Ecotoxicology and Environmental Safety 158 (2018) 78–86

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Intestinal damage, neurotoxicity and biochemical responses caused by tris (2-chloroethyl) phosphate and tricresyl phosphate on earthworm

Yang Yang[a], Yao Xiao[a], Yeqian Chang[a], Yibin Cui[b], Göran Klobučar[c], Mei Li[a,*]

[a] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210023, PR China
[b] Nanjing Institute of Environmental Sciences, Ministry of Environmental Protection, Nanjing 210042, PR China
[c] Faculty of Science, University of Zagreb, Department of Biology, Division of Zoology, Rooseveltov trg 6, 10000 Zagreb, Croatia

ARTICLE INFO

Keywords:
Histopathological examination
Oxidative stress
DNA damage
Integrated biological response
Eisenia fetida

ABSTRACT

Organophosphate esters (OPEs) draw growing concern about characterizing the potential risk on environmental health due to its wide usage and distribution. Two typical types of organophosphate esters (OPEs): tris (2-chloroethyl) phosphate (TCEP) and tricresyl phosphate (TCP) were selected to evaluate toxicity of OPEs to the soil organism like earthworm (*Eisenia fetida*). Histopathological examination (H&E), oxidative stress, DNA damage and RT-qPCR was used to identify the effects and potential mechanism of their toxicity. Hameatoxylin and eosin (H&E) demonstrated that intestinal cells suffered serious damage, and the observed up-regulation of chitinase and cathepsin L in mRNA levels confirmed it. Both TCEP and TCP significantly increased the DNA damage when the concentrations exceeded 1 mg/kg ($p < 0.01$), and a dose-response relationship was observed. In addition, TCEP and TCP also changed the acetylcholinesterase (AChE) activity and expression of genes associated with neurotoxic effects in earthworms even under exposure to low OPEs concentration (0.1 mg/kg). Moreover, genes associated with nicotinic acetylcholine receptors (nAChR) and carrier protein further demonstrated that highest concentration of TCEP (10 mg/kg) may have an overloading impact on the cholinergic system of *E. fetida*. Integrated Biological Response index (IBRv2) showed that TCEP exerted stronger toxicity than TCP under the same concentrations. We deduced that the observed intestinal damage, oxidative stress and neurotoxic effect might be the primary mechanisms of TCEP and TCP toxicity. This study provides insight into the toxicological effects of OPEs on earthworm model, and may be useful for risk assessment of OPEs on soil ecosystems.

1. Introduction

Organophosphate esters (OPEs) have been widely used as flame retardants (FRs), plasticizers and anti-foaming agents in various consumer products after the prohibition of using polybrominated diphenyl ethers (PBDEs) in recent years (Wei et al., 2015). It has been reported that the production of OPEs went more than 70,000 t in 2007 and was estimated to increase 15% per year in China (Hammel et al., 2016). Given that OPEs are mainly used as additive chemicals that just mixes with but do not chemically bond to polymer matrices, it tend to be migrated into the surrounding environment by volatilization, leaching and abrasion (Bollmann et al., 2012). Increasing concentrations of OPEs have been detected in various environmental compartments such as water, indoor dust, outdoor air and sediment (Bollmann et al., 2012; Cui et al., 2017; Malarvannan et al., 2015; Reemtsma et al., 2008; Salamova et al., 2014). Moreover, OPEs in biotic samples including fishes, birds, and human breast milk had also been reported (Sundkvist et al., 2010; Wei et al., 2015). Because of the widespread distribution, it draws growing concern about characterizing the potential risk of OPEs on environmental health.

Organophosphate esters own the same phosphate base unit and can be classified into three groups including chlororalkyl-, non-chlorinated alkyl-, aryl- phosphates with the difference of their substituent (Quintana et al., 2008). Previous studies showed that nearly sixty percent OPEs have medium to high persistence (> 1700 h) and/or long range transport propertities, including tris (2-chloroethyl) phosphate (TCEP), tricresyl phosphate (TCP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), triphenyl phosphate (TPhP), tris(2-butoxyethyl) phosphate (TBEP). (Suehring et al., 2016; Zhang et al., 2016b). Typically, TCEP, recognized as carcinogenic, highly toxic and environmentally persistent chemical (EC, 2009), has been prohibited for maternal and child supplies. Ta et al. (2014) showed that both TDCPP and TCEP

* Correspondence to: State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, 163 Xianlin Avenue, Nanjing 210023, Jiangsu Province, PR China.
E-mail address: meili@nju.edu.cn (M. Li).

https://doi.org/10.1016/j.ecoenv.2018.04.012
Received 4 January 2018; Received in revised form 4 April 2018; Accepted 6 April 2018

decreased PC12 rat cells growth, increased apoptosis and altered morphology within the concentration of 1–50 μM and 15–400 μM, respectively. Moreover, Li et al. (2017) showed that the environmentally relevant or greater concentrations TDCPP caused significant growth inhibition of *Daphnia magna* at the later stage (≥32 days). Until now, limited studies have described the toxicity of TCEP in soil organisms based on environmentally relevant concentration. Tricresyl phosphate (TCP), aryl OPE, mainly used as hydraulic fluid or lubricant in the industrial processes to regulate pore sizes, is proven to be a neurotoxic compound (de Ree et al., 2014). Wang et al. (2014) found that TCP was high toxic to zebrafish *Danio rerio* under 0.01–0.5 mg/L with induced antioxidative enzymes and enhanced DNA damage. Most toxic compounds could generate the reactive oxygen species (ROS) and alter the biochemistry and ultrastructure of exposed organisms, resulting in the oxidation of proteins and DNA damage (Zhang et al., 2016a). In addition, AChE activity has been widely used for assessing neurotoxicity of OPEs, while the baseline levels change greatly for different studies (Moser et al., 2015; Wang et al., 2015). Gene expression is proposed to provide potential mechanisms of actions of target chemicals for further determination of the cell/molecular process toxicity (Zhang et al., 2014a). It is therefore required to investigate the effects of these chemicals towards systematically toxicological detection methods such as effective enzymatic, histopathological biomarkers and related gene transcription to study the potential toxicity mechanisms based on environmentally relevant concentrations.

Soil is the major terrestrial environmental reservoir comprised of mainly mineral particles and organic matter, with a major sinks for hydrophobic organic compounds such as OPEs (van der Veen and de Boer, 2012; Wei et al., 2015). He et al. (2017) found that the ΣOPEs contents ranged from 10.1 to 315 ng/g dry weight (dw) and from 348 to 1369 ng/g dw in surface soil and street dust samples, respectively from Chongqing, China. Additionally, total concentrations ranging from 0.041 to 1.37 mg/kg dw OPEs were detected in all analyzed soil samples in Guangzhou, China. (Wan et al., 2016; Cui et al., 2017). The highest ΣOPEs concentration reported in the soil observed in Kathmandu Valley, Nepal was 27.5 mg/kg dw (Yadav et al., 2018). However, the latest studies on OPEs have mainly focused attention on aquatic environments (Li et al., 2017; Wang et al., 2017), with little attention being focused on their toxicity in terrestrial environments. Earthworm occupies a pivotal position within soil faunal biomass and plays a key character in metabolism, fertility, and structure of soils. *Eisenia fetida*, proposed to be a well-recognized test organism for evaluating environmental chemicals (Rico et al., 2016; Zhiqun et al., 2017), was selected to explore the potential toxicity of OPEs under environment-related concentration to provide more comprehensive environmental risk assessment data.

In the present study, we assessed toxicity of TCEP and TCP on the earthworm, *E. fetida*, by acute lethal testing, histopathological examination, and measurements of enzyme activities and DNA damage. According to the results of H&E and enzyme activities, 10 related genes were applied to identify the effects of TCEP exposure on gene expression in *E. fetida* and analyze potential mechanisms of toxic action. To our knowledge, the systematic analysis of OPEs basic toxicity on a terrestrial organism was rarely reported. The results of this study aims to enhance our awareness of OPEs contamination in soil and their potential risk for terrestrial organisms.

2. Materials and methods

2.1. Chemicals and earthworms

TCEP (purity > 97%) and TCP (mixture of isomers, purity > 98%, ortho-isomers < 1%) were obtained from TCI Development Co, Ltd. (Shanghai, China). The earthworm *E. fetida* were purchased from a local farming factory (Jurong, China) and cultured in artificial soil based on the OECD (1984). Healthy adult earthworms approximately 300–600 mg with well-developed clitellum were chosen for exposure experiments.

2.2. Artificial soil treatment and body weight measurements

Artificial soil treatment were conducted in a wide-mouth bottle (1 L) with 0.5 kg dry artificial soil (water content of 35% (w/w)) according to the OECD guidelines (OECD, 1984). Prior to the experiment, the earthworms were acclimatised in artificial soil under the same conditions described for culture maintenance for 24 h. Earthworms were then added to the bottles containing 0, 0.1, 1, and 10 mg/kg TCEP or TCP of dry soil. The bottles were covered with plastic foil that had small holes and placed in an incubator with continuous light source (12:12 h light: dark cycle) at 20 ± 1 °C for 14 days. Each bottle contained 10 earthworms and each treatment consisted of 6 bottles/replicates, named R1 to R6. One earthworm from each of the first three replicates (R1-R3) was sacrificed for the comet assay (three earthworms in total) and another one for the biochemical examinations (also three earthworms in total) after 3, 7 and 14 days exposure. After 3, 7 and 14 days exposure, three earthworms from R4 to R6 were taken out and checked for mortality and weighed for growth rate. At the end of 14 days exposure, three adult earthworms from replicates R4 to R6 were sampled for histopathological analyses of the intestinal epithelium and another three earthworms from each treatment with 3 replicates were sampled for RNA extraction. Every individual was extracted gently from the soil and placed on moist filter paper for 24 h to empty their gut content. Samples for histopathological analyses and comet assay were processed immediately. Samples for biochemical analyses were kept frozen until all samples were prepared and samples for RNA extraction were cut into pieces and kept frozen in sample protector for RNA (Takara Biotechnology, Dalian, China) solution.

2.3. Histopathological examination

The earthworms were placed on cold filter paper for 10 min prior to dissection (Sorour and Larink, 2001). The middle parts of body were transversely cut and then placed in a 10% formalin buffer (pH 7.0 ± 0.2) for at least 24 h. Tissues were then paraffin-embedded and sliced vertically for 4 μm thick with microtome. Sections of 4 μm thickness were subjected to hematoxylin-eosin (H&E) staining and examined by light microscopy (CKX31, Olympus, Japan) for histopathological assessment.

2.4. Comet assay

DNA damage of earthworm coelomocytes were determined by the comet assay. Coelomocytes extraction was carried out according to the non-invasive extrusion method of Eyambe et al. (1991) and the DNA strand breaks level was examined using the method reported by Singh et al. (1988). After being stained with ethidium bromide, slides were viewed with a fluorescent microscope (BX41, Olympus, Japan). Six slides were examined for each group, and at least 50 cells on each slide were analyzed. A digital camera (C-5050ZOOM, Olympus, Japan) was used to take photos and CASP software were used to analyze images by the method of Collins et al. (1995). The extent of DNA damage was determined by the percentage of tail DNA (% tDNA) and Olive tail moment (OTM).

2.5. GSH, AChE and 8-OHdG analyses

Sampled earthworms after 3, 7 and 14 days exposure were taken out from the refrigerator and cut into pieces with ice-cold PBS solution after being washed with distilled water. The mixture was homogenized in ice bath by ultrasonic processor (Biosafer 650–92, China), centrifuged at 3000 × *g* in 4 °C for 10 min and the supernatants were used for further analyses.