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Developmental circulatory failure caused by metabolites of organophosphorus flame retardants in zebrafish, *Danio rerio*

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Running title: Developmental effects of OPFR metabolites in zebrafish

Abstract

Organophosphate triesters are used worldwide as additives in flame retardants and plasticizer as a replacement of polybrominated diphenyl ethers. Increasing evidence on human exposure to and environmental contamination with organophosphorus flame retardants (OPFRs) requires an adequate toxicity assessment for this class of chemicals. While developmental toxicity of several OPFRs has been reported, developmental effects of OPFR metabolites have still to be understood. The present study aimed at characterizing developmental effects of OPFR metabolites using zebrafish embryos (*Danio rerio*). Triphenyl phosphate (TPHP) was most potent for inducing pericardial edema and reduction in blood flow in trunk vessels, followed by two of its metabolites, 3-hydroxylphenyl diphenyl phosphate and 4-hydroxylphenyl diphenyl phosphate, respectively. Other TPHP metabolites, such as diphenyl phosphate and 4-hydroxylphenyl phenyl phosphate, showed no substantial increase in circulatory failure at concentrations up to 30 μM. Tris(1,3-dichloro-2-propyl) phosphate showed circulatory failure at 30 μM, but its metabolite bis(1,3-dichloro-2-propyl) phosphate did not. Neither tris(2-chloroethyl) phosphate nor its metabolite bis(2-chloroethyl) phosphate, induced circulatory failure. The circulatory failure appeared to be enhanced with the increase in the octanol-water partition coefficients of OPFRs and their metabolites, suggesting that developmental circulatory failure posed by these chemicals could be estimated by their bioaccumulative potential. The present study demonstrated developmental circulatory failure of hydroxylated TPHP metabolites, which was almost equipotent to TPHP. Diester OPFR metabolites showed no major developmental toxicity at the concentrations used in this study. The

41 current results establish the foundation for further understanding the similarities and differences in

42 the toxic mechanisms between OPFRs and their metabolites.

1. Introduction

Due largely to persistency and toxicity, polybrominated diphenyl ethers have been replaced with other types of flame retardants, including organophosphate triesters. The production and use of organophosphorus flame retardants (OPFRs) have been growing since the past two decades. The production volume of worldwide OPFRs was 100,000 tons in 1992, and then reached 341,000 tons in 2007 (Greaves and Letcher, 2017). The amount of use of OPFRs in EU, the United States and Asia was 200,000 tons in 2007 (Xu et al., 2019). Five hundred thousand tons of OPFRs were consumed globally in 2011, and the expected consumption became 680,000 in 2015 (Hou et al., 2016).

The abundant use of OPFRs resulted in detection from a variety of samples, including indoor and outdoor dust, human urine and breast milk, and wildlife. For instance, tris(butoxyethyl) phosphate (TBOEP) was the major OPFR detected from indoor dust in many countries. For instance, the range of TBOEP in indoor dust samples was 1.40 to 230 μg/g in Japan, while in Belgium the range was 0.36 to 67.6 μg/g in house dust samples and 0.20 to 55.7 μg/g in store samples (Takigami et al., 2009; Van den Eede et al., 2011). Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and triphenyl phosphate (TPHP) were frequently detected in house dust in the Unites States, ranging from 0.197 to 39.5 and 0.0995 to 40.4 μg/g (Hoffman et al., 2015), and <0.09 to 56.1 μg /g and <0.15 to 1,800 μg/g, respectively (Stapleton et al., 2009). These OPFRs were also detected in consumer products and furniture. For instance, the median concentration of TPHP in nail polish purchased in North Carolina was 8.9 mg/g (Mendelsohn et al., 2016). Polyurethane foam

63 samples from the house sofa in North Carolina had 0.534 to 88.1 mg/g of TDCIPP and 0.0013 to 15
64 mg/g of TPHP (Hammel et al., 2017). Tris(2-chloroethyl) phosphate (TCEP) was one of the most
65 prevalent OPFRs detected from freshwater fish and aquatic birds. A monitoring study using Lake
66 Trout in Canadian lakes demonstrated that TCEP was detected from 75% of the samples, ranging
67 from <0.03 to 3.4 ng/g (McGoldrick et al., 2014). Temporal trend of TCEP contamination in herring
68 gull eggs in the Laurentian Great Lakes showed increase from average 0.25 ng/g wet weight in
69 1990's to average 1.62 ng/g wet weight in 2010 (Greaves et al., 2016). TCEP and TPHP were
70 detected in human breast milk samples collected from Japan (year 2009 – 2011, median values of
71 0.14 and 1.4 ng/g lipid weight, respectively), Philippines (year 2008, median values of 42 and 19
72 ng/g lipid weight, respectively) and Vietnam (year 2008, median value of 4.9 ng/g lipid weight for
73 TPHP) (Kim et al. 2014).
74 Previous studies reported that OPFRs have a wide range of toxicity in vertebrate species. One
75 of the major targets of developmental effects caused by OPFRs is the cardiac system, which can be
76 well studied by using zebrafish (*Danio rerio*) as a model animal, due largely to its transparency
77 during the early development (Dishaw et al., 2014; Du et al., 2015; Isales et al., 2015; McGee et al.,
78 2013, 2012; Mitchell et al., 2018; Wu et al., 2017; Zhong et al., 2019). TPHP was shown to have
79 developmental and cardiotoxicity in zebrafish embryos possibly through the retinoid X receptor
80 (RXR) related pathways (Isales et al., 2015; Mitchell et al., 2018), but independent of the aryl
81 hydrocarbon receptor (AHR) signaling (McGee et al. 2013). TDCIPP also posed pericardial edema
82 in zebrafish embryos (McGee et al., 2012). The effect of TCEP on developmental cardiac system in

83 zebrafish appears controversial, with one study showing deformity (Wu et al., 2017), but another
84 one having no clear toxicity (Dishaw et al., 2014). In addition, it has been reported that
85 bioaccumulative properties of OPFRs predicted by octanol-water partition coefficients (Log K*ow*)
86 were associated with lethality and developmental toxicity in zebrafish embryos (Dishaw et al.,
87 2014; Du et al., 2015).
88 Determination of OPFR metabolites in urine samples has been performed to assess and
89 monitor human exposure to these contaminants. A variety of OPFR metabolites, including bis(1,3-
90 dichloro-2-propyl) phosphate (BDCIPP), diphenyl phosphate (DPHP) and bis(2-chloroethyl)
91 phosphate (BCEP) were detected in urine samples. Geometric mean values of BDCIPP and DPHP
92 in children's urine were 5.6 and 3.0 ng/mL, with detection rate of 100% (Butt et al., 2014). In adult
93 urine samples, average concentrations of BCEP, BDCIPP and DPHP were 0.76, 0.46, and 1.1
94 ng/mL, respectively (Dodson et al., 2014). Urinary BDCIPP concentrations were shown to be 16.5
95 times higher in 2014 and 2015 than in 2002 and 2003 in the United States (Hoffman et al., 2017).
96 These biomonitoring studies confirmed that human exposure to OPFRs has been increasing since
97 the last decade and urinary levels of OPFR metabolites in children were higher than those in adults
98 (Butt et al., 2014; Van den Eede et al., 2014), possibly due to contribution of the hand-to-mouth
99 exposure in children (Butt et al., 2014).
100 Some OPFR metabolites were shown to elicit endocrine disruption and developmental toxicity.
101 For instance, 3-hydroxylphenyl diphenyl phosphate (HO-*m*-TPHP) and 4-hydroxylphenyl diphenyl
102 phosphate (HO-*p*-TPHP) were potent agonists to human estrogen receptor (ER) subtypes, ERα and

ERβ, with greater potency than their parental chemical TPHP, as revealed by the *in vitro* reporter gene transactivation assay (Kojima et al., 2016). BDCIPP appears to possess more teratogenic potency than TDCIPP, showing reduced survival and impaired development in zebrafish embryos (Noyes et al., 2015). DPHP-induced cardiac defects, which may not involve the retinoic acid receptor (RAR)/RXR signaling, were reported in zebrafish embryos (Mitchell et al., 2019). These findings highlight the potential health impact of developmental exposure to parental OPFRs and to their metabolites on cardiac and endocrine systems. However, the developmental effects of metabolites of OPFRs have still to be understood.

The aim of the present study was to investigate developmental effects of metabolites of TDCIPP, TPHP, and TCEP. These metabolites include HO-*m*-TPHP, HO-*p*-TPHP, DPHP, 4-hydroxylphenyl phenyl phosphate (HO-DPHP), BDCIPP and BCEP. The severity of pericardial edema and reduction in blood flow in trunk vessels caused by these OPFR metabolites is compared to that of parental OPFRs.

2. Materials and Methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), TDCIPP and TCEP were purchased from Wako Pure Chemical Industries (Osaka, Japan). TPHP was purchased from Sigma-Aldrich (Bornem, Belgium). OPFR metabolites, HO-*m*-TPHP, HO-*p*-TPHP, DPHP, HO-DPHP, BDCIPP and BCEP were custom synthesized by Dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany). Purity of all

123 standards was above 98% as measured by MS and NMR techniques. Isooctane was used for
124 preparing stock solutions of TPHP, TDCIPP and TCEP. Acetonitrile was used for preparing stock
125 solutions of HO-*m*-TPHP, HO-*p*-TPHP and HO-DPHP, while methanol was used to prepare stock
126 solutions of DPHP, BDCIPP and BCEP. These solvents were evaporated by the nitrogen blow-down
127 system and replaced with DMSO to be used for exposure study with zebrafish embryos. Serially
128 diluted working solutions (i.e., 1, 3, 10, and 30 mM) were prepared for all chemicals. Structures of
129 chemicals examined in the present study are shown in Fig. 1. Carboxymethyl cellulose (CMC) was
130 obtained from Sigma-Aldrich.
131
132 *2.2. Zebrafish breeding*
133 Zebrafish of AB wild type strain was used for all experiments. Adult zebrafish were reared in a
134 continuous flow system (REI-SEA, Iwaki, Tokyo) with filtered water at 28.5 ºC under a 14 h
135 light/10 h dark diurnal cycle. Water quality was regularly monitored and kept as described
136 previously (Kubota et al., 2019). Fertilized eggs were collected from group mating of 3 females and
137 4 males per tank, and rapidly transferred to petri dishes containing breeding water. Breeding water
138 was refreshed every 24 h, and any dead or dysgenesis embryos were removed every day until the
139 chemical exposure at 72 hours post fertilization (hpf).
140
141 *2.3. Chemical exposure and morphological assessment*
142 In our preliminary experiments, pericardial edema and reduction in blood flow in trunk vessels

were clearly observed by several of OPFRs and their metabolites when embryos were exposed between 72 and 96 hpf. Thus, we selected this exposure protocol for elucidating developmental effects in the present study. Embryos (n = 10) at 72 hpf were exposed to vehicle (0.1% DMSO, v/v) alone or various concentrations (i.e., 1, 3, 10, and 30 μM) of OPFRs or their metabolites, including TPHP, HO-*m*-TPHP, HO-*p*-TPHP, DPHP, HO-DPHP, TDCIPP, BDCIPP, TCEP and BCEP in 4 cm petri dishes containing breeding water for 24 h. Exposures were conducted by adding 4 μL of each working solution directly into the 4 mL of breeding water at a position distant from the embryos and then mixed through gentle but thorough swirling. Exposures were kept at static condition in the same exposure medium throughout the experiment. At 96 hpf, all exposed embryos were rinsed by fresh breeding water and placed laterally on the microscope slide with 3% CMC for immobilization. Pericardial edema and reduction of blood flow in the trunk vessel were evaluated by using an inverted microscope CKX53 (Olympus, Tokyo, Japan) equipped with a digital camera DP-73 (Olympus) and an imaging software cellSens® (Olympus). The severity of pericardial edema and reduction in blood flow was scored between 0 and 2, with "0" being no effect and "2" being severe effect (see Fig. 2 for pericardial edema), according to the previous study (Wincent et al., 2016) with slight modification. The modification was made to avoid potential misleading arisen from the subjective judgement; we used 3 levels of severity scoring index (i.e., no effect, mild effect, and severe effect) (see Fig. 2 for pericardial edema scoring). Severity of reduction in blood flow was defined as different degree of circulation of red blood cells passing through the particular region of the trunk vessel.

2.4. Statistics

R version 3.5.1 (R Core Team, 2018) and its packages (tidyverse (Wickham, 2017), nparcomp (Konietschke et al., 2015), and ggplot2 (Wickham, 2016)) were used for statistical analysis and graphical representations. In the present study, since the chemical effects on pericardial area and blood flow were reproducible among three replicate dishes, we conducted statistical analysis using n=30 embryos which were obtained from the combination of three separate experiments. Significant differences in numbers of affected and unaffected embryos between control group (DMSO) and exposed groups were determined by one way ANOVA followed by Dunnett's *post hoc* test. "Affected embryos" are represented by the pooled number of all living embryos with severity score 1 and 2 plus dead embryos. Dunnett type multiple comparisons for nonparametric relative contrast effects were performed with nparcomp packages in R. Significance of difference was set to 0.05.

3. Results

3.1. Circulatory failure caused by TPHP and its metabolites

TPHP and two of its metabolites, HO-*m*-TPHP and HO-*p*-TPHP, elicited pericardial edema at concentrations of 10 and 30 μM, culminating with death (Fig. 3). Similarly, reduction of blood flow in trunk vessels was seen for TPHP, HO-*m*-TPHP and HO-*p*-TPHP (Supplementary Fig. S1). At the highest concentration tested (30 μM), TPHP, HO-*m*-TPHP and HO-*p*-TPHP showed 33%, 100% and 80% of mortality, respectively. In embryos exposed to 10 μM HO-*m*-TPHP, mortality reached

183 37%, whereas 30% and 33% were without pericardial edema and reduction of blood flow,
184 respectively. All other embryos in the 10 μM HO-*m*-TPHP exposed group were affected, showing
185 pericardial edema and reduction in blood flow (Fig. 3). In embryos exposed to 10 μM HO-*p*-TPHP,
186 mortality was only 3%, while 63% and 67% of embryos were without pericardial edema and
187 reduction of blood flow, respectively. Embryos exposed to 10 μM TPHP were all affected, with
188 mortality being 13%. In contrast, neither DPHP nor HO-DPHP showed significant increase in
189 pericardial edema or blood flow reduction.
190
191 *3.2. Circulatory failure caused by TDCIPP and its metabolite*
192 As shown in Fig. 4, TDCIPP showed 43% of lethality at the highest concentration (30 μM),
193 whereas BDCIPP showed no lethality at any of concentrations tested (1 to 30 μM). Embryos which
194 survived in the 30 μM TDCIPP-exposed group were severely affected, showing 53% for pericardial
195 edema and 57% for reduction in blood flow. On the other hand, BDCIPP showed no positive signs
196 of pericardial edema or blood flow reduction even at the highest concentration tested, although 14%
197 of embryos exposed to the highest concentration showed mild pericardial edema. BDCIPP failed to
198 elicit reduction in blood flow at the highest concentration (Supplementary Fig. S2).
199
200 *3.3. Morphological assessment of embryos exposed to TCEP and its metabolite*
201 Unlike TPHP, TDCIPP or two of TPHP metabolites, neither TCEP nor its metabolite BCEP
202 elicited pericardial edema or blood flow reduction in trunk vessels, even at the highest

concentration tested (30 μM) (Fig. 4; Supplementary Fig. S2). Embryos exposed to BCEP posed no significant signs of edema.

3.4. Relationship between lipophilicity and circulatory failure of OPFRs and their metabolites

Data on log K*ow* for OPFRs and their metabolites were obtained from a literature by Du and co-workers (2015), EPISuite, and ACD/Labs in Chemspider (2019) (Table 1). TPHP had the highest log K*ow* value, followed by HO-*m*-TPHP and HO-*p*-TPHP. Log K*ow* values of DPHP and HO-DPHP were much lower than those of the three compounds mentioned above. As for chlorinated OPFR groups, log K*ow* values of TDCIPP and BDCIPP were considerably higher than those of TCEP and BCEP. The values and order of log K*ow* for OPFRs and their metabolites tested was as follows: TPHP (4.59) > HO-*m*-TPHP (4.22) > TDCIPP (3.65) > HO-*p*-TPHP (3.27) > DPHP (2.88) > BDCIPP (2.18) > TCEP (1.44) > BCEP (0.83) > HO-DPHP (0.51). As shown in Fig. 5, chemicals with high log K*ow* values showed increasing occurrence of pericardial edema and reduction in blood flow.

4. Discussion

The present study showed that waterborne exposure of zebrafish embryos to several metabolites of OPFRs elicited circulatory failure in a concentration-dependent manner, with potency almost equivalent to their parent chemical. TPHP and its metabolites HO-*m*-TPHP and HO-*p*-TPHP were most potent for inducing circulatory failure among chemicals tested, followed by

223 TDCIPP. Unlike HO-*m*-TPHP and HO-*p*-TPHP, the other two TPHP metabolites, DPHP and HO-
224 DPHP, showed no significant increase in circulatory failure at 30 μM or less. Similarly, a TDCIPP
225 metabolite BDCIPP was less potent for inducing circulatory failure in the present study. Neither
226 TCEP nor its metabolite BCEP showed substantial effect on the circulatory system at any of the
227 concentrations tested.

228 There have been several reports on developmental toxicity caused by metabolites of OPFRs in
229 zebrafish. Continuous exposure of enzymatically dechorionated embryos to DPHP beginning at 6
230 hpf showed no malformation at 120 hpf at concentrations up to 6.4 μM (Noyes et al., 2015). When
231 embryos were treated with a much higher concentration of DPHP (i.e., 1000 μM) beginning at 24,
232 30 or 48 hpf, cardiac defects were recorded (Mitchell et al., 2019). These results, together with ours,
233 indicate a weak potency of DPHP toward developmental toxicity, as compared to its parental
234 chemical, TPHP.

235 Exposure to BDCIPP resulted in mortality at 24 hpf, with much greater potency than its
236 parental chemical TDCIPP (Noyes et al., 2015). In our study, TDCIPP but not BDCIPP was potent
237 for causing circulatory failure, when hatched embryos were exposed during a period of 72 and 96
238 hpf. Such a difference in developmental toxicity may be explained by the combination of differing
239 endpoints (mortality vs circulatory failure) and developmental stages (before 24 hpf vs 96 hpf). In
240 general, pericardial edema caused by chemical exposure becomes prominent at 72 hpf or later.
241 Thus, it is unlikely that the same mechanism is involved in the developmental toxicity seen in the
242 study by Noyes and co-workers and ours.

We found an association between circulatory failure and log K*ow* values for not only parent OPFRs but also their metabolites. In particular, the higher bioaccumulative potential, the higher potency for inducing circulatory failure the chemicals will have. Based on chemical structures, TPHP, HO-*m*-TPHP and HO-*p*-TPHP have 3 phenyl rings, while DPHP and HO-DPHP possess 2 phenyl rings (Fig. 1). Such a structural difference can lead to differences in physiochemical properties and biological impacts, including hydrophobicity and developmental toxicity. As a result, TPHP, HO-*m*-TPHP and HO-*p*-TPHP are more hydrophobic and toxic than DPHP and HO-DPHP. Concerning chlorinated aliphatic OPFRs (i.e., TDCIPP and TCEP) and their metabolites (i.e., BDCIPP and BCEP), the severe effect on the circulatory system was pronounced only for the 30 μM TDCIPP-exposed group, while other chemicals with lower log K*ow* values failed to induce circulatory failure. Thus, developmental circulatory failure posed by OPFRs and their metabolites could be expected, at least in part, by the lipophilicity and bioaccumulative potential of chemicals of concern. Our results are in agreement with data obtained from previous studies where developmental toxicity caused by parental OPFRs was positively correlated with their log K*ow* values (Dishaw et al., 2014; Du et al., 2015; Sun et al., 2016). Here we expanded the relationship to including OPFR metabolites. These positive associations might be explained by the potential difference in the membrane permeability of chemicals which relies on the lipophilicity.

Multiple mechanisms of OPFRs-induced developmental and cardiotoxicity have been suggested to date by using zebrafish embryos. The inhibitory effect of TPHP on expression of genes involved in heart development (i.e., BMP4, NKX2-5, TBX5) led to heart malfunction, including

decreased heart rate and increased distance of sinus venosus and bulbus arteriosus (Du et al., 2015). An antagonist for RAR (BMS493) enhanced TPHP-induced cardiotoxicity (Isales et al., 2015). Conversely, cardiotoxicity induced by TPHP was mitigated by agonists for RAR (fenretinide) and for peroxisome proliferator-activated receptor gamma (PPARγ) (ciglitazone) (Isales et al., 2015; Mitchell et al., 2018). These results suggest that TPHP may affect cardiac development through the RXR related pathways, as RXR acts as a partner for dimerization with both RAR and PPARγ. Apart from this, cardiotoxicity induced by TPHP appears to be independent of the AHR-mediated pathway, because TPHP-induced cardiotoxicity was not rescued by co-exposure to an AHR antagonist (CH223191) (McGee et al., 2013).

A recent study by Dasgupta et al. (2019) reported that epiboly defects at very early stage were induced by 3.12 μM TDCIPP, and this adverse effect was mitigated by co-exposure to or pretreatment with ciglitazone (a PPARγ agonist) or 17β-estradiol (an estrogen receptor α (ERα) agonist). However, this inhibitory effect was not observed by other PPARγ agonists, including troglitazone and pioglitazone or by ERα ligands, including 17α-ethinylestradiol and genistein. Continuous exposure of zebrafish embryos to the same concentration of TDCIPP (3.12 μM) from 0.75 to 24 hpf decreased transcript levels of genes related to cardiac development (i.e., *bin1b*, *tnni2b.1*, *tnni2a.4*, *etc.*), and the same exposure increased pericardial edema at 72 hpf (Dasgupta et al., 2018). However, the same study also demonstrated that no pericardial edema was observed when embryos were exposed to the same concentration of TDCIPP during a period of post-gastrulation (~10 hpf). Thus, there could be a shared and somewhat distinct mechanism underlying

283 cardiac toxicity observed between TDCIPP and TPHP. Further investigation in a systematic
284 approach is necessary for elucidating mechanisms underlying developmental effects caused by
285 OPFRs and their metabolites, because these adverse effects could depend on chemical structures
286 and concentrations, timing and duration of exposure, and developmental stage of phenotypic
287 observations.

288 Our results clearly demonstrated that 24 hours of exposure beginning at 72 hpf was long
289 enough to induce circulatory failure. This applied to all OPFRs and their metabolites that elicited
290 circulatory failure, i.e., TPHP, HO-*m*-TPHP, HO-*p*-TPHP and TDCIPP. Studies on chemical
291 toxicology using zebrafish embryos are in general performed by continuous exposure beginning at
292 around 3 hpf when the maternal-to-zygotic transition occurs. A recent study by Alzualde et al.
293 (2018) showed that 30 and 100 μM TDCIPP exposures beginning at post-pharyngula period (after
294 48 hpf to 54 hpf) also caused bradycardia and mortality at 51 to 57 hpf, indicating that TDCIPP was
295 not considered cardiotoxic. Current results, together with the study by Alzualde et al. (2018)
296 highlight that a shorter period of exposure beginning at the later stage of development even after the
297 pharyngula period (24 – 48 hpf) is sufficient to elicit circulatory failure. This may give an ideal and
298 more readily achievable experimental protocol to further understand the adverse outcome pathway
299 of developmental effects of OPFRs and their metabolites on the circulatory system. However,
300 caution should be exercised for mechanistic investigation, because developmental effects of
301 chemicals might be affected by a variety of factors as discussed above, and the same phenotypic
302 outcome may not necessarily mean the same pathway involved in.

Conflict of interest

The authors declare no conflicts of interest in this work.

Acknowledgements

This study was supported by Grant-in-Aid for Young Scientists (A) (No. 15H05334 to A.K.) and Grant-in-Aid for Scientific Research (B) (No. 19H04275 to A.K.) from the Japan Society for the Promotion of Science. The sponsors had no involvement in performing or in the decision to publish this study.

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441

Table 1

Octanol-water partition coefficient (log*Kow*) values of OPFRs and their metabolites used in this study.

Chemical name	Abbreviation	Log *Kow* value	Reference
Triphenyl phosphate	TPHP	4.59	Du et al. (2015)
3-Hydroxylphenyl diphenyl phosphate	HO-*m*-TPHP	4.22	EPISuite
4-Hydroxylphenyl diphenyl phosphate	HO-*p*-TPHP	3.27	ACD/Labs
Diphenyl phosphate	DPHP	2.88	EPISuite
4-Hydroxylphenyl phenyl phosphate	HO-DPHP	0.51	ACD/Labs
Tris(1,3-dichloro-2-propyl) phosphate	TDCIPP	3.65	Du et al. (2015)
Bis(1,3-dichloro-2-propyl) phosphate	BDCIPP	2.18	EPISuite
Tris(2-chloroethyl) phosphate	TCEP	1.44	Du et al. (2015)
Bis(2-chloroethyl) phosphate	BCEP	0.83	EPISuite

442

443

444 **Figure legend**

445 Fig. 1. Chemical structures of the OPFRs and their metabolites used in the present study.

446

447 Fig. 2. Pericardial edema (PE) observed in zebrafish embryos exposed to OPFRs and their

448 metabolites. Severity of PE and reduction of blood flow (BF) were scored between 0 and 2, with

449 "0" being no effect and "2" being severe effect, according to the previous study (Wincent et al.,

450 2016) with some modification.

451

452 Fig. 3. Pericardial edema (PE) observed in zebrafish embryos exposed to TPHP and its metabolites.

453 Embryos were exposed to DMSO (control), or indicated concentrations of TPHP (A) or its

454 metabolites HO-m-TPHP (B), HO-p-TPHP (C), DPHP (D), or HO-DPHP (E) from 72 hpf to 96 hpf

455 (N=30). Severity of PE was scored between 0 and 2, with "0" being no effect and "2" being severe

456 effect, according to the previous study (Wincent et al., 2016), with some modification. Significant

457 differences in numbers of affected and unaffected embryos between control group (DMSO) and

458 exposed groups were determined by one way ANOVA followed by Dunnett's post hoc test, and are

459 shown by asterisk (**$p < 0.01$, ***$p < 0.001$). "Affected embryos" are represented by the pooled

460 number of all living embryos with severity score 1 and 2 plus dead embryos.

461

462 Fig. 4. Pericardial edema (PE) observed in zebrafish embryos exposed to TDCIPP, TCEP and their

463 metabolites. Embryos were exposed to DMSO (control), or indicated concentrations of TDCIPP (A)

464 or its metabolite BDCIPP (B), or TCEP (C) or its metabolite BCEP (D) from 72 hpf to 96 hpf

465 (N=30). Other conditions are the same as given in the legend of Fig. 3.

466

467 Fig. 5. Relationship between Log Kow of OPFRs and their metabolites and circulatory failure

468 scoring in zebrafish embryos exposed to these compounds. Data on pericardial edema (A) and

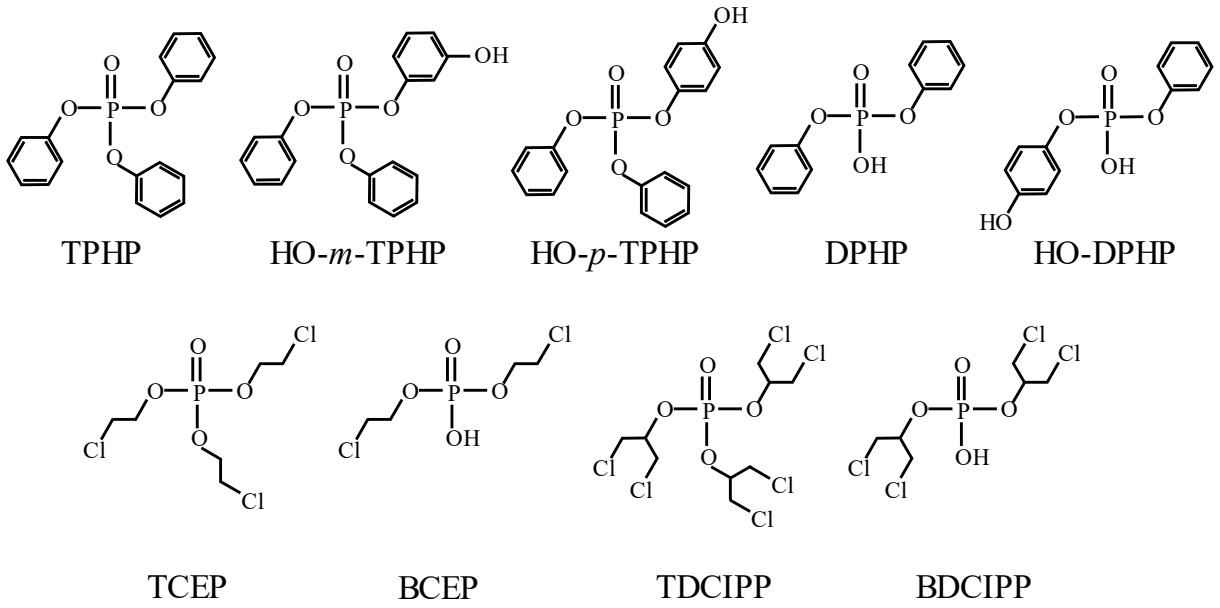
469 reduction in blood flow (B) in embryos exposed to the two highest concentrations (10 and 30 μM)

470 were shown for clarity. Color of the plots shows the two concentrations and size of plots shows

471 number of zebrafish embryos.

472

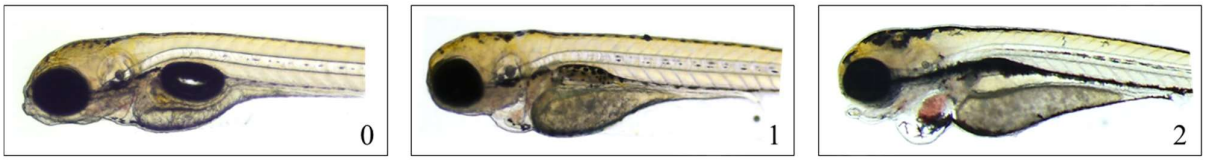
473



474

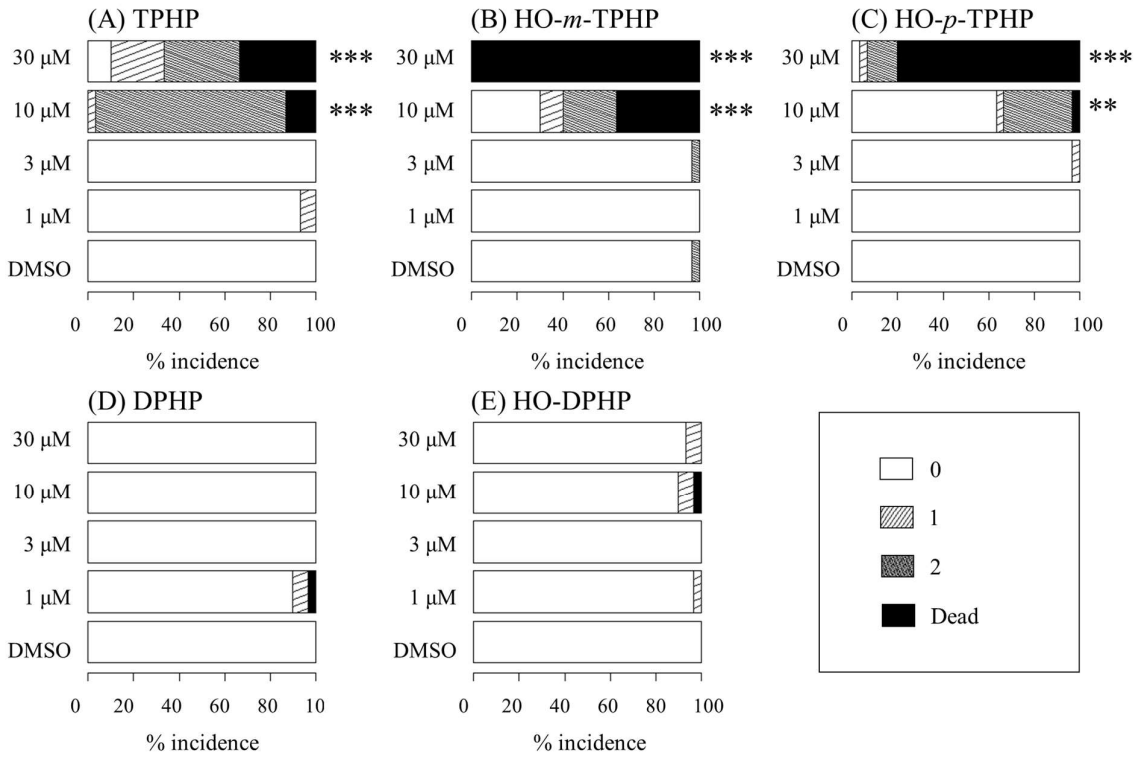
475 **Fig. 1**

476

477 

478 **Fig. 2**

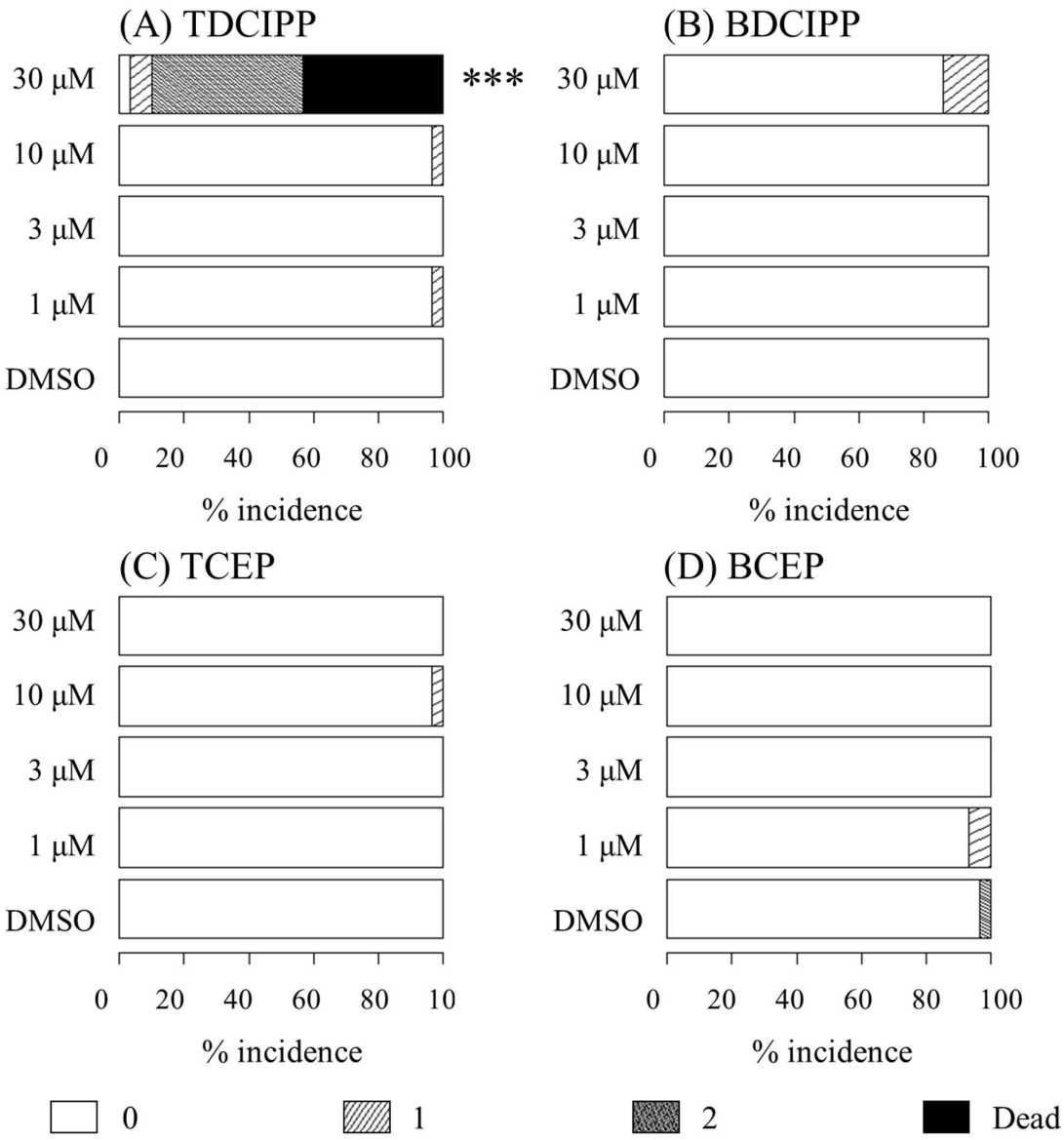
479



480

481 **Fig. 3**

482



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484 **Fig. 4**

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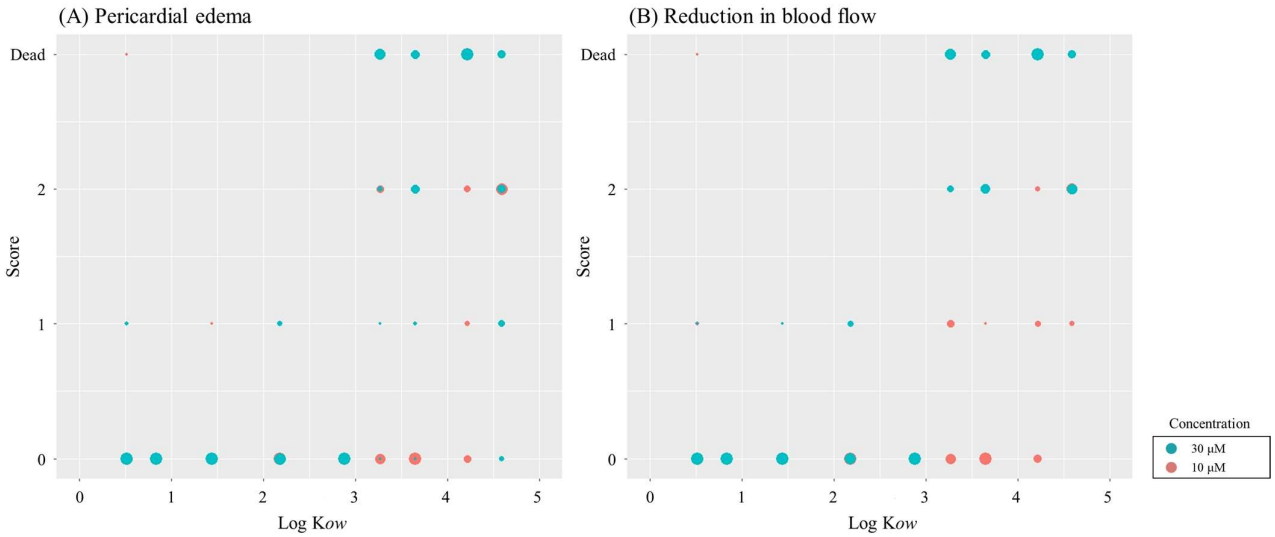
(A) Pericardial edema
(B) Reduction in blood flow
Dead
2
1
0
Score
0
1
2
3
4
5
Log Kow
Concentration
30 μM
10 μM

Fig. 5