# Thyroid disrupting effects of low-dose dibenzothiophene and cadmium in single or concurrent exposure: new evidence from a translational zebrafish model

Guzzolino E[1], Milella MS[1], Forini F[1], Borsò M[2], Rutigliano G[2], Gorini F[1], Zucchi R[2], Saba A[2], Bianchi F[1], Iervasi G[1] and Pitto L[1].

[1]Institute of Clinical Physiology, National Research Council, Pisa, Italy.

[2]Department of Pathology, University of Pisa, Pisa.

*Corresponding author:*

Letizia Pitto Institute of Clinica Physiology, CNR Research Area, via Moruzzi 1 56124 Pisa Italy. Phone number: +39 0503153090. E-mail: l.pitto@ifc.cnr.it

## Abstract

Thyroid hormones (THs) are major regulators of biological processes essential for correct development and energy homeostasis. Although thyroid disruptors can deeply affect human health, the impact of exogenous chemicals and in particular mixture of chemicals on different aspects of thyroid development and metabolism is not yet fully understood. In this study we have used the highly versatile zebrafish model to assess the thyroid axis disrupting effects of cadmium and dibenzothiophene, two environmental endocrine disruptors found to be significantly correlated in epidemiological co-exposure studies. Zebrafish embryos (5dpf) were exposed to low concentrations of Cd (from 0.05 to 2 µM) and DBT (from 0.05 to 1 µM) and to mixtures of them. A multilevel assessment of the pollutant effects has been obtained by combining in vivo morphological analyses allowed by the use of transgenic fluorescent lines with liquid chromatography mass spectrometry determination of TH levels and quantification of the expression levels of key genes involved in the Hypothalamic-Pituitary-Thyroid Axis (HPTA) and TH metabolism. Our results underscore for the first time an important synergistic toxic effect of these pollutants on embryonic development and thyroid morphology highlighting differences in the mechanisms through which they can adversely impact on multiple physiological processes of the HPTA and TH disposal influencing also heart geometry and function.



Keywords:

Dibenzothiophene, Cadmium, zebrafish embryos, Hypothalamic-Pituitary-Thyroid Ax disruption, Thyroid Hormone Receptors, cardiac defects

## 1. Introduction

Thyroid hormones play a pivotal role both during mammal development and in the adulthood by controlling growth, maturation, homeostasis and functions of key biological systems including cardiovascular, nervous and reproductive apparatuses {Li, 2014; Klein, 2007;Jabbar, 2017;Yazbeck, 2012;Gilbert, 2012}. For this reason, thyroid disruptor chemicals are gaining increasing interest as key determinants of adverse effects in fetal maturation and in the post-natal life in humans. However, despite the importance of thyroid system for human health, not all thyroid disruptors have so far been identified and the mechanisms of combined disruption remain largely inexplored. Among chemical disruptors, polycyclic aromatic hydrocarbons (PAHs) are the most prevalent global environmental contaminants {Samanta, 2002; Manzetti, 2013; Sinaei, 2019} with deleterious metabolic, cardiovascular and neurodevelopmental bioactivities {Moorthy, 2015;Incardona, 2005 ;Billiard, 2008}, observed either in humans and animal models {Rundle, 2012 ;Poursafa, 2018; Poursafa, 2017; Incardona, 2011; Perera, 2006; Perera, 2014}. A growing body of evidence indicates that PAHs may also affect thyroid function. Although the exact mechanism of action is not completely understood, PAHs seem to impact several aspects of thyroid gland biological functions including TH synthesis, accumulation and secretion {Izawa, 2007; Wu, 2004; Hua, 2007 }. In particular, PAH might interfere with TH receptor (THR) transcription, hence inhibiting thyroid function {Sun, 2008}.

Cadmium (Cd), one of the most harmful metallic elements, is a well-established endocrine disruptor and a widespread environmental pollutant as well {Tchounwou, 2012 }. {Khan, 2017; Rafati Rahimzadeh, 2017; Kim, 2018 }. Cd accumulates predominantly in liver, kidneys, and muscles, but also in the thyroid gland due to the presence of metallothioneins (MT), cysteine-rich proteins that bind Cd and represent a potent intracellular Cd detoxifier

{Klaassen, 2009;Li, 2015 }. The relationship between Cd exposure and Cd accumulation in thyroid gland is supported by the observation that thyroid Cd concentrations are higher in people living in Cd polluted areas than in those residing in non-contaminated areas {Uetani, 2006 }.

The vast majority of studies have examined the action of a single chemical disruptor at a time but humans are often exposed to mixtures of multiple chemical disruptors. Notably a recent study, which characterized the correlation profile and cluster patterns of seven classes of environmental endocrine disruptors (EEDs), found a significant correlation between Cd and PAHs {Chen, 2019}. Therefore, a better understanding of the impact of PAH and Cd co-exposure on the complete development and function of the thyroid system may be helpful in contrasting the noxious effects of such pollutants.

The use of zebrafish as model organism for environmental health studies has been steadily increasing over the past few decades {Lieschke, 2007; Dooley, 2000}.

The zebrafish model has several advantages including: high degree of homology with the human genome, ex utero embryonic development, short reproductive cycle, and possibility of population studies {Ali, 2011} {Howe, 2013}. Moreover, the transparence of the larvae and the possibility to easily generate transgenic lines expressing tissue-specific fluorescent proteins provide powerful tools to follow *in vivo* and in a non-invasive way the differentiation/development of numerous tissues/organs {Zon, 2005; Huang, 2003; Lawson, 2002}. Importantly in the context of this study, the toxicity of drugs and environmental pollutants as weel as thyroid gland development and functioning of the hypothalamus-pituitary-thyroid-axis (HPTA) have been shown to be well conserved between humans and zebrafish {Reimers, 2006; Brittijn, 2009; De Felice, 2012}, {Blanton, 2007;Fagman, 2010;Porazzi, 2009}.

These advantages have been exploited for toxicity texts of PAHs and Cd (inserire I riferimenti più salient tra quelli citati sotto). In spite of significant advances, several important aspects remain unaddress.

Based on such premises, this study aimed to investigate the impact of an important PAH compound, the 3 rings dibenzothiophene (DBT), and of Cd on different aspects of fetal development, with a focus on the HPTA, thyroid morphogenesis and function and cardiac development, since heart is a well-known target of TH action {Li, 2014; Klein, 2007; Jabbar, 2017} and alterations of sheared congenit factors have been identified at the root of cardiac and thyroid congenital defects in human and zebrafish studies {Opitz, 2015; Marelli, 2017}. For this purpose, zebrafish embryos were exposed to low concentrations of BDT and Cd, either in single or combined treatment. Our results indicate a novel multilevel synergic toxic impact of the pollutant exposure highlighting differences in their toxicity effects.

## 2. Materials ad methods

*2.1 Maintenance of Zebrafish lines and breeding.*

The zebrafish facility has held the authorization n°297/2012-A since 12/21/2012. All animal procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament regarding the protection of animals used for scientific purposes. Wild type AB, *Tg(myl7:EGFP)* and *Tg(tg:mcherry)* (kindly provided by Dr. Sabine Costagliola, University of Bruxelles, Belgium) zebrafish lines were used in this study.

Zebrafish were raised in Tecniplast housing systems (Zebtec, Standalone) in 14 hrs light and 10 hrs dark at 28°C. Zebrafish diet was purchased from SDS, Dietex, France and from Sparos, Portugal). Artemia magnetica was obtained from INVE acquatica, INVE

technologies, Belgium. Tricaine (3-amino benzoic acid ethylester) comes in a powdered form from Sigma (Cat.# A-5040).

Breeding

The day before treatments, adult fishes (about 1 years old) at a ratio of 2 males to 3 females for group mating fish were put in off-system breeding tanks at a density of 5 or less fish per liter of water, separated by sex using a divider. The breeding tank consists of an upper (breeding) and lower (embryo collection) chamber separated by plastic mesh to allow the eggs to fall avoiding access to the breeding adults. Early in the following morning, when the facility light switch on, the water tank was refreshed and divider removed. Three hours later, eggs from successful crosses were manually collected and analyzed under microscope to remove dead or deformed eggs while adult fishes were returned to system tanks. Selected eggs were distributed in plastic plates, 50 eggs/plates in 40 ml of water. Time 0 (0 hpf) was considered 1 hour after divider removal.

*2.2 Chemical solution preparation*

Dibenzothiophene and cadmium were obtained from Sigma Aldrich Corporation (USA). DBT and Cd were dissolved respectively in DMSO and in distilled water to make stock solutions at concentration of 50 mM. These stock solutions were kept at 4°C until they were used. The working solutions were freshly prepared by diluting the stock solutions with DMSO or deionized water before use. All the concentrations used were prepared $10^3$ folds concentrated, so the same volume was added to the plates in all the treatments. The range of concentrations we tested were:

DBT: 0.05, 0.1, 1,10 µM

Cd: 0.05, 0.1, 0.2, 2,10 µM

Cd 0.05 µM+DBT (0.05, 0.1, 1 µM)

Cd 0.2 µM+DBT (0.05, 0.1, 1 µM)

The final DMSO concentration in DBT and in co-exposure treatments was 0.1%, which is below the values considered toxic {Hallare, 2006 #1321;Christou, 2020 #1328}. The lack of significant DMSO effects, even in the presence of cadmium, was demonstrated by the absence of differences in survival and hatching rate in embryos exposed to increasing concentration of Cd or Cd+0.1% DMSO (Table 1). Moreover embryos exposed to increasing concentration of Cd or Cd+0.1% DMSO were also compared to evaluate an eventual contribution of DMSO to the Cd-induced thyroid malformations and skin defects. No differences were observed in any of these comparisons (data not shown).

*2.3 Toxicology tests*

At 4 hpf, solvent or chemical compounds at the different concentrations were added to the plates containing the eggs. During the exposure period, water with the different chemical concentrations were changed every day, to keep the concentration consistent.

After 24 hr dead embryos were removed and recorded for the survival rate calculation. Usually no further dead embryos were observed in the following days. At 48 hpf hatched embryos were recorded. The hatching % indicates the percentage of embryos which have hatched on the 48hpf survived embryos. Malformation analysis and cardiac imaging were performed on 3 days post fertilization (dpf) embryos. Thyroid imaging was performed on 5 dpf embryos. 5 dpf embryos were collected and stored at −80°C for subsequent thyroid hormone determination and gene transcription analysis. For RNA extraction embryos were frozen at -80°C in Quiazol (30-50 embryos /700µl of Quiazol). Three biological replicates for each exposure concentration were prepared. This means that all the breeding procedures were repeated at different times and starting from different combination of male and female fishes.

*2.3 Imaging*

Imaging was performed as described previously {Guzzolino, 2019 }. Briefly bright field optical imaging was performed with a Leica M80 microscope and a Nikon DS-Fi1 camera

using NIS-Elements F 3.0 software. The acquisition of fluorescent samples was performed with a Leica DM IL microscope and with a Nikon YFL microscope both equipped with a CoolSnap CF camera (Photometric). For thyroid imaging, *Tg(tg:mcherry)* embryos were previously anesthetized in 100mg/100ml of tricaine and embedded in 1% low melting agarose to allow a correct positioning of the body embryo. For heart beat analysis 3dpf *Tg(myl7:EGFP)* embryos were embedded in 1% low melting agarose and Videos were acquired and quantified with a Leica MZ10F microscope. For each embryos at least 3 videos of 30 seconds each were recorded from the Selected Regions of Interest fixed on the fluorescent atrium and ventriculum. In each video atrium and ventriculum beats were simultaneously recorded. The heart rate was successively measured analyzing the tracking signal obtained.

*2.4 TH quantification by HPLC-MS-MS method*

Instrumentation and operative conditions

For TH quantification, instrument layout was made up of an Agilent (Santa Clara, CA, USA) 1290 UHPLC system, including a binary pump, a column oven set to 20°C and a thermostated autosampler, coupled to an AB-Sciex (Concord, Ontario, Canada) QTrap 6500+ triple quadrupole mass spectrometer, equipped with an IonDrive Turbo V source operating in positive ion mode. The integrated switching valve was used to discard both head and tail of the HPLC runs. Chromatographic separations were carried out using a 110 Å, 2x50 mm, 3µm particle size, Gemini C18 column (Phenomenex, Torrance, CA), protected by a C18 Security Guard Cartridge. 5µL of each sample were injected into the UHPLC system and the chromatographic separation was carried out by a flow rate of 400 µl min-1 using methanol/acetonitrile (20/80 by volume) added with 0.1% formic acid as solvent A and water containing 0.1% formic acid as solvent B. Mobile phases' gradient conditions were as follow: 95 % solvent B from 0 to 3 min; 35 % solvent B from 3 to 8.5

min; 0 % solvent B from 8.5 to 9 min; 0 % solvent B from 9 to 11 min. The column was re-equilibrated to 95 % solvent B from 11 to 14 min.

System control, data acquisition and analyses were performed using an ABSciex Analyst® version 1.7 software.. Mass spectrometry selected reaction monitoring (SRM) method and all related parameters were set as previously described methods {Saba, 2010; Saba, 2014}.

Sample extraction

Formerly published methods {Saba, 2010; Saba, 2014; Chen, 2018 } have been slightly modified and used for the quantification of T3 and T4 in zebrafish larvae. Briefly, 100 fish larvae were resuspended in 200µL of aqueous buffer containing 200 ng of pronase enzyme. After that, appropriate amounts (0.76 pmol13C6-T3 and 0.64 pmol13C6-T4) of stable isotope labeled internal standards were added, samples were gently vortexed and then incubated 16h at 37°C. After incubation, samples were cooled down (RT), sonicated for 10 min, then 600 µL of ice-cold acetone were added and the mixture was kept at 4°C for 30min to allow proteins precipitation. After centrifugation at 22780 x g for 15 min, the supernatants were transferred to a new 2 mL Eppendorf tube and evaporated at 40 °C under a gentle stream of nitrogen. Dried samples were reconstituted with 500 µL of 0,1 M potassium acetate buffer (pH=4) prior to loading onto Agilent (Santa Clara, CA, USA) Bond-Elut Certify 130 mg SPE cartridges, antecedently conditioned by consecutive wetting with 2 mL of dichloromethane/isopropanol (75/25 by volume), 2 mL of methanol and 2 mL of 0,1 M potassium buffer (PH = 4). Each cartridge was washed with 3.5 mL of water, 2 mL of 0.1 M hydrochloric acid, 7 mL of methanol and 3.5 mL of dichloromethane/isopropanol (75/25 by volume). After complete dryness, samples were eluted with 2 mL of dichloromethane/isopropanol/ammonium hydroxide (70/26.5/3.5 by volume), dried under nitrogen. Dried eluates were derivatized adding 200 µL of 3.0 N hydrochloric acid in n-butanol and incubated for 60 min at 60 °C. This derivatization step allows the formation of

the corresponding butyl esters of thyroid hormones and their internal standards. Afterwards, samples were dried again as mentioned above and, then, reconstituted with 100 µL of acetonitrile/0.1 M hydrochloric acid (50/50 by volume) prior the injection into the HPLC-MS-MS system. Stock solutions of T3 and T4 were prepared at 1µg/mL concentration in methanol. Calibration curves were daily prepared by serial dilution with methanol at a concentration ranging from 0.05 to 25 ng/mL and derivatized with samples.

*2.5 RNA extraction and quantitative Real-time PCR.*

Total RNA was extracted from frozen embryos using the miRNeasy Mini kit (Qiagen). Embryos, which had been frozen in Quiazol immediately after the end of the treatment, were solubilized by repeated pipetting up and down of the suspension. After the solubilization step, the samples were centrifuged to remove the extremely few non-solubilized residues. Thereafter, the extraction was performed according to the manifacturer's instructions. RNA quantity and quality were analyzed using a NanoDrop-1000 spectrophotometer. The purity of each sample was between 1.8 and 2.0 (A260/A280 nm ratio). RNA integrity was evaluated running non-denaturing TBE 1,5% agarose gel. CDNA was retro-transcribed using Quantitec Reverse Transcription kit (Quiagen) following the manifacturer's instructions. Real-time PCR (qRT-PCR) was carried out using SsoAdvanced™ Universal SYBR® Green Supermix - Bio-Rad with Rotor Gene (Quiagen). PCR conditions were: 95°C 30" (1X); 95°C 5", 60°C 20" (40X); 1 cycle of melting. For analysis normalization 3 housekeeping genes were routinely analyzed: EF1α, rpl13a and 18S. A list of oligonucleotides used in this manuscript is reported in table S1.

*2.4 Statistic*

All the data are expressed as the mean ± standard error. The normality and homogeneity of the data were verified using Kolmogorov-Smirnov test and Levene's test, respectively. Two group comparisons were perfomed by Student's t test. For thyroid analyses Chi

square test was performed. Multiple group comparisons were performed by one-way Analysis of Variance (ANOVA) followed by Bonferroni post hoc test. Differences were considered statistically significant at a value of $p < 0.05$. Following a square root transformation of the data, a univariate linear model was run in SPSS to estimate the main effect and the interaction effect of the two pollutants on the gene expression levels of THRα and THRβ.

## 3.Results

*3.1 DBT and Cd exert synergistic adverse effects on embryo development*

Both single exposure to Cd or DBT and their co-exposure did not affect the survival and hatching rates of zebrafish embryos at any of the different concentrations or treatment combinations tested (Table 1).

| *Survived embryos at 24hpf (%)* | Cd 0 µM | Cd 0,05 µM | Cd 0,2 µM | Cd 2 µM | Cd 10 µM |
|---|---|---|---|---|---|
| $H_20$ | 94,11 ± 3,2 | 98,33 ± 1,67 | 94,78 ± 2,69 | 95,22 ± 3,25 | 92,44 ± 0,44 |
| DMSO | 95 ± 2,54 | 94,7 ± 0,1 | 93,3 ± 0,2 | 95 ± 5 | 92,08 ± 0,47 |
| DBT 0,05 µM | 98,89 ± 1,11 | 89,17 ± 4,17 | 96,67 ± 3,33 | 96,67 ± 3,33 | -- |
| DBT 0,1 µM | 96,67 ± 3,33 | 91,67 ± 2,39 | 87,16 ± 4,99 | 97,11 ± 1,97 | -- |
| DBT 1 µM | 98,89 ± 1,11 | 95 ± 5 | 89,15 ± 3,41 | 90,24 ± 3,97 | -- |
| DBT 10 µM | 91,33 ± 0,67 | -- | -- | -- | -- |

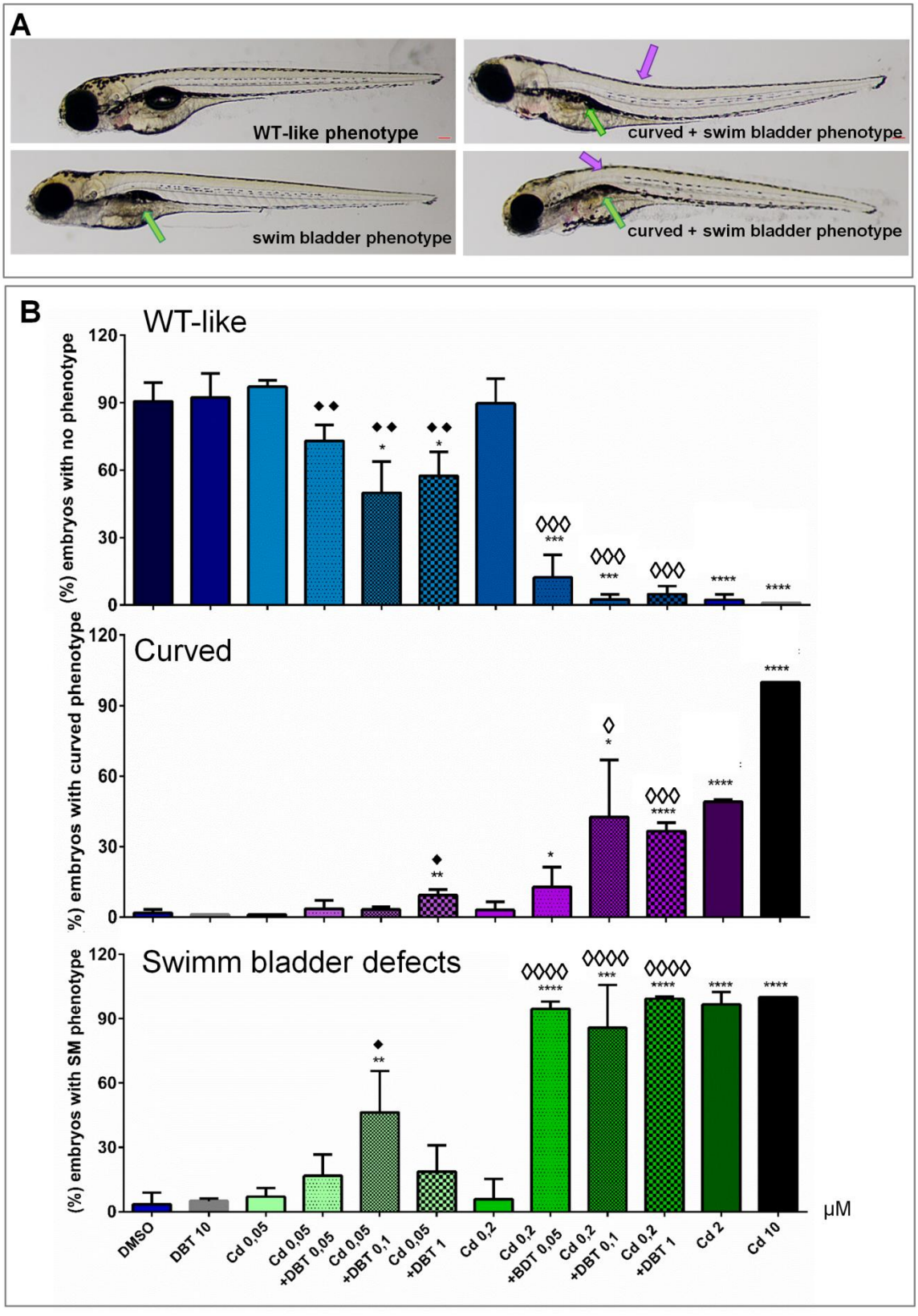

| *Survived embryos at 24hpf (%)* | Cd 0 µM | Cd 0,05 µM | Cd 0,2 µM | Cd 2 µM | Cd 10 µM |
|---|---|---|---|---|---|
| $H_20$ | 94,11 ± 3,2 | 98,33 ± 1,67 | 94,78 ± 2,69 | 95,22 ± 3,25 | 92,44 ± 0,44 |
| DMSO | 95 ± 2,54 | 94,7 ± 0,1 | 93,3 ± 0,2 | 95 ± 5 | 92,08 ± 0,47 |
| DBT 0,05 µM | 98,89 ± 1,11 | 89,17 ± 4,17 | 96,67 ± 3,33 | 96,67 ± 3,33 | -- |
| DBT 0,1 µM | 96,67 ± 3,33 | 91,67 ± 2,39 | 87,16 ± 4,99 | 97,11 ± 1,97 | -- |
| DBT 1 µM | 98,89 ± 1,11 | 95 ± 5 | 89,15 ± 3,41 | 90,24 ± 3,97 | -- |
| DBT 10 µM | 91,33 ± 0,67 | -- | -- | -- | -- |


**Table1. Quantification of survival rate at 24 hpf and hatching rate at 2 dpf, in embryos exposed to increasing concentrations of Cd and DBT or mixtures of them.** Values are shown as media ± SEM of ≥3 independent experiments where ≥ 60 embryos for each treated group were analyzed. ANOVA-1 test * P<0.05. Abbreviations: Cd: cadmium; DBT: dibenzothiophene; DMSO: dimethyl sulfoxide.

However, the morphological analysis revealed a progressive dose-dependent increase of embryo malformations, especially when co-treatment was considered (Fig. 1). As a single

pollutant, Cd at the highest concentrations (2 and 10 µM), resulted in a significant increase of embryos with slight axial-tail curvature and with delayed or failed inflation of the swim bladder, a recognized specific marker of developmental toxicity {Price, 2020} (Fig.1A,B). None of these morphological defects were induced by DBT exposure alone, with the exception of a trend versus failed inflation of swim bladder that was observed only at the highest DBT concentration tested and that did not reach the statistical significance in comparison to control (Fig.1A,B). Nonetheless, co-exposure to these substances dramatically decreased the susceptibility threshold to embryo malformations to a lower level than the sum of single pollutants, indicating a synergistic toxic effects of these chemicals (Fig.1B).

As a single pollutant, Cd showed not only a stronger toxicity compared to DBT but also wider effects. Cd concentration of 0.2 µM and above was particularly aggressive for embryo skin that is not protected by scales at this stage of development (Fig.S1A,B,C). Moreover, as already reported {Han, 2019 #6}, 0.05 µM Cd were able to affects the otolith development (Fig.S1A,B) in about 30% of embryos while almost the totality of embryo otoliths appeared smaller at 2 µM Cd concentration (data not shown). At any of the proven concentrations DBT did not induce these defects nor influenced the percentage of Cd-exposed embryos showing both skin and otolith defects (Fig S1 C and data not shown).




290

291 **Figure 1. Effects of Cd and/or DBT exposure on embryo morphology**. A) Bright field representative
292 images of 5 dpf embryos showing the different kind of phenotypes obtained after exposure to Cd, DBT or
293 their mixtures. Green arrows point to swim bladder defects, purple arrows point to axis defects. Scale bar

100 µm. B) Quantification of embryos with the indicated phenotypes All values reported were the mean of ≥3 independent experiments each of them including embryos from different clutch. ≥ 60 embryos for each treated group were analyzed. Since DBT has no effects at any of the tested concentrations, only the 10 µM concentration value was reported in the graphs. T test * P<0.05 ** P<0.01 *** P<0.001 **** P< 0.0001 vs DMSO 0.1%. ♦ P<0.05 ♦♦ P<0.01 *** vs Cd 0.05µM; ◊ P<0.05 ◊◊ P<0.01 ◊◊◊ P<0.001 ◊◊◊◊ P<0.0001 vs Cd 0,2 µM. Abbreviations: Cd: cadmium; DBT: dibenzothiophene; DMSO: dimethylsulfoxide; dpf: days post fertilization; WT: wild type-like morphology; SM swim bladder.

*3.2 DBT and Cd critically affect thyroid gland development and TH body concentration*

As first assessment of specific thyroid toxicity, we next focused on thyroid morphology alterations. To morphologically analyze thyroid development after Cd or/and DBT exposure, we exploited the transgenic zebrafish line *Tg(tg:mCherry)*, in which thyroid-specific expression of a membrane version of mCherry reporter gene allows live imaging of thyroid development {Opitz, 2012 #9}.

Following a single treatment, live imaging of embryos raised in the presence of Cd and DBT revealed that both toxicants affected the early stages of thyroid development even at the lowest concentrations and with a dose-dependent effect (Fig. 2). However two different phenotypes were induced by the two toxicants: in Cd treatments thyroid follicular cell populations lost spatial definition both in number and size and could appear partially fused (phenotype A, Fig. 2A, middle). DBT exposure resulted essentially in thyroid size reduction with a higher distance among thyroid follicular cell populations along the anteroposterior axis (phenotype B, Fig. 2A bottom). Co-exposure further reduced the number of embryos with normal thyroid development with grossly additive effect (Fig. 2A, top). However a more careful observation of the two different phenotype trends suggests a synergistic increase of phenotype A and a synergistic decrease of phenotype B in the mixtures. This effect becames more evident with the increase of DBT concentrations (Fig. 2 middle and bottom). Overall these data indicate that Cd and DBT affect thyroid development,

suggesting different mechanisms of action which can mutually influence each other when mixture are utilized.

As a second endpoint of thyroid toxicity, we assessed the total triiodothyronine (T3) and thyroxine (T4) levels, using HPLC-MS-MS. Several different clutches and treatments were collected and analyzed. T3 and T4 values obtained for untreated embryos were respectively 0,4 and 6 pg/larvae, in line with already reported values {Chen, 2018 #28;Walter, 2019 #32}. Quantification results in embryos exposed to single pollutants were more variable, although a general trend for T3 decrease was observed. However, co-exposure, even at the lower concentrations, consistently caused a significant increase of T4/T3 ratio, suggesting an alteration of TH production (Fig. 2C).

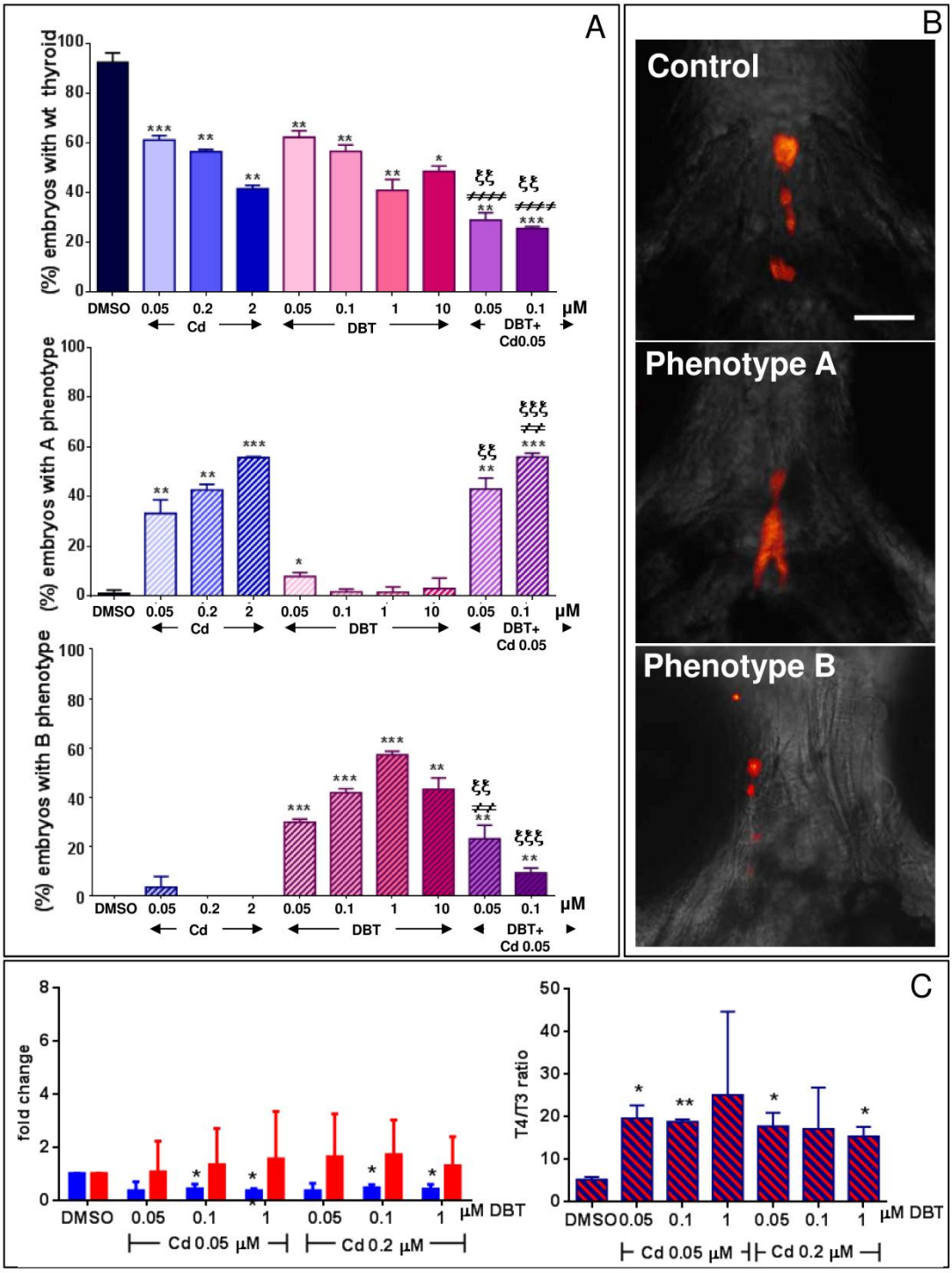



**Figure 2. Cd and/or DBT exposure cause alterations of thyroid development and activity.** A) Quantification ofthyroid defects induced by exposure or by co-exposure to increasing concentrations of DBT and Cd in 5dpf embryos. ≥ 30 embryos for each treated group were analyzed B) Fluorescence images of 5dpf *Tg(tg:mcherry)* embryos showing representative examples of thyroid defects. Chi square test * P<0.05 ** P<0.01 *** P<0.001 **** P< 0.0001 vs DMSO. ≠≠ P<0.01 ≠≠≠≠ P<0.0001 vs Cd 0,05 µM. ξξ P<0.01 ξξξ P<0.001 of DBT+Cd *versus* DBT only . Scale bar 50 µm C) 5dpf zebrafish wild type AB embryos of different clutches and from different treatments were collected and processed as described in methods. Mass

spectrometry analysis were performed to quantify the pg of total T3 and T4 hormones per embryos after the different treatments. Two mass spectrometry analysis on pools of three different treatments were performed. T3 and T4 values are reported as fold changes relative to the quantifications obtained from embryos exposed to the vehicle DMSO. * P<0.05, ** P<0.01 vs DMSO. WT: wild type-like morphology.

### *3.3 DBT and Cd differentially affect the HPT axes*

The observed alterations of the thyroid gland morphology and TH levels prompted us to analyze the expression of HPTA key hormones: ì) the thyroid-stimulating hormone (*tsh*), an essential regulator of thyroid differentiation, growth and function {Ortiga-Carvalho, 2016 #13} and ìì) the thyrotropin-releasing hormone (*trh)*, the main positive regulator of Tsh synthesis (Ortiga-Carvalho et al., 2016).

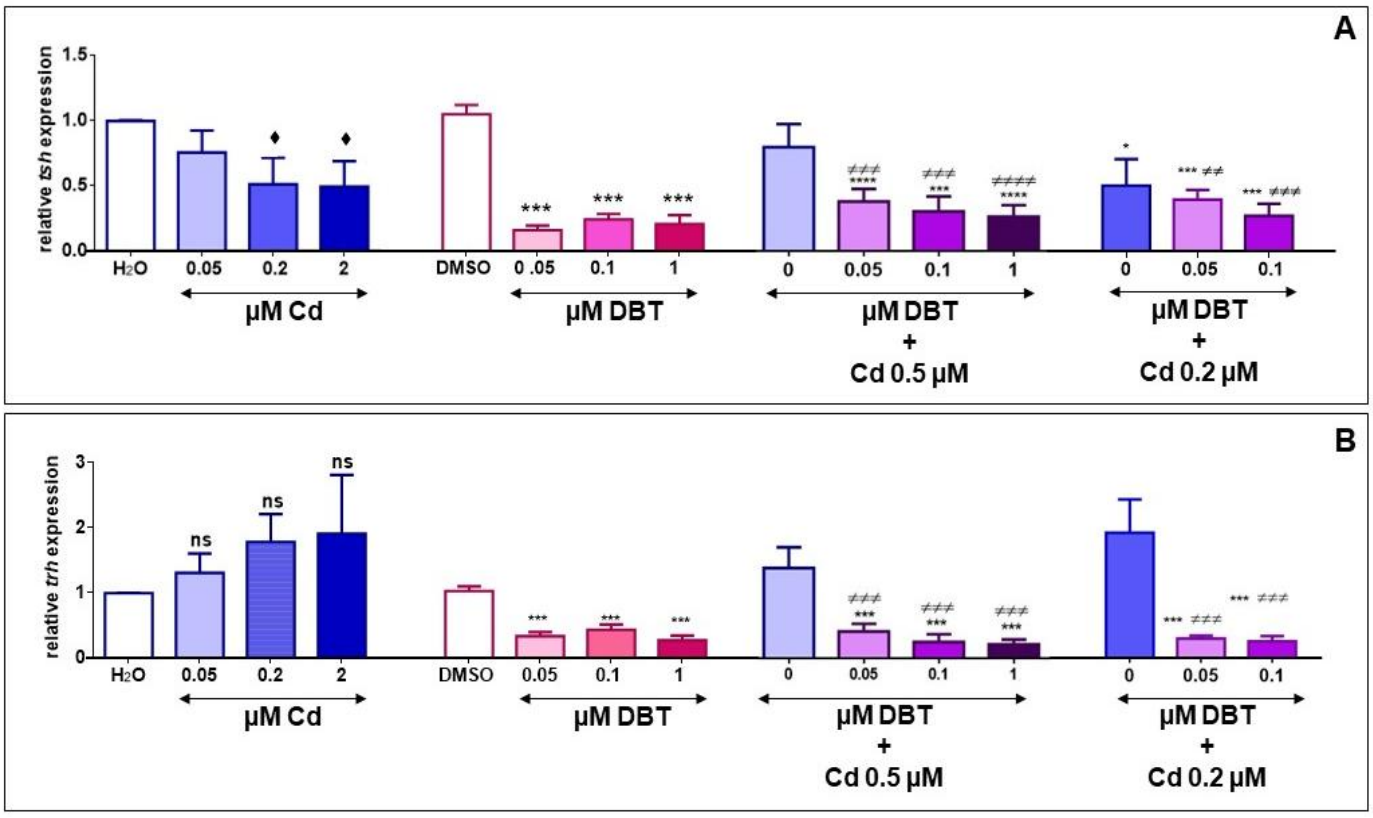



**Figure 3. Impact of Cd and/or DBT exposure on the expression of key genes of hypothalamus pituitary axis.** Relative mRNA expression levels of *tsh* (A) and *trh* (B) in 5dpf embryos exposed to increasing concentrations of Cd (left) DBT (center) and mixtures of them (right). ♦P versus $H_2O$, * P *versus* DMSO, ≠*P of* DBT+Cd *versus* Cd and ξ *P* of DBT+Cd *versus* DBT only.

Exposure to both toxicants significantly repressed *tsh* expression as quantified by Q-RT PCR analysis (Fig. 3A). The impact of DBT was particularly severe even at the lowest concentrations and without a dose-dependent effect (Fig.3A middle panel). Although Cd alone affected *tsh* expression, the presence of Cd was not able to further significantly decrease the *tsh* levels in DBT exposed embryos(Fig. 3A ). Besides the reduced *tsh* expression level, DBT exposed embryos showed decreased expression of *trh. Trh levels were not significantly modified by increasing concentration of Cd both in control or DBT exposed embryos.* Collectively, the observed alterations induced by DBT suggest a down-regulation of the HPTA, which is in line with the raised T4/T3 levels. In contrast, Cd exposure did not significantly affect *trh* levels nor significantly modified the DBT toxicity (Fig.3B), thus prompting for minimal impact of this toxicant when the HPTA signaling is concerned.

Since inflammation is one of the possible causes of *trh*/*tsh* alterations Ortiga-Carvalho et al., 2016), we then evaluated the expression of tumor necrosis factor alpha (*tnf*-α), interleukins (IL)*IL-1* and *IL-6* inflammation markers by Q-RT PCR in embryos exposed to all the different toxicant combinations. Exposed embryos did not show any significant changes in the expression of all these markers compared to those not exposed (not shown) suggesting that inflammation does not contribute to the observed hormonal changes at such concentrations of treatments.

To assess the contribution of aberrant thyroid gland production to the observed alterations in TH levels, we next analyzed the aftermaths of Cd and DBT exposure on two Tsh-regulated key proteins involved in TH biosynthesis: i) the sodium iodine symporter (*scl5a5*), which primarily transports iodide in the thyroid gland and ii) the thyroperoxidase (*tpo*), which catalyzes the addition of iodine to thyroglobulin. *Tpo* and *scl5a5* showed a similar trend to that of *tsh*, with a significant inhibition in the presence of all the DBT concentrations, a low or absent sensitivity to Cd exposure and a dosage-dependent down-

regulation in embryos co-exposed to increasing DBT concentrations and 0,2 µM Cd (Fig.4A,B). Overall, these findings indicate a depression of thyroid function, especially by DBT.

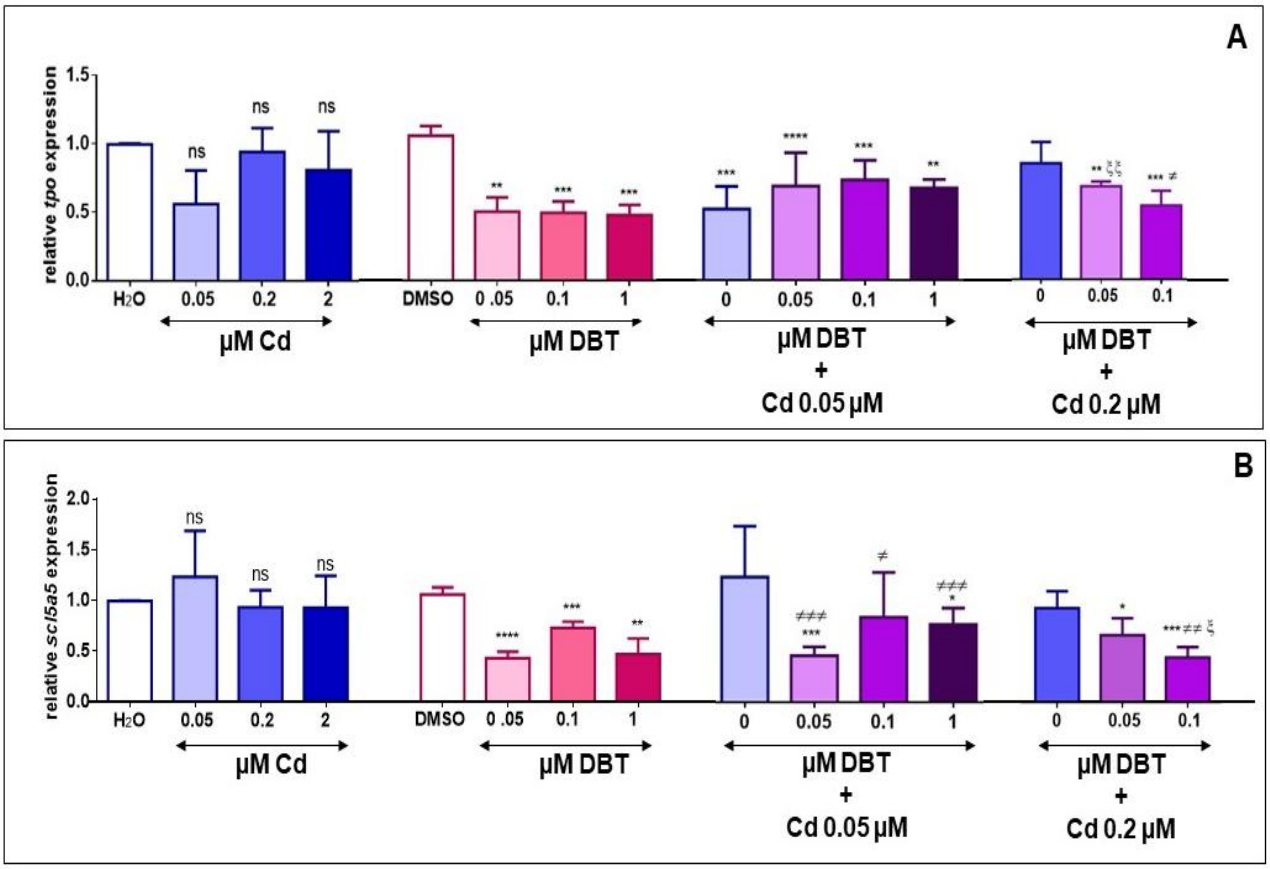



**Figure 4. Impact of Cd and/or DBT exposure on the expression of thyroid hormone biosynthesis genes.** Relative mRNA expression levels of *tpo (A)* and *scl5a5 (B)* in 5dpf embryos exposed to increasing concentrations of Cd (left) DBT (center) and mixtures of them (right). ♦P versus $H_2O$, * P *versus* DMSO, ≠*P of* DBT+Cd *versus* Cd only and ξ *P* of DBT+Cd *versus* DBT only.

## *3.4 DBT and Cd differentially affect TH peripheral disposal and THR expression*

Then we focused on crucial players of thyroid hormone peripheral metabolism and transport such as: i) deiodinases (*dios)*, which mediate either the activation or inactivation of T4 leading to T3 or rT3, respectively, ìi) uridine-5'-diphosphate-glucuronosyl transferases (*ugt*), which can impact TH homeostasis facilitating their biliary excretion

{Ritter, 1992 #136} and iii) the transthyretin (*ttr*), an important TH carrier (the main TH carrier in mammals, the thyroid-binding globulin protein, is absent in fish) but also a regulator of TH solubility and half-life in plasma. In particular, we tested the expression of *dio1,* the hepatic isoform, and *dio2* that is the isoform responsible for the intracellular production of T3. As shown in Fig. 5A, we detected a slight decrease of *dio1* only after co-exposure to 0.1 µM DBT and 0.2 µM Cd, while DBT but not Cd strongly inhibited *dio2* expression (Fig. 5B). The *ugt* expression was affected exclusively by DBT that significantly

stimulated *ugt* expression even at even at lower concentrations (0.05 and 0.1 µM, Fig. 5C).

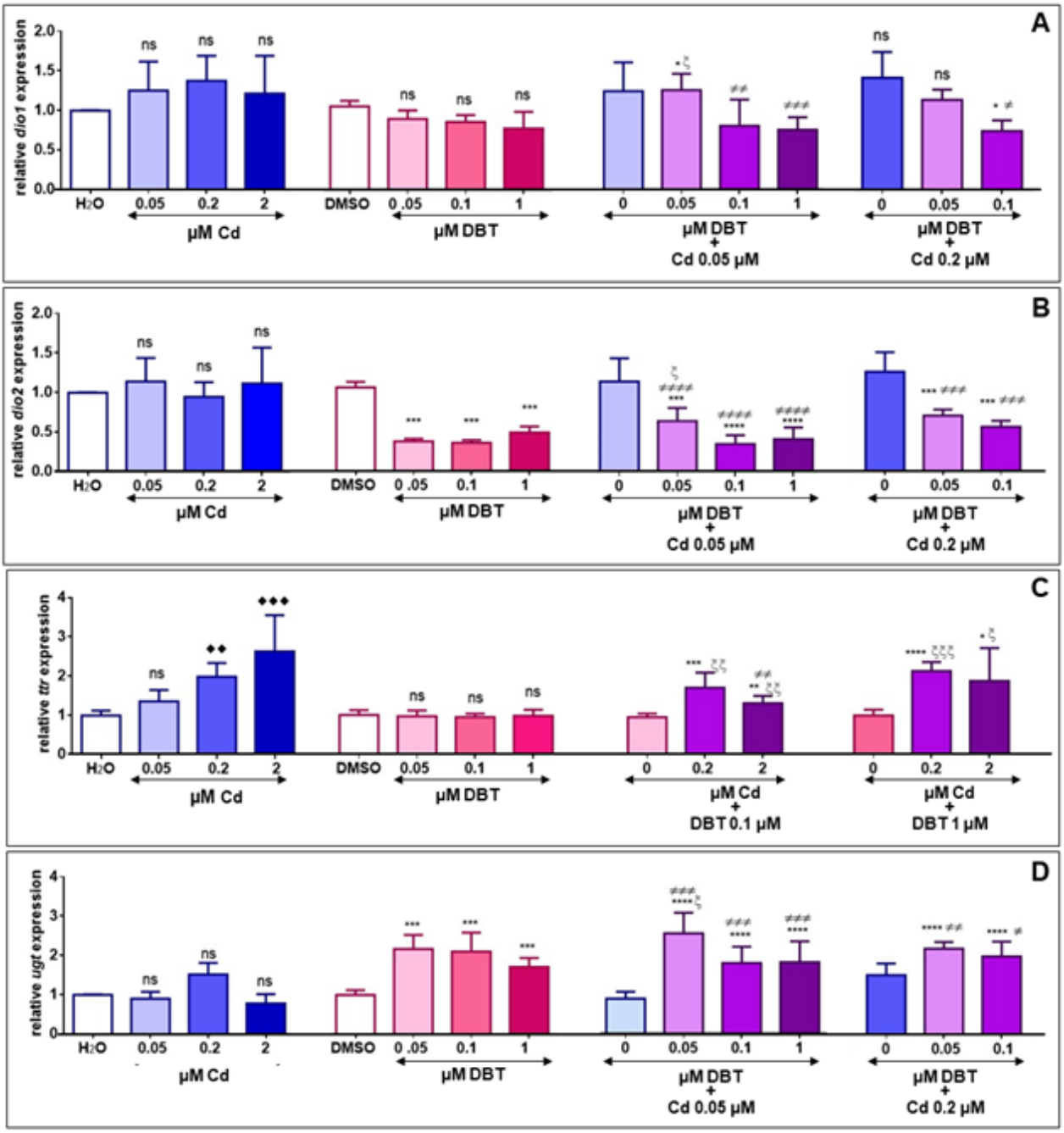



**Figure 5. Effects of Cd and/or DBT exposure on the expression of thyroid hormone peripheral metabolism genes.** Relative mRNA expression levels of *dio1* (A), *dio2* (B), *ugt* (C) and *ttr* (D) in 5dpf embryos exposed to increasing concentrations of Cd (left) DBT (center) and mixtures of them (right). ♦P versus $H_2O$, * P *versus* DMSO, ≠*P of* DBT+Cd *versus* Cd only ,and ξ *P* of DBT+Cd *versus* DBT only.

These data confirm the high impact of DBT on the overall HPTA signaling and on the peripheral disposal of THs. Importantly, the reduction in *dio2* expression may account, at

least partially, for the observed rise in T4/T3 ratio. Conversely, *ttr* expression was increased by Cd but not DBT (Fig.5D), suggesting a differential impact of the two toxicants on different processes of TH system biology.

Lastly, we analyzed the expression of THRs, the nuclear transcription factors required for T3-dependent regulation of gene expression. Unlike all the other genes analyzed, THR expression exhibited a positive linear correlation with increasing toxicant concentrations (Fig.6 and figure S2). Cd individually was able to cause a significant dose-dependent increase of THRα expression level (main effect: $p<0.05$) and, to a lesser extent, of THRβ expression levels (main effect: $p<0.05$) (Fig.6 turquoise lines). The univariate analysis excluded a main driver effect of DBT on THRs expression ratio (main effect: not significant), however a strong synergistic and dose-dependent effect on both receptors was highlighted after DBT and Cd co-exposure (Cd*DBT interaction effect: $p<0.0001$).

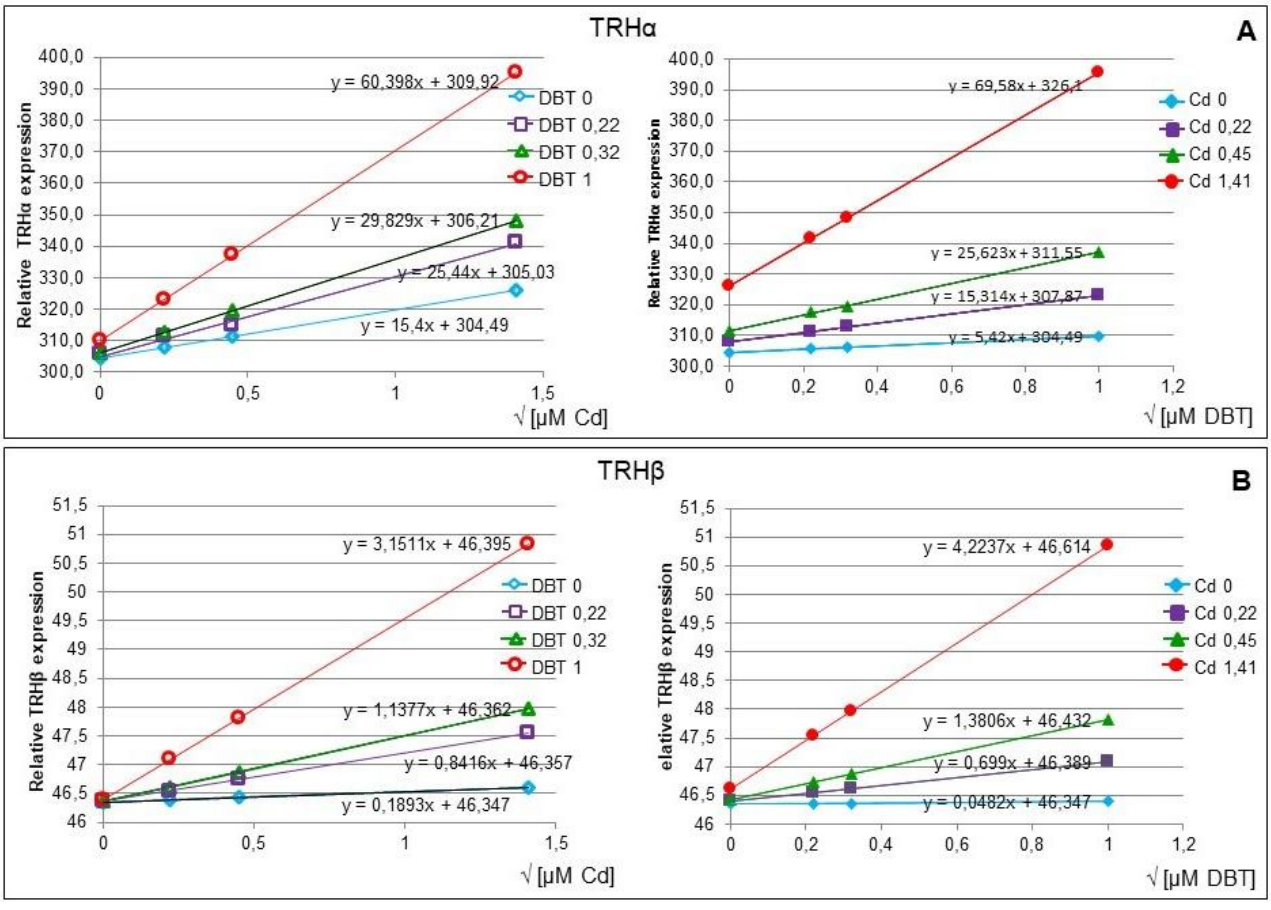


**Figure 6 Positive linear regression between Cd and/or DBT exposure and THRα and β expression.** Relative mRNA expression levels of THRα(A) and β(B) in 5dpf embryos exposed to increasing concentrations of Cd and DBT alone and mixtures of both toxicants. As assessed by univariate analysis, the data were well fitted by a linear model in which Cd as single pollutant increased gene expression of both receptors (Cd main effect: $p<0.05$) (turquoise lines). Also, a highly significant interaction of the two pollutants was observed (Cd*DBT interaction effect: $p<0.0001$), indicating a strong synergistic action. A square root transformation of the Cd and DBT concentration were used. In panel A, main effect Cd P<0,05 and DBT P=0,06, not significant; interaction effect P<0,001. In panel B, main effect Cd P<0,05 and DBT P=0,3, not significant; interaction effect P<0,001.

These data strongly support a strong impact of Cd as single contaminants and og both pollutants in combined exposure, on the expression of these two members of the nuclear receptor superfamily.

*3.5 DBT and Cd adversely impact heart development and function*

Next, we investigated the effect of toxicant exposure on cardiac development since aberrant cardiovascular development has been associated to thyroid dysgenesis in

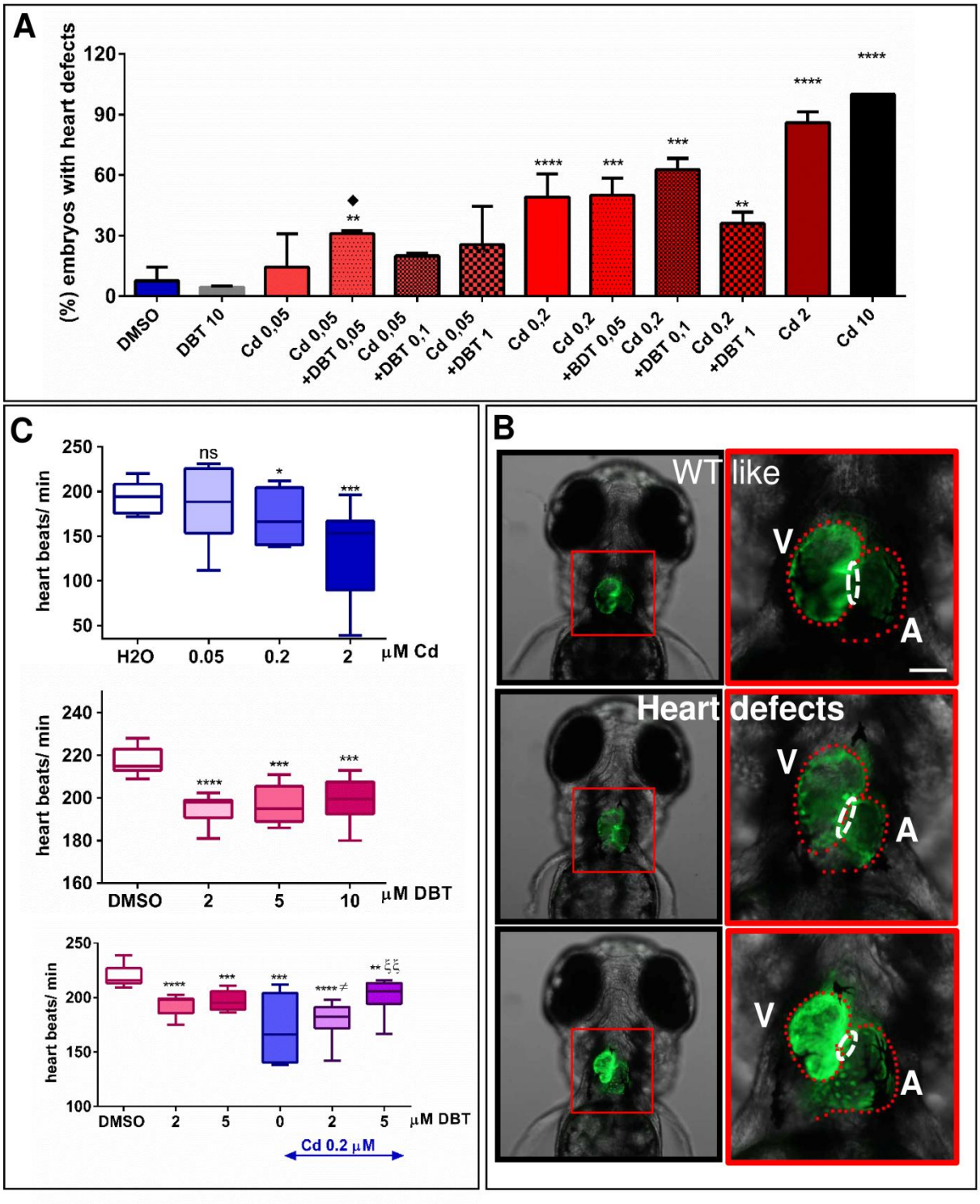



**Figure 7. Effects of Cd and/or DBT exposure on embryo cardiac morphology and function.** A) Quantification of cardiac defects induced by exposure to increasing concentrations of DBT and Cd and their mixtures. All values reported were the mean of 3 independent experiments. ≥ 50 embryos for each treated group were analyzed. B) Fluorescence images of 72 hpf *Tg(Myl7:EGFP)* embryos showing representative

examples of mild heart defect. Red dotted lines mark heart contours and white dotted line outline the orientation of the atrio-ventricular valve. Scale bar 50 µM. C) Quantification of heart beats/min in 5dpf embryos exposed to CD, DBT or mixtures of them. * *P versus* DMSO, ξ *P versus* Cd 0.2 and ≠*P versus* DBT at the corresponding dosages.

zebrafish {Opitz, 2015#140;Marelli, 2017 #146} and, conversely, THs are important for maturation of several organ systems, including the heart {Chattergoon, 2019 #143;Chattergoon, 2019 #145}. Transgenic line *Tg(Myl7:eGFP)*, in which cardiomyocytes express EGFP{Huang, 2003 #133}, was used for *in vivo* analysis of heart development in control and treated embryos. Live imaging of 72hpf embryos revealed the emergence of a significant number of cardiac defects in embryos exposed to Cd while DBT treatment did not cause any cardiac morphological alteration even at high dosages (Fig.7A). Exposure to 0,05 to 0,2 µM Cd concentrations determined a dose-dependent increase in mild cardiac defects, especially looping defects (Fig.7A,B). More severe defects such as alteration of ballooning, shape or size of chambers (Fig. S3) were always associated to extracardiac morphological embryo alterations suggesting that more general non-cardiac specific developmental molecular pathways may be affected by Cd toxicant. The addition of DBT to low Cd concentrations did not lead to a any further deterioration on heart development with the exception of an additional modest impact on cardiac ballooning (Fig.7 A,B). The analysis of the heartbeat frequency in 5dpf embryos showed that the progressive cardiac morphological alterations observed following Cd exposure were concomitant with a progressive decrease of the heartbeat frequency (Fig. 7C, up ). Interestingly, although not affecting the cardiac morphology, DBT exposure induced a decrease of heartbeats/min at 2 and 5 µM (Fig. 7B, middle), while at 10µM DBT we also observed cases of arrhythmias with occasional 2:1 AV block (not presented). These outcomes are suggestive of a condition of tissue hypothyroidism, which is consistent with the altered thyroid metabolism,

decreased *dio2* activity and reduced T3 levels. In co-exposed embryos, the presence of DBT seemed to slightly antagonize this effect (Fig. 7B, bottom).

## 4. Discussion

The current study point to show that in zebrafish DBT, Cd, synergistically impact zebrafish development and specifically thyroid development and metabolism. Zebrafish embryo/larval stages have been previously utilized to study the toxicity of different members of PAHs and, in particular, of the three ring DBT. DBT was generally used at concentrations that showed a strong impact on embryo morphology, causing several malformations such as serious dorsal curvature of the trunk and tail, and reduction of the head and cardiac edema. Our study starts from the observation that much lower DBT concentrations, which grossly preserve embryo body integrity, are nevertheless able {Walter, 2019, Marelli, 2017, Kim, 2016; Brar, 2010; Geier, 2018} impact on zebrafish morphology significantly increasing the toxicity of Cd in co-exposure experiments. We investigated the effects of Cd on zebrafish morphology in a range (0.05-10 μM) that can be considered in the low range of concentrations reported for zebrafish studies. Curved body axis and reduced or absent swimm bladder inflaction, exploited as developmental toxicity markers, were significantly affected by Cd as single toxicant only at concentrations higher than 0.2 μM. However the sensitivity threshold dramatically drop off in the presence of DBT showing a clear synergistic effect. It is worth noting that synergism osserved for these developmental toxicological endpoints do not mean common mechanisms of toxicity because largely different molecular signaling events are underlying these effects. Indeed development is a particularly susceptible period dependent from a highly coordinated and regulated network of transcription and signaling events. These endpoints therefore have

the advanyage to be particularly efficient and sensitive hazard discovery biosensors, but have the limits of a low specificity . {Buha, 2018 }

Our data show that Zebrafish embryos are particularly sensitive to Cd that causes skin alteration even at 0,05 µM dosage and induces 50% of curved embryos at 2 µM concentration. Worth noting, the lowest DBT tested concentration with no impact on embryo development, significantly increases the Cd impact on embryo morphology, revealing a adverse synergistic effect of these contaminants on embryo development.

Moreover our study show that DBT concentrations not affecting structural embryo development induce a significant increase in thyroid malformations as highlighted using the *Tg(tg:mcherry)* transgenic line. This result indicates that zebrafish, in the developmental window analyzed, can be particularly susceptible to TH-disrupting contaminants, as previously suggested {Walter, 2019 #150}. The lack of embryo malformations in the presence of thyroid alterations, which are known to affect Zebrafish development {Marelli, 2017 #151}, is only an apparent contradiction. During the first three days of zebrafish development (sufficient for the development of most organs), the embryo depends on the maternal THs stored in the yolk sac since thyroid gland is not yet formed. For this reason, during the first days of development thyroid alterations might have less impact on the overall embryo development. Cd exposure is also able to impact thyroid in zebrafish embryos, but several line of evidences suggest that the two pollutants can act through different mechanisms.

In vivo analysis show that different phenotypes are induced in a dose-dependent way by Cd and DBT exposure. The follicular-like thyroid structures appeared heavily deformed and often fused in Cd treated embryos (A phenotype) while DBT exposure resulted essentially in size reduction and higher dispersion along the vertical embryo axes of the follicular structure (B phenotype). Although the effects for combined exposure are

numerically additive, the progressive increase of A phenotype and the decrease of B phenotype observed with the increasing of DBT in the presence of Cd, suggests some interactions between the two disrupting pathways which foster the system toward the more severe phenotype.

Also the analysis by QRT-PCR of several enzymes of the HPT axis show different effects onsequent to Cd or DBT exposure. similar result was also observed at the morphological level on thyroid development.

In line with thyroid morphological alterations, both pollutants caused a significant decrease of *tsh* expression, which is generally used as marker to indicate whether environmental contaminants give rise to thyroid dysfunction {Zhai, 2014}. The impact of DBT on *tsh* and *trh*, weas high even at the lowest tested concentration. On the contrary  Cd and not significantly affected by the copresence of Cd. We also tested other genes of HPTA such as *tpo* or *sclc5a5* that showed a similar trend as *tsh*: significant down-regulation by DBT exposure, low or no effect after Cd exposure and a progressive decrease in expression at increasing concentrations of DBT in the presence of Cd. An opposite trend was found for *ttr* that seems positively affected only by Cd exposure. {Buha, 2018}

In fish also, HPTA regulates the concentration of THs that in turn exerts a negative feedback control not only on the pituitary, but also on the hypothalamus, inhibiting TRH secretion. Our study revealed that co-exposure to DBT and Cd in different dosage combinations caused an alteration of T4/T3 ratio confirming an impact of these pollutants on THs homeostasis. A significant decrease of global T3 level was observed in exposed embryos, while for T4 only a trend to increase was detectable due to a variability of the absolute hormone levels quantified in different experiments. The decrease in the transcription of *dio2*, the main player in the conversion of T4 to T3 (Ortiga-Carvalho et al.,2016), might be one of the causes of the observed T3 reduction. DBT but not Cd represses *dio2* expression and a progressive reduction of this enzyme was observed also

552 in embryos exposed to increasing DBT concentrations in the presence of Cd. Conversely,
553 neither Cd nor DBT impact *dio1* expression and exclusively the co-exposure to both
554 toxicants produced a weak but significant decrease.
555 Our THs quantification does not allow to discriminate between bound and free hormones.
556 Free and bound THs might have different concentrations and this may be one possible
557 explanation for the observed low levels of *trh* and *tsh* in presence of low T3. Moreover,
558 both Cd and DBT are known to adversely affect neurodevelopment {Ciesielski, 2012;
559 Sarma, 2017; Wang, 2016}, therefore this aspect could interfere with the neuronal control
560 of HPTA.
561 Alterations of HPTA have been observed during inflammatory diseases. In addition,
562 injection of cytokines such as IL-1 and IL-6 and TNF-α in animal models has been reported
563 to decrease TSH. Although both Cd and DBT have been shown to induce inflammatory
564 response, {Alshaarawy, 2013;Yang, 2016; Olszowski, 2012} we did not detect any
565 increase in IL-1 and IL-6 nor in TNF-α in any of the experimental group of treated embryos
566 compared to controls, probably because of the low dosages used.
567 Several of the genes we investigated exhibit non-linear responses to toxicant exposures.
568 Dose-dependent frequency of embryo malformations and non-linear responses of some
569 genes of thyroid patterns have been already described, for example, in Chinese toad
570 (*Bufogargarizans*) {Wu, 2017}. Several reports indicate non-linear responses after both Cd
571 and DBT exposure and imply also hormetic phenomena. In our study, the impact on the
572 expression of some thyroid genes caused by the exposure to increasing DBT
573 concentrations, did not significantly change from the low to the high dosages we tested. A
574 dose-dependence was more frequently identifiable in co-exposure experiments. Although
575 we did not explore a wide range of dosages, these trends are difficult to explain. {Buha,
576 2018}.

A different trend was shown by the two genes codifying for THs receptors, *thrα* and *thrβ*. A positive linear relationship was observed between the expression of *thrα* and *thrβ* and exposure to Cd. In addition, our analysis revealed a significant interaction of the two toxicants with regard to the expression of both receptors. THRs are the crucial mediators of THs activity. Therefore, the alterations of these receptors, which are responsible for sensing not only THs but also many other molecules, could interfere with the homeostasis and metabolism of the organism.

In zebrafish, repression of *thr* expression in thyroid receptor-morPAHnts has been shown to recapitulate the clinical features of RTHα or RTHβ patients {Marelli, 2016}. These findings, together with the observation that zebrafish and human THRs are functionally interchangeable, support a crucial role of THRs also in zebrafish. THRs are pivotal regulators of gene expression and exert their activity by binding to TR responsive element within targeting genes and recruiting co-activator or co-repressor chromatin remodeling complexes. Interestingly, THRs are engaged on chromatin DNA not only in their TH ligated form but also as ligand-independent form. The presence or the absence of the hormones can induce THRs to recruit alternatively activating promoter complexes or transcriptional corepressors in a highly dynamic process. Therefore, altered levels of THs and/or THRs, by affecting the ratio between ligated and unligated receptor forms, are expected to deeply impact global gene expression and function of tissues where the receptors are highly expressed.

Accordingly, in spite of increased THR expression, we found alterations of cardiac function that are indicative of tissue hypothyroidism. These results are consistent with the notion that unligated THRs act as transcriptional repressors of several T3-dependent cardiac genes involved in regulating the frequency, force and speed of heart contraction {Forini, 2019}.

In conclusion, our study highlights a synergic effect on thyroid metabolism between two environmental endocrine disruptors that epidemiological studies have shown to be significantly correlated, DBT and Cd. The synergism is detectable even at low dosages of Cd and DBT and particular evident at the level of THR modulation. Due to the pervasive activity that THRs have in the control of numerous biological activities, this result, obtained in the zebrafish model, if confirmed in higher vertebrates by further studies, could be of great translational relevance.

## 5. Acknowledgment

We are grateful to Dr. Sabine Costagliola (Univrsity of Bruxelles, Belgium) who kindly provided the *Tg(tg:mcherry)* zebrafish line. The authors acknowledge “CISUP—Centre for Instrumentation Sharing—University of Pisa” for having made available the Sciex QTrap 6500+ used for the mass spectrometric analysis. This work was supported by CISAS research program (International Centre for advanced studies on environment, ecosystems and human health—National Research Council—Ministry of Education, Universities and Research, Italy, Delibera CIPE n. 105/2015, 23 December 2015).

## 6. References

Ali, S., Champagne, D.L., Spaink, H.P., and Richardson, M.K. (2011). Zebrafish embryos and larvae: a new generation of disease models and drug screens. *Birth Defects Res C Embryo Today* 93(2), 115-133. doi: 10.1002/bdrc.20206.

Alshaarawy, O., Zhu, M.T., Ducatman, A., Conway, B., and Andrew, M.E. (2013). Polycyclic aromatic hydrocarbon biomarkers and serum markers of inflammation. A

626 positive association that is more evident in men. *Environmental Research* 126, 98-
627 104. doi: 10.1016/j.envres.2013.07.006.
628 Billiard, S.M., Meyer, J.N., Wassenberg, D.M., Hodson, P.V., and Di Giulio, R.T. (2008).
629 Nonadditive effects of PAHs on Early Vertebrate Development: mechanisms and
630 implications for risk assessment. *Toxicol Sci* 105(1), 5-23.
631 doi:10.1093/toxsci/kfm303.
632 Brar, N.K., Waggoner, C., Reyes, J.A., Fairey, R., and Kelley, K.M. (2010). Evidence for
633 thyroid endocrine disruption in wild fish in San Francisco Bay, California, USA.
634 Relationships to contaminant exposures. *Aquatic Toxicology* 96(3), 203-215. doi:
635 10.1016/j.aquatox.2009.10.023.
636 Brittijn, S.A., Duivesteijn, S.J., Belmamoune, M., Bertens, L.F.M., Bitter, W., De Bruijn,
637 J.D., et al. (2009). Zebrafish development and regeneration: new tools for
638 biomedical research. *International Journal of Developmental Biology* 53(5-6), 835-
639 850. doi: 10.1387/ijdb.082615sb.
640 Buha, A., Matovic, V., Antonijevic, B., Bulat, Z., Curcic, M., Renieri, E.A., et al. (2018).
641 Overview of Cadmium Thyroid Disrupting Effects and Mechanisms. *International*
642 *Journal of Molecular Sciences* 19(5). doi: 10.3390/Ijms19051501.
643 Chattergoon, N.N. (2019). Thyroid hormone signaling and consequences for cardiac
644 development. *J Endocrinol* 242(1), T145-T160. doi: 10.1530/JOE-18-0704
645 Chattergoon, N.N., Louey, S., Scanlan, T., Lindgren, I., Giraud, G.D., and Thornburg, K.L.
646 (2019). Thyroid hormone receptor function in maturing ovine cardiomyocytes. *J*
647 *Physiol* 597(8), 2163-2176. doi: 10.1113/JP276874.
648 Chen, L., Luo, K., Etzel, R., Zhang, X.Y., Tian, Y., and Zhang, J. (2019). Co-exposure to
649 environmental endocrine disruptors in the US population. *Environmental Science*
650 *and Pollution Research* 26(8), 7665-7676. doi: 10.1007/s11356-018-04105-x.

651 Chen, X., Walter, K.M., Miller, G.W., Lein, P.J., and Puschner, B. (2018). Simultaneous
652 quantification of T4, T3, rT3, 3,5-T2 and 3,3'-T2 in larval zebrafish (Danio rerio) as a
653 model to study exposure to polychlorinated biphenyls. *Biomed Chromatogr* 32(6),
654 e4185. doi: 10.1002/bmc.4185.
655 Ciesielski, T., Weuve, J., Bellinger, D.C., Schwartz, J., Lanphear, B., and Wright, R.O.
656 (2012). Cadmium Exposure and Neurodevelopmental Outcomes in US Children.
657 *Environmental Health Perspectives* 120(5), 758-763. doi: 10.1289/ehp.1104152.
658 De Felice, B., Copia, L., and Guida, M. (2012). Gene expression profiling in zebrafish
659 embryos exposed to diclofenac, an environmental toxicant. *Molecular Biology*
660 *Reports* 39(3), 2119-2128. doi: 10.1007/s11033-011-0959-z.
661 de Souza Anselmo, C., Sardela, V.F., de Sousa, V.P., and Pereira, H.M.G. (2018).
662 Zebrafish (Danio rerio): A valuable tool for predicting the metabolism of xenobiotics
663 in humans? *Comp Biochem Physiol C Toxicol PAHrmacol* 212, 34-46. doi:
664 10.1016/j.cbpc.2018.06.005.
665 Dooley, K., and Zon, L.I. (2000). Zebrafish: a model system for the study of human
666 disease. *Curr Opin Genet Dev* 10(3), 252-256. doi:10.1016/s0959-437x(00)00074-
667 5.
668 Forini, F., Nicolini, G., Pitto, L., and Iervasi, G. (2019). Novel Insight Into the Epigenetic
669 and Post-transcriptional Control of Cardiac Gene Expression by Thyroid Hormone.
670 *Front Endocrinol (Lausanne)* 10, 601. doi: 10.3389/fendo.2019.00601.
671 Geier, M.C., Chlebowski, A.C., Truong, L., Massey Simonich, S.L., Anderson, K.A., and
672 Tanguay, R.L. (2018). Comparative developmental toxicity of a comprehensive
673 suite of polycyclic aromatic hydrocarbons. *Arch Toxicol* 92(2), 571-586. doi:
674 10.1007/s00204-017-2068-9.
675 Gilbert, M.E., Rovet, J., Chen, Z., and Koibuchi, N. (2012). Developmental thyroid
676 hormone disruption: prevalence, environmental contaminants and

677 neurodevelopmental consequences. *Neurotoxicology* 33(4), 842-852. doi:
678 10.1016/j.neuro.2011.11.005.
679 Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., et al.
680 (2010). Identification and developmental expression of the full complement of
681 Cytochrome P450 genes in Zebrafish. *Bmc Genomics* 11. doi:10.1186/1471-2164-
682 11-643.
683 Guzzolino, E., Pellegrino, M., Ahuja, N., Garrity, D., D'Aurizio, R., Groth, M., et al. (2019).
684 miR-182-5p is an evolutionarily conserved Tbx5 effector that impacts cardiac
685 development and electrical activity in zebrafish. *Cellular and Molecular Life*
686 *Sciences*. doi: 10.1007/s00018-019-03343-7.
687 Han, J., Liu, K., Wang, R., Zhang, Y., and Zhou, B. (2019). Exposure to cadmium causes
688 inhibition of otolith development and behavioral impairment in zebrafish larvae.
689 *Aquat Toxicol* 214, 105236. doi: 10.1016/j.aquatox.2019.105236.
690 Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., et al. (2013).
691 The zebrafish reference genome sequence and its relationship to the human
692 genome. *Nature* 496(7446), 498-503. doi: 10.1038/nature12111.
693 Hua, G., Broderick, J., Semple, K.T., Killham, K., and Singleton, I. (2007). Rapid
694 quantification of polycyclic aromatic hydrocarbons in hydroxypropyl-beta-
695 cyclodextrin (HPCD) soil extracts by synchronous fluorescence spectroscopy
696 (SFS). *Environ Pollut* 148(1), 176-181. doi:10.1016/j.envpol.2006.10.040.
697 Huang, C.J., Tu, C.T., Hsiao, C.D., Hsieh, F.J., and Tsai, H.J. (2003). Germ-line
698 transmission of a myocardium-specific GFP transgene reveals critical regulatory
699 elements in the cardiac myosin light chain 2 promoter of zebrafish. *Developmental*
700 *Dynamics* 228(1), 30-40. doi: 10.1002/dvdy.10356.
701 Incardona, J.P., Carls, M.G., Teraoka, H., Sloan, C.A., Collier, T.K., and Scholz, N.L.
702 (2005). Aryl hydrocarbon receptor-independent toxicity of weathered crude oil

703 during fish development. *Environmental Health Perspectives* 113(12), 1755-1762.
704 doi: 10.1289/ehp.8230.
705 Incardona, J.P., Linbo, T.L., and Scholz, N.L. (2011). Cardiac toxicity of 5-ring polycyclic
706 aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor
707 2 isoform during zebrafish development. *Toxicol Appl PAHrmacol* 257(2), 242-249.
708 doi: 10.1016/j.taap.2011.09.010.
709 Izawa, H., Kohara, M., Watanabe, G., Taya, K., and Sagai, M. (2007). Effects of diesel
710 exhaust particles on the male reproductive system in strains of mice with different
711 aryl hydrocarbon receptor responsiveness. *J Reprod Dev* 53(6), 1191-1197. doi:
712 10.1262/jrd.19114.
713 Jabbar, A., Pingitore, A., Pearce, S.H., Zaman, A., Iervasi, G., and Razvi, S. (2017).
714 Thyroid hormones and cardiovascular disease. *Nat Rev Cardiol* 14(1), 39-55. doi:
715 10.1038/nrcardio.2016.174.
716 Khan, M.A., Khan, S., Khan, A., and Alam, M. (2017). Soil contamination with cadmium,
717 consequences and remediation using organic amendments. *Science of the Total*
718 *Environment* 601, 1591-1605. doi: 10.1016/j.scitotenv.2017.06.030.
719 Kim, K., Melough, M.M., Vance, T.M., Noh, H., Koo, S.I., and Chun, O.K. (2018). Dietary
720 Cadmium Intake and Sources in the US. *Nutrients* 11(1). doi:10.3390/nu11010002.
721 Kim, S., Sohn, J.H., Ha, S.Y., Kang, H., Yim, U.H., Shim, W.J., et al. (2016). Thyroid
722 Hormone Disruption by Water-Accommodated Fractions of Crude Oil and
723 Sediments Affected by the Hebei Spirit Oil Spill in Zebrafish and GH3 Cells.
724 *Environmental Science & Technology* 50(11), 5972-5980. doi:
725 10.1021/acs.est.6b00751.
726 Klaassen, C.D., Liu, J., and Diwan, B.A. (2009). Metallothionein protection of cadmium
727 toxicity. *Toxicol Appl PAHrmacol* 238(3), 215-220. doi: 10.1016/j.taap.2009.03.026.

728 Klein, I., and Danzi, S. (2007). Thyroid disease and the heart. *Circulation* 116(15), 1725-
729 1735. doi:10.1161/CIRCULATIONAHA.106.678326.
730 Lawson, N.D., and Weinstein, B.M. (2002). Arteries and veins: Making a difference with
731 zebrafish. *Nature Reviews Genetics* 3(9), 674-682. doi: 10.1038/nrg888.
732 Li, M., Iismaa, S.E., Naqvi, N., Nicks, A., Husain, A., and Graham, R.M. (2014). Thyroid
733 hormone action in postnatal heart development. *Stem Cell Res* 13(3 Pt B), 582-591.
734 doi: 10.1016/j.scr.2014.07.001.
735 Li, Y., Yang, H., Liu, N., Luo, J., Wang, Q., and Wang, L. (2015). Cadmium accumulation
736 and metallothionein biosynthesis in cadmium-treated freshwater mussel Anodonta
737 woodiana. *PLoS One* 10(2), e0117037. doi: 10.1371/journal.pone.0117037.
738 Lieschke, G.J., and Currie, P.D. (2007). Animal models of human disease: zebrafish swim
739 into view. *Nat Rev Genet* 8(5), 353-367. doi: 10.1038/nrg2091.
740 Manzetti, S. (2013). Polycyclic Aromatic Hydrocarbons in the Environment: Environmental
741 Fate and Transformation. *Polycyclic Aromatic Compounds* 33(4), 311-330. doi:
742 10.1080/10406638.2013.781042.
743 Marelli, F., Carra, S., Agostini, M., Cotelli, F., Peeters, R., Chatterjee, K., et al. (2016).
744 Patterns of thyroid hormone receptor expression in zebrafish and generation of a
745 novel model of resistance to thyroid hormone action. *Mol Cell Endocrinol* 424, 102-
746 117. doi: 10.1016/j.mce.2016.01.020.
747 Marelli, F., and Persani, L. (2017). How zebrafish research has helped in understanding
748 thyroid diseases. *F1000Res* 6, 2137. doi: 10.12688/f1000research.12142.1.
749 Moorthy, B., Chu, C., and Carlin, D.J. (2015). Polycyclic aromatic hydrocarbons: from
750 metabolism to lung cancer. *Toxicol Sci* 145(1), 5-15. doi: 10.1093/toxsci/kfv040.
751 Olszowski, T., Baranowska-Bosiacka, I., Gutowska, I., and Chlubek, D. (2012). Pro-
752 inflammatory properties of cadmium. *Acta Biochimica Polonica* 59(4), 475-482.

753 Opitz, R., Hitz, M.P., Vandernoot, I., Trubiroha, A., Abu-Khudir, R., Samuels, M., et al.
754 (2015). Functional zebrafish studies based on human genotyping point to netrin-1
755 as a link between aberrant cardiovascular development and thyroid dysgenesis.
756 *Endocrinology* 156(1), 377-388. doi: 10.1210/en.2014-1628.
757 Opitz, R., Maquet, E., Huisken, J., Antonica, F., Trubiroha, A., Pottier, G., et al. (2012).
758 Transgenic zebrafish illuminate the dynamics of thyroid morphogenesis and its
759 relationship to cardiovascular development. *Dev Biol* 372(2), 203-216. doi:
760 10.1016/j.ydbio.2012.09.011.
761 Ortiga-Carvalho, T.M., Chiamolera, M.I., Pazos-Moura, C.C., and Wondisford, F.E. (2016).
762 Hypothalamus-Pituitary-Thyroid Axis. *Compr Physiol* 6(3), 1387-1428. doi:
763 10.1002/cphy.c150027.
764 Perera, F.P., Chang, H.W., Tang, D., Roen, E.L., Herbstman, J., Margolis, A., et al. (2014).
765 Early-life exposure to polycyclic aromatic hydrocarbons and ADHD behavior
766 problems. *PLoS One* 9(11), e111670. doi: 10.1371/journal.pone.0111670.
767 Perera, F.P., Rauh, V., Whyatt, R.M., Tsai, W.Y., Tang, D., Diaz, D., et al. (2006). Effect of
768 prenatal exposure to airborne polycyclic aromatic hydrocarbons on
769 neurodevelopment in the first 3 years of life among inner-city children. *Environ*
770 *Health Perspect* 114(8), 1287-1292. doi: 10.1289/ehp.9084.
771 Poursafa, P., Dadvand, P., Amin, M.M., Hajizadeh, Y., Ebrahimpour, K., Mansourian, M.,
772 et al. (2018). Association of polycyclic aromatic hydrocarbons with cardiometabolic
773 risk factors and obesity in children. *Environ Int* 118, 203-210. doi: S0160-
774 4120(18)30450-1.
775 Poursafa, P., Moosazadeh, M., Abedini, E., Hajizadeh, Y., Mansourian, M., Pourzamani,
776 H., et al. (2017). A Systematic Review on the Effects of Polycyclic Aromatic
777 Hydrocarbons on Cardiometabolic Impairment. *Int J Prev Med* 8, 19. doi:
778 10.4103/ijpvm.IJPVM_144_17.

779 Rafati Rahimzadeh, M., Kazemi, S., and Moghadamnia, A.A. (2017). Cadmium toxicity and
780 treatment: An update. *Caspian J Intern Med* 8(3), 135-145. doi:
781 10.22088/cjim.8.3.135.
782 Reimers, M.J., La Du, J.K., Periera, C.B., Giovanini, J., and Tanguay, R.L. (2006).
783 Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants.
784 *Neurotoxicology and Teratology* 28(4), 497-508. doi: 10.1016/j.ntt.2006.05.007.
785 Ritter, J.K., Chen, F., Sheen, Y.Y., Tran, H.M., Kimura, S., Yeatman, M.T., et al. (1992). A
786 Novel Complex Locus Ugt1 Encodes Human Bilirubin, Phenol, and Other Udp-
787 Glucuronosyltransferase Isozymes with Identical Carboxyl Termini. *Journal of*
788 *Biological Chemistry* 267(5), 3257-3261.
789 Rundle, A., Hoepner, L., Hassoun, A., Oberfield, S., Freyer, G., Holmes, D., et al. (2012).
790 Association of childhood obesity with maternal exposure to ambient air polycyclic
791 aromatic hydrocarbons during pregnancy. *Am J Epidemiol* 175(11), 1163-1172. doi:
792 10.1093/aje/kwr455.
793 Saba, A., Chiellini, G., Frascarelli, S., Marchini, M., Ghelardoni, S., Raffaelli, A., et al.
794 (2010). Tissue distribution and cardiac metabolism of 3-iodothyronamine.
795 *Endocrinology* 151(10), 5063-5073. doi: 10.1210/en.2010-0491.
796 Saba, A., Donzelli, R., Colligiani, D., Raffaelli, A., Nannipieri, M., Kusmic, C., et al. (2014).
797 Quantification of thyroxine and 3,5,3'-triiodo-thyronine in human and animal hearts
798 by a novel liquid chromatography-tandem mass spectrometry method. *Horm Metab*
799 *Res* 46(9), 628-634. doi: 10.1055/s-0034-1368717.
800 Samanta, S.K., Singh, O.V., and Jain, R.K. (2002). Polycyclic aromatic hydrocarbons:
801 environmental pollution and bioremediation. *Trends Biotechnol* 20(6), 243-248. doi:
802 10.1016/s0167-7799(02)01943-1.
803 Sarma, S.N., Blais, J.M., and Chan, H.M. (2017). Neurotoxicity of alkylated polycyclic
804 aromatic compounds in human neuroblastoma cells. *Journal of Toxicology and*

*Environmental Health-Part a-Current Issues* 80(5), 285-300. doi: 10.1080/15287394.2017.1314840.

Sinaei, M., and Loghmani, M. (2019). Toxicity and Mechanisms of Action of Polycyclic Aromatic Hydrocarbon Pollution in Red Algae (Gracilaria corticata) from the Northern Coast of the Oman Sea. *Environmental Toxicology and Chemistry* 38(9), 1947-1953. doi: 10.1002/etc.4507.

Sun, H., Shen, O.X., Xu, X.L., Song, L., and Wang, X.R. (2008). Carbaryl, 1-naphthol and 2-naphthol inhibit the beta-1 thyroid hormone receptor-mediated transcription in vitro. *Toxicology* 249(2-3), 238-242. doi: 10.1016/j.tox.2008.05.008.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., and Sutton, D.J. (2012). Heavy metal toxicity and the environment. *Exp Suppl* 101, 133-164. doi: 10.1007/978-3-7643-8340-4_6.

Uetani, M., Kobayashi, E., Suwazono, Y., Honda, R., Nishijo, M., Nakagawa, H., et al. (2006). Tissue cadmium (Cd) concentrations of people living in a Cd polluted area, Japan. *Biometals* 19(5), 521-525. doi: 10.1007/s10534-005-5619-0.

Walter, K.M., Miller, G.W., Chen, X., Yaghoobi, B., Puschner, B., and Lein, P.J. (2019). Effects of thyroid hormone disruption on the ontogenetic expression of thyroid hormone signaling genes in developing zebrafish (Danio rerio). *Gen Comp Endocrinol* 272, 20-32. doi:10.1016/j.ygcen.2018.11.007.

Wang, Y.W., Chen, L.M., Gao, Y., Zhang, Y., Wang, C.F., Zhou, Y.J., et al. (2016). Effects of prenatal exposure to cadmium on neurodevelopment of infants in Shandong, China. *Environmental Pollution* 211, 67-73. doi: 10.1016/j.envpol.2015.12.038.

Wu, C., Zhang, Y.H., Chai, L.H., and Wang, H.Y. (2017). Oxidative stress, endocrine disruption, and malformation of Bufo gargarizans embryo exposed to sub-lethal cadmium concentrations. *Environmental Toxicology and PAHrmacology* 49, 97-104. doi: 10.1016/j.etap.2016.12.005.

831 Wu, M.T., Lee, L.H., Ho, C.K., Wu, S.C., Lin, L.Y., Cheng, B.H., et al. (2004).
832 Environmental exposure to cooking oil fumes and cervical intraepithelial neoplasm.
833 *Environ Res* 94(1), 25-32. doi:10.1016/s0013-9351(03)00118-x.
834 Yang, B.Y., Deng, Q.F., Zhang, W.Z., Feng, Y.Y., Dai, X.Y., Feng, W., et al. (2016).
835 Exposure to Polycyclic Aromatic Hydrocarbons, Plasma Cytokines, and Heart Rate
836 Variability. *Scientific Reports* 6. doi:10.1038/Srep19272.
837 Yazbeck, C.F., and Sullivan, S.D. (2012). Thyroid disorders during pregnancy. *Med Clin*
838 *North Am* 96(2), 235-256. doi: 10.1016/j.mcna.2012.01.004.
839 Zhai, W., Huang, Z., Chen, L., Feng, C., Li, B., and Li, T. (2014). Thyroid endocrine
840 disruption in zebrafish larvae after exposure to mono-(2-ethylhexyl) phthalate
841 (MEHP). *PLoS One* 9(3), e92465. doi: 10.1371/journal.pone.0092465.
842 Zon, L.I., and Peterson, R.T. (2005). In vivo drug discovery in the zebrafish. *Nature*
843 *Reviews Drug Discovery* 4(1), 35-44. doi: 10.1038/nrd1606.