Accepted Manuscript

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PII: S0166-445X(14)00329-4
DOI: http://dx.doi.org/doi:10.1016/j.aquatox.2014.11.001
Reference: AQTOX 3965

To appear in: Aquatic Toxicology

Received date: 5-8-2014
Revised date: 23-10-2014
Accepted date: 1-11-2014

Please cite this article as: Wang, Q., Lam, J.C.-W., Man, Y.-C., Lai, N.L.-S., Kwok, K.Y., Guo, Y., Lam, P.K.-S., Zhou, B., Bioconcentration, Metabolism and Neurotoxicity of the Organophorous Flame Retardant 1,3-dichloro 2-propyl phosphate (TDCPP) to Zebrafish, Aquatic Toxicology (2014), http://dx.doi.org/10.1016/j.aquatox.2014.11.001

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Bioconcentration, Metabolism and Neurotoxicity of the Organophorous Flame Retardant 1,3-dichloro 2-propyl phosphate (TDCPP) to Zebrafish

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Abstract

Organophosphate flame retardants are ubiquitous environmental contaminants; however, knowledge is limited regarding their environmental health risks and toxicity. Here, we investigated the effects of acute and long-term exposure to tris (1, 3-dichloro-2-propyl) phosphate (TDCPP) to the nervous system of zebrafish. Zebrafish embryos (2 h post-fertilization) were exposed to TDCPP (0–100 μg/L) for 6 months up until sexual maturation. Concentrations of TDCPP and its metabolic product (bis(1,3-dichloro-2-propyl) phosphate, BDCPP) were measured in the tissues of 5 day post-fertilization (dpf) larvae. There was no effect on locomotion, acetylcholinesterase activity, levels of the neurotransmitters dopamine and serotonin, and expression of mRNAs and proteins related to central nervous system development (e.g., myelin basic protein, α1-tubulin) in any exposure group. However, in adult fish, reductions of dopamine and serotonin levels were detected in the brains of females but not males. Downregulation of nervous system development genes was observed in both the male and female brain tissues. TDCPP concentrations were measured in adult fish tissues including the brain, and greater levels were detected in females. Our results showed that females are more sensitive to TDCPP stress than males in terms of TDCPP-induced neurotoxicity. We demonstrate that long-term exposure to lower concentrations of TDCPP in fish can lead to neurotoxicity.

Key words: TDCPP; bioconcentration and metabolism; neurotoxicity; gender-specific toxicity; zebrafish
**Abbreviations**

Acetylcholinesterase (AChE); Bis(1,3-dichloro-2-propyl) phosphate (BDCPP); Organophosphorus (OP); Days post-fertilization (dpf); Deuterated tri-n-propyl phosphate (d21-TPrP); Deuterated trimethyl phosphate (d9-TMP); Dimethylsulfoxide (DMSO); Glial fibrillary acidic protein (gfap); Hours post-fertilization (hpf); Liquid chromatograph (LC); Myelin basic protein (mbp); Organophosphorus flame retardants (OPFRs); Polybrominated diphenyl ethers (PBDEs); Standard error (SEM); Synapsin IIa (syn2a); Thyroid hormone (TH); Tris(2-chloro-1-methylethyl) phosphate (TCPP); Tris (1, 3-dichloro-2-propyl) phosphate (TDCPP).
1. Introduction

Organophosphorus flame retardants (OPFRs) have been used as additives in various household and industrial products, such as electronic equipment, plastic products, textiles, furniture and building materials (Reemtsma et al., 2008). Due to the adverse health effects caused by brominated flame retardants, such as polybrominated diphenyl ethers (PBDEs), many industrial countries have banned or phased-out PBDEs, and OPFRs have been proposed as alternatives (Stapleton et al., 2009). Consequently, the production and usage of OPFRs has increased and now they are ubiquitous environmental contaminants and they are also detectable in biota samples (Stapleton et al., 2009; van der Veen and de Boer, 2012). In river water, OPFR concentrations are generally at ng–μg/L levels (Martínez-Carballo et al., 2007; Regnery and Püttmann, 2010; Cristale et al., 2013), and highest concentrations of tris(2-chloro-1-methylethyl) phosphate (TCPP) were reported in United Kingdom rivers (up to 26 μg/L) (Cristale et al., 2013). OPFRs have been detected in river sediments too, and here they can reach up to 1300 μg/kg as reported for the River Schwechat in Austria (Martínez-Carballo et al., 2007). In marine fish, concentrations of OPFRs can reach 2000 ng/g lipid weight (Kim et al., 2011).

Tris (1, 3-dichloro-2-propyl) phosphate (TDCPP) is an OPFR that is commonly used as an additive in polyurethane foam padding used in furniture, children's foam products and in upholstery for automobiles (Dishaw et al., 2011, Stapleton et al., 2011, 2012). TDCPP is found in indoor dust at high concentrations (Stapleton et al., 2009), while in the aquatic environment, TDCPP concentrations in surface waters are typically <100 ng/L (van der Veen and de Boer, 2012). However, TDCPP at up to several μg/L can be detected in water at waste disposal sites; for example, 3 μg/L was detected in effluent from a
sewage treatment plant (Marklund et al., 2005). TDCPP is not readily degraded in water and it tends to persist and accumulate in the environment, which maybe bioavailable to fish. Indeed, up to 140 ng/g lipid weight of TDCPP has been measured in perch (Sundkvist et al., 2010).

In China, more than 70,000 tons of OPFRs were produced in 2007, and this accounted for 35% of global production (Reemtsma et al., 2008). In China, limited data show that the surface water concentration of TDCPP is 2.5–40 ng/L in the Songhua River and 0.62–5.54 μg/kg in sediment from Taihu Lake (Cao et al., 2012; Wang et al., 2011). Up to 251 μg/kg lipid weight of TDCPP has been reported in muscle samples of freshwater fish from the Pearl River Delta region in South China (Ma et al., 2013), where tributoxyethyl phosphate is the dominant OPFR and this can reach up to 8842 μg/kg lipid weight. Total OPFRs were detected at up to 1312 μg/kg dry weight in sludge samples collected from municipal wastewater treatment plants in the same region of South China (Zeng et al., 2014). OPFRs have also been detected in tap water in China (85.1–325 ng/L) (Li et al., 2014) and in office air (up to 147.7 ng/m³) (Yang et al., 2014). Thus, the production and application of OPFRs has resulted in environmental contamination.

Toxicological studies have shown that exposure to OPFRs has the potential to cause neurological, adverse reproductive, endocrine disruptive and systemic effects in animals (Dishaw et al., 2011; van der Veen and de Boer, 2012). In vitro reporter gene assays showed that TDCPP has potential endocrine-disrupting effects and can act as an androgen receptor (AR) antagonist (Kojima et al., 2013). TDCPP may have thyroid endocrine-disrupting effects as concentrations of this chemical in house dust correlate with decreased concentrations of circulating thyroid hormone (TH) in humans (Meeker
and Stapleton, 2009). TDCPP can cause developmental abnormalities during zebrafish embryogenesis (McGee et al., 2012; Fu et al., 2013; Liu et al., 2013a), including thyroid endocrine-disruption activity, as it reduced the levels of circulating T4 levels in zebrafish larvae (Wang et al., 2013). Moreover, in cultured chicken embryos TDCPP caused altered expression of TH-responsive genes (Crump et al., 2012; Farhat et al., 2013). TDCPP also disrupted sex hormone levels in human adrenocortical carcinoma cells (H295R) and impaired reproduction in zebrafish (Liu et al., 2012, 2013b). A recent DNA microarray study in chicken embryos injected with TDCPP demonstrated toxicity to lipid metabolism, including cholesterol, as well as a transcriptional dysregulation of genes involved in immune response (Farhat et al., 2014). In vitro studies show that TDCPP exhibits similar neurotoxic actions to organophosphorus (OP) pesticides (e.g., altered neurodifferentiation) (Dishaw et al., 2011) and exerts cytotoxic and neurotoxic effects to PC12 cells (e.g., decreased cell growth, increased apoptosis, altered cell morphology, and changes in gene expression) (Ta et al., 2014). However, knowledge of the toxicological and environmental health effects of TDCPP remains limited, particularly the effects of long-term exposures to low concentrations and whether TDCPP causes neurotoxicity in vivo.

The objectives of the present study were to investigate potential neurotoxicity of TDCPP in fish. As public health concerns have focused primarily on the potential hazardous effects resulting from long-term exposure, especially to infants and young children (Staskal et al., 2006), we examined the neurotoxicity of TDCPP exposure at an early developmental stage of zebrafish (5 days post-fertilization [dpf]) and after long-term exposure (6 months). Moreover, early developmental stages are typically more sensitive to toxicant stress, particularly the developing brain (Rice and Barone, 2000),
thus we examined the expression of several genes that are expressed exclusively in the nervous system during early developmental stages of zebrafish (Brosamle et al., 2002; Udvadia et al., 2001). These candidate genes can serve as biomarkers for developmental neurotoxicity by demonstrating their responsiveness to neurotoxicants (Fan et al., 2010). Acetylcholinesterase (AChE) activity is widely used as a biomarker for the presence of neurotoxicants in aquatic environments, and this assay has been used to detect OP pesticides (Payne et al., 1996). The evaluation of neurotransmitter parameters has emerged as an important strategy to assess neurochemical, behavioral and toxicological phenotypes in zebrafish (Rico et al., 2011), and the locomotion abilities of larvae can also indicate toxicity (Rao et al., 2005). Therefore, we also examined the neurotransmitter system, including AChE activity and neurotransmitter concentrations (e.g., dopamine and serotonin). Taken together, this information will further elucidate the environmental risks of OPFRs to aquatic organisms. Since little information is available regarding bioconcentrations of TDCPP and its metabolism in fish, we also assess the chemical accumulation of TDCPP in the whole body and the brain of zebrafish.

2. Materials and methods

2.1 Reagents

Reagents were purchased from the following sources: TDCPP (CAS#13674-87-8; >95.6% purity) from TCI Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); bis(1,3-dichloro-2-propyl) phosphate (BDCPP, >97% purity) from Wellington Laboratories, Ontario, Canada; deuterated tri-n-propyl phosphate (d21-TPrP, 99.2%),
deuterated trimethyl phosphate (d9-TMP, 99.9%) from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada), 3,4-dihydroxybenzylamine (DHB, >98.0% purity), dopamine (>98.5% purity) and serotonin (>98.0% purity) from Sigma–Aldrich, St. Louis, USA. TDCPP stock solutions and serial dilutions were prepared in dimethylsulfoxide (DMSO) (purity >99.9%; Amresco, Solon, OH, USA). All other chemicals used were of analytical grade.

2.2 Zebrafish maintenance and experimental design

Adult zebrafish (AB strain) were maintained and embryos were exposed to toxicants according to a published protocol (Yu et al., 2011). Briefly, normal embryos were randomly selected at the blastula stage (2 h post-fertilization [hpf]) and transferred into glass beakers containing 500 mL of TDCPP solution (0, 4, 20 and 100 μg/L). Experiments were carried out in triplicate for each exposure concentration and each beaker contained approximately 300 embryos. The selection of the lowest exposure concentration (4 μg/L) was based on a previous study (Wang et al., 2013), where decreased T4 levels in zebrafish larvae were observed after short-term exposure (144 h) to 10 μg/L TDCPP, and this concentration is also similar to that reported in sewage effluent (e.g., 3 μg/L) (Marklund et al., 2005). A subset of the 5-dpf larvae was sampled at random to measure locomotion activity, AChE activity, neurotransmitter levels, gene and protein expression, and chemical concentrations. Fish (10 days old) were placed in 20-L aquaria (50 larvae per aquaria) and at 40 dpf the larvae were transferred into 30-L aquaria (40 fry per aquaria). The fish were aerated, kept at 28°C and exposed to an artificial day-night cycle (14 h light, 10 h dark). During all the experimental period,
exposure media were prepared with dechlorinated carbon-filtered water and replaced completely with freshly prepared media daily. Control and experimental treatment groups received 0.001% (v/v) DMSO. After 6 months of exposure, survival and growth (weight) were recorded. Zebrafish were euthanized using an overdose of MS-222 (300 mg/L) by prolonged immersion until cessation of opercular movement and total weights and snout-to-vent lengths were recorded. Samples of brain were collected and preserved at -80°C for later chemical analyses. All studies were conducted in accordance with the guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China.

2.3 Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Adult fish were euthanized as above and brains were removed from the skull by dissection. Three brains were pooled to be used as one replicate for each treatment. The brain samples (n=3 replicates) and larval samples (30 larvae, n=3 replicates) were collected and preserved with RNAiso Plus according to the manufacturer’s instructions (Takara, Dalian, China). RNA extraction, purification, quantification and first-strand cDNA synthesis were performed following the protocols described by Chen et al. (2012). Oligonucleotide primers specific to each of the selected genes were identified using the online Primer 3 program (http://frodo.wi.mit.edu/) (Supplementary material, Table S1). The rpl8 gene was selected as the internal standard.

2.4 Protein extraction and Western blotting

A Western blot was performed as previously described using approximately 150
larvae and brain samples for each replicate (n=3 replicates; Chen et al. 2012). Briefly, 50 μg of each protein sample was separated by electrophoresis on a 12% sodium dodecylsulfate polyacrylamide gel. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. The membranes were further blocked with 5% fat-free milk for 2 h in tris-buffered saline (TBS) (10 mM tris, 150 mM NaCl, pH 8.0) and 0.1% Tween 20, before being incubated overnight at 4°C with primary antibodies against myelin basic protein (mbp; 1:300), α1-tubulin (1:100) or β-actin (1:1000). Both rabbit α1-tubulin antibody (Abcam, Cambridge, UK) and rabbit mbp (AnaSpec, Fremont, CA) have previously been verified as reactive and suitable for zebrafish studies (Thatcher et al. 2008; Buckley et al. 2010). After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:50,000) for 2 h at room temperature, the signal was detected by enhanced chemiluminescence. The relative optical density of each band was analyzed using Kodak film (Eastman Kodak Co., Rochester, NY) and Quantity One version 4.3 software (Bio-Rad, USA).

2.5 Neurotransmitter measurements

Monoamine extraction from both larvae (50, n=3 replicates) and from adult fish brains was performed as previously described (Wang et al., 2012; Sari et al., 2010). Before extraction, 1 ng of DHB was added as internal standard (Sari et al., 2010). After weighing and homogenizing in 400 μL of 1% formic acid dissolved in pre-cooled acetonitrile, the samples were incubated for 15 min at -20°C, before centrifuging at 12000×g for 20 min at 4°C. The supernatant was freeze-dried and reconstituted by vortexing in 50 μL of 50% acetonitrile/water (v/v). Samples were further centrifuged at
12000×g for 20 min at 4°C and the supernatant was transferred to a new tube and stored at -20°C until chemical analyses were performed.

Identification and quantification of analytes were carried out using an Agilent 1200 (Agilent, USA) liquid chromatograph equipped with a QQQ (Agilent, USA) tandem mass spectrometer. Dopamine and serotonin were used as the analytical standards. Agilent ZorbaxXDB-C18 (2.1×50mm, 1.8µm) was used for LC separation. Data were acquired with MassLynx 4.0 and processed with QuanLynx software for calibration and for quantification of analytes.

2.6 AChE activity in larvae and in adult fish brains

Adult fish were euthanized and brains removed as above. Three brains were pooled to be used as one replicate for each treatment. The brains (n=3 replicates) and larvae (50, n=3 replicates) were homogenized on ice in 60 volumes (v/w) of tris-citrate buffer (50 mM tris, 2 mM EDTA, 2 mM EGTA; pH 7.4, adjusted with citric acid) (Chen et al., 2012), and then centrifuged at 3000×g for 10 min at 4°C. AChE enzyme activity was measured by a commercial kit following the manufacturer’s instructions (Nanjing Keygen Biotech, Co, LTD). Protein concentration was measured using the Bradford method.

2.7 Locomotion activity measurement

Quantification of larval locomotion activity was performed using the Video-Track system (ViewPoint Life Sciences, Inc., Montreal, Canada) following a previously described method (Chen et al., 2012). The 5-dpf zebrafish larvae were placed into
24-well plate wells (1 larva per well) to measure swimming activity. Larvae were allowed to acclimate for 10 min before the test started. Swimming behavior was monitored for 20 min in continuous light and also in response to 40 min dark-to-light photoperiod stimulation (cycle of 10 min visible light followed by 10 min infrared light). Data (frequency of movements, distance traveled, and total duration of movement) were collected from 30 larvae per treatment every 60 s, and further analyzed using custom Open Office.Org 2.4 software.

2.8 Quantification of TDCPP and BDCPP

2.8.1 Extraction and clean-up

Actual TDCPP concentrations in the exposure solutions were measured at the embryo, larval and adult stages before and after renewal of the water solution (n=3 replicate tanks). TDCPP was extracted and analyzed as described previously (Wang et al., 2011).

TDCPP and BDCPP concentrations were determined in 5-dpf larvae (100, n=3 replicates), in adult fish (one individual fish) and brain tissue (three, n=3 replicates). d21-TPrP and d9-TMP were used as internal standards.

Zebrafish larvae samples, adult fish and brain tissues were weighed and spiked with 100 ng of d21-TPrP and d9-TMP as internal standards for TDCPP and BDCPP, and then 4 mL acetonitrile was added. Samples were homogenized, sonicated for 30 min, and centrifuged at 3000×g for 5 min. The supernatant was collected and the extraction was repeated two additional times using 3 mL acetonitrile. The supernatants were combined
and then concentrated to 1 mL under nitrogen. The extract was reconstituted in 125 mL water, transferred to solid-phase extraction cartridges (Bond Elut-PPL 200 mg, 3 mL), eluted with 2 ml acetonitrile and 2 mL methanol, and then diluted to 10 mL with methanol. The sample was mixed in 100 μL methanol:water (1:1) before instrumental analysis.

2.8.2 LC-MS/MS analysis

TDCPP and BDCPP identification and quantification was performed using a LC-tandem mass spectrometer system consisting of an Agilent 1290 Infinity LC (Agilent Technologies, Palo Alto, CA) coupled to an AB SCIEX QTRAP 5500 LC-MS/MS system.

2.8.3 Quality assurance and control

Procedural blanks were processed with every batch of samples to check for interference or contamination, but no contamination was observed during the analysis. The detection limit (DL) was defined as the mean level in the procedural blank plus three times the standard deviation. The DLs of TDCPP and BDCPP were 0.100 and 0.500 μg/kg wet weight, respectively. The DL of TDCPP analysis in water was 7.00 ng/L. For recovery measurements, fish, larvae and brain tissue samples were spiked with TDCPP and BDCPP standards. There were three replicates for each sample. For recovery of the deuterated standards, tissue samples were weighed and spiked with 100 ng of d21-TPrP and d9-TMP as internal standards prior to extraction. Recovery of spiked blank tissue samples for TDCPP and BDCPP was between 84±13% and 95%±18% (n= 4),
respectively. The recovery of the deuterated standards, spiked into tissues, was 73±13% for \textit{d21-TPrP} and 63±9% for \textit{d9-TMP} (n=9).

\subsection*{2.9 Statistical analysis}

All data were initially verified for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene’s tests, respectively. All data are reported as means ± standard error (SEM). Differences between the control and each exposure group were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s test. All analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA). P < 0.05 was considered to be statistically significant.

\section*{3. Results}

\subsection*{3.1 Toxicological end-points in the larvae and adult fish}

Larvae (5 dpf) exposed to TDCPP (<100 \textmu g/L) did not significantly affect hatching, malformation, survival and growth rates (see Supplementary data, Table S2). In the present study, we did not observe any developmental toxicity in 5-dpf larvae at <100 \textmu g/L, which is in agreement with several previous studies on TDCPP (Mcgee et al., 2012; Fu et al., 2013; Liu et al., 2013a; Wang et al., 2013) and indicates relatively low acute toxicity of TDCPP to developing fish.

For the groups exposed to different concentrations of TDCPP up to sexual maturity, there were no significant differences in survival rates (71.7%, 73.0%, 68.3% and 69.3%
at 0, 4, 20 and 100 μg/L TDCPP, respectively). At 4, 20 and 100 μg/L TDCPP, growth (weight) was significantly reduced in males by 10.1% (P = 0.005), 8.7% (P = 0.022) and 9.3% (P = 0.014) respectively, and in females by 13.7% (P = 0.002), 10.7% (P = 0.022) and 10.2% (P = 0.032) respectively.

3.2 Gene expression in larvae and adult fish brain

The expression of genes involved in central nervous system development, differentiation and growth were evaluated in larvae. Transcription of several selected genes (glial fibrillary acidic protein [gfap], synapsin IIa [syn2a], mbp, α1-tubulin) that are expressed exclusively in the nervous system during early developmental stages was not significantly altered by exposure to TDCPP, except for a small but significant upregulation of gap-43 (see Supplementary data, Table S3). In the brains of females, the transcription levels of mbp and α1-tubulin were significantly downregulated in the 20 and 100 μg/L exposure groups (Table 2). Transcriptional downregulation of syn2a was also observed in the 100 μg/L exposure group, while a significant transcriptional upregulation of gap-43 was observed in the 100 μg/L exposure group. Gfap gene expression was unchanged (Table 1).

In the brains of male fish, downregulation of gene transcription was only observed for α1-tubulin in 20 and 100 μg/L exposure groups, while a significant transcriptional upregulation of gap-43 was observed in the 100 μg/L group. Transcription of gfap, mbp and syn2a did not change significantly (Table 1).

Expression of α1-tubulin and mbp proteins was examined by Western blotting, but their expression was unchanged in the larvae (see Supplementary data, Figure S1). In the
female adult fish brains, significant reductions of α1-tubulin (Figure 1) and mbp proteins were observed in the 20 and 100 μg/L exposure groups (Figure 1). In the male brains, a significant reduction of α1-tubulin was observed in the 100 μg/L exposure group only (Figure 1).

3.3 Neurotransmitter contents and AChE activity

Dopamine and serotonin contents and AChE activity were not significantly affected in 5-dpf larvae exposed to TDCPP (see Supplementary data, Table S4). In the brains of females, dopamine contents were reduced significantly by 30.2% (P = 0.034), 45.5% (P = 0.003) and 28.3% (P = 0.046) in the 4, 20 and 100 μg/L TDCPP groups, respectively (Figure 2). Serotonin contents were also reduced significantly in the brain by 35.5% (P = 0.033), 34.2% (P = 0.039) and 32.9% (P = 0.047) in the females exposed to 4, 20 and 100 μg/L TDCPP, respectively (Figure 2). Long-term exposure did not affect neurotransmitter contents in male fish or AChE activity in the adult fish brains (see Supplementary data, Table S4).

3.4 Locomotion activity

There were no significant changes in 5-dpf larvae locomotion in any of the exposure groups compared with the control (see Supplementary data, Figure S2).

3.5 Quantification of TDCPP and BDCPP

The close correlation between the nominal and measured TDCPP concentrations (Table 2) indicated the stability of TDCPP in water, and this observation is consistent
with a previous study (Wang et al., 2013). No TDCPP was detected in the control group.

TDCPP contents (μg/kg wet weight) in the larvae exposed to this chemical increased with exposure concentration, while substantial amounts of its metabolite, BDCPP, were detected in exposed larvae (Figure 3A). The ratio of TDCPP: BDCPP was 2.45±0.03, 0.99±0.17 and 0.84±0.01 in 4, 20 and 100 μg/L exposure groups, respectively. No TDCPP or BDCPP was detected in the control group.

The total body burden and brain contents of TDCPP and BDCPP were measured in adult fish. In males, the total body burden of TDCPP and BDCPP showed a concentration-dependent relationship in 4, 20 and 100 μg/L exposure groups (Figure 3B). The ratio of TDCPP: BDCPP was 2.27±1.22, 6.95±0.8 and 5.56±3.22 in 4, 20 and 100 μg/L exposure groups, respectively. Brain tissue contained considerable amounts of TDCPP, but no BDCPP was detected (Figure 3B). Control males contained detectable amounts of TDCPP (0.9±0.4 μg/kg), while BDCPP content was <0.5 μg/kg wet weight.

In females, the measured total body burden of TDCPP and BDCPP also showed a concentration-dependent relationship in the 4, 20 and 100 μg/L exposure groups (Figure 3C). The ratio of TDCPP: BDCPP was 3.39±0.63, 3.23±2.04 and 8.73±3.83 in 4, 20 and 100 μg/L exposure groups, respectively. A similar deposition of TDCPP was also detected in female fish brain tissue (Figure 3C). Moreover, a small amount of BDCPP was detected in the brains from the highest exposure group (100 μg/L). Negligible levels of TDCPP (0.8±0.6 μg/kg) were detected, while BDCPP content was <0.5 μg/kg wet weight in control females.

4. Discussion
Although several recent studies have described the potential neurotoxicity of TDCPP to cultured PC12 cells, the potential neurotoxicity of TDCPP to animals is currently unknown. In addition, to our knowledge, few studies have been conducted to assess the impact of long-term exposure to low concentrations of OPFRs in fish. In the present study, we observed no acute toxicity to developing zebrafish larvae, but we did observe significant decreases in dopamine and serotonin within adult female zebrafish after long-term exposure to TDCPP, which further supports the importance of assessing the risks of long-term low exposures to environmental OPFRs. Furthermore, we found evidence for tissue burden and metabolism of TDCPP in the larvae and adult zebrafish, particularly in the brain.

In the present study, we observed high body burdens of TDCPP in fish, indicating bioavailability and bioaccumulation of this hydrophobic chemical under laboratory exposure conditions (log Kow = 3.76) (OEHHA, 2011). We also observed the metabolite BDCPP, which is the primary metabolite of TDCPP produced in mammals (Cooper et al., 2011; Carignan et al., 2013). The relatively high content of BDCPP indicates a high TDCPP biotransformation capacity by enzymes in the fish liver. In addition, unusually high TDCPP contents were detected in brain tissue, suggesting that it is able to cross the blood brain barrier and accumulates in brain suggesting that the brain may be a key target organ for TDCPP toxicity and this may have direct effects on nerve cells, leading to neurotoxicity. Furthermore, BDCPP was also detected in the brain and so more research is warranted concerning the toxic effects of TDCPP on the central nervous system. It should be mentioned that chemical analysis showed lower ratio of TDCPP: BDCPP in
larval stages, indicating that larvae may have high biotransformation potential. On the other hand, higher body burdens of TDCPP and BDCPP in larvae could also be due to larval fish having a lower capacity to excrete these chemicals. Indeed, a previous study with PBDE bioaccumulation in developing mice showed that young animals have a reduced ability to excrete PBDEs during development (Staskal et al., 2006). Hence, it is also necessary to investigate whether TDCPP preferentially distributes to lipophilic tissues, as larval stages possess higher lipid contents. Additional studies regarding the bioaccumulation kinetics of TDCPP in developing fish and also potential hazardous effects to early developmental stages are needed.

We observed higher contents of TDCPP in females than in males, indicating gender-dependent bioconcentration of TDCPP, but the mechanisms underlying this differential bioconcentration remain unknown. However, gender-dependent bioconcentration of organic toxicants has often been observed in fish species. For example, long-term exposure to low concentrations of PBDEs resulted in higher body burden in male zebrafish (Yu et al., 2011; Chen et al., 2012). This phenomenon was also observed in wild marine dolphins (*Stenella attenuate*) where males bioaccumulated significantly higher levels of PBDEs (Ko et al., 2014). In fish species, higher bioaccumulation of other toxicants (e.g., metals like mercury) is often observed in females (Burger et al., 2007). Hence, gender-specific bioconcentration of different types of toxicants is common, and this could be due to differences in uptake, metabolism, distribution and elimination, as well as gender differences in nutritional needs, feeding behavior and various physiological activities influencing contaminant accumulation (Burger et al., 2007). Furthermore, in vivo gender-specific receptors can also contribute
to gender differences in the bioaccumulation of pollutants (Monteverdi and Di Giulio, 2000). Hence, the gender-specific bioconcentration of toxicants could cause gender-specific sensitivity or toxicity (Burger et al., 2007; Deane et al., 2014). Given that TDCPP is relatively hydrophobic (log Kow=3.76), it is possible that the differences in uptake between males and females were driven by differences in the lipid content. In the present study, we observed that endpoints indicative of neurotoxicity were more sensitive in females than males, which could be partly explained by gender-specific sensitivity or toxicity.

In our present study, neurotransmitter levels were unchanged in larvae, which may indicate that the developing nervous system is not a target during acute exposure to low concentrations of TDCPP. It is well-known that the mechanism of OP toxicity is via inhibition of AChE activity, but we did not observe any effect on AChE activity in larvae and in the adult fish brain. This observation suggests that, unlike OPs, TDCPP is not a typical neuron toxin. In addition to affecting neurotransmitters, we hypothesize that the mechanism by which TDCPP causes neurotoxicity is by modifying proteins in the brain. Thus, we examined several genes and proteins that are expressed in the central nervous system during early developmental stages of the zebrafish and in adult fish brains.

We found that the expression of the selected genes associated with the central nervous system remained unchanged in the larvae (except for gap-43), but there was significant downregulation of these key genes (e.g., α1-tubulin, mbp, SYN2a) and proteins in adult fish. The α1-tubulin gene encodes an intermediate filament protein that forms an essential part of microtubules (Baas, 1997). Thus, if the transcriptional effect is also seen at protein level, alterations to α1-tubulin proteins or their levels would likely
affect the structure and function of microtubules, which may have dramatic effects on brain architecture and function. It should also be mentioned that downregulation of the \textit{\alpha}1-\textit{tubulin} gene expression (both at mRNA and protein level) was observed in both males and females, suggesting that it is very sensitive to TDCPP stress. Mbp, a major protein constituent of the myelin sheath, is encoded by \textit{mbp}, also a biomarker of myelination that is expressed in oligodendrocytes in the central and peripheral nervous systems (Lee and Fields, 2009). Perturbation of \textit{mbp} gene expression can impact myelination, which may lead to myelination deficiency and ultimately disrupt neuronal functions. We observed downregulation of \textit{mbp} gene expression only in females, which further indicates a gender-specific effect on myelination in oligodendrocytes. The \textit{gap-43} gene transcription was upregulated. \textit{gap-43} is frequently used as a marker in re-inducing axonal growth for regeneration after damage (Benowitz and Routtenberg, 1997). It is tempting to speculate that the upregulation of \textit{gap-43} represents an adaptive response to maintain overall brain growth necessary to offset the direct effects of toxicants (Alm et al., 2008). Our results are consistent with previous reports regarding upregulation of \textit{gap-43} either at transcriptional or protein level in the brain of mouse (Alm et al., 2006; Viberg et al., 2008) or in zebrafish (Chen et al., 2012) after exposure to PBDEs, which is a common response and most likely reflects a compensatory mechanism. \textit{SYN2a} plays an important role in both synaptogenesis and neurotransmitter release in mammals (Kao et al., 1998). We observed a small downregulation of \textit{SYN2a} gene transcription in females exposed to 100 \textmu g/L TDCPP, suggesting that it is not sensitive to TDCPP stress.

Our results show that the locomotion was unchanged in the larvae by exposure to TDCPP. Alterations of locomotion can result from changes in gene expression in the
central nervous system and/or by direct neurotoxic effects on neuronal cells, including changing neurotransmitters and AChE activity (Chen et al., 2012; Irons et al., 2013). In our present study, we did not observe any alterations in these parameters or locomotion, suggesting no acute developmental neurotoxicity to fish embryos and larvae.

In summary, the present report demonstrates that long-term exposure to TDCPP can result in neurotoxicity in fish. However, the mechanisms underlying alterations of gene expression in central nervous system, as well as how TDCPP affects neurotransmitters, are unknown. As TDCPP can bioaccumulate in the brain, it should be investigated how TDCPP can affect cell signaling pathways in the central nervous system. Current knowledge on the environmental behavior and toxicity of OPFRs is limited despite increasing production and application of these chemicals, which are now common environmental contaminants. Therefore, it is important to understand the environmental risks of OPFRs to aquatic ecosystems and their potential human health effects.
Acknowledgments

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB14040103), the National Natural Science Foundation of China to BZ (21237005), and PL (21207063, 41206080), and the Hong Kong Research Grants Council (CityU 160613). The authors sincerely thank the two anonymous reviewers for their constructive comments.

Note: The authors declare no competing financial interests.
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Table 1. Gene transcription levels in adult zebrafish brain exposed to TDCPP (0, 4, 20 and 100 μg/L) for 6 months.

The data represent the mean ± SEM of three replicates (3 brains pooled as one replicate sample) and are expressed as fold change relative to control. *P < 0.05; **P < 0.01 indicates a significant difference between the exposure groups and the control group.

<table>
<thead>
<tr>
<th>Zebrafish</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDCPP (μg/L)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><em>gfap</em></td>
<td>1.02±0.10</td>
<td>1.17±0.20</td>
</tr>
<tr>
<td><em>syn2a</em></td>
<td>1.01±0.06</td>
<td>1.14±0.16</td>
</tr>
<tr>
<td><em>mbp</em></td>
<td>1.01±0.10</td>
<td>0.88±0.16</td>
</tr>
<tr>
<td><em>α1-tubulin</em></td>
<td>1.01±0.09</td>
<td>0.78±0.10</td>
</tr>
<tr>
<td><em>gap-43</em></td>
<td>1.00±0.04</td>
<td>1.38±0.18</td>
</tr>
</tbody>
</table>
Table 2. Actual TDCPP concentrations in exposure solutions at embryo (2-dpf), larval (42-dpf) and adult (120-dpf) stages. The data (average before and after water changes) are expressed as mean ± standard error (SEM) of 3 replicate beakers (tanks). LOD: limit of detection.

<table>
<thead>
<tr>
<th>Nominal TDCPP (µg/L)</th>
<th>0</th>
<th>4</th>
<th>20</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Embryo</td>
<td>&lt;LOD</td>
<td>5.2 ± 0.6</td>
<td>23.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Larval</td>
<td>&lt;LOD</td>
<td>5.2 ± 0.3</td>
<td>24.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>&lt;LOD</td>
<td>4.6 ± 0.1</td>
<td>22.8 ± 0.9</td>
</tr>
</tbody>
</table>
Figure captions

**Figure 1.** Western blot analysis of protein levels in the adult zebrafish brain exposed to TDCPP (0, 4, 20 or 100 μg/L) for 6 months. (A) Representative Western blot of α1-tubulin and mbp in female zebrafish; (B) Representative Western blot of α1-tubulin and mbp in male zebrafish; (C) Quantification of the relative expression of α1-tubulin and mbp protein. The data represent similar results from three replicate samples and each replicate contained 150 larvae. The bands from three replicate samples were quantified by densitometry. The results were normalized to β-actin expression in each sample and are expressed as the mean ± SEM fold change relative to the control. *P < 0.05 and **P < 0.01 indicates significant differences between exposure groups and the corresponding control group.

**Figure 2.** Neurotransmitter content in female brain tissues exposed to TDCPP (0, 4, 20 or 100 μg/L) for 6 months. The neurotransmitter (dopamine, serotonin) levels are expressed as ng/g. ww. All data are expressed as mean ± SEM of three replicate samples; each replicate contained 3 brain tissues. *P < 0.05 and **P < 0.01 indicates significant differences between exposure groups and control group.

**Figure 3.** TDCPP and BDCPP contents in zebrafish and brain tissue after exposure to TDCPP. (A) TDCPP and BDCPP contents in the 5-dpf zebrafish larvae; (B) TDCPP and BDCPP contents in adult male zebrafish; (C) TDCPP and BDCPP contents in adult female zebrafish; For adult fish, the values represent the mean of three individual replicate fish. For larvae, TDCPP and BDCPP were measured in 50 larvae, with three replicate samples. The data are expressed as mean ± SEM.
Figure 1
Figure 2
Figure 3
Highlights:

- The acute and long-term exposure of TDCPP to zebrafish was investigated.

- Uptake of TDCPP was observed in larvae but did not cause developing neurotoxicity.

- Greater uptake of TDCPP was observed in female fish tissues including brain.

- TDCPP could be metabolized to BDCPP in larvae and adult fish.

- Long-term exposure of TDCPP resulted in gender-dependent neurotoxicity.