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Testing the sensitivity of the medaka Transgenic Eleuthero-embryonic THYroid-Specific assay (TETHYS) to different mechanism of action.

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Abstract

There are many concerns about the impacts of Endocrine-Disrupting Chemicals on both wildlife and human populations. A plethora of chemicals have been shown to interfere with the Hypothalamic–Pituitary–Thyroid (HPT) axis in vertebrates. Disruption of the HPT axis is one of main endocrine criteria considered for the regulation of chemicals, along with the estrogen axis, androgen axis and steroidogenesis (EATS). In response to these concerns, the Organization for Economic Cooperation and Development (OECD) initiated the validation of test guidelines (TGs) covering the EATS modalities. Regarding thyroid activity and/or disruption assessment, three OECD TGs are validated, all of them using amphibians. To date, no OECD TGs based on fish are available for the detection of Thyroid Active Chemicals (TACs). To fill this gap, we developed a new test for the detection of TACs, the TETHYS assay (Transgenic Eleuthero-embryonic THYroid-Specific assay). This assay uses a medaka (*Oryzias latipes*) transgenic line Tg(*tg:eGFP*) expressing Green Fluorescent Protein in the thyroid follicles, under the control of the thyroglobulin promoter. This assay is performed at eleuthero-embryonic life-stages with an exposure length of 72 h. In the present study, the following reference chemicals with known thyroid hormone system mechanism of action have been tested: methimazole, sodium perchlorate, sodium tetrafluoroborate, diclofenac, iopanoic acid, sobetirome, NH-3 and 1-850. Except for the thyroid receptor antagonists, all chemicals tested were identified as thyroid active, modifying the total fluorescence and the size of the thyroid follicles. To investigate the test specificity, we tested three chemicals presumed to be inert on the HPT axis: cefuroxime, abamectin and 17 α -ethinylestradiol. All were found to be inactive in the TETHYS assay. This promising New Approach Methodology can serve as a foundation for the development of a new OECD TG in the frame of regulatory assessment of chemicals for thyroid activity.

Key-words: Thyroid hormone disruption, *Oryzias latipes*, Screening test, Fish, New Approach Methodology, Thyroid follicles.

Introduction

It is crucial to develop screening methods for the identification of chemicals that disrupt the HPT axis *in vivo* (Köhrle and Frädrieh, 2021). Indeed, a multitude of fundamental functions such as developmental, physiologic, and metabolic processes are TH-dependent, therefore, thyroid hormone system (THS) disrupting chemicals that interfere with these processes are a major issue (Ren and Flamant, 2023). European Union (EU) regulations, such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, EC 1907/2006), the Plant Protection Products Regulation (EC 1107/2009), and the Biocidal Products Regulation (EU 528/2012)), mandate the identification of Endocrine-Disrupting Chemicals (EDCs). The EU's strategy for chemical assessment involves conducting a series of *in vitro* and *in vivo* assays to assess potential activity on the estrogen, androgen, and thyroid axes, including steroidogenesis (EATS) (OECD, 2018). A weight-of-evidence approach is subsequently employed to evaluate the potential hazards of EDCs based on a variety of information sources. This approach considers results from both *in vitro* and *in vivo* assays, epidemiological studies, and other forms of evidence to comprehensively identify chemicals having EDCs properties. The majority of assays utilized are OECD TGs.

Concerning the disruption of the THS, three specific OECD guidelines for non-target organisms have been validated. All three are based on thyroid hormone-dependent amphibian metamorphosis. The Amphibian Metamorphosis Assay (AMA) (OECD TG 231, 2009) and the Larval Amphibian Growth and Development Assay (LAGDA) (OECD TG 241, 2015) are conducted over 21 days or 16 weeks, respectively. Despite the fact these level 3 and level 4 OECD TG provide information regarding both endocrine activity and adversity (Dang, 2022), performing these tests can be time-consuming, expensive and necessitate specialized skills and knowledge related to breeding frogs. The third, the *Xenopus* Eleuthero-embryonic Thyroid Assay (XETA) (OECD TG 248, 2019) relies on using eleuthero-embryos of a transgenic *Xenopus* line expressing the green fluorescent protein (GFP) under the control of the promoter of the direct thyroid response gene *thbzip*. The XETA is a 72h assay which informs about thyroid activity but not the adverse effects due to its short period of exposure. This advantage makes this TG easy to implement in new laboratories and less expensive than other *in vivo* assays. The validation of the XETA marks a significant milestone as the first eleuthero-embryonic method validated by the OECD for assessing endocrine activity. The XETA paved the way for the validation of new OECD TGs using transgenic aquatic fish embryos including the EASZY, RADAR, and the REACTIV assays (OECD TG 250, 2021; TG 251, 2022; TG 252, 2024, respectively). These assays are sensitive to estrogenic activity (EASZY), estrogenic and steroidogenic activity (REACTIV), or androgenic and steroidogenic activity (RADAR). These four validated eleuthero-embryo TGs are currently employed in the regulatory assessment of potential endocrine activities for pesticides and biocides. An additional advantage of the use of eleuthero-embryonic life stages in the period after hatching but before the embryo can independently feed on external food sources that this ongoing embryonic development stage is not considered a protected life stage in certain regulatory jurisdictions, such as the EU (Directive 2010/63/EU), making it an ethical alternative to other assays using later developmental stages, aligning with the 3R principles (replacement, reduction, and refinement in animal testing).

Currently, there are no OECD test guidelines available for assessing THS disruption in fish, and EU regulators anticipate the development of new assays. Efforts have begun to introduce thyroid-related endpoints into existing OECD test guidelines, (Gölz et al., 2023; Pannetier et al.,

2023) but the need for validated *in vivo* screening assays persists (OECD, 2023). Some fish assays or models have been developed, such as the Thyroxine (T₄) Immunofluorescence Quantitative Disruption Test (TIQDT) (Raldúa et al., 2012; Raldua and Babin, 2009; Thienpont et al., 2011). This test is designed to quantify the thyroid activity of potential THS disrupting chemicals, using zebrafish (*Danio rerio*) eleuthero-embryos by quantification of T₄-content by immunofluorescence. It is currently undergoing validation by the EU Reference Laboratory for alternatives to animal testing (EURL-ECVAM) as the Zebrafish Eleuthero-embryo Thyroid Assay (ZETA). Zebrafish transgenic lines have been created using the promoter of TSH β to drive the expression of GFP (Ji et al., 2012). Two promising transgenic lines using the zebrafish *thyroglobulin* (*tg*) promoter—Tg(*tg:mCherry*) (Opitz et al., 2012) and Tg(*tg:nlsEGFP*) (Trubiroha et al., 2018)—have been reported, not only as tools for studying thyroid follicle morphogenesis but also for screening thyroid-active chemicals using eleuthero-embryonic life stages.

The THS is a complex endocrine pathway involving the synthesis of TH by the thyroid stimulated by the pituitary gland, the transport of TH in the blood and through the membrane of target cells and interaction of TH with thyroid hormone receptors (THR) to regulate the expression of the target genes (**SI, Figure S1**). TH synthesis is regulated by a negative feedback loop. In parallel, the plasmatic concentration of TH is regulated by hepatic clearance and the local concentration of the different TH is regulated by deiodinases enzymes. Thyroid active chemicals (TACs) could act and modify the activity of one or several of the enzymes, transporters or receptors involved in the THS. The major described Mechanism of Action (MoAs) for TACs are, therefore: inhibition of the sodium/iodine cotransporter (NIS), inhibition of thyroid peroxidase (TPO), inhibition of plasmatic transporters (as transthyretin (TTR), thyroxine-binding globulin (TBG), albumin), inhibition of membrane transporters (Monocarboxylate transporter (MCT8/10), organic anion transporter polypeptide (OATPs), etc.), inhibition of deiodinases, activation or inhibition of UDP-glucuronyl transferases (UDPGT) and agonism or antagonism of TRs (Crofton, 2008). Characterizing the MoAs covered by each available thyroid assay is a prerequisite to their application in the regulatory assessment of chemicals to allow a correct interpretation of the data in a weight of evidence approach to identify EDCs.

We recently developed a new assay at eleuthero-embryonic life stages using a novel medaka (*Oryzias latipes*) transgenic line. This transgenic line expresses GFP in thyrocytes, under the control of the medaka *tg* gene promoter. Previously two different zebrafish transgenic lines were successfully developed using zebrafish *tg* gene promoter. (Jarque et al., 2018; Opitz et al., 2012). The fluorescence expressed in the thyrocytes is inversely proportional to THS activity, reflecting the regulation of *tg* by the negative feedback loop of the HPT axis. In this system, GFP expression is, therefore, under the control of Thyroid Stimulating Hormone (TSH) synthesized by the pituitary in response to a decrease in the local TH concentration. In our previous publication, we exposed transgenic eleuthero-embryos for 72 h to activators (T₃ and T₄) or inhibitors (6-propylthiouracil, tetrabromobisphenol A) of the THS and observed that the thyrocytes can change their size and express lower or higher levels of fluorescence, respectively (Pesce et al., 2023). The objective of the present study was to expand the number of reference chemicals tested in order to consolidate evidence regarding the applicability domain of this new assay for the different thyroid MoAs.

Materials & Methods

Japanese medaka breeding

Medaka of the Tg(*tg:eGFP*) line (Pesce et al., 2023) were maintained in 54 or 96 L glass

Class	Concentrations range in $\mu\text{g/L}$ or mg/L (and $\text{nM-}\mu\text{M}$)	Decision criteria for highest tested [C]	Observed Toxic [C]*	Mortality or sublethal effect observed*
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aquaria in recirculating water using standard laboratory conditions ($26 \pm 1^\circ\text{C}$, 12 L:12 D for growth, with 14 L:10 D light cycle for breeding). Freshly fertilized eggs were gently collected from females in the hours following reproduction or collected at the bottom of the aquaria the day after reproduction. Collected embryos were manually separated from each other and maintained in embryo rearing medium with 0.01% methylene blue in a temperature-controlled incubator using a 12L:12D light cycle at 30°C . In these conditions embryos hatched within 7-8 days post-fertilization (dpf). Newly hatched embryos (0-days post-hatching, 0 dph) were collected. GFP positive heterozygous eleuthero-embryos were used for the exposure experiments. According to the French regulation, the use of this transgenic line was done under the authorization number DUO 12168. Watchfrog's animal facility registration number for the use of transgenic organisms is A1-1719.

Chemicals and solutions

The following chemicals were used for the exposure: methimazole (MMI), sodium perchlorate (NaPER), sodium tetrafluoroborate (NABF₄), diclofenac sodium salt (DCF), iopanoic acid (IOP), NH-3, 1-850, cefuroxime sodium salt, abamectin, and 17α -ethinylestradiol (EE2) (

Drugs to treat hyperthyroidism	0.1 - 100 mg/L (875 nM - 875 µM)	100 mg/L threshold	none	none
Explosives, manufacturing processes	0.5 - 100 mg/L (4 µM - 816 µM)	100 mg/L threshold	none	none
	0.5 - 100 mg/L	100 mg/L	none	none
Class	Concentrations range in µg/L or mg/L (and nM-µM)	Decision criteria for highest tested [C]	Observed Toxic [C]*	Mortality or sublethal effect observed*
Drugs to treat hyperthyroidism	0.1 - 100 mg/L (17 nM - 17 µM)	100 mg/L threshold	none	none
Radiologic contract media	0.01 - 2 mg/L (875 nM - 875 µM)	100 mg/L threshold	none	none
Explosives, manufacturing processes	0.5 - 100 mg/L (30 nM - 6 µM)	Solubility limit	none	none
Synthetic THR agonist	0.001 - 1 mg/L (4 nM - 816 µM)	100 mg/L threshold	none	none
Synthetic THR antagonist	(2 nM - 2 µM)	Solubility limit	none	none
Synthetic THR antagonist	0.0001 - 5 mg/L (0,21 nM - 10 µM)	Solubility limit	none	none
Antibiotic	0.01 - 100 mg/L (22 nM - 224 µM)	100 mg/L threshold	none	none
Insecticide	0.01 - 10 µg/L (11 pM - 11 nM)	MTC	100 µg/L	100% mortality
Synthetic estrogen	0.01 - 1 µg/L (33 pM - 3 nM)	Based on existing data	none	none

Table 1). All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except IOP which was purchased from TCI Chemicals (Zwijndrecht, Belgium), NH-3 from MedChemExpress (Sollentuna, Sweden) and 1-850 from Santa Cruz Biotechnology (Santa Cruz, California, USA). Readily water-soluble chemicals (MMI, NaPER, DCF) were directly dissolved in control medium (glass bottled Evian water). All chemicals purity were ≥98%. For IOP, EE2 and Abamectin, a concentrated stock solution was prepared in dimethyl sulfoxide (DMSO). For NH-3, a stock solution was prepared in ethanol. Stock solutions in DMSO or ethanol were aliquoted and stored at -20°C. The final solvent concentration in each exposure solution was 0.01% where DMSO was used, or 0.02% of ethanol for NH-3. Chemical structures are given in supplementary material (SI, Table 1).

Exposure

Exposures were carried out in six-well plates at 26°C with a 14L:10D photoperiod, for 72 h and solutions were renewed daily. Seven newly hatched eleuthero-embryos were placed in each well in 8 mL of exposure solution. Two wells were used per condition (n=14). A survival pre-test was performed to assess mortality and sublethal effects (immobility or difficulty swimming), this allowed the determination of the maximum tolerated concentration (MTC) for each chemical, which was considered as the highest tested concentration resulting in less than two eleuthero-embryos exhibiting mortality or sublethal effects.

Manufacturing processes	0.5 - 100 mg/L (4 µM – 910 µM)	100 mg/L threshold	none	none
Nonsteroidal anti-inflammatory drug	0.01 - 100 mg/L (31 nM - 314 µM)	100 mg/L threshold	none	none
Radiologic contract media	0.01 - 10 mg/L (17 nM - 17 µM)	100 mg/L threshold	none	none
Synthetic THR agonist	0.01 - 2 mg/L (30 nM – 6 µM)	Solubility limit	none	none
Synthetic THR antagonist	0.001 - 1 mg/L (2 nM - 2 µM)	Solubility limit	none	none
Synthetic THR antagonist	0.0001 – 5 mg/L (0,21 nM – 10 µM)	Solubility limit	none	none
Antibiotic	0.01 - 100 mg/L (22 nM – 224 µM)	100 mg/L threshold	none	none
Insecticide	0.01 - 10 µg/L (11 pM - 11 nM)	MTC	100 µg/L	100% mortality
Synthetic estrogen	0.01 - 1 µg/L (33 pM - 3 nM)	Based on existing data	none	none

Table 1 indicates the highest exposure concentration for each chemical. According to OECD test guidelines using eleuthero-embryos, the highest exposure concentration was defined by the solubility limit of the test chemical, the MTC for acutely toxic chemicals, or a maximum of 100 mg/L, whichever value was the lowest (OECD TG 248, 2019; TG 251, 2022; TG 252, 2024). For each assay including chemicals dissolved in solvent, both a solvent control and a test medium control group (Evian water only) were included. In all assays including a solvent control, no statistically significant differences were observed between the test medium control group and the solvent control group.

Table 1: Information about chemicals tested: name, abbreviation, N°CAS, concentrations tested, and decision criteria for the highest tested concentration. MTC = Maximum tolerated concentration. Abbr. = abbreviation. *: Toxic concentration, mortality or sublethal effects were determined only for concentrations below 100 mg/L or solubility limit.

Chemical name	Abbr.	N° CAS	Class	Concentrations range in µg/L or mg/L (and nM-µM)	Decision criteria for highest tested [C]	Observed Toxic [C]*	Mortality or sublethal effect observed*
Methimazole	MMI	60-56-0	Drugs to treat hyperthyroidism	0.1 - 100 mg/L (875 nM - 875 µM)	100 mg/L threshold	none	none
Sodium perchlorate	NaPER	7601-89-0	Explosives, manufacturing processes	0.5 - 100 mg/L (4 µM - 816 µM)	100 mg/L threshold	none	none
Sodium tetrafluoroborate	NABF ₄	13755-29-8	Manufacturing processes	0.5 - 100 mg/L (4 µM - 910 µM)	100 mg/L threshold	none	none
Diclofenac sodium salt	DCF	15307-79-6	Nonsteroidal anti-inflammatory drug	0.01 - 100 mg/L (31 nM - 314 µM)	100 mg/L threshold	none	none
Iopanoic acid	IOP	96-83-3	Radiologic contract media	0.01 - 10 mg/L (17 nM - 17 µM)	100 mg/L threshold	none	none
Sobetirome	GC-1	21111-0-63-3	Synthetic THR agonist	0.01 - 2 mg/L (30 nM - 6 µM)	Solubility limit	none	none
NH-3	NH-3	44741-5-26-1	Synthetic THR antagonist	0.001 - 1 mg/L (2 nM - 2 µM)	Solubility limit	none	none
1-850	1-850	25131-0-57-3	Synthetic THR antagonist	0.0001 - 5 mg/L (0,21 nM - 10 µM)	Solubility limit	none	none
Cefuroxime sodium salt	CEF	56238-63-2	Antibiotic	0.01 - 100 mg/L (22 nM - 224 µM)	100 mg/L threshold	none	none
Abamectin	ABA	71751-41-2	Insecticide	0.01 - 10 µg/L (11 pM - 11 nM)	MTC	100 µg/L	100% mortality
17α-ethinylestradiol	EE2	57-63-6	Synthetic estrogen	0.01 - 1 µg/L (33 pM - 3 nM)	Based on existing data	none	none

Image acquisition

Eleuthero-embryos were anesthetized with 200 mg/L of tricaine (n° CAS 886-86-2, Pharmaq, Overhalla, Norway) and positioned dorsally in order to image the ventral pharyngeal region. The fluorescence of thyroid follicles was revealed using an Olympus CoolLED pE-300^{white} fluorescence source at 20% intensity, with U-F19002 GFP long-pass filters (ex475/40, em515LP). Images were captured with a 20 ms exposure time and a gain of 0.5, at 6.3x magnification using an Olympus MVX10 stereomicroscope equipped with a DP74 camera and CellSens Dimension software (Olympus, Tokyo, Japan). Each eleuthero-embryo was imaged individually (**Figure 1A**).

Image analysis

FIJI software (Schindelin et al., 2012) was used to perform the following analysis as previously described (Sébillot et al., 2014). RGB images were split in three channels: red, green and blue. In order to remove background fluorescence resulting from endogenous pigments, the intensity of the red layer was subtracted from the green layer and a threshold of 35–255 was applied to the resulting images. The thresholded images showed only the fluorescence resulting from the GFP signal in the thyroid follicles. All images were analyzed automatically by this process. To eliminate any artefactual fluorescence, the region of interest containing the thyroid follicles was manually selected for each image (**Figure 1B**). Details of image analysis are given in supplementary data (**SI, figure S1**).

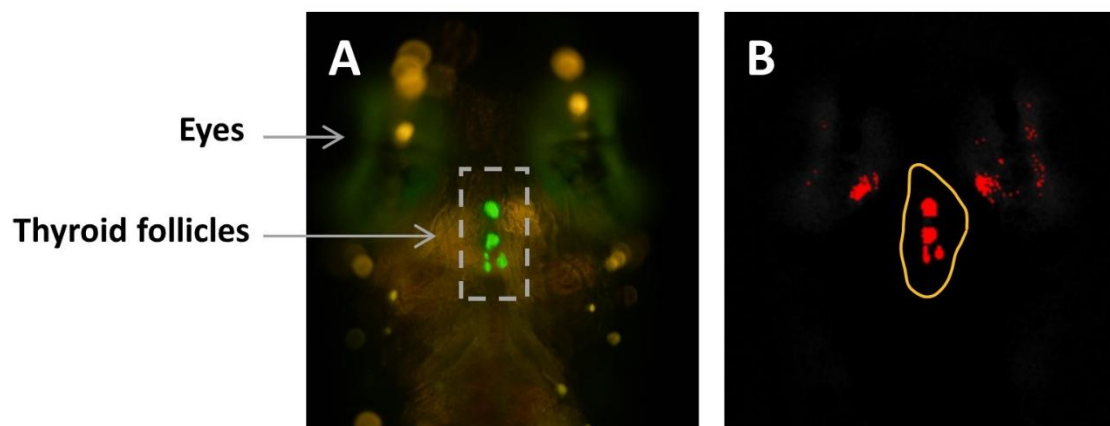


Figure 1: Ventral view of the anterior part of a Tg(tg:eGFP) eleuthero-embryo imaged under fluorescence light (A). An image before processing. (B) An image post-processing. Red represents pixels with a gray value in the interval of the 35-255 threshold. The yellow line represents the manual selection of the thyroid follicles area.

Data analysis and statistics

The total fluorescence of the region of interest of each processed image was determined. This value refers to the sum of the fluorescence level of all pixels included in the region of interest which have an intensity within the thresholded range (35-255). This value integrates both changes in the size of the region of interest and changes in fluorescence intensity. Total fluorescence values of each image were then normalized to the mean of the total fluorescence values from the control group for each run. The data for the three runs were pooled before the statistical analysis. A D'Agostino-Pearson normality test was performed prior to a one-way

ANOVA and Dunnett multiple-comparison when data followed a Gaussian distribution. For non-parametric data, a Kruskal-Wallis test followed by a Dunn's multiple-comparison test was used. Each exposed group was compared to the control. EC50 values were estimated using log(concentration) response curves of total fluorescence. All statistical tests were carried out with GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA).

Results and discussion

Exposure to inhibitors of THs synthesis

In the present study, we exposed Tg(*tg:eGFP*) transgenic eleuthero-embryos to a concentration range of three inhibitors of THs synthesis. MMI is used to treat hyperthyroidism by inhibiting TPO. This key oxidoreductase is present in the apical membrane of thyrocytes oxidizing the anion iodide (I⁻) to iodine and facilitating iodine's addition to tyrosine residues of thyroglobulin to synthesize T₃ and T₄ (Paul et al., 2014). NaPER and NABF₄ are known to inhibit NIS, therefore, inhibit iodide intake into thyrocytes (Concilio et al., 2020). Perchlorates are used in rocket fuel, explosives and fireworks and have been used to treat hyperthyroidism (Wolff, 1998). NABF₄ is used in various industries, serving as a powerful fluorinating agent.

All three compounds are highly soluble and were tested up to the maximum concentration of 100 mg/L. No (sub-)lethal effects were observed at any concentrations during the experiments. We observed a concentration-dependent increase in the total fluorescence of thyroid follicles when exposed to increasing concentrations of the three TH synthesis inhibitors (**Figure 2A-C**). The lowest observable effect concentration (LOEC) was 10 mg/L for NaPER and MMI, and 25 mg/L for NABF₄, which aligns with the hierarchical inhibitory effect of NaPER over NABF₄ as an NIS inhibitor (Concilio et al., 2020). The concentration-response relationship indicates that a maximum measurable effect was reached for the highest concentrations showing that biological saturation was presumably reached with these chemicals. An EC50 was calculated of 7.8, 9.725, and 13.86 mg/L for MMI, NaPER and NABF₄, respectively.

The sensitivity of our medaka model for MMI and NaPER could be compared to published data for a similar transgenic model, the Tg(*tg:mCherry*) zebrafish in a study using the same endpoint and the same exposure duration (Erreur ! Source du renvoi introuvable.). The EC50 for MMI for the transgenic zebrafish model was 31.86 mg/L (Jarque et al., 2018). When exposed to MMI, the increased expression of *tg* measured by qPCR showed an EC50 of 54.3 mg/L (Fetter et al., 2015). The EC50 determined for the fluorescence variation induced by potassium perchlorate with the zebrafish transgenic model was 18.71 mg/L. We tested sodium perchlorate in the present study, but it is more likely that the difference of EC50 is due to the sensitivity of the test system than the counter ion of perchlorate. For the two chemicals, the sensitivity appears to be higher for the medaka transgenic line. This could potentially be explained by technical differences. The method used in Jarque et al., (2018) is automatized in 96-well plates and analyzed using the VAST system (Union Biometrica, Geel, Belgium). Although the automatization may present a considerable gain in time for the loading and positioning of the eleuthero-embryos, it could potentially lead to a decreased sensitivity and explain why a higher EC50 was obtained for the zebrafish model, as manual positioning and imaging might increase the sensitivity.

Total fluorescence is composed of the fluorescent area multiplied by its mean fluorescence intensity. It's important to keep in mind that these two values can be influenced by the other: an

increase in the mean fluorescence could increase the area detected within the selected threshold, therefore, the fluorescent area could increase, and vice-versa. Nevertheless, considering these two parameters independently could inform on the physiological response of the follicles. Both total area and mean intensity of follicles increased when exposed to TH synthesis inhibitors (**SI, Figure S2**), indicating that both the size of the follicles and the level of expression of *tg* increased. This observation is in line with the compensatory mechanism that is known to occur in response to TH synthesis inhibition. It has been well described that MMI and perchlorate can induce thyroid hyperplasia and/or hypertrophy in fish, amphibians and other vertebrates as an adaptative response (Degitz et al., 2005; Fetter et al., 2015; Furin et al., 2015; Hooth et al., 2001; Opitz et al., 2009; Ramhøj et al., 2022; Tietge et al., 2005). These goitrogenic effects allow an increase in TH synthesis by the thyroid follicles counterbalancing the reduction in TH production.

This physiological change is the consequence of an increase in TSH production in response to the induced local decrease in THs. The up-regulation of the transcription of *tsh* and *tg* is associated with hyperplasia and hypertrophy of thyroid follicular cells (Honglian et al., 2020).

Exposure to a TH plasmatic transport inhibitor

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug, widely used around the world, and persistent through water sewage treatment. DCF sodium salt is highly soluble in water and was tested up to the maximum concentration of 100 mg/L. This concentration induced an increase in the total fluorescence (**Figure 2D**). The EC50 is 50.3 mg/L (**Table 2 : Comparison of LOEC, EC50 and activity of different chemicals tested among TETHYS, thyroglobulin fluorescent assay in zebrafish and XETA. In italic: Only one concentration has been tested, not representative of the LOEC. NT: Not tested or not published.**

). Several mechanisms of action of DCF on the THS have been described. *In vitro*, DCF displays an inhibitory effect on Oatp1c1-mediated T₄ uptake (Westholm et al., 2009), and THRB-antagonistic activity (Zloh et al., 2016). DCF has an anti-thyroid effect in *Xenopus* embryos in the XETA assay (Reis et al., 2024). In zebrafish embryos, it has been shown that DCF exposure reduces T₄ levels while T₃ levels stay constant and increase the expression of thyroid-related genes (*pax8*, *tshr*, *dio1*, *dio2*, *nis*, *tg*, *thra*, *thrb*) (Wang et al., 2022). DCF is also known to inhibit the formation of transthyretin amyloid fibrils *in vitro*, therefore, reducing the function of this thyroid-binding protein. According to Yamauchi (2021), the *ttr* gene was identified in several teleost fish including zebrafish, but may have been lost in some teleost groups and was not found in the beloniformes species studied, including four different medaka species (Yamauchi, 2021). This might explain why altered thyroid-related gene expression in zebrafish is observed at lower concentrations of DCF (between 15.5 to 139.5 µg/L) (Wang et al., 2022), than the concentrations that significantly increase the fluorescence in this current study. The increase in fluorescence observed in this study may result from one of the MoAs described for DCF which do not involve transthyretin such as: inhibition of organic anion transporter polypeptide 1C1 (OATP1C1), THRB-antagonistic activity, or inhibition of other thyroid-binding proteins. DCF is also known to inhibit thyroxine-binding globulin (TBG), but this plasmatic transport protein appears to be inexistent in amphibians and teleost fish (Kinouchi et al., 2016; Rabah et al., 2019). To our knowledge, specificity of DCF to interact with non-specific TH-binding proteins as albumin or high-density lipoproteins in fish (or other binding proteins not yet described) is not documented.

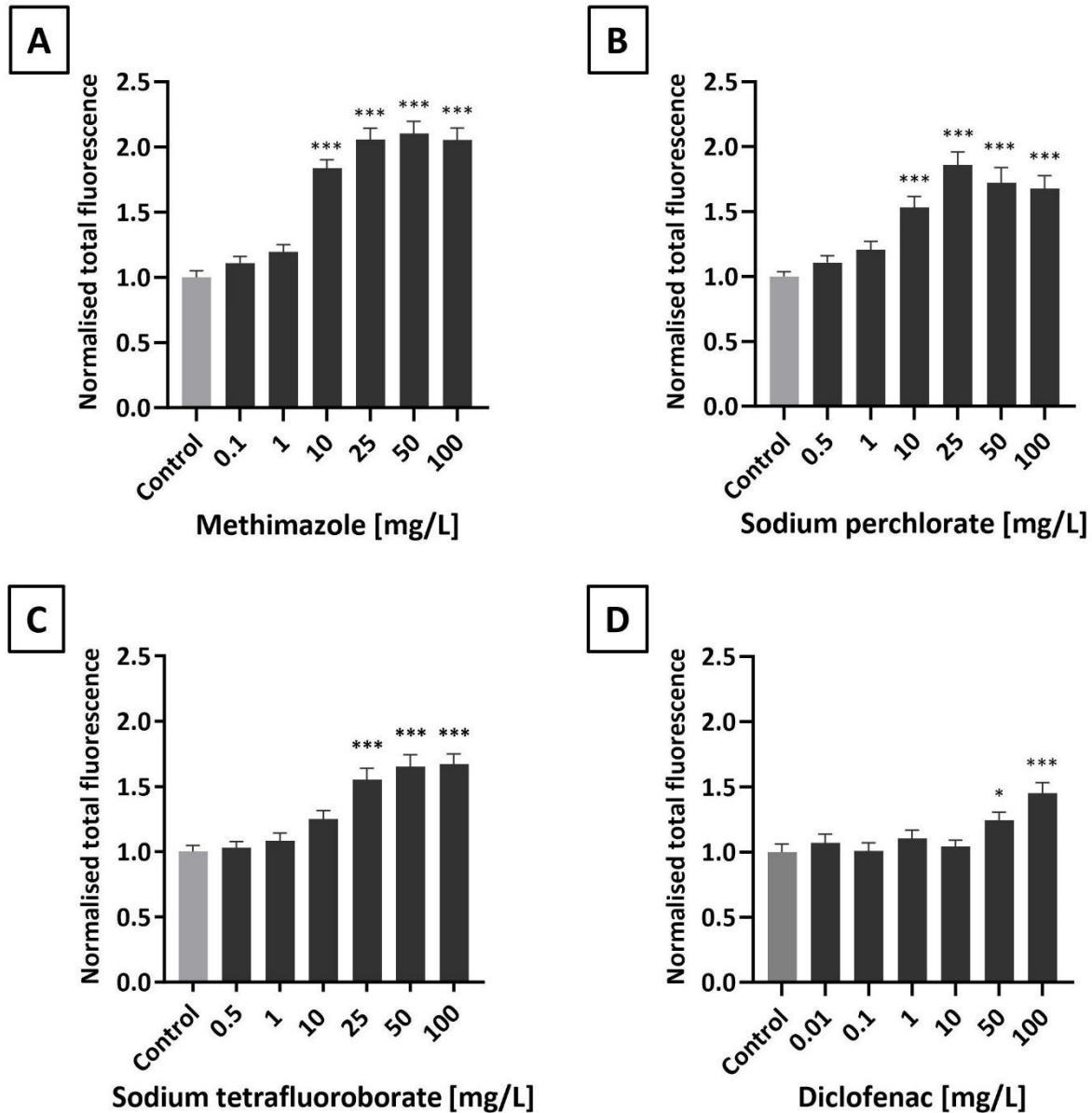


Figure 2: Mean of total fluorescence of thyroid follicles normalized to the control group. Tg(*tg:eGFP*) medaka eleuthero-embryos were exposed to inhibitors of TH synthesis, MMI (A), NaPER (B), NABF₄ (C), and to one TH transport inhibitor, Diclofenac (D) for 72 h, n= 42 (n=14 per run, with three independent runs performed and pooled). *: P<0.05; ***: P < 0.001. Mean ± SEM are given.

Exposure to an inhibitor of deiodinase activity

IOP was developed as a radiocontrast medium used for cholecystography. This iodine rich chemical is known to be a potent inhibitor of DIO1 and DIO2 deiodinases (Braga and Cooper, 2001; Fekkes et al, 1982; Stinckens et al, 2020). *In vitro* assays using human and *X. laevis* deiodinases showed that DIO1 and DIO2 were inhibited, with IOP failing to inhibit DIO3 under the tested conditions (Haselman et al, 2022). In addition, IOP has been shown to disrupt OATP1 activity *in vitro* (Westholm et al, 2009). IOP is the only deiodinase inhibitor with reported *in vivo* effects. In zebrafish eleuthero-embryos, it has been observed that IOP exposure from 48 h to 120

h decreased T_3 levels and increased T_4 levels (Van Dingenen et al., 2023). The same observation was made for *X. laevis*, T_3 levels were decreased but T_4 levels increased after 11 days of exposure (Haselman et al., 2022). The exposure of eleuthero-embryos to IOP for 72 h in the present study induced a decrease in total fluorescence, in a concentration dependent-manner (**Figure 3**). The EC50 was estimated to be 0.89 mg/L with a LOEC of 0.5 mg/L (Erreur ! Source du renvoi introuvable.). Observing a decrease in fluorescence could suggest that TH concentration increased in the brain, increasing the negative feedback loop. This is in agreement with the T_4 increase observed in other species.

Exposure to a TR agonist and antagonist

THs are transported into target cells via specific TH transmembrane transporters (MCT8/10 and OATPs). THs down-regulate the synthesis of TSHb in the pituitary gland in several fish species (Pradet-Balade et al., 1999; Yoshiura et al., 1999) maintaining the homeostasis through the negative feedback loop. There is currently no evidence how THs act on the hypothalamus to decrease TRH or CRH synthesis. In our previous study, we confirmed that natural agonists of TRs (T_3 and T_4) can be detected by the TETHYS assay. In this present study, we tested sobetirome (GC-1), a synthetic agonist used as a pharmaceutical which bind specifically to THRB (Saponaro et al., 2020). We observed a significant decrease in the total fluorescence of eleuthero-embryos exposed to GC-1, in a concentration dependent-manner (**Figure 3B**). The LOEC was 0.5 mg/L and the EC50 was estimated to be 0.27 mg/L (Erreur ! Source du renvoi introuvable.).

We then investigated if the biological effect of a TR-antagonist can be revealed by the TETHYS assay. We tested the chemicals NH-3 and 1-850, both are synthetic chemicals developed to have TR-antagonistic properties (Nguyen et al., 2002; Schapira et al., 2003). NH-3 inhibits the recruitment of TR coactivators and corepressors by preventing the folding of TR to form the cofactor-binding area (Nguyen et al., 2002). Schapira et al., (2003) suggest that 1-850 interacts directly with THR ligand-binding domain. In the test medium, NH-3 and 1-850 have solubility limits of 1 mg/L and 2 mg/L. Exposing the eleuthero-embryos at these concentrations did not induce any signs of lethal or sublethal toxicity. We did not observe any statistically significant changes in the total fluorescence level of the eleuthero-embryos following exposure to NH-3 or 1-850 (**Figure 3C-D**).

Several hypotheses could explain the lack of detectable activity of these TH-antagonists by the TETHYS assay. Hypothesis 1: The specificity of both chemicals for THR in medaka may differ from their specificity for THR from humans or amphibians. Also, limited information is available on the selective inhibition for the different types of THR in medaka (THRB, THRAA, THRAB), especially during eleuthero-embryonic life stage. Hypothesis 2: Our model is the reflection of *tg* transcription activity and *tg* is not directly regulated by TH, but by TSH via the regulation of *tg* transcription factors (Mascia et al., 2002). T_3 negatively regulates TSH transcription, therefore, TH-antagonists might regulate positively TSH transcription (therefore indirectly *tg* transcription). It means that TH-antagonists should interact with TSH transcription, therefore, be able to reach pituitary gland. It could be possible that these two compounds are not efficiently transported into the pituitary gland and will not affect the negative feedback loop. It has been documented, notably in zebrafish, that the vascularization developed around certain brain structures, such as neurohypophyseal capillaries, presenting permeable pores called fenestrae can facilitate exchanges of nutriment and hormones (Anbalagan et al., 2018). To date there are

no studies indicating if NH-3 or 1-850 could enter the pituitary gland *via* fenestrae in any animal model. Hypothesis 3: The concentrations tested may not be sufficient to reach an effective concentration allowing a competition between the antagonists and T₃ in the medaka brain. This could also be the consequence of a low solubility limit for these molecules.

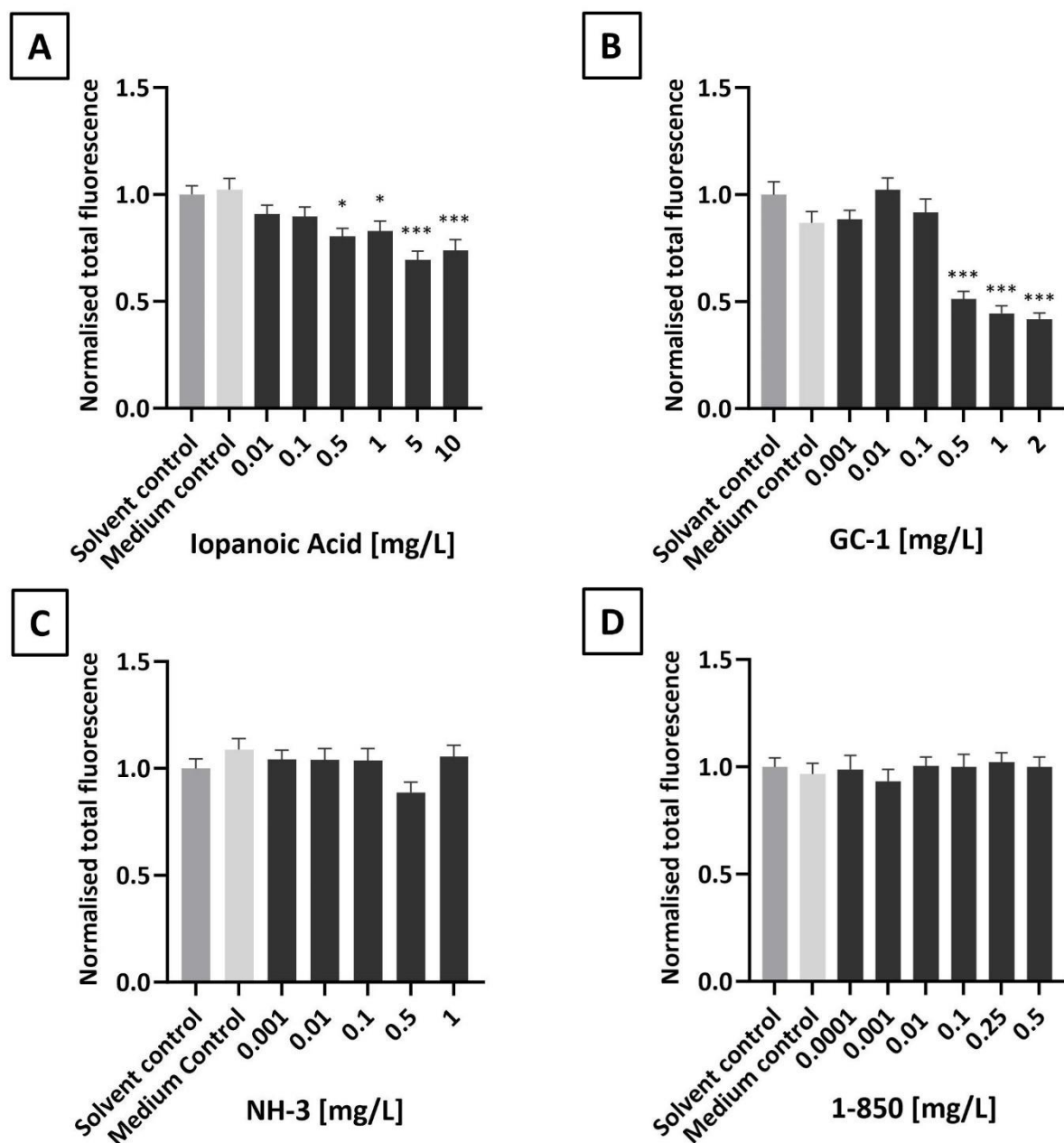


Figure 3: Mean of total fluorescence of thyroid follicles normalized to the control group. Tg(*tg:eGFP*) medaka eleuthero-embryos were exposed to IOP (A), GC-1 (B), NH-3 (C) and 1-850 (D) for 72 h, n= 42 (n=14 per run, with three independent runs performed and pooled). All groups including the control contain the same percentage of DMSO 0.01%. *: P < 0.05; ***: P < 0.001. Mean \pm SEM are given.

Exposure to thyroid-inactive chemicals

Three biologically active chemicals with no reported thyroid activity, cefuroxime, abamectin and EE2, were selected to evaluate the specificity of the assay. Cefuroxime is an antibiotic, abamectin is an insecticide, and EE2 is a potent synthetic agonist of the estrogen receptor. Abamectin was identified as an inactive reference chemical for thyroid activity after being part of the Endocrine Disruptor Screening Program (EDSP) of the US Environmental Protection Agency (EPA). The testing concluded in an absence of thyroid-related effects in *in vivo* screening assays including the AMA, and male and female pubertal rat assays (EPA, 2009a, 2009b; Wegner et al., 2016). In medaka, exposure to concentrations as low as 10 ng/L of EE2 from post-hatching stage to sexual maturity is sufficient to alter reproductive behaviours and induce testis-ova in 68% of males (Balch et al., 2004). Caldwell et al. (2008) reviewed the effects of EE2 in aquatic species, and showed that concentrations of 1 to 488 ng/L altered physiological endpoints in fish. For this study, we intentionally chose a higher concentration (1 µg/L) for the maximum EE2 test concentration. No signs of sublethal toxicity were observed in the eleuthero-embryos exposed to all test concentrations for the three chemicals during the exposure phase. None of the tested concentrations of these chemicals induced a statistically significant total fluorescence change (**Figure 4A-D**). Despite the fact that crosstalk has been described between the thyroid and estrogenic axis (Myosho et al., 2021), in this model, the GFP expression controlled by the *thyroglobulin* promoter appeared to be insensitive to agonists of the estrogen receptor. This absence of thyroid activity is in accordance with results obtained with estradiol (E2) used as a reference thyroid inactive chemical during the multi-laboratory testing during the OECD validation of the AMA and XETA TGs where it was found negative in both assays (OECD TG 231, 2008; TG 248, 2018).

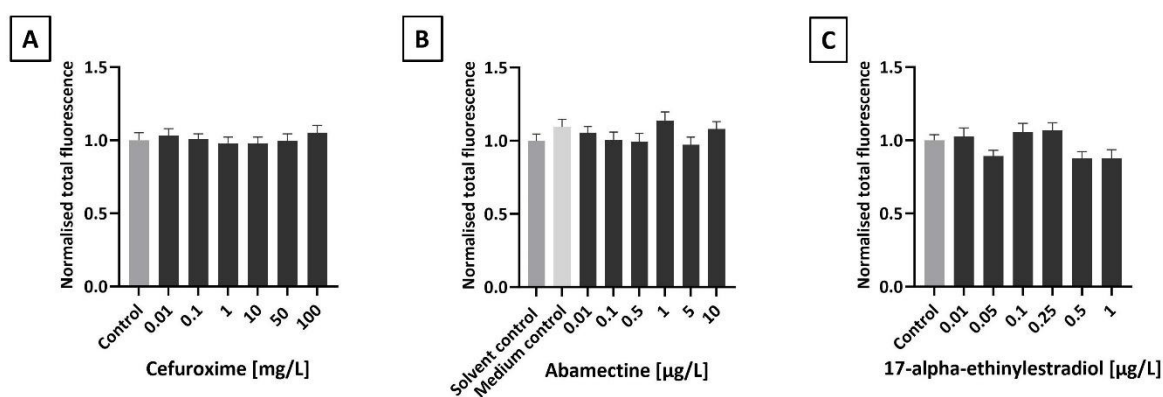


Figure 4: Mean of total fluorescence of thyroid follicles normalized to the control group. Tg(*tg:eGFP*) medaka eleuthero-embryos were exposed to THS inert chemicals : cefuroxime (A), abamectin (C), EE2 (D) for 72 h, n= 42 (n=14 per run, with three independent runs performed and pooled). Mean ± SEM are given.

Table 2 : Comparison of LOEC, EC50 and activity of different chemicals tested among TETHYS, thyroglobulin fluorescent assay in zebrafish and XETA. In italic: Only one concentration has been tested, not representative of the LOEC. NT: Not tested or not published.

		TETHYS		<i>Tg(tg:mCherry)</i> zebrafish		XETA		AMA	
Abbr.	Mechanism of action	Activity detected	LOEC	Activity detected	LOEC	Activity detected	LOEC	Activity detected	LOEC (histology or dev stage)
T ₃ *	natural THR-agonist	Active (✓)	5 µg/L	NT	\	Active ^d	3.25 µg/L	NT	\
T ₄ *	natural THR-agonist	Active (✓)	1 µg/L	Active ^a (✓)	5 µg/L	Active ^d	10-100 µg/L	Active ^{h,i}	1 µg/L (his, ds)
PTU*	TPO inhibition DIO inhibition	Active (✓)	8 mg/L	Active ^b (✓)	34 mg/L	Active ^d	10-30 mg/L	Active ^h	1.25 mg/L (his) 10 mg/L (ds)
TBBPA	TH transport and DIO1 inhibition, THR-(ant)agonist	Active (✓)	5 mg/L	NT	\	Active ^{e,f}	0.55 mg/L	NT	\
MMI	TPO inhibition	Active (✓)	10 mg/L	Active ^c (✓)	Not given	Active ^{e,g}	100 mg/L	Active ⁱ	16 mg/L (ds) 4 mg/L (his)
NaPER	NIS inhibition	Active (✓)	10 mg/L	Active ^{b,c} (✓)	27.3 mg/L	Inactive ^e		Active ^h	65 µg/L (his)
NABF ₄	NIS inhibition	Active (✓)	25 mg/L	NT	\	Inactive	Not published	NT	\
DCL	TH transport inhibition and THR-antagonist	Active (✓)	50 mg/L	NT	\	Active ^j	6,25 mg/L	NT	\
IOP	DIO1/2 inhibition	Active (✓)	0.5 mg/L	NT	\	Active ^g	3.125 mg/L	Active ^h	0.75 mg/L (ds) 1.5 mg/L (his)
GC-1	THR-agonist	Active (✓)	0.5 mg/L	NT	\	Active ^e	0,03 mg/L	NT	\
NH-3	THR-antagonist	Inactive	\	NT	\	Active ^{d,e}	1 mg/L	NT	\
1-850	THR-antagonist	Inactive	\	T	\	Not tested	\	NT	\

^a (Trubiroha et al., 2018)

^b (Jaka et al., 2023)

^c (Jarque et al., 2018)

^d (OECD, 2018)

^e (Fini et al., 2007)

^f (Fini et al., 2012)

^g (Du Pasquier et al., 2024)

^h (OECD, TG 231, 2008)

ⁱ (Coady et al., 2010)

^j (Reis et al., 2024)

Table 3 : Comparison of the activity of chemicals presumed to be inert for thyroid signaling chemicals which were tested with the TETHYS, thyroglobulin fluorescent assay in zebrafish, XETA and AMA. NT: Not tested or not published.

		TETHYS	Tg(tg:mCherry) zebrafish	XETA	AMA
Abbr.	Mechanism of actions	Activity detected	Activity detected	Activity detected	Activity detected
CEF	Inert on thyroid signaling	Inactive	NT	Inactive ^d	NT
MET	Inert on thyroid signaling	Inactive	NT	Inactive ^d	Inactive ^k
ABA	Inert on thyroid signaling	Inactive	NT	Inactive ^d	Inactive ^k
EE2	Inert on thyroid signaling	Inactive	NT	NT	Inactive ^k

^d

(OECD, TG 248, 2018)

^k (US EPA, 2015)

Table 4: Summary of the MoAs detected in the TETHYS and XETA assays.

Mechanism of action	TETHYS results	XETA results
TPO inhibition	Active	Equivocal
NIS inhibition	Active	Inactive
DIO inhibition	Active	Active
Inhibition of plasmatic TH transport	Active	Active
TR agonist	Active	Active
TR antagonist	Inactive	Active
Inert on THS (expected to be inactive)	Inactive	Inactive

Advantages of the TETHYS assay

The hormonal systems control major physiological functions. They are evolutionarily conserved across vertebrates and composed of glands secreting hormones transported into blood to act on the cells of target organs through hormone receptors. Endocrine active chemicals can modify the activity of one or several of the enzymes, transporters or receptors involved in a given endocrine pathway. Endocrine signalling results from the interaction of multiple and coordinated mechanisms. *In vitro*, isolated MoAs may not reveal disruption as multiple interactions along the signalling pathway are sometimes required. Chemicals found inactive *in vitro* can be metabolized into endocrine active chemicals *in vivo*. Identifying endocrine active chemicals, therefore, requires a complete endocrine test system and an ability of the test system to metabolize xenobiotics. The embryonic stages of aquatic vertebrates present a unique opportunity to fulfil these requirements by presenting an entire endocrine system associated with metabolic competency amenable to use in an *in vitro* like test system. These advantages are part of the new TETHYS assay using transgenic medaka eleuthero-embryos which is described in the present study.

The OECD TGs using eleuthero-embryos are considered as screening assays at level 3 of the OECD conceptual framework for the identification of endocrine disruptors (OECD, 2018). These TGs (OECD, TG 250, 2021; TG 251, 2022; TG 252, 2024) have been designed to classify chemicals into potentially endocrine active or inactive for a hazard-based regulation but are not meant to identify adverse effects or toxicity values for risk assessment (e.g., NOEC or ECx). However, it is important to consider that NOEC and ECx values could be determined using these assays if the experimental design is adapted by increasing the number of tested concentrations and with the possible requirement of a preliminary range-finding test. The data shown in the present study and in Pesce et al. (2023), indicate that similarly to the recently validated eleuthero-embryo assays (OECD, TG 250, 2021; TG 251, 2022; TG 252, 2024), the TETHYS assay could be used both in an experimental design with three test concentrations to identify thyroid active chemicals and with a greater number of test concentrations to calculate ECx values. Another advantage of the eleuthero-embryos assays is the ability to provide some indications regarding the MoA of chemical in addition to identifying endocrine activity. In the case of the TETHYS assay, an increase of fluorescence is the signature of a MoA leading to the decrease of TH availability (inhibition of synthesis via TPO or NIS, inhibition of plasmatic or membrane transport) while a decrease of fluorescence is induced by an increase of thyroid signalling (agonism of TR, potentially inhibition of clearance, etc.). In any case, *in vitro* mechanistic assays are required to confirm the MoA of chemicals that are found to be active *in vivo*. Compared to amphibian assays with longer exposure phases, such as the AMA, one obvious advantage of the TETHYS is the reduced time and cost of the assay. When comparing the sensitivity of both assays, histopathological endpoints in the AMA are more sensitive than the fluorescence endpoints used in the transgenic fish assay (Erreur ! Source du renvoi introuvable.). However, the primary aim of level 3 assays is to identify endocrine activity. Notably, all chemicals that tested positive in the AMA also showed activity in the TETHYS assay (Table 2), demonstrating that the TETHYS is an efficient and reliable alternative.

The use of fish eleuthero-embryos rather than amphibians offers logistical benefits, as more research laboratories are experienced in working with fish. Since medaka embryos are highly robust and can be shipped without affecting their development, unlike zebrafish embryos, research laboratories can receive transgenic medaka embryos and perform the TETHYS assay without needing an on-site fish husbandry facility.

Limitations of the TETHYS assay

Limitations of the assay need to be underlined. It is possible that the TETHYS assay might not be able to detect chemicals that bind to TRs but that cannot be transported across the blood-brain barrier for binding TRs localized in the hypothalamus or pituitary gland. Therefore, *in vitro* transactivation assays with both THRA and THRB, or a XETA test may be required in parallel. A TETHYS assay combined with a XETA is a particularly relevant combination of assays as MoAs poorly detected in the XETA such as NIS inhibition or TPO inhibition are efficiently detected in the TETHYS, while TR antagonism is efficiently detected in the XETA (

Table 4). Nevertheless, it must be underlined that few environmental chemicals are described in the literature with agonist or antagonist activity toward TRs only. For instance,

from 39 tested <i>in vitro</i> none of them showed	Mechanism of action	TETHYS results	XETA results	pesticides <i>in vitro</i> , them agonist or
	TPO inhibition	Active	Equivocal	
	NIS inhibition	Active	Inactive	
	DIO inhibition	Active	Active	
	Inhibition of plasmatic TH transport	Active	Active	
	TR agonist	Active	Active	
	TR antagonist	Inactive	Active	
	Inert on THS (expected to be inactive)	Inactive	Inactive	

antagonist activity toward THRA1 (Zekri et al., 2021). This is in accordance with the conclusion of the Tox21 screen (Paul-Friedman et al., 2019). In this study, 8,305 chemicals from the Tox21 library were screened. Only one direct agonist and three antagonists were found. Chemicals displaying agonist or antagonist activity, as they are often similar in three-dimensional form to THs, might often interact with other TH-binding proteins and display other MoAs. For instance, amiodarone, is known to be a TH-antagonist but also inhibits OAPT1C1 activity (Westholm et al., 2009). The detection of TAC by the TETHYS that display additional MoAs should now be investigated, such as xenobiotics affecting the hepatic clearance of THs, while interacting with UDP-GT.

Regarding all fluorescence-based assays, it is essential to note that chemicals emitting fluorescence at the same wavelength as GFP may result in a false positive outcome. Consequently, it is imperative to avoid testing such chemicals. To mitigate this risk, it is advisable to conduct a preliminary assessment of the fluorescence exhibited by the test chemical in solution, a practice recommended for both the XETA and RADAR assays. While several publications support the notion that early life stages of medaka demonstrate metabolic competence, the available data are currently insufficient to draw definitive conclusions about the extent of this metabolic competency. Consequently, testing at eleuthero-embryo stages could potentially yield different sensitivity outcomes compared to testing at later developmental stages for certain chemicals. It is crucial to recognize that the assay provides valuable insights into the thyroid activity of a chemical. The direction of fluorescence changes offers indications about the potential MoA. However, it is essential to emphasize that the assay cannot precisely identify the exact MoA or potential adverse physiological effects.

Conclusion:

Following the testing of a set of chemicals acting via different MoA on THS, our results from the TETHYS assay demonstrate the ability of this new *in vivo* screening method to be sensitive and specific to THS. Using an eleuthero-embryonic model is a valuable tool in the frame of the development of NAMs. Making use of the TETHYS assay to test new chemicals of high concern will supplement findings from other assays and species, useful for the development of Integrated Approaches to Testing and Assessment in the reglementary framework of thyroid disruption assessment. There is no indication that the TETHYS assay cannot be used for the environmental samples (such as ground and surface water, waste water samples, etc.) in the near future.

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Declaration of competing interest:

The authors declare the following competing financial interests. GL has financial interests in Laboratoire WatchFrog, which plans to commercialize the TETHYS assay. AT, BRD, GF and DDP are currently employed by Laboratoire WatchFrog. EP was employed by Laboratoire WatchFrog while she was involved in the present study. LMS has a collaboration contract with the cosmetics company L'OREAL.

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Supporting Information

Testing the sensitivity of the medaka Transgenic Eleuthero-embryonic THYroid-Specific assay (TETHYS) to different mechanism of action.

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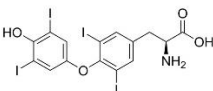
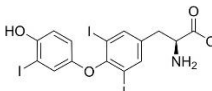
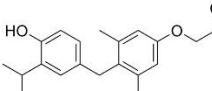
Figure S3: Mean of total area or mean intensity of thyroid follicles normalized to the control group for Tg(tg:eGFP) medaka eleuthero-embryos exposed to inhibitors of TH synthesis inhibitors.....S4

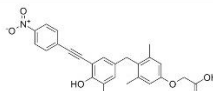
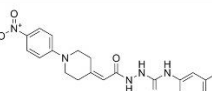
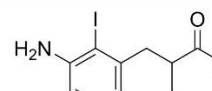
Figure S4: Mean of total area or mean intensity of thyroid follicles normalized to the control group for Tg(tg:eGFP) medaka eleuthero-embryos exposed to an inhibitor of TH binding to TH binding proteinsS4

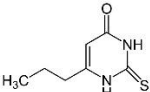

Figure S5: Mean of total area or mean intensity of thyroid follicles normalized to the control group for Tg(tg:eGFP) medaka eleuthero-embryos exposed to IOP.....S5

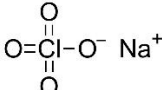
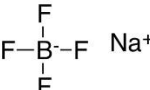
Figure S6: Mean of total area or mean intensity of thyroid follicles normalized to the control group for Tg(tg:eGFP) medaka eleuthero-embryos exposed to THR-agonist or antagonists.....S5

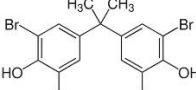
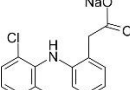
Figure S7: Mean of total area of thyroid follicles normalized to the control group for Tg(tg:eGFP) medaka eleuthero-embryos exposed to chemicals presumed to be inert on the thyroid axis.....S6

Agonists of thyroid receptors		
T4*	T3*	GC-1
		
MW: 776.87	MW: 650.97	MW: 328.41

Antagonists of thyroid receptors		Inhibitor of deiodinases
NH-3	1-850	IOP
		
MW: 650.97	MW: 473.52	MW: 570.935

Inhibitors of TPO	
6-propylthiouracil (PTU)*	Methimazole (MMI)
	
MW: 650.97	MW: 776.87

Inhibitors of NIS	
Sodium perchlorate (NaPER)	Sodium tetrafluoroborate (NABF4)
	
MW: 122.44	MW: 109.79

Inhibitors of THs transport inhibition (transmembrane and/or plasmatic)	
TBBPA*	Diclofenac sodium salt
	
MW: 343.9	MW: 318.13

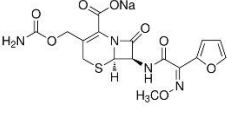
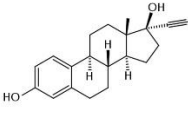
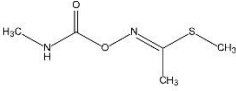
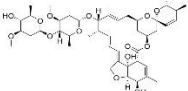
Inerts on the TH axis	
Cefuroxime sodium salt	EE2
	
MW: 446.37	MW: 296.40
Methomyl	Abamectin
	
MW: 165.23	MW: 873.08

Table S1: Structures of chemicals tested in the TETHYS assay. This table includes chemicals tested in Pesce et al., 2023 (*).

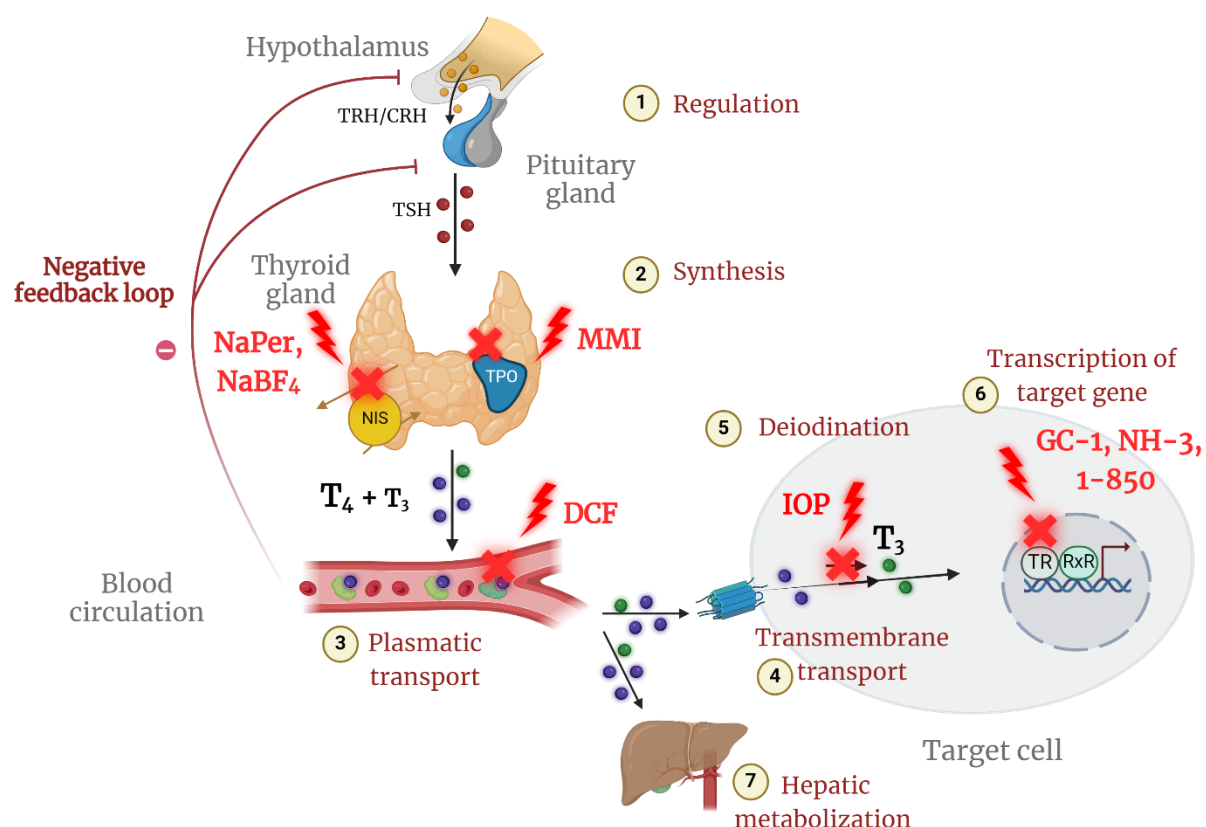


Figure S1: Thyroid hormone system signaling pathway and thyroid-disrupting chemicals tested in this study.

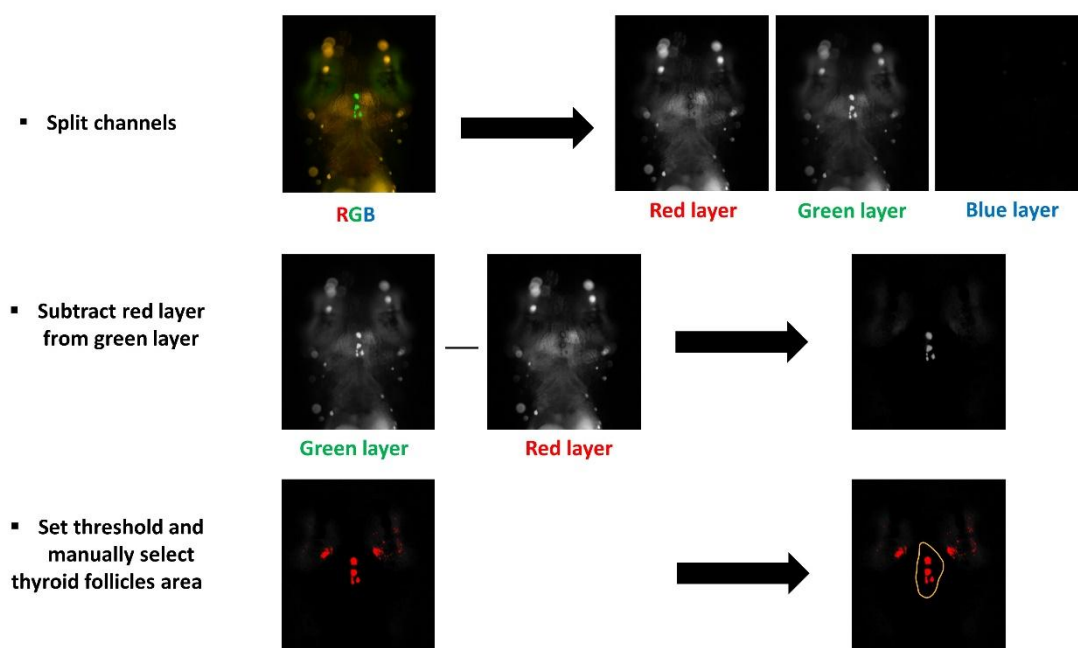


Figure S2: Image analysis pipeline using FIJI.

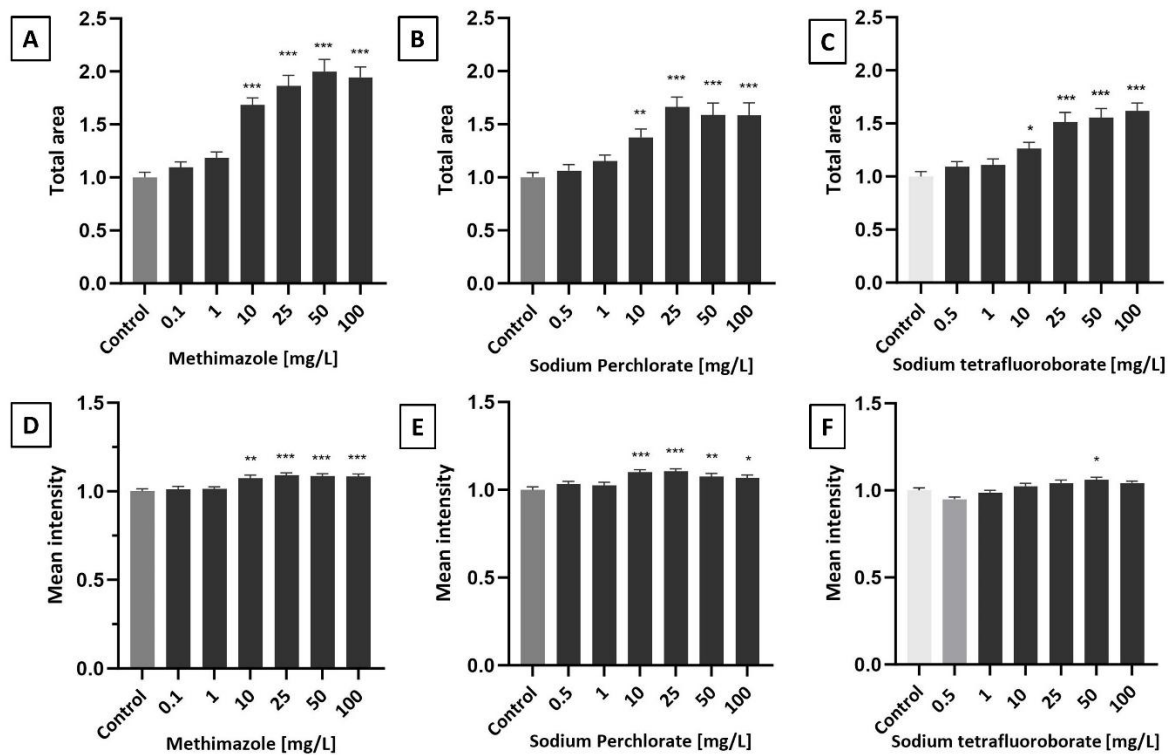


Figure S3: Mean of total area of thyroid follicles normalized to the control group for Tg(*tg:eGFP*) medaka eleuthero-embryos exposed to inhibitors of TH synthesis, MMI, NaPer, NaBF₄ (A,B,C) or mean intensity normalized to control group (D,E,F) for 72 hours, n= 42 (n=14 per run, with three independent runs performed and pooled). *: P<0.05; **: P<0.01; ***: P < 0.001. Mean ± SEM are given.

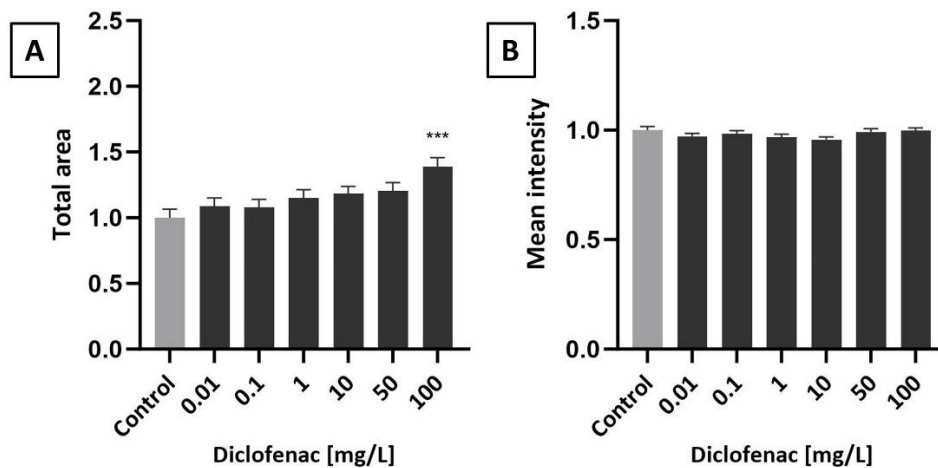


Figure S4: Mean of total area of thyroid follicles normalized to the control group for Tg(*tg:eGFP*) medaka eleuthero-embryos exposed to an inhibitor of TH binding to TH binding proteins, diclofenac sodium salt for 72 hours, n= 14 per group (n=14 per run, with three independent runs performed and pooled). ***: P < 0.001. Mean ± SEM are given.

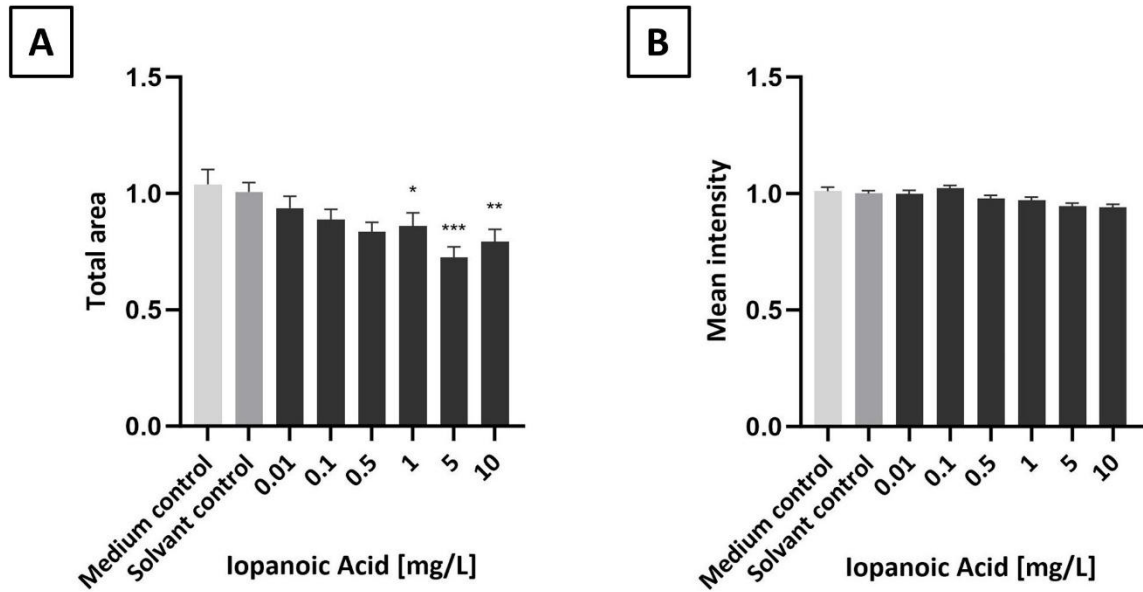


Figure S5: Mean of total area of thyroid follicles normalized to the control group for Tg(*tg:eGFP*) medaka eleuthero-embryos exposed to IOP (A) or mean intensity normalized to control group (B) for 72 hours, n= 42 (n=14 per run, with three independent runs performed and pooled). ***: P < 0.001. Mean \pm SEM are given.

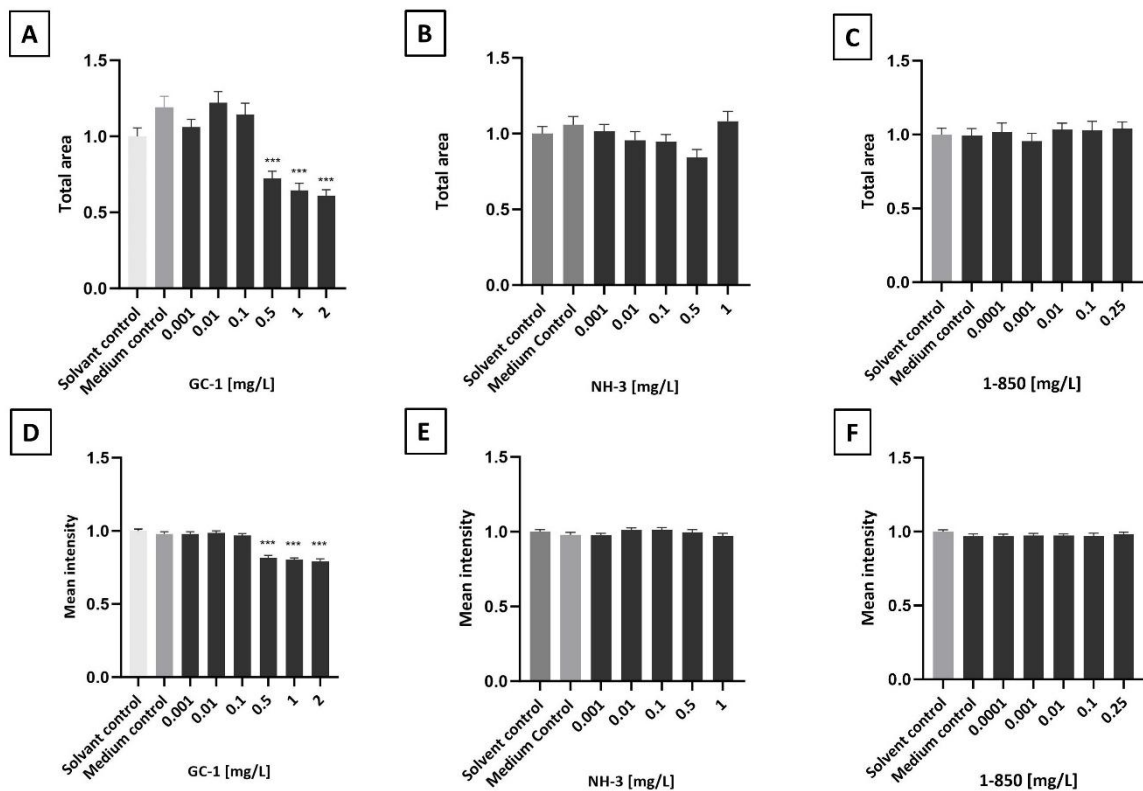


Figure S6: Mean of total area of thyroid follicles normalized to the control group for Tg(*tg:eGFP*) medaka eleuthero-embryos exposed to GC-1 (A), NH-3 (B) or 1-850 (C), or mean intensity normalized to control group (C,D,F) for 72 hours, n= 42 (n=14 per run, with three independent runs performed and pooled). ***: P < 0.001. Mean \pm SEM are given.

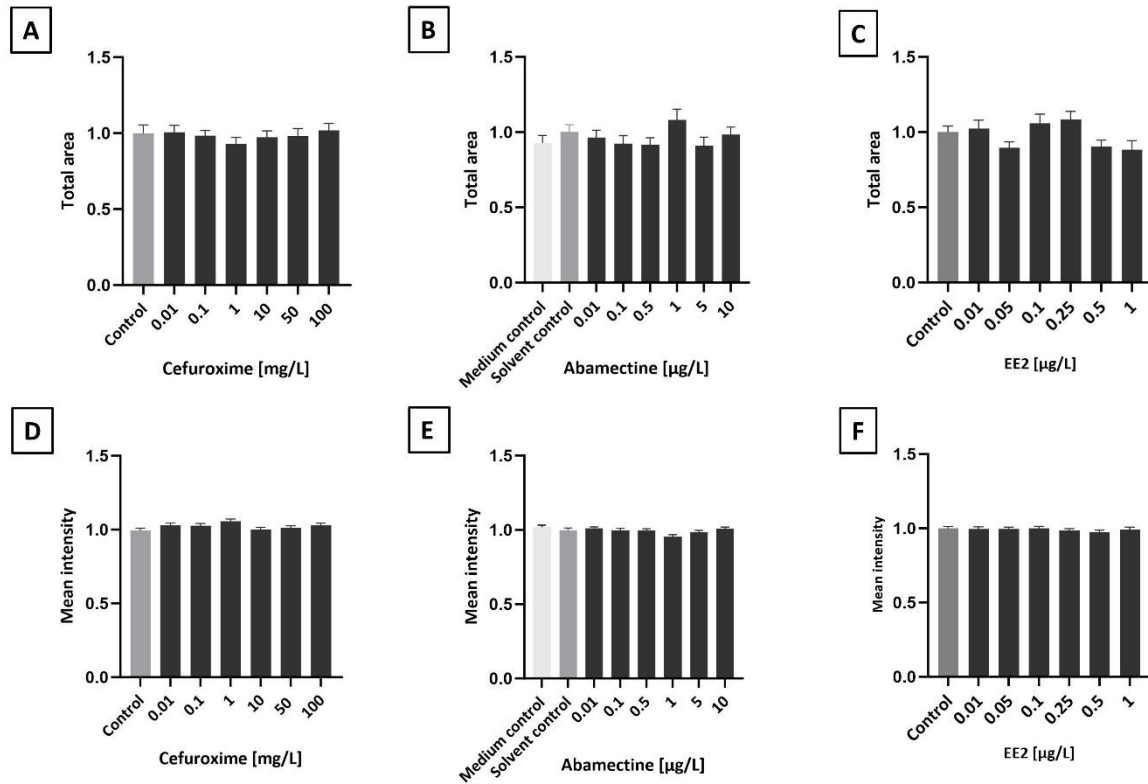


Figure S7: Mean of total area of thyroid follicles normalized to the control group for Tg(*tg:eGFP*) medaka eleuthero-embryos exposed to chemicals presumed to be inert on the thyroid axis, (A,B,C) or mean intensity normalized to control group (B,D,E) for 72 hours, n= 42 (n=14 per run, with three independent runs performed and pooled). ***: $P < 0.001$. Mean \pm SEM are given.