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Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish

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Abstract

As polybrominated diphenyl ethers are phased out, numerous compounds are emerging as potential replacement flame retardants for use in consumer and electronic products. Little is known, however, about the neurobehavioral toxicity of these replacements. This study evaluated the neurobehavioral effects of acute or developmental exposure to *t*-butylphenyl diphenyl phosphate (BPDP), 2-ethylhexyl diphenyl phosphate (EHDP), isodecyl diphenyl phosphate (IDDP), isopropylated phenyl phosphate (IPP), tricresyl phosphate (TMPP; also abbreviated TCP), triphenyl phosphate (TPHP; also abbreviated TPP), tetrabromobisphenol A (TBBPA), tris (2-chloroethyl) phosphate (TCEP), tris (1,3-dichloroisopropyl) phosphate (TDCIPP; also abbreviated TDCPP), tri-*o*-cresyl phosphate (TOCP), and 2,2-,4,4′-tetrabromodiphenyl ether (BDE-47) in zebrafish (*Danio rerio*) larvae. Larvae (n ≈ 24 per dose per compound) were exposed to test compounds (0.4–120 μM) at sub-teratogenic concentrations either developmentally or acutely, and locomotor activity was assessed at 6 days post fertilization. When given developmentally, all chemicals except BPDP, IDDP and TBBPA produced behavioral effects. When given acutely, all chemicals produced behavioral effects, with TPHP, TBBPA, EHDP, IPP, and BPDP eliciting the most effects at the most concentrations. The results indicate that these replacement flame retardants may have developmental or pharmacological effects on the vertebrate nervous system.

Keywords

Zebrafish; Neurotoxicity; Flame retardants; Developmental; Acute

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1. Introduction

There is widespread human and wildlife exposure to flame retardants, making these chemicals a priority for both human and ecological health assessments. As some classes of flame retardants (e.g., polybrominated biphenyl ethers PBDEs) are being phased out due to bioaccumulation and toxicity, others have been introduced as replacements for use in furniture, electronics, textiles, automotive products, and construction materials. A recent longitudinal study compared levels of PBDEs and the emerging alternative flame retardants in indoor dust and children's hand wipes; findings suggest that exposure to these alternative flame retardants are predicted to be as high as PBDE exposure (Stapleton et al., 2014). Despite documented human and wildlife exposure to these newer compounds (Segev et al., 2009; Dishaw et al., 2014b; Ezechiáš et al., 2014; Wei et al., 2015; Gao et al., 2014), there is sparse information on the possible human health or ecological toxicity of many of these replacements.

Neurotoxicity is a primary concern associated with emerging alternative flame retardants due to their organophosphorus backbone. Structurally related compounds have previously been shown to affect brain development (Carr et al., 2013, 2014; Dishaw et al., 2011; Slotkin et al., 2006, 2009; Slotkin and Seidler, 2005, 2011). Hence, it is important to deploy a test system for rapid assessment of nervous system perturbations. The zebrafish model is positioned to address these concerns, as there is a basic understanding of nervous system development (reviewed in Blader and Strähle, 2000; Guo, 2009; Young et al., 2011; Guo, 2004), as well as techniques for rapidly evaluating the effects of chemical exposures on the zebrafish nervous system (Bang et al., 2002; Bichara et al., 2014; Ellis and Soanes, 2012; Green et al., 2012). Many investigators concentrate on evaluating behavior, because, to a large extent, behavior integrates nervous system function, making it an appropriate, approachable, and apical endpoint for screening, demonstrating excellent concordance with mammalian neurotoxicity (Kokel et al., 2010; Kokel and Peterson, 2011; Levin et al., 2003, 2004; Sallinen et al., 2009; Selderslaghs et al., 2013, 2010; Anichtchik et al., 2004; Fernandes et al., 2014; Fetcho and Liu, 1998; Nishimura et al., 2015). The developmentally neurotoxic PBDE flame retardants (Costa and Giordano, 2007; Costa et al., 2014) have been phased out and replaced with other halogenated (primarily chlorinated) and organophosphorus based chemicals (see Table 1 for chemicals, abbreviations and structures). There is some preliminary evidence that member(s) of both the halogenated and organophosphorus classes (BDE-47, TBBPA, TDCIPP, and TPHP) perturb the thyroid system in developing zebrafish (Chan and Chan, 2012; Kim et al., 2015; Liu et al., 2013). There is also evidence that developmental exposure to either TDCIPP or TCEP produces developmental neurotoxicity as assessed by changes in locomotor activity at 6 days post-fertilization (dpf) in larval zebrafish (Dishaw et al., 2014a, 2014b). To a large extent, however, our knowledge about the general toxicity and/or developmental neurotoxicity of most of the organophosphorus flame retardants is incomplete.

The studies described in this present paper, in conjunction with its companion paper (Behl et al., 2015), have been conducted to address this key gap in the flame retardant literature. Here, we have assessed the behavioral toxicity of a group of 11 different chemicals: one

(BDE-47) is a flame retardant that is being phased out and is known to be developmentally neurotoxic; many others (TBBPA,TCEP,TDCIPP BPDP, EHDP, IDDP,IPP,TMPP,TPHP) are currently being used, or are emerging, as flame retardants; and one (TOCP) is a neurotoxic compound with a structure similar to some of the organophosphorus flame retardants (see Table 1 for a list of the 11 chemicals that are the subject of these experiments). All chemicals were assessed for lethality and teratogenic endpoints [data presented in Behl et al. (2015), as well as developmental neurotoxicity and acute behavioral toxicity (data presented herein)].

2. Materials and methods

2.1. Chemicals

Table 1 lists the chemicals used in this study, their CAS #s, abbreviations, and structures and the supplier and purity of each chemical are listed in ,Supplemental Table 1. Stock solutions of each chemical were prepared in dimethyl sulfoxide (DMSO; obtained from Sigma-Aldrich, St. Louis, MO). The National Toxicology Program supplied the chemicals, and are from the same source as those used in our companion paper (Behl et al., 2015). Subsequent serial dilutions for the stock plates, used to dose the experimental plates, were also prepared in DMSO (final DMSO concentration was 0.4% (*v/v*) in all wells, including controls).

2.2. Experimental animals

All studies were carried out in accordance with the guidelines of, and approved by, the Institutional Animal Care and Use Committee at the U.S. EPA National Health and Environmental Effects Research Laboratory.

Wild type adult zebrafish (*Danio rerio*), undefined, outbred stock originally obtained from Aquatic Research Organisms, Hampton, NH, 03842 and EkkWill Waterlife Resources, Ruskin, FL 33575 were housed in an AAALAC-approved animal facility with a 14:10 h light:dark cycle (lights on at 08:30 h). Adult fish were kept in flow-through colony tanks (Aquaneering Inc., San Diego, CA) with a water temperature of 28 °C. For egg collection, adults were placed in a 15 L (static) breeding tank the afternoon before egg collection. Eggs were collected the next morning approximately 1 h after the lights came on (08:30 h).

2.3. General embryo rearing

All embryos were gathered from the breeder tank and placed in a 26 °C water bath until washing. Embryos were washed (Westerfield, 2000) twice with 0.06% bleach (*v/v*) in 10% Hanks' Balanced Salt Solution (13.7 mM NaCl, 0.54 mM KCl, 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 130 μM $CaCl_2$, 100 μm $MgSO_4$ and 420 μm $NaHCO_3$; all salts obtained from Sigma-Aldrich, St. Louis, MO) (hereafter referred to as "10% Hanks'") for 5 min each wash, and rinsed in 10% Hanks' between washes.

2.4. Chemical exposure during development

After washing, fertilized eggs were placed, one embryo per well, into 10% Hanks' solution in the upper insert of a 96-well mesh microtiter plate (Multiscreen™ catalog #MANMN4050, Millipore Corp., Bedford, MA). After all embryos were plated, the upper

mesh insert was moved to a new 96-well plate lower insert that contained the chemical dilutions. To dilute the chemicals, 1 μl from the stock plate was added to each well in the lower insert. Then 150 μl of 10% Hanks' was added to each well. The upper mesh insert containing the embryos was blotted on filter paper (Whatman GF/B paper (fired), Brandel, Gaithersburg, MD), and placed in the lower insert. Finally, an additional 100 μl of 10% Hanks' was added to the upper insert. Each plate was then sealed with a non-adhesive material (Type A, BioRad, Hercules, CA), covered with the lid, and wrapped in Parafilm™ to minimize evaporation. The fish were then placed in an incubator, maintained on a 14:10 light:dark cycle at 26 ± 0.1 °C, where they were reared for 6 days.

The embryos were dosed on day 0 (initial plating) and day 1 post fertilization (see Fig. 1 for Experimental Design). The day after plating (day 1 post fertilization; 1 dpf) the 250 μL of 10% Hanks' solution with the appropriate chemical concentration in each well was completely renewed. The fish then remained undisturbed in the incubator for three days. On day 5 they were transferred to 10% Hanks' only (without chemical). The 10% Hanks' was also renewed on day 6, prior to behavioral testing and assessments (see Fig. 1). Therefore the animals had been rinsed in 10% Hanks' twice to remove any residual chemical before they were tested on day 6.

The overall experimental design was to first assess the overt toxicity of each chemical (i.e., Range Finding) followed by a more detailed assessment of the developmental neurotoxicity of the chemical at non-overtly toxic concentrations (Developmental Behavioral Assessment). The design of both studies is depicted in Fig. 1. The Range Finding study did not include any behavioral assessment and was only used to determine the concentrations used in the Developmental Behavioral Assessment neurotoxicity study. The same concentrations of chemical were also used for an Acute Behavioral Assessment on day 6 pf (described below).

2.5. Range finding

For behavioral testing, the fish needed to be alive, hatched and not malformed. Therefore, in order to select appropriate concentrations for each chemical, we conducted a range finding study to assess each chemical for overt toxicity (i.e., death, malformations, or non-hatching). Zebrafish embryos were dosed, as described above, with 8 different concentrations (semi-log spacing, $n \geq 4$ per concentration), with a maximum possible concentration of 120 μM. The Developmental Behavioral Assessment was conducted so that the highest concentration used for behavioral testing was at or just below the overtly toxic concentrations, determined from the Range Finding Study. If the Range Finding Study did not elicit any overt toxicity, the highest nominal concentration for the behavioral assessments was 120 μM.

2.6. Developmental behavioral assessment

In order to dose the fish for behavioral testing, a stock plate for each chemical was made (see "Chemicals" section). The location of each chemical concentration was randomized on the stock plate (96 well glass plate which mirrored the dosing on the experimental plate containing the embryos/larvae), and therefore the dose groups on the experimental plate were also randomized. All concentrations of each chemical accompanied by vehicle controls

were included on every stock plate and experimental plate. In addition, a positive technical control was included on each plate: either chlorpyrifos (procured from Battelle, Columbus, OH; CAS # 2921–88–2; final nominal concentration was 11.5 μM) or heptachlor (procured from Chem Service, Inc., West Chester, PA; CAS# 76–44–8; final nominal concentration was 2 μM), as our preliminary data indicated that either chemical would produce a behavioral effect when given to zebrafish larvae either in a developmental or acute dosing scenario. Moreover, both chemicals have been shown to produce developmental neurotoxicity in mammals (Moser et al., 2001; Richardson et al., 2008; Slotkin et al., 2006; Slotkin and Seidler, 2005). All testing was performed on 6 dpf larvae in the same 96-well plate in which they had been dosed and reared. Recording fish behavior was essentially as described by MacPhail and coworkers (MacPhail et al., 2009). The 2 h testing paradigm consisted of two periods of light/dark/light separated by a 40 min dark phase. The luminance of the first light/dark/light portion of the testing paradigm was 0.09 lx in both light phases and the luminance of the second light/dark/light period was 5.0 lx in both light phases. Luminance was taken at the level of the recording platform using a photometer (model Dr.-2250–1, 2B silicon detector, TC 284 photometric filter, Gamma Scientific, San Diego, CA).

The morning of testing (day 6), the rearing solution was renewed, then plates were moved to the darkened behavioral testing room in which the ambient temperature was the same as the rearing incubator (26 °C). For all experiments, testing occurred between 4 and 8 h after light initiation for the zebrafish larvae, in order to encompass the most stable behavioral period for the fish (MacPhail et al., 2009). For testing, the plate was transferred to a light box that provided both infrared and visible light, and the movement of each fish was monitored using a behavior-recording system [Noldus Information Technology, Leesburg, VA (www.noldus.com)]. The testing paradigm consisted of a 20 min acclimation phase in the dark (Basal phase; data not presented nor analyzed), followed by 10 min of light (0.09 lx), 10 min of darkness, 10 min of light (0.09 lx), 40 min of darkness, 10 min of light (5.0 lx), 10 min of darkness, and 10 min of light (5.0 lx). Prior research in this laboratory (Irons et al., 2010; MacPhail et al., 2009; Padilla et al., 2011), and in several others (Burgess and Granato, 2007; Emran et al., 2007; Prober et al., 2006; Fernandes et al., 2012), has demonstrated that 1) exposure of zebrafish larvae to light has a characteristic effect of increasing locomotor activity when darkness is reinstated, and 2) the level of light during the light phase determines the level of increase in activity during the subsequent dark phase. Thus, the basal phase was included to minimize any behavioral disruption due to transfer of a plate to the recording platform. Data were collected during this acclimation phase but were not analyzed further because of uncertainties, and therefore lack of specification and stimulus control, regarding the variables that could influence the activity.

2.7. Analysis of fish movement

Fish movement (locomotion) was tracked from videos using Ethovision XT (Noldus Information Technology) software Version 8.5. Tracking rate was 5 samples/s (i.e., an image was captured every 200 ms). A dynamic subtraction method was used to detect objects that were darker than the background, with a minimum object size of 10 pixels. Tracks were analyzed for total distance moved (cm). An input filter of 0.135 cm (minimum

distance moved) was used to remove system noise. All locomotion data is expressed as distance moved (cm) per 2 min segment of testing.

2.8. Lethality and malformation assessment

We have demonstrated that malformed 6 dpf zebrafish larvae do not behave normally in our behavioral paradigm(Padilla et al., 2011),there-fore all malformed larvae were eliminated from any behavioral analysis. All animals that were dead, not hatched or displayed overt toxicity (e.g., edema, curved spine, small eyes, small head, abnormal position in the water column) were excluded based on post-testing assessments (Fig. 1); only animals that appeared normal were used for the behavioral analysis. Assessments were made by visual inspection of the larvae under a dissection scope (Olympus SZH10 Research Stereo) at 6 dpf immediately after behavioral testing.

2.9. Acute challenge and behavioral assessment

After washing, fertilized eggs were plated as described above (see “Chemical Exposure During Development” section). The test chemical, however, was not added at this stage of the procedure (day 0 – initial plating); only 250 μL of 10% Hanks’ solution was used. The unexposed, plated embryos were then placed in an incubator, maintained on a 14:10 light:dark cycle at 26 ± 0.1 °C, and remained undisturbed for 4 days. On day 5 pf, each well was completely renewed with fresh 10% Hanks’, and the plate was returned to the incubator. On day 6 pf, the plate was removed from the incubator and dosed with the same stock plate that was used for the developmental behavioral assessment (see “Developmental Behavioral Assessment” section). Immediately after dosing, the plate of larvae was transferred to the darkroom, with an ambient temperature of 26 °C, and remained undisturbed for 30 min in the dark to allow the larvae to re-adjust after disruption from movement and light due to dosing. Next, the larvae were exposed to three identical, sequential 26 min testing periods: 6 min of darkness, 10 min of light (5.0 lx), 10 min of darkness. The three tests occurred at the following times: 1) 0.5 h after dosing, 2) 1.5 h after dosing, and 3) 2.5 h after dosing. Larval locomotor activity was analyzed as described above (see “Developmental Behavioral Assessment” section). After all three periods of testing were completed, fish were assessed for hatching, death, and malformations, and only normal animals were included in the acute behavioral exposure analyses.

2.10. Statistical analyses

All data were analyzed using Statview© (SAS Institute, Inc., Cary, NC; version 5.0.1). The data were first assessed using a repeated-measures analysis of variance (ANOVA) with time and dose as the independent variables and locomotor activity (distance moved/time) as the dependent variable. All of the results of this repeated-measures ANOVA for both the developmental and acute (time of peak effect) assessments are presented in Supplemental Table 2. Significance was set at $p \leq 0.05$. In the case of a significant time by dose interaction (time x dose interaction), step-down ANOVAs were performed to assess lower order effects. This involved first assessing that there was a significant effect of dose at each two minute behavioral interval, and if so, Fisher’s PLSD comparisons were conducted to compare between dosage groups. All data are presented as mean ± standard error of the mean (SEM). The number of independent observations are given in the figure legends. Underneath each

behavioral graph is a detailed grid showing each dose level of the chemical and whether a significant increase or decrease in activity was noted at each 2 min interval and at which dose(s). Only statistically significant results are discussed throughout the paper, and if there were fewer than 14 normal larvae in a dosage group, that group was not included in the analysis.

3. Results

3.1. Developmental exposure effects

3.1.1. Positive controls (Fig. 2)—Both developmental exposure to heptachlor (2 μM final concentration) or chlorpyrifos (11.5 μM final concentration) produced changes in locomotor activity when the animals were tested on day 6 pf. Developmental exposure to either chemical decreased activity primarily in the dark phases of testing, although heptachlor also produced an exaggerated hyperactivity during the initial 2 to 4 min after the light to dark transition and decreased activity in the final light phase. Developmental exposure to either chemical produced extended hypoactivity during the 40 min dark phase.

3.1.2. Test chemicals (Fig. 3 and ,Supplemental Table 3)—Developmental exposure to three of the test chemicals did not produce any effects on locomotor activity: TBBPA, BPDP, and IDDP. One chemical, EHDP, only produced very weak effects. We considered these effects weak because they did not occur at adjacent doses, nor at adjacent 2 min time segments, and only occurred in 2 out of the 50 possible time segments. Developmental exposure to either TOCP or IPP produced hyperactivity in animals when tested on day 6 pf, and those effects were primarily noted in the dark phases. Larvae that were devel-opmentally exposed to BDE-47, TCEP or TMPP showed primarily hypoactivity when tested on day 6 pf, and those effects were also mainly noted in the dark phases, although both BDE-47 and TCEP also showed decreased activity in the second (0.09 lx) light phase. Animals treated with TDCIPP during development showed a mixed behavioral response dependent on dose, with the lower doses showing increased activity and the higher doses showing decreased activity; both effects were seen in the dark phases. Animals treated with TCEP during development also showed a mixed behavioral response that appeared to be dependent on brightness of the light during the light phases: increased activity during the lower light phase (0.09 lx), but decreased activity during the brighter phase (5.0 lx).

3.2. Acute exposure effects

3.2.1. Positive controls (Fig. 2)—Acute exposure to chlorpyrifos (11.5 μM final concentration) produced dramatic changes in locomotor activity: chlorpyrifos-treated larvae were hyperactive during all time segments and were generally unresponsive to changes in lighting conditions. Larvae acutely exposed to heptachlor (2 μM final concentration) were hyperactive during the light phase and hypoactive during the dark phase, most apparent in the final two testing periods (1.5 or 2.5 h after dosing).

3.2.2. Time of peak effect—We assessed three different periods after acute dosing because we had no prior knowledge about the time of peak effect for these chemicals. The time of peak effect was defined as the test period that had the most 2 min time segments

with significant changes in locomotor activity. In practice, this was determined by summing the number of 2 min time segments that showed significant changes within each test period. In some instances, there were only very minor differences between sums at adjacent test periods. The time of peak effect for each chemical is circled in each graph in Fig. 4 and also noted in ,Supplemental Table 4. No chemical showed a time of peak effect at 0.5 h; all of the chemicals showed a time of peak effect at either 1.5 or 2.5 h. The time of peak effect for TPHP, IPP, EHDP, TCEP, and TOCP occurred during the second testing period (1.5 h after chemical treatment). By contrast, the time of peak effect for TBBPA, IDDP, TMPP, BPDP, TDCIPP, and BDE-47 occurred during the third testing period (2.5 h after chemical treatment). It is possible that some chemicals may exhibit times of peak effect beyond 2.5 h, but this experiment did not evaluate effects at later times.

3.2.3. Test chemicals (Fig. 4 and ,Supplemental Table 4)—All chemicals produced changes in locomotor activity. In general, acute exposure to the test chemicals decreased activity during the dark phases and increased activity in the light phases. Exceptions to this pattern were BPDP, EHDP and TOCP: these compounds elicited hyperactivity in the basal dark phases. In addition, TOCP and TCEP did not cause hypoactivity in the non-basal dark phases. Overall, when considering the number of significant changes across doses at the time of peak effect, BDE-47, TCEP and IDDP produced the fewest acute behavioral effects, while TPHP, TBBPA, EHDP, IPP and BPDP produced the most behavioral effects after acute exposure.

3.2.4. Comparison of the developmental and acute effects—Table 2 compares the pattern of effects at the time of peak effect for the acute testing paradigm to the corresponding dark/5.0 lx/dark period of the developmental testing paradigm. This portion of the developmental testing schedule was chosen because it was exactly the same schedule and light level as the acute testing schedule. In general, the pattern of behavioral changes was different after acute exposure as compared to developmental exposure, supporting the idea that the developmental effects are not due to residual chemical present at the time of testing. Interestingly, BDE-47 was the only chemical that showed more extensive (i.e., many time segments were affected at many doses) behavioral effects after developmental exposure than after acute exposure: acute exposure to BDE-47 elicited only a few effects at a couple of time segments in the last dark phase, while developmental exposure produced extensive hypoactivity in both dark phases. In contrast, EHDP elicited extensive acute effects with hyperactivity in the basal phase and the light phase and hypoactivity in the dark phase as compared to only minimal (i.e., few time segments affected at few doses) hypoactivity in the second dark phase after developmental exposure. IPP, likewise, only produced minimal developmental effects, but extensive acute effects. TMPP produced extensive hypoactivity in the second dark phase after acute and developmental exposure, but the hyperactivity in the light phase appeared only after acute exposure. TPHP also produced extensive hypoactivity in the second dark phase after both acute and developmental exposure, but the hypoactivity in the first dark phase and the light phase were only present after acute exposure. Developmental exposure to TCEP produced hypoactivity in the light phase, while acute exposure to the same chemical did not cause abnormal activity during the light phase but did elicit hypoactivity in the first dark phase. Unlike any of the other chemicals,

developmental exposure to TDCIPP produced hyperactivity at low doses, and just two instances of hypoactivity during the final dark phase. Acutely, however, TDCIPP showed significant hypoactivity at the highest doses during the final dark phase and also hyperactivity at the high doses during the light phase. TOCP exposure did not produce any hypoactivity, but rather increased activity in the first dark phase and the light phase, with many more effects seen at the lower doses after the acute exposure as compared to the developmental exposure.

4. Discussion

Every flame retardant tested perturbed the behavior of 6 day old zebrafish larvae. When tested acutely, all of the chemicals produced behavioral effects, while developmental exposure to most, but not all, of the chemicals affected behavior. With the exception of TOCP, chemicals that produced behavioral effects after developmental exposure did so at exposure concentrations 4 to 10 times lower than the concentration that produced overt (lethality and malformations) toxicity, with the majority of the lowest effective levels below 10 μM (Table 3). Acute exposure to every chemical tested produced behavioral effects at concentrations 2 to 12 times lower than the developmentally toxic concentrations, with the majority of the lowest effect levels in the very low micromolar range. In many cases, a no-effect level was not achieved, meaning that the actual lowest effect level may be below that shown in Table 3. Because of the concordance between zebrafish and mammalian developmental pathways, the zebrafish model is used to understand developmental disorders in humans (e.g., The Deciphering Developmental Disorders Study, 2015) and is also employed extensively in ecotoxicological studies as a surrogate for other fish species (Belanger et al., 2013; Braunbeck et al., 2005; Knöbel et al., 2012; Lammer et al., 2009). Therefore, to build on the initial zebrafish screening tests presented here, these organophosphorus flame retardants should be examined for developmental neurotoxicity in mammals and for additional relevant effects across ecosystems.

In order to put our results in context with previously published data, we discuss each chemical individually below. Unfortunately, the toxicity of many of these compounds in either mammals or fish is largely unexamined in the literature. One recently published paper (Noyes et al., 2015), however, examined the overt and behavioral toxicity of many of these chemicals allowing us to make some comparisons, though differences in procedures are noted. One of the most drastic differences was that Noyes and coworkers reported a LEL (lowest effect level) when gauging overt toxicity (lethality and dysmorphology). Because, in some cases, their pattern of overt toxicity was not dose-dependent, we have elected not to use those data for comparison with our Point of Departure calculation (based on dose dependency) for overt toxicity.

BDE-47 is developmentally neurotoxic to mammals, likely through disturbing the function of the thyroid axis during development (reviewed in Costa and Giordano, 2007; Gee and Moser, 2008; Talsness et al., 2008). Studies in zebrafish have also shown behavioral (Chen et al., 2012; Noyes et al., 2015) and potential thyroid effects (Chan and Chan, 2012). Our results on the behavior of zebrafish devel-opmentally exposed to BDE-47 are almost superimposable with the behavioral profile reported by Chen and coworkers (Chen et al.,

2012), likely because of our similar experimental designs, including rinsing out the BDE-47 at least 24 h before testing. Both laboratories found that the primary effect was decreased activity in the dark, and the lowest dose affected was in the low micromolar range (around 4–5 μM). These results differ from that of Noyes et al. (2015) who reported decreased activity under both light and dark conditions in larval zebrafish treated with BDE-47; these differences are likely due to the fact that those investigators did not remove the larvae from the chemical before testing,so the behavioral measures may be confounded by concomitant acute exposure.

BPDP failed to produce any behavioral effects following developmental exposure, though we did note behavioral changes when the larvae were dosed acutely (increased activity in the light accompanied by decreased activity in the dark). Our results closely resemble the behavioral changes noted by the only other behavioral study (Noyes et al., 2015), and further reinforce the idea that their behavioral results at 5 days may be a combination of both developmental and acute exposure.

We found extensive behavioral changes after acute exposure to **EHDP**, but only marginal behavioral changes after developmental exposure, in agreement with the marginal behavioral changes noted by Noyes and co-workers (Noyes et al., 2015).

We found that **IDDP** did not affect zebrafish development (maximum concentration 120 μM (Table 3). No behavioral differences were noted after developmental exposure in our study, and acute exposure decreased activity in the dark at the higher doses. Marginal to no behavioral changes were also noted by Noyes and coworkers (Noyes et al., 2015). There is one report (McGee et al., 2013) showing no developmental toxicity in zebrafish due to IPP (tri-ITP) exposure, but their highest exposure concentration was 10 μM; our Point of Departure (POD) for overt toxicity was around 5 μM (Table 3). The behavioral changes noted by Noyes et al. (2015) closely resemble behavioral changes precipitated by acute IPP dosing in our study: increased activity in the light and decreased activity in the dark, a pattern which contrasts with the dark hyperactivity pattern seen after developmental exposure in the present study.

TMPP (also known as tricresyl phosphate TCP) is a mixture of para, meta, and ortho isomers of tricresyl phosphate, as well as some other related chemicals. It has been studied in relation to organophosphate-induced delayed neuropathy (OPIDN), but with few studies relating to developmental toxicity or developmental neurotoxicity. Noyes and co-workers (Noyes et al., 2015) reported decreased activity in both the light and dark phases at 6.4 and 64 μM, where as we found decreased activity in the dark only when the fish were dosed developmentally, while acute exposure elicited increased light activity and decreased dark activity. Even though TOCP is a component of TMPP, the developmental and acute behavioral effects of TMPP are distinct from those of TOCP in the present study (,Supplemental Tables 3 and 4), indicating that the TOCP toxicity is not the major influence in the spectrum of toxicity produced by TMPP.

There is a growing body of literature on **TPHP** (also abbreviated as TPP), a component of FM550 (Firemaster 550). Numerous groups have shown that TPHP affects thyroid

development and function in zebrafish: Kim and coworkers (Kim et al., 2015) noted gene expression changes precipitated in the sub-micromolar range (0.6 to 1.5 μM) accompanied by increased T3 and T4 levels also at very low concentrations (40 μg/L = 0.123 μM), and Liu and coworkers (Liu et al., 2013) also found gene expression changes in larval zebrafish indicative of thyroid receptor perturbations at micromolar exposure concentrations. A range of potencies for overt toxicity (i.e., lethality and malformations) has been reported by various groups: the highest was an LC_{50} of 29.6 mg/L (90.7 μM) (Liu et al., 2013). Other estimates clustered with our POD for overt toxicity of 2 μM: TPHP-induced malformations and lethality were reported at 2 μM (McGee et al., 2013) or 0.5 μM (500 μg/L) (Kim et al., 2015). Whereas Noyes and coworkers (Noyes et al., 2015) reported decreased activity in both light and dark at the higher (64 and 6.4 μM) TPHP concentrations, the larvae that were treated develop-mentally with TPHP in our study showed behavioral changes at much lower concentrations. After acute dosing, TPHP produced decreased activity in the dark phases.

Developmental exposure to **TBBPA** in zebrafish produces myriad effects: the compound changes the rate of yoke absorption (Kalasekar et al., 2014), acts as an obesogen and PPAR-γ agonist (Riu et al., 2014), and at high concentrations, alters gene expression of hypothalamic-pituitary-thyroid axis (Chan and Chan, 2012). There are conflicting reports on whether TBBPA has estrogenic activity in larval zebrafish with one group (Chow et al., 2013) reporting increased vitellogenin (an estrogenic biomarker in zebrafish) expression at high TBBPA concentrations, but another group reporting no estrogenic activity (Song et al., 2014). Many different laboratories (Kalasekar et al., 2014; McCormick et al., 2010; Noyes et al., 2015; Song et al., 2014; Yang et al., 2014) have reported that TBBPA produces malformations and lethality in developing zebrafish in the low micromolar ranges in agreement with our reported POD of 4.6 μM (Table 3). We did not observe changes in behavior after developmental exposure, but we did note marked changes in behavior after acute exposure, which correlates with what has been reported in mammals: TBBPA is, at most, a weak developmental neurotoxicant in mammals (Cope et al., 2015; Eriksson et al., 2001; Lilienthal et al., 2008), but it does appear to produce extensive behavioral changes after an acute exposure in mice (Nakajima et al., 2009).

Previously published work indicates that **TCEP** may not be as toxic to developing zebrafish as many of the other organophosphorus flame retardants (Dishaw et al., 2014a; McGee et al., 2012) even at concentrations over 100 μM; our data aligns with existing literature, as we saw no developmental overt toxicity even when the animals were exposed to 120 μM TCEP (Table 3). Two laboratories have behaviorally tested zebrafish larvae exposure to TCEP during development and found decreased activity in the dark at 31.4 and 100 μM (Dishaw et al., 2014a) or decreased activity in the dark and light at 64 μM (Noyes et al., 2015). Our results extend those observations by reporting changes in behavior after exposures to that level of TCEP and also to concentrations as low as 12 μM. Acute exposure to TCEP produced transient changes in behavior that abated by 2.5 h after dosing.

There is extensive use of and exposure to **TDCIPP** (also abbreviated TDCPP; reviewed in (Dishaw et al., 2014b), and there have been numerous studies of this compound in zebrafish. Our overt toxicity POD of 8.9 μM (Table 3) is very similar to the overt toxicity threshold of 10 μM reported by Dishaw and coworkers (Dishaw et al., 2014a), and the LC_{50} of 16.25 μM

(7.0 mg/L) reported by Liu and coworkers (Liu et al., 2013). Two studies (Dishaw et al., 2014a; Noyes et al., 2015) reported decreased dark activity in zebrafish larvae treated with TDCIPP during development, a pattern that we noted, but we also detected increased dark activity at even lower doses. When larvae were exposed to TDCIPP acutely, activity increased during the light phases and decreased during the dark phase. Chronically dosing zebrafish for the first 6 months of life at sub-micromolar levels, Wang and coworkers (Wang et al., 2015) did not note any cholinesterase inhibition or locomotor effects after 7 days of exposure, but did report that after 6 months of exposure, there were many nervous system changes (i.e., dopamine, serotonin, myelin basic protein and α1-tubulin was decreased), providing evidence for the neurotoxic nature of this chemical, possibly due to thyroid disrupting activity during development at sub-micromolar concentrations (Liu et al., 2013).

Two of the positive controls chosen for this study, BDE-47 and TOCP, provoked opposite behaviors in zebrafish, though the observed patterns are aligned with what would be expected from a developmentally neurotoxic chemical (BDE-47) and a chemical that is primarily toxic to the adult (TOCP). One would expect a chemical that is developmentally neurotoxic to have effects on behavior when administered develop-mentally: BDE-47 produced such effects down into the low micromolar levels. In contrast, TOCP, which is not regarded as a developmentally neurotoxic chemical (Funk et al., 1994; Harp et al., 1997; Moretto et al., 1991; Pope et al., 1992), only produced developmental behavioral effects at doses very close to teratogenic/lethal doses. Conversely, BDE-47 produced only very weak acute effects, while TOCP (weak cholinesterase inhibitor; (reviewed in Weiner and Jortner, 1999) produced more marked acute effects—a hyperactive profile very similar to the hyperactive acute profile precipitated by chlorpyrifos (another cholinesterase inhibitor) which was used as one of our technical positive controls. Another relevant comparison is between BDE-47 and TBBPA. It is interesting to note that in the one study (Eriksson et al., 2001) that compared the neurodevelopmental toxicity of BDE-47 and TBBPA side by side in developing mice, BDE-47 was positive and TBBPA tested negative, which aligns with the results of our zebrafish developmental neurotoxicity tests. This concordance supports the position suggested by many other studies (Kokel et al., 2010; Kokel and Peterson, 2011; Parker et al., 2013; Selderslaghs et al., 2013): that the zebrafish is a good model for developmental neurotoxicity screening and prioritization.

The acute data reported on these chemicals are novel, because to our knowledge, there have been only sporadic reports of acute pharmacological testing in vertebrates with these chemicals. The observed effects in the low micromolar dosing range suggest that these chemicals are neuropharmacologically active. It is also possible, however, that the acutely exposed larvae are behaving differently for reasons not related directly to the nervous system, such as simple dermal irritation or olfactory cues. Regardless of the cause, abnormal behavior in fish has the potential to perturb the population and community through altered preycapture or predator-avoidance relationships (Weis et al., 2001; Renick et al., 2015). To extrapolate the acute effects of these chemicals in zebrafish to human health, it would be very informative to conduct studies to elucidate the underlying mechanism(s) of the altered behavior. Few investigators have considered the possibility that exposure to these chemicals in furniture, toys, cars, or electronics may cause rapid (as opposed to developmental) behavioral changes in humans or wildlife.

As a class, the organophosphorus flame retardants maybe as toxic as the chemicals they are replacing. To rank the chemicals in an objective manner, we calculated a potency score $[(\text{percentage of 2 min intervals affected}) \times (\frac{1}{lower\ dose\ affected})]$ for the developmental and acute behavioral effects of each chemical (Supplemental Table 5); a higher score signifies both a higher proportion of 2 min intervals with behavioral changes and lower doses at which effects were observed. When exposed developmentally, two of the organophosphorus flame retardants ranked higher than BDE-47: TPHP > IPP > **BDE-47** > TMPP > TDCIPP > EHDP > TCEP. After acute exposure, TPHP was still the highest ranked chemical, and IPP was in the top three: TPHP > EHDP = TBBPA > IPP = BPDP > TMPP > TDCIPP > IDDP= TCEP = **BDE-47**. Based on both their relatively high acute and developmental potencies, TPHP and/or IPP would be excellent candidates for developmental neurotoxicological testing in mammalian models.

Although these present studies were not conducted in order to make extrapolations to the human condition, general observations may be attempted. Lacking information on the internal dose of each chemical to the zebrafish, we are therefore limited to using the nominal concentration for exposure estimation purposes. In general, 1 mg/l of each of the chemicals is a low μM concentration, which is in the general range of the lowest concentrations tested in the present paper. To equate the 1 mg/l to what is reported in the human studies, we can consider 1 mg/l equivalent to 1 μg/ml or 1 μg/g. In general, very little is known about human exposure to some of these chemicals (e.g.,BPDP, IDDP, IPP), but there have been some reports for the others. Body burden (hair, urine, serum) of these chemical is reported in approximately the pg to ng/g range (Cooper et al., 2011; Kim and Oh, 2014; Liu et al., 2015; Meeker et al., 2013; Reemtsma et al., 2011), which is at least 1000 times lower than the lowest exposure concentration used in the present study. In some cases, however, higher levels are reported in some humans in the lower μg/g range (Cooper et al., 2011; Liu et al., 2015; Reemtsma et al., 2011); these body burden concentrations may overlap with some of the lower concentrations used in the present study. That is assuming, however, that the internal concentration in the zebrafish larva equates with the nominal concentration. Because these chemicals are, in general, highly lipophilic (Table 1), it likely that the internal concentration of these chemicals in the zebrafish is orders of magnitude higher than the nominal concentration (Dishaw et al., 2014a; Padilla, 2013; Petersen and Kristensen, 1998), thereby decreasing the applicability to the human condition. Another confounder is that exposure to these chemicals in the real world does not occur in isolation; the real world exposures are most likely via multiple flame retardants (Stapleton et al., 2009), and no information exists on the toxicity of these chemicals using mixture dosing scenarios, although the zebrafish model would be an excellent one with which to assess the combinatorial toxicology of these chemicals.

The work presented here utilized the zebrafish model to obtain rapid and relevant data concerning the developmental and acute neurotoxicity of emerging flame retardant replacement compounds. These compounds were intended to replace a class of known neurotoxicants, PBDEs, yet this study suggests that the alternatives may also be neurotoxic, either acutely and/or developmentally. Given that several of these compounds are already being used and have been detected in households and the environment, additional studies

should be conducted to characterize their toxicities and to understand their potential effects on human and ecological health.

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disclaimer

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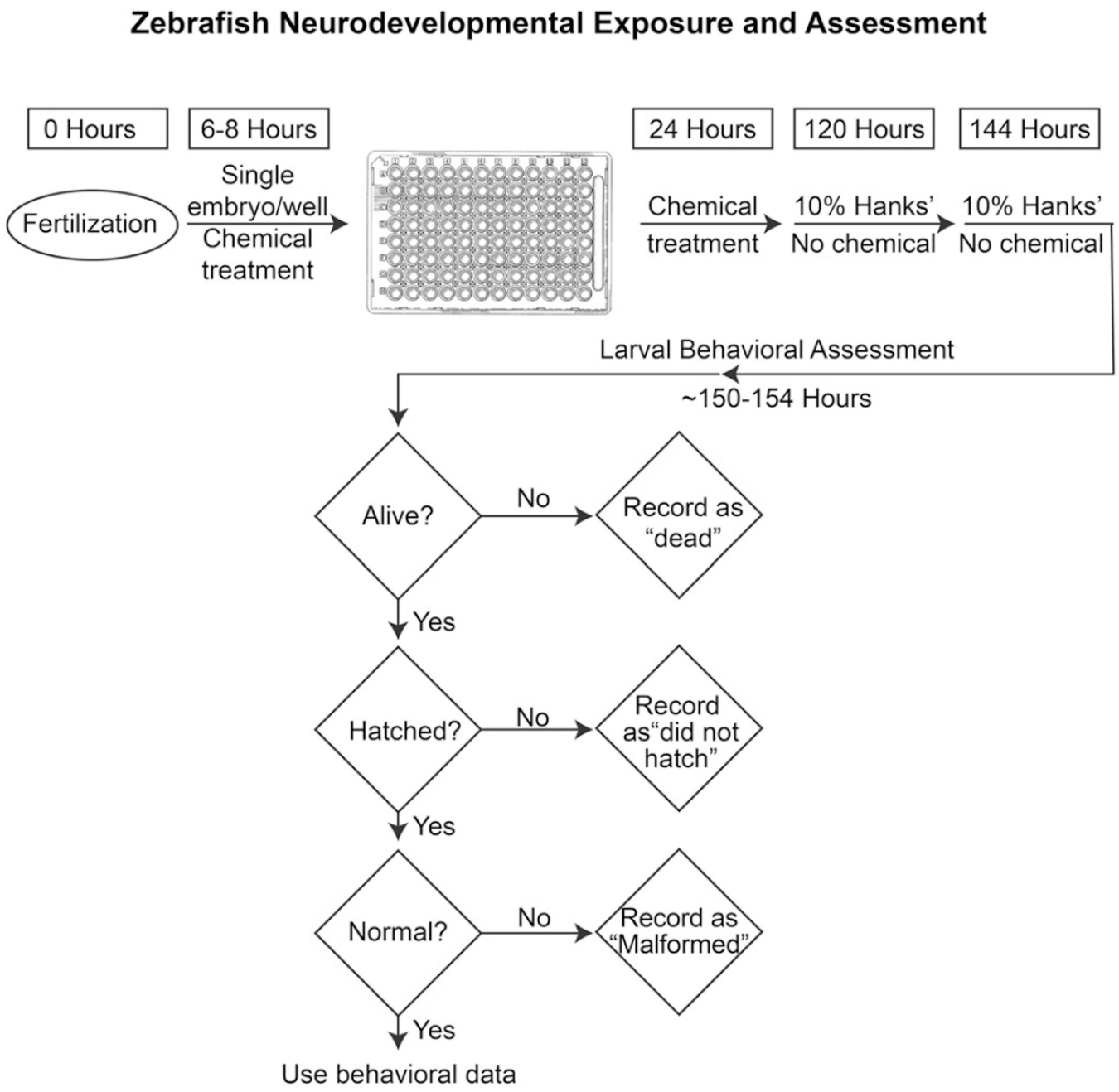


Fig. 1.

Schematic depicting overall experimental design. Rectangles at the top indicate time post fertilization and text below the rectangles describes the treatment at that time. The diamond shapes show assessment categories and indicate that behavioral assessments are only included for normal fish.

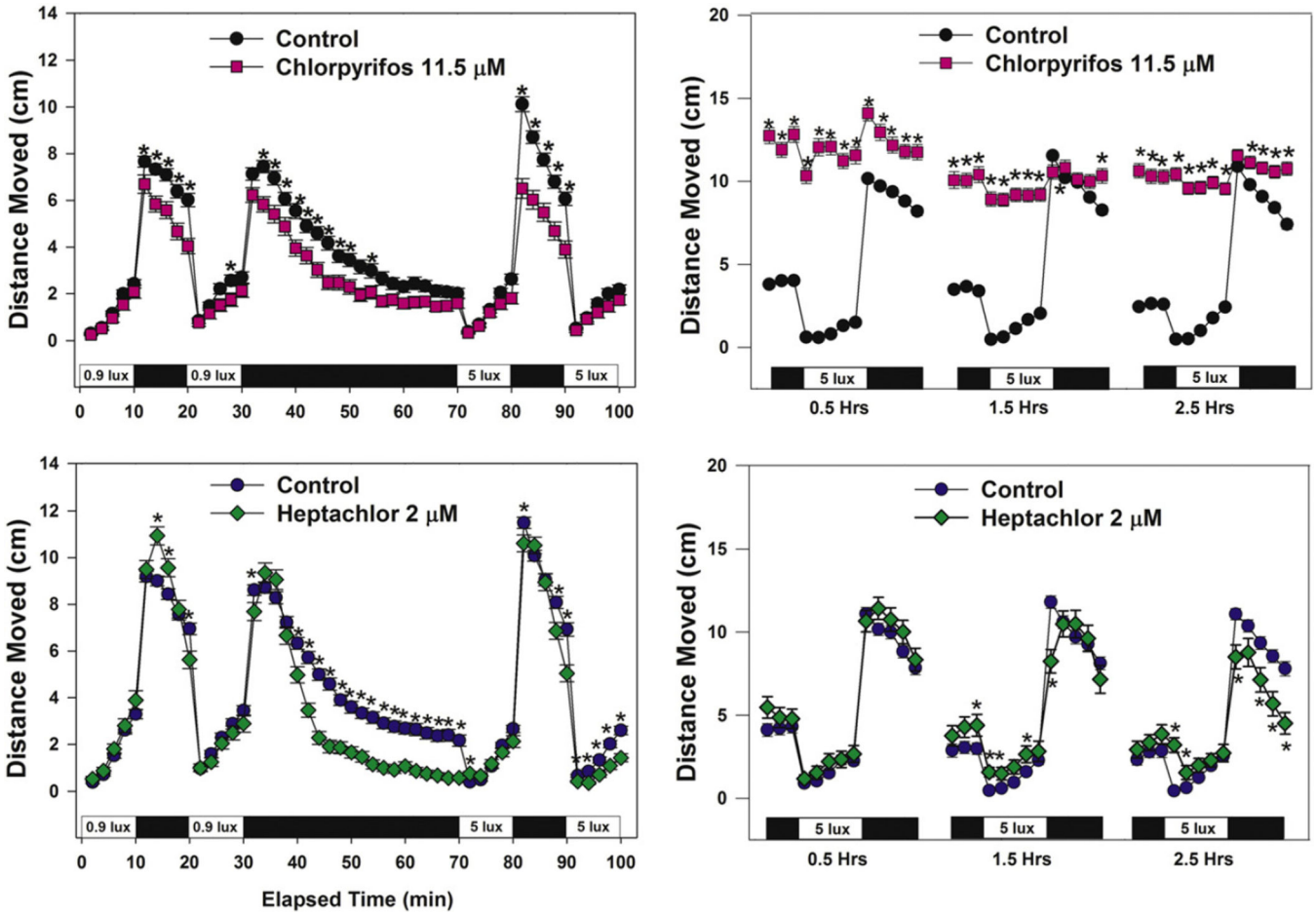
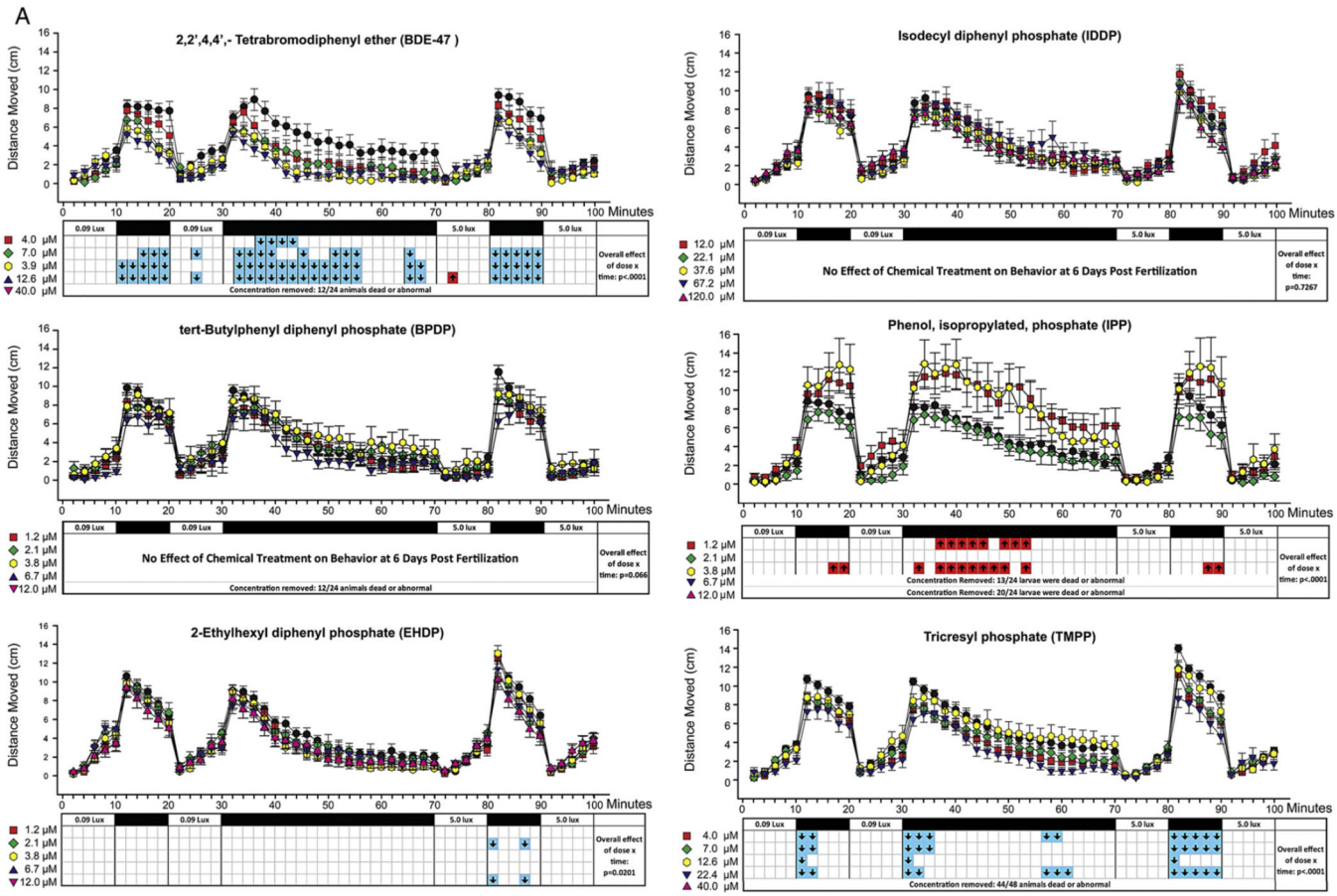


Fig. 2.

Control (DMSO) and positive technical control (heptachlor or chlorpyrifos) behavioral profiles for acute and developmental exposure. Data points represent average distance moved (cm) for each 2 min block. Developmental profiles are shown on the left, and the acute profiles are presented on the right. The graphs showing chlorpyrifos (11.5 μM) as a positive control are on the top, and graphs showing heptachlor (2 μM) as a positive control are on the bottom. The bar at the bottom indicates when behavior occurred in the dark (black rectangles) or light (white rectangles). The lux level during the light phase is also indicated in the white rectangles. Statistically significant differences are indicated with an asterisk (*). Sample sizes: (**A**) control (n = 215) and chlorpyrifos (n = 83); (**B**) control (n = 379) and heptachlor (n = 179); (C) control (n = 265) and chlorpyrifos (n = 132); (**D**) control (n = 129) and heptachlor (n = 58). Mean ± SEM.



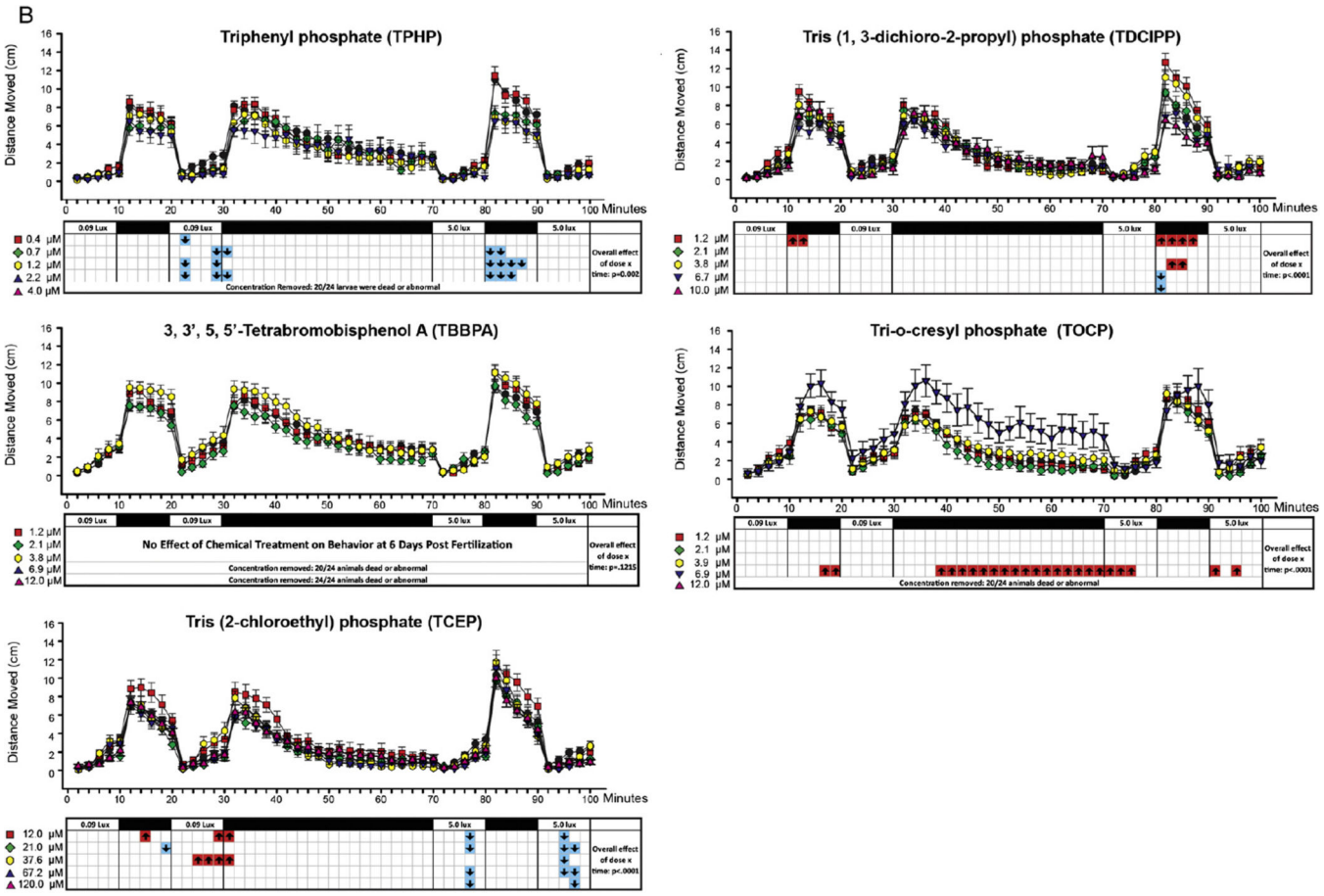
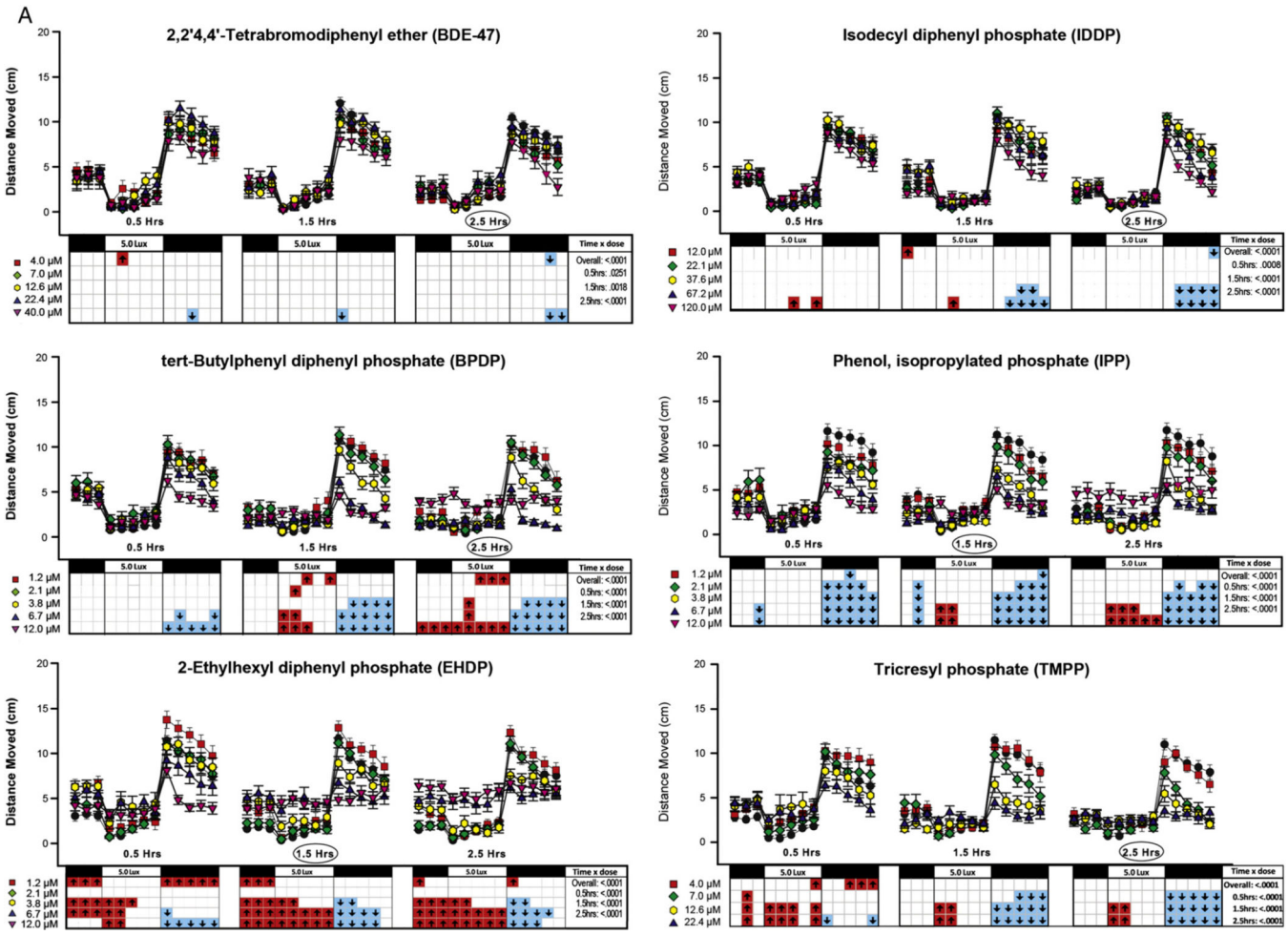


Fig. 3.

Developmental Exposure Produces Behavioral Changes. The results for each chemical are presented as a line graph with the grid below the line graph indicating the statistical results. Boxes with an up arrow (↑) (red box in the on-line version; dark gray box otherwise) denote a significant increase from control, while boxes with adown arrow (↓) (blue box in the on-line version; light gray box otherwise) denote a significant decrease from control. Overall p-values are located to the right of the grid. Below the graph and above the statistics grid is alight/dark bar indicating when behavior occurred in the dark (black rectangles) or light (white rectangles). The lux level during the light phase is also indicated in the white rectangles. The filled black circles (●) represent the DMSO control data in every graph. The key to the left of each grid indicates the symbol for each dose of the treatment chemicals. Sample sizes: BDE-47: Control (n = 45), 4.0 μM (n = 21), 7.0 μM (n = 20), 12.6 μM (n = 23), 22.4 μM (n = 20), 40.0 μM (n = 12; data not shown); **BPDP**: Control (n = 44), 1.2 μM (n = 24), 2.1 μM (n = 22), 3.8 μM (n = 19), 6.7 μM (n = 20), 12.0 μM (n = 12;data not shown); **EHDP**: Control (n = 48), 1.2 μM (n = 24), 2.1 μM (n = 24), 3.8 μM (n = 23), 6.7 μM (n = 24), 12.0 μM (n = 24); **IDDP**: Control (n = 47), 12.0 μM (n = 23), 22.1 μM (n = 22), 37.6 μM (n = 20), 67.2 μM (n = 22), 120.0 μM (n = 20); **IPP**: Control (n = 45), 1.2 μM (n = 20), 2.1 μM (n = 19), 3.8 μM (n = 20), 6.7 μM (n = 11) data not shown, 12.0 μM (n = 4; data not shown); **TMPP**: Control (n = 105), 4.0 μM (n = 48), 7.0 μM (n = 46), 12.6 μM (n = 47), 22.4 μM (n = 26), 40.0 μM (n = 4; data not shown); **TPHP**: Control (n = 41), 0.4 μM (n

= 20), 0.7 μM (n = 19), 1.2 μM (n = 21), 2.2 μM (n = 15), 4.0 μM (n = 4; data not shown); **TBBPA**: Control (n = 42), 1.2 μM (n = 20), 2.1 μM (n = 20), 3.8 μM (n = 22), 6.7 μM (n = 4; data not shown), 12.0 μM (n = 0; data not shown); **TCEP**: Control (n = 44), 12.0 μM (n = 21), 21.1 μM (n = 24), 37.6 μM (n = 22), 67.2 μM (n = 22), 120.0 μM (n = 23); **TDCIPP**: Control (n = 43), 1.2 μM (n = 20), 2.1 μM (n = 21), 3.8 μM (n = 21), 6.7 μM (n = 21), 10.0 μM (n = 14); **TOCP**: Control (n = 90), 1.2 μM (n = 21), 2.1 μM (n = 20), 3.9 μM (n = 39), 6.9 μM (n = 30), 12.0 μM (n = 4; data not shown). Mean ± SEM.



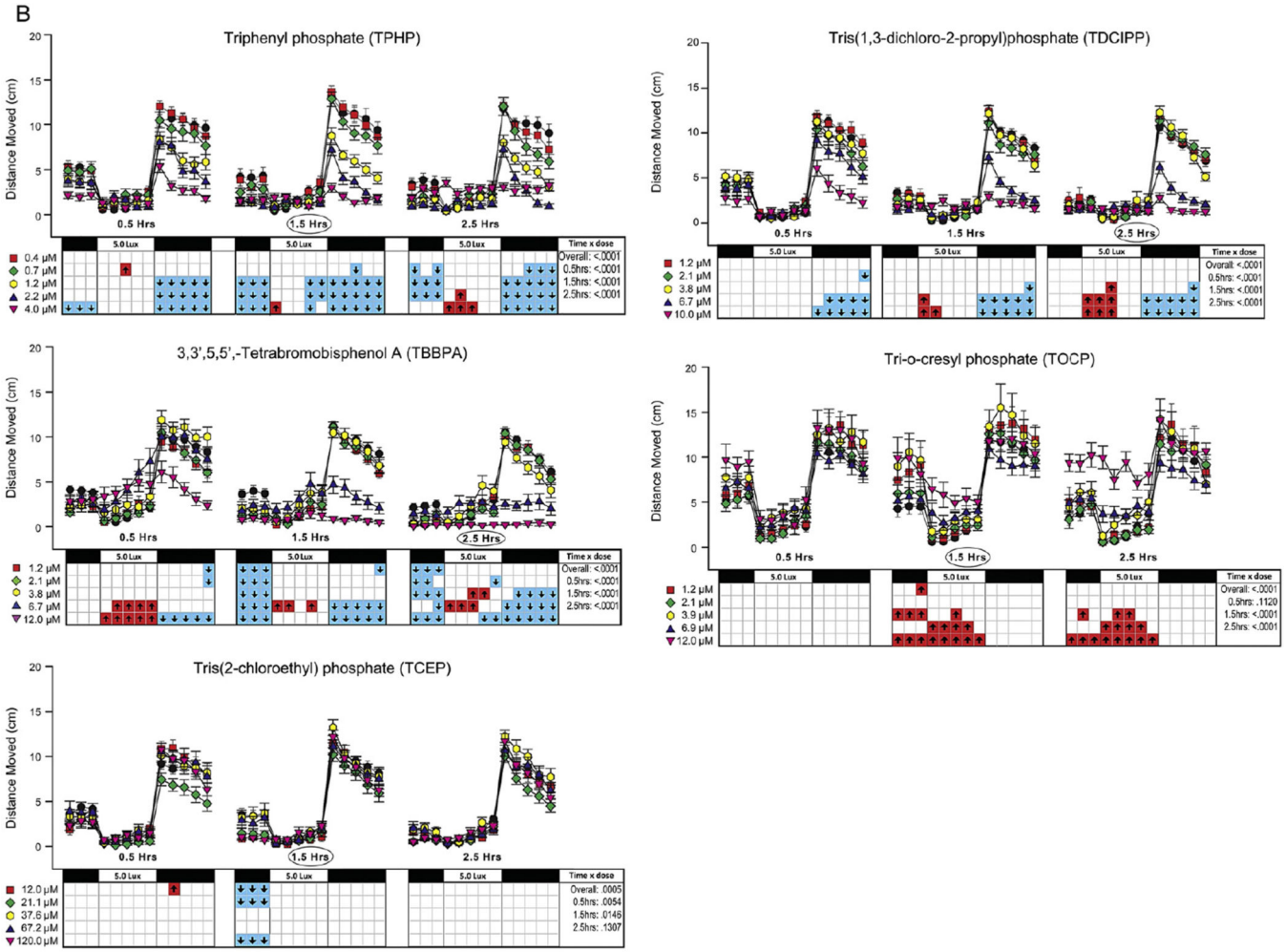


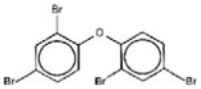
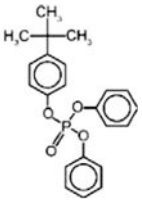
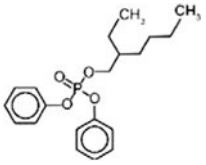
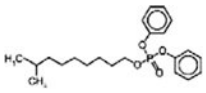
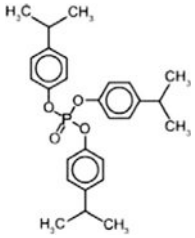
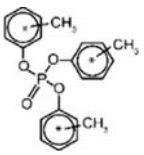
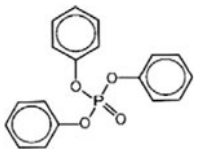
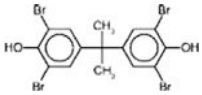
Fig. 4.

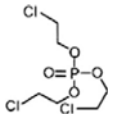
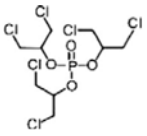
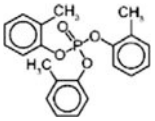
Acute Exposure Produces Behavioral Changes. The results for each chemical are presented as a line graph with the grid below the line graph indicating the statistical results. Boxes with an up arrow (↑) (red box in the on-line version; dark gray box otherwise) denote a significant increase from control, while boxes with a down arrow (↓) (blue box in the on-line version; light gray box otherwise) denote a significant decrease from control. The circled time-period on the graph indicates the time of peak of effect. Overall p-values for the entire assessment, as well as for each individual testing period are located to the right of the grid. Below the graph and above the statistics grid is a light/dark bar indicating when behavior occurred in the dark (black rectangles) or light (white rectangles). The lux level during the light phase is also indicated in the white rectangles. The filled black circles (●) represent the DMSO control data in every graph. The key to the left of each grid indicates the symbol for each dose of the treatment chemicals. Sample sizes for the acute data: **BDE-47**: Control (n = 39), 4.0 μM (n = 20), 7.0 μM (n = 19), 12.6 μM (n = 18), 22.4 μM (n = 22), 40.0μM (n = 21); **BPDP**: Control (n = 45), 1.2 μM (n = 22), 2.1 μM (n = 21), 3.8μM (n = 21), 6.7 μM (n = 23), 12.0 μM (n= 24); **EHDP**: Control (n = 46), 1.2μM (n = 24), 2.1 μM (n = 22), 3.8μM (n = 23), 6.7 μM (n = 23), 12.0μM (n = 18); **IDDP**: Control (n = 47), 12.0 μM (n = 22), 22.1μM

(n = 21), 37.6μM (n = 23), 67.2μM (n = 22), 120.0 μM (n = 23); **IPP**: Control (n = 42), 1.2μM (n = 24),2.1μM (n = 22), 3.8 μM (n = 22), 6.7μM (n = 23), 12.0 μM (n = 19); **TMPP**: Control (n = 50), 4.0μM (n = 20), 7.0μM (n = 22), 12.6 μM (n = 21), 22.4 μM (n = 21); **TPHP**: Control (n = 37), 0.4 μM (n = 22), 0.7 μM (n = 22), 1.2 μM (n = 19), 2.2 μM (n = 21), 4.0 μM (n = 20); **TBBPA**: Control (n = 45), 1.2 μM (n = 24), 2.1 μM (n = 23), 3.8 μM (n = 22), 6.7 μM (n = 21), 12.0 μM (n = 22); **TCEP**: Control (n = 44), 12.0 μM (n = 22), 21.1 μM (n = 22), 37.6 μM (n = 22), 67.2 μM (n = 24), 120.0 μM (n = 24); **TDCIPP**: Control (n = 43), 1.2 μM (n = 20), 2.1 μM (n = 21), 3.8 μM (n = 21), 6.7 μM (n = 23), 10.0 μM (n = 20); **TOCP**: Control (n = 44), 1.2 μM (n = 22), 2.1 μM (n = 23), 3.9 μM (n = 22), 6.9 μM (n = 23), 12.0 μM (n = 22). mean ± SEM.

Table 1

Physiochemical Characteristics of the Chemicals Tested.

Chemical Name	Abbreviation	Characteristics	Structure
2,2′4,4′-Tetrabromodiphenyl ether	BDE-47	CAS# 5436–43–1 MW† = 485.79 Log P†† = 6.77	
tert-Butylphenyl diphenyl phosphate	BPDP*	CAS# 56,803–37–3 MW = 382.39 Log P = 6.61	
2-Ethylhexyl diphenyl phosphate	EHDP	CAS# 1241–94–7 MW = 362.4 Log P = 6.30	
Isodecyl diphenyl phosphate	IDDP*	CAS# 29,761–21–5 MW = 390.45 Log P = 7.28	
Phenol, isopropylated, phosphate (3:1)	IPP	CAS# 68,937–41–7 MW = 390.00 Log P = 9.07	
Tricresyl phosphate	TMPP*	CAS# 1330–78–5 MW = 371.39 Log P = 6.34	
Triphenyl phosphate	TPHP	CAS# 115–86–6 MW = 326.28 Log P = 3.065	
3,3′,5,5′-Tetrabromobisphenol A	TBBPA	CAS# 79–94–7 MW = 543.87 Log P = 7.20	

Chemical Name	Abbreviation	Characteristics	Structure
Tris(2-chloroethyl) phosphate	TCEP	CAS# 115–96–8 MW = 285.49 Log P = 1.63	
Tris(1,3-dichloro-2-propyl)phosphate	TDCIPP	CAS# 13,674–87–8 MW = 490.9 Log P = 3.65	
Tri-*o*-cresyl phosphate (this is the ortho isomer of TMPP)	TOCP	CAS# 78–30–8 MW = 368.36 Log P = 6.34	

Both molecular weight and LogP were obtained from EPI SuiteTM (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm).

*Mixture.

†MW = Molecular Weight.

††LogP = Octanol/water Partition Coefficient.

Table 2

Comparison of statistical results for 2 min intervals from both developmental and acute behavioral testing. The time of peak effect period was used for the acute data while the 26 min around the first 5.0 lx period (including the dark 6 min before and 10 min after) was used for developmental. Boxes with an up arrow (↑) (red box in the on-line version; dark gray box otherwise) indicate a significant increase from control, while boxes with a down arrow (↓) (blue box in the on-line version; light gray box otherwise) indicate a significant decrease from control. A light/dark bar indicates when behavior occurred in the dark (black rectangles) or light (white rectangles). The lux level during the light phase is also indicated in the white rectangles.

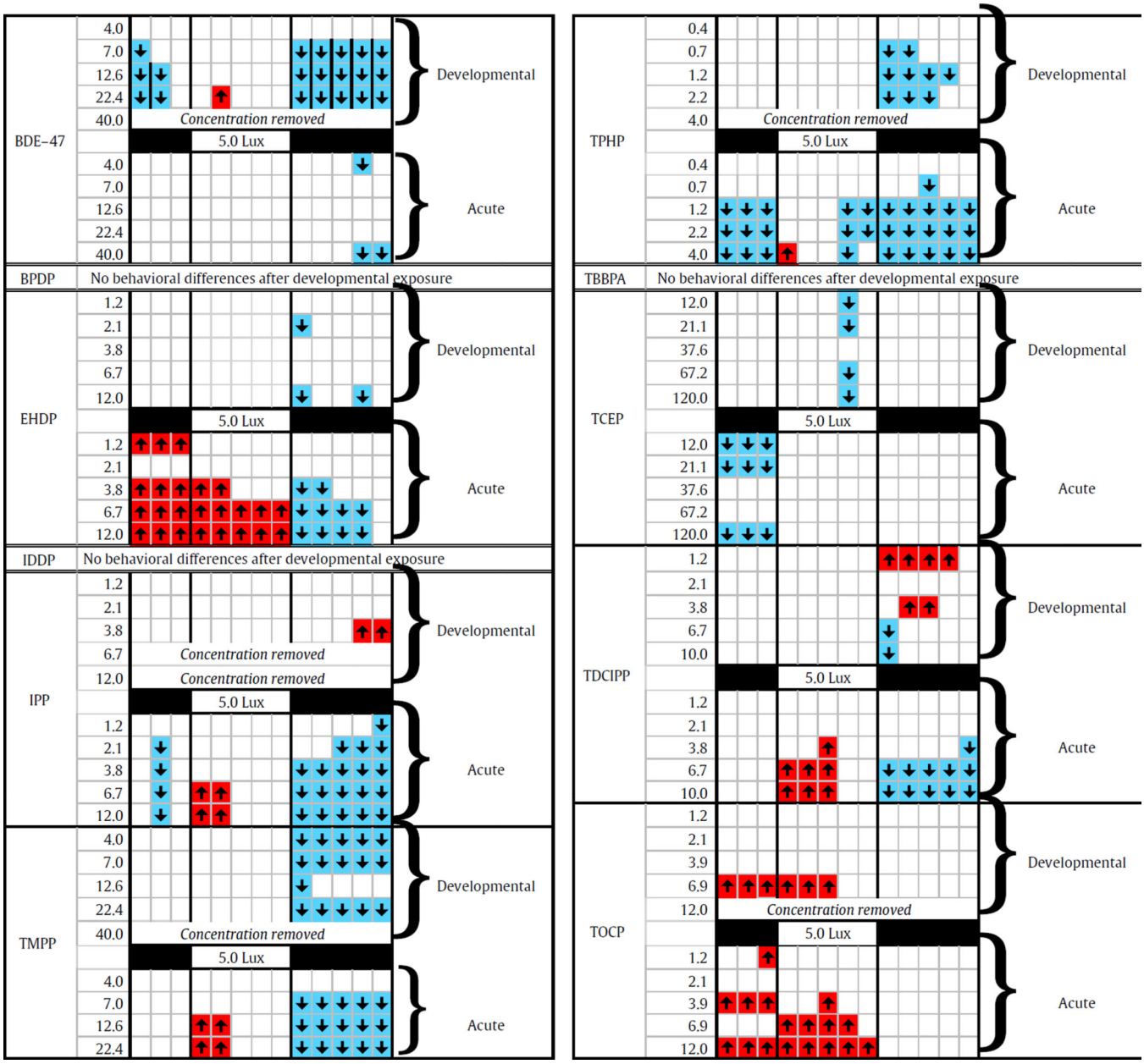


Table 3

Comparison of the Acute and Developmental Toxicity of the Tested Chemicals.

Chemical	Overt Toxicity (POD) (from Behl et al., 2015)	Developmental Behavioral LOEL	Acute Behavioral LOEL (at time of Peak Effect)	Overt Toxicity divided by Develop LOEL	Overt Toxicity divided by Acute LOEL
BDE-47	28.2	4.0	4.0	7.0	7.0
BPDP	9.8	No effect	1.2		8.2
EHDP	15.3	2.1	1.2	7.3	12.7
IDDP	No effect	No effect	12.0		>10
IPP	4.9	1.2	1.2	4.1	4.1
TMPP	15.4	4.0	4.0	3.9	3.9
TPHP	2.0	0.4	0.7	4.9	2.8
TBBPA	4.6	No effect	1.2		3.8
TCEP	No effect	12.0	12.0	>10	>10
TDCIPP	8.9	1.2	3.8	7.4	2.3
TOCP	3.7	6.9	1.2	0.5	3.1