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# Simple, Rapid Zebrafish Larva Bioassay for Assessing the Potential of Chemical Pollutants and Drugs to Disrupt Thyroid Gland Function

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Thyroid function may be altered by a very large number of chemicals routinely found in the environment. Research evaluating potential thyroid disruption is ongoing, but there are thousands of synthetic and naturally occurring drugs and chemicals to be considered. European and United States policies call for the development of simple methodologies for screening endocrine-disrupting chemicals. Zebrafish are widely used as a model organism for assessing drug effects because of their small size, high fecundity, rapid organogenesis, morphological and physiological similarities to mammals, and ease with which large-scale phenotypic screening is performed. A zebrafish-based short-duration screening method was developed to detect the potential effect of chemicals and drugs on thyroid function. This method used a T4 immunofluorescence quantitative disruption test (TIQDT) to measure thyroid function. The 3 day exposure window protocol, from day 2 to day 5 postfertilization (dpf), avoided any potential side effects on thyroid gland morphogenesis. Methimazole, propylthiouracil, and potassium perchlorate, three well-known goitrogens, totally abolished T4 immunoreactivity in thyroid follicles in a dose-specific manner. Amiodarone, a human pharmaceutical with a reported cytotoxic effect on thyroid follicular cells, also decreased T4 levels. Moreover, exposure to 50 nM 3,3',5-triiodothyronine induced a significant decrease in T4 immunoreactivity as did DDT, 2,4-D, and 4-nonylphenol. In conclusion, these data indicated that TIQDT may be useful for obtaining initial information about the ability of environmental pollutants and drugs to impair thyroid gland function as well as assessing the combined effects of endocrine disruptors.

## Introduction

There is growing evidence that endocrine-disrupting compounds target the thyroid system (1–4). Concern about these thyroid disrupting chemicals (TDC) has increased because of the critical role played by thyroid hormones (TH) during

nervous system development (5, 6). Multiple steps in the complex TH regulatory network are disrupted by TDC. For instance, there are many examples of TDCs disrupting key processes involved in the synthesis of TH in thyroid follicular cells (thyrocytes) such as iodide uptake by the sodium–iodide symport or iodide organification by thyroid peroxidases (4, 7, 8). Binding TH to thyroid transport proteins is an important target for TDCs such as polychlorinated biphenyls and polybrominated diphenyl ethers (9, 10). Many classes of TDCs alter thyroid hormone homeostasis by inducing catabolic pathways, e.g., hepatic UDP-glucuronyl-transferases (11, 12). Furthermore, various xenobiotics are known to inhibit the hepatic outer ring deiodinase activity, involved in the metabolic activation of thyroxine (T4) to the biologically active 3,5,3'-triiodothyronine (T3) (7). Finally, some thyroid disruptors alter TH signaling through the thyroid receptors either directly by binding to the receptor or indirectly through interactions with other accessory proteins (13).

The complexity of TH function and regulation make it unlikely that a single assay will detect chemicals acting on any or all of these pathways (2, 14). The current endpoints proposed for thyroid toxicity in *in vivo* mammalian assays by the Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (U.S. EPA) include thyroid weight and histopathology as well as hormone measurements (T4 and TSH as well as, in some cases, T3). Thyroid weight and histopathology indicate stimulation by TSH over time. McNabb et al. (15) recently targeted a more sensitive and easily measured thyroid endpoint, which, however, requires further development and confirmation. Specifically, the T4 content of the bobwhite quail thyroid gland was measured in response to ammonium perchlorate exposure and was found to be far more sensitive to perchlorate exposure than either serum T4 concentration or thyroid weight (14, 15). Measuring intrathyroidal T4 may be an important and easily captured endpoint for thyroid toxicity as thyroid gland function integrates most of the potential action modes of TDC.

The origin and growth of the thyroid gland in zebrafish has been extensively studied and described (16–18). Whole-mount T4 immunohistochemistry and [<sup>125</sup>I] uptake were proposed as tools for assessing the thyroid gland activity in this species (19, 20). This paper describes the development of a zebrafish-based short-duration screening assay for chemicals and drugs that alter thyroid gland function. The assay is based on (1) 3 day exposure of 2 day postfertilization (dpf) zebrafish larvae to chemicals or drugs, (2) whole-mount T4 immunofluorescence detection of 5 dpf treated larvae, and (3) quantitative analysis of the T4 immunofluorescence signal assayed on thyroid follicles as a measure of thyroid function. The assay was developed using a group of compounds well-known to impair thyroid gland function by different mechanisms: methimazole (MMI), 6-n-propyl-2-thiouracil (PTuracil), potassium perchlorate (KClO<sub>4</sub>), amiodarone, and exogenous T3. MMI and PTuracil are goitrogens, which reduce TH synthesis by inhibiting thyroperoxidase enzyme function (21). KClO<sub>4</sub>, another goitrogen, inhibits iodide uptake through the follicular sodium–iodide symport (8). Amiodarone, an iodine-rich, benzofuranic derivative, widely used to treat tachyarrhythmias, has cytotoxic effects on thyrocytes (22, 23). Finally, administration of exogenous T3 induces thyroid toxicity via a secondary mechanism, the inhibition of TSH $\beta$  mRNA expression (24, 25).

After this initial step using model compounds, the assay was tested for its capacity to screen environmentally relevant

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compounds. 2,4-Dichlorophenoxyacetic acid (2,4-D), 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (DDT), and 4-nonylphenol (4-NP) were selected as they are usually considered to be TDCs (1, 7, 26–30). Reports concerning the effect of methylmercury (MeHg) on thyroid function are contradictory (31–33). Two environmentally relevant compounds with no reported effects on the thyroid system, fenoxycarb and atrazine (34–36), were included as negative controls to assess the specificity of the assay. Our data strongly suggest that the T4 immunofluorescence quantitative disruption test (TIQDT) may be used to assess exposure to environmental pollutants and as an *in vivo* bioassay for screening chemicals and drugs.

## Materials and Methods

**Zebrafish Embryo and Larva Maintenance.** Zebrafish (*Danio rerio*) embryos and larvae were obtained by natural mating and raised at 28.5 °C with a 12 L:12 D photoperiod in embryo water [90 µg/mL of Instant Ocean (Aquarium Systems, Sarrebourg, France), 0.58 mM CaSO<sub>4</sub>, and 2H<sub>2</sub>O, dissolved in reverse osmosis purified water]. Animal stages were recorded as dpf or hours postfertilization (hpf).

**Chemicals and Larva Treatment.** Amiodarone hydrochloride, dimethylsulfoxide (DMSO), MMI, MeHg (II) chloride, KClO<sub>4</sub>, PTuracil and T3 were purchased from Sigma-Aldrich (St. Louis, MO). Atrazine, 2,4-D, DDT, fenoxycarb, and 4-NP were from Riedel-de Haën (Seelze, Germany).

In order to avoid potential confounding factors, such as disruption of thyroid gland morphogenesis, chemical exposure was from 48 hpf to 120 hpf, ending before the first feeding. Thus, 48 hpf larvae were transferred to 24 well multiplates (1 larva per well in 2 mL medium) and exposed under semistatic conditions. Larvae were transferred to a new plate with freshly prepared test solutions every other day.

A first set of experiments were devoted to testing the suitability of our 3 day zebrafish-based assay to identify the effect of selected model TDCs on the thyroid gland. The following concentrations of TDCs were used in initial tests to check the performance of the assay: 1.5 mM MMI, 0.65 mM PTuracil, 1.98 mM KClO<sub>4</sub>, 1 µM amiodarone, and 50 nM T3. These concentrations of the first three compounds have been reported to abolish T4 immunoreactivity in the thyroid follicles of zebrafish larvae after 5 days of treatment (20). The concentrations of amiodarone and exogenous T3 used have been reported to affect TH-regulated genes in zebrafish larvae (37). In this set of experiments, eight larvae per condition were exposed on two 24 well microplates (four wells per compound and four additional wells for the control larvae in each microplate, and five larvae per condition were randomly selected for TIQDT.

The second stage consisted of three independent dose-response experiments using the following concentrations of goitrogens: 0.01, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 mM MMI; 0.01, 0.06, 0.18, 0.29, 0.41, 0.53, and 0.65 mM PTuracil; and 0.01, 0.18, 0.54, 0.90, 1.26, 1.62, and 1.98 mM KClO<sub>4</sub>. The concentrations of the three compounds used in the dose-response experiments ranged from the highest concentration with no effect to the lowest concentration that totally abolished T4 immunostaining in 5 dpf zebrafish. In each experiment, eight larvae were exposed for each compound and concentration as described above, and six larvae per condition were randomly selected for TIQDT.

Finally, the ability of the assay to screen environmentally relevant compounds was tested using the following concentrations of chemicals: 0.2, 2.3, and 23.2 µM atrazine; 0.2, 2.5, and 24.9 µM 2,4-D; 0.1, 1.4, and 14.1 µM DDT; 0.2, 1.7, and 16.6 µM fenoxycarb; 0.2, 2.3, and 22.7 µM 4-NP; and 0.2, 2.0, and 19.9 µM MeHg. The atrazine, 2,4-D, DDT, and 4-NP concentrations selected were in the low range of those used

recently for neurotoxicity screening in zebrafish (38). For this screening, eight larvae were exposed per compound and concentration as described above, and a minimum of five larvae per condition were randomly selected for TIQDT.

All dilutions are reported as nominal concentrations. 1000x stock solutions of the various compounds were prepared in DMSO on the day of the experiment. Embryos exposed to 0.1% DMSO were used as a vehicle control. All concentrations tested for MMI, PTuracil, KClO<sub>4</sub>, amiodarone, T3, atrazine, 2,4-D, fenoxycarb, MeHg, and 4-NP were below the water solubility values indicated in the SRC PhysProp Database ([www.syrres.com/esc/physprop.htm](http://www.syrres.com/esc/physprop.htm)). Although no precipitate was observed in the range of DDT concentrations used, values were above its reported water solubility. Nevertheless, in most of the cyprinid toxicology studies reported in the ECOTOX DataBase ([http://cfpub.epa.gov/ecotox/quick\\_query.htm](http://cfpub.epa.gov/ecotox/quick_query.htm)), DDT exhibits a linear dose-response, even at concentrations well above its reported water solubility.

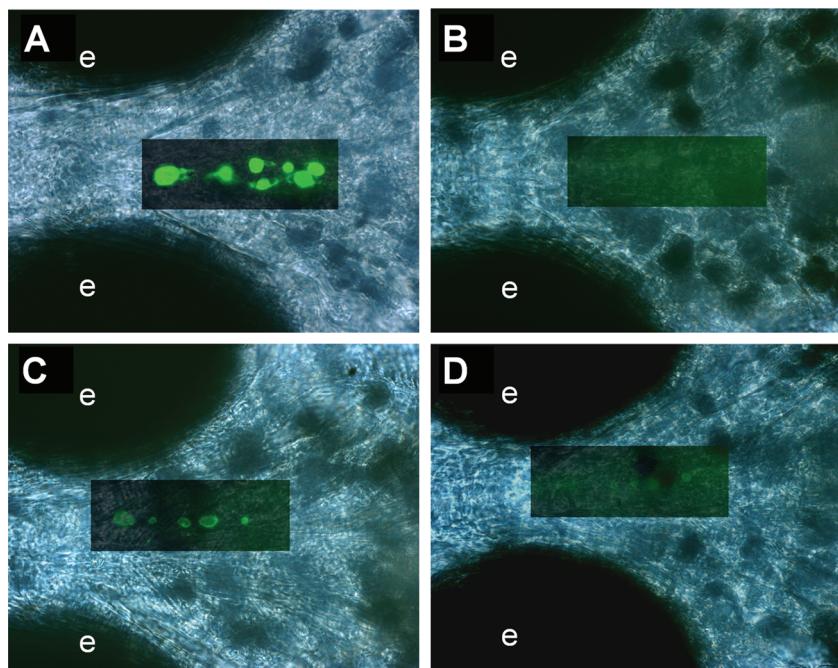
Cumulative mortality at the end of the exposure period was assessed for the various concentrations of MMI, PTuracil, KClO<sub>4</sub>, atrazine, 2,4-D, DDT, fenoxycarb, 4-NP, and MeHg. The maximum tolerated concentration (MTC) was defined as the concentration for which no lethality was observed above that seen in vehicle treated siblings (39). The seven concentrations selected for the dose-response study using the three goitrogens and the MTC of each compound used in the screening of environmentally relevant compounds were selected for TIQDT.

**Whole-Mount Immunohistochemistry.** Larvae were fixed in 4% paraformaldehyde at 4 °C overnight, washed several times in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 0.02 M PO<sub>4</sub>), gradually transferred to 100% methanol, and stored at –20 °C until analysis. Whole-mount immunohistochemistry in control and chemical-exposed larvae was performed using standard methods (40) ([http://zfin.org/zf\\_info/zfbook/zfbk.html](http://zfin.org/zf_info/zfbook/zfbk.html)). After rehydration, larvae were pretreated with 0.1% collagenase (Sigma C-9891) in PBS at room temperature for 15 min, immersed in blocking a buffer (4% goat serum, 1% BSA, 1% DMSO, 0.8% Triton X-100, and 0.1% Tween-20 in PBS) for 2 h, and incubated overnight at 4 °C in 1:4000 diluted primary antibody against T4 (polyclonal rabbit anti T4, MP Biochemicals, Illkirch, France). Larvae were washed with a blocking buffer and incubated with a fluorescent secondary antibody (1:300 dilution, Alexa Fluor 488, Invitrogen, Eugene, OR) at room temperature for 2 h.

**Microscopy and Image Capture.** Differential interference contrast and fluorescence images were obtained using a Nikon Eclipse E1000 (Nikon, Champigny sur Marne, France) microscope fitted with Nomarski optics and a Nikon Intensilight C-HGFI unit. Images were acquired with a Nikon DXM1200 camera and LUCIA G software (version 4.81) and saved as high-resolution (3840 pixels × 3005 pixels) tagged image file format (TIFF). All image series for quantification were taken the same day, using the same settings.

**Linearity of Detection System and Lamp Emission Stability.** A FocalCheck fluorescence microscope test slide 1 (Invitrogen) was used for checking the linearity of the microscope detection system (Figure S1 Supporting Information (SI)). All the average pixel intensity (API) values determined for the T4 immunofluorescence signals of our biological samples were inside of the linear range of the detection system. Periodic imaging of microbeads, using the same acquisition parameters, was used to evaluate the microscope excitation source stability by plotting the intensity signal of the beads over time (Figure S1 of the Supporting Information). No difference in API was observed ( $P > 0.05$ ), demonstrating the stability of the excitation source.

**Quantitative Analysis of Whole-Mount T4 immunofluorescence Signal.** Quantitative analysis of whole-mount T4 immunofluorescence signals was performed following the



**FIGURE 1.** Whole-mount T4 immunofluorescence staining superposed on brightfield illumination of thyroid follicles in (A) 5 dpf control (0.1% DMSO used as a vehicle control), (B) 1.5 mM MMI-treated larvae, (C) 1  $\mu$ M amiodarone-treated larvae, and (D) 50 nM T3-treated larvae. The protocol used a 3 day exposure window, from 2 dpf to 5 dpf. Heads of representative larvae are shown in ventral view with the anterior part on the left. Abbreviation: e, eye.

protocol summarized in Figure S2 of the Supporting Information, using free-processing ImageJ software [National Institutes of Health (NIH), <http://rsb.info.nih.gov/ij/>].

**Statistical Analyses.** Statistics were analyzed using SigmaStat 3.1 software (Systat Software Inc., San Jose, CA). Values are presented as mean  $\pm$  SEM. Normality of the distributions was tested with the Kolmogorov–Smirnov test. The assumption of equal variances for all data sets was checked using the Bartlett test. The significance of the intergroup differences was determined by one-way ANOVA followed by the post hoc Dunnett multiple comparison test. The significance level was set at 0.05.

## Results

T4 whole-mount immunofluorescence identified the effects of model TDC on thyroid gland function in a 3 day zebrafish-based bioassay. First, we checked whether the design of this short-duration screening assay, using a 3 day exposure period, starting at 2 dpf, produced detectable changes in the T4 immunofluorescence signal in thyroid follicles after TDC exposure. In zebrafish, the first differentiated thyroid follicle appears around 2.5 dpf, and thyroid follicular tissue then grows along the ventral pharyngeal midline. By 5 dpf, the second phase of thyroid relocation has produced follicles oriented longitudinally along the ventral aorta, from the second pharyngeal arch element, the ceratohyal, to ceratobranchial III. In control larvae, these follicles exhibited a T4 immunofluorescence signal (Figure 1A). Treatment with 1.5 mM MMI completely abolished T4 immunoreactivity (Figure 1B) as did 0.65 mM PTuracil and 1.98 mM KClO<sub>4</sub> (data not shown). Larvae exposed to 1  $\mu$ M amiodarone showed a clear decline in T4 immunoreactivity (Figure 1C). Treatment with 50 nM T3 also induced a marked reduction in the T4 immunoreactivity of the thyroid follicles (Figure 1D).

Our short-duration TIQDT was then used to perform dose–response experiments with three well-known goitrogens: MMI, PTuracil, and KClO<sub>4</sub>. Thyroid T4 content as indicated by T4 immunoreactivity decreased significantly

at increasing goitrogen concentrations (Figure 2). In terms of thyroid toxicity, PTuracil exhibited an extremely steep dose–response curve: 0.18 mM induced total abolition of T4 immunoreactivity, while 0.06 mM caused no measurable effects. The No Observed Effect Concentration (NOEC) for MMI and KClO<sub>4</sub> was 0.01 mM, whereas concentrations of 0.75 and 0.18 mM, respectively, induced total abolition of T4 immunoreactivity. Neither phenotypic effect nor mortality were detected at any of the goitrogen concentrations used in the dose–response experiments.

TIQDT was then performed after exposing larvae to well-known environmental pollutants. Three concentrations of each compound, 0.05, 0.50, and 5.00 mg/L, were evaluated for lethality. The MTC of each chemical compound was then selected for TIQDT (Table S1 of the Supporting Information). Water supplemented with 0.1% DMSO, used as a chemical vehicle, had no discernible effect on larval development (Figure S3A of the Supporting Information). The morphology of overall animals exposed to the chemicals at the following concentrations was generally well preserved: 23.2  $\mu$ M atrazine, 24.9  $\mu$ M 2,4-D, and 2.3  $\mu$ M 4-NP (Figure S3B,C,F of the Supporting Information). However, larvae exposed to 1.4  $\mu$ M DDT and 0.2  $\mu$ M MeHg exhibited lethargic behavior, associated with a deflated swimming bladder (Figure S3D,G of the Supporting Information). This latter trait was also observed in those incubated with 1.7  $\mu$ M fenoxy carb (Figure S3E of the Supporting Information). This compound, together with 4-NP and MeHg, induced kyphosis and a tendency to slower yolk sac resorption (Figure S3E–G of the Supporting Information). MMI, included as a goitrogen TIQDT control, did not induce any morphological effect at the selected dose of 1.5 mM (Figure S3H of the Supporting Information).

Our data demonstrated that, even if the number of thyroid follicles varied from one individual to another, the intragroup variability of API was small enough to detect statistically significant effects for some of the compounds applied to the target tissue (Figure 3). Whole-mount TIQDT demonstrated a significant decrease ( $P < 0.05$ ) relative to control in the T4 immunoreactivity of thyroid follicles, following exposure to

2,4-D, DDT, and 4-NP (Figure 3). API decreased from  $18.29 \pm 2.69$  in control larvae versus  $9.20 \pm 2.40$  in 2,4-D-,  $8.68 \pm 3.36$  in DDT-, and  $8.98 \pm 1.31$  in 4-NP-treated larvae. The presence of a limited amount of T4 immunofluorescence signal in several follicles located in the outflow tract region of the heart in TDC-treated larvae demonstrated that there was no disruption of the presence and alignment of thyroid follicles along the anteroposterior axis in the hypopharyngeal area. As previously described (Figures 1B and 2), exposure to 1.5 mM MMI completely abolished the T4 immunoreactivity of thyroid follicles.

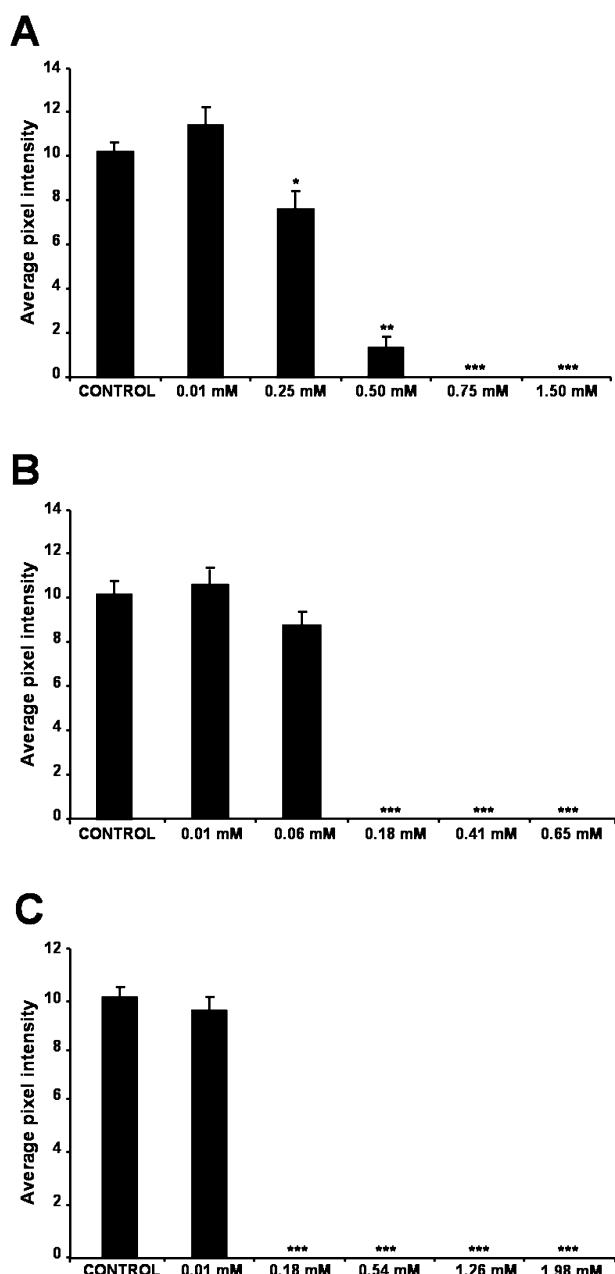
## Discussion

A simple and rapid zebrafish larva screening assay has been developed to detect compounds with the potential to disrupt thyroid gland function. The endpoint selected for the assay was thyroid T4 content, assessed using TIQDT. In bobwhite quail chicks exposed to  $\text{KClO}_4$ , thyroidal T4 content was demonstrated to be a more sensitive index of thyroid gland function than plasma thyroid hormone concentrations or thyroid weight (15). This endpoint also offers an alternative, less labor-intensive, more easily quantified assessment of thyroid gland function than histological examinations (15) and has been recently proposed as an important and easily obtained endpoint for thyroid toxicity (14).

Quantitative immunohistochemistry, based on chromogenic and fluorescence techniques, is currently widely used, e.g., in neurosciences (41, 42). Immunofluorescence signals are more sensitive (43, 44) and were recently used in neurotoxicity screening on whole-mount zebrafish embryos to assess the effect of pollutants on axon tracts (38). In this study, we demonstrated that it was possible to obtain quantitative data on thyroid follicles, using whole-mount T4 immunofluorescence imaging. TIQDT used a microscope equipped with a precentered fiber mercury lamp illuminator and an image selection process similar to a previously published procedure (44).

The 3 day exposure window protocol has two major advantages. First, starting exposure at 2 dpf avoids any potential side effects of the chemicals on thyroid gland morphogenesis. During normal zebrafish development, immunostaining against T4 revealed a first thyroid follicle at around 55 hpf (16, 17), but early exposure to some compounds, like the pharmaceutical clofibrate, disrupts the second phase of thyroid relocation by impairing elongation of the ventral aorta (45). Second, as exposure ends at 5 dpf, the protocol is considered a nonanimal-based alternative method by Directive 86/609/EEC.

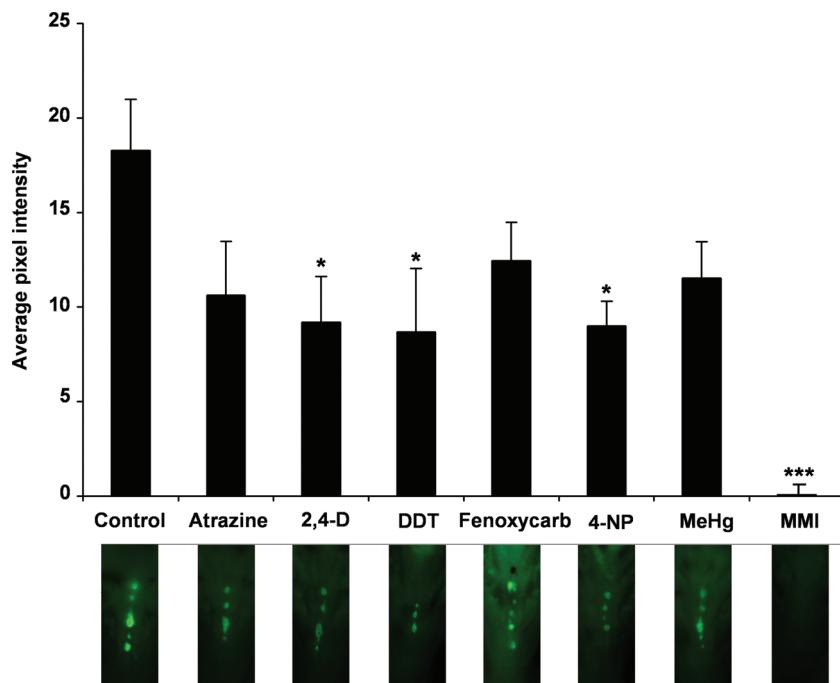
Several compounds well-known to impair thyroid gland function by different mechanisms were selected for initial development of the TIQDT procedure. Our results with goitrogens, MMI, PTuracil, and  $\text{KClO}_4$ , and amiodarone, a thyrotoxic compound, demonstrated that a 3 day exposure of zebrafish larvae was long enough to induce a significant decrease in the T4 content of thyroid follicles. Another concern was when the thyroid axis started to function during zebrafish development. Chemicals acting on the thyroid gland as a secondary mechanism will only be detected in the bioassay if the thyroid axis is functional as early as 48–120 hpf (24). In vertebrates, it is clearly established that thyroid hormones (T4 and T3) exert a negative feedback on pituitary TSH release and hypothalamic TRH neuron activity (14). It has been demonstrated that exogenous TH depresses the uptake of radioiodide by the zebrafish thyroid gland, presumably by suppressing thyrotropin synthesis in the pituitary (19). Several studies in teleost fish (25) have consistently shown that *in vivo* administration of exogenous T3 or T4 resulted in substantial inhibition of TSH $\beta$  expression. TIQDT revealed that treatment with exogenous T3 induced



**FIGURE 2.** Increasing doses of the following goitrogens: (A) MMI, (B) PTuracil, and (C)  $\text{KClO}_4$  induced a sharp decrease in T4 immunoreactivity in the thyroid follicles of 5 dpf zebrafish larvae exposed for 3 days. Animals exposed to 0.1% DMSO were used as a control. Columns represent the arithmetic mean of API measured in thyroid follicles from three independent experiments, with six larvae analyzed per experiment. Symbols: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\* no signal detected.

a marked decrease in follicular T4 immunoreactivity. Although we did not measure TSH levels directly, our data suggest that T3 negative feedback on the thyroid axis was already functional during the developmental window selected for the bioassay.

The results of the dose-response experiments using goitrogens show that the sensitivity of our 3 day screening assay is at least as good as the 5 day assay previously described by Elsalani and Rohr (20). NOEC and the lowest concentration inducing the total abolition of T4 immunostaining after MMI exposure were 0.3 and 1.5 mM using the 5 day protocol and 0.01 and 0.75 mM in the present study, respectively. Following the TIQDT protocol, T4 immunoreactivity of the larvae exposed to 0.25 mM MMI, a concentration under the NOEC



**FIGURE 3.** TIQDT-screening of environmentally relevant compounds for their ability to disrupt thyroid gland function. API of T4 immunofluorescence signals in thyroid follicles from 5 dpf zebrafish larvae treated with 2,4-D, DDT, 4-NP, and MMI exhibited a significant decrease, compared to that of animals exposed to 0.1% DMSO used as a vehicle control. Toxicant concentrations were the same as those in Figure S3 of the Supporting Information. Images below every column show T4 immunofluorescence signals of thyroid follicles in the larva with an API value close to the average. Images are ventral views with the anterior part at the top. Symbols: \*,  $P < 0.05$  and \*\*\*, no signal detected. The number of larvae analyzed per compound were as follows: five for atrazine, DDT, and MMI; six for 2,4-D and 4-NP; and seven for control, fenoxy carb, and MeHg.

for the 5 day assay, was about 20% lower than in the control. Consistent with our previous results, exposure of zebrafish to 1 mM MMI from 4 to 20 dpf inhibited the incorporation of radiolabeled iodide into thyroid hormone by 98% (19). NOEC and the lowest concentration inducing total abolition of T4 immunostaining after PTuracil exposure were 0.001% and 0.01% (10 and 100 mg/L) in the 5 day assay (20), and 0.001% and 0.003% (10 and 30 mg/L) in the present study, respectively. Other available data on adult zebrafish found that the low-observed effect concentration (LOEC) for thyroid histology in animals exposed to PTuracil for 3 weeks was 10 mg/L, although the effects were clearest at 100 mg/L (46). The results of experiments using 3 week old animals showed a similar sensitivity in detecting thyroid toxicity to those obtained in nonanimal based 3 day TIQDT assay. NOEC and the lowest concentration inducing the total abolition of T4 immunostaining after  $KClO_4$  exposure were 0.0025% and 0.025% (25 and 250 mg/L) in the 5 day assay and 0.0001% and 0.0025% (1 and 25 mg/L) in the present study, demonstrating that TIQDT is a highly sensitive method for detecting the thyroid toxicity of perchlorate. Several previous studies have used different endpoints to assess the thyroid toxicity of perchlorate in adult zebrafish. Thus, after an 8 week exposure to 18 mg/L of perchlorate, an environmentally relevant concentration, disrupted histology of the thyroid follicles was associated with hypertrophy, angiogenesis, hyperplasia, and colloid depletion (47). TIQDT showed a similar sensitivity to this compound, with total abolition of T4 immunoreactivity after exposure to 25 mg/L for only 3 days. Perchlorate in water has not been clearly demonstrated to accumulate in whole-body zebrafish (48). However, the possibility of tissue-specific accumulation of perchlorate in thyroid follicles has not been eliminated. In fact, the sensitivity of the different endpoints in zebrafish to perchlorate and other goitrogens increases with exposure time. Thus, the LOEC for the colloidal T4 ring, the most sensitive endpoint in that study, was 1131 and 11 ppb after 2 and 12 week

exposure, respectively. Measuring the colloidal T4 ring in adult zebrafish after a 12 week perchlorate exposure is thus several orders of magnitude more sensitive than TIQDT. However, considering that 12 weeks constitutes a generation for zebrafish (from egg to egg), this may be considered a chronic assay, and the cost and time involved make it unsuitable for a Tier I screening procedure.

In the OECD Test Guidelines, the term “screening assay” refers to protocols designed to obtain initial information on the ability of a compound to interfere with the thyroid system (14). In contrast, the term “test” or “testing assay” describes protocols aimed at establishing whether a substance affects the thyroid system, determining the consequences for the organism studied, and establishing a dose-response relationship between the substance and the effect observed in the test. For this reason, in our initial testing of the suitability of TIQDT for screening environmentally relevant pollutants, we decided to select only one concentration per compound instead of more extensive testing to generate dose-response curves for each substance. Similarly, recent screening using zebrafish embryos to detect pharmacological and toxicological effects, only one concentration was tested for each compound (38, 49, 50). It was, therefore, very important to choose the right concentration. On the one hand, testing a high but sublethal concentration was likely to increase the number of false positives as any effect on thyroid function would potentially be nonspecific, i.e., an indirect effect of systemic toxicity. On the other hand, testing low concentrations was likely to increase the number of false negatives. Our main concern in designing screening for thyroid disruptors was to avoid false negatives, so TIQDT was carried out using atrazine, 2,4-D, DDT, fenoxy carb, 4-NP, and MeHg at MTC.

Consistent with previous studies on mammals and teleosts, the thyroid gland T4 content decreased significantly in zebrafish larva treated with 2,4-D, DDT, and 4-NP. Wistar rats exposed to 2,4-D had significantly lower serum levels of

T4 and T3 (26), while rats exposed to the three chemical isomers of this herbicide all exhibited lower circulating TH levels and thyroid weights (27). The insecticide DDT has been classified as an environmental factor capable of causing thyroid dysfunction (1). DDT inhibits TSH-stimulated cAMP production *in vitro* (28, 29). Mullet (*Liza parsia*) exposed to DDT exhibited a decrease in thyroid epithelial cell height, degeneration of epithelial cells, and depletion of the colloid (30). *In vitro* studies have shown that 4-NP has a direct effect on thyroid function (7). After 5 days of incubation with 10 μM 4-NP, the iodine uptake of FTRL-5 cells, a model of normal nontransformed rat thyrocytes, was significantly inhibited. 4-NP also inhibited human TPO (7). Moreover, *in vivo* studies in rats demonstrated that treatment with low and high doses of 4-NP reduced the epithelium versus colloid ratio by about 50% (7). 4-NP is suspected to induce a hypothyroid condition in exposed fish (51). Other chemicals enrolled in TIQDT, i.e., atrazine, fenoxy carb, and MeHg, did not significantly alter thyroid follicle T4 signals. No differences were observed in TSH, T4, or thyroid histology in male Wistar rats treated with atrazine or its metabolites (34, 35). Fenoxy carb is a juvenile hormone-like insecticide, with a chemical structure resembling that of TH (36), but there are no previous data about the potential effects of this xenobiotic on the thyroid gland. Reports concerning the effect of MeHg on thyroid function are contradictory. Plasma T4 and T3 levels increased in rainbow trout (*Oncorhynchus mykiss*) (31) and decreased in catfish (*Clarias batrachus*) (32) after exposure to MeHg. This contaminant induced flattened thyroid epithelial cells in rats but had no effect on TPO (33).

It is important to note that TIDQT results indicate thyroid gland function status and not the modality of action of the molecule tested, i.e., specific or nonspecific, direct or indirect. For instance, the concentrations of DDT and 4-NP used in the screening also induced other phenotypic effects unrelated to thyroid toxicity, including deflated swimming bladder, kyphosis, and slower yolk resorption. Thus, although the decrease in follicular T4 content detected by TIDQT following exposure to these two compounds is consistent with other findings, it is not possible to infer from TIQDT whether the effect on the thyroid system is direct or indirect because of systemic toxicity. Concentration-response data would be very useful in addressing this concern in cases of overlaying general toxicity.

In conclusion, the results presented here suggest that prefeeding zebrafish larvae may be used as an early warning model for identifying environmental pollutants and drugs that have a disrupting effect on thyroid gland function. This simple, rapid, cost-effective test represents a promising alternative, innovative method for screening environmental pollutants and drugs. The zebrafish, a model organism of vertebrate development and organogenesis, is receiving increasing attention as a model for human disease, drug discovery, and toxicological studies (52, 53). One major advantage of the zebrafish larva model is that the complex dynamic, interactive, multiorgan events that occur *in vivo* remain intact. Our initial results suggest that TIQDT may be suitable for inclusion in a Tier I (54) screening battery.

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## Supporting Information Available

Figures of quality control of quantitative image analysis, quantitative analysis of whole-mount T4 immunofluorescence signals, and phenotypes and a table listing the cumulative percentage mortality of zebrafish embryos and larvae exposed to increasing concentrations of selected environmental pollutants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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