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**Molecular mechanisms and tissue targets of brominated flame retardants, BDE-47 and TBBPA, in embryo-larval life stages of zebrafish (*Danio rerio*).**

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## Highlights

- Zebrafish thyroid system is vulnerable to disruption by BFRs during early development
- BFRs disrupt thyroid axis of zebrafish embryo-larvae at multiple mRNA transcripts
- Effects of BFRs on the thyroid system are tissue- and developmental stage-specific
- Implications of transcriptional changes targets involved in tissue development

## Abstract

Brominated flame retardants are known to disrupt thyroid hormone (TH) homeostasis in several vertebrate species, but the molecular mechanisms underlying this process and their effects on TH-sensitive tissues during the stages of early development are not well characterised. In this study, we exposed zebrafish (*Danio rerio*) embryo-larvae to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and tetrabromobisphenol A (TBBPA) via the water for 96 h from fertilisation and assessed for lethality, effects on development and on the expression of a suite of genes in the hypothalamic-pituitary-thyroid (HPT) axis via both real time quantitative PCR (qRT-PCR) on whole body extracts and whole mount *in situ* hybridisation (WISH) to identify tissue targets. The 96-h lethal median concentration (96h-LC<sub>50</sub>) for TBBPA was 0.9 µM and mortality was preceded by retardation of development (smaller animals) and morphological deformities including, oedemas in the pericardial region and tail, small heads,

swollen yolk sac extension. Exposure to BDE-47 did not affect zebrafish embryo-larvae survival at any of the concentrations tested (1 – 100  $\mu$ M) but caused yolk sac and craniofacial deformities, a curved spine and shorter tail at the highest exposure concentration. TBBPA exposure resulted in higher levels of mRNAs for genes encoding deiodinases (*dio1*), transport proteins (*ttr*), the thyroid follicle synthesis protein paired box 8 (*pax8*) and glucuronidation enzymes (*ugt1ab*) and lower levels of *dio3b* mRNAs in whole body extracts, with responses varying with developmental stage. BDE-47 exposure resulted in higher levels of *thrb*, *dio1*, *dio2*, *pax8* and *ugt1ab* mRNAs and lower levels of *ttr* mRNAs in whole body extracts. TBBPA and BDE-47 therefore appear to disrupt the TH system at multiple levels, increasing TH conjugation and clearance, disrupting thyroid follicle development and altering TH transport. Compensatory responses in TH production/ metabolism by deiodinases were also evident. WISH analyses further revealed that both TBBPA and BDE-47 caused tissue-specific changes in thyroid receptor and deiodinase enzyme expression, with the brain, liver, pronephric ducts and craniofacial tissues appearing particularly responsive to altered TH signalling. Given the important role of TRs in mediating the actions of THs during key developmental processes and deiodinases in the control of peripheral TH levels, these transcriptional alterations may have implications for TH sensitive target genes involved in brain and skeletal development. These findings further highlight the potential vulnerability of the thyroid system to disruption by BFRs during early developmental windows.

Keywords: brominated flame retardant, thyroid, endocrine, fish, toxicology, development

## 1. Introduction

Over the last two decades, there has been growing concern over the levels of brominated flame retardants (BFRs) in the environment due to their ability to disrupt the thyroid hormone (TH) system [1]. THs play a key role in a wide range of vertebrate physiological functions both during early development and in adult life stages, including influencing the maturation of bones [2], the gonads [3] and the central nervous system [4]. Given the detrimental effects that can result from subtle changes in TH status, particularly during crucial developmental windows [5], even relatively low environmental levels of BFRs pose a potential risk to the health of humans and wildlife.

BFRs have been commercially important high production compounds since their introduction to the global market in the 1970s and are used routinely in industrial and consumer products in an effort to reduce fire-related injury and property damage [6]. Polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA) are amongst the most extensively used BFRs worldwide [6]. PBDEs are a family of 209 possible congeners that can be divided into 10 congener groups (mono- to decabromodiphenyl ethers). Commercial PBDE mixtures are made up of congeners with varying numbers of bromine atoms on their two phenyl rings and are classified according to their average bromine content; penta-, octa-, and deca-BDEs. The congener BDE-47 (2,2',4,4'-tetrabromodiphenyl ether) was one of the main components of the now banned commercial penta-BDE mixtures, accounting for approximately 40% of the product by weight [6, 7].

Since PBDEs are blended physically rather than bonded chemically to the polymer, they subsequently migrate into the environment and over the past four decades they have become ubiquitous environmental contaminants. BDE-47 is often the most commonly detected PBDE congener in the aqueous phase of environmental water samples [8] and often dominates the PBDE profiles of human tissues, marine mammals, birds and birds eggs, invertebrates and fish [9-13]. The production and usage of penta-BDE commercial mixtures were prohibited globally in 2004 and officially labelled as Persistent Organic Pollutants (POPs) in 2009 [14]. Controls on the use of the penta-BDE products are only just beginning to yield declines in the lower brominated congener concentrations in environmental samples [15]. Nonetheless, global contamination occurs today as a result of the continued use and disposal of older products, manufactured prior to the restrictions, which still contain PBDEs. In 2016, over a decade since the introduction of restrictions, there is a continued presence of penta-BDE constituents in indoor air and dust [16] and they remain the dominant PBDEs detected in both human samples [up to 141  $\mu\text{g}/\text{kg}$  lipid weight (lw) in placental tissue] and wildlife samples (up to 1 mg/kg lw in freshwater fish) [17-20].

TBBPA is used as a reactive and additive flame retardant in printed circuit boards and electronic enclosures, respectively. It is the most widely used BFR (representing approximately 60% of the total BFR market) [6], with global production estimated to be over 200,000 metric tonnes a year [21]. TBBPA has been identified in dust, sewage sludge, river sediments and the water phase of lakes and rivers [22-26]. Despite TBBPA's relatively short half-life [27] which suggests little potential for bioaccumulation, it has been detected globally, in some cases at high levels, in many

biotic samples including human breast milk and plasma (up to 37.3  $\mu\text{g}/\text{kg lw}$ ) [28], suggesting both recent and continuous use.

Several BFRs are structurally similar to thyroxine (T4), the precursor of the biologically active TH 3,3',5-triiodo-L-thyronine (T3), and concerns have been raised about their effect on the thyroid system of both mammalian and non-mammalian vertebrates [1, 29]. Indeed, numerous *in vivo* studies have shown that both TBBPA and PBDEs can alter the circulating levels of both T4 and T3 in a range of vertebrate species, including fish [30, 31]. The thyroïdal system is centrally driven by the hypothalamic-pituitary-thyroid (HPT) axis and under peripheral control in all vertebrates. The HPT axis is regulated as a negative feedback mechanism in which the level of thyroid-stimulating hormone (TSH) secreted by the pituitary controls the production and release of T4 by the thyroid follicles. In the peripheral system, TH activity is tightly regulated by the metabolising enzymes iodothyronine deiodinases, type I, II and III (D1, D2 and D3), which can modulate TH signalling in individual tissues as well as controlling serum TH concentrations. D2 catalyses the outer ring deiodination (ORD) of T4 to produce the bioactive T3. In contrast, D3 catalyses the inner ring deiodination (IRD) of T4 and T3 producing the inactive metabolites reverse T3 (rT3) and 3, 3'-diiodo-L-thyronine (T2), respectively. D1 is a kinetically inefficient enzyme that is capable of catalysing both IRD and ORD [32, 33].

Until recently, evaluating thyroid disruption by environmental chemicals mainly relied on measures of circulating TH levels, thyroid size or histopathology. It is important, however, to note that the thyroid system can maintain normal physiological functions in response to TH level perturbations by changing the production and/or metabolism

of THs by the thyroid gland itself, in target tissues or in the liver [34, 35]. Thyroid-disrupting chemicals (TDCs) may not cause obvious changes to TH levels, but may nonetheless alter TH homeostasis. Consequently, identifying tissue-specific responses to environmental contaminants is fundamental to building our understanding on the effect mechanisms of BFRs. Several studies have demonstrated that BFRs can elicit localised effects on the expression of thyroid related genes in adult fathead minnows (*Pimephales promelas*) and Chinese rare minnows (*Gobiocypris rarus*) [30, 36], however isolating organs and/or tissues of interest from embryos and larvae for studies on gene expression is more challenging. Whole mount *in situ* hybridisation (WISH), however, allows for sites of expression of target genes to be detected in whole zebrafish embryo-larvae and this approach has been used to illustrate a significant up-regulation of deiodinase encoding *dio1* mRNA levels in the periventricular region of the brain and *dio3b* mRNA in the pronephric ducts of zebrafish embryos exposed to the hydroxylated metabolite 6-OH-BDE-47 [37].

The overall aim of this study was to assess the toxicological effects of two important BFRs, TBBPA and BDE-47, on zebrafish during early development. The first objective was to examine the acute toxicity of both compounds on zebrafish embryo-larvae, in terms of induced mortalities and deformities, in order to calculate lethal and effect concentration (LC and EC values). The second objective was to examine the effect of these compounds on the expression of a suite of genes in the HPT axis, with the goal of highlighting potential effect mechanisms and target tissues of thyroid disruption. Given the plasticity of the TH system, we further undertook to examine the effects of TBBPA across different developmental life stages. We used a

combination of real time quantitative PCR (qRT-PCR) assays to quantify changes in gene transcript levels in whole body extracts and WISH to assess changes in tissue gene expression patterns.

## 2. Materials and Methods

### 2.1 Materials and reagents

Tetrabromobisphenol A (TBBPA; CAS 79-94-7; purity 97%) and 3,3',5-Triiodo-L-thyronine (T3; CAS 6893-02-3; purity  $\geq 95\%$ ) were purchased from Sigma-Aldrich (Gillingham, UK). 2,2',4,4'-tetra-bromodiphenyl ether (BDE-47; purity 99.8%) was provided by Ulrika Winnberg, Jorke Kamstra and Kees Swart on behalf of Dr. Juliette Legler from VU University Amsterdam, The Netherlands. Stock solutions of chemicals were prepared by dissolving them in dimethylsulfoxide (DMSO).

### 2.2 Zebrafish maintenance

Adult zebrafish [*casper* (*mitfa*; *roy*) mutant strain] were obtained from breeding stocks at the University of Exeter. The *casper* mutants lack melanocytes and iridophores, thus facilitating the visualisation of gene expression via WISH. Fish were maintained at  $28 \pm 1$  °C in a 12:12 h light: dark cycle in a closed flow-through system. Embryos were collected approximately 1 hour post fertilisation (hpf) from breeding colonies, washed twice with embryo culture water with the addition of methylene blue ( $10^{-5}$  %) to prevent fungal growth [38]. We found no effects of methylene blue on thyroid signalling in fish reported in the literature. Eggs were incubated in culture water without methylene blue. Embryo culture water was aerated artificial freshwater made according to the ISO-7346/3 guidelines (ISO water

diluted 1:5, pH 6.5-7.5, air saturation 95-100%) [39]. Embryos were examined under a stereomicroscope and only those fertilised were selected for subsequent experimental work. All fish were maintained under approved protocols, according to the UK Home Office regulations for the use of animals in scientific procedures.

### **2.3 Acute toxicity of TBBPA and BDE-47**

TBBPA exposures were carried out at 0, 0.18, 0.46, 0.92, 1.38 and 2.7  $\mu\text{M}$  (in DMSO, not exceeding 0.01% of the culture medium). BDE-47 exposures were carried out at 0, 1, 5, 10, 50 and 100  $\mu\text{M}$  (in DMSO, not exceeding 0.1% of the culture medium). Controls were incubated in DMSO at 0.01% (TBBPA) and 0.1% (BDE-47). BDE-47 has a low water solubility hence the requirement for a higher DMSO concentration. Twenty fertilised embryos were randomly allocated into glass tanks containing 50 ml of each treatment concentration and half of the exposure solutions were replaced every 24 h with freshly prepared solutions. Exposures were conducted for 96 h starting from approximately 1-2 hpf. The number of dead embryo-larvae and phenotypic deformities (compared to normal development [40]) were recorded every 24 h before removing any dead embryo-larvae. Experiments were carried out in triplicate and repeated three times.

### **2.4 Effect of TBBPA and BDE-47 on gene transcripts in the HPT axis**

TBBPA exposures were carried out at concentrations of 0, 0.04, 0.18 and 0.46  $\mu\text{M}$  (in DMSO, not exceeding 0.01% of the culture medium) and BDE-47 exposures were carried out at concentrations of 0, 0.1, 1 and 10  $\mu\text{M}$  (in DMSO, not exceeding 0.1% of the culture medium). Again, a higher DMSO concentration was used in the BDE-47 exposure due to its low water solubility. These concentrations were chosen

based on range-finding tests to determine concentrations that were sub-lethal and they are lower than, or similar to, the concentrations used in previous studies reported in the literature [41, 42]. Control groups were incubated in DMSO at 0.01% (TBBPA) and 0.1% (BDE-47). Fifty fertilised embryos were randomly allocated into glass tanks containing 50 ml of each treatment concentration and half of the exposure solutions were replaced every 24 h with freshly prepared solutions. TBBPA exposures were conducted for 48, 96 and 120 h from fertilisation. BDE-47 exposures were conducted for 96 h from fertilisation only, as this compound was obtainable in amounts sufficient for analyses at one life developmental stage only.

At the desired developmental stage for the TBBPA and BDE-47 exposures, 40 individuals from each treatment were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, washed and dechorionated in PBS and stored at -20 °C in 100% methanol for WISH experiments. The remaining 10 embryo-larvae from each treatment group were pooled, frozen in liquid nitrogen and stored at -80 °C for qRT-PCR analyses. Experiments were carried out in triplicate and repeated three times. An overview of the experimental design is provided in Figure 1.

## **2.5 Transcript profiling by quantitative real-time PCR (qRT-PCR)**

qRT-PCR was used to quantify the transcript profiles of several target genes in the HPT axis of zebrafish (whole body samples) including: thyroid receptors (*thraa* and *thrb*), thyroid-stimulating hormone (*tshb*), deiodinases type I, II and III (*dio1*, *dio2*, *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). Ribosomal protein I8 (*rpl8*) was used as a control gene for normalisation purposes and its stable

expression has been validated (Fig. S1). qRT-PCR assays for each target gene were optimised as previously described [43] and detailed information for each assay is provided in the supplementary information (Table S1). Results are expressed as mean fold changes  $\pm$  standard error of the mean.

## 2.6 Whole mount *in situ* hybridisation (WISH)

WISH was used to examine tissue-specific changes in gene expression for several genes of interest in the HPT axis including: thyroid receptors (*thraa* and *thrb*) and deiodinases (*dio1*, *dio2* and *dio3b*), with modifications to the protocol of Thisse and Thisse (2008) [44]. For details on the methodologies for gene probe synthesis (including vector information) and WISH are provided in the supplementary material (Supplemental Material and Methods, Tables S2 & S3).

## 2.7 Statistical analyses

All statistical analyses were conducted in R (R Studio, 1.1.423) [45]. LC<sub>x</sub> and EC<sub>x</sub> values and their 95% confidence intervals (CI), for TBBPA and BDE-47 were calculated using generalised linear models (GLM) with binomial error structures and probit links according to Finney, 1971 [46]. Concentrations were log transformed ( $\log_{10}$ ) to linearise the data.

To examine the effect of TBBPA and BDE-47 exposures on gene transcripts in the HPT axis, qRT-PCR data was analysed using general linear mixed models (GLMM) with Gaussian error structures. GLMMs were performed with the *lme4* package within R [47]. P values were obtained using maximum likelihood tests of the full model (with BFR treatment incorporated as a fixed effect) against a reduced model

(without BFR treatment incorporated). BFR concentrations were incorporated as a fixed effect into the model. As a random effect, random intercepts for each experiment (since each experiment was repeated 3 times) were incorporated into the model. When an overall significant effect of the BFR concentrations was identified, pairwise comparisons to determine which groups differed were conducted using a multiple comparison analysis of means (Tukey contrasts) with the *multcomp* package within R [48]. Prior to analysis, gene expression data was scrutinised by Chauvenet's criterion to detect outliers for each gene and these were subsequently removed [49]. In addition, data were tested for equal variance and for normality using the Shapiro–Wilk test. Non-normal data were subjected to variance-stabilising log transformations. All statistical models were checked for homoscedasticity and normality of residuals. For all statistical analyses, differences were considered significant at  $p < 0.05$ . All graphed data were plotted using the *ggplot2* R package [50].

### 3. Results

#### 3.1. Acute toxicity of TBBPA and BDE-47

Exposure to TBBPA for 96 h led to a significant increase in mortalities and deformities of zebrafish embryo-larvae ( $p < 0.001$ ; Fig 2; Table S4). The 96h-LC<sub>50</sub> with 95% confidence intervals for TBBPA was 0.9  $\mu\text{M}$  (0.8 – 1  $\mu\text{M}$ : Fig. 2A). The 96h-EC<sub>50</sub> with 95% confidence intervals for TBBPA, based on deformities, was 0.7  $\mu\text{M}$  (0.6 – 0.9  $\mu\text{M}$ : Fig. 2B). At the higher TBBPA concentrations ( $> 0.92 \mu\text{M}$ ), mortality was preceded by retardation of development (smaller animals) and morphological deformities (oedemas in the pericardial region and tail, small heads, swollen yolk sac

extension; Fig. 2C). Deformities were observed in  $86 \pm 11\%$  of surviving individuals exposed to  $0.92 \mu\text{M}$  TBBPA, including oedema, short body, curved spine, swollen yolk sac, small eyes and craniofacial deformities (Fig. 2D).

Exposure to BDE-47 (1 -  $100 \mu\text{M}$ ) had no significant effect on the mortality of zebrafish embryo-larvae compared with controls (Fig. 3A). Exposure to BDE-47 for a 96-h period led to a significant increase in the proportion of zebrafish deformities ( $p < 0.05$ ). The 96h-EC<sub>50</sub> with 95% confidence intervals for BDE-47, based on deformities, was  $38.2 \mu\text{M}$  ( $10.4 - 475.9 \mu\text{M}$ ; Fig. 3B). The mean number of deformities were  $42 \pm 11\%$ ,  $51 \pm 10\%$  and  $68 \pm 12.0\%$  in groups exposed to BDE-47 at 10, 50 and  $100 \mu\text{M}$ , respectively. At these higher BDE-47 concentrations, deformities included oedema, short tail and head deformities in 24 hpf embryos (Fig. 3C). At 96 hpf, curved spines, yolk sac deformities and craniofacial deformities were also observed (Fig. 3D). See Table 1 for the full list of LC<sub>x</sub> and EC<sub>x</sub> values for both TBBPA and BDE-47.

### **3.2. Effect of TBBPA on gene transcripts in HPT axis**

#### **3.2.1. TBBPA: Whole body transcript levels (qRT-PCR)**

The effect of TBBPA (pools of  $n=10$  larvae) on the transcription of genes in the HPT axis were quantified at 48, 96 and 120 hpf via qRT-PCR and results are shown in Figure 4 (A-J). TBBPA had no effect on *thraa*, *thrb*, *dio2*, *crhb* and *tshb* mRNA levels in whole body extracts at any of the developmental stages examined relative to controls. There was no effect of TBBPA on the expression levels of *dio1* at 48 and 120 hpf in embryo-larvae samples. After 96 h, however, exposure to  $0.18 \mu\text{M}$  TBBPA resulted in a significantly higher (1.5-fold) level of *dio1* mRNA in compared with

controls ( $p < 0.01$ ). After 48- and 120-h exposures, TBBPA had no significant effect on the levels of *dio3b* at any of the concentrations tested. After 96 h, however, TBBPA exposure resulted in significantly reduced mRNA levels of *dio3b* in a concentration-dependent manner (1.7, 2.8- and 3.2-fold lower in 0.04, 0.18 and 0.46  $\mu\text{M}$  treatment groups) compared to the control, respectively ( $p < 0.05$ ). Exposure to 0.46  $\mu\text{M}$  TBBPA resulted in significantly higher levels of *ttr* after a 48-h exposure, with mRNA levels 2-fold higher compared with the control ( $p < 0.05$ ), but with no effects thereafter (96- and 120-h exposures). *Pax8* transcript levels were higher (1.5-fold) after a 48-h exposure to all TBBPA concentrations tested compared with the control ( $p < 0.01$ ), but this was not the case after 96- and 120-h exposures. *Ugt1ab* mRNA levels increased, in a concentration-dependent manner, following 48- and 96-h exposures to TBBPA ( $p < 0.05$ ). After 48 h, *ugt1ab* mRNA levels were 1.7- and 5.1-fold higher in the 0.18 and 0.46  $\mu\text{M}$  TBBPA treatment groups, respectively, compared to controls and 2-fold higher in the 0.18 and 0.46  $\mu\text{M}$  treatment groups compared with controls after 96 h. No significant effect of TBBPA exposure on *ugt1ab* transcription was detected after 120 h.

### 3.2.2. TBBPA: Tissue specific transcript expression (WISH)

Following a 48-h exposure to TBBPA, *thraa* expression appeared to be higher in the brain for all three concentrations and in the branchial arches for the 0.18 and 0.46  $\mu\text{M}$  exposures, compared with controls (Fig. 5A-D). In 96 hpf larvae, exposure to 0.18 and 0.46  $\mu\text{M}$  TBBPA resulted in higher (and concentration related) expression of *thrb* mRNA in the liver, brain, jaw cartilage and swim bladder. There was also an enhanced expression in the otic vesicles in animals treated with 0.46  $\mu\text{M}$  TBBPA compared with the controls (Fig. 5E-H). *Thrb* expression in the pronephric ducts

appeared to be reduced in TBBPA treated individuals (Fig. 5E-H) with 56% of control larvae exhibiting *thrb* expression in the pronephric ducts compared with only 15% of larvae exposed to 0.46  $\mu$ M TBBPA. TBBPA had no apparent effect of the expression pattern of *thraa* and *thrb* in 120 and 48 hpf embryos-larvae, respectively, compared with controls (Fig. S2A-H).

For all TBBPA exposure concentrations, WISH analysis illustrated that *dio1* expression was enhanced in the brain of TBBPA exposed animals compared with controls at 48 hpf (Fig. 6A-D). At 96 hpf, *dio1* expression was enhanced in the liver and intestinal bulb in TBBPA exposed larvae (Fig. 6E-H). After a 48-h exposure to TBBPA, a greater proportion of embryos displayed a detectable *dio2* expression in the pituitary gland; 52% in control versus 74% of embryos treated with 0.46  $\mu$ M TBBPA (Fig. 6I-L). After 96 h, TBBPA exposure (0.04, 0.18 and 0.46  $\mu$ M) resulted in enhanced *dio2* signalling in the liver, brain and intestinal bulb compared with control larvae (Fig. 6M-P). At 96 hpf, enhanced *dio2* signalling was also observed in the pituitary gland of TBBPA treated larvae; 17% in control versus 34% of larvae treated with 0.46  $\mu$ M TBBPA (Fig. S3A-D).

Via WISH, no effect of TBBPA was seen on the expression pattern of *dio3b* in 48 hpf embryos (Fig. S3E-H) but in 96 hpf larvae expression of *dio3b* was reduced in the liver (0.46  $\mu$ M; Fig. 7A-D).

### **3.3 Effect of BDE-47 on gene transcripts in HPT axis**

#### **3.3.1 BDE-47: Whole body transcript levels (qRT-PCR)**

The effect of BDE-47 exposure on the transcription of genes in the HPT axis of whole body 96 hpf larval samples is shown in Figure 8 (A-J). BDE-47 had no significant effect on the expression of *thraa*, *crhb* and *tshb*. Compared with controls, exposure to BDE-47 significantly induced whole body transcript levels of *thrb* (1.9-fold at 10  $\mu$ M;  $p < 0.001$ ), *dio1* (1.6-fold at 1  $\mu$ M;  $p < 0.001$ ), *dio2* (1.4-fold at 1  $\mu$ M;  $p < 0.01$ ), *dio3b* (1.5-fold at 10  $\mu$ M;  $p < 0.01$ ), *pax8* (2.2- and 2.3-fold at 1 and 10  $\mu$ M, respectively;  $p < 0.01$ ) and *ugt1ab* (2.1-fold at 1  $\mu$ M;  $p < 0.01$ ). Transcript levels of *ttr* were significantly reduced in BDE-47 exposed larvae compared with controls (1.4-fold at 10  $\mu$ M;  $p < 0.05$ ).

### 3.3.2. BDE-47: Tissue specific transcript expression (WISH)

Via WISH, enhanced *thraa* expression was observed in the brain, liver, pronephric ducts and branchial arches of larvae exposed to BDE-47 (1 and 10  $\mu$ M) (Fig. 9A-D). *Thrb* expression on the other hand was seen to be suppressed in the brain, otic vesicle, intestinal bulb and liver, while expression in the pronephric ducts was enhanced in larvae exposed to 1 and 10  $\mu$ M BDE-47 (Fig. 9E-H).

*Dio1* expression was enhanced in the pronephric ducts and liver of BDE-47 exposed larvae compared to controls (1 and 10  $\mu$ M; Fig. 10A-D). A greater proportion of BDE-47-exposed larvae showed *dio3b* expression in the pronephric ducts compared to controls as determined by WISH; 28% and 7% of larvae exposed to 1 and 10  $\mu$ M respectively versus none in controls (Fig. 10E-H).

## 4. Discussion

This study adopted zebrafish embryo-larvae as a biological model to evaluate TBBPA and BDE-47 induced developmental toxicity and gene expression changes in the HPT axis.

### 4.1. Acute toxicity of TBBPA and BDE-47

We show that exposure to high concentrations of TBBPA for 96-h was lethal to zebrafish embryo-larvae, with an LC<sub>50</sub> value of 0.9 µM that is in general accordance with similar studies conducted previously [51, 52]. Neither of those studies however calculated an LC<sub>50</sub> value to allow for direct toxicity comparisons with our findings.

Lee *et al.*, (1993) reported an 96h-LC<sub>50</sub> value of 3 mg TBBPA L<sup>-1</sup> (5.5 µM) for adult zebrafish suggesting a greater sensitivity to TBBPA in early life stages [53].

Consistent with previous studies, here we also observed various morphological deformities for exposure to TBBPA, including pericardial and tail oedemas, small heads and swollen yolk sac extensions, which may be a result of apoptosis and/or oxidative damage [51, 52]. Based on deformities in surviving zebrafish, we estimated an 96h-EC<sub>50</sub> of 0.7 µM for TBBPA, slightly lower than the 96h-EC<sub>50</sub> (based on hatching) of 1.1 mg L<sup>-1</sup> (2.0 µM) reported previously [41].

BDE-47 up to 100 µM was not lethal to zebrafish larvae in the 96-h exposures, consistent with the low acute toxicity of PBDEs observed in rodents [54]. There appear to be considerable differences in sensitivities between fish species with regards to lethal concentrations of BDE-47. In previous studies, zebrafish embryo-larvae showed significant mortalities after exposure to BDE-47 up to 5 mg L<sup>-1</sup> (10.3 µM) albeit that the exposure period was longer (168 h) and the mortalities occurred

after 100 h [55]. In contrast, a relatively low 96h-LC<sub>50</sub> of 14.13 µg L<sup>-1</sup> (0.03 µM) has been reported for turbot (*Psetta maxima*) larvae [56], which may indicate considerable differences in sensitivities between fish species. We found, however, deformities such as short tails, oedemas, curved spines and craniofacial deformities for exposure to BDE-47, as also reported previously [55, 57] with a comparable 96h-EC<sub>50</sub> for deformities (38.2 µM) with that reported for hatching (20.3 mg L<sup>-1</sup> [41.8 µM]) [41].

#### 4.2. Effects of TBBPA and BDE-47 on TR expression

Here we found that *thraa* mRNA levels in whole body extracts were unaffected by exposures to TBBPA and BDE-47 during early zebrafish development, comparable to previous studies with zebrafish larvae exposed to BDE-47 and DE-71 for 96 hpf and 14 dpf, respectively [41, 58]. In contrast, TRα transcript levels were enhanced in zebrafish larvae exposed to TBBPA for 96 hpf [41] as well as larvae exposed to TBBPA and BDE-47 later in development (from the point of hatching) [41]. Exposure to TBBPA did not alter *thrb* RNA levels at any of the developmental stages examined in the present study, consistent with previous finding with similar stage zebrafish embryo-larvae [41]. We did, however, observe that exposure to BDE-47 resulted in elevated *thrb* mRNA levels in 96 hpf larvae. While previous studies found no effect of BDE-47 or DE-71 on TRβ transcript levels in whole body zebrafish larvae samples exposed from fertilisation, others observed reduced mRNA levels in larvae exposed to BDE-47 from the point of hatching [41, 58]. Taken together, these results suggest that TR regulation by BFRs may vary depending on the specific developmental window in which exposure occurs [59] and may differ depending on the receptor and exposure compound in question.

Through WISH analyses, we observed tissue-specific alterations to both *thraa* and *thrb* expression in zebrafish embryo-larvae following exposure to TBBPA and BDE-47. Following a TBBPA exposure, *thraa* expression was higher in the brain and branchial arches of exposed 48 hpf embryos compared to controls, while *thrb* expression was enhanced in the liver, brain, jaw cartilage, otic vesicle and swim bladder of 96 hpf larvae. As far as we are aware, no study to date has examined tissue specific changes to TR gene expression in fish (either in adults via PCR or in larvae via WISH) exposed to TBBPA. Similar to that seen for the TBBPA exposures, *thraa* expression was found to be enhanced in the brain and branchial arches, as well as the liver and pronephric ducts, of 96 hpf larvae exposed to BDE-47. These results are consistent with the findings of increased *thra* transcript levels in the brain of adult fathead minnows following dietary exposure to BDE-47 and BDE-209 for 21 and 28 days, respectively [30, 60]. In contrast, *thrb* expression was suppressed in the liver, brain, intestinal bulb and otic vesicle of 96 hpf larvae exposed to BDE-47 in our study, though expression was induced in the pronephric ducts. Interestingly, a dietary exposure to BDE-47 reduced *thrb* mRNA levels in the brains of adult fathead minnows, but no changes were observed in the liver [61], whilst in a similar study with BDE-209 the opposite occurred [60]. TRs mediate the thyroid system's genomic control over several key developmental processes, including growth [62], auditory [63], eye [64], brain [65] and skeletal [66] development [67]. Whether the BFR-induced changes in TR transcripts observed here translate to altered TH-dependant developmental processes remains unclear. As far as we are aware, only one study has examined the effect of overexpression of TR $\alpha$ 1 in zebrafish and report disrupted hindbrain patterning in embryos [68]. Based on the observed transcriptional changes

of TRs, our results suggest that TH signalling in the brain, as well as the liver, intestine and jaw cartilage are sensitive to TBBPA and BDE-47 during early zebrafish development.

The mechanisms by which TBBPA and BDE-47 altered both TR transcripts in this study are not fully understood. Changes may be a result of BFR-induced alterations in circulating and/or local TH levels or a result of a direct interaction with TRs or their co-repressors/activators (since the genes encoding TRs themselves contain TREs). Since several *in vitro* studies have demonstrated that BFRs have limited agonistic effects on TR-mediated gene transcription [69-71], it seems plausible that the increases in *thraa* and *thrb* transcription following TBBPA and BDE-47 exposures were a result of increased local T3 levels.

#### **4.3. Effects of TBBPA and BDE-47 on deiodinase expression**

Exposure to both TBBPA and BDE-47 resulted in higher levels of *dio1* transcripts in whole body samples of 96 hpf zebrafish larvae, in agreement with results observed in zebrafish larvae and adults exposed to DE-71, BDE-209 and BDE-47 [58, 72, 73]. In contrast, other studies found that *dio1* transcript levels were not altered in zebrafish juveniles exposed to BDE-47 or zebrafish larvae exposed to TBBPA, respectively, though the exposure period in the BDE-47 study began at a later stage in development [42, 74]. Interestingly, here TBBPA had no effect on *dio2* levels in whole zebrafish embryo-larvae at any stage examined, while exposure to BDE-47 led to higher *dio2* levels in 96 hpf larvae. These results are in line with previous studies, where no change in *dio2* levels were detected in zebrafish larvae exposed to TBBPA for 5 d from fertilisation [42], while increased *dio2* levels in larvae were

detected following exposure to BDE-47 and DE-71 for 14 d from fertilisation [58, 73]. Whilst D1 is capable of both activating and deactivating THs, in fish it has been suggested that the former is its primary function, in contrast to its regulation and function in mammals [75, 76]. D2 is considered, however, to be the major TH-activating enzyme. Therefore, the increased levels of *dio1* and *dio2* transcripts observed here provide strong evidence of an induced systemic hypothyroid condition in the TBBPA and BDE-47 exposed zebrafish larvae [76-78]. This likelihood is supported by the reduced *dio3b* mRNA levels observed in whole larval (96 hpf) samples exposed to TBBPA (0.18 and 0.46  $\mu$ M groups), since D3 is the major inactivating pathway of THs and thyroidal status often parallels hepatic D3 activity, increasing during hyperthyroidism and decreasing during hypothyroidism [33, 79].

WISH analysis provided us with a more detailed insight into the tissue specific changes and the mechanisms underlying thyroid disruption to *dio1*, *dio2* and *dio3b* expression in zebrafish embryo-larvae following BFR exposure. We observed higher *dio1* expression in the brain of embryos (48 hpf) and in the liver and intestinal bulb of larvae (96 hpf) exposed to TBBPA, while higher levels of *dio2* expression were observed in the pituitary of embryos (48 hpf) and in the brain, pituitary and intestinal bulb of larvae (96 hpf). As far as we aware, no previous studies have examined tissue specific changes in the expression of genes encoding the deiodinase enzymes following exposure to TBBPA. The increased expression of *dio2* in the pituitary of TBBPA exposed embryo-larvae is not fully understood. In fish under normal physiological regulations, reduced circulating TH levels lead to increased stimulation and secretion of TSH from the pituitary through the negative feedback system of the HPT axis [35]. An increased conversion of T4 into T3 in the pituitary

(catalysed by increased D2 levels) may deplete local T4 levels, inducing the negative feedback loop and increasing the production of THs by the thyroid follicles. In support of the qPCR results, the higher expression of *dio1* and *dio2*, along with the reduced expression of *dio3b*, in the liver of TBBPA exposed larvae provides strong evidence of systemic hypothyroidism [76]. In addition, the elevated *dio1* and *dio2* mRNA levels in the brain and intestinal bulb are likely associated with reduced local TH levels and may be compensatory responses in order to maintain local TH homeostasis during the development of neurological and gastrointestinal tissues. In BDE-47 exposed larvae, enhanced *dio1* expression was observed in the pronephric ducts and liver. This is in contrast to a previous study with zebrafish, which found no significant changes in *dio1* transcript levels in the liver of adults exposed to BDE-47 for 180 days from fertilisation, though *dio2* levels were significantly increased [73]. Interestingly, we found that BDE-47 had no effect on *dio3b* expression in the liver but higher levels were observed in the pronephric ducts. Increased D3 activity as a result of increased *dio3b* expression in the pronephric ducts may reflect increased local T3 production (*dio1* expression also increased in pronephric ducts). Our results suggest that altering the expression of deiodinase genes could be a potential mechanism by which TBBPA and BDE-47 disrupt/correct TH homeostasis, though observed effects vary depending on the life stage examined, exposure period and compound tested.

#### **4.4. Effects of TBBPA and BDE-47 on the expression of further genes in the HPT Axis**

Elevated *ugt1ab* mRNA levels were detected in whole zebrafish samples exposed to TBBPA (at 48 hpf and 96 hpf) and BDE-47 (at 96 hpf), similar to the effect observed with zebrafish exposed to DE-71 [58]. UDPGTs play an important role in TH

metabolism in the liver and increased *ugt1ab* expression could lead to the increased elimination of THs, a plausible explanation for the altered TH signalling observed throughout the HPT axis here [80]. Previous findings in mammals and fish have reported reduced circulating levels of T4, combined with increased UDPGT activity and/or transcription, following exposure to BFRs [58, 81].

Elevated levels of *pax8* transcripts were observed here in zebrafish embryo-larvae following exposures to TBBPA (at 48 hpf) and BDE-47 (at 96 hpf). Similar findings have been reported in zebrafish embryos/larvae exposed to various other pollutants [58, 82], although to our knowledge no studies have yet examined the effect of TBBPA on *pax8* transcription. Pax8 proteins are essential for the late differentiation of the thyroid follicular cells during development [83], therefore our results suggest the promotion of thyroid primordium growth and possibly a compensation mechanism for reduced TH levels, induced by the BRFs tested.

TTR is proposed to be the major TH carrier protein in fish [84] and plays a key role in maintaining extra-thyroidal stores of TH and regulating its supply to various target tissues [85]. Here we found that TBBPA significantly increased *ttr* transcripts levels in 48 hpf embryos while BDE-47 significantly decreased *ttr* transcript levels in 96 hpf larvae. Our results are consistent with previous studies which have found increased and decreased *ttr* mRNA levels in zebrafish larvae following exposure to TBBPA [41] and BDE-47, respectively [41, 73]. Altered levels of TTR proteins in exposed individuals, may decrease or increase the amount of TTR available to bind free TH. In the case of BDE-47, the reduced *ttr* levels may lead to excess unbound TH, which would be more susceptible to hepatic catabolism, resulting in a greater clearance

rate and a decrease in circulating TH concentrations. The impacts of the increased *ttr* levels in TBBPA exposed larvae may be more complex, since TBBPA has been shown to potently compete with T3 for TTR binding sites. Increased TTR protein levels could potentially lead to increased substrate available for TBBPA binding, making the displaced and unbound THs in the plasma more susceptible to hepatic metabolism and suppress circulating levels of T4/T3 [86]. The altered *ttr* mRNA expression by TBBPA and BDE-47, suggest that while induction of UDPGTs may be partly responsible for disrupting systemic TH levels, other mechanisms may also be involved.

Corticotropin-releasing hormone (CRH) and thyroid-stimulating hormone (TSH) regulate THs synthesis and secretion in fish through a negative feedback mechanism within the HPT axis, triggered via alteration in circulating T4 levels [87]. Interestingly, in the present study, both TBBPA and BDE-47 had no effect on *tshb* and *crhb* transcript levels in zebrafish embryo-larvae. This is in agreement with previous studies that found no effect on *tshb* mRNA levels in 5 dpf zebrafish larvae exposed to TBBPA and juvenile zebrafish exposed to BDE-47 for 40 days [42, 74]. However, the expression of *crhb* and *tshb* has been shown to be elevated in adult fathead minnows exposed to BDE-47 [61] and both adult and larval zebrafish exposed to various BFRs, including TBBPA and BDE-47 [41, 73]. Increased *crhb* and *tshb* expression in these studies were accompanied by reduced T4 levels. Interestingly, recent studies have reported lower *crhb* and/or *tshb* transcript levels in zebrafish adults, embryos and larvae exposed to TBBPA or BDE-47 [41, 88], associated with increased T4 and T3 levels [88]. From these studies, it is evident that BFRs have varying effects on the expression of *crh* and *tsh* genes in fish,

highlighting the importance of examining the effect of TDC on multiple levels of the HPT axis.

#### **4.5. Combining qRT-PCR and WISH to enhance our understanding of tissue specific targets of BFRs.**

Given the wide range of tissues influenced by THs during early development and the complex nature involved in regulating the TH system, it is important to assess the effect of TDCs on the spatial expression of targeted thyroid-related genes. To date, assessing transcriptional changes in fish embryo-larvae following exposure to BFRs have largely relied on qRT-PCR, while tissue-specific effects have been limited to adult studies where isolating organs/tissues of interest was relatively straightforward. In the present study, we simultaneously examined the effects of TBBPA and BDE-47 on the transcription of several thyroid-related genes using WISH and qRT-PCR. While qRT-PCR provides quantitative data on gene expression and is sensitive at detecting even small changes in mRNA levels, when carried out on pooled homogenates of whole embryo-larvae, transcriptional changes in small and/or localised tissues may not be detected if diluted by non-responsive organ/tissues of the whole animal. Only recently has WISH been used in ecotoxicology studies as a means of examining gene expression changes on a spatial scale and identifying tissue-specific markers of exposure during early stages of development [37, 89]. Interestingly here, the transcription of several genes in the HPT axis appeared unaffected by BFR exposures when examined using qRT-PCR but tissue-specific effects were observed using WISH. These included *thraa* (at 48 hpf; TBBPA and at 96 hpf; BDE-47) *dio1* (at 48 hpf; TBBPA), *dio2* (at 48 and 96 hpf; TBBPA) and *dio3b* (at 96 hpf; BDE-47). Here we demonstrate the value of WISH as an approach, in

conjunction with qRT-PCR, to examine the mechanistic basis of the effects of TDCs on early-life stage zebrafish.

#### 4.6. Developmental stage-specific effects

We also examined the effect of TBBPA exposure on thyroid gene transcripts at three early developmental stages and found that effects were developmental stage specific. TBBPA-induced alterations to *dio1* and *dio3b* transcription were observed in 96 hpf larvae while *ttr* and *pax8* mRNAs were altered in 48 hpf embryos only. Finally, *ugt1ab* expression was altered at 48 and 96 hpf. TBBPA had no effect on any of the genes examined following 120-h exposures. This study is one of only a few that examined the effect of BFRs on thyroid-related gene transcription at different life stages. Similar to our findings, several genes in the HPT axis were found to be transiently altered in the liver and/or brain of adult male fathead minnows exposed to BDE-209 [60]. The TH system is regulated by sophisticated compensatory mechanisms both centrally (negative feedback loop in HPT axis) and peripherally (by deiodinases), which, if adequate, can normalise both serum and peripheral TH concentrations [90]. These compensatory mechanisms may explain why no transcriptional effects were detected here in 120 hpf larvae. It is also important to note that increased endogenous T3 levels are associated with the embryo-larval transition of teleost fish, and this natural increase may intensify the effects of BFRs on the thyroid system at this life stage. We observed that zebrafish at 96 hpf, a period coinciding with increased TH levels, were particularly sensitive to TBBPA exposure.

## Summary

In summary, the present study demonstrates that the BFRs, TBBPA and BDE-47, can disrupt the thyroid axis of zebrafish embryo-larvae at multiple levels by altering mRNA transcript levels of genes encoding deiodinases, thyroid synthesis proteins, transport proteins and glucuronidation enzymes. We have also shown that both compounds induced tissue-specific transcriptional changes for several genes in the HPT axis, with TH signalling in the brain, liver, pronephric duct and craniofacial tissues appearing particularly sensitive to TBBPA and BDE-47 exposures (Fig. 11). Furthermore, TH disruption by TBBPA appeared more pronounced in larvae at 96 hpf, compared to 48 and 120 hpf. These results demonstrate the effects of BFRs on the thyroid system should not be generalised across tissues or developmental stages in fish species. Future work should focus on understanding the consequences of chronic low level exposure to BFRs and examine whether the BFR-induced changes in TR transcript levels in the brain and skeleton as observed here translate into health consequences for wild fish populations and humans.

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## Figures

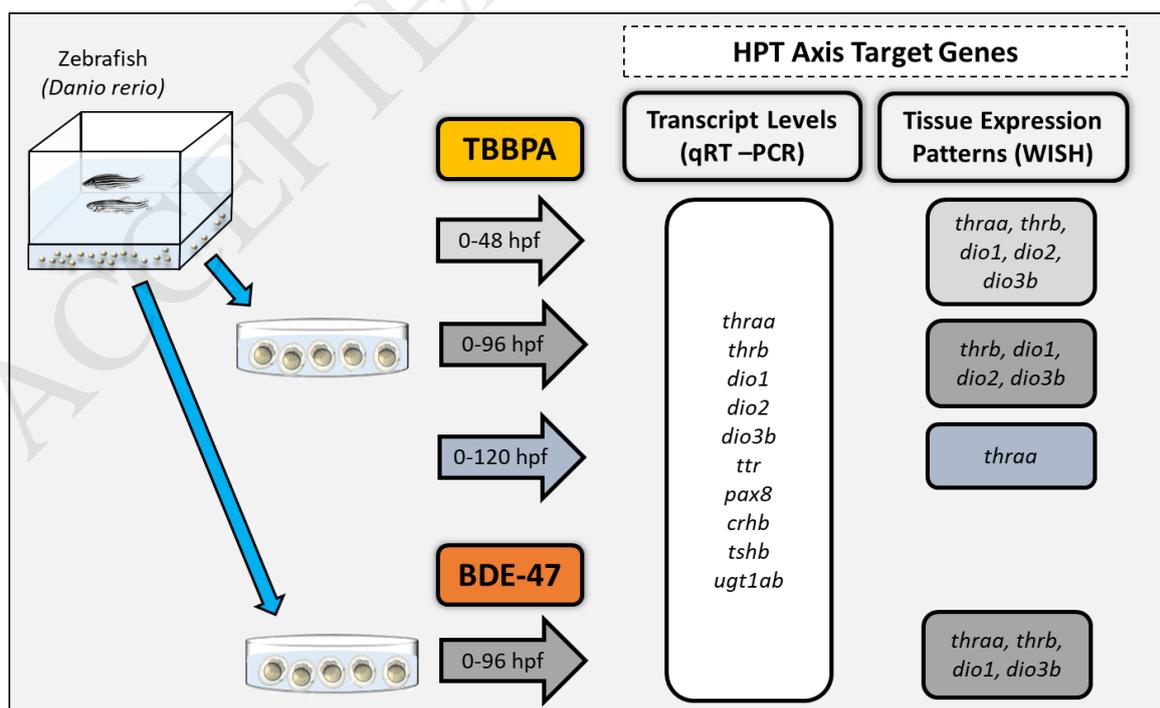


Figure 1. Overview of the experimental design of this study, showing the HPT axis target genes analysed with WISH and qRT-PCR assays following exposures of zebrafish embryo-larvae to TBBPA or BDE-47 exposures.

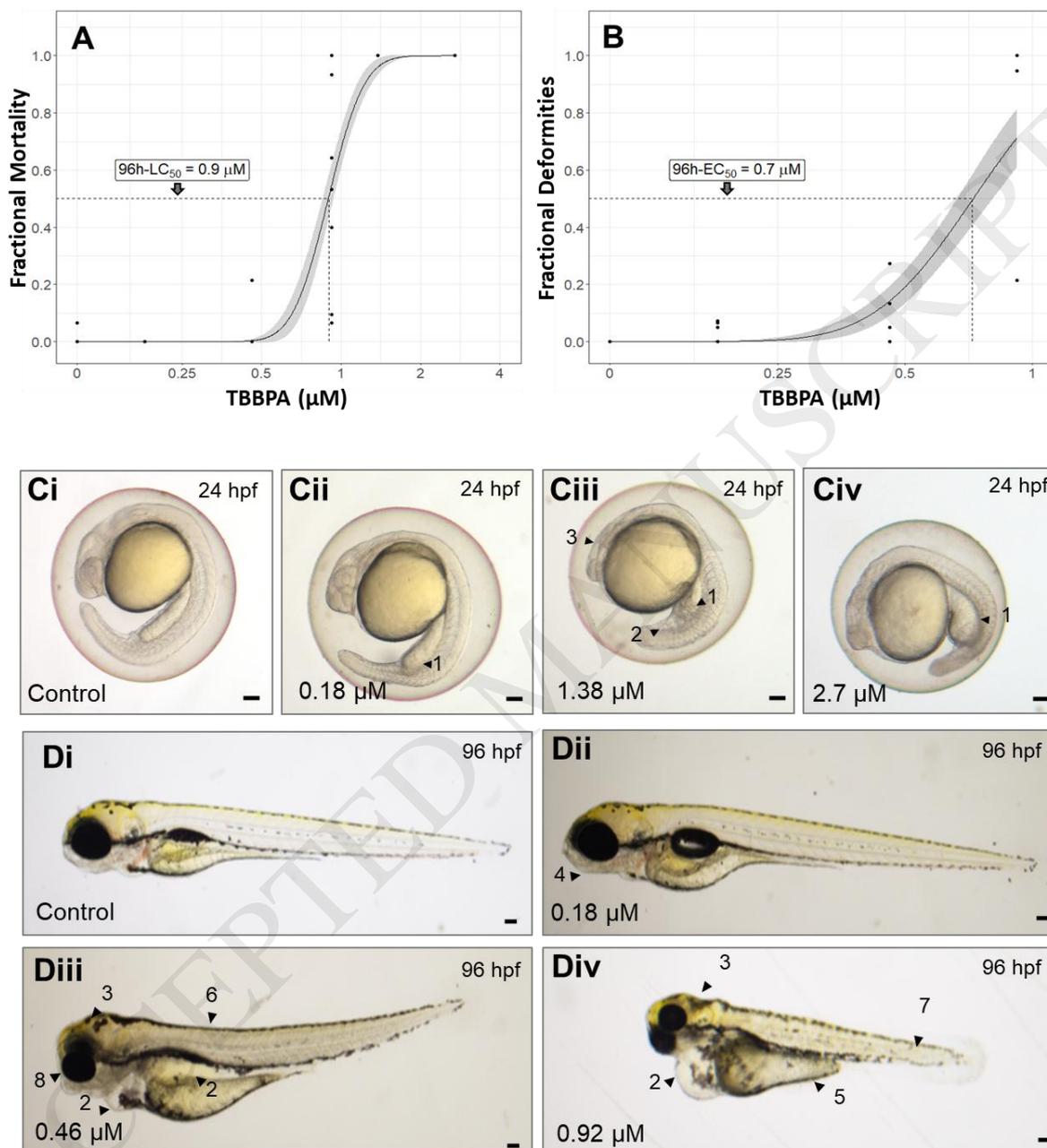


Figure 2. Effects of TBBPA on developing zebrafish embryo-larvae. Dose response curves showing (A) mortality and (B) deformities in zebrafish embryo-larvae after 96-h exposures to TBBPA (at nominal concentrations between 0.18 and 2.7 µM). Each point on the graphs represents the relative mortality and relative deformities in an individual replicate glass dish containing 15-20 individuals and the lines represent

the best-fit model for the data, calculated using generalised linear models in R (model output summarised in Table S5A). Representative images of zebrafish embryos at 24 hpf exposed to (Ci) Control (Cii) 0.18  $\mu\text{M}$ , (Ciii) 1.38  $\mu\text{M}$  and (Civ) 2.7  $\mu\text{M}$  and at 96 hpf exposed to (Di) Control (Dii) 0.18  $\mu\text{M}$ , (Diii) 0.46  $\mu\text{M}$  and (Div) 0.92  $\mu\text{M}$  showing- 1. swollen yolk sac extension, 2. oedema, 3. head deformity, 4. lower jaw deformity, 5. yolk sac deformity, 6. bent spine, 7. short tail and 8. smaller than normal eyes. Scale bar=100  $\mu\text{m}$ .

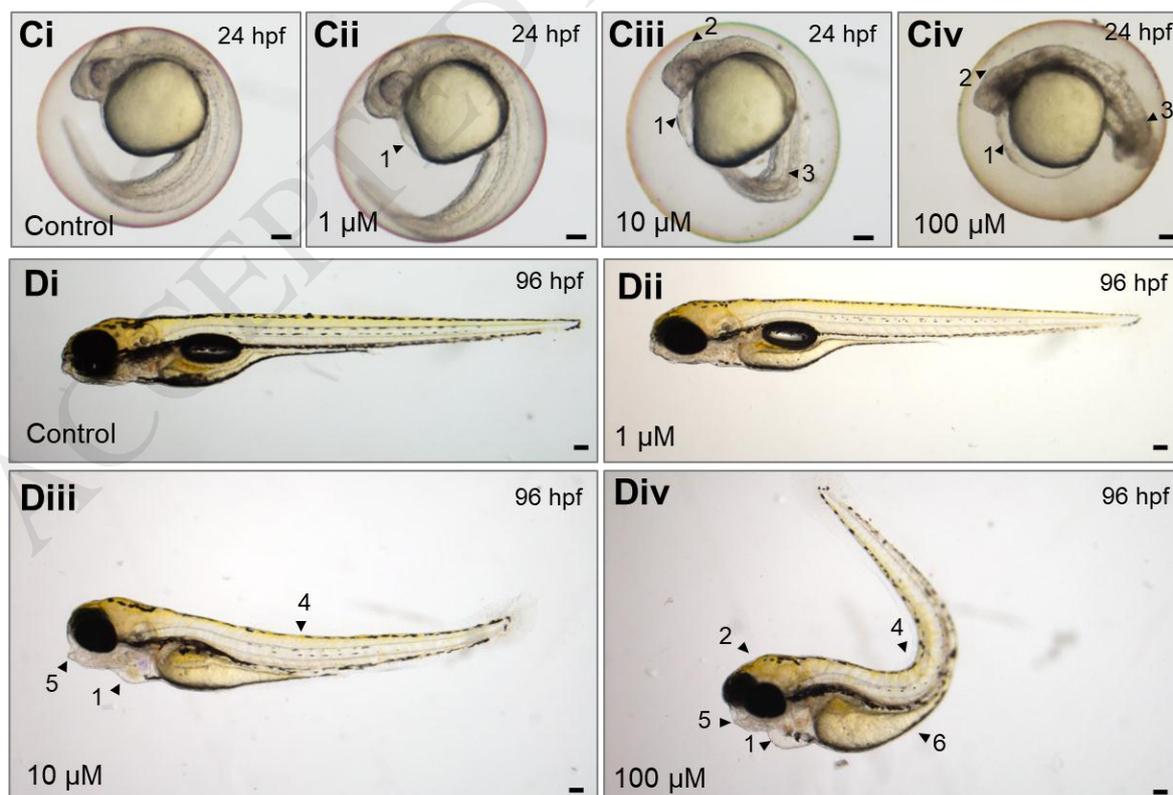
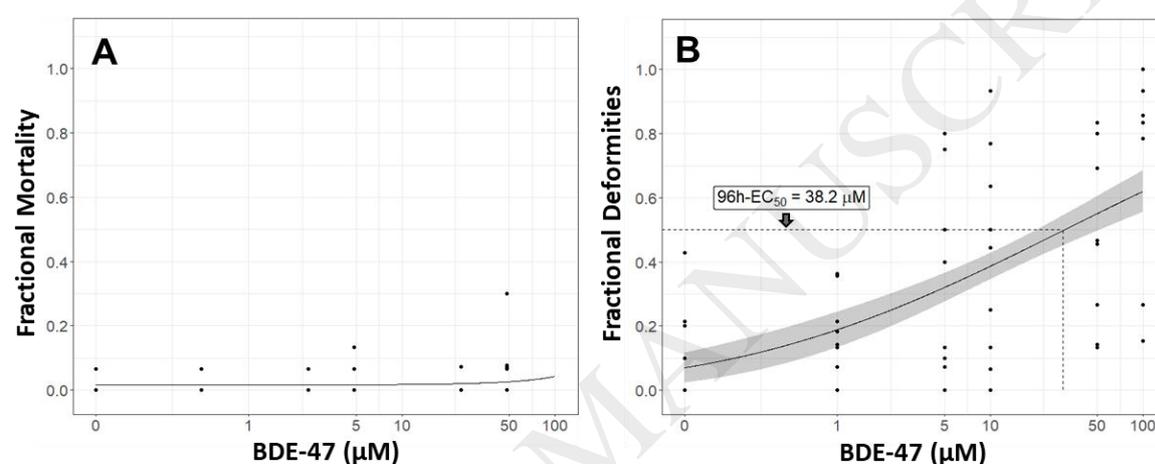


Figure 3. Effects of BDE-47 on developing zebrafish embryo-larvae. Dose response curves showing (A) mortality and (B) deformities in zebrafish embryo-larvae after 96-h exposures to BDE-47 (at nominal concentrations between 1 and 100  $\mu\text{M}$ ). Each point on the graphs represents the relative mortality and relative deformities in an individual replicate glass dish containing 15-20 individuals and the lines represent the best-fit model for the data, calculated using generalised linear models in R (model output summarised in Table S5B). Representative images of zebrafish embryo-larvae at (C) 24 hpf and (D) 96 hpf following exposure to (i) Control (ii) 1  $\mu\text{M}$ , (iii) 10  $\mu\text{M}$  and (iv) 100  $\mu\text{M}$  showing— 1. oedema, 2. head deformity, 3. short tail, 4. bent spine, 5. lower jaw deformity and 6. yolk sac deformity. Scale bar=100  $\mu\text{m}$ .

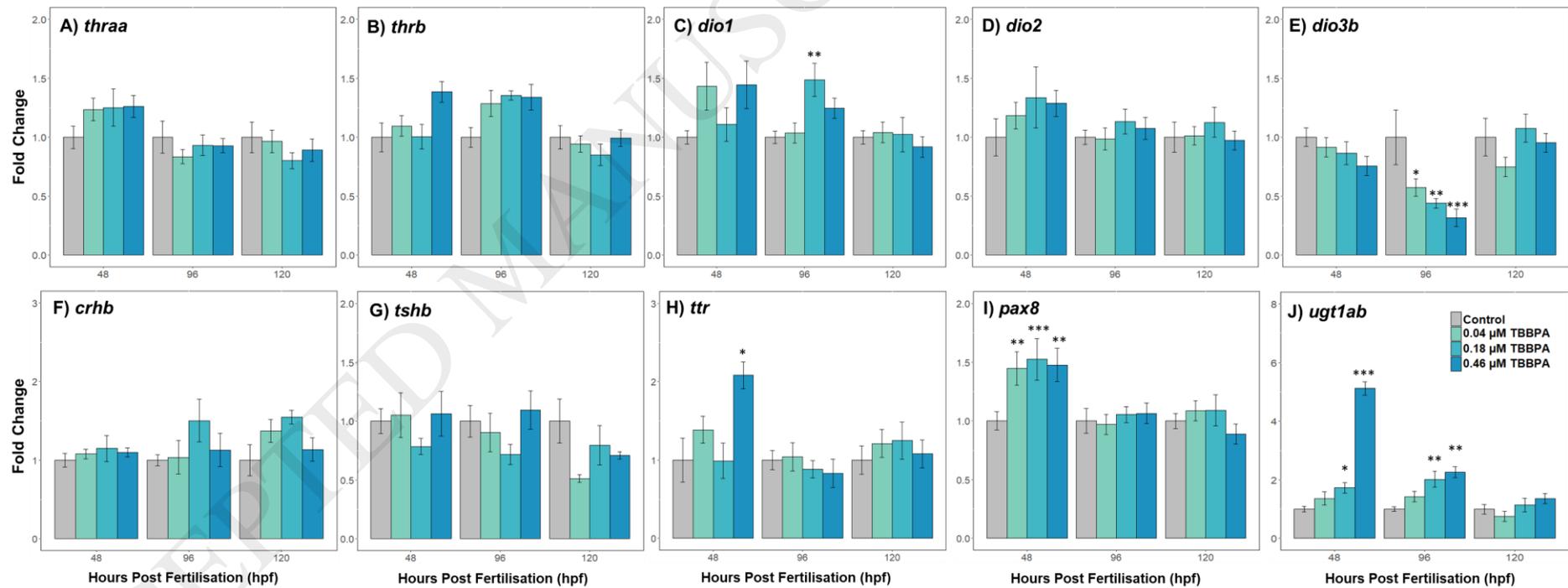


Figure 4. Transcript profile of genes in the HPT axis of zebrafish embryo-larvae exposed to TBBPA. Changes in (A) thyroid receptor alpha (*thraa*), (B) thyroid receptor beta (*thrb*), (C) deiodinase type I (*dio1*), (D) deiodinase type II (*dio2*), (E) deiodinase type III (*dio3b*), (F) corticotropin-releasing hormone (*crhb*), (G) thyroid-stimulating hormone (*tshb*), (H) transthyretin (*ttr*), (I) paired box 8 (*pax8*) and (J) uridine diphosphate-glucuronosyltransferase (*ugt1ab*) mRNA levels in whole zebrafish following exposure to TBBPA (0, 0.04, 0.18 and 0.46 μM) for up to 120 hpf. Transcript profiles were determined using qRT-PCR and the relationship between transcript expression and TBBPA concentration was assessed using linear mixed models (model output summarised in Table S6).

Plotted data are presented as mean fold changes (normalised against the expression of the control gene *rpl8*)  $\pm$  SEM compared to the corresponding control group. Outliers, identified as described in the text, were excluded from the analysis, resulting in a replication of n= 6-9 samples per treatment group. Significance codes: \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

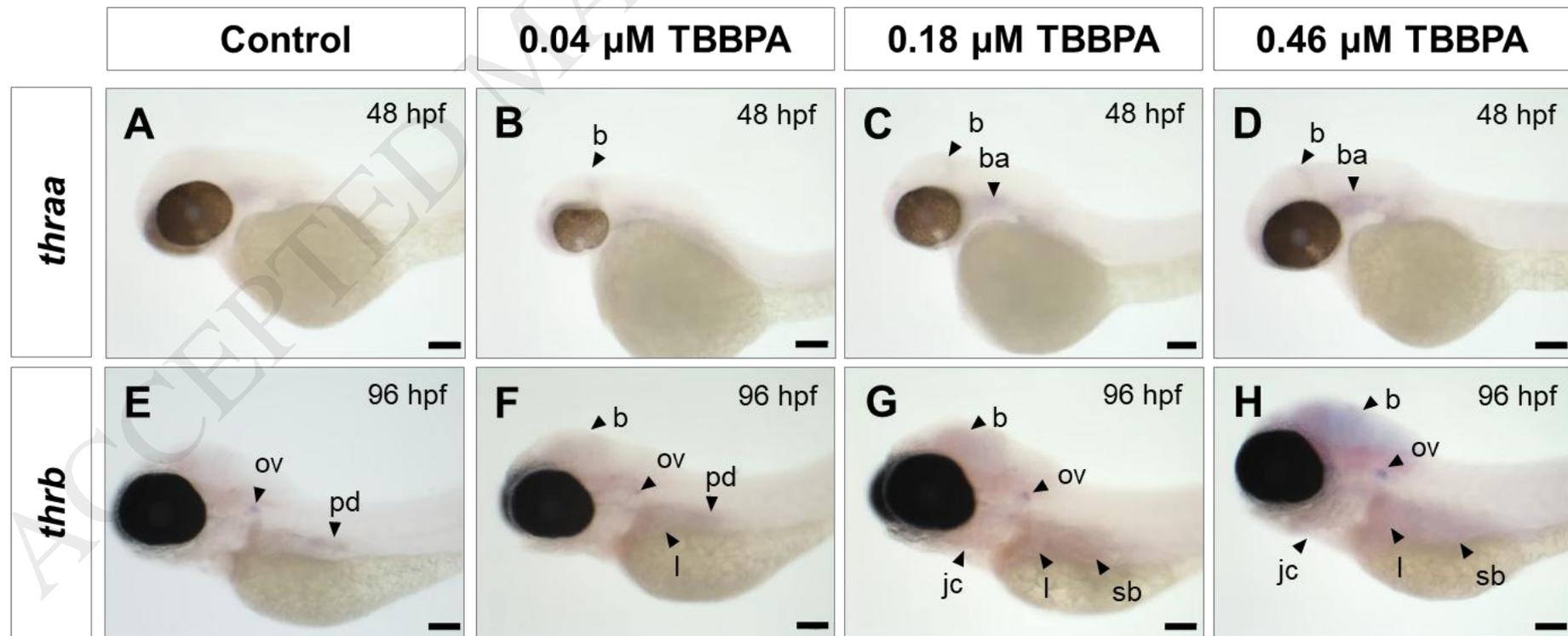


Figure 5. WISH images of TR mRNA expression patterns in zebrafish embryo larvae following exposure to TBBPA. Representative images of (A-D) *thraa* mRNA expression patterns in zebrafish embryos at 48 hpf and (E-H) *thrb* mRNA expression patterns in zebrafish larvae at 96 hpf treated with TBBPA. Lateral (A-D) and dorsal (E-H) views of whole embryo-larvae are shown with

anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, ba=branchial arches, ov=otic vesicle, l=liver, sb=swim bladder, pd=pronephric duct, jc=jaw cartilage. Scale bar=100  $\mu$ m. A summary of the variability in mRNA expression in the different zebrafish tissues is provided in Table S7.

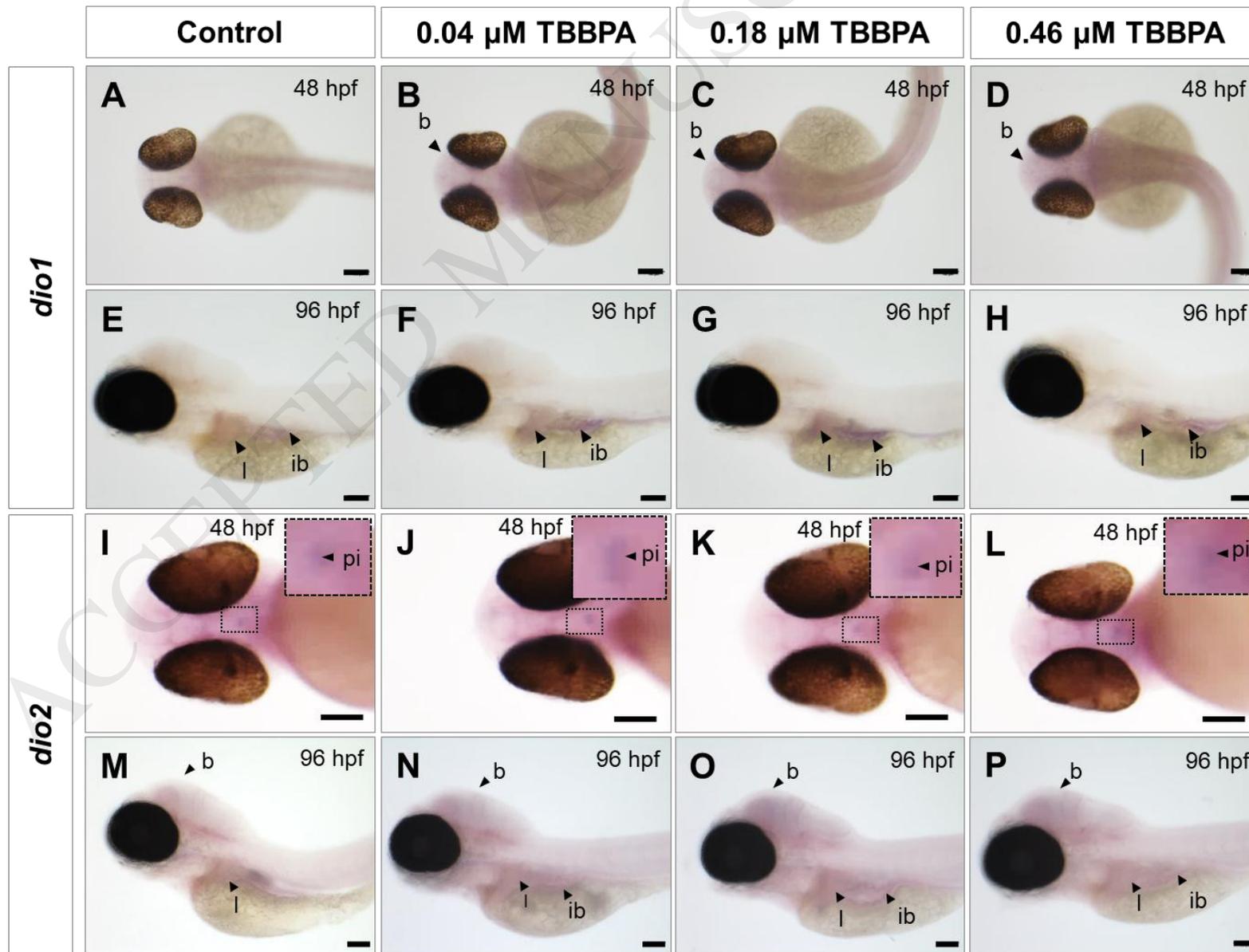


Figure 6. WISH images of deiodinase type I and type II mRNA expression patterns in zebrafish embryo larvae following exposure to TBBPA. Representative images of mRNA expression patterns in zebrafish embryo-larvae treated with TBBPA showing (A-D) *dio1* at 48 hpf, (E-H) *dio1* at 96 hpf, (I-L) *dio2* at 48 hpf and (M-P) *dio2* at 96 hpf. Dorsal (A-D), lateral (E-H, M-P) and ventral (I-L) views of whole embryo-larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver, ib=intestinal bulb, b=brain, pi=pituitary. Scale bar=100  $\mu$ m. A summary of the variability in mRNA expression in the different zebrafish tissues is provided in Table S8.

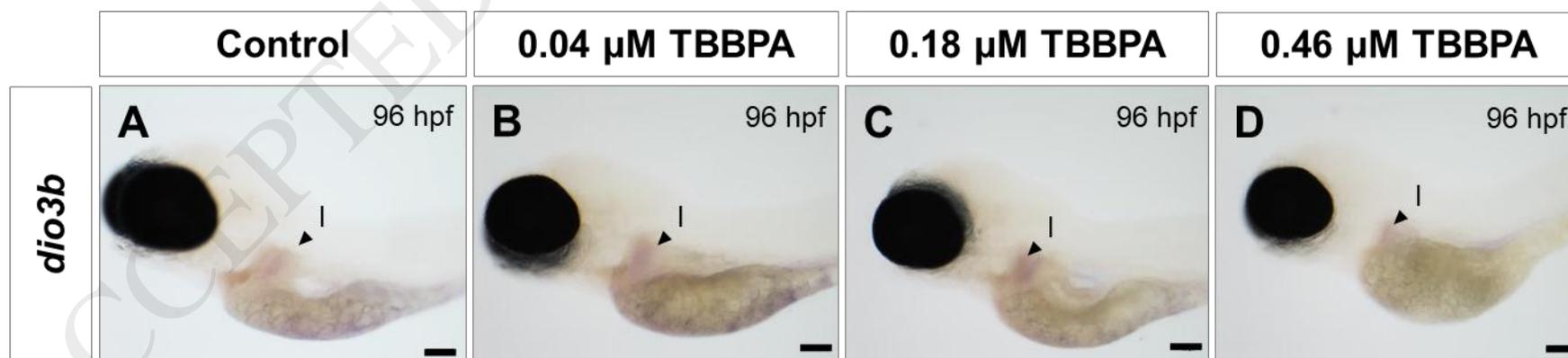


Figure 7. WISH images of deiodinase type III mRNA expression patterns in zebrafish larvae following exposure to TBBPA. (A-D) Representative images of *dio3b* mRNA expression patterns in 96 hpf zebrafish larvae treated with TBBPA. Lateral views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver. Scale bar=100  $\mu$ m. A summary of the variability in mRNA expression in the different zebrafish tissues is provided in Table S8.

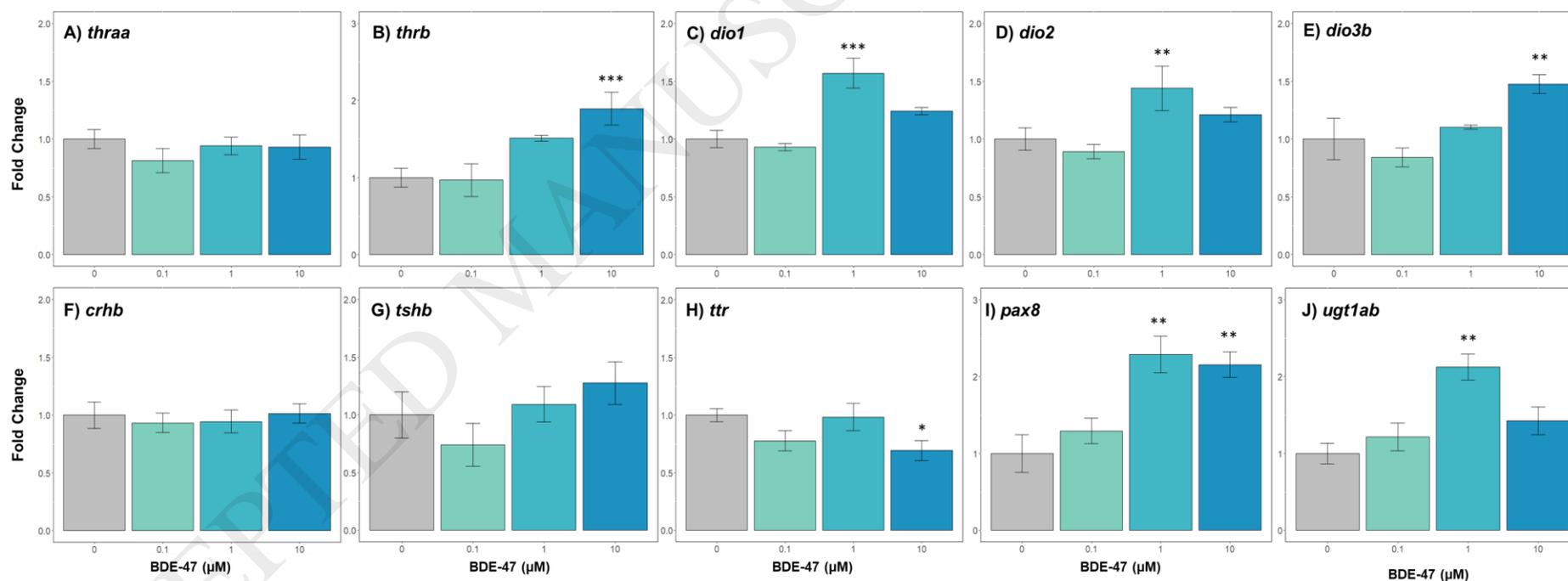


Figure 8. Transcript profile of genes in the HPT axis of zebrafish larvae exposed to BDE-47. Changes in (A) thyroid receptor alpha (*thraa*), (B) thyroid receptor beta (*thrb*), (C) deiodinase type I (*dio1*), (D) deiodinase type II (*dio2*), (E) deiodinase type III (*dio3b*), (F) corticotropin-releasing hormone (*crhb*), (G) thyroid-stimulating hormone (*tshb*), (H) transthyretin (*ttr*), (I) paired box 8 (*pax8*) and (J) uridine diphosphate-glucuronosyltransferase (*ugt1ab*) mRNA levels in whole zebrafish following exposure to BDE-47 (0, 0.1, 1 and 10 µM) for 96 hpf. Transcript profiles were determined using qRT-PCR and the relationship between transcript expression and TBBPA concentration was assessed using linear mixed models (model output summarised in Table S9). Plotted data are presented as mean fold changes (normalised against the expression of the control gene *rp18*)  $\pm$  SEM compared to the corresponding control

group. Outliers, identified as described in the text, were excluded from the analysis, resulting in a replication of  $n = 5-6$  samples per treatment group. Significance codes: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

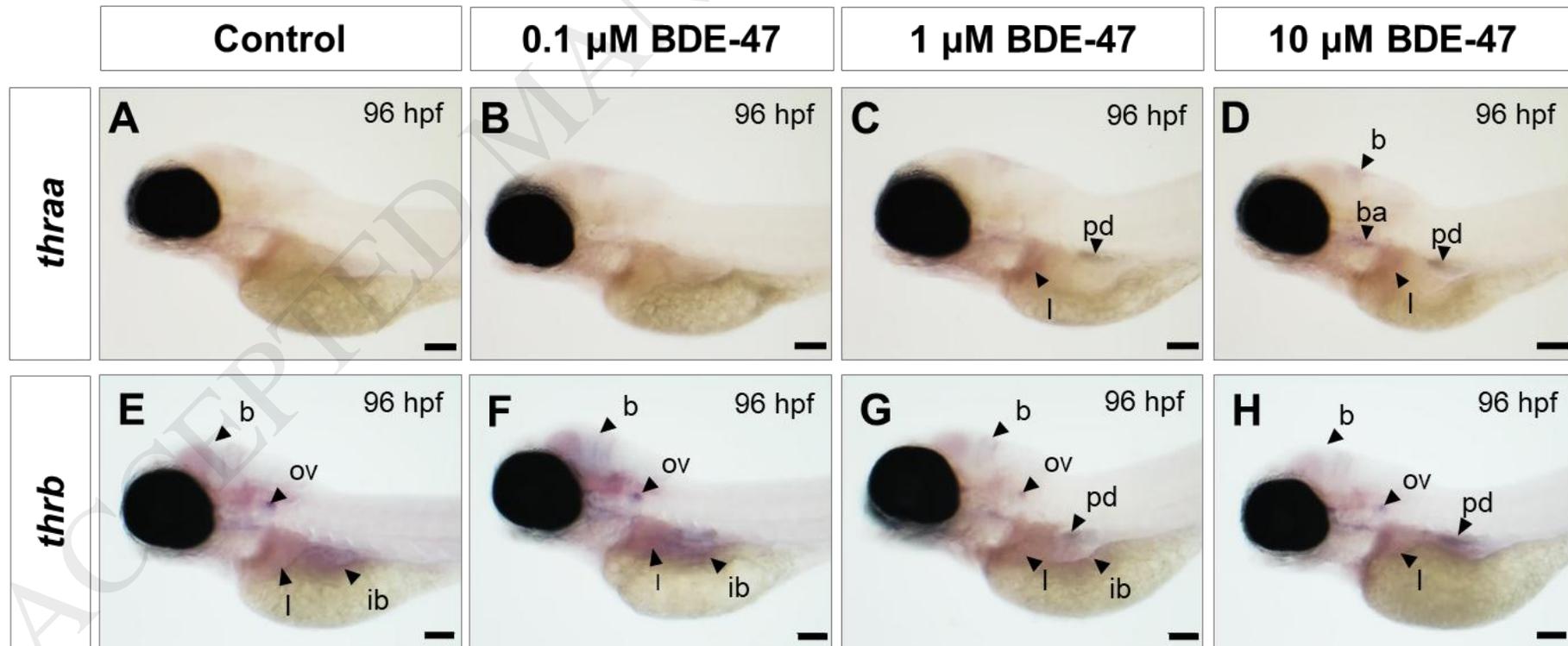


Figure 9. WISH images of TR mRNA expression patterns in zebrafish larvae following exposure to BDE-47. Representative images of (A-D) *thraa* and (E-H) *thrb* mRNA expression patterns in 96 hpf zebrafish larvae treated with BDE-47 (0.1, 1 and 10  $\mu\text{M}$ ). Lateral views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads.

b=brain, ba=branchial arches, pd=pronephric ducts, l=liver, ov=otic vesicle, ib=intestinal bulb. Scale bar=100  $\mu$ m. A summary of the variability in mRNA expression in the different zebrafish tissues is provided in Table S10.

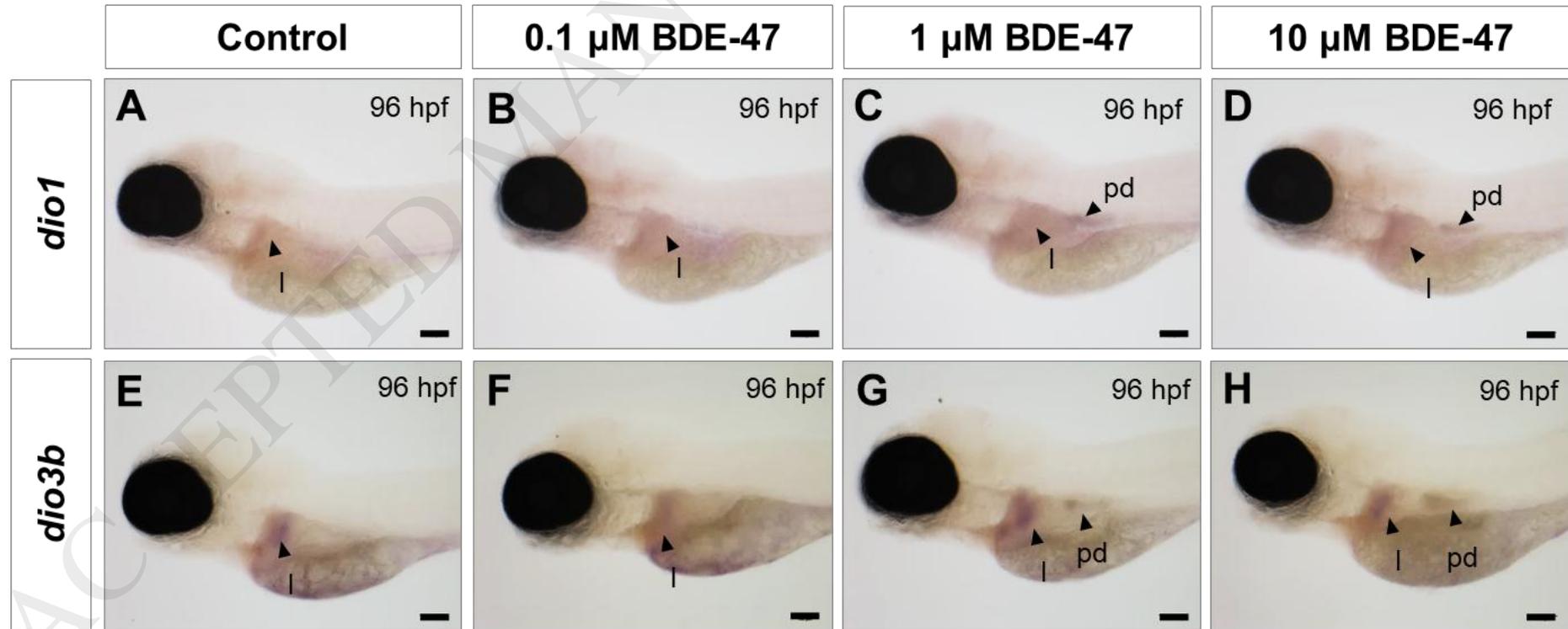


Figure 10. WISH images of deiodinase type I and type III mRNA expression patterns in zebrafish larvae following exposure to BDE-47. Representative images of (A-D) *dio1* and (E-H) *dio3b* mRNA expression patterns in zebrafish larvae at 96 hpf treated with BDE-47 (0.1, 1 and 10  $\mu$ M). Lateral views of whole larvae are shown with anterior to the left and focal areas of expression are indicated

by black arrowheads. b=brain, pd=pronephric ducts and l=liver. Scale bar=100  $\mu$ m. A summary of the variability in mRNA expression in the different zebrafish tissues in provided in Table S11.

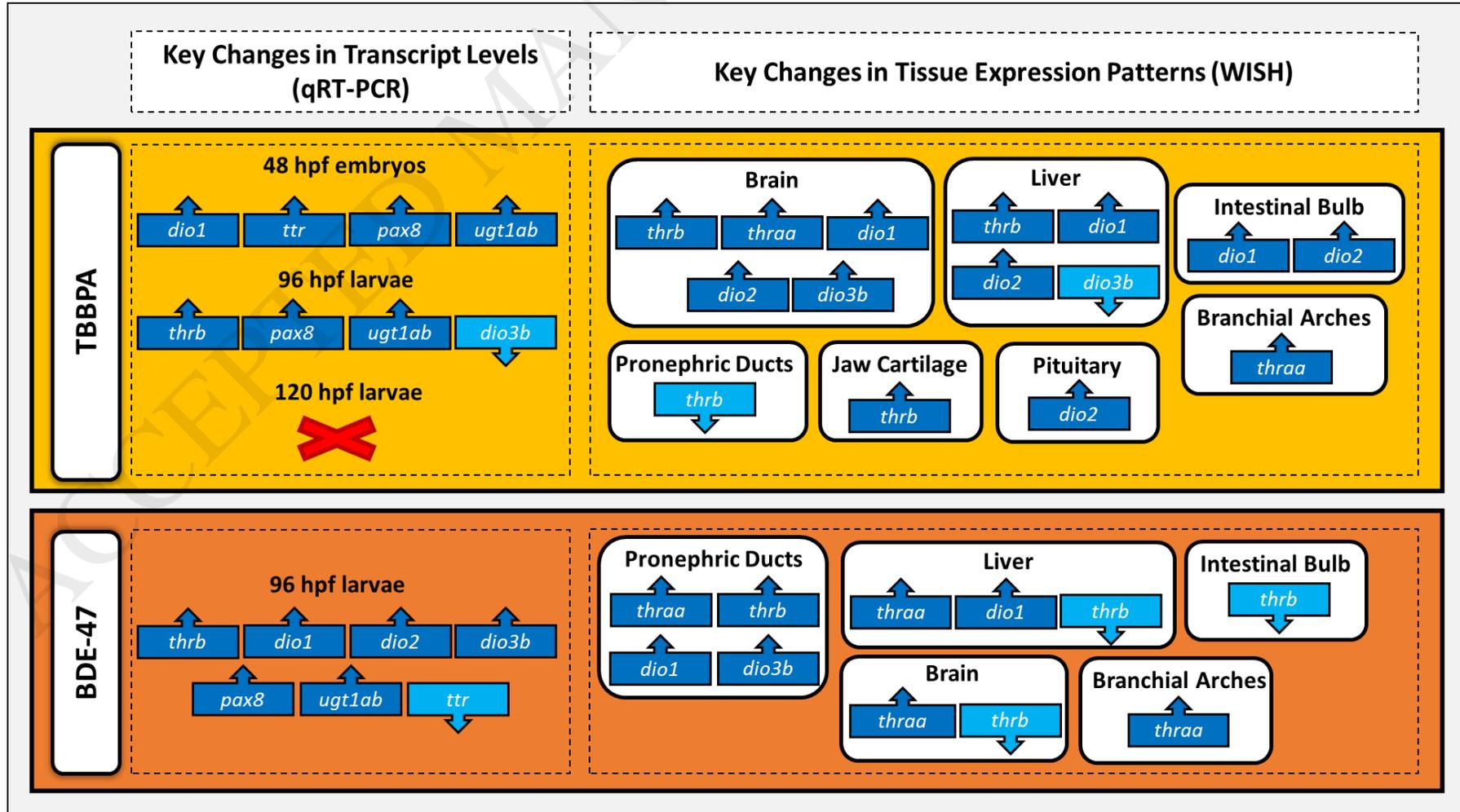


Figure 11. Overview of the key changes in the transcript level (measured via qRT-PCR) and tissue expression pattern (measured via WISH) of HPT axis target genes in zebrafish embryo-larvae exposed to TBBPA and BDE-47. Dark blue arrow blocks indicate transcript levels were up-regulated and light blue arrow blocks indicate transcript levels were down-regulated.

**Tables**

Table 1: LC<sub>x</sub> and EC<sub>x</sub> values (μM) with corresponding 95% confidence intervals for zebrafish embryo-larvae following 96-h exposures to TBBPA and BDE-47.

	<b>TBBPA</b>	<b>BDE-47</b>
<b>LC<sub>10</sub></b>	0.6 (0.5-0.7)	NC
<b>LC<sub>50</sub></b>	0.9 (0.8-1)	NC
<b>LC<sub>90</sub></b>	1.3 (1.2-1.5)	NC
<b>EC<sub>10</sub></b>	0.4 (0.3-0.5)	0.03 (0-0.23)
<b>EC<sub>50</sub></b>	0.7 (0.6-0.9)	38.2 (10.4-475.9)
<b>EC<sub>90</sub></b>	1.25 (0.9-2.2)	NC