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Joint toxic effects of triazophos and imidacloprid on zebrafish (*Danio rerio*)☆

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ABSTRACT

Pesticide contamination is more often found as a mixture of different pesticides in water bodies rather than individual compounds. However, regulatory risk evaluation is mostly based on the effects of individual pesticides. In the present study, we aimed to investigate the individual and joint toxicities of triazophos (TRI) and imidacloprid (IMI) to the zebrafish (*Danio rerio*) using acute indices and various sublethal endpoints. Results from 96-h semi-static test indicated that the LC_{50} values of TRI to *D. rerio* at multiple life stages (embryonic, larval, juvenile and adult stages) ranged from 0.49 (0.36–0.71) to 4.99 (2.06–6.81) mg a.i. L^{-1}, which were higher than those of IMI ranging from 26.39 (19.04–38.01) to 128.9 (68.47–173.6) mg a.i. L^{-1}. Pesticide mixtures of TRI and IMI displayed synergistic response to zebrafish embryos. Activities of carboxylesterase (CarE) and catalase (CAT) were significantly changed in most of the individual and joint exposures of pesticides compared with the control group. The expressions of 26 genes related to oxidative stress, cellular apoptosis, immune system, hypothalamic-pituitary-thyroid and hypothalamic-pituitary-gonadal axis at the mRNA level revealed that zebrafish embryos were affected by the individual or joint pesticides, and greater changes in the expressions of six genes (*Mn-sod*, *CXCL-C1C*, *Dio1*, *Dio2*, *tsh* and *vtg1*) were observed when exposed to joint pesticides compared with their individual pesticides. Taken together, the synergistic effects indicated that it was highly important to incorporate joint toxicity studies, especially at low concentrations, when assessing the risk of pesticides.

1. Introduction

Although pesticides have been widely used in most sectors of the agricultural production during the past few decades, they can reach surface water through spray drift and run-off after rain events (Morrissey et al., 2015; Zheng et al., 2016). Effects of pesticides on non-target organisms in ecological environment have been the subject of worldwide concern in recent years (Chagnon et al., 2015; Matozzo et al., 2018). Triazophos [TRI; O,O-diethyl-*O*-(1-phenyl-1H-1,2,4-triazol-3-yl)] has been widely used in agriculture for controlling insect pests, and it is a known organophosphorus insecticide (Chen et al., 2014). Due to its long-term and intensive usage, TRI residues can be often detected in water bodies (Kumar et al., 2016). High toxicity of TRI to aquatic organism, including fish, shrimp, freshwater cladolera and crab, has been reported in many studies, and this insecticide may induce neutotoxic effect, teratogenicity, oxidative damage, histological and MiRNA expression alteration in fish (Wang et al., 2010; Mustafa et al., 2014; Zhu et al., 2014; Liu et al., 2015). In addition, TRI in combination with chlorpyrifos-methyl has synergistic effects on *Daphnia magna* and *Luminescent bacteria* (Mansour, 2015). Therefore, extensive use of TRI detrimental to aquatic organisms has attracted public concern.

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Imidacloprid [IMI; 1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine] is the most widely used neonicotinoid insecticide, and its use continues to increase (Furlan and Kreutzweiser, 2015). Detected aquatic concentration indicates that the measured level of IMI is increased from 14 μg L^{-1} up to 0.3 mg L^{-1} in surface waters, while the estimated concentrations for accidental spills reach high values going from 1.8 up to 7.3 mg L^{-1} (Morrissey et al., 2015). IMI has high toxicity to Mysid shrimp as well as aquatic insect midges, black flies and mosquito larvae (Stoughton et al., 2008; Roessink et al., 2013; Smit et al., 2015). Although IMI has low toxicity to fish, amphibians and Daphnia, it can induce immune system suppression, DNA damage and neurobehavioral impairment in fish (Jemec et al., 2007; Tišler et al., 2009; Crosby et al., 2015; Ge et al., 2015). Given IMI's widespread, environmental persistence and high water solubility, there is a significant need for research on the aquatic toxicity of mixtures of IMI with other pesticides (Scheil and Köhler, 2009; Anderson et al., 2015; Kunce et al., 2015).

Aquatic organisms in natural environments are commonly exposed to chemical mixtures rather than individual compounds (Key et al., 2007; Grung et al., 2015). The behavior of chemical mixtures may not correspond to that predicted from data for individual compounds (Chen et al., 2016). Therefore, the evaluation of mixture toxicity is very important for risk assessment, which is a more realistic approach to evaluate the toxicity of chemicals in the ecosystem (Scheil and Köhler, 2009; Guo et al., 2017). Recently, zebrafish (*Danio rerio*) is an important model organism in ecotoxicological research because of its inherent advantages as follows: low cost, short reproductive cycle, production of numerous transparent and synchronously developing embryos (Ge et al., 2015; Martinez-Sales et al., 2015). However, the effects of pesticide mixtures of TRI and IMI on zebrafish remain largely unexplored (Scheil and Köhler, 2009; Wang et al., 2017).

It has become more and more common to use pesticide mixtures to improve efficacy and overcome resistance to an individual insecticide in the agricultural practice (Zhang et al., 2010). TRI and IMI are often applied together in tank mixtures, leading to their simultaneous presence in the same water samples (Svendsen et al., 2010; Anderson et al., 2015). Because organophosphates have been shown to synergize the effect of neonicotinoids in the target pests, joint effects of TRI and IMI on non-target aquatic organisms are of increasing concern (LeBlanc et al., 2012). Previous toxicological studies on TRI and IMI have mainly focused on their individual toxic effects with little attention to joint toxicity and possible interactions between them (Zhu et al., 2014; Crosby et al., 2015; Ge et al., 2015). Therefore, the study aimed to investigate the potential joint effect of TRI and IMI on the zebrafish, with attention to the acute lethal testing, enzyme activity and gene expression. The multi-level approach, including physiological, biochemical and molecular level tests, will help us assess the potential toxic interactions to aquatic organisms of pesticide mixtures.

2. Materials and methods

2.1. Chemicals

Both TRI (purity 95%, CAS: 24017-47-8) and IMI (purity 95.3%, CAS: 138261-41-3) were supplied by Nanjing Red-sun Chemical Co., Ltd. (Nanjing, Jiangsu, China). The stock solutions of technical products were prepared in dimethyl sulfoxide (DMSO; purity > 99.9%; Amresco, Solon, OH, USA) and Tween-80 and then stored in the dark at 4 °C.

All stock solutions were further diluted to desired concentrations using standard water containing 2 mmol L^{-1} Ca^{2+}, 0.5 mmol L^{-1} Mg^{2+}, 0.75 mmol L^{-1} Na^{2+} and 0.074 mmol L^{-1} K^{+}. All working solutions were freshly prepared prior to analysis. Cytochrome P450 (Cyp450) kit was purchased from GENMED SCIENTIFICS INC. U.S.A (Shanghai, China). Trizol reagent, reverse transcriptase kit and the SYBR Green system were obtained from Takara (Dalian, Liaoning, China). Other kits for index assays were provided from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

2.2. Zebrafish maintenance and embryo collection

Adult zebrafish of wild type AB-strain were obtained from China Zebrafish Resource Center (Wuhan, Hubei, China). These zebrafish were kept in charcoal-filtered water at 26 ± 1 °C with a photoperiod of 14 h/10 h (light/dark) in the flow-through system. The fish were fed twice daily with live brine shrimp. Isolation boards were used to separate female and male adult fish (female/male ratio was 1/2) in spawning boxes overnight. The eggs were harvested and assessed under microscope. Fertilized eggs (embryos with normal development of a blastula) were used for exposure experiments. Unfertilized eggs or eggs with evident irregularities during cleavage or injuries of the chorion were discarded.

Embryos at about 2 h post-fertilization (hpf) or larvae at 72 h post-hatching were used for embryonic and larval toxicity tests, respectively. Juvenile zebrafish (about 1-month-old) and adult zebrafish (about 3-month-old) were fasted for 1 day prior to evaluation. All animal protocols and procedures were approved by the Independent Animal Ethics Committee at the Zhejiang Academy of Agricultural Sciences and conducted in accordance with current Chinese legislation.

2.3. Multiple life stage assays

The toxicities of TRI and IMI to multiple life stages of *D. rerio* were assessed according to OECD test guidelines with slight modifications (OECD, 1992, 2013). In order to maintain the appropriate concentration of pesticide and water quality, all of the test solutions were refreshed daily. The external circumstances, such as temperature, humidity and light cycle, were identical to breeding environment during the 4-day treatment. Details about toxicity tests of pesticides to various developmental stages of zebrafish were given in the Supplemental Information.

2.4. Joint toxicity test

The joint toxicity of TRI and IMI was evaluated with embryonic zebrafish. To directly compare toxicity of the individual compounds with their mixtures, concurrent testing was carried out based on a previously described protocol. In order to examine the interaction of TRI and IMI, their mixtures were prepared at an equitoxic ratio (50% of the 96-h LC_{50} of each pesticide). Complete concentration-response relationships were experimentally obtained by changing the total concentration of each mixture and keeping all above-mentioned ratios constant. For each tested concentration, the experiment was performed in triplicate.

2.5. Evaluation of joint toxicity

In order to determine the joint toxicity, the additive index (AI) value was calculated based on the LC_{50} values of individual pesticides and their mixtures (Marking, 1985). This method defines an AI for the joint effect of chemical mixture. The biological activity (S) of pesticide mixture of A and B was determined by the equation as follows:

$$S = (Am/Ai) + (Bm/Bi)$$