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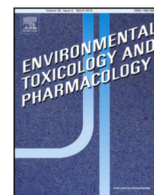

# Thyroid endocrine disruption of azocyclotin to *Xenopus laevis* during metamorphosis

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ABSTRACT

Organotin compounds are ubiquitous contaminants that are frequently detected in the environment and in biota, which raises concern about their risk to wildlife and human health. In the present study, Nieuwkoop & Faber stage 51 *Xenopus laevis* tadpoles were exposed to different concentrations of azocyclotin (0, 0.02, 0.1 and 0.5 μg/L) for 21 days, during which time the tadpoles underwent morphological development. Exposure to azocyclotin caused an inhibitory effect on the pre-metamorphic development of *X. laevis* (e.g., a shortened hind limb length). Azocyclotin induced an alteration of the triiodothyronine ($T_3$) content, which indicated thyroid endocrine disruption. Real-time PCR was performed to examine the expression levels of the genes involved in the thyroid hormone (TH) signaling pathway. Significant down-regulation of the *type 2 deiodinase* gene was observed, which may be partially responsible for the decreased $T_3$ concentrations. Furthermore, the expression of $T_3$ responsive genes, including *thyroid hormone receptor, basic transcription element binding protein, 2tromelysins-3* and *matrix metalloproteinase 2*, were down-regulated in tadpoles, suggesting that azocyclotin induced a decrease in the $T_3$ contents and, in turn, affected the mRNA expression of downstream genes involved in multiple physiological responses. Chemical analysis showed that azocyclotin could accumulate in *X. laevis* after 21 days of exposure. In conclusion, the results of the present study showed that azocyclotin could alter the mRNA expression of genes involved in TH signaling as well as the thyroid hormone concentrations in *X. laevis* tadpoles, leading to endocrine disruption of thyroid system, and that azocyclotin had obvious inhibitory effects on *X. laevis* metamorphosis.

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## 1. Introduction

Over the last 50 years, organotin compounds (OTCs) have been used in a broad range of applications for consumer and industrial products (Hoch, 2001), but they have mainly been used as biocides in agriculture for wood or crop protection and in antifouling paints to protect ships (Furdek et al., 2012). Consequently, considerable amounts of OTCs have entered various ecosystems due to complex transport processes, and currently, OTCs are ubiquitous environmental contaminants (Kördel and Stein, 1997). OTCs may have entered the aquatic environment via spray drift, runoff or leaching, and undesirable effects of this harmful substance will pose a risk for aquatic organisms (Kördel and Stein, 1997). OTC concentrations are generally at (ng Sn)/L levels in water samples (Radke et al., 2013; Furdek et al., 2012). OTCs have been detected in sediments as well, and here, they can reach up to 3161 ng cation/g dry weight (d.w.), as reported for the Gdynia Harbor (Radke et al., 2013).

Many OTCs act as endocrine disruptors of non-target organisms, disturbing the enzyme mediated conversion of steroid hormones (Appel, 2004). Previous studies have shown that OTCs have high toxicity to embryos of various organisms (Wu et al., 2014; Higley et al., 2013). For example, triphenyltin and tributyltin could induce multiple teratogentic effects in *Xenopus tropicalis* embryos at environmentally relevant concentrations (Guo et al., 2010; Yu et al., 2011). In addition, a study suggested that triphenyltin significantly affected survival and metamorphic development in *Lithobates sylvaticus* larvae (Higley et al., 2013). Tributyltin can delay metamorphosis, cause growth inhibition in larvae, and disrupt gonadal differentiation in *Xenopus laevis* at environmentally relevant concentrations (Shi et al., 2014). Although these studies demonstrate the disruptive effects of OTCs on metamorphic development in amphibians, the impact of OTC exposure on *X. laevis* metamorphosis remains poorly understood, particularly with regard to thyroid hormone (TH) signaling. The metamorphosis of anuran amphibians is controlled by a complex interaction of several hormones,

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and thyroid hormone is believed to be the major stimulatory hormone that induces metamorphosis (McDiarmid and Altig, 1999; Furlow and Neff, 2006; Tamura et al., 2015). Thyroid hormone signaling is suggested to be involved in the chemical-induced toxicity of metamorphosis in amphibians (Shi et al., 2014). Thus, the metamorphosis of the tadpole is a good experimental model to screen chemicals that may interfere with the function of the thyroid system and the hypothalamic–pituitary–thyroid (HPT) axis (OECD, 2009).

Azocyclotin [tri(cyclohexyl)-1*H*-1,2,4-triazol-1-yltin] has been used extensively as a biocide and acaricide in many agricultural applications due to its biocidal properties (Niu et al., 2011). The mode of action of azocyclotin is to disrupt ATP formation by inhibiting oxidative phosphorylation (Van Leeuwen et al., 2010). A previous study reported that azocyclotin (135 μg/L) that was applied as a soil slurry had severe effects on biocenosis under experimental conditions (Fliedner et al., 1997). Azocyclotin may also be a respiratory inhibitor and is one of the most toxic compounds to fish (i.e., $LC_{50} < 0.1$ mg/L). Azocyclotin can be quickly hydrolyzed to cyhexatin after application. The metabolite cyhexatin is also a non-systemic acaricide that was banned in 1987 in China, and it may be more toxic than the parent compound (Sinclair and Boxall, 2003). However, there is limited information currently available on the endocrine disruptive effects and mechanisms of azocyclotin (Sánchez-Bayo, 2012). Agricultural land provides habitation for many amphibian species, and previous studies provide strong evidence that agricultural chemicals have the potential to affect amphibian populations (Mann et al., 2009; Wagner et al., 2013; Brühl et al., 2011). Given that azocyclotin has been extensively used in many agricultural applications, azocyclotin could pose a risk to amphibians. Therefore, in the present study, we used *X. laevis* tadpoles that were undergoing metamorphosis to evaluate the effects of azocyclotin on TH-dependent development and the underlying mechanisms of TH disruption.

## 2. Materials and methods

### 2.1. Materials and reagents

Azocyclotin [tri(cyclohexyl)-1*H*-1,2,4-triazol-1-yltin] was purchased from the Zhejiang Heben Pesticide & Chemicals Co., Ltd. (CAS: 41083-11-8; 95% purity). These chemicals were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and stored at −20 °C. Azocyclotin standard was obtained from the Zhejiang Heben Pesticide & Chemicals Co., Ltd. (CAS: 41083-11-8; 95% purity). Trizol reagent and a thyroid hormone enzyme-linked immunosorbent assay kit were obtained from Invitrogen (Carlsbad, CA USA) and IBL-America, respectively.

### 2.2. Animals and chemical exposure protocol

*X. laevis* maintenance and tadpole exposure were performed according to the manufacturer's guidelines for the Amphibian Metamorphosis Assay (AMA; OECD, 2009). Briefly, five pairs of adult females and males were held in large tanks and injected with approximately 900 IU and 600 IU of human chorionic gonadotropin (hCG), respectively. The hCG was dissolved in a 0.6% saline solution. The bottom of each tank had a plastic mesh to permit the egg masses from falling to the bottom of the tank. Adults were injected in the late afternoon, and eggs were obtained at mid-morning on the next day. The eggs were maintained and then developed into stage 51 tadpoles within 17 days after fertilization. During the pre-exposure phase, tadpoles were acclimated to the conditions of the exposure phase in glass beakers containing charcoal-filtered tap water. The test was conducted at 22 ± 2.0 °C, and the day-night cycle was 12 h light and 12 h dark. The water quality parameters were pH 7.0 ± 0.5, less than 5 μg/L chlorine and chloramines, less than 0.2 mg/L ammonia, and more than 4 mg/L of dissolved oxygen. Nieuwkoop & Faber (NF) stage 51 (Nieuwkoop and Faber, 1956) *X. laevis* tadpoles were exposed to different concentrations of azocyclotin (0, 0.02, 0.1 and 0.5 μg/L) for 21 days, during which time the tadpoles were undergoing pre-metamorphic development (OECD, 2009). At the termination of the test, the minimum median developmental stage of the controls reached stage 57, and the stage distribution differed by less than 4 stages. Exposure concentrations of azocyclotin were selected based on a range-finding study to determine the concentrations that slightly decreased the survival rates. The control groups received 0.01% DMSO (v/v). Four replicates of each treatment were performed in 10-L glass beakers that each contained 6 L of the exposure solutions. For all of the treatment groups, the exposure solutions were renewed daily, and the tadpole density was 20 tadpoles per glass beaker. Tadpoles were fed with small portions of Artemia three times every day during the pre-exposure period and during an exposure period of 21 days. The survival and malformation rates, hind limb length, snout to vent length (SVL) and developmental stage were recorded during the exposure period. During the 21-day exposure, a total of 20 tadpoles for each replicate were humanely euthanized in 180 mg/L MS-222 and sampled for body wet weight, chemical analysis, mRNA expression and thyroid hormones measurement.

### 2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from three tadpoles that were randomly chosen from every glass beaker with RNAiso plus (Takara, Dalian, China) following the manufacturer's instructions, and total RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA) to remove genomic DNA. Total RNA quality was determined by measuring the 260/280 absorbance ratio and by agarose–formaldehyde gel electrophoresis. First-strand cDNA was synthesized using a PrimeScript® RT Reagent Kit (Takara, Dalian, China), and qRT-PCR was carried out using a SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) and analyzed on an ABI StepOnePlus™ Real-time PCR System (PerkinElmer Applied Biosystems, Foster City, CA, USA) in triplicate. The amplification program was 95 °C for 1 min, 40 cycles at 95 °C for 5 s, annealing for 30 s, and 72 °C for 30 s. A melting curve was used to examine the specificity of the PCR at the end of each qRT-PCR reaction. The fold change of gene expression was normalized to *ef1α* mRNA using the $2^{-\Delta\Delta Ct}$ method. The *ef1α* gene was selected as the internal standard and did not vary upon chemical exposure (data not shown). The gene primer sequences were identified using the online Primer 3 program (http://frodo.wi.mit.edu/), and the sequence are shown in Table S1.

### 2.4. Thyroid hormone extraction and assay

The extraction and measurement of thyroid hormone was performed as described next. The heads of three tadpoles were homogenized in 4 mL of ELISA buffer (provided by the kit). After centrifugation (6000 ×g, 4 °C) for 15 min, the supernatants were obtained immediately for the $T_3$ and thyroxine ($T_4$) measurements. The TH levels were measured using a commercial kit (Uscnlife, Wuhan, China) based on the competitive binding enzyme immunoassay technique following the manufacturer's instructions. The kits were certified for use with *X. laevis* samples by validating the parallelism between a series of diluted and spiked samples in reference to a standard curve. The detection limits for $T_3$ and

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