Science of the Total Environment 715 (2020) 137069

Contents lists available at ScienceDirect

Science of the Total Environment

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

Fluoxastrobin-induced effects on acute toxicity, development toxicity, oxidative stress, and DNA damage in *Danio rerio* embryos

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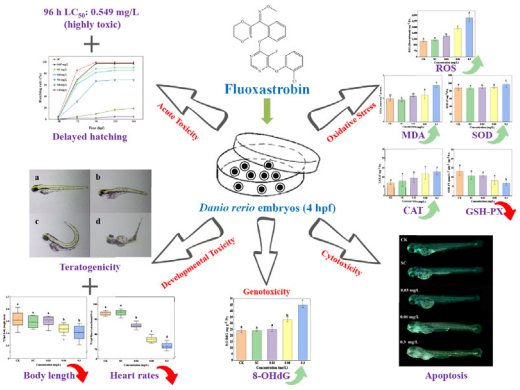
HIGHLIGHTS

- Fluoxastrobin (FLUO) has high acute toxicity to embryos of *Danio rerio*.
- FLUO may lead embryos to delayed hatching.
- FLUO induced severe development toxicity to embryos and larvae of *Danio rerio*.
- FLUO induced oxidative stress, dose-related genotoxicity and cytotoxicity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 October 2019
Received in revised form 30 January 2020
Accepted 31 January 2020
Available online 01 February 2020

Editor: Daniel Wunderlin

Keywords:
96 h LC_{50}
Delayed hatching
Teratogenicity
8-OHdG
AO-EB staining
Apoptosis

ABSTRACT

Strobilurin fungicides (SFs), the most commonly used fungicides, pose threats for controlling fungal diseases. The fungicides were monitored in aquatic ecosystems and may have negative effects on nontarget organisms. This project was undertaken to monitor the toxic effects of fluoxastrobin (FLUO) on *Danio rerio* embryos and to evaluate the SF risks in aquatic ecosystems. The 96-hour median lethal concentration (96 h LC_{50}), hatching rates, and morphological abnormalities were used to analyze acute toxicity and teratogenicity of FLUO to *Danio rerio* embryos at an FLUO dose of 0.549 mg/L (95% confidence limits: 0.423 to 0.698 mg/L); the results showed that FLUO has high toxicity in embryos that is analogous to the toxicity observed in adult *Danio rerio*. Fluoxastrobin may lead embryos to delayed hatching at concentrations >0.6 mg/L, and it may lead to teratogenicity (i.e., pericardial edema and spinal curvature). Based on the 96 h LC_{50} results, the following parameters were evaluated in *Danio rerio*: development-related indicators (body length and heart rates), reactive oxygen species (ROS) levels, lipid peroxidation (LPO) levels, the levels of three antioxidants, 8-hydroxy-2-deoxyguanosine (8-OHdG), and apoptosis. The results elucidated that FLUO inhibition of spinal and heart development may be induced by oxidative stress. In addition, FLUO induced a notable climb in ROS content, LPO, the activated activity of superoxide dismutase (SOD) and catalase (CAT), and it inhibited glutathione peroxidase (GSH-PX) activity. Fluoxastrobin led to DNA damage (i.e., a notable climb of 8-OHdG contents and apoptotic cells). Collectively, FLUO posed threats to *Danio rerio* embryos at multiple levels, and this investigation could be a reminder for people to be more judicious in SF-use to avoid or relieve SF toxicity to nontarget organisms.

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https://doi.org/10.1016/j.scitotenv.2020.137069
0048-9697/

1. Introduction

Strobilurin fungicides (SFs), using strobilurin A as the lead material, pose threats to controlling fungal diseases (Bartlett et al., 2002; Li et al., 2018). This kind of fungicide inhibits fungal mitochondrial respiratory activity by influencing electron transfer (Zhang et al., 2018b). Because of their high efficiency, SFs (i.e., azoxystrobin (AZOX, CAS No. 131860-33-8), pyraclostrobin (PYRA, CAS No. 175013-18-0), picoxystrobin (PICO, CAS No. 117428-22-5), and trifloxystrobin (TRIF, CAS No. 141517-21-7)) have been the most popular fungicides known with market share >20% in 2014 (Li et al., 2018). In addition, new SFs including fluoxastrobin (FLUO), are regularly used (Zhang et al., 2018a, 2019).

With the application of SFs, prior studies have noticed that SFs may exist and can be monitored in aquatic ecosystems. Though very little has been found in the literature on FLUO concentrations in aquatic environments, TRIF has been monitored and was found to have concentrations of 170 and 0.73 μg/L in the water of a Chinese paddy and in southeastern Australia, respectively (Cao et al., 2015; Wightwick et al., 2012). In addition, PYRA has been monitored and was found to have concentrations of 17.24, 1.61, and 0.10 μg/L in the water of a Chinese paddy, Nebraska's Rainwater Basin Wetlands, and southeastern Australia, respectively (Guo et al., 2016; Mimbs et al., 2016; Wightwick et al., 2012). Besides, extensive studies also noted that SFs had negative effects on nontarget organisms in the water (Cui et al., 2017; Jia et al., 2018; Jiang et al., 2018; Lu et al., 2017).

Danio rerio (OECD 203, 1992) has been extensively applied to the study of external contaminants, mainly considering its rapid reproductive capacity and genetic similarity with humans. Our prior studies (Zhang et al., 2017, 2018a) elucidated PYRA- and FLUO-induced oxidative stress and DNA damage to *Danio rerio* adults. Li et al. (2018) studied the acute, developmental toxicity and oxidative stress of three SFs (PYRA, TRIF, and PICO) to zebrafish (*Danio rerio*) embryos.

Compared with adult fish, embryos could be used to more sensitively screen the toxicity of contaminants (Sun et al., 2019; Yang et al., 2018). Little was found in the literature on SF toxicity in relation to developmental toxicity, oxidative stress, genotoxicity and apoptosis in *Danio rerio* embryos (Li et al., 2018).

Thus, this project was undertaken to the monitor toxicity of FLUO to *Danio rerio* embryos to evaluate the SF risks in aquatic ecosystems studying multiple endpoints: 96-hour median lethal concentration (96 h LC_{50}), hatching rates, development-related indicators (teratogenicity, body length, and heart rates), reactive oxygen species (ROS), lipid peroxidation, three antioxidants, 8-hydroxy-2-deoxyguanosine (8-OHdG), and apoptosis. The mechanism of genotoxicity and cytotoxicity induced by FLUO could be elucidated in further studies. This investigation could also be a reminder for people to be more judicious in SF-use to avoid or relieve SF toxicity to nontarget organisms.

2. Materials and methods

2.1. Chemicals

Fluoxastrobin (Fig. S1, 99.3% pure, CAS No. 361377-29-9) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared using Dimethyl sulfoxide (DMSO, 99.9% pure) purchased from Sigma (Missouri, USA), and they were stored at 4 °C. The other chemicals (analytical grade) were purchased from Solarbio Science & Technology Co. (Beijing, China) and Kaitong Chemical Reagent Co., Ltd. (Tianjin, China). Assay kits for measuring of total protein, LPO, the activity of three antioxidants, 8-OHdG contents and apoptosis were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China), Hengyuan Biological Technology Co., Ltd. (Shanghai, China), and Solarbio Science & Technology Co. (Beijing, China).

2.2. Fish handling and embryo collection

The zebrafish (*Danio rerio*) was purchased from the China Zebrafish Resource Center (Wuhan, China). Fish handling and embryo collections were performed as per Sun et al. (2019). Fish were fed dry flakes twice a day, and they were kept on an automatic photoperiod (14 h light/10 h dark) and were maintained at approximately 28 ± 1 °C. Dead fish and leftover food were removed timely. The fish were handled for 2 weeks before formal experiments. Paired adult fish were placed in an incubator overnight and used for spawning on the following morning. Then, the embryos were collected and rinsed with hatching fluid. Four hours post-fertilization (hpf), embryos (healthy and fertilized) were selected for formal experiments (Yang et al., 2018) using stereomicroscopes (Phenix Optics Co., Ltd., Jiangxi, China).

2.3. Test of FLUO acute toxicity to embryos

Acute toxicity to fish embryos was performed per OECD 236 (2013). The tested concentrations (FLUO: 0.05, 0.1, 0.4, 0.6, 0.8, 1.0 mg/L) for lethality and hatching rates were set based on the preliminary experiments and the 96 h LC_{50} of FLUO to adult zebrafish (Zhang et al., 2018a). DMSO (100 μL) was added to 100 mL of prepared hatching solution and was used as the solvent control (SC) with a final concentration of approximately 0.1% (*v*/v) (Cao et al., 2019; Huang et al., 2019). One embryo of the selected embryos in Section 2.2 was randomly placed in the well of a 24-well plate with 2 mL of SC and exposure solution and was incubated for 96 h. Each 24-well plate was maintained in an automatic photoperiod (14 h light/10 h dark) and at approximately 26 ± 1 °C. To evaluate the FLUO toxicity in relation to lethality and hatching rates of embryos, the deaths were recorded at 96 hpf and hatching rates were recorded at 48, 72, 96, 120, and 144 hpf, respectively ($n = 48$). Euthanasia method (rapid chilling) was used to handle with the zebrafish after each trial according to the current Chinese legislation and the American Veterinary Medical Association (2020) Guidelines for the Euthanasia of Animals.

2.4. Measurement for teratogenicity, body length, and heart rates of Danio rerio induced by FLUO

The tested concentrations (FLUO: 0.05, 0.1, 0.4, 0.6, 0.8, 1.0 mg/L) for teratogenicity were set. DMSO (100 μL) was added to 100 mL of prepared hatching solution and was used as the SC with a final concentration of approximately 0.1% (v/v). One embryo of the selected embryos in Section 2.2 was randomly placed in the well of a 24-well plate with 2 mL of SC and exposure solution and was incubated for 96 h. Each 24-well plate was maintained in an automatic photoperiod (14 h light/10 h dark) and at approximately 26 ± 1 °C. According to Oliveira et al. (2017) and Li et al. (2018), larvae malformations (e.g. pericardial edema (PE), spinal curvature, and tail formation) were observed and recorded at 72 hpf using photographed under stereomicroscopes (Phenix Optics Co., Ltd., Jiangxi, China).

To evaluate the FLUO development toxicity to embryos, the body length and heart rates were recorded ($n = 10$) at 72 hpf as per Li et al. (2018). Tested concentrations were based on 1/20, 1/10, and 1/2 dilutions of 96 h LC_{50} to embryos, so the concentrations of FLUO (0.03, 0.06, and 0.3 mg/L) were used for developmental toxicity. Twenty of the selected embryos from Section 2.2 were randomly placed in a culture dish with 20 mL of deionized water as a control check (CK), DMSO and exposure solution, and they were incubated for 72 h. To maintain appropriate FLUO concentrations and avoid interference from bacterial pollution, half of the solutions in each culture dish were refreshed daily, and the dead individuals were timely removed. Each culture dish was maintained in a photoperiod (14 h light/10 h dark) and at approximately 26 ± 1 °C. The body length of the larvae was observed and photographed under stereomicroscopes. The heartbeat rates were evaluated by the number of heartbeats per 30 s.

2.5. Exposure for oxidative stress and DNA damage induced by FLUO

To evaluate the oxidative stress induced by FLUO in larvae, ROS contents, LPO, and the activity of three antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX)) were monitored. To evaluate the DNA damage of larvae induced by FLUO, 8-OHdG contents and apoptosis were measured as per Sun et al. (2019). The design for the measurement of oxidative stress and DNA damage was similar to the measurement of body length. The concentrations of 1/20, 1/10, and 1/2 of 96 h LC_{50} to embryos were set for measurement of oxidative stress and DNA damage. All trials were performed in triplicate. Two hundred embryos were randomly selected and placed in each conical flask (250 mL) with 50 mL CK, DMSO and exposure solution for 120 h, respectively. To maintain appropriate FLUO concentrations and avoid interference from bacterial pollution, half of the solutions in each culture dish were daily refreshed and the dead individuals were timely removed. Each conical flask was maintained in a photoperiod (14 h light/10 h dark) and at approximately 26 ± 1 °C. At 120 hpf, all living larvae (about six hundred) from each concentration were pooled, all the larvae were used and rinsed using hatching solution. Then, the prepared larvae were ground (1:9, *w*/*v*) in phosphate buffer solution (PBS) at pH = 7.4 for further study.

2.6. Measurement of oxidative stress induced by FLUO

The prepared homogenates in Section 2.5 were centrifuged at 1000*g* and 4 °C for 10 min (Eppendorf 5810R, Eppendorf AG, Germany), and the supernatant was recentrifuged at 20,000*g* and 4 °C for 20 min (Zhang et al., 2018a). Then, the precipitate was resuspended. The resultant solution was kept on ice during the whole routine and used for the measurement of ROS contents. The ROS contents were measured using a ROS assay kit (Beyotime Biotech. Inc., Shanghai, China), and the data were collected under the fluorescence spectrometer with excitation and emission at 488 and 522 nm (RF-5301PC, Shimadzu, Japan), respectively.

The supernatant was prepared from the homogenates in Section 2.5 after centrifugation at 12,857*g*, 4 °C for 10 min, which enabled measurement of protein contents, LPO, and enzyme activity (Li et al., 2018; Sun et al., 2019); these data were collected according to the instructions of the kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The resultant solution was kept on ice during the whole procedure. The total protein contents were measured using a total protein quantitative assay kit and analysis of Coomassie brilliant blue staining at 595 nm (UV/VIS spectrophotometer, UV-2600, Shimadzu, Japan).

Malondialdehyde (MDA), which is produced during fatty acid degradation, was important for evaluating LPO (Li et al., 2018). MDA contents were measured at 532 nm and in units of nmol/mg protein using a microscale MDA assay kit using the thiobarbituric acid method. Superoxide dismutase (SOD) activity was monitored at 550 nm and in units of U/mg protein using a total SOD assay kit using the hydroxylamine method. The SOD content that could induce 50% SOD inhibition in a 1 mL reaction solution was established as a SOD activity unit (U) of one. The catalase (CAT) activity was monitored using a CAT assay kit using visible light at 405 nm and in units of U/mg protein. The CAT content induced the breakdown of 1 μmol H_2O_2 per second was one CAT activity unit (U). The glutathione peroxidase (GSH-PX) activity was monitored using a GSH-PX assay kit as per the colorimetric method at 412 nm and in units of μmol L^{-1} min^{-1} mg^{-1} protein.

2.7. Measurement of DNA damage induced by FLUO

To evaluate DNA damage to *Danio rerio* larvae induced by FLUO, 8-OHdG contents and apoptosis were monitored. The supernatant was prepared from the homogenates prepared in Section 2.5 after centrifugation at 12,857*g* and 4 °C for 10 min. The resultant solution was kept on ice during the entire procedure. A prior study (Guo et al., 2014) noted the significance of 8-OHdG in evaluating DNA damage. The 8-OHdH contents were monitored using an 8-OHdG ELISA kit (Hengyuan Biological Technology Co., Ltd., Shanghai, China) at 450 nm for 10 min (Multiskan MK3, Thermo Fisher Scientific, Massachusetts, USA) and in units of ng/g protein. The total protein contents were assessed as in Section 2.6.

As per Sun et al. (2019) and Wang et al. (2018), apoptosis was monitored using acridine orange (AO)-ethidium bromide (EB) double dyeing kit (Solarbio Science & Technology Co., Beijing, China). At 96 hpf, ten larvae from each trial in Section 2.5 were rinsed twice with PBS (pH = 7.4) and dyed with 20 μL of AO and EB working fluid (1/1, *v*/v) for 5 min and then rinsed twice with PBS. Tritricaine (MS-222) was used for 3 min to anesthetize larvae for better observation. Then, the apoptosis of larvae was observed and photographed under an inverted fluorescence microscope (xioVert. A1, Carl Zeiss AG, Oberkochen, Germany).

2.8. Statistics

All experiments had three or more replicates. The figures were produced with Excel 2016 (Microsoft, WA, USA) and Origin 2019 (OriginLab Corporation, Massachusetts, USA). Homogeneity of variance test was carried out for a one-way analysis of variance (ANOVA) using SPSS 22.0 (SPSS Inc., IL, USA). The least significant difference test (LSD, one kind of equal variances assumed) with $p < 0.05$ was selected as post-hoc test.

3. Results and discussion

3.1. Fluoxastrobin toxicity to survival and hatching rates

No death was observed in the SC group. The result suggested that 96 h LC_{50} was 0.549 mg/L (95% confidence limits: 0.423 to 0.698 mg/L). According to the toxicity grading standard illustrated by Lin et al. (2014), it was suggested that fluoxastrobin showed high toxicity to *Danio rerio* embryos. Zhang et al. (2018a) also studied FLUO toxicity in *Danio rerio* adults, and the 96 h LC_{50} was 0.510 mg/L indicating that FLUO had high toxicity to *Danio rerio* adults (Lin et al., 2014), which was similar to the present study.

The toxicity to embryos was slightly lower than it was to adult fish, which may be caused by sensitivity differences between adult and embryo fish to pollutants. The results verified the statement of Sun et al. (2016) that the embryo chorion and membrane could protect fish from the harm of pollutants. Li et al. (2018) studied the toxicity of other SFs (PYRA, TRIF, and PICO) to *Danio rerio* embryos with 96 h LC_{50} values of 0.061, 0.055, 0.086 mg/L. They also inferred that the three tested fungicides had high toxicity (Lin et al., 2014). Both the studies of Zhang et al. (2018a) and Li et al. (2018) reported data that were similar to what was seen in the present study (0.549 mg/L). Additionally, FLUO has a lower toxicity than PYRA, TRIF, and PICO in *Danio rerio* embryos. Kaushik and Kaushik (2007) believed the structures of pesticides (e.g. as double-triple bond, aromaticity, presence of the epoxide ring, the nature of substituents) are the primary factors for its activity. Besides, they stated the solubility may also have a direct or indirect influence. Thus, the differences among the toxicity of different SFs may be due to different chemical structures (Fig. S1) and water solubility (Table S1).

Prior studies (Cao et al., 2016; Ren et al., 2018) have noted the hatching rates are the indicator commonly used in evaluation for embryo development and the toxicity of pollutants. The embryo hatching rates of *Danio rerio* exposed to fluoxastrobin were listed in Fig. 1. Fluoxastrobin induces inhibition of embryo hatching rates. Most embryos did not hatch at 48 hpf, especially at high concentrations (0.8 and 1.0 mg/L), and there were no hatching fish. The hatching rates showed a time-related increase, and almost all embryos in the exposure groups stopped incubation; more specifically, all the embryos in SC hatched by 96 hpf. Until 144 hpf, all the living embryos all hatched even at the highest

concentration (1.0 mg/L). The hatching rates also showed dose-related inhibition, especially at concentrations >0.6 mg/L, at which embryos showed delayed hatching. Hatching enzymes, which are zinc-containing enzymes, have the ability to decompose eggshells. However, high exposure doses of pesticides may interfere with the structure and function of hatching enzymes and inhibit hatching (Henn and Braunbeck, 2011). In addition, the relationship between hatching inhibition and the relative levels of genes encoding hatching enzymes could be assessed in further studies. Li et al. (2018) studied the toxicity of PYRA, TRIF, and PICO to embryo hatching rates of *Danio rerio*. They inferred that the three fungicides notably showed dose-related hatching inhibition at 72 hpf, which is analogous to the data from the current trial.

3.2. Fluoxastrobin toxicity to teratogenicity, body length, and heart rates

Prior studies (Cao et al., 2016) have noted the morphological abnormalities are the indicator commonly used in evaluating the teratogenicity of pollutants. The malformation rates of FLUO to *Danio rerio* at 72 hpf were recorded in Table S2. No teratogenicity was observed in the SC group. The cumulative rates of abnormal development were 7.32%, 10.5%, 5.56% in lower exposure trials (0.05, 0.1, and 0.4 mg/L), showing no notable dose-related difference. The cumulative rates of abnormal development were 26.7% and 25.0% in 0.6 and 0.8 mg/L, respectively. One hundred percent of teratogenicity was observed at the highest concentration (1.0 mg/L) treatment group. Similar to hatching rates, rates of abnormal development showed an increase at concentrations >0.6 mg/L. The specific malformations including PE and spinal curvature were photographed and are shown in Fig. 2. Li et al. (2018) studied the toxicity of PYRA, TRIF, and PICO to morphological abnormalities of *Danio rerio* embryos. They also found that the three fungicides induced PE in larvae at 72 hpf similar to the present study, indicating that SFs may have potent toxicity to the heart. They inferred that the fungicides showed a notable development inhibition of larvae. As per Bangeppagari et al. (2014), teratogenicity caused by pollutants may inhibit the hatching-related enzyme activity. The specific enzymes could be studied in the future study.

Prior studies (Liu et al., 2013; Mu et al., 2013) have noted the heart rates is the indicator commonly used in evaluating pollutant toxicity to heart function. Heart rates at 72 hpf showed a dose-related reduction. The 30 s heart rates of larvae treated with the doses of 0.03, 0.06, and 0.3 mg/L were significantly reduced to approximately 91.1%, 81.5%, and 76.8% of what was observed in the CK group, respectively. As per Yang et al. (2018), nonlethal doses of pollutants could also disturb heart functions. Li et al. (2018) studied the toxicity of PYRA, TRIF, and PICO to heart rates of *Danio rerio* embryos, and they also found consistent inhibition to heart rates of larvae, which is analogous to the data from the current trial. They also believed that the potential target of SFs may be the fish heart, which was also be speculated due to the teratogenicity induced PE. Mitochondria is the main source to supply the energy to keep cardiomyocytes at the contractile state (Li et al., 2018). However, Zhang et al. (2018a) have elucidated FLUO inhibited the mitochondria activity. Li et al. (2018) also found PYRA, another SFs, showed the inhibit of the expression of *uqcrc* (related gene to mitochondrial complex III). Thus, SFs may interfere with the complex-related mitochondria to show the toxicity to heart development in *Danio rerio*.

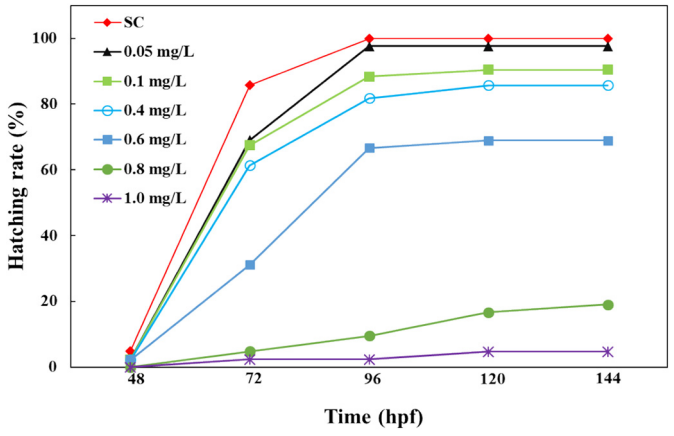

Fig. 1. The hatching rates of *Danio rerio* exposed to fluoxastrobin. SC, solvent control; Hpf, hour post fertilization.

Yang et al. (2018) noted the significance of body length in evaluating growing fish larvae and embryos. The toxicity of fluoxastrobin to body length and heart rates of *Danio rerio* at 72 hpf are listed in Fig. 3. No death or teratogenicity of embryos or larvae were monitored in the CK or SC. No notable change was found between the SC and CK groups; thus, DMSO has little impact on body length and heart rates. No notable change in body length was monitored at the lowest dose (0.03 mg/L) when compared to the CK group, while body length was reduced at 0.06 and 0.3 mg/L. The reduced body length was independent of hatching rates, which was evidenced by the results of Yang et al. (2018), who speculated development in larvae spine may be affected by diverse biological processes. Li et al. (2018) studied the toxicity of PYRA (0.03, 0.0375, 0.047, 0.0586 mg/L), TRIF (0.03, 0.0375, 0.047, 0.0586 mg/L), and PICO (0.06, 0.069, 0.079, 0.091 mg/L) to body lengths of *Danio rerio* embryos. They also found that the three fungicides showed a consistent reduction of the body length of larvae. Specifically, PYRA and TRIF showed a notable decline from 0.03 to 0.0586 mg/L, which is analogous to the data from the current trial (from 0.03 to 0.06 mg/L).

3.3. Fluoxastrobin toxicity to ROS and MDA contents

When there is an imbalance between antioxidant and oxidant, oxidative stress occurs (Sun et al., 2019). The teratogenicity of fish embryos may also be due to oxidative stress (Yan et al., 2018). Li et al. (2018) noted the significance of oxidative stress in evaluating the aquatic toxicity of three SFs (PYRA, TRIF, and PICO) to zebrafish (*Danio rerio*) embryos. The ROS and MDA content following treatment with FLUO at 120 hpf are listed in Table 1. No notable change was observed between the SC and CK groups; thus, DMSO has little impact on oxidative stress and DNA damage.

Li et al. (2018) believed mitochondrion was the main ROS source. The ROS content exists in a dynamic redox equilibrium in organisms, but the equilibrium can be impacted if mitochondria are impaired or the ROS are not timely cleaned by antioxidants (Liu et al., 2014). Hydrogen peroxide (H_2O_2) content, which is one type of ROS, was monitored according to the dichlorofluorescein-diacetate method as per Han et al. (2016). The ROS contents at 120 hpf showed a dose-related climb. Notable changes were observed in the 0.03, 0.06, and 0.3 mg/L groups. The fluoe- at the highest dose (0.3 mg/L) climbed to over twice the levels that were observed in the CK group. The results verified the report from our previous study (Zhang et al., 2018a), which stated that FLUO had dose-related toxicity to adult zebrafish. Li et al. (2018) studied the toxicity of PYRA, TRIF, and PICO in relation to ROS contents in *Danio rerio* embryos, and they speculated that SFs led to a notable increase in ROS contents, which is analogous to the results of the current trial. Sun et al. (2019) also inferred that there may be a relationship between ROS contents and teratogenicity based on a prior study by Hansen and Harris (2013), who believed that the balance of ROS contents benefited for the normal development of embryos and larvae. The disharmony of ROS contents was considered one of the potent indicators of oxidative damage (Li et al., 2018), thus, MDA contents and antioxidants (SOD, CAT, and GSH-PX) were also monitored.

Similar to ROS contents, MDA contents at 120 hpf showed an increase, with levels that were 1.12-, 1.17- and 1.59-fold as those in the CK group. Significant changes were observed in the 0.3 mg/L treatment groups. The results verified the proposal of Draper and Hadley (1990), who speculated that the activated MDA contents may reflect the degree

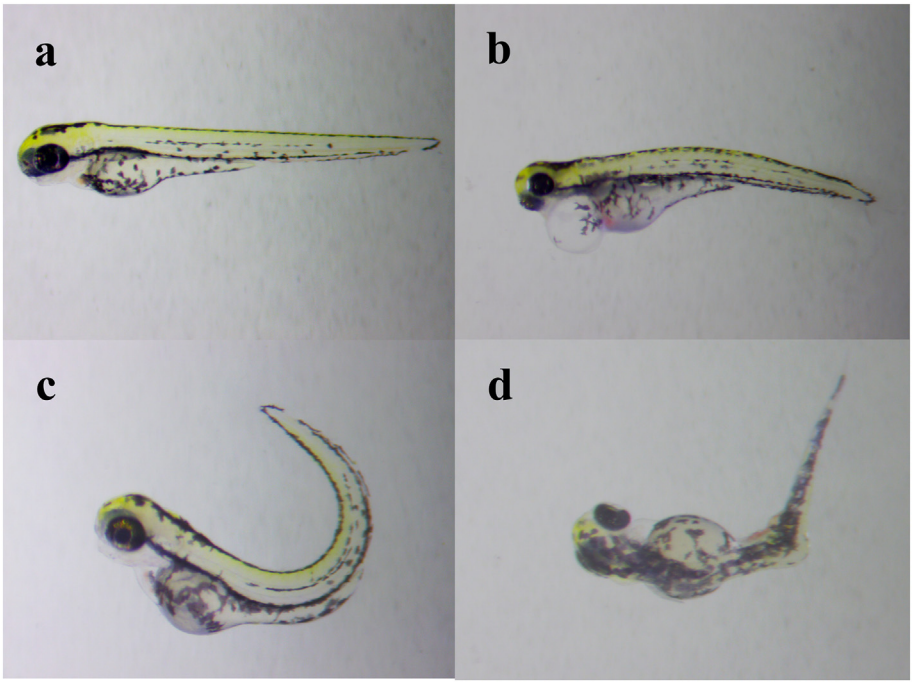

Fig. 2. Teratogenicity of fluoxastrobin to embryo development of *Danio rerio* at 72 hpf (a: normal larvae; b: pericardial edema; c, d: spinal curvature). Hpf, hour post fertilization.

of cell damage induced by free radicals. Li et al. (2018) stated that PYRA had dose-related toxicity to *Danio rerio* embryos. In addition, they also speculated that TRIF and PICO led to notable increases in MDA contents, analogous to our previous studies (Zhang et al., 2017, 2018a), which stated that PYRA and FLUO exhibited LPO in adult zebrafish. Taken together, the similar increases in ROS and MDA contents indicated MDA may accordingly induced LPO caused by extra ROS (Yan et al., 2018), thus, MDA was considered the potent indicator for evaluation of oxidative damage in organisms (Sun et al., 2019).

3.4. Fluoxastrobin toxicity to antioxidants

The enzyme activity for SOD, CAT, and GSH-PX induced by FLUO at 120 hpf are listed in Table 1. Prior studies (Li et al., 2018; Sun et al., 2019) have noted the significance of SOD activity in evaluating oxidative stress, considering SOD could induce ROS to H_2O_2. The SOD activity at 120 hpf showed a slight increase in the 0.03 and 0.06 mg/L. Significant increase were observed in the 0.3 mg/L group. Li et al. (2018) believed that PYRA, TRIF, and PICO led to notable inhibition of SOD enzymatic activity which is in contrast to the current trial. The diverse toxicity responses may be caused by a distinct structure of fungicides or different observation times. Our previous study (Zhang et al., 2018a) also stated that FLUO promoted SOD activity in adult *Danio rerio*, which is analogous to the data of the current trial.

Dogan et al. (2011) noted the significance of CAT activity in the antioxidant system and in protecting organisms from the harm of ROS, considering that CAT could break down H_2O_2 to generate H_2O and O_2. Similar to SOD activity, CAT at 120 hpf showed an increase in activity with rates that were 1.38-, 1.71- and 1.85-fold as those in the CK group. No significant changes were observed in 0.03, 0.06, and 0.3 mg/L treatment groups. The increased CAT activity may be a response aimed at fixing the disrupted ROS equilibrium in embryos and

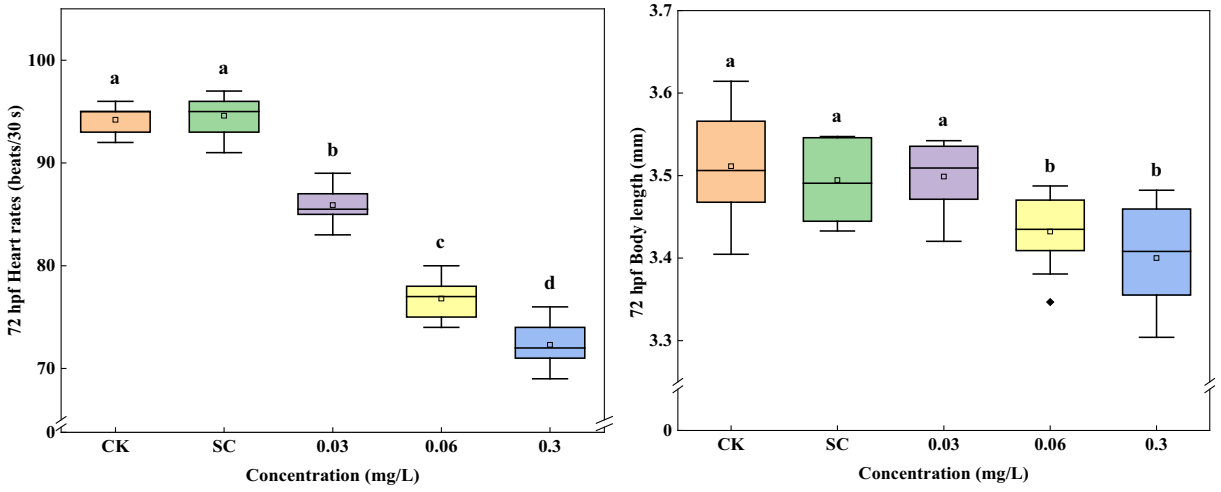

Fig. 3. Toxic effects of FLUO on heart rates and body length of *Danio rerio* larvae at 72 hpf ($n = 10$). The five lines of the box plot from bottom to top indicate the minimum, lower quartile, median, upper quartile, and maximum. The small check in the box plot represents mean values. One-way analysis of variance was performed following the least significant difference test ($p < 0.05$). CK, control check; SC, solvent control; Hpf, hour post fertilization.

Table 1
Oxidative stress induced by FLUO in *Danio rerio* larvae at 120 h-post fertilization (hpf, $n = 3$).

Concentration (mg/L)	ROS (fluoe-intensity/mg Pr)		MDA (nmol/mg Pr)		SOD (U/mg Pr)		CAT (U/mg Pr)		GSH-PX (μmol/(L × min × mg Pr))	
	Means ± SD	*p*	Means ± SD	*p*	Means ± SD	*p*	Means ± SD	*p*	Means ± SD	*p*
CK	$1.06 * 10^3 \pm 48.3$	–	1.98 ± 0.206	–	89.3 ± 5.94	–	6.96 ± 2.36	–	13.2 ± 2.52	–
SC	$1.14 * 10^3 \pm 64.4$	0.153	1.87 ± 0.135	0.507	85.4 ± 1.53	0.988	10.2 ± 3.03	0.264	10.7 ± 3.53	0.382
0.03	$1.41 * 10^3 \pm 39.3$	0.000635**	2.21 ± 0.0780	0.141	87.9 ± 2.17	0.494	9.57 ± 2.45	0.230	10.0 ± 3.62	0.425
0.06	$1.92 * 10^3 \pm 57.5$	0.000383**	2.32 ± 0.349	0.211	90.2 ± 2.24	0.225	11.9 ± 3.05	0.0915	8.15 ± 3.42	0.108
0.30	$2.61 * 10^3 \pm 234$	0.000357**	3.14 ± 0.169	0.00161**	104 ± 7.28	0.0225*	12.9 ± 1.65	0.0529	6.87 ± 1.19	0.0170*

One-way analysis of variance was performed following the least significant difference test. CK, control check; SC, solvent control; Pr, protein.
* $p < 0.05$.
** $p < 0.01$.

larvae. Li et al. (2018) also stated that PICO promoted CAT activity in *Danio rerio* embryos. However, they also speculated that PYRA and TRIF led to notable inhibition of CAT when treated at 0.04 mg/L, which is in contrast to the data from the current trial. The diverse toxicity responses may thus be induced by the distinct structure of fungicides. Taken together, the increases of SOD and CAT activities showed no significant difference with the control group exception SOD activity at the highest concentration (0.3 mg/L) of FLUO. Thus, FLUO might provoke a meaningful increase in SOD activity in a longer period of time (Sun et al., 2019).

Singh et al. (2011) noted the significance of GST activity in the antioxidant system and in protecting organisms from the harm of ROS, considering that GSH-PX could induce H_2O_2 to become H_2O. GSH-PX activity at 120 hpf showed the inhibition. Significant inhibition was observed at the highest concentration (0.3 mg/L), where the enzyme activity was approximately half of what it was in the CK group. The results verified the statement of Wang et al. (2015) that the inhibition of GSH-PX activity indicated a protective mechanism in *Danio rerio* larvae that may be regulated and could lead to more oxidative damage.

3.5. Fluoxastrobin toxicity in relation to DNA damage

Sun et al. (2019) believed there was a connection between DNA damage and oxidative stress. They elucidated the target of the oxidative attack with most biological significance is DNA. The gene *apex1* is considered involved in oxidative stress and regulation of the pathway of DNA repair. The gene *polb* is considered involved in reparation of damaged areas and regulation of the pathway of DNA repair (Pei and Strauss, 2013). Sun et al. (2019) elucidated pollutants may inhibit the expression of *apex1* and *polb*, which could further lead to DNA damage in zebrafish larvae. Previous studies (Chang et al., 2002; Du et al., 2018) also speculated oxidative stress may damage the DNA mismatch repair. Zhang et al. (2018a) also reported that DNA damage was considered a key indicator in evaluating damage induced by pollutants. Topal et al. (2017) and Wang et al. (2018) noted the significance of 8-OHdG in evaluating oxidative stress and DNA damage. The values of 8-OHdG production induced by FLUO at 120 hpf are listed in Table 2. Similar to MDA, 8-OHdG values at 120 hpf showed a significant dose-related with 1.18- and 1.86-fold as the values observed in the CK group at 0.06 and 0.3 mg/L, which indicated that FLUO has genotoxicity. No significant change was observed in the lowest concentration (0.03 mg/L) group, which may be due to DNA repair.

Sun et al. (2019) believed that excess ROS may have toxicity that induces apoptosis. Rates of apoptosis induced by FLUO in *Danio rerio* larvae at 96 hpf are listed in Fig. 4. No significant change was observed between the SC and CK groups; thus, DMSO has little impact on apoptosis. Apoptotic cells in *Danio rerio* larvae showed dose-related increases and were mainly located in the spine area, which indicated that FLUO has cytotoxicity. The results of apoptotic cells were similar to the teratogenicity results found in Section 3.2 and further verified the statement of Wang et al. (2018) that apoptosis is connected to developmental toxicity. Collectively, DNA damage was induced by FLUO in diverse pathways. Prior studies (Han et al., 2016; Zhang et al., 2017, 2018a) also indicated that AZOX, PYRA, and FLUO have toxic impacts on DNA damage according to the comet assay, which are results analogous to the ones from the current trial. The mechanism of genotoxicity and cytotoxicity induced by FLUO could be elucidated in further studies.

4. Conclusions

The present study provided insight into FLUO-induced toxicity, and the data clearly stated that FLUO posed threats to *Danio rerio* embryos at multiple levels.

(1) Fluoxastrobin exhibits high acute toxicity to *Danio rerio* embryos.
(2) Fluoxastrobin may lead embryos to delay hatching, and the relationship between hatching inhibition and relative gene expression levels of hatching enzymes could be analyzed in further studies.
(3) Fluoxastrobin exhibits development toxicity, including increasing the rates of abnormal development and inhibition of spinal and heart development, which may be induced by oxidative stress.
(4) Fluoxastrobin induces a significant increase in ROS content and MDA content, activated SOD activity, and it inhibits GSH-PX activity.
(5) Fluoxastrobin leads to DNA damage, including a notable increase in 8-OHdG content and the number of apoptotic cells.

Declaration of competing interest

None.

Table 2
The 8-OHdG contents induced by FLUO in *Danio rerio* larvae at 120 hour-post fertilization (hpf, $n = 3$).

Concentration (mg/L)	8-OHdG (ng/g Pr)	
	Means ± SD	*p*
CK	25.2 ± 0.625	–
SC	24.5 ± 0.342	0.288
0.03	24.2 ± 0.982	0.336
0.06	29.7 ± 1.835	0.0411*
0.30	46.9 ± 0.914	0.0000551**

One-way analysis of variance was performed following the least significant difference test. CK, control check; SC, solvent control; Pr, protein.
* $p < 0.05$.
** $p < 0.01$.

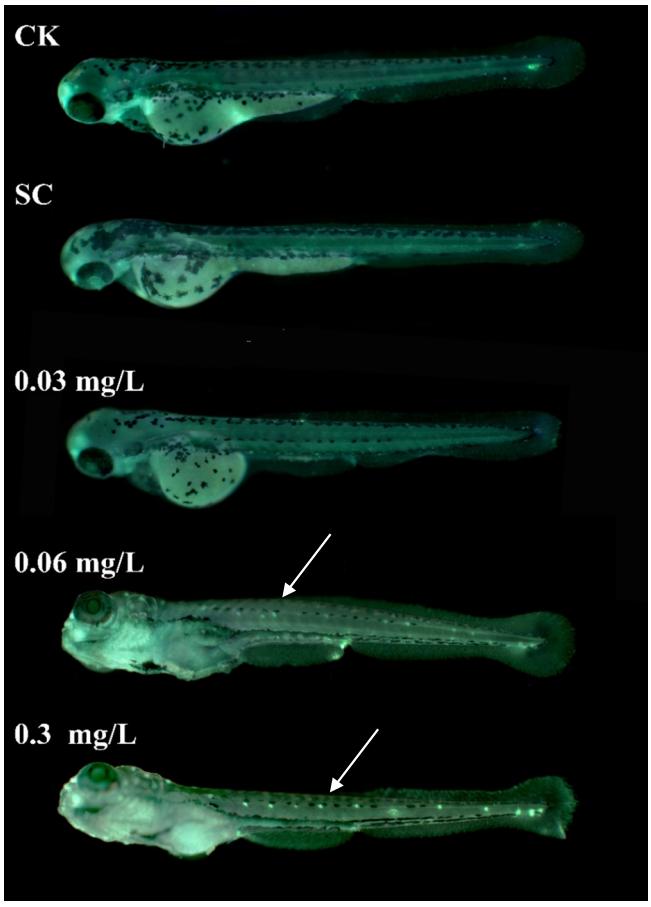

Fig. 4. Apoptosis induced by FLUO in *Danio rerio* larvae at 96 hpf.

Acknowledgements

Funding

This work was supported by the National Key Research and Development Program of China [grant number 2016YFD0800202]; the National Natural Science Foundation of China [grant numbers 41771282, 41701279]; the Natural Science Foundation of Shandong Province, China [grant numbers ZR2017MD005, ZR2017BB075]; and the Special Funds of Taishan Scholar Project of Shandong Province, China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137069.

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