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# Comparison of triadimefon and its metabolite on acute toxicity and chronic effects during the early development of *Rana nigromaculata* tadpoles

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ABSTRACT

Pesticides are one of major causes for amphibian population declines and the behavior of pesticide metabolite products to amphibians has become a rising concern. In this study, the acute toxicity and the chronic effects of triadimefon and triadimenol (the metabolite of triadimefon) on *Rana. nigromaculata* were investigated. In the acute assay, significant differences were observed in antioxidant enzyme activities and malondialdehyde levels between the triadimefon and triadimenol. The 96 h-acute toxicity of triadimefon (25.97 mg/L) and triadimenol (34.55 mg/L) to tadpoles was low. In 28d-chronic exposure, we studied the relative expression of tadpoles genes related to thyroid hormone-dependent metamorphic development, histological examination of liver and some biological index, including wet weight, snout-to-vent length (SVL) and development stages. The results revealed that the effects of triadimefon and triadimenol on tadpole development are driven by a disruption of the hormonal pathways involved in metamorphosis. Interestingly, triadimefon was more harmful on *R. nigromaculata* than triadimenol at high dose, whereas the reverse result was observed at low doses. According to the relative expression of thyroid hormone-dependent genes, we also found that the two compounds may have different mechanisms of toxic action on R. nigromaculata. Our study developed a pragmatic approach for use in the risk assessment of pesticide and its metabolite , and increased the information and understanding of the impacts of fungicides and other potential endocrine disrupting environmental contaminants on amphibians.

## 1. Introduction

Triadimefon (Fig. S1)
[1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone, CAS 43121-43-3] is a 1,2,4-triazole fungicide to control powdery mildews and fungi on agricultural crops. Triadimefon (Fig. S1) can be enzymatically reduced to triadimenol
[β-(4-chlorophenoxy)-R-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol, CAS 55219-65-3], which is also a kind of agricultural fungicide. Because of their excellent antifungal activity, they have been the most major class of fungicides for many years (Manclús et al., 2008).

Generally , pesticide metabolite products are less toxic to biota than their parent compounds. Differences between many pesticide metabolite products and their parent in the environmental behavior, where pesticide metabolite products may have increased mobility contrast to the parent (Kolpin et al., 2001), might also mean that low-toxicity metabolite product may be still potential to exert an adverse effect on the environment. Therefore, it is necessary to access pesticide metabolite products during the environmental risk assessment process.

Many research have reported that triadimefon and triadimenol can potentially exert effects on wildlife and humans (Zarn et al., 2003). Acute oral median lethal dose (LD50) of Bayleton (92.6% triadimefon) , the technical formulation of triadimefon , is 569 mg/kg in rats and 500 mg/kg in rabbits and dogs. The EPA has classified triadimenol as moderately toxic pesticides, similar to triadimefon on toxicity for rainbow trout and bluegill. LC50s (the lethal concentration that causes 50% mortality compared with the control) of triadimefon are 19 and 15 mg/L, respectively (Kenneke et al., 2009). Therefore, it is necessary to evaluate the fate of those compounds in the environment as well as the effect of their toxicity to humans and various wildlife species.

Due to its high chemical and photochemical stability, low biodegradability and easy transport in the environment, triadimefon and triadimenol are persistent in soil and water. There has not reported

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about detection of triadimefon in sediments, which is probably due to its relatively fast bio-transformation to triadimenol in sediment (Singh, 2005). Whereas, triadimefon is very stable in water with a pH of 3.0, 6.0, or 9.0 (Garrison et al., 2011). The estimated expected environmental concentration (EEC) for triadimefon is 41 μg/L. Detection of Triadimenol in water samples was up to a few micrograms per liter (Kahle et al., 2008).

Amphibian species, developing in aquatic environment, can absorb chemicals through their permeable skin, increasing their susceptibility to the effects of environmental contaminutesants, some of which could cause abnormal sex differentiation and gonadal development (Hayes et al., 2010; Qin et al., 2003). *Rana nigromaculata* (*Pelophylax nigromaculatus*), mainly distributed in East Asia, has been used as toxicological experimental species for some advantages such as easy-maintained in laboratory, low natural death rate and the relatively short generation time. It reported that early developmental stages of marine invertebrates were more responsive to toxicants than adults (Bishop et al., 1991). Therefore, we collected *R. nigromaculata* on early developmental stages as experimental materials.

In this study, we conducted acute and chronic toxicity test to comprehensively assess the toxicity of triadimefon and triadimenol to *R. nigromaculata*. LC50 is an index to assess a pesticide's acute toxicity, and a lower value of LC50 indicates a more potent toxicity. On the other hands, amounts of reports have revealed that the low levels of pesticides might exert long-term effect (Ray and Richards, 2001). Low pesticide levels can influence amphibians by disrupting thyroid hormone signaling, because amphibian metamorphosis is in part controlled by thyroid hormone. In addition, thyroid hormone-dependent metamorphic development is also used to identify thyroid disruptors (Heimeier and Shi, 2010).

The purpose of this study was to investigate comparatively the toxicological effects of triadimefon and triadimenol on *R. nigromaculata* during the early development. We assayed 96 h-acute toxicity to measure LC50 and oxidative stress including superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPX) activities and malondialdehyde (MDA). After 28 days exposure, we studied the relative expression of tadpoles genes related to thyroid hormone-dependent metamorphic development. Besides, some biological indexes, such as wet weight, snout-to-vent length (SVL) , development stages were recorded to learn more about the toxicological effects of triadimefon and triadimenol on *R. nigromaculata.*

## 2. Materials and methods

### 2.1. Chemicals

Triadimefon (purity > 98.5%) and triadimenol (purity > 99.0%) were provided by ICAMA (Institute for the Control of Agrochemicals , MOA). Acetone , dimethyl sulfoxide (DMSO) , isopropyl alcohol , ethanol , chloroform and sodium iodide used in the experiment were analytical grade, purchased from Yili Fine Chemicals (Beijing, China). 3-aminobenzoic acid ethyl ester (MS - 222) and formalin were purchased from J&K Chemical (Beijing, China) and Xilong Chemical (Guangdong, China), respectively. Chemicals for RNA Extraction and Quantitative RT-PCR assay were purchased from Tiangen (Beijing, China). PCR primers were synthesized by Sangon Biotech (Beijing, China).

### 2.2. Animal collection and husbandry

*R. nigromaculata* tadpoles were provided by State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-environmental Sciences. Before the experiment, tadpoles were acclimated to laboratory conditions and maintained in 5 L-beaker containing charcoal-filtered tap water at 24 ± 1 °C with a 12 h light/12 h dark cycle and fed three times with Labdiet Frog Diet (Labdiet, America) daily. The dechlorinated tap water quality was as follows: dissolved oxygen concentration > 5 mg/L, pH 7.3–7.8, and water hardness ($CaCO_3$), approximately 150 mg/L. Tadpoles were not fed during acute toxicity testing, and fed three times with Labdiet Frog Diet daily in chronic toxicity. The tadpoles were staged according to the Gosner system (Gosner, 1960).

### 2.3. Acute toxicity experiment

Ten randomly selected tadpoles at Gosner stage 26 were placed in 500 mL glass beakers filled with filtered water. Triadimefon or triadimenol was dissolved in DMSO as a stock solution, and this solution was added into the 500 mL glass beakers to achieve a series of desired concentrations. According to the pre-experiment, the concentrations of triadimefon and triadimenol were designed from 20 mg/L to 25 mg/L and from 20 mg/L to 40 mg/L. Each concentration was tested in three parallel experiments. The control solvent group received the same amount of DMSO. The medium was changed daily using freshly prepared solutions. Housing and breeding conditions were described as above. At the point of 48 h, 72 h and 96 h, mortality was recorded to establish an LC50.

### 2.4. Determination of oxidative stress

Survival frog exposed to 96 h acute toxicity were collected and homogenized with phosphate buffer (5.0 mmol/L, pH 7.8) after being euthanized. The disrupted cells were centrifuged at 10,000 g for 10 min at 4 ℃ and than supernatant protein extract was stored at 80 ℃. Bovine serum albumin was used as a standard to determine the total soluble protein concentration.

The SOD activity was assayed based on its capacity to inhibit production of nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). One unit of SOD activity was assayed by measuring amount of enzyme that caused 50% inhibition of the initial rate of NBT reduction at 560 nm. CAT activity was defined as the rate of $H_2O_2$ reduction at 20 ℃, measured by changes in absorbance at 240 nm for 1 min (e: 436 mmol/cm) according to Aebi (1984). The contents of GST and GPx were measured by GST and GPx test kits, respectively, both of which were purchased from Nanjing Jiancheng Bioengineering Institude. GPx activity was measured at 412 nm by quantifying the rate of oxidation of reduced GSH to oxidized glutathione, and was expressed as unit $mg^{-1}$ protein. One unit of GPx activity was defined as the amount of enzyme depleting 1 μmol GSH in 1 min. Glutathione-S-transferase (GST) enzyme activity was measured spectrophotometrically by the standard substrate, 1-chloro-2,4-dinitrobenzene (CDNB) conjugated with GSH and was expressed as unit $mg^{-1}$ protein. One unit of GST activity was defined as the amount of enzyme depleting 1 μmol GSH within 1 min.

MDA content was assayed following the method of Packer and Health (Heath and Packer, 1968). The reaction mixtures containing 5% TCA, including 0.5% TBA and the supernatant were maintained in boiling water for 15 min, and then centrifuged at 4500 rpm for 10 min. The MDA content was calculated based on $A_{532}$–$A_{600}$ using an extinction coefficient of 155 mmol/cm.

### 2.5. Chronic toxicity experiment

To investigate the chronic toxicity of triadimefon and triadimenol on *R. nigromaculata*, we exposed tadpoles to one of three concentrations of triadimefon or triadimenol (0.1, 1, and 10 mg/L). Each treatment contained three replicate tanks with 15 tadpoles and 5 L test water per replicate. The test water was completely replaced every three days. Housing and breeding conditions were described as above. After 28d exposure, the survival rate of the tadpoles in each treat was recorded. After euthanasia in 100 mg/L MS-222, weight, the body length and development stages of tadpoles were recorded. The livers of all tadpoles