Neurotoxicology and Teratology 55 (2016) 16–22

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

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

# Developmental exposure of zebrafish larvae to organophosphate flame retardants causes neurotoxicity

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ARTICLE INFO

*Article history:*
Received 6 December 2015
Received in revised form 14 March 2016
Accepted 20 March 2016
Available online 24 March 2016

*Keywords:*
Nervous system
Acetylcholinesterase (AChE) activity
Gene transcriptional analysis
Locomotor behavior
Early life stage
Aquatic organism

ABSTRACT

With the gradual ban on brominated flame retardants (FRs), the application of organophosphate flame retardants (OPFRs) has increased remarkably. Considering the structural similarity between OPFRs and organophosphate pesticides, hypotheses that OPFRs may interfere with neurodevelopment as organophosphate pesticides are reasonable. In this study, the neurotoxicity of three OPFRs, including tri-n-butyl phosphate (TNBP), tris (2-butoxyethyl) phosphate (TBOEP) and tris (2-chloroethyl) phosphate (TCEP), was evaluated in zebrafish larvae and then compared with the neurotoxicity of organophosphate pesticide chlorpyrifos (CPF). The results showed that similar to CPF, exposure to OPFRs for 5 days resulted in significant changes in locomotor behavior, either in free swimming or in photomotor response. However, given the transcriptional changes that occur in nervous system genes in response to OPFRs and CPF, as well as the altered enzyme activity of AChE and its mRNA level, the underlying mechanisms for neurotoxicity among these organophosphate chemicals might be varied. In summary, the results confirm the potential neurodevelopmental toxicity of OPFRs and underscore the importance of identifying the mechanistic targets of the OPFRs with specific moieties. Furthermore, as the neurobehavioral responses are well conserved among vertebrates and the exposure of children to OPFRs is significant, a thorough assessment of the risk of OPFRs exposure during early development should be highly emphasized in future studies.



## 1. Introduction

Because of the increased use of flammable plastics and electronic devices, along with stricter legislation of fire-safety standards, flame retardants (FRs) have been extensively used at large scales (Hendriks and Westerink, 2015; Noyes et al., 2015; Wei et al., 2015). However, due to extensive reports on their persistence, long-range atmospheric transport, bioaccumulation and/or toxicity, brominated flame retardant substances, which used to be the dominant class of FRs, were banned or phased out in many countries (van der Veen and de Boer, 2012; Wei et al., 2015). Accordingly, organophosphate flame retardants (OPFRs) were proposed as alternatives, resulting in dramatically increased production and usage in recent years. In 1992, OPFRs use globally totaled only 100,000 tons, whereas the consumption amounted to 500,000 tons in 2011 and is expected to reach 680,000 tons in 2015 (van der Veen and de Boer, 2012; Wei et al., 2015). In addition to their broad application, OPFRs are mainly utilized as additives which results in steady emission into the environment throughout the lifetimes of the products (Marklund et al., 2005; van der Veen and de Boer, 2012). Nowadays, OPFRs are ubiquitous environmental contaminants, and their concentrations are sometimes reported to be even higher than those of brominated FRs in diverse environmental matrices (Hendriks and Westerink, 2015). More recently, studies have begun to document the increasing exposure and bioaccumulation of OPFRs in wildlife and humans (Noyes et al., 2015; Wei et al., 2015).

Water is a preferred medium of distribution for OPFRs (Verbruggen et al., 2006). Although great variation exists in their physical properties, OPFRs are typically water-soluble (Li et al., 2014; van der Veen and de Boer, 2012), and can enter the aquatic environment via different paths. Wastewater discharge is the primary entry pathway into surface water, and then groundwater is affected through infiltration (Wei et al., 2015). The other important pathway was precipitation, especially for

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http://dx.doi.org/10.1016/j.ntt.2016.03.003
0892-0362/

the areas away from the emission sources, including the remote lake water and marine offshore waters (Wei et al., 2015). The first studies on the occurrence and fate of OPFRs in the aquatic environment were published in the late 1970s (Saeger et al., 1979; Sheldon and Hites, 1978). Since that time, a number of published studies have documented the rising levels of OPFRs in waters, including sludge treatment plant influents and effluents, river water, marine water, groundwater, and even drinking water, with concentrations ranging from several ng/L to tens of μg/L (Li et al., 2014; Wei et al., 2015). As reviewed in (Wei et al., 2015), the highest ΣOPFRs levels were found in raw water from a Japanese sea-based solid waste disposal site in which the concentration of the most prominent compound, TCEP, reached up to 87.4 μg/L (Kawagoshi et al., 1999), whereas in river water, the highest ΣOPFRs concentrations (113–26,300 ng/L) were found along the River Aire, UK (Cristale et al., 2013). Therefore, the considerable concentrations of OPFRs in the aquatic environment indicate that aquatic organisms, especially fish, appear to be at increasing risk.

During the early development stages, the brain undergoes a series of extraordinary changes and therefore is extremely sensitive to many environmental contaminants (Rice and Barone, 2000). Interference with one or more of these development processes can thereby negatively affect brain function and behavior, resulting in persistent neurological deficits into later life (Fan et al., 2010). Other classes of organophosphates, such as organophosphate pesticides, have been confirmed to interfere with neurodevelopment (Eaton et al., 2008). Considering the structural similarity between OPFRs and organophosphate pesticides, exposure to OPFRs in early life may adversely affect the developing nervous system as well. However, the data regarding neurotoxicity of OPFRs is relatively scarce or paradoxical. For instance, previous studies have been performed on the neurotoxicity of triphenyl phosphate (TPHP) but with different conclusions. Both Andresen et al. (2004) and Ni et al. (2007) reported that TPHP is possibly neurotoxic. In contrast, Pakalin et al., (2007) only mentioned a low neurotoxicity, and Danish EPA (Lassen and Lokke, 1999) found no evidence for TPHP neurotoxicity in animal experiments. In a human epidemiology study, no symptoms or physical or laboratory findings were detected on the operators in a TPHP production plant (Agency for Toxic Substances and Disease Registry, 2009). The lessons learned from the adverse effects of brominated FRs clearly underline the caution needed when using OPFRs as an alternative when they have not yet been fully characterized (Hendriks and Westerink, 2015); therefore, the knowledge regarding toxicological properties of OPFRs, including neurotoxicity, are urgently required.

The objective of the present study, therefore, was to investigate the impacts of OPFRs on developmental neurotoxicity in the early life stage of zebrafish. In the past two decades, a vast amount of zebrafish research has been conducted. The small size, high fecundity and rapid external development of the zebrafish make it a favored vertebrate model for rapidly screening chemicals (Dishaw et al., 2014). As mounting evidence suggests the neurobehavioral responses of zebrafish resemble that of rodents (Dishaw et al., 2014; Noyes et al., 2015), developmental research employing zebrafish is becoming increasingly common in behavioral neurotoxicology. In this study, the effects of three OPFRs with different substituents, including halogenated (tris (2-chloroethyl) phosphate, TCEP), alkyl (tri-n-butyl phosphate, TNBP) and butoxy (tris (2-butoxyethyl) phosphate, TBOEP), were evaluated and compared with that of organophosphate pesticide chlorpyrifos (CPF), a widely recognized developmental neurotoxicant. In addition to the locomotor behaviors, the enzyme activity and gene transcription of acetylcholinesterase (AChE), as well as the transcriptional response of several marker genes related to the nervous system, were examined to reveal the potential underlying mechanism. This work aimed to test the hypothesis that the OPFRs and the organophosphate pesticides might share similar impairments on the neurodevelopment due to their structural similarity, and was anticipated to advance our knowledge about the toxicity of OPFRs to aquatic organisms.

## 2. Materials and methods

### 2.1. Chemicals

The following OPFRs tested were all purchased from Sigma-Aldrich: TNBP (CAS: 126-73-8; purity > 99%), TBOEP (CAS: 78-51-3; purity: 94%), and TCEP (CAS: 115-96-8; purity: 97%). The abbreviations of OPFRs followed the naming convention established by Bergman et al. (2012). CPF (CAS: 2921-88-2; purity > 99%) was obtained from Sigma-Aldrich as well. The stock solutions and serial dilutions were prepared in HPLC DMSO. All other chemicals were analytical or HPLC grade.

### 2.2. Fish husbandry and chemical exposure

The zebrafish (*Danio rerio*) originated from the Institute of Hydrobiology of the Chinese Academy of Science (Wuhan, China). The fish maintenances were conducted according to the method of Westerfield (2000) with minor modification (Sun et al., 2010). Adult fish pairs were transferred into mating tanks in the morning, 1 h prior to onset of the light cycle. After spawning, fertilized embryos were collected, disinfected (Westerfield, 2000), and maintained under the same conditions as the adults for the following test procedures.

The embryos (<2 h post fertilization (hpf)) were randomly transferred into individual wells of 96-well plates (Corning, NY, USA) containing 100 μL of chemical solution. Based on the results of a previous range-finding study, the exposure concentrations were as the following: TNBP at 25, 125, 625 and 3125 μg/L, TBOEP at 50, 250, 1250 and 6250 μg/L, and TCEP at 50, 250, 1250 and 6250 μg/L; and control groups were administered 0.01% (vol/vol) DMSO. Moreover, CPF-exposed groups (10, 30, 100 and 300 μg/L) were also included. Twenty embryos in one plate were used for one replicate, with triplicate plates for each treatment. The test duration was 5 days with daily solution changes. The fish were observed three times daily, and any dead ones were recorded and removed. No treatment-related effects were found in the numbers of dead or malformed larvae for any chemical tested.

### 2.3. Locomotor behavior assay

After 5 days of exposure, the locomotor activity was measured by the Zebralab Video-track system (ViewPoint Life Science, France) in the afternoon (at 14:00) as the activity was relatively stable during this time span and the variability between larvae was lowest (MacPhail et al., 2009). After 10 min of acclimation, free swimming activities under continuous visible light (30 min) and the swimming responses to a 10 min dark-to-light transitions were monitored (Chen et al., 2012a; Jin et al., 2015a, 2015b). The data of the dead and malformed larvae were excluded from subsequent analysis.

### 2.4. Gene transcription analysis

After locomotor behavior assay, the total RNA of whole larvae was extracted using RNAiso Plus (Takara, Dalian, China). About fifteen larvae were pooled into one sample, and four biological replicates were used for each treatment. First-strand cDNA was synthesized using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), and real-time PCR with SYBR Green detection (Toyobo, Osaka, Japan) was performed using a Mastercycler ep realplex system (Eppendorf, Hamburg, Germany). The primer sequences for the target genes (namely *ache*, *elavl3*, *gap43*, *gfap*, *mbp*, *shha*, *syn2a* and *α1-tubulin*) involved in the nervous system, as well as the related information, were summarized in the Supplemental data, Table S1. The mRNA levels were expressed relative to the transcription level of the reference gene *β-actin*, and the quantification of target gene transcription was calculated using the comparative cycle threshold (Ct) method (Livak and Schmittgen, 2001).