Chemosphere 192 (2018) 14–23

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Single and joint toxicity assessment of four currently used pesticides to zebrafish (*Danio rerio*) using traditional and molecular endpoints

Yanhua Wang, Shenggan Wu, Jine Chen, Changpeng Zhang, Zhenlan Xu, Gang Li, Leiming Cai, Weifeng Shen*, Qiang Wang**

State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Key Laboratory for Pesticide Residue Detection of Ministry of Agriculture, Laboratory (Hangzhou) for Risk Assessment of Agricultural Products of Ministry of Agriculture, Institute of Quality and Standard for Agro-products, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, Zhejiang, China

HIGHLIGHTS

- Pyraclostrobin was the most toxic pesticide to zebrafish.
- Embryonic stage was a vulnerable period in the multiple tested stages of zebrafish.
- Six out of 11 pesticide mixtures exhibited synergistic effects on zebrafish.
- Combined pesticides impacted expressions of 5 genes more severely than their individuals.

ARTICLE INFO

Article history:
Received 22 August 2017
Received in revised form
23 October 2017
Accepted 24 October 2017

Handling Editor: David Volz

Keywords:
Combined effects
Pesticide
Aquatic toxicity
mRNA expression
qPCR

ABSTRACT

Pesticides usually present in mixtures in surface waters, although they are traditionally regulated on an individual basis in aquatic ecosystems. In this study, we aimed to investigate the lethal and transcriptional responses of individual and combined pesticides (iprodione, pyrimethanil, pyraclostrobin and acetamiprid) on zebrafish (*Danio rerio*). Semi-static toxicity test indicated that the greatest toxicity to the four life stages (embryonic, larval, juvenile and adult stages) of *D. rerio* was detected from pyraclostrobin, followed by iprodione and pyrimethanil. In contrast, the lowest toxicity to the organisms was found from acetamiprid. Most of the selected pesticides exerted greater toxicities to *D. rerio* of embryonic stage compared with other life stages. Synergistic responses were observed from all binary mixtures of iprodione in combination with pyrimethanil or acetamiprid and ternary mixtures of iprodione+pyraclostrobin in combination with pyrimethanil or acetamiprid. The expressions of 16 genes related to cell apoptosis pathway, oxidative stress response, innate immunity and endocrine disruption at the mRNA level showed that zebrafish embryos were affected by the individual or combined pesticides. The expressions of *P53*, *Tnf*, *TRβ*, *Tsh* and *Cyp19a* exhibited greater changes upon exposure to combined pesticides compared with individual pesticides. Taken together, increased toxicity might be triggered by the simultaneous presence of several pesticides in the aquatic environment, which seriously damaged the non-target organisms.

1. Introduction

Pesticides are widely used in agricultural production (Furlan and Kreutzweiser, 2015), which undoubtedly enhance food production and improve quality of the product (Doruchowski et al., 2017). Despite numerous merits, application of pesticides in agriculture has become a major risk to wildlife due to their toxicity and bioaccumulation (Cui et al., 2015). Moreover, pesticides are often present in mixtures in natural environment instead of single compounds (Phyu et al., 2011; Schreiner et al., 2016). It is known that pesticide mixtures can cause changes in their toxicity. Therefore, the actual field situations will be underrated based on experiments using only single pesticides (Nowell et al., 2014; Wang et al., 2017), leading to underestimated ecological risk of

* Corresponding author.
** Corresponding author.
E-mail addresses: weifsh@126.com (W. Shen), wq13575733860@126.com (Q. Wang).

https://doi.org/10.1016/j.chemosphere.2017.10.129
0045-6535/

compounds (Pérez et al., 2013; Chen et al., 2015). It is urgently necessary to assess the effects of pesticide mixtures when evaluating their ecological risks on ecosystems.

Aquatic toxicity assay has become a common method to assess the negative influence of toxic chemicals on the water environment (Watson et al., 2014). Fish are an essential component for the aquatic environment in conventional ecotoxicity test (Dai et al., 2014; Jeffries et al., 2015). Zebrafish (*Danio rerio*) has become an efficient and commonly used biological model for chemical toxicity screening due to its several advantages, such as easy handling, short life span, high fecundity, and high homology to mammalian species (Hill et al., 2005; He et al., 2014). It becomes urgently necessary to assess the negative influence of pesticides on *D. rerio* at different life stages since these animals are actually exposed to chemicals at different life stages or even during the whole life stage (Mu et al., 2013; Yang et al., 2016). Although many recent studies have been carried out on zebrafish, but only the effects of single pesticides have been investigated in most of these studies (Jin et al., 2009; Pamanji et al., 2015; Zhu et al., 2015; Gómez-Canela et al., 2017). However, in natural ecosystems, aquatic organisms are usually exposed to chemical mixtures instead of single compounds (Phyu et al., 2011; Ku et al., 2015; Tufi et al., 2016). Therefore, a realistic risk assessment highly requires accurate knowledge of mixture toxicity (McCarty and Borgert, 2006; Hernández et al., 2013).

Iprodione, pyrimethanil, pyraclostrobin and acetamiprid are commonly used pesticides for different fruits, vegetables, grain crops, ornamental trees and lawns (Radice et al., 2001; Seeland et al., 2012; Furlan and Kreutzweiser, 2015; Cui et al., 2017). These pesticides are persistent in water due to their high chemical and photochemical stability, low biodegradability and easy transportation in the environment (Zubrod et al., 2014; Bonmatin et al., 2015). Moreover, these compounds are linked to a potential source of risks to living organisms since they have been detected in several types of water circulating in the ecosystem (Zhao et al., 2008; Miao et al., 2012; Li et al., 2014). There is a growing awareness among ecologists regarding their harmful effects on aquatic ecosystem (Rodney, 2013; Nowell et al., 2014). In the present study, we investigated the acute toxicity of four pesticides to different life stages (embryonic, larval, juvenile and adult stages) of zebrafish and their combined effects on the sensitive life stage. As gene expression is usually more sensitive than traditional toxicity endpoints, it has been increasingly used in ecotoxicology as an early warning. There is a great need to develop gene expression analysis to improve traditional toxicological tests. Therefore, the alterations of gene expression related to cellular apoptosis, oxidative stress, immune response and endocrine system after individual and combined exposures were also analyzed to elucidate the potential mechanism of joint toxicity at the molecular level. The results provided new insights into the toxicological mechanism of these pesticide and their mixtures against aquatic organisms.

2. Materials and methods

2.1. Maintenance of zebrafish and egg collection

The parental and adult fish of wild-type AB strain were collected from National Zebrafish Resource Center of China. The animals were cultured in the flow-through feeding equipment (made by Esen Corp., China) at 26 ± 1 °C with a 14 h/10 h (light/dark) cycle. Zebrafish were fed with live brine shrimps (*Artemia nauplii*) two times a day. A dissecting microscope was employed to select fertilized and normally developed eggs at the eight-cell stage [approximately 2 h post-fertilization (hpf)]. Larvae that survived for 72 h post-hatching were used for larval toxicity test. Juvenile zebrafish (about 1-month-old) and adult zebrafish (about 3-month-old) were fasted for 1 day prior to assessment. All procedures were approved by the Independent Animal Ethics Committee at the Zhejiang Academy of Agricultural Sciences and conducted in accordance with current Chinese legislation. Details of zebrafish egg collection were given in the Supplemental Information.

2.2. Chemicals and reagents

The toxicities of four pesticides were examined in this study, including one dicarboximide fungicide iprodione, one anilinopyrimidine fungicide pyrimethanil, one strobilurin fungicide pyraclostrobin and one neonicotinoid insecticide acetamiprid. Iprodione [96% technical product (TC)] was obtained from Jiangsu Huifeng Agrochemical Group (Yancheng, Jiangsu, China). Pyrimethanil (98% TC) was provided by Jiangsu Kuaida Agrochemical Co., Ltd. (Rudong, Jiangsu, China). Pyraclostrobin (98% TC) was supplied from Shandong Hailir Chemical Co., Ltd. (Weifang, Shandong, China). Acetamiprid (96.2% TC) was purchased from Hebei Weiyuan Biochemical Industrial Group (Shijiazhuang, Hebei, China). It was worth noting that only active ingredients, not commercial formulations, were used because our study attempted to address toxic effects of the chemical compounds but not the additives in commercial products.

Stock solutions of above-mentioned four pesticides were prepared with acetone AR and Tween-80 and then stored at 4 °C prior to further analyses.

2.3. Individual pesticide toxicity experiments

The toxicity of individual pesticides to multiple life stages of *D. rerio* was carried out according to OECD test guideline with a slight modification (OECD, 1992, 2013). In order to maintain the appropriate concentration of pesticide and water quality, the exposure solution was changed every 24 h. The external conditions (temperature, humidity and light cycle) were kept the same as the culture environment during 4-day exposure. Details about toxic effects of pesticides to the zebrafish at various life stages were given in the Supplemental Information.

2.4. Combined toxicity test

The combined toxicity of pesticides was assessed with zebrafish embryos. The toxicity of individual chemicals was directly compared with their mixtures by concurrent testing of the individual pesticides and their mixtures based on a previously described procedure (Wang et al., 2017). Mixture toxicity of equitoxic ratio was tested. The test procedure of combined toxicity was given in the Supplemental Information.

2.5. Evaluation methods for combined toxicity

A probit analysis developed by Chi (1997) was employed to determine the acute toxicity of pesticides to *D. rerio*. Significant level of mean separation ($P < 0.05$) was set based on non-overlap between the 95% confidence interval of two LC_{50} values. Marking's additive index (AI) was used to evaluate combined toxicity (Marking, 1985). The description of AI method was given in the Supplemental Information.

2.6. Sub-acute toxicity test on zebrafish embryos

A total of 300 normal zebrafish embryos were randomly distributed into glass beakers containing 500 mL of test solutions