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Ecotoxicity evaluation of azoxystrobin on *Eisenia fetida* in different soils

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ABSTRACT

Azoxystrobin, a widely used broad-spectrum strobilurin fungicide, may pose a potential threat in agricultural ecosystems. To assess the ecological risk of azoxystrobin in real soil environments, we performed a study on the toxic effects of azoxystrobin on earthworms (*Eisenia fetida*) in three different natural soils (fluvo-aquic soil, black soil and red clay soil) and an artificial soil. Acute toxicity of azoxystrobin was determined by filter paper test and soil test. Accordingly, exposure concentrations of chronic toxicity were set at 0, 0.1, 1.0 and 2.5 mg kg^{-1}. For chronic toxicity test, reactive oxygen species, activity of antioxidant enzymes (superoxide dismutase, catalase and peroxidase), detoxifying enzyme (glutathione transferase), level of lipid peroxidation (malondialdehyde) and level of oxygen damage of DNA (8-hydroxydeoxyguanosine) in earthworms were determined on the 7th, 14th, 21st, 28th, 42nd and 56th days after treatment. Both acute and chronic toxic results showed azoxystrobin exhibit higher toxicity in natural soil than in artificial soil, indicating that traditional artificial soil testing method underestimate ecotoxicity of azoxystrobin in a real agricultural environment on the earthworm population. Combining with the analysis of soil physicochemical properties, the present experiment provided scientific guidance for rational application of azoxystrobin in agricultural production systems.

1. Introduction

Azoxystrobin is one of the broad-spectrum strobilurin fungicides, which has a highly effective prevention and cure effect on a variety of fungal diseases, such as wheat septoria, septoria leaf spot, wheat leaf rust, rye leafrust, powdery mildew, downy mildew, stripe rust, etc. (Furzer et al., 2006; Hamdy, 2007; Rodrigues et al., 2013). Due to its high efficiency, azoxystrobin has been widely used in agricultural production. However, the residues of azoxystrobin on crops and in soil have attracted attention. Hou et al. (2016) measured the residue of azoxystrobin in soil and found that the initial residue of azoxystrobin in test field of Jilin Province, China was 9.54 mg kg^{-1}, and the residue of azoxystrobin in test field of Beijing, China was 8.57 mg kg^{-1}. Bartlett et al. (2002) demonstrated that the half-life of azoxystrobin is between 7 and 56 days. Bending GD et al. (2006) measured that the degradation half-life of azoxystrobin was 1–8 weeks, and the degradation of azoxystrobin was significantly related to soil pH. For every increase in pH, the 25% degradation time of azoxystrobin would decrease by 3.6–4.25 weeks. Therefore, azoxystrobin could have negative ecological influences on organisms other than the target fungi.

There have been relevant research studies focused on fish (Cao et al., 2018; Jiang et al., 2018; Liu et al., 2013; Olsvik et al., 2010), algae (Garanzini and Menone, 2015; Kunz et al., 2017; Lu et al., 2017), soil microorganisms (Bacmaga et al., 2015; Guo et al., 2015; Howell et al. 2014; Wang et al., 2015, 2018) and earthworms (Han et al., 2014; Kohlschmid and Ruf, 2016; Leitao et al., 2014; Wang et al., 2012). Studies mentioned above have shown that azoxystrobin does pose potential ecological risks to none-target organisms.

Earthworms as terrestrial invertebrate which have the highest biomass, were used in toxicity test frequently. Kohlschmid and Ruf

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(2016) compared the sensitivity of earthworms and soil arthropods to earthworms to azoxystrobin, and the results showed that earthworms were more sensitive to azoxystrobin. Wang et al. (2012) measured the acute toxicity of azoxystrobin in *Eisenia fetida* by filter paper test and found that the 48 h LD_{50} was 2.72 μg cm^{-2}, and the 7 d and 14 d LD_{50} of artificial soil test were 362.4 and 327.4 mg kg^{-1}. Han et al. (2014) exposed *Eisenia fetida* cultured in artificial soil to azoxystrobin at concentrations of 0, 0.1, 1, and 10 mg kg^{-1} for 28 days and found that ROS was accumulated in earthworms, oxidative stress was promoted enzyme activity, and DNA damage was caused. In previous studies (Han et al., 2014; Wang et al., 2012), acute and chronic toxicity tests of azoxystrobin on earthworms were performed in artificial soil. Results showed azoxystrobin could cause dose related acute toxic effects and chronic oxidative stress in earthworms cultivated in artificial soil. However, artificial soil tests are designed to compare toxicities between different contaminants. Due to that artificial soil has significantly different physicochemical properties compared to natural soil, it may lead to different toxicities of contaminant in natural soil and artificial soil. As a result, it is not accurate to use artificial soil test results to represent the actual situation after azoxystrobin enters natural soil. Therefore, we performed toxicity tests of azoxystrobin on earthworms in three typical natural soils (fluvo-aquic soil, black soil and red clay soil) in China and compared the results with those performed in artificial soil. These three soils as typical soil type in China, each of them has distinctive soil properties, which could represent the natural condition in soil ecosystem.

In the present study, toxic effects of azoxystrobin on earthworms (*Eisenia fetida*) was evaluated in three different natural soils (fluvo-aquic soil, black soil and red clay soil) and an artificial soil. Acute toxicity of azoxystrobin was determined by filter paper test and soil test. Exposure concentrations of chronic toxicity were set at 0, 0.1, 1.0 and 2.5 mg kg^{-1}. For chronic toxicity test, reactive oxygen species, activity of antioxidant enzymes (superoxide dismutase, catalase and peroxidase), detoxifying enzyme (glutathione transferase), level of lipid peroxidation (malondialdehyde) and level of oxygen damage of DNA (8-hydroxydeoxyguanosine) in earthworms were determined on the 7th, 14th, 21st, 28th, 42nd and 56th days after treatment.

In order to compare the chronic toxicity of azoxystrobin to earthworms in different soils, the Integrated Biomarker Response Index (IBR) was used in the present study to evaluate the toxicity differences between different soils according to Sanchez et al. (2013).

The present study is aimed to provide toxicity data of azoxystrobin on earthworms in three typical natural soils and reference for the selection of fungicide in actual agricultural production. Furthermore, the present study could serve as a reminder that only artificial data is not accurate and comprehensive to evaluate ecological risks of chemicals. Therefore, toxic tests of chemicals in natural condition are instructive as well.

2. Materials and methods

2.1. Chemicals

Azoxystrobin used in the present study was purchased from Jingbo Agrochemical Technology Co. Ltd (Shandong, China). Chemicals used in the experiment were analytical grade and purchased from Sigma-Aldrich Co. LLC (St. Louis, Missouri, USA), Beijing Solarbio Science & Technology Co. Ltd. (Beijing, China), and Tianjin Kaitong Chemical Reagent Co. Ltd. (Tianjin, China).

2.2. Earthworms and soil

Earthworms (*Eisenia fetida*) were purchased from Shandong Rizhao earthworm breeding base (Shandong, China). After a two-week cultivation under experimental condition, healthy earthworms weighed between 300 and 600 mg were selected for experiments.

Fluvo-aquic soil was sampled from Dezhou, Shandong Province, China (36.78°N, 116.54°E). Black soil was sampled from Changchun, Jilin Province, China (43.80°N, 125.40°E). Red clay soil was sampled from Nanning, Guangxi Province, China (22.74°N, 109.31°E). Artificial soil was composed of 70% quartz sand, 20% kaoline and 10% dry sphagnum according to the guideline of Organisation for Economic Co-operation and Development (OECD, 1984). Physical and chemical properties of tested soils are measured according to Bao (2000) and detailed as below: the pH was measured with a water: soil ratio 2.5: 1; conductivity was measured with a water: soil ratio 5: 1; cation exchange capacity was measured using 1 M ammonium acetate exchange method; organic carbon and organic matter was measured by the potassium dichromate method; effective nitrogen was measured by alkaline hydrolysis diffusion method; effective phosphorus was measured by sodium hydrogen carbonate solution-Mo-Sb anti spectrophotometric method; rapidly available potassium was measured using ammonium acetate extraction-flame photometry method. The results are listed in Table 1.

2.3. Experimental design

First, filter paper test was performed to preliminarily estimate the percutaneous toxicity of azoxystrobin. According to OECD guideline, filter papers were spread without overlap on customized glass tubes with diameter of 3 cm and length of 8 cm. Azoxystrobin was dissolved in acetonitrile and diluted to different concentrations (0, 0.1, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 8.0 and 10.0 μg cm^{-2}) and 1 mL acetonitrile-azoxystrobin solution was added on the filter paper. After the acetonitrile was fully volatilized, 1 mL of deionized water was added to keep the filter paper moist. Each glass tube contained 1 earthworm and 10 replicates were set in each concentration. All glass tubes were cultivated at 20 ± 2 °C. LD_{50} was calculated after 24 and 48 h.

Then the soil test was performed for acute and chronic toxicity. Fresh soil was sieved with a 2.5 mm diameter sieve to remove stones and plant tissue. According to the moisture content of each type of soil, the equivalent of 500 g dry soil was weighted to ensure the comparability between different soils. Each glass breaker contained 500 g (dry weight) soil. Acetonitrile-azoxystrobin solutions of corresponding concentrations were added into 50–100 g of the soil. After the acetonitrile was fully volatilized, the contaminated soil was mixed with the rest of the soil. Soil water content was controlled within 60–70% of the maximum field capacity by adding distilled water. All glass breakers were cultivated at 20 ± 2 °C. For acute toxicity test, each glass breaker contained 10 earthworms and 3 replicates for each concentration. LD_{50} was calculated after 7 and 14 d. For chronic toxicity test, each glass breaker contained 20 earthworms and 3 replicates for each concentration (0, 0.1, 1.0 and 2.5 mg kg^{-1}). Earthworms were randomly selected to determine oxidative damage on day 7, 14, 21, 28, 42 and 56.

Table 1
Physical and chemical properties of tested soils.

Physical and chemical properties	Fluvo-aquic soil	Black soil	Red clay soil	Artificial soil
pH	7.95	6.07	5.47	5.87
Conductivity (μs cm^{-1})	183	78.4	60.5	232
Cation exchange capacity (cmol kg^{-1})	15.6	23.7	11.7	8.90
Organic carbon (g kg^{-1})	7.73	17.0	3.19	24.8
Organic matter (%)	1.33	2.93	0.72	4.28
Effective nitrogen (mg kg^{-1})	76.9	139	35.7	385
Effective phosphorus (mg kg^{-1})	26.9	18.5	4.79	34.0
Rapidly available potassium (mg kg^{-1})	132	141	51.8	31.2
Sand (%)	20.1	39.0	14.9	70.0
Silt (%)	57.5	29.0	13.8	10.0
Clay (%)	22.4	32.0	71.3	20.0

2.4. Determination of azoxystrobin residue during chronic-toxicity test period

Azoxystrobin concentrations in three natural soils were determined on day 0, 7, 14, 21, 28, 42 and 56. Soil samples were treated by the QuEChERS (quick, easy, cheap, effective, rugged and safe) method for extraction and purification (González-Curbelo et al., 2014). The extracting solutions were used to detect azoxystrobin concentration by gas chromatography (7890B, Agilent, USA). Limit of detection was 1.42 $\times$ 10^{-11} g and recovery rate was 114.07%–116.07%.

2.5. Preparation of earthworm samples

Sample 1: For each exposed concentration, three earthworms were selected randomly from three breakers. After being cultivated in culture dish for one night to empty the intestinal canal, earthworms were ground with PBS and then centrifuged (Eppendorf, Centrifuge 5804, Germany) at 10000 rpm (4 °C) for 10 min. The supernate was used to determine SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), GST (glutathione transferase) activity and MDA (malondialdehyde) content.

Sample 2: Same method as sample 1 was applied, three earthworms from three breakers with empty intestinal canals were ground with PBS and centrifuged at 3000 g (4 °C) for 10 min. The supernate was centrifuged at 20000 g (4 °C) for 20 min. The sediment was resuspended with 1 mL PBS. Final solution was used to determine ROS (reactive oxygen species) content.

Sample 3: Similar to the methods for samples 1 and 2, three earthworms were ground with PBS and then centrifuged at 6500 rpm (4 °C) for 15 min. The supernate was used to determine 8-OHdG (8-hydroxy-2-deoxyguanosine) content.

Protein content of each sample was determined with a spectrophotometer (UV-2550, Shimadzu, Japan) according to Bradford (1976). Standard curves were run to calculate protein content (mg mL^{-1}).

2.6. Determination of oxidative damage

Reactive oxygen species (ROS) content was determined using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) method (Guo et al., 2015). 2′,7′-dichlorofluorescein is the product of reaction of ROS and DCFH-DA, which can be detected by a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan). MDA content was determined according to Han et al. (2014) by evaluating thiobarbituric acid chromogenic reaction. The UV absorbance were read at 532 nm. DNA oxidative damage indicator (8-OHdG) was determined using enzyme linked immunosorbent assay kit (Shanghai Hengyuan Biological Technology Co., Ltd, China). The samples were read at 450 nm by microplate reader (MK3, Thermo Fisher, USA).

2.7. Determination of enzyme activity

SOD activity was determined by assessing the inhibition of nitroblue tetrazolium chloride (NBT) photochemical reduction according to the method of Giannopolitis and Ries (1977). The UV absorbance was measured at 560 nm.

CAT activity was determined using the method of Xu et al. (1997) by measuring the decomposition of H_2O_2. The dynamic changes of UV absorbance were read every 5 s for 1 min.

POD activity was determined by the method of Kochba et al. (1977). The reaction solution contained guaiacol and H_2O_2 was mixed with the enzyme extraction and the dynamic changes of UV absorbance at 470 nm were recorded every 30 s for 3 min.

GST activity was determined according to Habig et al. (1974) by monitoring the reaction rate of GSH and 1-chloro-2,4-dinitro-benzene (CDNB) catalyzed by GST. UV absorbance of the reaction product were read every 30 s for 3 min.

2.8. Statistical analysis

SPSS (Version 22.0) was used for data processing and then expressed as means ± standard deviation (SD). Comparison of data was conducted by the one-way analysis of variance (AVOVA) using least significant differences ($p<0.05$) test. Excel 2016 was used to draw the figures.

To compare the toxic difference of azoxystrobin in natural soil and artificial soil, data of the present experiment and data from our previous study (Han et al., 2014) at same concentration groups, were used to calculate IBR index based on Sanchez et al. (2013). Values of IBR stand for toxicity level of azoxystrobin as an integrated index.

3. Results and discussion

3.1. Acute toxicity of azoxystrobin on earthworm by filter and soil test

In filter paper test, no earthworm died in control group to prove the acetonitrile was fully volatilized in all treatments. After earthworm exposure to azoxystrobin for 24 h, the LD_{50} was 2.64 (2.15–3.12) μg cm^{-2}; after 48 h, the LD_{50} was 0.47 (0.24–0.73) μg cm^{-2}. Compared to data from Wang et al. (2012), where the LD_{50} of 48 h was 2.72 (2.22–3.19) μg cm^{-2}, azoxystrobin showed bigger toxicity in the present experiment. The main reason for the toxicity difference is that we used the glass tube recommended by OECD with diameter of 3 cm to perform the experiment while Wang et al. (2012) used culture dish with diameter of 9 cm. Earthworms are more able to escape from the contaminated filter paper in culture dish, which can make the results inaccurate. However, filter paper test can only evaluate epidermis toxicity of azoxystrobin, so further toxic mechanisms should be detected. Therefore, soil acute toxicity test was performed and toxicity in different soils was compared. The results of soil acute toxicity test are shown in Table 2.

As shown in Table 2, toxicity order was: red clay soil > fluvo-aquic soil > black soil > artificial soil. Azoxystrobin showed bigger toxicity in the three natural soils.

In the present study, the 7 d LD_{50} of azoxystrobin in artificial soil was 607.2 (544.8–675.6) mg kg^{-1} and 14 d LD_{50} was 528.7 (471.2–588.9) mg kg^{-1}. In the study of Wang et al. (2012), the 7 d LD_{50} of azoxystrobin in artificial soil was 362.4 (302.1–517.5) mg kg^{-1} and the 14 d LD_{50} was 327.4 (279.9–439.0) mg kg^{-1}. In the same artificial soil formulation, azoxystrobin in the present study showed lower toxicity than in the study of Wang et al. (2012). This difference could be a result of different experimental operations or different earthworm properties. In contrast, however, our study did find a result for the significantly higher toxicity of azoxystrobin in natural soil.

3.2. Azoxystrobin residue during chronic-toxicity test period

Azoxystrobin in three natural soils with exposure concentration of 2.5 mg kg^{-1} was verified on day 0 to ensure the exposure concentration was accurate and was also monitored throughout the experimental period. As shown in Fig. 1, azoxystrobin gradually degraded during the experiment but persisted at low levels even at the end of the study. Bartlett (2002) demonstrated the half-life period of azoxystrobin in soil ranged from 7 d to 56 d. Bending et al. (2006) confirmed this result and verified the degradation of azoxystrobin in soil was related to pH. For

Table 2
Acute toxicity test results of azoxystrobin on earthworms in soil.

Soil	7-d LD_{50} (mg kg^{-1}) (95% confidence interval)	14-d LD_{50} (mg kg^{-1}) (95% confidence interval)
Fluvo-aquic soil	53.8 (43.5–66.0)	32.5 (18.6–44.8)
Black soil	266.6 (235.8–297.3)	241.0 (185.2–244.1)
Red clay soil	37.4 (33.7–42.2)	21.3 (19.8–28.7)
Artificial soil	607.2 (544.8–675.6)	528.7 (471.2–588.9)

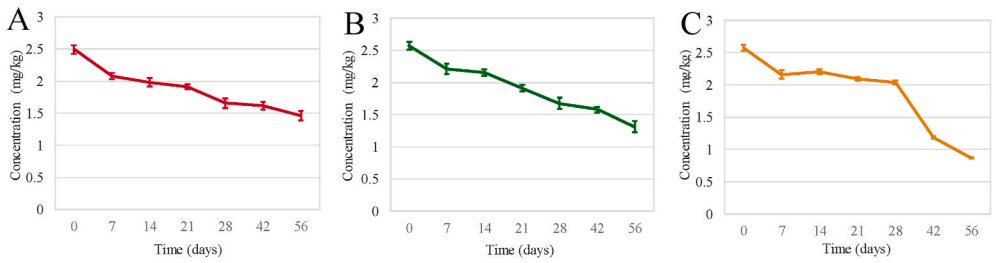

Fig. 1. Azoxystrobin residue change in fluvo-soil (A), black soil (B) and red clay soil (C) during chronic-toxicity experimental period (exposure concentration: 2.5 mg kg^{-1}). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

each unit increase in pH, the 25% degradation time decreased by 3.6–4.25 weeks, which is consistent with the concentration change in our three natural soils. Results confirmed accuracy of the experimental operation and provided evidence of following toxic behavior of azoxystrobin.

3.3. Antioxidant enzyme activity of earthworm after exposure to azoxystrobin

Superoxide anion radical in organisms could be scavenged by SOD, which is the primary antioxidant enzyme (Singh et al., 2006). Afterwards, byproduct H_2O_2 was continuously decomposed by CAT to protect the organisms (Gill and Tuteja, 2010). As shown in Fig. 2, in general, the activity of SOD in earthworms was increased in all three types of soil, except for the 7th day in red clay soil, where there was no significant difference in SOD activity between the control group and the azoxystrobin treatment group. This shows that in general, exposure to azoxystrobin in the three soils activated SOD activity in earthworms.

In this test, based on the changes of SOD activity in earthworms exposed to azoxystrobin in different soils, azoxystrobin can stimulate the increase of SOD activity in earthworms at low concentrations (0.1 mg kg^{-1}), but at the initial stage of exposure, the change of SOD activity is

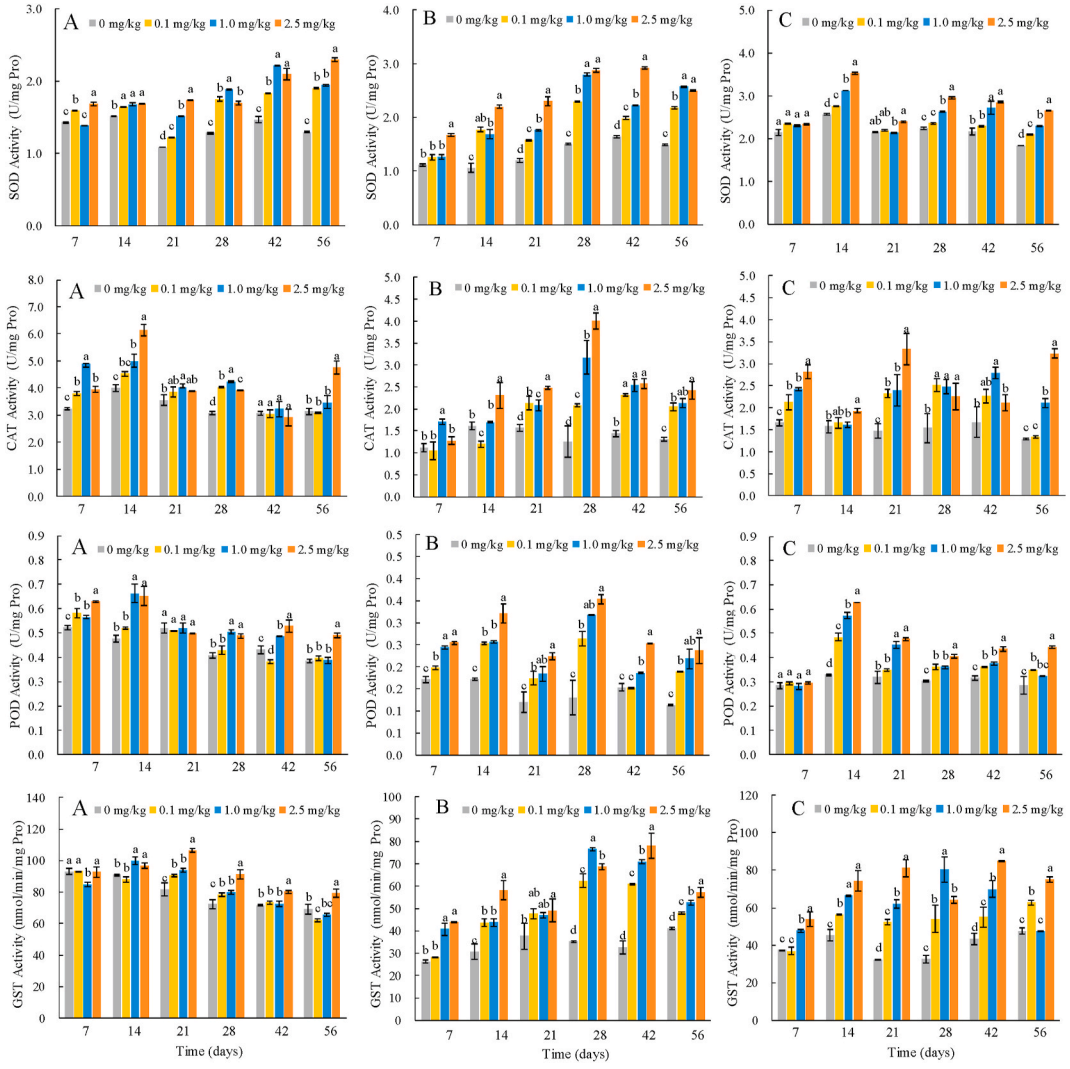

Fig. 2. Effect of azoxystrobin on the enzyme activity in earthworms in fluvo-aquic soil (A), black soil (B) and red clay soil (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

not significant, and the increase of SOD activity is more obvious with the increase of exposure time. This shows that after exposure to azoxystrobin, the SOD in the earthworm is activated to remove excess ROS. Carina et al. (2018) studied the toxicity of the herbicides diuron and fluroxyline to *Andersei* earthworms and found that fluroxyline significantly affected the SOD gene expression level in earthworms. Xue et al. (2009) studied the toxicity of tetrabromobisphenol A on earthworms and found that the SOD activity in earthworms was increased and used to remove excess ROS. Zhang et al. (2018) studied the subchronic toxicity of fluoxastrobin to earthworms in artificial soils. The SOD activity in the body changed first and then was inhibited, indicating that fluoxastrobin first activated SOD to remove ROS, and then the accumulation of ROS was beyond the range of SOD removal, and the activity of SOD was inhibited instead. Han et al. (2014) studied the chronic toxicity of azoxystrobin to earthworms in artificial soil. The activity of SOD in earthworms showed an upward trend during the 28-day exposure period, which was consistent with the results of this test.

From the general trend of CAT activity (Fig. 2), azoxystrobin promoted CAT activity in earthworms in three types of soils. The maximum of this promotion effect appeared on the 14th, 28th and 21st days in fluvo-aquic soil, black soil, and red clay soil, respectively.

CAT is a very efficient antioxidant enzyme, and its catalytic efficiency increases with increasing hydrogen peroxide concentration. CAT activity was found in fluvo-aquic soil, black soil, and red clay soil on the 14th day, and the maximum appeared on 28 days and 21 days.

In the three types of soil, CAT was activated in earthworms exposed to azoxystrobin. It may be that SOD decomposes ROS in the earthworm into H_2O_2, which stimulates the increase of CAT activity. It confirmed that azoxystrobin had effect against earthworms and caused oxidative stress. Han et al. (2014) studied the chronic toxicity of azoxystrobin to earthworms in artificial soil and found no significant change in CAT activity in earthworms. Zhang et al. (2018) studied the subchronic toxicity of fluoxastrobin to earthworms in artificial soil and found that CAT in earthworms showed a trend of first inhibition and then activation. Compared with the above two tests, the CAT activity in earthworms was significantly increased in this test. It is speculated that it may be because azoxystrobin is more toxic to earthworms in natural soils than in artificial soils, causing earthworms to generate hydrogen peroxide more, and in turn, stimulating CAT activity. In this case, CAT activity shows an upward trend and is used to remove excess hydrogen peroxide.

The main function of POD is to catalyze the oxidation reaction between H_2O_2 and hydrogen donors (Vidossich et al., 2012), and it can remove hydrogen peroxide, phenols and amines (Chance et al., 1995). According to Fig. 2, POD activity in earthworms exposed to azoxystrobin showed an upward trend. There was no significant difference between the concentrations of POD activity in fluvo-aquic soil and black soil. The relationship between POD activity and azoxystrobin concentration on the 14th day of exposure in red clay soil was significant. The change mechanism of POD is similar to that of CAT, so POD activity also appears to be activated, which can also explain that earthworms exposed to azoxystrobin in three types of soil showed oxidative stress effects. In the study of Han et al. (2014), POD activity in earthworms exposed to azoxystrobin in artificial soils was constantly activated. Song et al. (2009) found in the study that atrazine had an oxidative stress effect on earthworms and activated the POD activity in earthworms, presumably because the accumulation of ROS in the earthworms caused the hydrogen peroxide content of its decomposition products to increase, which stimulated increased POD activity and removed excess hydrogen peroxide. The results of the above experiments are consistent with this study (Fig. 2), and together show that the increase in POD activity can be used as one of the indicators of oxidative stress on earthworms.

Glutathione s-transferase (GST) is a detoxifying enzyme that scavenges ROS to reduce oxidative damage. GST also has a detoxifying effect, which can be combined with lipophilic toxic substances such as bile acid, dyes, hormones, etc., to make it into a hydrophilic substance, and then excreted through metabolism to achieve detoxification. As shown in Fig. 2, the effect of azoxystrobin on GST activity in earthworms in three soils was mainly activation, except for the 14th, 21st and 42nd days in red clay soil. No significant dose-toxic effects showed during most other exposure cycles. GST is an important detoxifying enzyme in earthworms. The increase in GST activity indicates that toxic substances are produced in earthworms, which stimulates GST to protect the body more efficiently. Therefore, the increase in GST activity also confirmed that earthworms produced more peroxides after being exposed to azoxystrobin and were subjected to oxidative stress. In the study of Han et al. (2014), GST activity in earthworms exposed to azoxystrobin in artificial soils increased with increasing exposure concentration and exposure time. Lin et al. (2010) found in the study that the antibacterial agent triclosan caused an increase in GST activity in earthworms. This study showed that GST can catalyze the reaction of triclosan with GSH, thereby reducing the toxicity of triclosan to earthworms. In this test, azoxystrobin itself and ROS in earthworms may cause increased GST activity. The activation of GST activity indicates that there are many toxic substances in the earthworm body that can be used as a substrate to bind GSH and confirms the toxicity of azoxystrobin to earthworms.

3.4. Oxidative damage of earthworm after exposure to azoxystrobin

Reactive oxygen species (ROS) is an intermediate product of normal cell metabolism, mainly including superoxide anions, hydroxyl radicals, and hydrogen peroxide (Lepetsos et al., 2016). It participates in the mitochondrial respiratory chain enzyme reaction and plays a role in regulating cell differentiation and apoptosis (Dayem et al., 2017). Normally, the production and elimination of ROS in the body is in a dynamic balance, but exposed to external stimuli or pressure, excess ROS will be generated, beyond the range that the body can clear itself of; this excess ROS will react with biological macromolecules, causing damage to the body (Kim et al., 2008).

In the present study, the oxidative stress of azoxystrobin on earthworms in three kinds of soil was not obvious on the seventh day of exposure (Fig. 3). On days 14, 21 and 28, azoxystrobin began to show oxidative stress on earthworms in all three soils, but the relationship between this effect and concentration is not clear. From the 42nd day, azoxystrobin in all three soils began to have a significant effect on the ROS content in earthworms, and this effect was related to the concentration of azoxystrobin, which showed a significant dose-toxic effect. The ROS content increased with the increase of azoxystrobin concentration. It shows that the earthworms exposed to azoxystrobin in all three types of soil have oxidative stress effects, and the ROS content in the body tends to increase. With the increase of azoxystrobin concentration and the extension of the test period, the oxidative stress produced by the earthworm makes the stimulus effect more obvious. Han et al. (2014) studied the effect of azoxystrobin on the ROS content in earthworms in artificial soil. The exposure concentration was the same as in this test, and the exposure period was 7, 14, 21 and 28 days. It was found that except for low concentrations (0.1 mg kg^{-1}), the ROS content of the azoxystrobin treatment group was significantly higher than that of the control group, which was consistent with the change trend of ROS content in the present study.

Lipid peroxidation (LPO) come up with disorder of metabolism and have malonaldehyde as a final product. Therefore, malonaldehyde can reflect the degree of lipid peroxidation of the body and can be used as an index to evaluate the degree of oxidative damage caused by pollutants to the organism (Duryee et al., 2010). As shown in Fig. 3, MDA content of earthworms exposed to azoxystrobin in fluvo-aquic soils increased first and then decreased with the extension of the exposure period. The MDA content in black soil and red clay soil followed the exposure period. This shows that earthworms in the fluvo-aquic soil were first damaged by lipid oxidation. Later, due to the combined action of antioxidant enzymes and detoxifying enzymes in the body, the content of ROS decreased, and the effect on lipid macromolecules also decreased. In black soil and red clay soil, the MDA content was increasing during the

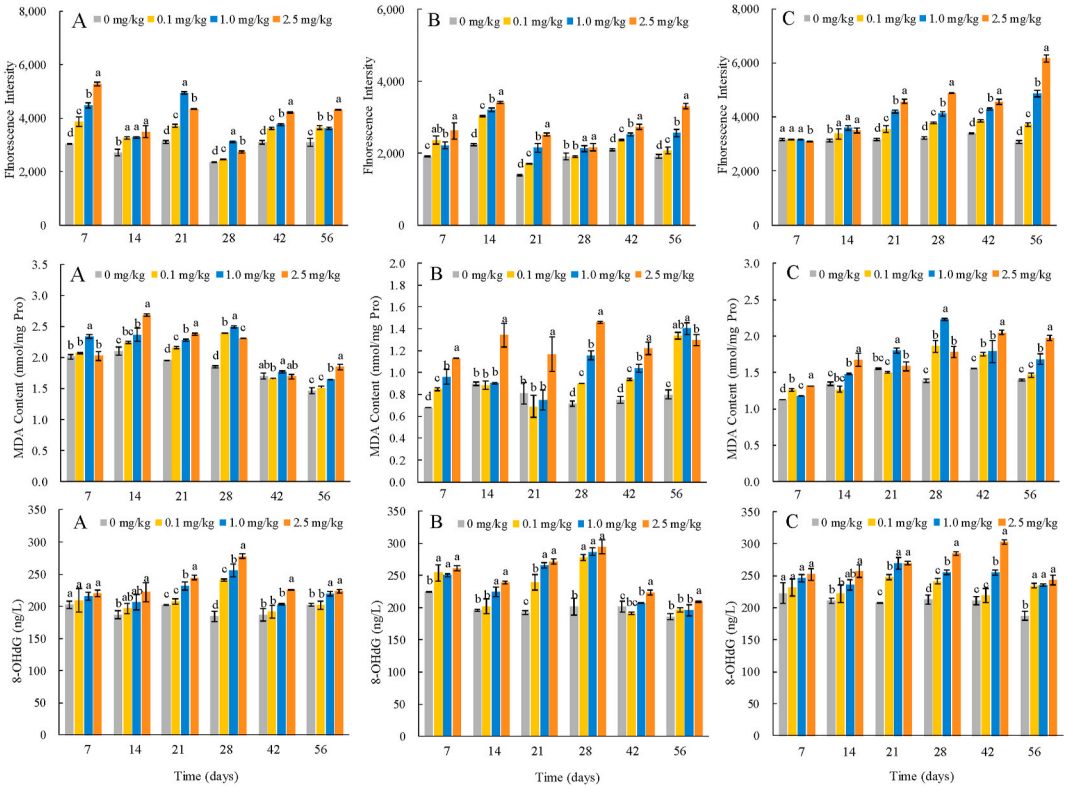

Fig. 3. Effect of azoxystrobin on the oxidative damage on earthworms in fluvo-aquic soil (A), black soil (B) and red clay soil (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

poisoning cycle. It may be because the antioxidant enzymes and detoxifying enzymes in the earthworm are not enough to remove excess ROS, which causes the earthworm toxicity in the black soil and red clay soil to be more severe. Zhang et al. (2018) studied the toxicity of fluoxastrobin to earthworms in artificial soils and observed that MDA showed a trend of first an increase and then a decrease in earthworms exposed to fluoxastrobin, as compared with exposure to fluvo-aquic soil in this study. The change trend of MDA content in earthworm after azoxystrobin was the same.

8-hydroxy deoxyguanosine (8-OHdG) is the product of the eighth carbon atom of guanine in DNA molecules attacked by ROS. Several studies have confirmed that 8-OHdG can be used as an indicator of DNA oxidative damage to assess the toxicity of pollutants to earthworms. In this test, the analysis of 8-OHdG from earthworms in different soils was comprehensively analyzed. Based on Fig. 3, the effect of azoxystrobin on 8-OHdG showed an overall upward trend, and each concentration showed a significant increase on the 28th day of exposure. The effect of azoxystrobin on 8-OHdG is longer. Zhang et al. (2014) exposed *Eisenia foetida* to chlorinated flame retardants and found that the content of 8-OHdG, an oxidative stress marker, increased. Yamashita et al. (2008) exposed earthworms to soils containing metal (Cd, Ni), and immunohistochemical analysis showed that 8-OHdG accumulation was detected only in *E. fetida* seminal vesicles treated with 10 μg Cd for 3 months. The above research is consistent with the change trend of 8-OHdG content in this experiment. Based on the conclusions obtained from the above studies, it can be inferred that azoxystrobin has caused DNA oxidative damage to earthworms in three types of soil.

3.5. Comparison of chronic toxicity in different soils using IBR

In order to compare the toxicity of azoxystrobin on earthworms in different soils, this study introduces a comprehensive biomarker response index (IBR), which standardizes the toxicity index data in different soils and calculates the magnitude of the toxicity. The chronic toxic data of azoxystrobin on earthworms in artificial soil was taken from the previous research (Han et al., 2014) of our laboratory. According to the composite index, the larger the IBR value, the greater is the chronic toxicity. The standardized values of the indicators are shown in Table 3. The sum of the standardized values of all indicators in each soil is the value of IBR. As shown in Fig. 4, the IBR values of chronic toxicity of azoxystrobin to earthworms in fluvo-aquic, black, red, and artificial soils were 8.01, 13.68, 9.93, and 4.75, respectively. Thus, the order of chronic toxicity of azoxystrobin on earthworms in different soils was: black soil > red clay soil > fluvo-aquic soil > artificial soil.

In the soil with low organic matter and low pH, azoxystrobin has a longer degradation half-life, so it will affect various physiological indicators of earthworms throughout the test cycle, and the toxicity is more obvious with the extension of the test cycle. Bending et al. (2006) determined that the degradation half-life of azoxystrobin in soil is 1–8 weeks, and that the degradation of azoxystrobin is significantly related to the pH of the soil. For each unit increase in pH, the 25% degradation time of azoxystrobin will reduce over 3.6–4.25 weeks. That is, the higher the soil pH, the shorter the azoxystrobin stays in the soil. The research by Bending et al. (2006) confirmed the hypothesis that pH affects the half-life of azoxystrobin in soil, and thus affects the chronic toxicity of earthworms.

Table 3
Standardized values of indicators in earthworms exposed to azoxystrobin in fluvo-aquic soil, black soil, red clay soil and artificial soil.

Indicators	Fluvo-aquic Soil	Black Soil	Red Clay Soil	Artificial Soil
ROS	2.649	1.002	2.354	0.607
SOD	1.963	3.143	0.807	1.369
CAT	0.654	1.913	0.987	0.508
POD	0.613	2.603	0.502	0.718
GST	0.251	1.958	2.275	0.578
MDA	1.884	3.060	3.004	0.968

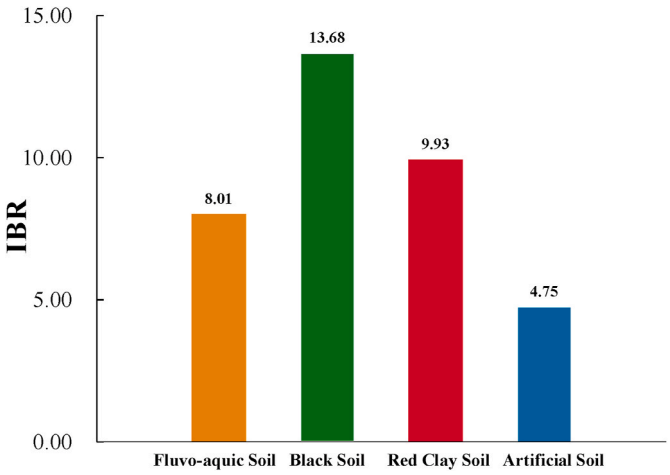

Fig. 4. IBR index of all indicators of earthworms after exposure to azoxystrobin in different soils and standardized values of indicators in earthworms exposed to fluvo-aquic soil (A), black soil (B), red clay soil (C) and artificial soil (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

For acute toxicity, azoxystrobin showed higher toxicity on earthworm in natural soils than in artificial soil. Specifically, the toxic order was: red clay soil > fluvo-aquic soil > black soil > artificial soil.

In chronic toxicity test, ROS content increased in earthworms exposed to azoxystrobin with dose-effect relationship. Oxidative damage is considered to be the main mechanism of how azoxystrobin chronically affects earthworms. In a result, antioxidant enzyme activities were activated. In addition, MDA content and 8-OHdG content increased as well, indicating oxidative damage including LPO and DNA damage. According to IBR index, chronic toxicity order of azoxystrobin on earthworm in different soils was: black soil > red clay soil > fluvo-aquic soil > artificial soil.

In conclusion, based on acute and chronic toxicity results, artificial soil tests may underestimate ecotoxicity of chemicals to earthworms. Artificial soil tests as a traditional way to access environmental ecological risk may therefore not be accurate.

Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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