al., 2014). A major transformation pathway is the *O*-methylation of TBBPA under oxic conditions to form TBBPA mono- and dimethyl ethers (MeO-TBBPA and diMeO-TBBPA, respectively). In fact, diMeO-TBBPA was detected at even higher levels than TBBPA in mussels (Watanabe et al., 1983) and sediments (Sellström and Jansson, 1995), although its origin in the mussels and sediments is unclear. Studies of the toxicity of TBBPA metabolites are important to better understand the mechanisms of TBBPA toxicity in terrestrial and other organisms and to accurately evaluate the environmental risks of TBBPA. Previous reports demonstrated differences in the toxicity of the debromination and methylation metabolites of TBBPA vs. the parent compound in aquatic organisms (Debenest et al., 2010; McCormick et al., 2010), but whether this is also the case in terrestrial organisms is unknown. While it seems likely that the fate and metabolism of organic compounds will determine their specific toxicity in organisms, this has yet to be demonstrated for TBBPA.

Earthworms occupy an important position in the terrestrial ecosystem and are thus a frequently used bioindicator for soil pollution risk assessments. The bioaccumulation and toxicity of xenobiotics are often studied in epigeic *Eisenia fetida*, a standard test species (OECD, 2004, 2010). However, conclusions based only on the results from *E. fetida* are unlikely to reflect the true risk of soil pollutants because this earthworm species is not typically found in mineral soils and its sensitivity to pollutants is very different from that of other ecological earthworm groups (Chen et al., 2017; Fourie et al., 2007; Pelosi et al., 2013; Qiu et al., 2014). Thus, soil-dwelling, local earthworm species may be better indicators of soil health. *Metaphire guillelmi* is an anecic and geophagous species widely found in China and the internal behavior of several chemicals in its body strongly differed from that in *E. fetida* (Chen et al., 2017; Li et al., 2016; Wang et al., 2014). In a previous study we showed that *M guillelmi* used strategies different from those of *E. fetida* with respect to the uptake and subcellular distribution of cadmium, such that it was much more sensitive to cadmium toxicity than *E. fetida* (Chen et al., 2017). *M. guillelmi* also differed from *E. fetida* in its uptake and depuration of organic pollutants, as demonstrated for hexabromocyclododecane (HBCD) (Li et al., 2016) and atrazine (Wang et al., 2014). Thus, parallel studies in *M. guillelmi* and *E. fetida* will provide important information on species-specific sensitivity to TBBPA and the respective underlying mechanisms.

In this work, we used the radio-tracer ^{14}C-TBBPA to investigate the uptake kinetics of TBBPA in *M. guillelmi* and *E. fetida*. By following the tissue distribution of the radioactivity in both species, we were able to identify the different routes of TBBPA uptake. In addition, we characterized the metabolites of TBBPA and determined the kinetics of their formation in the two earthworm species to establish the relevant metabolic pathways. Finally, we evaluated the toxicity of TBBPA and its main metabolites in the earthworm bodies. Our results will provide insights into the internal mechanisms of TBBPA toxicity dependent on earthworm species.

2. Materials and methods

2.1. Chemicals

Uniformly ^{14}C-ring-labeled TBBPA (^{14}C-TBBPA) was synthesized from ^{14}C-ring-labeled phenol, which was first converted to ^{14}C-ring-labeled BPA and then brominated (Li et al., 2015a). ^{14}C-TBBPA has a specific radioactivity of 7.4×10^8 Bq/mmol and a chemical purity of 99%. Nonlabeled TBBPA was purchased from Sigma-Aldrich (97% purity).

MeO-TBBPA and diMeO-TBBPA were synthesized according to the method of George and Häggblom (2008), with modifications [see Supporting Information (SI)]. In brief, TBBPA in acetone was reacted with iodomethane (CH_3I, ≥98% purity, Habo Medical Technology, China) and potassium carbonate under reflux conditions. By modifying the ratio of TBBPA to CH_3I and the reaction time, a mixture of MeO-TBBPA and diMeO-TBBPA was obtained. Separation of the mixture on a preparative silica gel column yielded the two compounds, each with a purity >99%.

2.2. Earthworms

E. fetida and *M. guillelmi* were obtained from an earthworm breeding farm in Jurong, Zhenjiang, China and acclimated in non-spiked soil at 20 °C for at least 2 weeks before use. Adult (with clitellum) earthworms with a fresh weight of 0.50–0.60 g (*E. fetida*) and 2.50–3.00 g (*M. guillelmi*) were depurated for 24 h in the dark on moist filter paper prior to the experiments. The gravimetrically determined lipid contents (Qi and Chen, 2010) of *E. fetida* and *M. guillelmi* were 10.5% and 6.2% (dry-weight based), respectively.

2.3. Soils and spiking procedure

A clay loam soil (34.7% clay, 36.1% silt, and 29.2% sand) was sampled from a paddy rice field in Wujin, Changzhou, China. The soil had a pH of 6.91 (0.01 M $CaCl_2$), an organic carbon (OC) content of 0.32%, and a maximum water-holding capacity (MWHC) of 60%. Sterilized soil was prepared by autoclaving the soil at 120 °C for 2 h thrice on three consecutive days. Worm-processed soils (WPSs) were obtained by incubating one *M. guillelmi* individual (WPS-M) or five *E. fetida* individuals (WPS-E) in 30 g soil (dry weight, 40% MWHC) for 14 days at 20 °C. All soils were air-dried, gently ground, and sieved to 2 mm prior to use.

The soils were spiked with a methanolic solution of ^{14}C-TBBPA and nonlabeled TBBPA, yielding radioactivity and TBBPA concentrations of 1.5 kBq g^{-1} and 10 mg kg^{-1} soil (dry weight), respectively. The addition of nonlabeled TBBPA was to increase the accumulated amount of TBBPA and its metabolites in the earthworms for identification. The soils were thoroughly mixed and left to stand in a fume hood overnight to evaporate the solvent. To verify the homogeneity of the TBBPA distribution in the soils, the radioactivity of three randomly taken soil subsamples was determined by combustion on a biological oxidizer (see SI). The recovery was 98%, with a standard deviation (SD) of 1.7%. The soil moisture was adjusted to 40% MWHC using ultrapure water and equilibrated for several hours. In soil incubation experiments (see SI), the fate of ^{14}C-TBBPA in soils, including its mineralization and distribution as extractable residues and nonextractable residues [fulvic acid (FA), humic acid (HA) and humin bound residues], was analyzed (see SI). The metabolite analyses were carried out using the same method as in the earthworms (see below).

2.4. Uptake experiments

Spiked soils prepared as described above were incubated at 20 °C for 14 days to mimic the aging of TBBPA in soils in nature. Uptake experiments were then started in six 1000-mL glass jars each containing 180 g (dry mass) of the spiked soil. Seven *M. guillelmi* or 35 *E. fetida* individuals were transferred to each of triplicate jars. The jars were closed with a rubber lid and incubated at 20 °C in the dark for 21 days. During the exposure time, the lids were opened for several minutes every day to ensure sufficient