Letter

Vision, color vision, and visually guided behavior: the novel toxicological targets of 2,2#,4,4#-tetrabromodiphenyl ether (BDE-47)

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Environ. Sci. Technol. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acs.estlett.7b00010 • Publication Date (Web): 08 Feb 2017

Just Accepted

Environmental Science & Technology Letters is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Vision, Color Vision, and Visually Guided Behavior: the Novel Toxicological Targets of 2,2′,4,4′-Tetrabromodiphenyl Ether (BDE-47)

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ABSTRACT. Our published studies have revealed that 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) could disrupt retina morphologies and related gene expressions of zebrafish larvae. Then, its possible effects on fish vision needed to be uncovered since sensory systems especially eyes were vital to the wildlife. In this paper two tests for vision development (opsin gene expression and photoreceptor immunostaining) and two tests for visually guided behaviors (optokinetic response and looming-evoked escape) were designed to investigate the potential visual impairments and subsequent ecological consequences caused by BDE-47 exposure in 6 dpf zebrafish larvae. The short wavelength sensitive cone opsins and rhodopsin were significantly inhibited by BDE-47 exposure. Meanwhile, BDE-47 exposure significantly reduced larval optokinetic responses with blue light stimuli, and induced less larvae to exhibit escape response with looming stimuli, which confirmed the adverse consequences of visual impairments in zebrafish. Our results indicated that BDE-47 exposure impaired zebrafish larval vision (including color vision) development, and further altered larval behaviors guided by vision, which provided adequate evidence to prove that vision system was a novel and urgent toxicological target of environmental pollutants.

INTRODUCTION

Behaviors are animal adaptive responses to external stimuli, which involves two elements, information and decision. Sensory systems are responsible for collecting ecological information to initiate purposive behavior.[1] Especially, vision occurrence brings functional and evolutional benefits for vast majority of animals.[2] Behavioral strategies adopted by animals rely on visual information input, and for example, locating and tracking the prey is usually visually mediated concluded by the comparison between tectum-ablated and wild-type fish in darkness, as well as blind mutants.[3] The normal development of visual structure and establishment of visual function are critical to animal survival, growth, and reproduction, which are all key processes for the maintenance of populations and ecosystem.

Zebrafish are regarded as an optimal model system in vertebrate vision development research, for their numerous advantages such as high dependency on vision, good genetic and morphological manipulation, and simple and convenient behavioral screening methodologies.[4] The zebrafish vision system develops rapidly to search for food and avoid predators in a short duration after hatching.[5] Early at 10-12 hours post-fertilization (hpf), eye primordium establishes at the anterior of fish.[6] Typical retinal layers eventually form at 5 days post-fertilization (dpf), and larvae are able to catch moving prey even from 4 dpf.[7] Compared to the mammal models, the cone-dominant retina of zebrafish could produce richer color vision and higher acuity,[8,9] which will provide more reliable cues for object detection and identification.[10]

Recently, new clues have emerged to suggest the adverse effects of environmental pollutants on animal vision, for example 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and the commercial mixture DE-71. DE-71 exposure could cause biochemical changes in the eye and photosensitive behavioral alterations at 15 dpf zebrafish larvae.[11] We observed that the locomotion of 6 dpf

zebrafish larvae was significantly decreased by nominal 500 μg/l BDE-47 exposure, which exclusively happened at the periods of light switching, and further found BDE-47 changed the retinal morphological structures and identified BDE-47-responsive transcripts functioning in visual perception and retina formation.[12,13] Besides PBDEs, $Aroclor_{1254}$ and pentachlorophenol were also reported to impair zebrafish vision development,[14,15] reminding us vision was potentially vulnerable to more than a few agents. Therefore, the further study on the effects of vision system may provide evidence to uncover the possible relationship between environmental pollution and animal/human visual impairments or diseases.

To explore the effects of BDE-47 exposure on visual functions in zebrafish larvae, we designed two tests for vision development (opsin gene expression and photoreceptor immunostaining) and two tests for visually guided behaviors (optokinetic response and stimuli-evoked escape) using previous exposure protocols.[13] The experimental designs and analytic methods were improved to reflect the factor of color vision considering zebrafish dominant cones. Our results confirmed that BDE-47 exposure impaired larval vision including color vision and related irritable behaviors, and proposed vision system as an emerging toxic target which would lead to a profound influence on the survival of wildlife.

MATERIALS AND METHODS

Fish and chemical exposure. Healthy wild-type Tuebingen zebrafish *(Danio rerio*) at 4 to 6 months were chosen. BDE-47 exposure performed from 3-4 hpf to 6 dpf at 28.5 °C, and all treatments were replicated three times. All animal protocols were in accordance with guidelines approved by the Animal Ethics Committee of Tongji University. The pretreatment and

instrument determination of BDE-47 in water and fish were performed following previous literatures.[11,16,17] More details were given in the Supporting Information.

Quantitative real-time PCR (qRT-PCR). After exposure, about 60 larvae from each group were homogenized for qRT-PCR experiment. Total RNA extraction and PCR amplification were performed according to manufacturer's instructions. The threshold cycle values for selected genes and housekeeping RPL13a were used to calculate the relative RNA amounts. Fold changes (FC) of tested genes were defined as the ratio of RNA amounts in treatment versus control. More details were given in the Supporting Information and Table S1.

Immunostaining staining of zebrafish photoreceptors. For each group, 12 larvae were fixed and dissected to prepare the frozen slides of eye. The specific markers for rods and cones were labeled using corresponding antibodies to detect the effects of BDE-47. Images were taken with an IX-70 confocal laser-scanning microscope (Olympus, Japan). More details were given in the Supporting Information.

Optokinetic response (OKR) test. OKR tests of zebrafish larvae were performed using VisioBox platform (Viewpoint, FR). Larvae were fixed in a 35-mm petri dish which was placed in a round chamber of VisioBox. To investigate the effects of BDE-47 exposure on zebrafish color vision, we counted larval saccadic movements in response to three primary colors. For the control and treatment, 6 larvae were tested in each color and the test duration was 1 min. More details were given in the Supporting Information and Table S2.

Escape behavior test. Escape behavior induced by looming stimuli was previously described.[18] Petri dishes containing larvae were arranged in a circular pattern, and the looming stimuli expanded from center of the circle and disappeared after 1.6 s. Six larvae were tested

each time, and for each larva eight trials were performed to calculate escape probability. More details were given in the Supporting Information and Figure S1.

Data analysis. Statistical analyses of raw experimental data were performed with SPSS program 19 (IBM, USA), and the outcome data were presented as mean ± standard error of the mean (SEM). Significant differences between the control and each treatment were determined by one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's multiple comparison test. For all analysis, the statistical criterion for a significant difference was $p<0.05$ (and $|FC|>1.5$ only for qRT-PCR). The graphical charts were illustrated using Origin 2016 (Originlab, USA) and Microsoft Excel 2016.

RESULTS AND DISCUSSION

The actual BDE-47 contents in water and fish. The BDE-47 concentrations in water and fish were listed in Table S3. Actual BDE-47 concentrations in water (47.87±5.41 μg/l at 6 d) were apparently lower than their nominal value (500 μg/l) although our test solutions were half renewed daily. The actual contents of poorly water-soluble chemicals in waterborne exposure would be seriously interrupted by the absorption of polystyrene microplates[19] which were applied in zebrafish embryo toxicity tests. Meanwhile, BDE-47 in 6 dpf larval tissues were 48.84±2.48 μg/g wet weight.

Altered expression patterns of visual opsin genes. Opsins are the light-sensitive proteins which were first discovered as one essential component of vertebrate visual pigments in photoreceptors. Even though non-visual opsins expressed outside the retina, their functions were always associated with photosensitivity and circadian rhythm of lives.[20,21] Zebrafish larvae have nine known visual opsins, including eight cone opsins (*opn1sw1*, *opn1sw2*, *opn1mw1*, *opn1mw2*,

130 *opn1mw3*, *opn1mw4*, *opn1lw1*, *opn1lw2*) and one rod opsin (*rho*),[22] which were all investigated
131 in the present study.

132 The influences of BDE-47 exposure on larval opsin gene expressions were shown in Figure S2
133 arranged by their max absorption wavelengths (note that the max absorption wavelengths of cone
134 opsins were not of cone pigments).[22] Our previous sequencing data (GSE59968) were served as
135 the reference to compare the expression levels of the tested visual opsins genes using qRT-
136 PCR.[13] The results showed a good consistence with each other for those highly-expressed
137 transcripts. Four mid-short wavelength sensitive opsins, *opn1sw1*, *opn1sw2*, *opn1mw1*, and *rho*
138 were significantly inhibited by BDE-47. Because of the low background levels of remaining
139 genes, the impacts of their expression changes were too subtle and not discussed here. The
140 expression changes of cone and rod opsins indicated an impaired spectral sensitivity to blue-
141 green light and dim light.[23]

142 **The developmental defects in retina photoreceptors.** To further correlate the BDE-47 with
143 visual impairments, we investigated the integrity of zebrafish photoreceptor cell layers (rods and
144 cones) after exposure. The expression changes of rhodopsin and zpr-1 were identified by
145 confocal microscopy. Abundant immunofluorescent staining of rhodopsin, a marker for rod
146 photoreceptors, was constantly observed in the inner/outer segment (IS/OS) layer of all control
147 larvae (Figure 1a-1b), whereas the staining of rhodopsin revealed greatly decreased expression in
148 the eye sections of the majority of zebrafish treated with BDE-47 (Figure 1c-1d). We next
149 determined the morphogenesis of cone photoreceptors in these models using an antibody against
150 ZPR-1, a specific marker for double cone containing red and green sensitive photoreceptors,
151 labeling the cone OS was clearly detected in the IS/OS layer of all zebrafish studied (Figure S3).

The decreased rhodopsin fluorescence and unaffected double cones were consistent with the data from qRT-PCR.

(Insert Figure 1 here)

Zebrafish rod photoreceptors could be histologically detected early at 4-5 dpf,[5,24] although they needed about ten more days to reach their morphological and functional maturity. Existing electroretinogram evidence suggested the dark-adapted spectral sensitivity of 6-15 dpf larvae was primarily from ultraviolent (UV)-cone input before rods were well developed,[25] which would also interrupt larval scotopic behavior because UV sensitive opsin (*opn1sw1*) was inhibited by BDE-47 exposure.

Reduced sensitivity to short wavelength light. OKR response which serves to produce stabilized high-resolution images on vertebrate retina is a classic test of visual behavior in zebrafish larvae.[26] Considering that cone opsins are responsible for daylight vision with color sensation, we upgraded black light in black-white stripes to different color lights. Zebrafish have a tetrachromatic vision: red, green, blue/violet, and UV. Our OKR tests were performed under the stimulation of three color lights except UV due to limitation of the equipment. BDE-47 exposure significantly reduced the larval OKR response to the blue light not the mid-long waveband lights (Figure 2). Additionally, larval left eyes had fewer movements than right eyes after BDE-47 exposure, while wild-type larvae had balanced movements of two eyes to different colors. This phenomenon of left-right asymmetry was postulated to relate with retinoic acid signaling considering its roles in vision formation and left-right axis establishment.[27,28] We also investigated the saccadic movement angles per time using VisioBox, however, no significant difference was observed between control and BDE-47 treatment (Table S4).

(Insert Figure 2 here)

The results of OKR and qRT-PCR indicated the impacts of BDE-47 on zebrafish color vision. As a surface swimmer, zebrafish live in a short wavelength-dominant environment and has an explicit preference to blue/green ambient color,[29] in accordance with the high expression of short wavelength sensitive opsins which were reported to help zebrafish acquire more photons to detect adjacent predators and preys.[30] Thus, BDE-47 exposure induced the reduction of larval response to blue light, which may severely threaten their survival.

The reduced response rates to looming stimuli. The larval escape response to the potential predators was tested using a "looming" stimuli which expanded and approached to larvae on a collision course. The animal response to looming is conserved across species, and represents one of the crucial defensive strategies for avoiding predation.[31] In principle, this process was controlled by a series of events of visual-motor system where retina ganglion cells (RGCs) were mainly responsible for detecting approach motion.[1,32] Looming-evoked escape response is thereby an excellent candidate test emphasizing the roles of visual factors in behavior of animals living in real ecosystem.

In the results, the amounts of responsive fish significantly reduced with BDE-47 exposure (Figure 3), reflecting BDE-47 impaired larval behavioral capacity with sensing ambient visual stimuli. Furthermore, larval response time to stimuli were not retarded; the relationship between response time and visual angles, which was commonly used in studies of looming-evoked visual pathway mechanisms,[1] were also not influenced (Figure S4). The effects existed only in response rate, not action mode or response speed. We estimated it was because RGCs processing had not been disrupted, which meant the reason of escape caused by BDE-47 primarily occurred at the

196 frontend of the pathway engaging visual information input, not RGCs and the backend (e.g.
197 motor and central nervous system).

198 (Insert Figure 3 here)

199 Taken together, we reported that BDE-47 exposure on zebrafish embryos/larvae disrupted
200 larval vision, color vision, and visually guided behaviors, and proposed vision system as an
201 urgent focus especially in ecological toxicology. The ecological and human health impacts of
202 anthropogenic pollution are our primary reason to concern about environmental issues. By far
203 most of current environmental toxicological studies were guided by some hotspots derived from
204 high-profile human diseases (e.g. cancers). Under this condition, the differences of demands
205 between animal survival and human welfare are also worth attention. For instance, sensory
206 dysfunction could only interfere with life quality for most people, but is a matter of wildlife
207 survival as it decided the information capacity involved in emergent behavioral strategies.

208

209 **ASSOCIATED CONTENT**

210 **Supporting Information**. Supporting Information Available: Supporting experimental section
211 with a detailed description, Figure S1-S4, and Table S1-S4 (PDF). This material is available free
212 of charge via the Internet at http://pubs.acs.org.

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Author Contributions

TX, DY, and QZ conceived and designed the study. YL performed immunofluorescence experiments. RP performed looming tests. TX and RP performed OKR tests. JZ and BZ performed qRT-PCR experiments. BZ determined the concentrations of BDE-47 in exposure. TX and YL drafted the initial manuscript, and TX, YL, JZ, and DY contributed to the preparation of the final manuscript. All authors read and approved the final version of this manuscript. TX and YL contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

The authors thank Funing Li from Institute of Neuroscience, Chinese Academy of Sciences for kindly assisting with larvae escape response test. The work was supported by the National Natural Science Foundation of China (21477086, 21507080, and 21577104), Natural Science of foundation of JiangSu province (BK20160133), and the Collaborative Innovation Center for Regional Environmental Quality.

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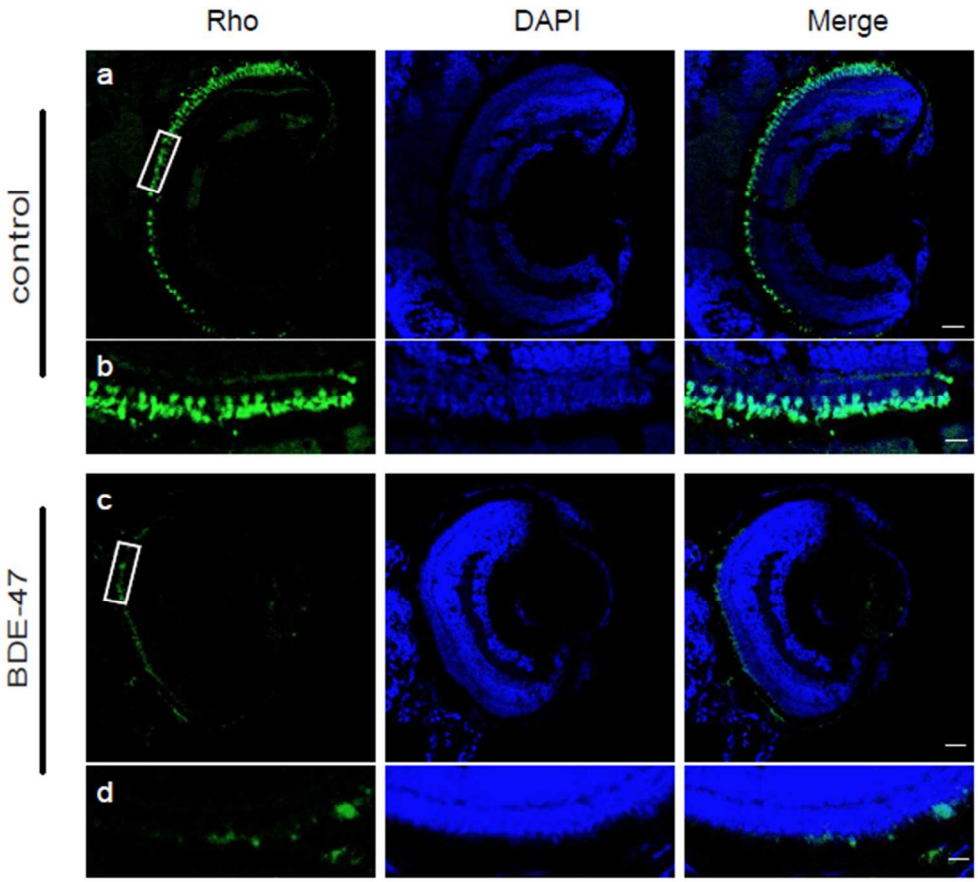
315 FIGURE LEGENDS

316 **Figure 1.** Immunostaining of rhodopsin on retinal frozen sections of zebrafish at 6 dpf was from
317 the exposure group and control group. Robust staining was found in the rhodopsin-expressing
318 rod IS/OS layers of control larvae (a-b). Reactivity of rhodopsin was decreased in BDE-47
319 treatment zebrafish (c-d) compare with the control. (b) and (d) were shown in higher
320 magnification in above box area. Scale bar: 20 μm.

321 **Figure 2.** The difference of larval OKR responses between BDE-47-treated and control group
322 (n=6). The response was represented by times of ocular saccadic movements, and left and right
323 eye of larvae were counted separately. The light sources are divided into three colors: blue (blue
324 columns), green (green columns), and red (red columns). The asterisk "*" indicated $p<0.05$
325 compared with control.

326 **Figure 3.** Larval escape response test. The numbers of (a) responsive individuals (n=48) and (b)
327 trials (n=8) were all reduced under the exposure of BDE-47. The asterisk "*" indicated $p<0.05$
328 compared with control.

329



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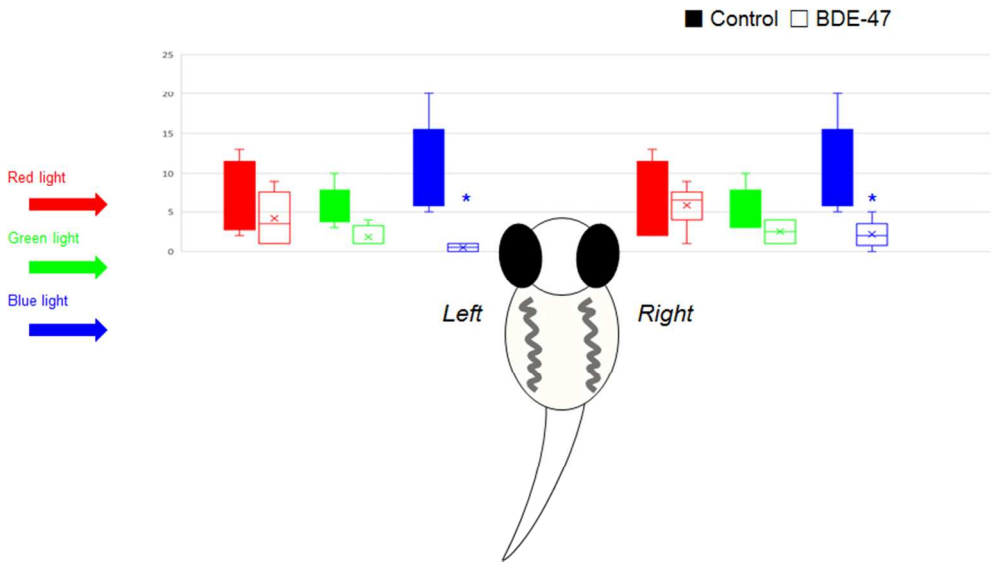

Figure 2. The difference of larval OKR responses between BDE-47-treated and control group (n=6). The response was represented by times of ocular saccadic movements, and left and right eye of larvae were counted separately. The light sources are divided into three colors: blue (blue columns), green (green columns), and red (red columns). The asterisk "*" indicated $p<0.05$ compared with control.

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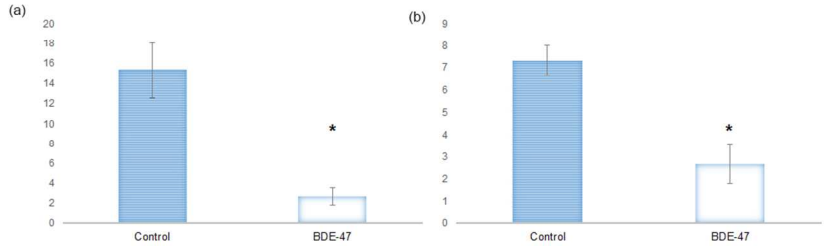

Figure 3. Larval escape response test. The numbers of (a) responsive individuals (n=48) and (b) trials (n=8) were all reduced under the exposure of BDE-47. The asterisk "*" indicated $p<0.05$ compared with control.

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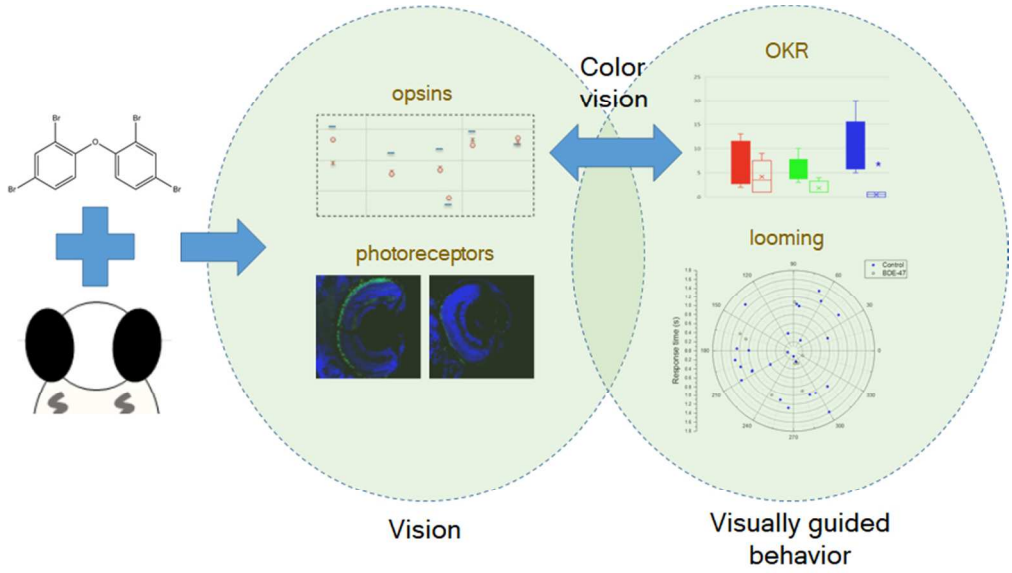

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77x44mm (300 x 300 DPI)