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# Effects of two strobilurins (azoxystrobin and picoxystrobin) on embryonic development and enzyme activities in juveniles and adult fish livers of zebrafish (*Danio rerio*)

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## HIGHLIGHTS

- Both AZOX and PICO had dose- and time-dependent effects on embryonic development.
- Both AZOX and PICO affected antioxidant/detoxification enzymes and MDA content.
- AZOX and PICO caused significant oxidative stress that differed between the sexes.
- PICO caused higher embryonic development toxicity and oxidative stress than AZOX.
- The ability of males to detoxify AZOX/PICO was stronger than that of the females.

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## ABSTRACT

Azoxystrobin and picoxystrobin are two primary strobilurin fungicides used worldwide. This study was conducted to test their effects on embryonic development and the activity of several enzyme in the zebrafish (*Danio rerio*). After fish eggs were separately exposed to azoxystrobin and picoxystrobin from 24 to 144 h post fertilization (hpf), the mortality, hatching, and teratogenetic rates were measured. Additionally, effects of azoxystrobin and picoxystrobin on activities of three important antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD)] and two primary detoxification enzymes [carboxylesterase (CarE) and glutathione S-transferase (GST)] and malondialdehyde (MDA) content in zebrafish larvae (96 h) and livers of adult zebrafish of both sexes were also assessed for potential toxicity mechanisms. Based on the embryonic development test results, the mortality, hatching, and teratogenetic rates of eggs treated with azoxystrobin and picoxystrobin all showed significant dose- and time-dependent effects, and the 144-h $LC_{50}$ values of azoxystrobin and picoxystrobin were 1174.9 and 213.8 $\mu g\ L^{-1}$, respectively. In the larval zebrafish (96 h) test, activities of CAT, POD, CarE, and GST and MDA content in azoxystrobin and picoxystrobin-treated zebrafish larvae increased significantly with concentrations of the pesticides compared with those in the control. We further revealed that azoxystrobin and picoxystrobin exposure both caused significant oxidative stress in adult fish livers and the changes differed between the sexes. Our results indicated that picoxystrobin led to higher embryonic development toxicity and oxidative stress than azoxystrobin in zebrafish and the male zebrafish liver had stronger ability to detoxify than that of the females.



## 1. Introduction

Strobilurins are one of the most important and promising classes of fungicides (Sauter et al., 1999; Bartlett et al., 2002). Azoxystrobin and picoxystrobin are two important strobilurin fungicides that are widely applied for plant disease control worldwide (Russell, 2005; Casida and Durkin, 2016). Asstrobilurins, azoxystrobin and picoxystrobin both inhibit mitochondrial respiration via blocking electron transfer at the Qo center of cytochrome *b* and *c*1 (BCPC, 2011).

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In recent years, much information on azoxystrobin residues in runoff water, which range in concentration from 1 to 30 $\mu g\,L^{-1}$, is available in the literature (Berenzen et al., 2005; Deb et al., 2010; Battaglin et al., 2011). Thus, studies of the effects of strobilurins on environmental nontarget organisms have attracted increasing attention in recent years. Much literature information is available on the adverse effects of azoxystrobin on a wide range of nontarget organisms; for example, zebrafish (Bony et al., 2010; Cao et al., 2016; Han et al., 2016), grass carp (Liu et al., 2013), copepods (Gustafsson et al., 2010; Van Wijngaarden et al., 2014), Atlantic salmon smolts (Olsvik et al., 2010), several species of macrophyte, phytoplankton and macroinvertebrate (Zafar et al., 2012), and food webs across trophic levels (Dawoud et al., 2017). Additionally, picoxystrobin causes adverse effects on earthworms (Wang et al., 2012; Schnug et al., 2015) and transient negative effects on soil microbial activities (Stenrød et al., 2013).

Zebrafish (*Danio rerio*) are recommended as a model organism for ecotoxicological tests by the Economic Co-operation and Development (OECD) (OECD, 1992, 2013) and are also widely used in various toxicity laboratory studies worldwide in recent years (Ducharme et al., 2015; Han et al., 2016; Glaberman et al., 2017). Early studies focused on the acute toxicity of strobilurins in zebrafish adults. Recent more in-depth studies on the toxicological effects of strobilurins in zebrafish, for example, oxidative stress, genotoxicity and early-life stage effects, continue to generate substantial attention. The adverse effects of oxidative stress and genotoxicity of azoxystrobin on zebrafish livers were recently reported (Han et al., 2016). However, little information has been reported on the effects of picoxystrobin on zebrafish livers. Most of the toxicological effects of azoxystrobin and picoxystrobin are from a focus on adult zebrafish, whereas little information is available on juveniles.

The present studies were designed to assess the toxic effects of two strobilurins (azoxystrobin and picoxystrobin) on embryonic development (mortality, hatching rates, and teratogenetic rates). Additionally, the effects of azoxystrobin and picoxystrobin on activities of three important antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD)] and two primary detoxification enzymes [carboxylesterase (CarE) and glutathione S-transferase (GST)] and content of malondialdehyde (MDA) in zebrafish larvae (96 h) and livers of adult zebrafish of both sexes were also assessed.

## 2. Materials and methods

### 2.1. Test chemicals and reagents

Azoxystrobin (95% TC; CAS Number: 131860-33-8) and picoxystrobin (98% TC; CAS Number: 117428-22-5) were both purchased from Beijing Huarong Biological Hormone Factory, China. Catalase assay kit (CAT-2-Y), superoxide dismutase assay kit (SOD-2-Y), peroxidase assay kit (POD-2-Y), carboxylesterase assay kit (CARE-2-W), glutathione S-transferase assay kit (GST-2-W) and malondialdehyde content assay kit (MDA-2-Y) were obtained from Suzhou Keming Biotechnology Co., Ltd., China.

### 2.2. Test organisms

Fertilized eggs used for the embryonic development test and enzyme activities determination in zebrafish juveniles were obtained as follows. Five-month-old adult zebrafish with a male and female ratio of 1:1 were obtained from the China Zebrafish Resource Center (CZRC). The male and female zebrafish at the ratio of 1:1 were placed in a spawning aquarium with a clapboard to separate them overnight (approximately 10 h in the dark). Grids were also placed at the bottom of the spawning aquarium to prevent the eggs from being predated by the adult zebrafish. The following morning, the clapboard was removed, and light irritation was adopted for spawning. Half an hour later, the fertilized eggs were collected and washed with the system water three times. Then, the normal fertilized eggs at 3 h post fertilization (hpf) were chosen for the test under an Olympus BX63 microscope (Olympus, Japan).

Zebrafish (*Danio rerio*) at 2 months old (male and female ratio, 1:1), obtained from Beijing Renhe Aquarium Products Company, China, were used for determination of enzyme activities in adult fish livers. All zebrafish were domesticated under recirculating, charcoal-dechlorinated tap water system at $26 \pm 1\,^{\circ}C$ with 12 h of illumination daily for at least two weeks before the test. The pH of the zebrafish system water ranged from 7.5 to 8.0 and the dissolved oxygen concentration was maintained at 7–9 $mg\,L^{-1}$. Brine shrimp were used to feed the fish twice per day until 24 h before the test and the feces and residuals were removed from the water in a timely manner. The healthy adult zebrafish with an average length of $2.43 \pm 0.38$ cm and weight of $0.217 \pm 0.054$ g were chosen for the test.

### 2.3. Embryonic development test

Embryonic development toxicity was tested according to OECD Guideline 210 (OECD, 2013) with some modifications. A group of twenty fertilized eggs at 3 hpf were exposed to each test concentration in a standard 24-well plate (one egg and 2 mL of solution per well; Corning Incorporated, USA), and the spare four wells were treated as controls (the system water). For the $LC_{50}$, embryos were exposed to azoxystrobin at nominal concentrations of 0, 150, 300, 500, 1000, 1500, and 2000 $\mu g\,L^{-1}$ or picoxystrobin at nominal concentrations of 0, 15, 25, 50, 100, 200, and 400 $\mu g\,L^{-1}$. An additional group of 20 embryos were exposed to the solvent acetone solutions on a separate 24-well plate, which served as a solvent control. A positive control at the fixed concentration of 4 $mg\,L^{-1}$ 3,4-dichloroaniline was performed with each egg batch used for testing. Exposure studies were repeated three times, and the exposure solution in each well was renewed every 24 h to maintain a relatively constant test chemical concentration. The plates were placed in an incubator at $26 \pm 1\,^{\circ}C$. The mortality, hatching rates, and teratogenetic rates of the embryos were checked under an Olympus BX63 microscope (Olympus, Japan) at 24, 48, 72, 96 and 144 hpf.

### 2.4. Enzyme activitiesin the larval zebrafish study

A standard 6-well plate (Corning Incorporated, USA) with 30 fertilized eggs at 3 hpf and 10 mL of test solution per well was used for the larval zebrafish study. Test concentrations were based on preliminary $LC_{50}$ results of the adult acute toxicity test (data not given), and the highest dose was set at the previous $LC_{50}/6$. The test solutions were a series of concentrations of azoxystrobin (nominal concentrations of 0, 0.25, 2.5, 25, and 250 $\mu g\,L^{-1}$) and picoxystrobin (nominal concentrations of 0, 0.02, 0.2, 2, and 20 $\mu g\,L^{-1}$). Exposure studies were repeated three times and the solution in each well was also renewed every 24 h to maintain a relatively constant test chemical concentration and water quality. The plates were placed in an incubator at $26 \pm 1\,^{\circ}C$ for 96 h, and the dead eggs or larval zebrafish were removed promptly during the exposure. Then, the treated larval zebrafish were used for enzyme activity (CAT, POD, CarE, and GST) and MDA content assays according to the manufacturer's recommendations (Suzhou Keming Biotechnology Co., Ltd., China). The activities of CAT, POD, CarE, and GST are expressed as U $mg^{-1}$ based on protein content. The MDA content is expressed as nmol $mg^{-1}$.