Environmental Pollution xxx (2017) 1–7

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Antioxidant gene expression and metabolic responses of earthworms (*Eisenia fetida*) after exposure to various concentrations of hexabromocyclododecane☆

Yajuan Shi [a], Xiangbo Xu [b, *], Juan Chen [a, c], Ruoyu Liang [a, c], Xiaoqi Zheng [b], Yajing Shi [d], Yurong Wang [d]

[a] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
[b] School of Environment & Natural Resources, Renmin University of China, Beijing 100872, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] School of Biomedical and Chemical Engineering, Liaoning Institute of Science and Technology, Benxi 117004, China

ARTICLE INFO

Article history:
Received 9 January 2017
Received in revised form
28 August 2017
Accepted 9 September 2017
Available online xxx

Keywords:
HBCD
Earthworms
Gene expression level
Metabolomics
Principal component analysis

ABSTRACT

Hexabromocyclododecane (HBCD), a ubiquitous suspected contaminant, is one of the world's most prominent brominated flame retardants (BFRs). In the present study, earthworms (*Eisenia fetida*) were exposed to HBCD. The expression of selected antioxidant enzyme genes was measured, and the metabolic responses were assessed using nuclear magnetic resonance (NMR) to identify the molecular mechanism of the antioxidant stress reaction and the metabolic reactions of earthworms to HBCD. A significant up-regulation ($p < 0.05$) of superoxide dismutase (SOD) gene expression was detected, with the highest gene expression level of SOD appearing at a dose of 400 mg kg^{-1} dw (2.06-fold, $p < 0.01$). However, the glutathione transferase (GST) gene expression levels did not differ significantly ($p > 0.05$). Principal component analysis (PCA) of the metabolic responses showed that all groups could be clearly differentiated, and the highest concentration dose group was the most distant from the control group. Except for fumarate, the measured metabolites, which included adenosine triphosphate (ATP), valine, lysine, glycine, betaine and lactate, revealed significant ($p < 0.05$) increases after 14 days of exposure to HBCD. HBCD likely induces high levels of anaerobic respiration, which would result in high levels of ATP and lead to the disintegration of proteins into amino acids, including valine and lysine, to produce energy. The observed changes in osmotic pressure were indicative of damage to the membrane structure. Furthermore, this study showed that NMR-based metabolomics was a more sensitive tool than measuring the gene expression levels for elucidating the mode of toxicity of HBCD in earthworm exposure studies.

1. Introduction

Hexabromocyclododecane (HBCD) is one of the world's most ubiquitous brominated flame retardants (BFRs), as well as tetrabromobisphenol A (TBBPA) and decabromodiphenyl ether (BDE209) (Zegers et al., 2005). As a bromide flame retardant additive, HBCD is widely used in high impact polystyrene, textile coatings, and fire-retardant materials (POPRC, 2011). The global production of HBCD was approximately 23,000 tons in 2011 (Programme, 2011). The production and use of HBCD has been steadily increasing due to the growth of market demand in the civil construction industry, and the demand is expected to continue to increase into the next decade (Zhu et al., 2015). However, HBCD was listed in Annex A of the Stockholm Convention on Persistent Organic Pollutants (POPs) in May 2013, implying that HBCD should be phased out gradually all over the world (Zhu et al., 2015).

HBCD easily separates from other material constituents and migrates into the environment. Currently, HBCD has been detected in various environmental media, including river water, soil, air, sea water, food and human serum, even in arctic regions (Roosens et al., 2009; Ni and Zeng, 2013; Ichihara et al., 2014; Kim and Oh, 2014; Ryan and Rawn, 2014; Barghi et al., 2016; Law et al., 2005). Total

☆ This paper has been recommended for acceptance by Prof. von Hippel Frank A..

* Corresponding author.
E-mail address: xuxiangbo.hi@163.com (X. Xu).

https://doi.org/10.1016/j.envpol.2017.09.039
0269-7491/

HBCD concentrations range from 0.2 to 6.9 μg kg^{-1} dry weight in soil (Law et al., 2008). HBCD is transported into the soil from the sludge generated by the collection of waste water from sewage pipe networks and from landfill and fertilizer runoff. From the contaminated soil, this chemical is absorbed by crops, threatening human health. HBCD can be bio-accumulated, is environmentally persistent, is transported over large distances, and has the potential to significantly damage human health and the environment (Xiang et al., 2014). The overall toxicity of HBCD has been reported using several model species. The gene expression level of heat shock protein 70 (Hsp70) was significantly up-regulated when earthworms (*Eisenia fetida*) were exposed to HBCD at 400 mg kg^{-1} (Shi et al., 2015). In addition, the mRNA expression of cytochrome P450s (CYPs) in chicken (*Gallus gallus*) hepatocytes was up-regulated after exposure to HBCD (Crump et al., 2008). Moreover, the liver function and thyroid hormone status of female rats were also affected by HBCD (Miller et al., 2016). In several in vitro studies, increased production of reactive oxygen species (ROS) induced cell apoptosis in HepG2 cells and human L02 hepatocytes that were exposed to high concentrations of HBCD (An et al., 2013, 2014; Hu et al., 2009).

The earthworm, a representative macro-invertebrate, is sensitive to chemicals and is available commercially. This species has been validated as a model species for investigations of the toxic effects of chemicals on soil ecosystems (OECD, 1984). Earthworms defend against oxidative stress and remove ROS by up-regulating the transcription of relevant genes, such as superoxide dismutase (SOD), glutathione transferase (GST) and catalase (CAT) (Xie et al., 2011; Wang et al., 2015; Xu et al., 2015; Hu et al., 2016). The regulation of metabolites also protects cells from toxic chemicals. NMR-based metabolomics provides a promising method of molecular analysis and is a reproducible technique for the direct identification of potential biomarkers of environmental contaminants in earthworms (Bundy et al., 2008; Brown et al., 2010; Mckelvie et al., 2010; Whitfield Åslund et al., 2011a, b; Lankadurai et al., 2012; Yuk et al., 2013). Principal component analysis (PCA) can be used to identify changes in the endogenous metabolites after HBCD exposure (Mckelvie et al., 2009). In contaminant-exposed earthworms, changes in metabolites such as alanine, leucine, glutamate, asparagine, valine, isoleucine, phenylalanine and glutamate, the sugars glucose and maltose, the osmolyte betaine, adenosine triphosphate (ATP), and fumarate have all been implicated as potential bio-indicators (Mckelvie et al., 2009; Lankadurai et al., 2012, 2013a, 2015; Brown et al., 2014). The metabolomic responses of HBCD-exposed earthworms have not yet been investigated.

The earthworm intensely bioaccumulates HBCD, such that the HBCD concentrations in earthworm can be 20 times higher than concentrations in artificially contaminated soil after 21 days of exposure to HBCD (Li et al., 2015). Using earthworms as a model species, the toxicity of HBCD has been examined in a few studies. Although growth was not significantly inhibited by HBCD, the stress-response gene (Hsp70) of earthworms was significantly up-regulated (Shi et al., 2015). However, little is known regarding the antioxidant gene expression and metabolic responses in earthworms exposed to HBCD, and these topics warrant more thorough study.

In this study, the gene expression levels of SOD and GST and the metabolic response (ATP, valine, lysine, glycine, fumarate, betaine and lactate) assessed using NMR were determined in HBCD-exposed earthworms to characterize the toxicity of HBCD. Antioxidant enzymes such as SOD and GST can protect organisms from oxidative damage by removing ROS. Changes in the levels of metabolites are indicative of the earthworm reaction to exposure to HBCD. This study may thus aid in understanding the molecular mechanism of the antioxidant defense and the metabolic mechanisms of the earthworm response to HBCD and provide a scientific database for assessing risk to soil ecosystems. Furthermore, this study provided early markers of toxic damage and evaluated NMR-based metabolomics as a routine ecotoxicological tool for the assessment of the toxicity of HBCD in soil environments.

2. Materials and methods

2.1. Tested earthworms and chemicals

Mature earthworms (0.35–0.45 g) with well-developed clitella were purchased from an earthworm company (Runfeng Company) in Beijing. Before the start of the test, healthy earthworms were placed in artificial soils to acclimatize them for 24 h. To void the earthworms' gut contents before the start of the exposure test, the earthworms were rinsed in deionized water and moved to petri dishes on damp filter paper (in the dark at 20 ± 1 °C, 24 h).

Hexabromocyclododecane (HBCD) (CAS No.3194-55-6, 95.0% purity) was purchased from TCI Chemicals (Tokyo, Japan). Monobasic sodium phosphate buffer solution ($NaH_2PO_4 \cdot 2H_2O$, 99.3% purity), N-hexane (analytical grade) and sodium azide (99.5% purity) were produced by Sinopharm Chemical Reagent Co, Ltd. (SCRC) (Shanghai, China). D_2O (99.9% purity), NaOD (99.5% purity), and 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS, 97% purity) were purchased from Sigma Aldrich (California, USA). All aqueous solutions were produced using reagent water produced using a Milli-Q Gradient system (Millipore Company, Bedford, USA).

2.2. Toxicological tests

The method for cultivating the earthworms was in accordance with the Guidelines of the OECD (OECD, 1984). The formula for the artificial soils was 70% quartz sand, 20% kaolin clay and 10% sphagnum peat; the moisture content was adjusted to approximately 35% by adding deionized water, and the pH was 6.0 ± 0.5 (adjusted with calcium carbonate).

HBCD was dissolved with acetone (20 mL) and thoroughly mixed into the artificial soils at concentrations of 0, 50, 100, 200, 400 and 600 mg kg^{-1} dry soil (four translucent beakers per group). A mass of 750 g of the wet artificial soil was added to each beaker. The controls (CK) and solvent controls (CKS) comprised four replicates in which HBCD was replaced by deionized water and acetone, respectively. Before adding the earthworms, each beaker containing the artificial soils was placed in an exhaust hood for 2–4 days to allow the acetone to completely evaporate. The beakers, each of which contained 10 earthworms, were placed in an incubation chamber (culture temperature 20 ± 1 °C, relative humidity 83 ± 3%, and continuous illumination at 400–800 lx) throughout the test period.

After exposure for 14 days, 6 earthworms of each replicate were randomly selected for the determination of the levels of gene expression and metabolic responses. Each earthworm was then cleaned using deionized water, stored in petri dishes with moist filter paper to clear out their gut contents for 24 h and ground immediately in liquid nitrogen.

2.3. Gene expression levels

The TRIzol Reagent (Invitrogen, California, USA) was used according to the manufacturer's instructions to isolate the total RNA. The absorbance ratios (A260/280) were confirmed to be between 1.8 and 2.0, and agarose gel electrophoresis (2%) was used to check the RNA purity and integrity. According to the manufacturer's instructions, superscript TMIIreverse transcriptase (Invitrogen, USA)

Please cite this article in press as: Shi, Y., et al., Antioxidant gene expression and metabolic responses of earthworms (*Eisenia fetida*) after exposure to various concentrations of hexabromocyclododecane, Environmental Pollution (2017), https://doi.org/10.1016/j.envpol.2017.09.039