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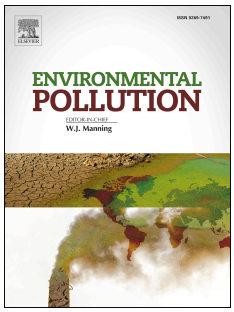

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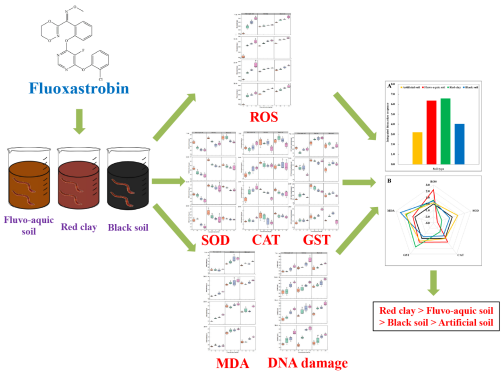

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Applying fungicide on earthworms: Biochemical effects of *Eisenia fetida* exposed to fluoxastrobin in three natural soils

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Abstract

Fluoxastrobin is one of the most widely used strobilurin fungicides, however, application of the fungicides may result in soil residues leading to environmental damage including oxidative stress and damage to sentinel organisms (i.e. earthworms). While this has been demonstrated in artificial soil, the biochemical response of *Eisenia fetida* exposed to fluoxastrobin in natural soils is unclear. This study utilized three typical natural soils (fluvo-aquic soils, red clay, and black soils) to evaluate the biochemical response of *Eisenia fetida* exposed to fluoxastrobin (0.1, 1.0, 2.5 mg/kg) including the production of reactive oxygen species, impact on three enzyme activities, lipid peroxidation, and 8-hydroxydeoxyguanosine after a 4-week exposure. The effects of fluoxastrobin on *Eisenia fetida* in different soils were assessed using an integrated biomarker response (IBR). The findings may be possible to state that the toxic effects of fluoxastrobin in artificial cannot exactly represent that in natural soils. Specifically, the fluoxastrobin subchronic toxicity was highest in red clay and lowest in black soil among the three natural soils. Furthermore, the 8-OHdG content was more sensitive to fluoxastrobin in all six environmental indicators of the present study.

Capsule: The toxicity of fluoxastrobin to *Eisenia fetida* in natural soils were different from those in artificial soil with the toxicity order: Red clay > Fluvo-aquic soil > Black soil > Artificial soil.

Keywords: Strobilurin fungicide; Fluvo-aquic soil; Red clay; Black soil; Integrated biomarker response (IBR)

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Introduction

Strobilurin fungicides, a new formulation following triazole fungicides, are effective agents in controlling fungal disease. The mechanism of action, (i.e. fluoxastrobin transfers electrons between cytochromes b and C_1) inhibits respiratory via mitochondria (Zhang et al., 2018d), which led to the wide use of strobilurin fungicides to protect a variety of crops from fungal disease. However, a consequence of their use is the potential for strobilurin fungicides to accumulate in with the potential to exert toxicological impact on other nontarget receptors (Kohlschmid and Ruf, 2016; Wang et al., 2015). One such strobilurin fungicide is fluoxastrobin (**Fig. S1**), a relative stable fungicide with a half-life of 16-119 days (Zhang et al., 2019). However, very little was found in the literature about the question of fluoxastrobin environmental toxicity.

Earthworms (*Eisenia fetida*) were defined as the soil model animals by the Organization for Economic Cooperation and Development (OECD 222, 2004). Short-term acute toxicity studies have identified that fluoxastrobin exhibits low toxicity to *Eisenia fetida* (14 d LD_{50} >1,000 mg/kg). Although high LD_{50} values have been reported, lower dose (0.1, 1.0, 2.5 mg/kg) exposure of fluoxastrobin to *Eisenia fetida* for 28 days in artificial soils (Zhang et al., 2018d) may lead to the induction of oxidative stress and damage.

Klára and Jakub (2012) identified that differences in POP bioavailability and bioaccumulation may arise when artificial and natural soils are utilized in *Eisenia fetida* toxicity tests. A similar result was observed by Gestel et al. (2011) when molybdenum bioaccumulation in *Eisenia Andrei* was assessed in diverse natural soils. This raises the question as to whether the biochemical response of *Eisenia fetida* exposed to fluoxastrobin in artificial soil is representative of effects that may be observed in natural soils.

Three typical natural soils (fluvo-aquic soils, red clay, and black soils) were chosen to evaluate the biochemical response of *Eisenia fetida* exposed to fluoxastrobin at low dose (0.1, 1.0, 2.5 mg/kg) over 28 days using endpoints including production of reactive oxygen species (ROS), impact on activities of superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST), lipid peroxidation (LPO), and 8-hydroxydeoxyguanosine (8-OHdG).

Integrated Biomarker Response (IBR, v2) was considered to evaluate the ecological and environmental conditions

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(Samanta et al., 2018; Sanchez-Hernandez, 2019; Wang et al., 2011). To clearly compare the fluoxastrobin toxicity to *Eisenia fetida* in different soil types, the ROS contents, LPO, and enzyme activity of 2.5 mg/kg after a 4-week exposure were selected to calculate the IBR index. The results of each indicator in artificial soil were as per our previous study (Zhang et al., 2018d). The current study aims to determine whether the biochemical effects of *Eisenia fetida* exposed to fluoxastrobin in artificial soils are representative of those in natural soils.

Materials and methods

Chemicals

Fluoxastrobin (CAS 361377-29-9; 99.3% purity) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), while acetonitrile (chromatographical purity) was purchased from Tedia Co. Inc. (Ohio, USA). The other chemicals of analytical purity are listed in **Table S1**. 8-OHdG was evaluated using the Earthworm 8-OHdG ELISA kit (hengyuan biological technology Co. Ltd., Shanghai, China).

Soil, Eisenia fetida, exposure concentration and time

The fluvo-aquic soils, red clay, and black soils were collected from Dezhou (Shandong Province, 36.78°N, 116.54°E), Nanning (Guangxi Province, 22.74°N, 109.31°E) and Changchun (Jilin Province, 43.80°N, 125.40°E), respectively. Soils were sieved to < 2-mm prior to exposure studies. **Table S2** details the physical-chemical properties of the test soils (Zhang et al., 2018a).

Toxicity tests used 1 L beakers containing 500 g (dry weight) of test soil. The moisture content was adjusted to 60% water-holding capacity. Perforated plastic wrap was used to seal each beaker to maintain moisture and gas exchange. A 28-day toxicology test (7, 14, 21, and 28 d) was performed with the final doses of fluoxastrobin in test soils (1 L beakers) of 0, 0.1, 1.0, and 2.5 mg/kg dry soil as per Zhang et al. (2018d).

Eisenia fetida were chosen as sentinel soil organisms for exposure studies with fluoxastrobin. *Eisenia fetida* were purchased from a supplier (Rizhao, China) and cultured for 2 weeks (Zhang et al., 2018d). Healthy *Eisenia fetida*

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(weight ranging from 0.3 to 0.5 g) with visible clitellum were chosen at random for sensitivity and subchronic toxicity tests.

The principle of using animals in toxicological tests was adopted in the present study (Zhang et al., 2018c). Each control and treatment contained 20 earthworms. Then, the incubator (RXZ-500B-LED, Ningbo Jiangnan Instrument Factory, China) was used to culture each beaker at 20 ± 1 □ for 1/1 of light/dark till 28 days. Each toxicological exposure trial contained three replicates.

Furthermore, it was considered that the earthworm sensitivity test would usefully testify the reliability of subchronic toxicity test. The details of the sensitivity test were shown in *Supporting Information*.

Assessment of ROS contents

ROS content was measured using the DCFH-DA method (Zhang et al., 2018d), using a reactive oxygen species assay kit purchased from Beyotime Biotech. Inc. (Shanghai, China). Earthworms from control and exposure treatments (n=3) were selected for ROS content determination randomly at each sample time point. A fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan) was used to quantify ROS content.

Assessment of protein contents, enzyme activities, and malonaldehyde (MDA) content

At each sample time point, earthworm from control and treatments (n=3) was selected at random for enzyme extraction (Zhang et al., 2018d). Prepared enzymes were used for the determination of protein content, enzyme activity, and MDA content.

Protein contents were measured using the method of Bradford (1976) with concentrations quantified using ultraviolet-visible spectrophotometer (UV-2600, Shimadzu, Japan) was used.

Enzyme activity was determined as per Zhang et al. (2018d). The UV-2600 was used to quantify activities of SOD, CAT, and GST. The units of SOD, CAT, and GST were U/mg pr (U: enzyme levels inhibited 50% NBT photochemical), U/mg pr (U: enzyme levels inhibited 50% H_2O_2), and nmol/min/mg pr, respectively.

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Box and Maccubbin (1997) consider that MDA content is a suitable biomarker of LPO. MDA content was also assessed using the thiobarbituric acid method (Zhang et al., 2018d) expressed as nmol/mg pr.

DNA damage assessment

Guo et al (2014). consider that 8-OHdG content is a suitable biomarker of DNA damage, which was determined using an Earthworm 8-OHdG ELISA kit following the manufacturer's instructions. Earthworms from control and exposure treatments (n=3) were chosen for DNA damage determination at random at each sample time point. The ELISA (Multiskan MK3, Thermo Fisher Scientific, Massachusetts, USA) was used to quantify 8-OHdG.

Statistics

The box plot was drawn using Origin 2019 (OriginLab Corporation, Massachusetts, USA). Each toxicological exposure trial contained three replicates. The five lines from top to bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. The small check in the box plot represents mean values. Statistical Package for Social Sciences ($V_{22.0}$, SPSS Inc., USA) was used to conduct a one-way analysis of variance (ANOVA) between control and exposure treatments. Specifically, the least significant difference test was adopted with the significance of $p < 0.05$.

Results of indicators of *Eisenia fetida* exposed to fluoxastrobin at 0.1, 1.0, and 2.5 mg/kg on day 28 were calculated for IBR index using the EXCEL software (Microsoft, Redmond, WA, USA). The calculation details were listed in the section "1.3 Calculation of integrated biomarker response (IBR) index" of the "*Supporting Information*".

Results and discussion

ROS contents

ROS content in organisms exists in a dynamic balance. However, once ROS is unable to be cleaned by antioxidant enzymes, the balance is impacted (Brendler-Schwaab et al., 2005). 2',7'-dichlorofluorescein (DCF), the reaction product of ROS and DCFH-DA, can be used to evaluate ROS content due to its fluorescence activity (Zhang et al.,

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2018d). **Figure 1** illustrates ROS content in *Eisenia fetida* exposed to fluoxastrobin in three natural soils at three low dose exposure concentrations.

<**Fig. 1**>

On days 7 and 14, the ROS contents at diverse exposure concentrations were all notably greater than those in control in all three diverse natural soils. The results showed a dose-response relationship. However, no notable discrepancy was found between 1.0 and 2.5 mg/kg treatments in red clay and between 0.1 and 1.0 mg/kg in black soils. On day 21, the ROS contents at diverse exposure concentrations were all notably greater than those in control in all three diverse natural soils. The results showed a significant dose-response relationship. However, no notable discrepancy was found between 1.0 and 2.5 mg/kg treatments in red clay. On day 28, the ROS contents at diverse exposure concentrations were all notably greater than those in control in all three diverse natural soils. The results showed a significant dose-response relationship. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments in fluvo-aquic soils.

Previously we studied the subchronic effect (at 0.1, 1.0, 2.5 mg/kg) of fluoxastrobin on *Eisenia fetida* in artificial soil (a mixture of kaolin, quartz sand, and peat as per OECD) (OECD 222, 2004, OECD 207, 1984, Zhang et al., 2018d). Similar results were obtained (i.e. values greater than unexposed *Eisenia fetida* and dose-response), however ROS content in artificial soil (813.8-1103 fluo-intensity/mg Pr) was lower compared to values determined in natural soils. Our previous study (Zhang et al., 2017) stated ROS can cause oxidative damage including LPO and DNA damage, which were also evaluated in the present study.

Activities of the antioxidant enzyme (SOD and CAT)

The activities of enzymes can be adopted to evaluate environmental pollution (Song et al., 2009; Zhang et al., 2014). ROS can be inactivated by antioxidant enzymes (SOD and CAT) (Guo et al., 2016; Hu et al., 2016; Nel et al., 2006), which are the first line of defense for cellular protection (Liu et al., 2018; Yan et al., 2015). SOD is responsible for the

dismutation of O_2^- to H_2O_2, which is detoxified by CAT (Zhang et al., 2018b). **Figures 2, 3** illustrate SOD and CAT activities in *Eisenia fetida* exposed to fluoxastrobin in three natural soils, respectively.

<**Figs. 2, 3**>

On day 7, SOD activities following exposure to fluoxastrobin at all concentrations were notably lower than those measured in control soils with the exception of that in the black soil at the lowest exposure dose (0.1 mg/kg). No notable discrepancy was observed between 1.0 and 2.5 mg/kg treatments in all three natural soils. On day 14, the SOD activities showed a notable decline from the control to exposure groups in all three diverse natural soils except for those in 0.1 mg/kg treatments of black soils. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments and between 1.0 and 2.5 mg/kg in all three diverse natural soils. On day 21, the SOD activities showed a notable decline from the control to exposure groups except for those in 0.1 mg/kg treatments in all three diverse natural soils. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments in red clay. On day 28, the SOD activities showed a notable decline from the control to exposure groups at the doses of 1.0 and 2.5 mg/kg in fluvo-aquic soil and red clay. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments in red clay, between 1.0 and 2.5 mg/kg in all three diverse natural soils, and among 0.1, 1.0, and 2.5 mg/kg treatments in black soils. The similar finding in each exposure time was observed by Han et al. (2016) when they studied the effects of another strobilurin-type fungicide on zebrafish at (1, 10, and 100 μg/L). We also studied the subchronic of fluoxastrobin on *Eisenia fetida* in artificial soil (Zhang et al., 2018d), while the SOD activities in exposure groups were all notably greater than those in control at diverse exposure time.

On day 7, the CAT activities showed a notable decline from the control to exposure groups at the dose 2.5 mg/kg in red and black soils, while those in fluvo-aquic soils were greater than in control. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments in fluvo-aquic soils and red clay. On day 14, the CAT activities showed a notable decline from the control to exposure groups in red clay, while those in fluvo-aquic soils were notably greater than in control. No notable discrepancy was found among the exposure groups and the controls in black soil. On day 21, the

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CAT activities showed a notable decline from the control to exposure groups in red and black soils, while those in fluvo-aquic soils showed a significant dose-response climb. On day 28, the CAT activities showed a notable decline from the control to exposure groups in red and black soils, while those in fluvo-aquic soils were greater than in control. The results showed a significant dose-response relationship in red clay. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments in fluvo-aquic soils and among 0.1, 1.0, and 2.5 mg/kg treatments in black soils. Another important finding was that the CAT activities showed a notable decline from the control to exposure groups in artificial soil (Zhang et al., 2018d), which was similar to the results in red and black soils, differed from those in fluvo-aquic soils. The difference among the results of SOD and CAT activities in artificial and natural soils may state the toxicity of fluoxastrobin in different soils was different. This may be due to the different soil types, including pH, TOC, and so on.

GST activity

As Zhu et al. (2011) stated, GST (detoxifying enzyme) can catalyze the nucleophilic coupling of some endogenous or exotic harmful substances with the mercaptol dipole of the modified glutathione, and increase its hydrophobicity so that it can easily cross the cell membrane and expel it after being decomposed, so as to achieve the purpose of detoxification. **Figure 4** illustrated GST activities in *Eisenia fetida* exposed to fluoxastrobin in three natural soils at low exposure concentrations.

<**Fig. 4**>

On day 7, the GST activities at diverse exposure concentrations remain stable in control in fluvo-aquic and black soils except for those of 0.1 mg/kg treatments in fluvo-aquic soils and 1.0 mg/kg in black soils, while those in red clay were notably greater than in control. No notable discrepancy was found between 1.0 and 2.5 mg/kg treatments in all three diverse natural soils. On day 14, the GST activities at diverse exposure concentrations remain stable in control in fluvo-aquic and red clay except for those of 2.5 mg/kg treatments in fluvo-aquic soils and 1.0 mg/kg in red clay, while those in black soils were notably greater than in control. No notable discrepancy was found among 0.1, 1.0, and 2.5 mg/kg treatments in black soils. On day 21, the GST activities at diverse exposure concentrations were all notably

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209 greater than those in control in fluvo-aquic and black soils except for those of 2.5 mg/kg treatments in black soils, while
210 those in red clay were notably lower than in control. No notable discrepancy was found between 0.1 and 1.0 mg/kg
211 treatments in fluvo-aquic and black soils and between 1.0 and 2.5 mg/kg in red clay. On day 28, the GST activities
212 showed a climb from the control to exposure groups in fluvo-aquic and black soils except for those of 2.5 mg/kg
213 treatments in black soils. No notable discrepancy was found among 0.1, 1.0, and 2.5 mg/kg treatments in fluvo-aquic
214 soils, and between 0.1 and 1.0 mg/kg treatments in red clay. The results in black soils showed reached a maximum in
215 1.0 mg/kg treatments (145.9 nmol/min/mg pr) and then reduced. No notable regularity was found in GST activities in
216 all three diverse natural soils, which may be due to the complex process of the stimulation of ROS and detoxifying
217 effects. The aforementioned results showed that GST activities may increase due to stimulation of ROS and LPO (Dong
218 et al., 2009). The values of GST activities in artificial soil were higher compared to those in control not including 0.1
219 mg/kg on days 21 and 28 (Zhang et al., 2018d). Glutathione S-transferase activities reached a maximum in 1.0 mg/kg
220 treatments and then reduced at each exposure time. These results were similar to those in black soils at each exposure
221 time except for those on day 7.
222
223 *MDA contents*
224 Box and Maccubbin (1997) believed ROS can cause LPO, which was evaluated using MDA contents as the biomarker.
225 **Figure 5** illustrated MDA contents in *Eisenia fetida* exposed to fluoxastrobin in three natural soils.
226 <**Fig. 5**>
227
228 On day 7, the MDA contents at diverse exposure concentrations were all notably greater than those in control in
229 red clay and black soils. Furthermore, no notable discrepancy was found among 0.1, 1.0, and 2.5 mg/kg treatments and
230 the controls in fluvo-aquic soils and between 0.1 and 1.0 mg/kg in black soils. On day 14, the MDA contents at diverse
231 exposure concentrations were all notably greater than those in control in all three diverse natural soils except for those
232 in 0.1 mg/kg treatments of fluvo-aquic and black soils. On day 21, the MDA contents at diverse exposure concentrations

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were all greater than those in control in all three diverse natural soils except for those in 0.1 mg/kg treatments of red clay, and in 0.1 and 1.0 mg/kg treatments of black soils. On day 28, the MDA contents at diverse exposure concentrations were all notably greater than those in control in all three diverse natural soils except for those in 1.0 and 2.5 mg/kg treatments of fluvo-aquic soils and in 0.1 mg/kg treatments of black soils. No notable discrepancy was found between 0.1, and 2.5 mg/kg treatments in both fluvo-aquic and red clay.

The excess ROS could induce LPO, and both of the pollutants and LPO could induce DNA damage and product excess ROS (Evert et al., 2004, Zhang et al., 2017). We studied the fluoxastrobin' subchronic toxicity to *Eisenia fetida* in artificial soil (Zhang et al., 2018d). The notable increase was only found in 1.0 mg/kg treatments on day 7. The MDA contents were notably higher than those in control except for those in 0.1 mg/kg on day 14, which was similar to the results in fluvo-aquic and black soils. The results in artificial soils reached a maximum of 1.0 mg/kg treatments and then reduced on day 21, which was different from those in the present study. Notable increases were found in 1.0 and 2.5 mg/kg treatments on day 28. These results may state that fluoxastrobin toxicity to *Eisenia fetida* in artificial soil was different from that in natural soils.

DNA damage (8-OHdG contents)

Previous studies (Box and Maccubbin, 1997; Wood et al., 1990) indicated that ROS can cause DNA-protein crosslinking and damage DNA strands including breaks and space structure changes. Deoxyguanosine, one DNA nucleoside component, can be altered to 8-OHdG, which is considered a biomarker to evaluate oxidative stress and DNA damage (Guo et al., 2014). **Figure 6** illustrates the 8-OHdG content in *Eisenia fetida* exposed to fluoxastrobin in the three natural soils.

<**Fig. 6**>

On days 7 and 14, the 8-OHdG contents at diverse exposure concentrations were all notably greater than those in control in all three diverse natural soils except for those in 0.1 mg/kg treatments in all three diverse natural soils. No

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notable discrepancy was found between 1.0 and 2.5 mg/kg treatments in black soil on day 7 and in fluvo-aquic and red clay on day 14. On days 21 and 28, the 8-OHdG contents at diverse exposure concentrations were all notably greater than those in control in all three diverse natural soils except for those in 0.1 mg/kg treatments of fluvo-aquic soils on day 21 and in black soils on day 28. No notable discrepancy was found between 0.1 and 1.0 mg/kg treatments in both fluvo-aquic and black soils and between 1.0 and 2.5 mg/kg in red clay on day 28.

The excess ROS could induce the production of 8-OHdG (Zhang et al., 2014). The formation of 8-OHdG indicated that there was oxidative and DNA damage following exposure to fluoxastrobin in *Eisenia fetida* (Aguirre-Martinez et al., 2013). Another thing stands out in the present study is that in all six environmental indicators of the present study, 8-OHdG content was more sensitive to fluoxastrobin.

Differential toxicity among artificial soil and three test natural soils

Integrated Biomarker Response (IBR) was considered the method to evaluate the ecological and environmental conditions (Samanta et al., 2018, Wang et al., 2011). The ROS content, MDA content, and SOD, CAT, GST activity of 2.5 mg/kg after 4-week exposure were selected to calculate the IBR index (**Figure 7**). The values of the fluoxastrobin toxicity to *Eisenia fetida* in artificial soil were as per our previous study (Zhang et al., 2018d).

<**Fig. 7**>

In **Figure 7A,** the IBR values which stand for the fluoxastrobin toxicity were 3.20, 6.35, 6.58, and 4.03 in artificial soil, fluvo-aquic soil, red clay, and black soil, respectively. The higher the IBR value, the higher the fluoxastrobin toxicity. Thus, the fluoxastrobin subchronic toxicity to *Eisenia fetida* showed a climb form the artificial soil to the natural soils. The subchronic toxicity was highest in red clay and lowest in black soil among the three natural soils.

In **Figure 7B,** the crossover points of the orange (red, green, and blue) circle, and the coordinate axis were the end point of each indicator. The length between the original point and end point in the star plot represents the standardized

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281 value of each index. The black circle was a baseline (zero). The values greater than zero means indicator stimulate,
282 lower than zero means indicator inhibit. The responses of biomarkers indicated the increase of GST activity, ROS and
283 MDA contents in all the artificial and natural soils except for the decrease of GST activity in black soil. The toxic
284 effects were the decrease of SOD and CAT activity in all the artificial and natural soils except for the increase of CAT
285 activity in fluvo-aquic soil and the increase of SOD activity in artificial soil. Besides, the ROS contents in red clay were
286 similar to those in black soil, the GST activities in artificial soils were similar to those in fluvo-aquic soils, and the
287 MDA contents in artificial soils were similar to those in red clay.

288 As the IBR results stated that the subchronic toxic effects of fluoxastrobin to *Eisenia fetida* in natural soils were
289 different from those in artificial soil. Specifically, the fluoxastrobin subchronic toxicity was highest in red clay and
290 lowest in black soil among the three natural soils. Thus, we considered the toxicology test of fluoxastrobin in artificial
291 soils could not exactly evaluate that in a real environment. The previous study argued that the toxicity of three test
292 pesticides in the field was higher than that in the laboratory (Schnug et al., 2014).

293 Furthermore, the toxic manifestation of fluoxastrobin existed differences in three test natural soils. This important
294 finding stated that the terrestrial toxicity of the test pollutant may be affected by physicochemical properties (Amorim et
295 al., 2005, Stepnowski et al., 2007).

296 Taken together, we evaluated the biochemical responses of *Eisenia fetida* exposed to fluoxastrobin in natural soils
297 with outcomes significantly different from toxicity observation in artificial soils. Though, fluoxastrobin also induced
298 oxidative and DNA damage in *Eisenia fetida* in natural soils at different levels, as the IBR results stated that the
299 fluoxastrobin subchronic toxicity was highest in red clay and lowest in artificial soils. Here come questions? Which one
300 or more physicochemical properties of natural soils affect the toxicity of fluoxastrobin? What is the toxicity of
301 fluoxastrobin to soil organisms in the other natural soils? Are the regularities similar to other pesticides or even other
302 environmental pollutants? All these questions may be arbitrated in future studies.
303

Declarations of interest: None

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Figures

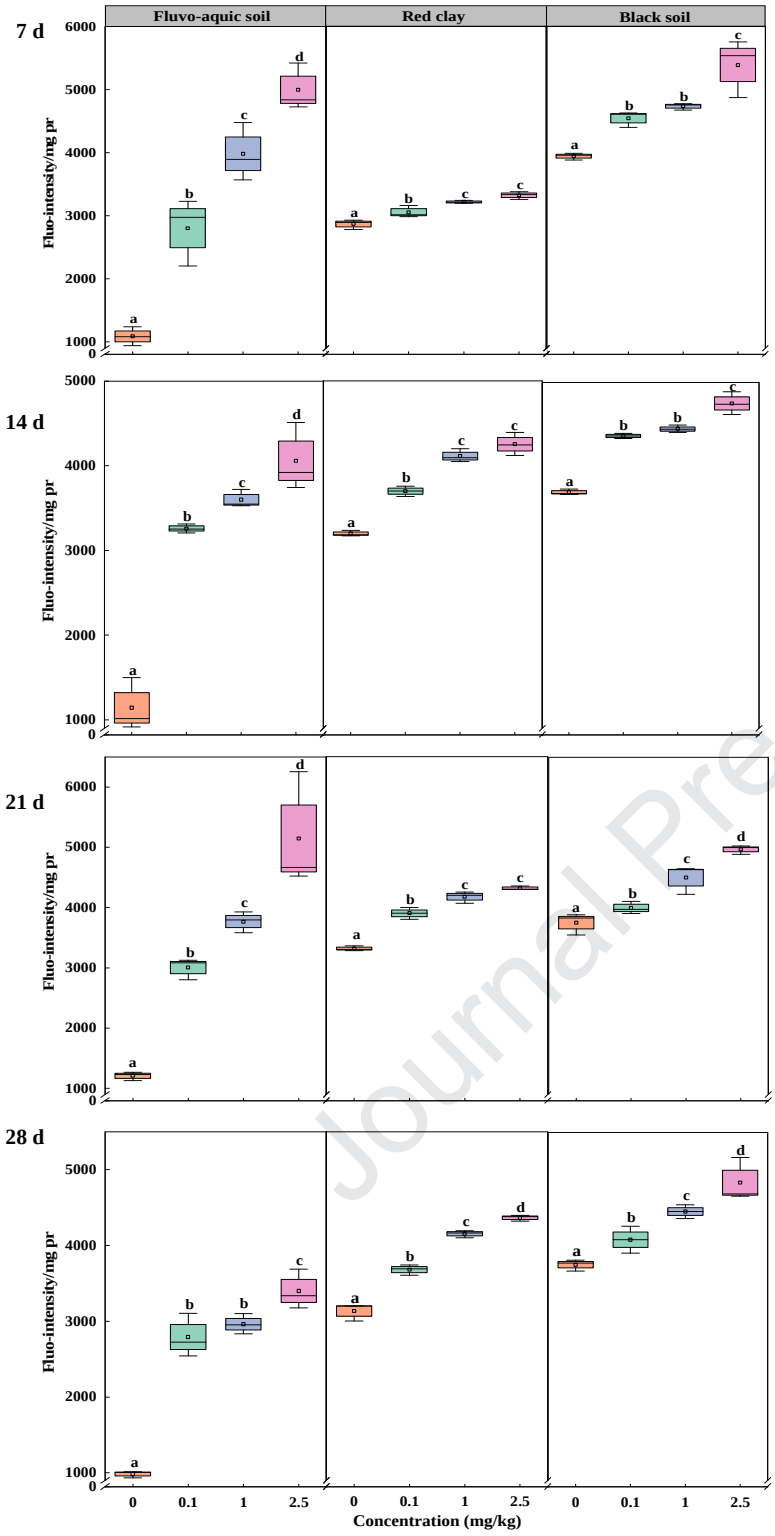

Fig. 1. The ROS content in *Eisenia fetida* exposed to fluoxastrobin in fluvo-aquic soils, red clay, and black soils at diverse concentrations. The small check in the box plot represents mean values of three replicates. The five lines from top to bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. Small letter represents significant difference ($p < 0.05$) among 0 mg/kg and other exposure groups. Pr, protein.

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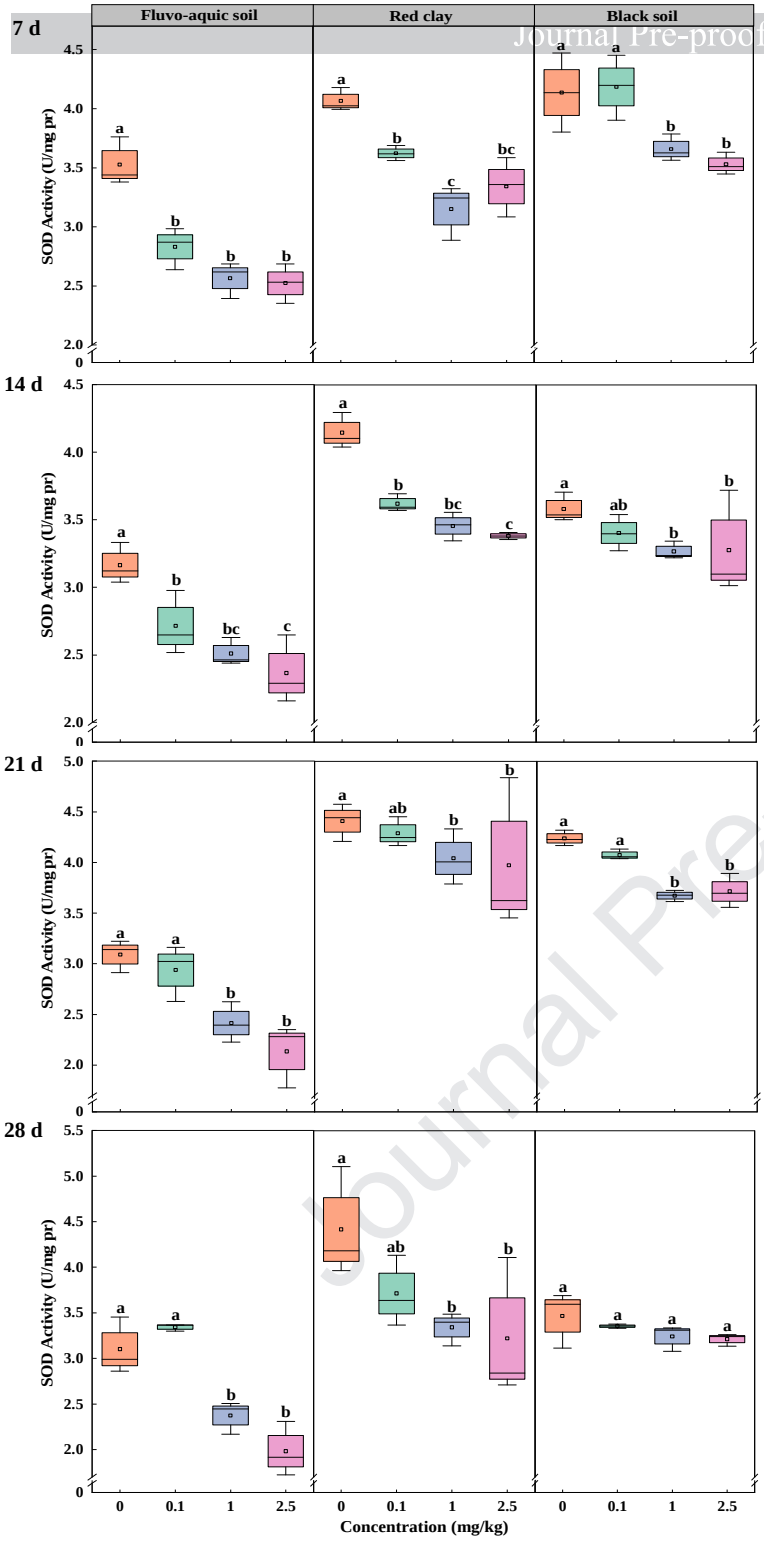

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410 **Fig. 2.** SOD activities in *Eisenia fetida* exposed to fluoxastrobin in fluvo-aquic soils, red clay, and black soils at diverse

411 concentrations. The small check in the box plot represents mean values of three replicates. The five lines from top to

412 bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. Small letter

413 represents significant difference ($p < 0.05$) among 0 mg/kg and other exposure groups. Pr, protein.

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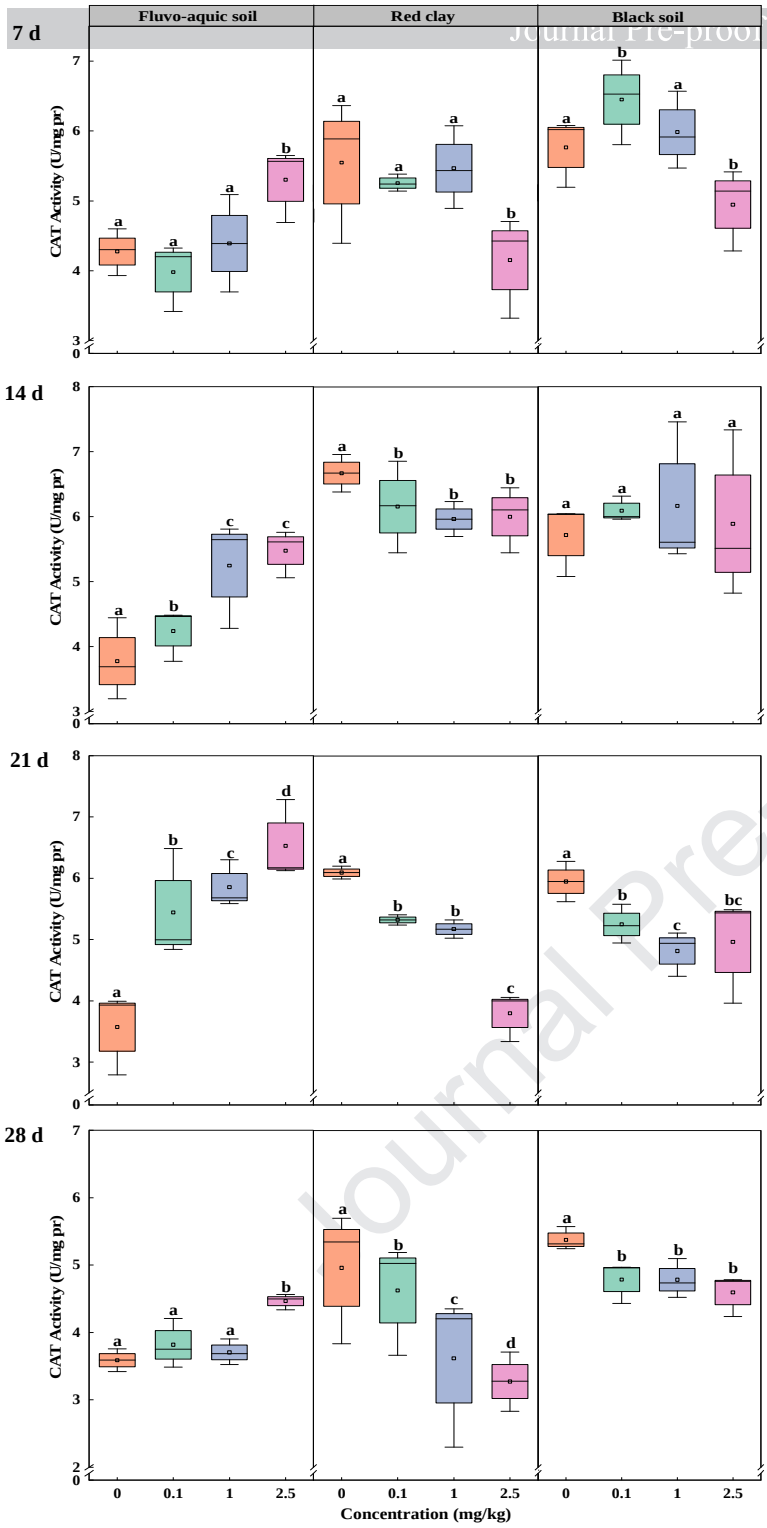

Fig. 3. CAT activities in *Eisenia fetida* exposed to fluoxastrobin in fluvo-aquic soils, red clay, and black soils at diverse concentrations. The small check in the box plot represents mean values of three replicates. The five lines from top to bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. Small letter represents significant difference ($p < 0.05$) among 0 mg/kg and other exposure groups. Pr, protein.

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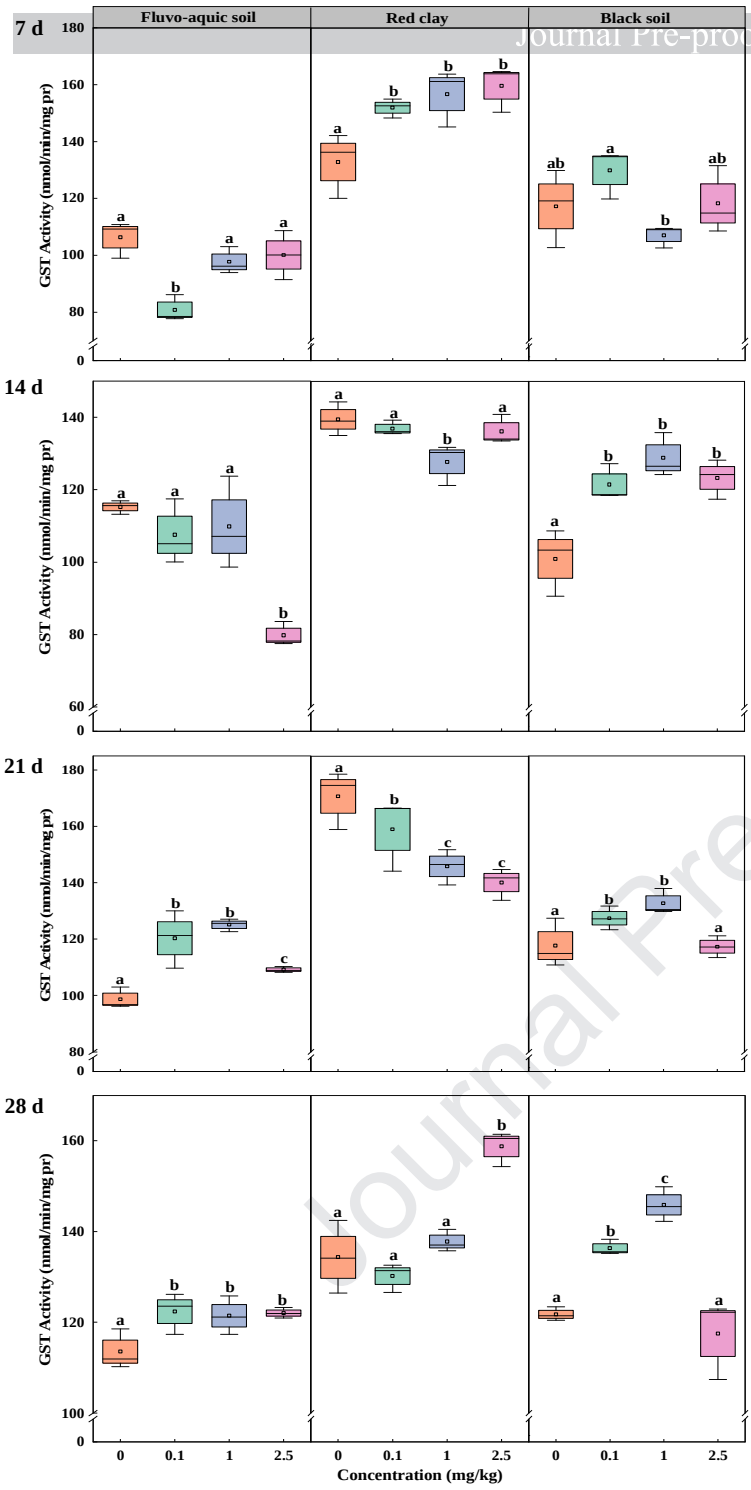

Fig. 4. GST activities in *Eisenia fetida* exposed to fluoxastrobin in fluvo-aquic soils, red clay, and black soils at diverse concentrations. The small check in the box plot represents mean values of three replicates. The five lines from top to bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. Small letter represents significant difference ($p < 0.05$) among 0 mg/kg and other exposure groups. Pr, protein.

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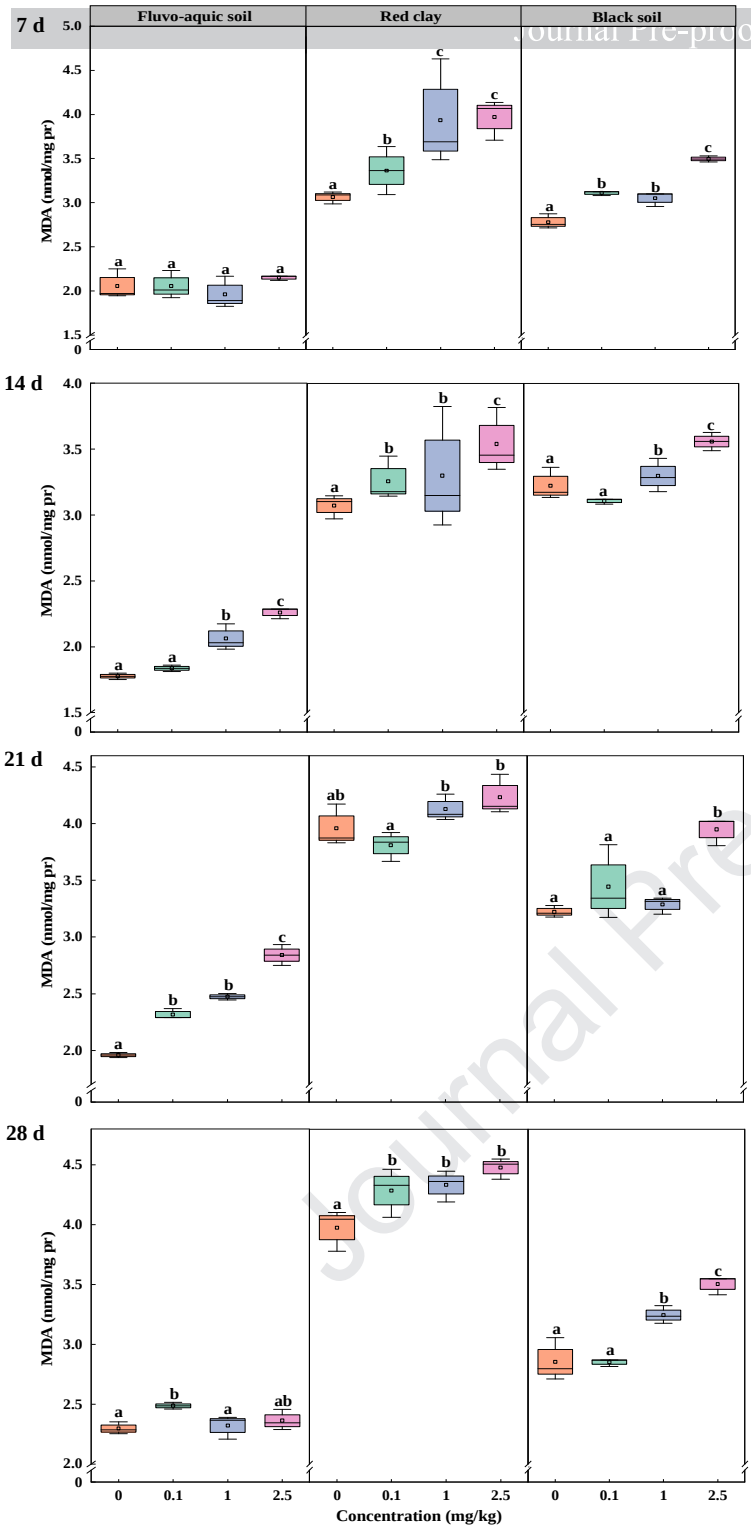

Fig. 5. The MDA content in *Eisenia fetida* exposed to fluoxastrobin in fluvo-aquic soils, red clay, and black soils at diverse concentrations. The small check in the box plot represents mean values of three replicates. The five lines from top to bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. Small letter represents significant difference ($p < 0.05$) among 0 mg/kg and other exposure groups. Pr, protein.

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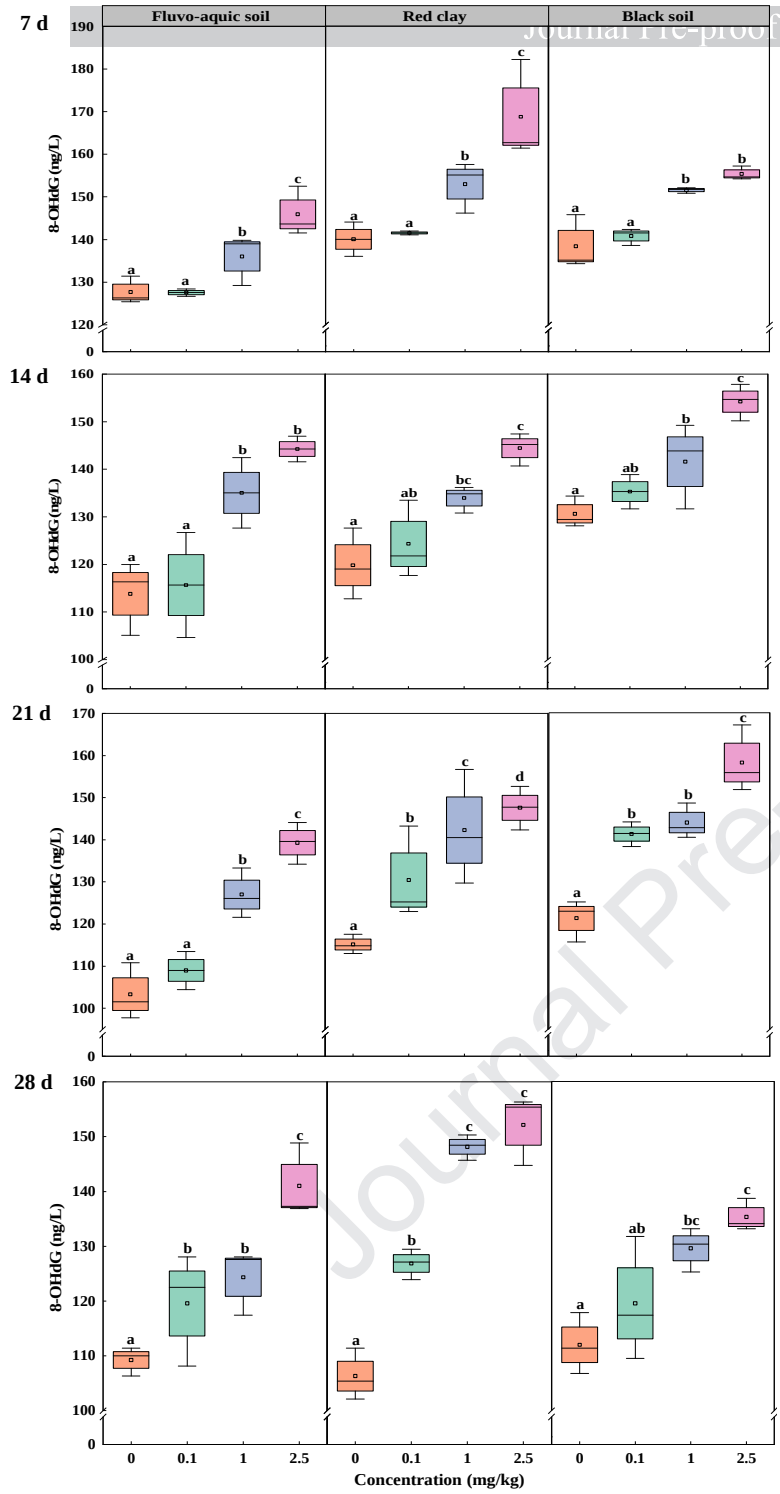

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Fig. 6. The 8-OHdG content in *Eisenia fetida* exposed to fluoxastrobin in fluvo-aquic soils, red clay, and black soils at diverse concentrations. The small check in the box plot represents mean values of three replicates. The five lines from top to bottom represent the maximum, means + standard error (SE), median, means – SE, and minimum. Small letter represents significant difference ($p < 0.05$) among 0 mg/kg and other exposure groups.

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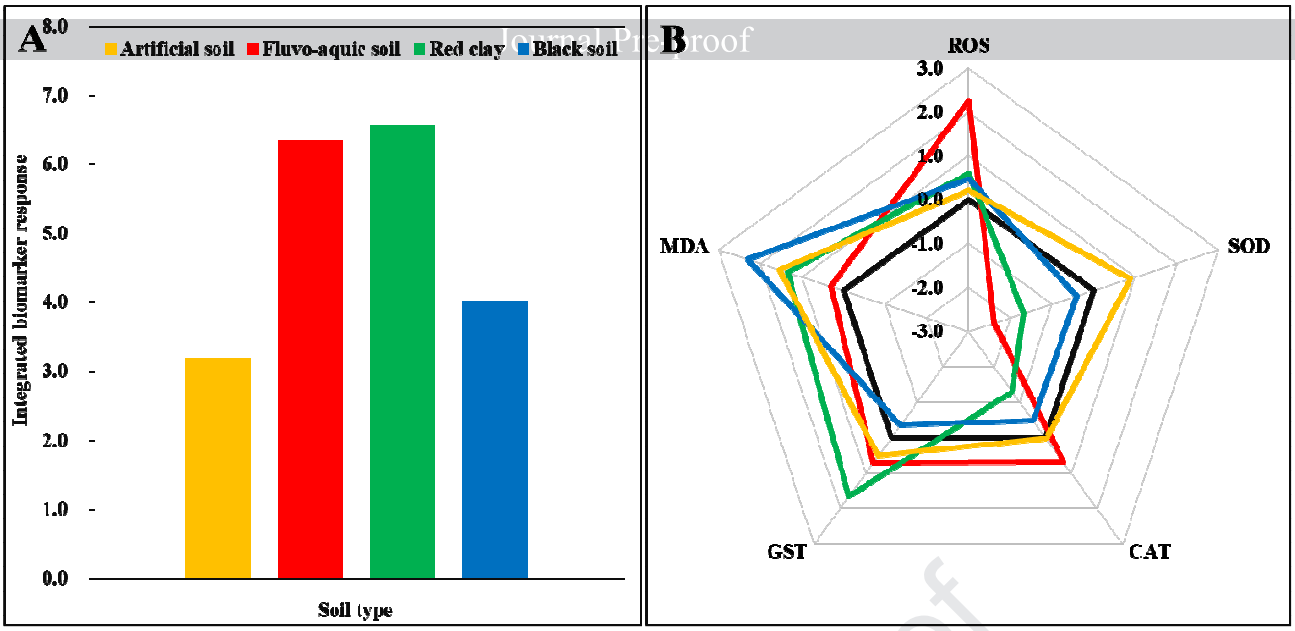

451 **Fig. 7.** Integrated biomarker response index (A) among all the test toxicology indicators exposed fluoxastrobin to

452 *Eisenia fetida* at 2.5 mg/kg in different soil types on day 28. The values (B) greater than zero means indicator stimulate,

453 lower than zero means indicator inhibit (Orange line: artificial soil; red line: fluvo-aquic soil; green line: red clay; blue

454 line: black soil).

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Highlights

Effects of fluoxastrobin on earthworms in different types of soils were compared.

Subchronic toxicity of fluoxastrobin to *Eisenia fetida* was systematically evaluated.

Toxicity in natural soils may not represent that in artificial soil evaluated by IBR.

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Conflicts of interest

The authors declare that they have no conflict of interest. All of the authors have read and approved the manuscript. This work has not been published previously, nor is it being considered by any other peer-reviewed journal.

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