Published in final edited form as:

Neurotoxicol Teratol. 2015 ; 52(0 0): 210–219. doi:10.1016/j.ntt.2015.07.001.

Neurotoxicity of FireMaster 550® in zebrafish (*Danio rerio*): Chronic developmental and acute adolescent exposures

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Abstract

BACKGROUND—FireMaster® 550 (FM 550) is the second most commonly used flame retardant (FR) product in consumer goods and has been detected in household dust samples. However, neurobehavioral effects associated with exposure have not been characterized in detail. We investigated the behavioral effects of FM 550 in zebrafish to facilitate the integration of the cellular and molecular effects of FM 550 with its behavioral consequences. The effects of developmental FM 550 exposure on zebrafish larvae swimming shortly after the end of exposure as well as the persisting effects of this exposure on adolescent behavior were studied. In addition, the acute effects of FM 550 on behavior with exposure during adolescence in zebrafish were studied.

METHODS—Developmental exposure to 0, 0.01, 0.1 or 1 mg/L of FM 550 via immersion spanned 0–5 days post fertilization, with larval testing on day 6 and adolescent testing on days 40–45. Acute adolescent (45 dpf) exposure was to 0, 1.0 or 3.0 mg/L of FM 550 via immersion, for 24 hrs, with testing 2 hr or 1 week later. The vehicle condition was colony tank water with .0004% (developmental) or .0012% (adolescent) DMSO. Zebrafish behavior was characterized across several domains including learning, social affiliation, sensorimotor function, predator escape, and novel environment exploration.

RESULTS—Persisting effects of developmental FM 550 exposure included a significant ($p < 0.01$) reduction in social behavior among all dose groups. Acute FM550 exposure during adolescence caused hypoactivity and reduced social behavior (p 's < 0.05) when the fish were tested 2 hr after exposure. These effects were attenuated at the 1 week post exposure testing point.

DISCUSSION—Taken together, these data indicate that FM 550 may cause persisting neurobehavioral alterations to social behavior in the absence of perturbations along other

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behavioral domains and that developmental exposure is more costly to the organism than acute adolescent exposure.

Keywords

Zebrafish; FireMaster 550; Flame retardant; Behavior; Cognition; Developmental; Acute

1. INTRODUCTION

Chemical flame retardants (FRs) are added to a wide range of household products to reduce flammability and meet fire safety standards (Stapleton et al., 2005; Stapleton et al., 2011; Ren et al., 2013). Once commonly used, halogenated FRs, like the polybrominated diphenyl ethers (PBDEs), have been largely phased out due to concerns over toxicity. These FRs can escape from the products to which they were applied and accumulate in the environment, including household dust, permitting human exposure via inhalation, ingestion and dermal absorption (Stapleton et al., 2012a; Stapleton et al., 2008b; Lorber, 2008). A number of studies have shown that children and adults have measureable body burdens of the PBDEs commonly used as FRs (Stapleton et al., 2012a; Stapleton et al., 2008a; Hites, 2004; Lunder et al., 2010). Moreover, epidemiological reports have associated PBDE exposure during gestation with learning disabilities and behavioral problems later in life (Roze et al., 2009; Herbstman et al., 2010; Eskenazi et al., 2013). These effects have been modeled using rodents, where learning and memory impairments (Viberg et al., 2002; Eriksson et al., 2002; Viberg et al., 2003; Viberg, 2003; Viberg et al., 2007; Viberg, 2009) and altered locomotor activity (Chou et al., 2010; Usenko et al., 2011; Chen et al., 2012; Macaulay et al., 2015) emerge following exposure to PBDEs or their metabolites (i.e. Macaulay et al., 2015).

FireMaster® 550 (FM 550) was introduced as an alternative flame retardant mixture to replace those commonly used PBDE mixtures in polyurethane foam (Stapleton et al., 2008b) as FM 550 was marketed as safer and less bioaccumulative than its predecessor (PentaBDE) (Stapleton et al., 2008b). FM 550 is a mixture of brominated and organophosphate flame retardants: the brominated components include 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) and bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate (BEH-TEBP) (Stapleton et al., 2014) and the organophosphate components include triphenylphosphate (TPHP) and several TPHP analogs with varying degrees of aryl isopropylation (collectively, iTPHP) (Van der Veen & de Boer, 2012). Currently, FM 550 is estimated to be the second most common FR mixture applied to polyurethane foam (Stapleton et al., 2009; Stapleton et al., 2011) and polyurethane foam samples from consumer goods (e.g. furniture, baby products) have been shown to contain these constituents (Stapleton et al. 2009, Stapleton et al., 2011). FM 550, just like PBDEs, can escape the products to which it was applied, accumulating in the environment. FM 550 constituents (EH-TBB and BEH-TEBP) have been detected in household dust samples and outdoor sources (Ali et al. 2012; Dodson et al. 2012; Ma et al. 2012; Stapleton et al. 2008b; Stapleton et al., 2009), and routes of human exposure are expected to be similar to those for PBDEs (Hoffman et al., 2014).

Specific components of FM 550, e.g. TPHP and to some extent TDCPP, have been shown to have reproductive toxicity (Liu et al., 2013) and FM 550 has been shown to be cardiotoxic (McGee et al., 2013) and act as an endocrine disrupter (Patisaul et al., 2013). However, relatively little is known about the toxicity of FM 550 more generally (Dishaw et al., 2014), and no studies to our knowledge have investigated the behavioral effects of exposure to this mixture. The present study seeks to characterize the behavioral effects associated with exposure to FM 550 within the context of a zebrafish model. Because very little is known about the potential neurotoxicity of FM 550, both acute exposures (24 hrs), administered during adolescence, and sub-chronic developmental exposures (0–6 dpf) were conducted to offer a more complete profile of toxicity. Moreover, for both exposure windows, behavioral testing commenced shortly after exposure terminated and after an extended depuration period. Zebrafish were assessed via the use of a neurobehavioral test battery, elements of which have been successfully used to characterize dysfunction in zebrafish following numerous toxicants and drugs of abuse (see Bailey et al., 2013 for a review).

2. METHODS

2.1: Subjects

2.1.1: Breeding and Egg Collection—Zebrafish (AB* strain) were bred onsite from progenitors originally obtained from the zebrafish international resource center (ZIRC, Eugene, OR, USA). Random pair-wise mating of zebrafish breeders was conducted and all breeders were kept in large (N=15) spawning groups at a male to female ratio of 2:1. Zebrafish embryos were collected at the beginning of the 14-h light cycle on the morning following the pairing of adult breeders (0 days post fertilization (dpf)). Collected eggs were inspected under a dissection microscope at 2-hours post fertilization (hpf), and those unfertilized or showing obvious malformations were excluded. Larvae were then randomly distributed among all aqueous exposure conditions (described below), housed in glass dishes, and placed inside incubators maintained at a constant temperature of 28.5°C and on a 14:10 h light/dark cycle until 5-days post fertilization (dpf).

2.1.2: Housing and Husbandry—Zebrafish 6-dpf and older were housed in 3-L tanks on a circulating rack system in a colony room arranged on a 14:10 h light/dark cycle. Water temperature was maintained at approximately 28.5°C and salinity and pH were monitored biweekly. A mixture of de-ionized H₂O, sea salt (Instant Ocean, 9.0-g/5 gal H₂O), and neutral regulator (Seachem, 2.5-g/5 gal H₂O) served as aquarium water. All fish were fed twice daily: 24-hr old brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) in the morning and solid food (at increasingly large particle size as they grew) (Brine Shrimp Direct Golden Pearl; TetraMin® Tropical Flakes, Blacksburg, VA, USA) in the evening. On days when fish were subjected to elements of the behavioral test battery the evening feeding was provided after the completion of testing. Behavioral testing among adolescents spanned the hours of 11:00 AM and 5:00 PM, with exact testing time counterbalanced among exposure groups. All behavioral testing of larvae was completed between the hours of 3:00 PM and 5:00 PM.

2.2: Chemical exposures

2.2.1. Acute Exposure—Adolescent zebrafish (45 dpf), an age chosen based on detection limits of tracking software, were exposed for 24 hrs to 0, 1.0 or 3.0 mg/L of FM 550 at a density of 2–3 fish/L. A stock solution of 256 mg/ml FM 550 was provided by H. Stapleton, Duke University from a sample donated by Great Lakes Chemical (West Lafayette, IN, USA). Zebrafish were transferred from their home tank into an entirely glass tank (containing no plastic or epoxy sealants) containing 1L of aquarium water spiked with FM 550 stock at quantities to achieve the doses listed above. Vehicle (.0012% DMSO) control fish were handled similarly. Feeding was withheld until the end of exposure and all exposure tanks were housed in the colony room to ensure a proper light cycle experience. At the end of exposure, fish were carefully rinsed into clean water three times over the course of two hours. At two hours post exposure, fish were run on behavioral assays (described below). Exposures were staggered to permit exactly 2 hr intervals between exposure and testing. Exposure water was not reused. Two groups of zebrafish (duplicated exposures), spanning one week of exposure/testing time, constituted the acute exposure cohort destined for behavioral testing immediately following the end of exposure. These methods were repeated for a separate group of fish, destined for behavioral testing one week following the end of exposure. See Table 1 for a summary of the experimental design.

2.2.2. Developmental Exposure—Beginning at 4-hpf, larvae were exposed to 0.01, 0.1 or 1.0 mg/L FM 550 (from stock solution described above) or vehicle control (.0004% DMSO) in glass Petri dishes for 5 days (4 hpf – 5 dpf). These doses were chosen with the anticipation that they would span a behaviorally active dose range, as there is at present very little existing literature on which to anchor a dose range. Exposure was conducted at a density of 60 eggs/40-mL aqueous solution (Easton & Goulson, 2013), which was done in duplicate. A total of 4 plates (replicates) per exposure group provided a cohort of duplicate exposures for larval testing and, separately, adult testing. Eggs or larvae in each plate were inspected under a dissection microscope daily during exposure and eggs with arrested development or obvious malformations were excluded. The stress associated with post-experimental transfer of larvae out of the 96-well plate, an arrangement required for larval activity assessment (described below), prohibits the later use of those same fish on adult behavioral assays, therefore two cohorts of developmental exposures (each duplicated) were required to achieve a larval testing and adolescent testing group. Regardless of cohort assignment (i.e. larval or adult testing), all zebrafish were transferred to fresh aquarium water at 5-dpf, ending the exposure period. See Table 1 for a summary of the experimental design.

2.3: Behavioral assessments

2.3.1. Larval Motility Assay—On 5-dpf all fish designated for larval testing were distributed pseudo-randomly across 96-well plates containing 45 μ L of un-dosed aquarium water per well. In this way, each exposure group was represented within a 96-well plate. The 96-well plates housed the fish for 24-hrs, until 6-dpf, at which time they were subjected to behavioral testing. Larval swimming activity (i.e. distance traveled) and the capacity to adapt to changing environmental stimuli (i.e. alternating periods of light and dark) was assessed (see Ahmed et al, 2012; Willemsen & Van der Linde, 2010). DanioVision™

hardware running EthoVision XT® tracking software was used (Noldus, Wageningen, The Netherlands), which tracks movement of individual larvae during alternating periods of white light (“100% illumination”, 5,000 lux) and dark (“0% illumination”, <1 lux). An initial acclimation dark period of 10 min was followed by 2 phases of a 10 min light/10 min dark period, and larval motion was tracked 30 times/sec over the course of the 50 min trial. Video data was further processed using EthoVision XT® (Noldus, Wageningen, The Netherlands), to calculate total distance traveled for each individual larvae during every min of the trial.

2.3.2. Adolescent Neurobehavioral Test Battery—Distinct behavioral assays were used to assess function in adult zebrafish that had been exposed to FM 550 during early development or acutely during adolescence. The test battery is made up of procedures designed to assess startle habituation, novel environment exploration, social affiliation, predator escape/avoidance, spatial discrimination, and (for the acute exposure group only) a light/dark motility assay; all are described below.

2.3.2.1. Startle Habituation: Typical zebrafish (and other animals) exhibit a diminished response to a repeated stimulus, “habituation”. Habituation curves, therefore, provide information about neuroplasticity and adaptation to an environmental stimulus. Here, habituation learning and sensorimotor function were quantified by exploiting a tap-elicited startle reflex. These methods have been described in detail elsewhere (see Eddins et al., 2009), but briefly, eight fish were tested simultaneously in a 2×4 array of swim arenas (exposure group was counterbalanced within each session of 8 fish). Below each arena was a centrally located push solenoid that delivered a sudden physical tap to the test environment when activated. A digital video camera was centrally positioned above the arena display, 75-cm above the water level. The video output from the camera was imported into a computer running EthoVision™ tracking software, which allowed for each fish to be located 6 times/sec. Motor startle responses were assessed for 10 tap-trials with 1 min intervals between taps to determine the initial startle response and subsequent habituation with repeated stimulus presentation. The dependent measures were distance traveled (cm) for the 5-s before and after each stimulus delivery.

2.3.2.2. Novel Environment Exploration: Anxiety-like behavior and exploration of an unfamiliar environment were assessed using a novel tank arrangement, which has also been previously described (see Bencan & Levin, 2008; Levin & Cerutti, 2008). When placed in an unfamiliar tank, zebrafish, like many other prey fish, avoid the more exposed upper regions of the tank and instead dive to the bottom, or “bottom dwell”. As zebrafish acclimate to a tank they gradually explore the upper regions of the tank. Across each minute of a 5-min trial, the distance from the tank floor (a measure of spatial location) and the total distance traveled were quantified to assess tank exploration.

2.3.2.3. Social Affiliation: To assess social behavior, a shoal location reversal task based off a procedure developed by Gerlai and colleagues (Saverino & Gerlai, 2008; Fernandes et al., 2015) was used to quantify social affiliation. Typically, zebrafish exhibit robust shoaling behavior and will rapidly approach a group of moving conspecifics (i.e. a “shoal”) and

engage in a characteristic pattern of swimming that is generally characterized by hyperactivity (relative to isolation conditions) and pause/dart swim behavior (i.e. “shoaling”). The testing apparatus was a 46-cm clear rectangular tank positioned beneath a video camera and between two monitors. Each monitor was programmed to show 1 min video clips of a group of 10–15 live shoaling zebrafish (AB* strain), filmed previously in the lab.

Individual fish were isolated in tanks surrounded by opaque dividers; after 1-h of isolation, they were transferred to the testing tank. Each trial began with a 1-min control period, after which time one monitor displayed a 1-min video of shoaling fish. Subsequently, the shoal location was reversed: the opposite monitor began to display the shoal video while the first monitor transitioned to a stationary control image, matched for color and illumination to the video image. This reversal process took place a total of 3 times in the 5-min trial, for a total of two left presentations and two right presentations (starting location was counterbalanced between subjects). EthoVision™ software was used to calculate distance of a test fish from the active shoal and total distance traveled.

2.3.2.4. Predator Escape and Avoidance: To analyze fear-like behavior and escape in zebrafish, the image of an expanding blue dot was used to simulate the approach of a predator. The dot, which increased in diameter from 1.3-cm to 30.5-cm in 5-sec, was displayed on a computer monitor located on one side of a rectangular 1.5-L tank. A single fish occupied the test tank and following a 1-min tank acclimation, the video alternated between a 1-min “predator ON” condition (in which the stimulus was presented 12 times) and a 1-min “predator OFF” condition (in which the screen was blank). Each 5-min trial was comprised of four alternations between conditions (“on”-“off”-“on”-“off”) and an initial 1-min acclimation/baseline period (see Luca & Gerlai, 2012). Typically, zebrafish will rapidly flee the presentation of the stimulus and restrict swimming to the tank end farthest from the “predator” stimulus; however, when the stimulus is off, control fish will typically explore the tank space and occasionally approach the tank wall associated with the predator stimulus. Utilizing the on/off paradigm allows for the examination of both escape (when the stimulus is on) and avoidance (when the stimulus is off). Total distance traveled and tank location measures (i.e. distance from the “predator”) were used to quantify behavior under this arrangement.

2.3.2.5. Spatial discrimination learning: A three-chamber tank was developed in our laboratory to assess spatial learning and memory in zebrafish (Arthur and Levin, 2001, Eddins et al., 2009, Levin and Cerutti, 2008, Levin and Chen, 2004, Levin et al., 2003 and Levin et al., 2006). The apparatus is made from a cylindrical pipe cut in half length-wise and divided into three chambers, the central start chamber and two choice chambers located to the right and left of the central alley. The central starting alley provides access each side chamber when movable door-openings are adjusted (i.e. rotatable plastic partitions were movable along Plexiglas rods through both sides of the apparatus). Three thick horizontal black stripes along one wall of the chamber serve as a visual cue to provide an axis of right–left orientation, so that the fish may discriminate right from left. Prior to training, 5 preference assessment trials were used to establish a side bias. After the preference

assessment, 10 test trials were initiated in which the fish were trained to select their non-preferred side as determined by the preference assessment. At the start of each trial, zebrafish were individually placed in the central alley. After 60 s, the partitions to both of the side chambers were simultaneously opened. After correct choices, the partition was closed and drawn a maximum distance from the fish, creating the largest possible tank space for the fish. After incorrect choices, the partition between the start chamber and the end wall was drawn to provide a space one cm wide for 30 seconds. Choice accuracy and response latency were recorded for each trial.

2.3.2.6. Light/Dark Motility: For the acute exposure cohort, a modified version of the larval swimming assay was used in adolescent zebrafish. The methods are exactly as described above for the larval assessment, except the trial was abbreviated to 20 mins in duration with 5 min intervals of illumination change and fish were housed in 6-well plates (diameter = 34.8mm) instead of 96-well plates.

2.4 Statistical Analysis

Dependent measures corresponding to each assay are described above. When appropriate, log transformations were performed on raw data if variability increased proportionally with magnitude or if distributions were positively or negatively skewed. All statistical analyses were performed using Superanova/Statview (SAS; Cary, NC, USA) and all graphs were made using SigmaPlot (SYSTAT Software Inc., Richmond, CA, USA). The Type 1 error rate (α) was set at 0.05 for all omnibus tests and Dunnett's post hoc comparisons. A repeated-measures analysis of variance (RMANOVA) was performed for each dependent variable of interest. Dose served as between-subject factors; tap number, session min, or condition served as within subject factors. When appropriate, e.g. where the adjustment to the degrees of freedom was less than 0.8, Huyn-Feldt or Greenhouse-Geiser adjustments to degrees of freedom were used to account for lack of sphericity in the dataset.

Regarding sample size(s) – following developmental exposure, all surviving 6 dpf fish were run on the larval swimming assay. Due to logistical constraints, a random subsection of these animals were selected for the complete test battery during adolescence. All animals dosed acutely during adolescence were run on the test battery. See Table 1 for N's per exposure group.

3. RESULTS

3.1. Survival

Survival and evidence of malformation were tracked daily (0–6 dpf) using a dissection microscope for developmentally exposed fish, and survival was tracked (0–24 hr) visually for acutely exposed fish. No fish died as a result of acute exposure. Developmental exposure (0–5 dpf) to 1 mg/L FM 550 (across both dose replications) increased mortality and rates of malformation (resulting in exclusion) significantly compared to controls ($p < 0.05$); 72% (86/120) of 1 mg/L animals died or were excluded due to malformations during the exposure window compared with 32% (38/120) of control, 33% (40/120) 0.01 and 36% (44/120) 0.1 mg/L animals. Malformations that resulted in exclusion included any structural deformity

visible under a dissection microscope. Spine curvature and edema (both pericardial and yolk sac) were the modal malformation observed. This exclusion rate among control animals is comparable to other experiments in our laboratory and likely reflects, to some degree, the misidentification of viable eggs on 0 dpf. These death and malformation/exclusion counts start accumulating following initial dosing (at 4 hpf), and end on 6 dpf.

3.2. Developmental Exposure: Behavioral Effects

3.2.1. Larval Swimming—No effect of FM 550 was detected on larval swimming under different conditions of illumination. A main effect of illumination condition (baseline, light, dark) was detected ($p < 0.05$), indicating all fish swam more during the dark conditions than during the light conditions, which is the typical response pattern under these conditions (see Fig. 1).

3.2.2. Novel tank exploration—A main effect of time (min) on total distance traveled ($F(4,356)=11.49$, $p < 0.001$) and on tank location (or, distance from the tank floor) ($F(4,356)=25.57$, $p < 0.001$) was detected. There was no significant effect of FM 550 dose on behavior under this test indicating that all fish explored the novel tank similarly, all displaying the typical bottom dwelling followed by exploration swim pattern (see Fig. 2 A–B).

3.2.3. Startle habituation—An interaction between dose and time (tap sequence) on distance traveled five-seconds following the stimulus delivery ($F(12,412)=1.76$, $p < 0.05$) was detected. A main effect of time (tap sequence) on distance traveled before the stimulus delivery (i.e. baseline) was also detected ($F(4,12)=3.64$, $p < 0.01$). The highest exposure group (1 mg/L) failed to engage in the typical habituation response to a repeated stimulus, seen among control animals ($p < 0.05$). During baseline conditions, all groups engaged in more swimming as the session progressed (see Fig. 2 C–D).

3.2.4. Social Affiliation—A main effect of stimulus condition (presence of shoal stimulus) ($F(1,77)=16.66$, $p < 0.001$) on distance from the active shoal and an interaction of stimulus condition with dose ($F(3,77)=3.75$, $p < 0.01$) on distance from the active shoal were detected (Fig 3 B). Also, a main effect of dose on total distance traveled ($F(3,77)=3.51$, $p < 0.05$) was detected, which was driven by the 0.1 mg/L dose (Fig 3 D). Here, all groups spent more time near the shoal when the shoal was present. All of the FM 550 animals, however, swam significantly farther from the shoal image than the control animals ($p < 0.05$). In sum, the control fish swam more and did so closer to the shoal (our two markers of shoaling) when the shoal was present than did the exposed fish (see Fig. 3 A–D).

3.2.5. Predator Escape—Only a main effect of condition (baseline, “ON”, “OFF”) ($F(2,134)=78.23$, $p < 0.001$) on distance from the predator stimulus was detected (Fig 5 A–C), indicating all fish fled the predator image when it was presented and explored the tank when it was absent. No effect of FM 550 exposure was detected on distance from the predator (Fig 4 AC) or on total distance traveled (Fig 4 D–F) during this test.

3.2.6. Spatial Discrimination Learning—No significant effects of FM 550 exposure were detected during this operant learning procedure.

3.3. Acute Exposure: Behavioral Effects

3.3.4. Testing 2 hr after exposure

3.3.4.1. Novel tank exploration: A main effect of FM 550 dose was detected on both tank location (or, distance from tank floor) ($F(2,49)=3.53$, $p<0.04$) and on total distance traveled ($F(2,49)=4.30$, $p<0.02$), driven by hypoactivity among the low dose (1.0 mg/L) group ($p<0.05$). Similarly, there was a main effect of time (min) on tank location ($F(4,196)=11.78$, $p<0.001$) and total distance traveled ($F(4,196)=9.01$, $p<0.001$), and there was an interaction between FM 550 dose and time on total distance traveled ($F(8,196)=2.16$, $p<0.04$), again occurring because of the reduced swimming across the entire trial among the low dose exposure fish (see Fig. 5 A–B)

3.3.4.2. Startle habituation: No significant effects of FM 550 exposure were detected during baseline, or following stimulus presentations on this test (all p 's >0.05) (Fig 5 C).

3.3.4.3. Social affiliation: A main effect of dose was detected on both distance to the shoal ($F(2,47)=4.76$, $p<0.02$) and total distance traveled ($F(2,47)=5.30$, $p<0.01$), such that the control group swam a greater distance in the presence of the shoal and swam closer to the shoal stimulus than exposed fish ($p<0.05$) (see Fig. 6 A–B).

3.3.4.4. Predator Escape: No significant effects of FM 550 exposure were detected on this assay.

3.3.4.5. Spatial Discrimination Learning: No effect of FM 550 exposure was detected on accuracy or latency to responding during this task.

3.3.4.6. Light/Dark motility: There was a significant interaction between illumination condition and FM 550 dose ($F(2,45)=3.53$, $p<0.04$), such that exposed fish swam comparable distances under both light and dark conditions whereas the control group swam more during the dark condition than the light condition ($p<0.05$) (Fig 5 D).

3.3.5. Testing 1 week after exposure—No effects of FM 550 exposure were detected, among any groups, for any of the assays when tested one week following acute exposure.

3.4. Summary

The results from each exposure windows and behavioral testing time are summarized in Table 2.

4. DISCUSSION

Flame retardants, added to polyurethane foam, are found in a wide range of consumer products. From these products, FRs can leech into the environment and accumulate indoors, often at detectable levels in household dust (Stapleton et al., 2012a; Stapleton et al., 2012b; Hoffman et al., 2014). Thus, human exposure occurs when contaminated dust is inhaled,

ingested or absorbed dermally. FireMaster® 550, a mixture of brominated and organophosphate flame retardants, is among the most commonly used mixtures applied to polyurethane foam (Stapleton et al., 2009; Stapleton et al., 2011) and was introduced as a safer and less bioaccumulative alternative to the PBDE FRs. Little is known regarding the consequences of exposure to this mixture, including what lasting effects of early life exposure may exist, despite its widespread use. The purpose of this study, therefore, was to begin characterizing the functional impact of exposure to the mixture FM 550 at various life stages and exposure windows.

Developmental exposure of zebrafish to 0.01 – 1.0 mg/L FM 550 in their water did not alter swimming ability (as measured by distance traveled during the session) or reactivity to changes in ambient illumination in 6 dpf larvae. Behavior during this test was not a good predictor of developmental FM 550-induced performance deficits as measured by the neurobehavioral test battery during adolescence. Weeks following the cessation of exposure, developmental FM 550 exposure caused a significant reduction in shoaling behavior (at all doses) and with diminished habituation to a startle stimulus (highest dose). Measures of function that are largely motoric (distance traveled during the predator escape/avoidance and to some degree distance traveled in the novel tank) were not found to be affected by these developmental FM 550 exposures, paralleling the larval data. These data highlight the limited capacity of a simple, high throughput, larval screen for behavioral toxicity. This assay has been described elsewhere as an early screen for behavioral toxicity (e.g. Padilla et al., 2011) but is likely predictive of motor toxicity alone, failing to capture more subtle or complex functional domains.

Only shoaling behavior was significantly affected by all doses of FM 550 following developmental exposure; accordingly, it is discussed here as the most sensitive indicator of FM 550's lasting neurobehavioral toxicity. However, it is possible that "sociability" or "social affiliation" per se is not the target, rather sensitivity to rewarding stimuli maybe the underlying mechanism on which FM 550 acts. In fact, the shoaling assay is unique among those used here as it is the only one that employs an appetitive stimulus. Predator escape, spatial discrimination and startle habituation explicitly utilize aversive stimuli to provoke behavior change, although along different dimensions. Even the novel tank swimming test capitalizing on the stress associated with entry into a new environment to measure function. If FM 550, as a mixture or any constituent working alone, diminishes the reinforcing efficacy of appetitive stimuli, this would be expected to manifest only on the shoaling assay. While the battery, taken as a whole, is able to disentangle a number of alternate explanations for behavior change – for example, fleeing during the predatory escape tasks makes visual impairment an unlikely explanation for reduced shoaling – it is not able to systematically identify the most likely behavioral mechanism underlying a given set of effects. This endeavor requires thoughtful probes into possible behavioral mechanisms. In this case, one would explicitly examine the appetitive control of stimuli over behavior across a range of rewarding stimuli (e.g. food, drugs of abuse, temperature/water quality variables, etc.).

In several ways the acute effects of exposure to FM 550 during adolescence mimicked the long-term effects following developmental exposure. First, shoaling behavior was altered compared to controls similarly for both exposure conditions. Animals exposed to 1 or 3

mg/L FM 550 for 24 hrs swam less overall and did so farther from the image of the conspecifics compared to control fish. Second, an aspect of motoric function was similarly spared by acute and developmental exposures. After acute exposure, all fish fled the image of a predator similarly – engaging in a heightened swim velocity, and all fish regardless of dose. Finally, acute FM 550 was not found to cause significant changes in operant learning (as measured by the spatial discrimination procedure). However, the acute effects of FM 550 did diverge from those caused by developmental exposure on novel tank swimming (in which acute exposure resulted in hypoactivity and bottom dwelling at the lowest dose) and startle habituation (in which developmental exposure muted habituation learning at the highest dose).

The acute effects of FM 550 exposure during adolescence, however, were transient, not being apparent when testing occurred 1 week following exposure. This suggests the effects captured at the 2 hr testing point might have been the result of intoxication, as exposed fish performed comparably to controls once (presumably) most of the active forms of these chemicals were excreted over the course of a one week interval. This interpretation might also be useful for understanding the divergences between the acute effects and those following developmental exposure (noted above). It is also worth noting that there were no signs of overt toxicity following this exposure regimen in this population – all fish were free from injury or signs of lethargy and feeding behavior remained normal. Similarly, no fish died or were moribund at any point during or following acute exposure.

While very little is known about effects on neurodevelopment or behavior, TPHP and iTPHPs have been associated with perturbed embryogenesis and reproductive toxicity in zebrafish (Liu et al., 2013; Wang et al., 2013; Liu et al., 2013; McGee et al., 2012) and humans (Honkakoski et al., 2004) and thyroid dysfunction in zebrafish (Liu et al., 2013). Similarly, EH-TBB and BEH-TEBP affect reproduction in vitro (Mankidy et al., 2013; Saunders et al., 2013) and disrupt thyroid function in rodents (Patisaul et al., 2013; Springer et al., 2012), and PBDEs have behavioral effects, including changes in motor function in fish (Chou et al., 2010; Usenko et al., 2011; Chen et al., 2012; Macaulay et al., 2015). Moreover, FM 550 exposure is associated with increased body weight and changes in maze exploration in rats (Patisaul et al., 2013) and has been shown to cause pronounced cardiotoxicity in zebrafish (McGee et al., 2013). In addition to endocrine disruption per se, the organophosphate constituents of FM 550 may act similarly to organophosphate pesticides via AChE blockade, which are known to be neurotoxic (for reviews see Gonzalez-Alzaga et al., 2014 or Munoz-Quezada et al., 2013) and have been shown to cause behavioral impairments in zebrafish (Levin et al., 2003; Levin et al., 2004; Eddins et al., 2010; Sledge et al., 2011). At least in terms of alterations to motoric function in zebrafish, the present results are consistent with the behavioral effects of PBDEs in zebrafish and, depending on the assay used, with the effects of the organophosphate pesticides. Therefore, any of these mechanisms, or others not yet identified, may underlie the behavioral effects demonstrated here. The behavioral consequences of FM 550 identified in the current study can be used to help identify the molecular and cellular actions of FM 550 key for producing functional impairment.

The absence of death or injury following acute exposure was not shared among fish exposed developmentally to 1.0 mg/L FM 550. This dose caused significant death and malformation during 0–6 dpf, indicating that embryonic/larval zebrafish are more sensitive to FM 500 toxicity than are fully developed adolescents. This, however, poses an interpretative limitation for the results from the 1.0 mg/L (0–6 dph) exposure group as the high death rate created a nonprobability sample for this dose. This, of course, limits the generalizability of these data as we cannot assume probability sampling. Related, it is possible that later selection of the eggs (e.g. 5–6 hpf) could have decreased the mortality rate overall, across all groups.

The current studies provide a starting point for additional investigations into the nature and mechanism of FM 550's toxicity. A number of future studies are suggested as follow-up to these data including the testing of doses intermediate to those used here, longer-term testing that includes the whole life span (e.g. late adulthood or old age), and the effects of these chemicals on other functional domains (e.g. substance abuse, interval timing, etc.) known to be sensitive to the effects of contaminants.

Acknowledgments

The authors would like to thank Dr. Heather Stapleton from Duke University for providing a stock solution of FM 550 and Anthony Oliveri for assisting with exposures and animal husbandry. This work was funded in part by the Duke Superfund Center (ES010356) and an RJR Goldberg Toxicology Fellowship.

References

- Ahmad F, Noldus L, Tegelenbosch R, Richardson M. Zebrafish embryos and larvae in behavioural assays. *Behaviour*. 2012; 149:1241–1281.
- Ali N, Dirtu AC, Van den Eede N, Goosey E, Harrad S, Neels H, t Mannetje A, Coakley J, Douwes J, Covaci A. Occurrence of alternative flame retardants in indoor dust from New Zealand: indoor sources and human exposure assessment. *Chemosphere*. 2012; 88(11):1276–1282. [PubMed: 22551874]
- Arthur D, Levin ED. Chronic inhibition of alpha4beta2 nicotinic receptors in the ventral hippocampus of rats: impacts on memory and nicotine response. *Psychopharmacology (Berl)*. 2002; 160(2):140–145. [PubMed: 11875631]
- Bailey J, Oliveri A, Levin ED. Zebrafish model systems for developmental neurobehavioral toxicology. *Birth Defects Res C Embryo Today*. 2013; 99(1):14–23. [PubMed: 23723169]
- Bencan Z, Levin ED. The role of alpha7 and alpha4beta2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. *Physiol Behav*. 2008; 95(3):408–412. [PubMed: 18671990]
- Chen X, Huang C, Wang X, Chen J, Bai C, Chen Y, Yang D. BDE-47 disrupts axonal growth and motor behavior in developing zebrafish. *Aquatic Toxicology*. 2012; 120–121:35–44.
- Chou C, Hsiao Y, Ko F, Cheng J, Cheng Y, Chen T. Chronic exposure of 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) alters locomotion behavior in juvenile zebrafish (danio rerio). *Aquatic Toxicology*. 2010; 98(4):388–395. [PubMed: 20416957]
- Dishaw LV, L JM, Roberts SC, Stapleton HM. Exposures, mechanisms, and impacts of endocrine-active flame retardants. *Curr Opin Pharmacol*. 2014; 19:125–133. [PubMed: 25306433]
- Dodson RE, Perovich LJ, Covaci A, Van den Eede N, Ionas AC, Dirtu AC, Brody JG, Rudel RA. After the PBDE phase-out: a broad suite of flame retardants in repeat house dust samples from California. *Environ Sci Technol*. 2012; 46(24):13056–13066. [PubMed: 23185960]
- Easton AH, Goulson D. The neonicotinoid insecticide imidacloprid repels pollinating flies and beetles at field-realistic concentrations. *PLoS One*. 2013; 8(1):e54819. [PubMed: 23382980]

- Eddins D, Petro A, Williams P, Cerutti DT, Levin ED. Nicotine effects on learning in zebrafish: the role of dopaminergic systems. *Psychopharmacology (Berl)*. 2009; 202(1–3):103–109. [PubMed: 18716760]
- Eddins D, Cerutti D, Williams P, Linney E, Levin ED. Zebrafish provide a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure: comparison with nicotine and pilocarpine effects and relationship to dopamine deficits. *Neurotoxicol Teratol*. 2010; 32(1):99–108. [PubMed: 19268529]
- Eriksson P, Viberg H, Jakobsson E, Orn U, Fredriksson A. A brominated flame retardant, 2,2',4,4',5-pentabromodiphenyl ether: uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development. *Toxicol Sci*. 2002; 67(1):98–103. [PubMed: 11961221]
- Eskenazi B, Chevrier J, Rauch SA, Kogut K, Harley KG, Johnson C, Trujillo C, Sjodin A, Bradman A. In utero and childhood polybrominated diphenyl ether (PBDE) exposures and neurodevelopment in the CHAMACOS study. *Environ Health Perspect*. 2013; 121(2):257–262. [PubMed: 23154064]
- Fernandes Y, Rampersad M, Jia J, Gerlai R. The effect of the number and size of animated conspecific images on shoaling responses of zebrafish. *Pharmacol Biochem Behav*. 2015
- González-Alzaga B, Lacasaña M, Aguilar-Garduño C, Rodríguez-Barranco M, Ballester F, Rebagliato M, Hernández AF. A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. *Toxicol Lett*. 2014; 230(2):104–21. [PubMed: 24291036]
- Herbstman JB, Sjodin A, Kurzor M, Lederman SA, Jones RS, Rauh V, Needham LL, Tang D, Niedzwiecki M, Wang RY, Perera F. Prenatal exposure to PBDEs and neurodevelopment. *Environ Health Perspect*. 2010; 118(5):712–719. [PubMed: 20056561]
- Hites RA. Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ Sci Technol*. 2004; 38(4):945–956. [PubMed: 14998004]
- Hoffman K, Fang M, Horman B, Patisaul HB, Garantziotis S, Birnbaum LS, Stapleton HM. Urinary tetrabromobenzoic acid (TBBA) as a biomarker of exposure to the flame retardant mixture Firemaster(R) 550. *Environ Health Perspect*. 2014; 122(9):963–969. [PubMed: 24823833]
- Honkakoski P, Palvimäki JJ, Penttilä L, Vepsäläinen J, Auriola S. Effects of triaryl phosphates on mouse and human nuclear receptors. *Biochem Pharmacol*. 2004; 67(1):97–106. [PubMed: 14667932]
- Levin ED, Chen E. Nicotinic involvement in memory function in zebrafish. *Neurotoxicol Teratol*. 2004; 26(6):731–735. [PubMed: 15451037]
- Levin ED, Chrysanthis E, Yacisin K, Linney E. Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination. *Neurotoxicol Teratol*. 2003; 25(1):51–57. [PubMed: 12633736]
- Levin ED, Swain HA, Donerly S, Linney E. Developmental chlorpyrifos effects on hatchling zebrafish swimming behavior. *Neurotoxicol Teratol*. 2004; 26(6):719–723. [PubMed: 15451035]
- Levin ED, Limpuangthip J, Rachakonda T, Peterson M. Timing of nicotine effects on learning in zebrafish. *Psychopharmacology (Berl)*. 2006; 184(3–4):547–552. [PubMed: 16175402]
- Liu C, Wang Q, Liang K, Liu J, Zhou B, Zhang X, Liu H, Giesy JP, Yu H. Effects of tris(1,3-dichloro-2-propyl) phosphate and triphenyl phosphate on receptor-associated mRNA expression in zebrafish embryos/larvae. *Aquat Toxicol*. 2013; 128–129:147–157.
- Liu X, Ji K, Jo A, Moon HB, Choi K. Effects of TDCPP or TPP on gene transcriptions and hormones of HPG axis, and their consequences on reproduction in adult zebrafish (*Danio rerio*). *Aquat Toxicol*. 2013; 134–135:104–111.
- Lorber M. Exposure of Americans to polybrominated diphenyl ethers. *J Expo Sci Environ Epidemiol*. 2008; 18(1):2–19. [PubMed: 17426733]
- Lunder S, Hovander L, Athanassiadis I, Bergman A. Significantly higher polybrominated diphenyl ether levels in young U.S. children than in their mothers. *Environ Sci Technol*. 2010; 44(13):5256–5262. [PubMed: 20540541]
- Ma Y, Venier M, Hites RA. 2-Ethylhexyl tetrabromobenzoate and bis(2-ethylhexyl) tetrabromophthalate flame retardants in the Great Lakes atmosphere. *Environ Sci Technol*. 2012; 46(1):204–208. [PubMed: 22128844]

- Macaulay LJ, Bailey JM, Levin ED, Stapleton HM. Persisting effects of a PBDE metabolite, 6-OH-BDE-47, on larval and juvenile zebrafish swimming behavior. *Neurotoxicology and Teratology*. 2015
- Mankidy R, Ranjan B, Honaramooz A, Giesy JP. Effects of Novel Brominated Flame Retardants on Steroidogenesis in Primary Porcine Testicular Cells. *Toxicol Lett*. 2013
- McGee SP, Konstantinov A, Stapleton HM, Volz DC. Aryl phosphate esters within a major PentaBDE replacement product induce cardiotoxicity in developing zebrafish embryos: potential role of the aryl hydrocarbon receptor. *Toxicol Sci*. 2013; 133(1):144–156. [PubMed: 23377616]
- McGee SP, Cooper EM, Stapleton HM, Volz DC. Early zebrafish embryogenesis is susceptible to developmental TDCPP exposure. *Environ Health Perspect*. 2012; 120(11):1585–1591. [PubMed: 23017583]
- Muñoz-Quezada MT, Lucero BA, Barr DB, Steenland K, Levy K, Ryan PB, Iglesias V, Alvarado S, Concha C, Rojas E, Vega C. Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review. *Neurotoxicology*. 2013; 39:158–68. [PubMed: 24121005]
- Padilla S, Hunter DL, Padnos B, Frady S, MacPhail RC. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. *Neurotoxicol Teratol*. 2011; 33(6):624–30. [PubMed: 21871562]
- Patisaul HB, Roberts SC, Mabrey N, McCaffrey KA, Gear RB, Braun J, Belcher SM, Stapleton HM. Accumulation and endocrine disrupting effects of the flame retardant mixture Firemaster(R) 550 in rats: an exploratory assessment. *J Biochem Mol Toxicol*. 2013; 27(2):124–136. [PubMed: 23139171]
- Ren Z, Bi X, Huang B, Liu M, Sheng G, Fu J. Hydroxylated PBDEs and brominated phenolic compounds in particulate matters emitted during recycling of waste printed circuit boards in a typical e-waste workshop of South China. *Environ Pollut*. 2013; 177:71–77. [PubMed: 23466734]
- Roze E, Meijer L, Bakker A, Van Braeckel KN, Sauer PJ, Bos AF. Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environ Health Perspect*. 2009; 117(12):1953–1958. [PubMed: 20049217]
- Saunders DM, Higley EB, Hecker M, Mankidy R, Giesy JP. In vitro endocrine disruption and TCDD-like effects of three novel brominated flame retardants: TBPH, TBB, & TBCO. *Toxicol Lett*. 2013; 223(2):252–259. [PubMed: 24064184]
- Saverino C, Gerlai R. The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. *Behav Brain Res*. 2008; 191(1):77–87. [PubMed: 18423643]
- Sledge D, Yen J, Morton T, Dishaw L, Petro A, Donerly S, Linney E, Levin ED. Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. *Neurotoxicol Teratol*. 2011; 33(6):742–751. [PubMed: 21745564]
- Springer C, Dere E, Hall SJ, McDonnell EV, Roberts SC, Butt CM, Stapleton HM, Watkins DJ, McClean MD, Webster TF, Schlezinger JJ, Boekelheide K. Rodent thyroid, liver, and fetal testis toxicity of the monoester metabolite of bis-(2-ethylhexyl) tetrabromophthalate (tbph), a novel brominated flame retardant present in indoor dust. *Environ Health Perspect*. 2012; 120(12):1711–1719. [PubMed: 23014847]
- Stapleton HM, Sjodin A, Jones RS, Niehuser S, Zhang Y, Patterson DG Jr. Serum levels of polybrominated diphenyl ethers (PBDEs) in foam recyclers and carpet installers working in the United States. *Environ Sci Technol*. 2008a; 42(9):3453–3458. [PubMed: 18522133]
- Stapleton HM, Allen JG, Kelly SM, Konstantinov A, Klosterhaus S, Watkins D, McClean MD, Webster TF. Alternate and new brominated flame retardants detected in U.S. house dust. *Environ Sci Technol*. 2008b; 42(18):6910–6916. [PubMed: 18853808]
- Stapleton HM, Misenheimer J, Hoffman K, Webster TF. Flame retardant associations between children's handwipes and house dust. *Chemosphere*. 2014; 116:54–60. [PubMed: 24485814]
- Stapleton HM, Dodder NG, Offenberger JH, Schantz MM, Wise SA. Polybrominated diphenyl ethers in house dust and clothes dryer lint. *Environ Sci Technol*. 2005; 39(4):925–931. [PubMed: 15773463]

- Stapleton HM, Eagle S, Sjodin A, Webster TF. Serum PBDEs in a North Carolina toddler cohort: associations with handwipes, house dust, and socioeconomic variables. *Environ Health Perspect.* 2012a; 120(7):1049–1054. [PubMed: 22763040]
- Stapleton HM, Klosterhaus S, Keller A, Ferguson PL, van Bergen S, Cooper E, Webster TF, Blum A. Identification of flame retardants in polyurethane foam collected from baby products. *Environ Sci Technol.* 2011; 45(12):5323–5331. [PubMed: 21591615]
- Stapleton HM, Kelly SM, Allen JG, McClean MD, Webster TF. Measurement of polybrominated diphenyl ethers on hand wipes: estimating exposure from hand-to-mouth contact. *Environ Sci Technol.* 2008; 42(9):3329–3334. [PubMed: 18522114]
- Stapleton HM, Sharma S, Getzinger G, Ferguson PL, Gabriel M, Webster TF, Blum A. Novel and high volume use flame retardants in US couches reflective of the 2005 PentaBDE phase out. *Environ Sci Technol.* 2012b; 46(24):13432–13439. [PubMed: 23186002]
- Stapleton HM, Harner T, Shoeib M, Keller JM, Schantz MM, Leigh SD, Wise SA. Determination of polybrominated diphenyl ethers in indoor dust standard reference materials. *Anal Bioanal Chem.* 2006; 384(3):791–800. [PubMed: 16385413]
- Usenko CY, Robinson EM, Usenko S, Brooks BW, Bruce ED. PBDE developmental effects on embryonic zebrafish. *Environmental Toxicology and Chemistry.* 2011; 30(8):1865–1872. [PubMed: 21560146]
- van der Veen I, de Boer J. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. *Chemosphere.* 2012; 88(10):1119–1153. [PubMed: 22537891]
- Viberg H. Exposure to polybrominated diphenyl ethers 203 and 206 during the neonatal brain growth spurt affects proteins important for normal neurodevelopment in mice. *Toxicol Sci.* 2009; 109(2):306–311. [PubMed: 19380496]
- Viberg H, Fredriksson A, Eriksson P. Neonatal exposure to the brominated flame retardant 2,2',4,4',5-pentabromodiphenyl ether causes altered susceptibility in the cholinergic transmitter system in the adult mouse. *Toxicol Sci.* 2002; 67(1):104–107. [PubMed: 11961222]
- Viberg H, Fredriksson A, Eriksson P. Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. *Toxicol Appl Pharmacol.* 2003; 192(2):95–106. [PubMed: 14550744]
- Viberg H, Fredriksson A, Eriksson P. Changes in spontaneous behaviour and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabrominated diphenyl ether (PBDE 209). *Neurotoxicology.* 2007; 28(1):136–142. [PubMed: 17030062]
- Viberg H, Fredriksson A, Jakobsson E, Orn U, Eriksson P. Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development. *Toxicol Sci.* 2003; 76(1):112–120. [PubMed: 12915714]
- Wang Q, Liang K, Liu J, Yang L, Guo Y, Liu C, Zhou B. Exposure of zebrafish embryos/larvae to TDCPP alters concentrations of thyroid hormones and transcriptions of genes involved in the hypothalamic-pituitary-thyroid axis. *Aquat Toxicol.* 2013; 126:207–213. [PubMed: 23220413]
- Willemsen R, Van der Linde H. Zebrafish larvae activity: A study of locomotive behavior with DanioVision. Noldus Information Technology. 2010

Figure 1. Developmental Exposure, Larval Testing: Motility

Swimming activity in alternating 10-min blocks of light and dark. Dotted line denotes phase change. FM 550 exposure groups are plotted with open symbols, the control group is plotted with filled circles, each data point corresponds to a 1-min average of activity. The rectangular blocks along the x-axis indicate illumination condition (filled=dark, open=light). Error bars represent standard error of the mean.

Figure 2. Developmental Exposure, Adolescent Testing: Tank exploration and habituation to a startle

Results from the neurobehavioral test battery are plotted for the developmental (0–6 dpf) exposure group. Panels A–B represent distance from the floor (A) or total distance traveled (B) during the novel tank exploration assay. Each bar represents 1-min of the session, exposure group is plotted along the x-axis. Panels C–D represent distance traveled before (C) and after (D) the presentation of a tap stimulus during the startle habituation assay. Each bar represents the average distance traveled for each 2-tap block during the session, with exposure group plotted along the x-axis. All error bars (A–D) represent standard error of the mean and “*” denotes difference from control.

Figure 3. Developmental Exposure, Adolescent Testing: Shoaling

Results from the neurobehavioral test battery are plotted for the developmental (0–6 dpf) exposure group. Panels A and C correspond to baseline behavioral during the shoaling procedure, B and D represent behavior in the presence of shoaling conspecifics (video). Distance to the shoal (A–B) and total distance traveled (C–D) are plotted by exposure group. All error bars represent standard error of the mean and “*” denotes difference from control.

Figure 4. Developmental Exposure, Adolescent Testing: Predator escape/avoidance

Results from the neurobehavioral test battery are plotted for the developmental (0–6 dpf) exposure group. Behavior during the predator escape/avoidance procedure is shown during baseline (A & D), in the presence of the predator stimulus (B & E) and in its absence (C & F). The top row (A–C) plots distance from the predator (cm) stimulus by exposure condition and the bottom row (D–F) plots total distance traveled (cm) by exposure condition. Error bars represent standard error of the mean.

Figure 5. Acute Exposure, Adolescent Testing 2-hr Following Exposure: Exploration, habituation to a startle and motility

Results from the neurobehavioral test battery are plotted for the acute (24 hr) exposure group, tested 2 hr after exposure. Panels A–B correspond to distance from the floor (A) and total distance traveled (B) during the novel tank exploration assay, each bar represents the mean for one min of the trial. Panel C shows distance traveled following the tap presentation during the startle habituation assay, each bar represents the average of two consecutive tap presentations. Panel D plots swimming during the light/dark adolescent motility assay, the filled bars correspond to swimming in a dark environment and the open bars to swimming in a brightly lit environment. Exposure condition is plotted along the x-axis. All error bars represent standard error of the mean and “*” denotes difference from control.

Figure 6. Acute Exposure, Adolescent Testing 2-hr Following Exposure: Shoaling

Results from the neurobehavioral test battery are plotted for the acute (24 hr) exposure group, tested 2 hr after exposure. Distance to the active shoal (A) and total distance traveled (B) in the presence of the conspecifics is plotted by exposure group. Error bars represent standard error of the mean and “*” denotes difference from control.

Table 1

Experimental Design

Exposure Age (exposure window)	Testing Time (chort)	N ^a
Developmental (0–5 dpf)	6 dpf (larval)	76–82; except 1 mg/L = 34
Developmental (0–5 dpf)	50–55 dpf (adolescent)	30–35; except 1 mg/L = 11
Adolescent (24 hr)	2 hr after exposure (immediate)	16
Adolescent (24 hr)	1 wk after exposure (delayed)	16

^aTotal N per exposure group that completed behavioral testing

Table 2Summary of the Behavioral Toxicity of FM550 in Adolescent Zebrafish^a

Assay	Effect Present ^b	Assay	Effect Present ^b	
<i>Developmental Exposure</i>	<i>Adolescent Testing</i>	<i>Acute, Adolescent Exposure</i>	<i>2 Hour Testing</i>	<i>1 Week Testing</i>
Novel Tank Exploration		Novel Tank Exploration		
Distance Traveled	--	Distance Traveled	↓	--
Distance from floor	--	Distance from floor	↓	--
Startle Habituation		Startle Habituation		
Baseline	↑	Baseline	--	--
Startle Response	↑	Startle Response	--	--
Shoaling		Shoaling		
Distance Traveled	↓	Distance Traveled	↓	--
Distance from Shoal	↓	Distance from Shoal	↓	--
Predator Escape/Avoidance		Predator Escape/Avoidance		
Escape	--	Escape	--	--
Avoidance	--	Avoidance	--	--
Spatial Discrimination Learning		Spatial Discrimination Learning		
Accuracy	--	Accuracy	--	--
Latency	--	Latency	--	--
		Light/Dark Sensitivity		
		Distance Traveled	↓*	--

^aSignificant main effect of dose or dose by condition/time interaction is included^bp<0.05; "↑" indicates an increase from control, "↓" indicates a decrease from control, "↓*" indicates divergent, condition-dependent effects