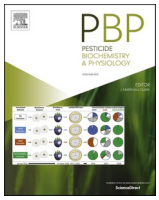
Pesticide Biochemistry and Physiology 187 (2022) 105198

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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



Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product

Marija Kovačević, Nikolina Stjepanović, Davorka K. Hackenberger, Željka Lončarić, Branimir K. Hackenberger *

University of Osijek, Department of Biology, Cara Hadrijana 8A, HR-31000 Osijek, Croatia

A R T I C L E I N F O

Keywords:
Survival
Reproduction
Oxidative stress
Energy available
Strobilurin

A B S T R A C T

Due to the often-excessive usage of fungicides, increasing attention is being paid to their impact on soil and non-target organisms. Risk assessments are usually based on the pure active ingredient and not on the formulated products applied in the environment. The aim of this study was therefore to investigate how azoxystrobin, the best-selling strobilurin fungicide, affects non-target soil organisms *Enchytraeus albidus*. To investigate the effects of the different types of azoxystrobin, *E. albidus* was exposed to the pure active ingredient, AZO_AI, and the formulated product, AZO_FP. Survival, reproduction, and molecular biomarkers of *E. albidus* were determined for different exposure durations (seven and 21 days). AZO_FP (LC_{50} = 15.3 $mg_{a.i.}$/kg) showed a slightly stronger effect on survival than AZO_AI (LC_{50} = 16.8 $mg_{a.i.}$/kg), yet the impact on reproduction was much stronger. Namely, while the tested concentrations of AZO_AI ($EC_{50}\geq$ 8 $mg_{a.i.}$/kg) had almost no effect on reproduction, AZO_FP (EC_{50} = 2.9 $mg_{a.i.}$/kg) significantly inhibited reproduction in a dose-dependent manner. Changes in enzyme activities (superoxide dismutase, catalase, glutathione-s-transferase) and malondialdehyde levels in both treatments indicated oxidative stress. Although AZO_FP had a stronger negative effect, the impact depended on the exposure time and the tested concentration. The higher toxicity of AZO_FP was a consequence of increased bioavailability and activity of the active ingredient due to the presence of adjuvants. Overall stronger adverse effects of AZO_FP suggest that the toxicity of azoxystrobin in the agricultural environment on the enchytraeid population may be underestimated. Furthermore, the results of this study highlighted the importance of comparing the toxicity of the active ingredient and the formulated product.

1. Introduction

To ensure high crop yields and protection against plant diseases and pests, plant protection products (PPPs) are commonly used in modern agriculture. Among the PPPs sold in the European Union (EU) in 2019, fungicides represented 40% of sales (Eurostat). Strobilurin fungicides (Sfs) are one of the essential fungicides used in large quantities world-wide due to their efficacy. SFs act by blocking ATP production and thus impairing mitochondrial respiration (Bartlett et al., 2002). Azoxystrobin (AZO) is one of the most important and highly efficient broad-spectrum SFs. Due to its wide use, AZO enters the aquatic and soil ecosystem through agricultural and urban runoff. The presence of azoxystrobin residues was detected in 22 out of 317 soil samples collected across Europe (Silva et al., 2019). The reported median concentration of azoxystrobin was 0.03 mg/kg, while the maximum concentration reached 0.25 mg/kg. Moreover, concentrations of AZO of up to 9.5 mg/kg were detected in Chinese soils (Xu et al., 2021). Namely, due to the increase in average humidity and temperatures, the possibility of fungal disease development in crops has increased, which has consequently led to an increased application of all PPPs, and especially fungicides.

Among SFs, AZO has attracted scientific attention because of its overapplication and ecotoxicity. AZO has been shown to affect various non-target organisms, including mammals, amphibians, aquatic, and soil organisms (Zhang et al., 2020). While the impact on aquatic organisms have been extensively studied (for a review see Rodrigues et al., 2013), most of the research among soil organisms has been conducted on earthworms (Wang et al., 2012; Han et al., 2014; Zhang et al., 2018; Zhang et al., 2020; Xu et al., 2021; Wu et al., 2021). AZO tested as an active ingredient was highly toxic (Wang et al., 2012) and caused oxidative stress to earthworm *Eisenia fetida*, leading to lipid peroxidation and DNA damage (Han et al., 2014). However, testing only the active ingredient may lead to an underestimation of the ecotoxicological

* Corresponding author.
E-mail address: hackenberger@biologija.unios.hr (B.K. Hackenberger).

https://doi.org/10.1016/j.pestbp.2022.105198
Received 5 June 2022; Received in revised form 28 July 2022; Accepted 4 August 2022
Available online 8 August 2022

effects of AZO. Namely, due to recommendations in regulatory guidelines, the use of pure active ingredients instead of formulated products is common. Nevertheless, it has been shown that the effect of a formulated product is often stronger than that of a pure active ingredient (Marques et al., 2009; Mesnage et al., 2014; Gomes et al., 2021). Recent research has also highlighted the importance of studies that include both a pure active ingredient and a formulated product (Gomes et al., 2021).

Enchytraeids are recognised as indicators of soil quality (Didden and Römbke, 2001; Castro-Ferreira et al., 2012; Pelosi and Römbke, 2016). They play an important role in soil ecology, especially in soils under tillage pressure, where the number of earthworms is reduced. Namely, enchytraeids affect the decomposition of organic matter, bioturbation, and the circulation of nutrients in the soil, thus improving soil structure, porosity, and quality (Briones, 2014; Maraldo et al., 2011). Due to their features, *Enchytraeus albidus* and *E. crypticus* are commonly used in ecotoxicology tests. *E. albidus*, the larger of the two species, can be found in the surface soil layers, especially in soils with a high content of organic matter. It can be found in diverse habitats, from temperate regions to the arctic (Christensen and Dózsa-Farkas, 2006). Due to its wide distribution, this species is considered a more relevant indicator organism compared to the earthworms *Eisenia fetida* and *E. andrei* (Römbke and Moser, 1999). Moreover, *E. albidus* has a generation time of 33 days at 18 °C (ISO, 2004), which allows the performance of the reproduction test in six weeks (OECD, 2016). Furthermore, in addition to the effect on survival and reproduction, it is possible to determine the effect on a wide range of endpoints and molecular biomarkers. However, existing information on the impact of strobilurin fungicides on this species is insufficient. Recent results addressed only the impact of AZO on *E. crypticus* (Leitão et al., 2014; Gomes et al., 2021). In addition to effects on survival and reproduction, a hatching delay has been demonstrated (Kovačević et al., 2021a). However, the impact on molecular biomarkers of enchytraeids has not been studied. Biomarkers can be used as an early warning system to detect stress following exposure to specific toxicants or stressors (Lam and Gray, 2003). The assessment of biomarkers at different levels of an organism's organization provides better insight into response to contaminants.

The multixenobiotic resistance mechanism (MXR) found in aquatic and soil organisms, including enchytraeids (Kovačević et al., 2021b), serves as a defence system and acts by pumping harmful substances out of the cell (Kurelec, 1992). Its activity can be assessed by measuring changes in the accumulation of fluorescent substrates. MXR effects fungicide toxicity by active pumping fungicides out of the cell, thus reducing their accumulation. However, if the MXR system is inhibited, the fungicides accumulate in the cell and toxic effects occur. Simultaneously, when an organism comes into contact with harmful substances, free radicals are produced and antioxidant defence is activated. The role and effectiveness of the first phase of detoxification depend on the activation of antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT). If the first phase does not eliminate all harmful molecules, second phase enzymes, such as glutathione-s-transferase (GST), are activated. Sometimes, however, the action of toxic substances is too strong and lipid peroxidation (LPO) occurs, indicating long-term damage. To obtain a more comprehensive picture of the effect of the toxicant, it is important to also assess the impact on the energy status of the organism. Namely, available energy and energy balance are crucial for key processes in an organism such as basal metabolic rate, growth, and reproduction (Novais et al., 2013). Therefore, measuring protein, lipid, and carbohydrate content are essential to evaluate available energy reserves.

In this research, the impact of AZO on *Enchytraeus albidus* was evaluated to obtain new information on the differences between the toxicity of the pure active ingredient and the formulated product. Therefore, multiple endpoints were assessed. In addition to survival and reproduction, effects on MXR activity, oxidative status, and available energy reserves were measured.

2. Material and methods

2.1. Test organism and soil

As a model organism, the soil annelid *Enchytraeus albidus* (Oligochaeta: Enchytraeidae) from the culture maintained in the laboratory at the Department of Biology in Osijek (Croatia) were used (OECD 220, 2016; ISO 16387, 2012). The culture was established in 2014 from organisms provided by the Complutense University of Madrid. The organisms were kept in moist soil in a climate room and fed ad libitum with oatmeal. The temperature was set at 20 ± 1 °C, relative humidity at 60% with a photoperiod of 16 h of light and 8 h of darkness.

All experiments were carried out in artificial soil (AS) (OECD 220, 2016) that consists of air-dried quartz sand (70%), kaolin clay (20%) and sphagnum peat (10%). As prescribed in the guidelines, the soil pH was modified by adding $CaCO_3$ up to 6 ± 0.5.

2.2. Test materials and spiking

The active ingredient azoxystrobin (AZO_AI) (Pestanal®, analytical standard, ≥ 98.0%) and the fungicide formulated product Quadris® (AZO_FP) (Syngenta) were used. AZO_FP based on the active substance azoxystrobin (25%) contains adjuvants 1,2-benzisothazol-3(2H)-one (0.025–0.05%), naphthalene and alkyl naphthalene sulfonic acid formaldehyde condensate and sodium salts (1 ≤ 10%). The recommended application dose for AZO_CP in the field is 1 L/ha or 0.17 mg $_{a.i.}$/kg $_{soil}$.

Priory the final experiment, a range-finding test was conducted. Tested concentrations were 0, 1, 2, 3, 4, 5, 6, 8, 10, 14, 18, 22, 25, 52, 75, 100 and 150 mg $_{a.i.}$/kg $_{soil}$ for AZO_AI and AZO_FP. Concentrations for the final test were selected based on the recommended application doses for AZO_FP and according to the results of the range-finding test. The concentrations used in the test were 0, 0.085, 0.17, 1.45, 2.7, 4 and 8 mg $_{a.i.}$/kg $_{soil}$ for AZO_AI and AZO_FP. The AZO_AI was prepared by dissolving in organic solvent (acetone) and diluted to the tested concentrations. After dissolving, the required concentrations of AZO_AI were added in 2 mL of acetone to each replicate, homogeneously mixed, and left for 24 h to evaporate. Solvent controls were run in parallel with the test. The soil moisture was adjusted with water until 60% of the water holding capacity (WHC). As AZO_FP is water-soluble, a stock solution was prepared and diluted. The required concentrations of AZO_FP were dissolved in water and separately added to each replicate in the amount required for 60% of the water holding capacity (WHC). The soil was mixed homogeneously and allowed to equilibrate for 24 h before the start of the tests. Clean soil with the required amount of water was used as a control.

2.3. Test procedures and sample preparation

The range-finding test was conducted in three replicates per test concentration. 10 adult organisms with well-developed clitellum were exposed to different concentrations of AZO_FP and AZO_AI for seven days. To determine LC values survival was determined after 24 h, 48 h, 72 h, 96 h and seven days.

The reproduction test was carried out according to the standardised enchytraeid reproduction test (ERT) guidelines (ISO, 2012; OECD, 2016). Ten adults with well-developed clitellum were introduced into each test vessel containing 20 g of moist soil and food supply (autoclaved rolled oats). The test was carried out at 20 ± 1 °C and a photoperiod of 16: 8 h for six weeks (42 days). Water and food were replenished weekly, according to weight loss. Five replicates per test concentration were used. After 21 days, the surviving adults were extracted and counted, pooled per replicate, weighted, and stored at −80 °C. 21 days after removing adults (42 days after beginning the experiment), the juvenile organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through the mesh (63 μm) to separate enchytraeids from soil and

facilitate counting with a stereomicroscope.

Simultaneously with the ERT, a seven-day exposure with identical settings was conducted. To provide a better understanding of the effects of fungicides and to allow a comprehensive consideration of changes in multiple endpoints at different periods.

Parallel with the above tests, ten replicates with ten organisms per test concentration were used to measure MXR activity. MXR activity was measured on day seven (5 replicates per test concentration) and day 21 (5 replicates per test concentration) as the accumulation of Rh123 according to Kovačević et al. (2021b).

Oxidative status and energy reserves were measured in the whole organisms that were pooled per replicate and homogenized (IKA RW20 digital homogenizer) in a cold potassium phosphate buffer (0.1 M, pH 7.4) (1: 15, w: v ratio). Post-mitochondrial fraction (S9) was obtained after centrifugation of homogenates for 30 min at 9000*g* and 4 °C. Homogenates and S9 fractions were stored at −80 °C until further analysis. Homogenates were used for measuring MDA, lipid and carbohydrate content while in S9 protein content and activities of enzymes SOD, CAT and GST were measured.

2.4. Oxidative status

SOD was measured according to McCord and Fridovich (1969), CAT activity was determined with the method of Claiborne (1985) and GST activity was evaluated with the method described by Habig et al. (1974). LPO was determined as the malondialdehyde (MDA) content according to Gagne (2014). All enzyme activities and MDA contents were calculated per protein content and expressed relative to their respective control.

2.5. Energy reserves

To determine the available energy reserves, the total protein, lipid, and carbohydrate content were quantified. The method described by Bradford (1976) was used to measure protein content in the S9 fraction, while the lipid and carbohydrate contents were determined in homogenate using the methods described by Frings et al. (1972) and Jermyn (1975). For transforming energy sources into energetic equivalents, the enthalpy combustion method described by De Coen and Janssen (1997, 2003) was used. Results were expressed relative to their respective control.

2.6. Data analysis

Data analyses were performed with statistical software R version 4.3.0 (R Development Core Team, 2021) and RStudio (RStudio Team, 2021). Data normality was tested using the Shapiro-Wilk test and homogeneity of variance was assessed by the Bartlett test. As data were normally distributed, ANOVA, followed by a Dunnett post hoc test ($p \leq 0.05$) was used. Effect concentration (ECx) and lethal concentration (LCx) were calculated by fitting the curve in the package *drc* to the three-parameter log-logistic models (function LL.3) (Ritz et al., 2015).

3. Results

The test validity criteria described in OECD 220 (2016) were met. Namely, adult mortality in the reproduction test was below 10% and the number of juveniles was higher than 50. The range-finding test showed a slightly stronger negative effect of AZO_FP on survival (Table 1). The results of the reproduction experiment (21 days of exposure) showed a significant inhibition (ANOVA, $p < 0.05$) of reproduction with the highest treatment of AZO_AI (8 $mg_{a.i.}/kg_{soil}$) and most AZO_FP treatments (1.45, 2.7, 4, and 8 $mg_{a.i.}/kg_{soil}$) (Fig. 1). Additionally, the calculated EC_{50} values show that AZO_CP has a stronger adverse effect on reproduction compared to AZO_AI (Table 1). Moreover, an increased number of unhatched cocoons was observed in some AZO_FP treatments

Table 1
Lethal (LCx) and effect (ECx) concentrations expressed as $mg_{a.i.}/kg_{soil}$ calculated for *Enchytraeus albidus* after exposure to azoxystrobin as the pure active ingredient (AZO_AI) and formulated product (AZO_FP). Results are expressed as LC/EC and the 95% confidence intervals.

	Exposure time	LC_{10}	LC_{50}	LC_{90}
AZO_AI	**24 h**	16.31 (15.20–17.42)	20.54 (19.76–21.32)	29.84 (26.03–32.93)
	48 h	19.89 (13.73–16.05)	18.76 (18.19–19.33)	23.64 (21.84–25.44)
	72 h	12.66 (11.69–13.63)	17.65 (17.30–17.99)	21.53 (20.50–22.63)
	7 days	11.95 (11.21–12.70)	16.76 (16.51–17.01)	19.14 (18.27–20.00)
AZO_CP	**24 h**	12.19 (11.41–12.98)	18.44 (16.62–20.26)	33.72 (29.97–37.48)
	48 h	11.67 (11.17–12.17)	18.12 (17.47–17.76)	31.58 (29.36–33.80)
	72 h	11.61 (10.98–12.50)	16.98 (16.47–17.50)	30.84 (28.12–33.56)
	7 days	10.65 (10.14–11.17)	15.29 (14.88–15.71)	26.98 (24.99–28.97)
		EC_{10}	**EC_{50}**	**EC_{90}**
AZO_AI	**21 day**	> 8	> 8	> 8
AZO_CP	**21 day**	1.23 (0.40–2.06)	2.94 (2.41–3.73)	6.21 (3.84–7.78)

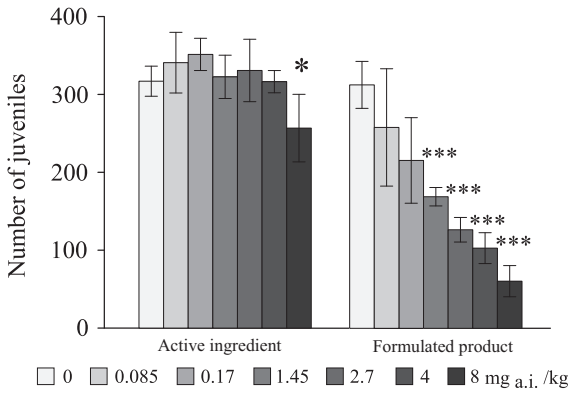


Fig. 1. The number of juveniles after exposure of *Enchytraeus albidus* to azoxystrobin as a pure active ingredient (AZO_AI) and formulated product (AZO_FP) presented as the number of juveniles. Results are expressed as the average number of juveniles ± SD.

(> 15 at 1.45 and > 8 at 2.7 $mg_{a.i.}/kg_{soil}$) and highest AZO_AI treatment (8 $mg_{a.i.}/kg_{soil}$).

In addition to population biomarkers, both AZO_AI and AZO_FP demonstrated negative effects on cellular and molecular biomarkers. MXR activity was only affected after seven days of exposure to AZO_AI and AZO_FP. AZO_AI significantly induced (ANOVA, $p < 0.05$) MXR activity at lower concentrations (0.17, 1.45 $mg_{a.i.}/kg_{soil}$), while AZO_FP inhibited MXR activity and caused the accumulation of R123 within cells at 4 and 8 $mg_{a.i.}/kg_{soil}$ (Fig. 2).

Moreover, exposure to both AZO_AI and AZO_FP induced changes in measured enzyme activities (Fig. 3). Namely, both substances tested led to a significant induction (ANOVA, $p < 0.05$) of SOD and CAT activities after seven days of exposure. After 21 days of exposure, SOD and CAT activities returned to control levels. While AZO_AI induced GST activity after seven days, AZO_FP caused induction after 21 days. The MDA content increased significantly (ANOVA, $p < 0.05$) after 21 days of exposure to AZO_AI (2.7 $mg_{a.i.}/kg_{soil}$) and AZO_FP (4 and 8 $mg_{a.i.}/kg_{soil}$).

The total available energy reserves after exposure to AZO_AI and AZO_FP in different time intervals are shown in Fig. 4. In organisms from

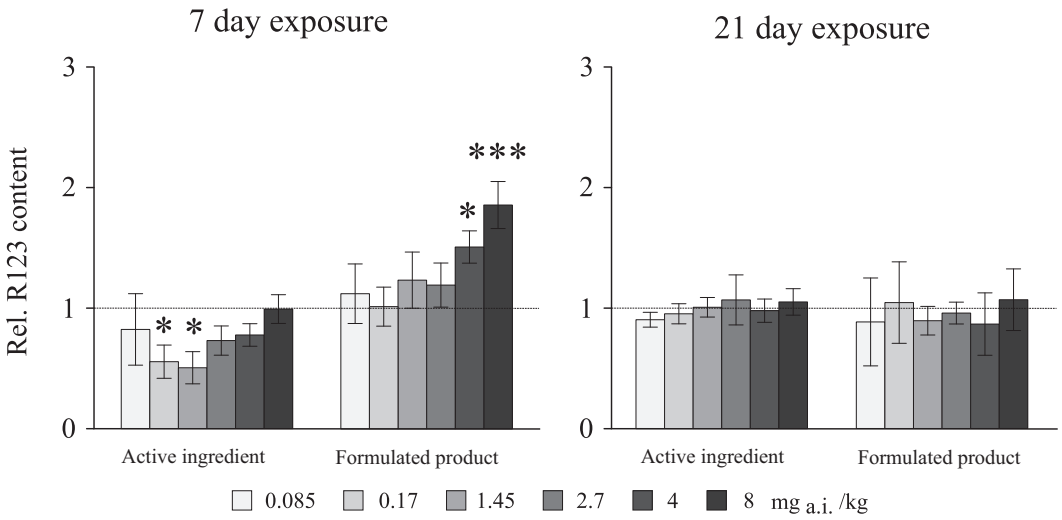


Fig. 2. Rh123 relative content in *Enchytraeus albidus* after exposure to azoxystrobin as a pure active ingredient (AZO_AI) and formulated product (AZO_FP). Results are relative to the corresponding control and expressed as average ± SD.

the control treatment, the ratios of individual energy reserve fractions were similar at different time points. Thus, proteins accounted for about 50% of the total available energy, lipids 40%, and carbohydrates 10%. No changes were observed in the total available energy reserves after exposure to AZO_AI. However, there was a shift in the proportion of each energy reserve fraction. Namely, after seven days of exposure, a significant decrease (ANOVA, $p < 0.05$) in protein and carbohydrate content was observed, while lipids significantly increased (ANOVA, $p < 0.05$) (up to 54% of total available energy reserves). After 21 days of exposure, lipids decreased significantly (ANOVA, $p < 0.05$) (up to 25% of total available energy), while proteins and carbohydrates were at the control level. The highest concentration of AZO_FP caused a significant increase (ANOVA, $p < 0.05$) in total available energy reserves after seven and 21 days of exposure. Namely, a significant increase (ANOVA, $p < 0.05$) in the amount of energy available occurred after seven days due to the increase in lipid content, while the increase after 21 days was the result of the increase in protein, lipid and carbohydrate content.

4. Discussion

The effects of AZO on *E. albidus* were evaluated by measuring various endpoints after seven and 21 days of exposure. Although no significant differences were observed between the impact of AZO_AI and AZO_CP on survival, species-specific sensitivity between *E. albidus* and *E. crypticus* was observed. *E. crypticus* has been reported as a more tolerant species to toxicants than *E. albidus* (Pokarzhevskii et al., 2003). Consequently, *E. albidus* (LC_{50} = 15.29 $mg_{a.i./kg_{soil}}$) showed higher susceptibility to AZO_FP than *E. crypticus* (LC_{50} ≥150 $mg_{a.i./kg_{soil}}$) (Kovačević et al., 2021a). Similarly, Gomes et al. (2021) reported an LC_{50} of 39 $mg_{a.i./kg_{soil}}$ after exposure of *E. crypticus* to AZO_AI, also indicating a higher susceptibility of *E. albidus* (LC_{50} = 20.54 $mg_{a.i./kg_{soil}}$). The differences in toxicity between the species are attributed to peculiarities in life cycle duration (Pokarzhevskii et al., 2003). Except for survival, both AZO_AI and AZO_FP affected the reproduction rate of *E. albidus*. Although AZO_AI has an impact on reproduction only at the highest tested concentration (8 $mg_{a.i./kg_{soil}}$), AZO_CP caused a decrease in reproduction at concentrations higher than 0.17 $mg_{a.i./kg_{soil}}$. The negative impact of the formulated products on *E. crypticus* reproduction has been shown in previous studies with EC_{50} values ranging from 37 $mg_{a.i./kg_{soil}}$ to 99.2 $mg_{a.i./kg_{soil}}$ (Gomes et al., 2021; Leitão et al., 2014; Kovačević et al., 2021a). Moreover, the increased number of unhatched cocoons observed in some treatments suggests an effect on embryonic development. The same effect was observed in *E. crypticus* (Kovačević et al., 2021a). Namely, at lower concentrations, AZO_FP caused impairment of embryonic development, while at higher concentrations the reproduction rate was reduced. Zebrafish (*Danio rerio*) embryotoxicity test showed that azoxystrobin alters mitochondrial bioenergetics and causes developmental toxicity (Yang et al., 2021). Moreover, early developmental stages are expected to have less robust and established antioxidant defence systems which may lead to high levels of oxidative stress that result in genotoxicity (Zhang et al., 2020). Additionally, the higher toxicity of AZO_CP may be caused by adjuvants, which increase the bioavailability of active ingredients and allow them to pass more easily through cocoon and bind to target sites and consequently increasing toxicity to non-target organisms (Pereira et al., 2009).

The survival of organisms in an environment with a high concentration of pollutants depends on the MXR mechanism and the efflux of a toxicant from the cell (Epel et al., 2008). The function of the ABC transporters in the MXR mechanism can be affected by pesticides. Namely, some pesticides act as chemosensitizers and can inhibit or induce the activity of the MXR system in enchytraeids (Kovačević et al., 2021b) and earthworms (Velki and Hackenberger, 2013). Furthermore, the activity of the MXR system is extremely sensitive to the concentrations of available substrates. Namely, while a small amount of substrate activates the MXR system, higher concentrations inhibit it (Velki and Hackenberger, 2013). Therefore, the opposite response of the MXR mechanism observed after seven days of exposure to AZO_AI and AZO_CP could be associated with the different availability of the active ingredient. In other words, adjuvants in formulated products enhance the absorption and stability of the active ingredient, thus promoting its pesticidal action (Mesnage and Antoniou, 2018). Moreover, the main adjuvants of AZO_FP, 1,2- benzothiazole-3(2H)-one (EU regulation No. 528/2012, 2022; IMAP, 2020; EPA, 2021) and naphthalene (EPA, 2008), have no adverse effects on non-target soil organisms at the concentrations present in this study, but certainly, enhance AZO uptake and stability. Since inhibition of MXR activity causes longer retention of toxicants in the organism, it leads to higher toxicity of AZO_CP.

Changes in antioxidant enzyme activities can indicate the mechanism by which organisms protect themselves from toxic effects. Although the influence of AZO on enchytraeid enzyme activity has not been investigated, the research carried out with the earthworm *E. fetida* indicates the importance of the enzymes SOD and GST (Han et al., 2014; Xu et al., 2021). SOD is part of the primary antioxidant defence mechanism and acts in the conversion of the superoxide anion radical (O^{2-}) to hydrogen peroxide (H_2O_2) (Ighodaro and Akinloye, 2018). SOD induction has been reported in earthworms exposed to AZO_AI (Han et al.,

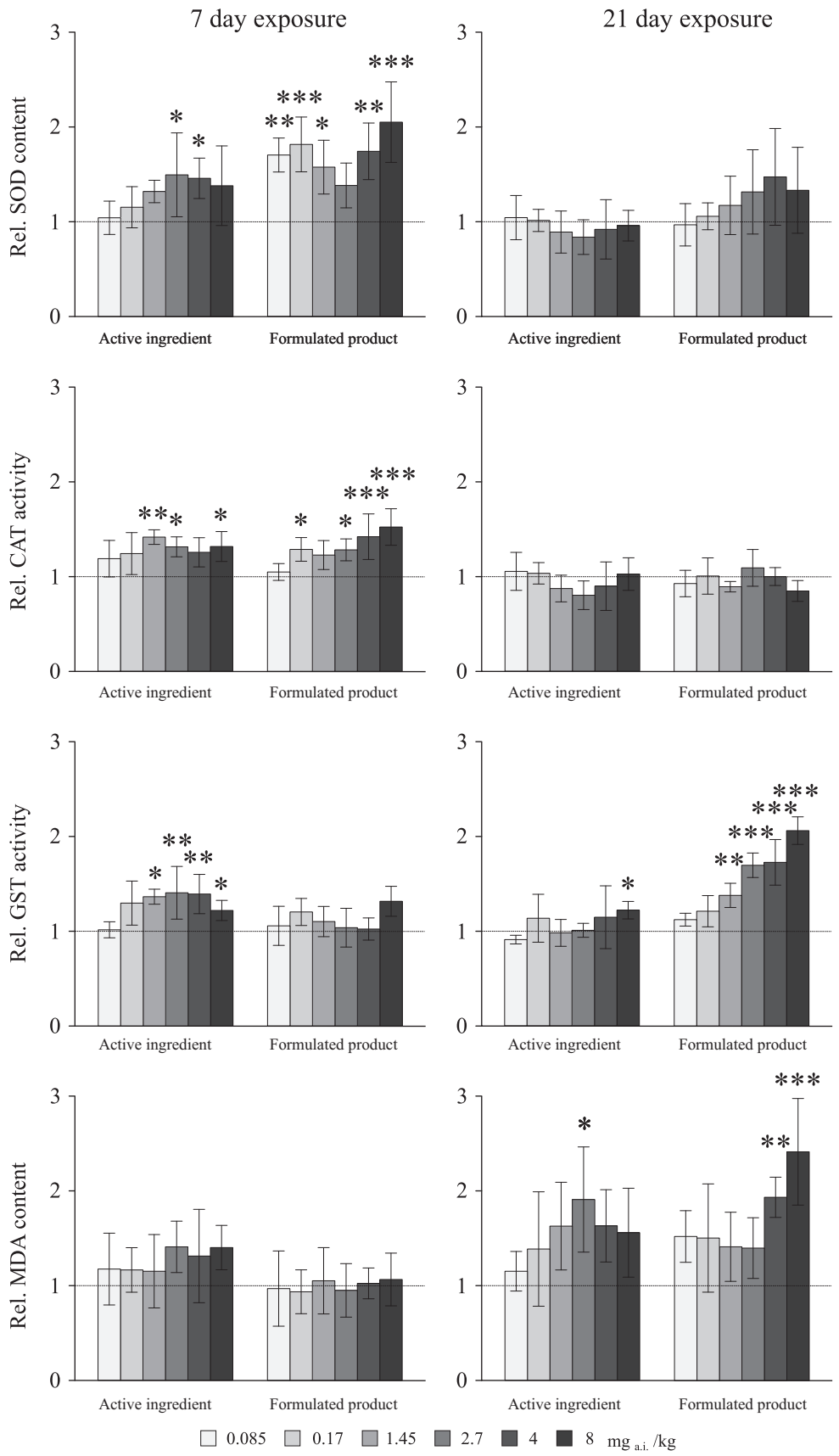


Fig. 3. Differences in biomarker responses of SOD, CAT, GST and MDA in *Enchytraeus albidus* after exposure to azoxystrobin as the pure active ingredient (AZO_AI) and formulated product (AZO_FP) presented as the number of juveniles. Results are relative to the corresponding control and expressed as average ± SD.

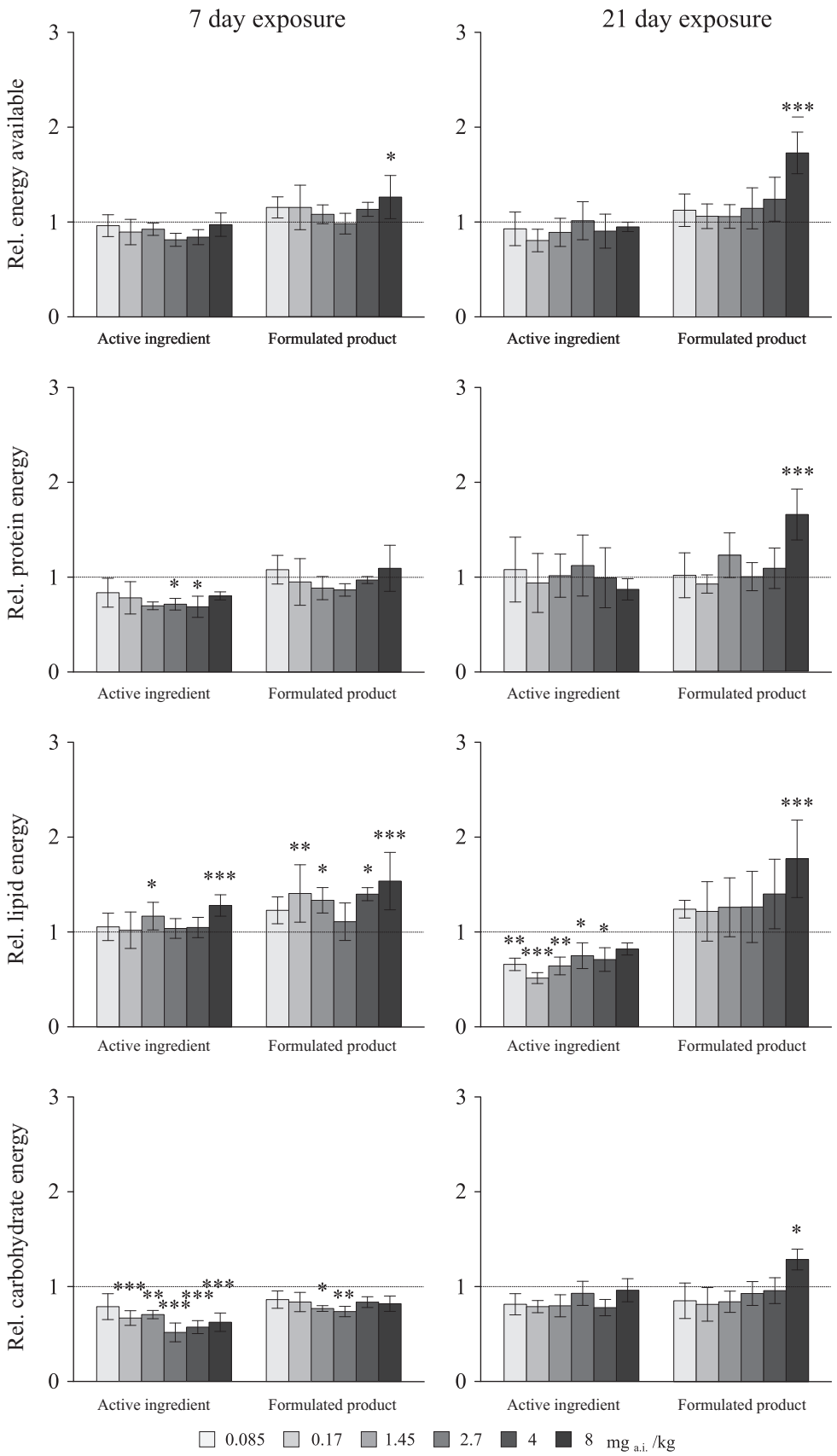


Fig. 4. Available energy reserves (A, B), protein (C, D), lipid (E, F) and carbohydrate (G, H) energy in *Enchytraeus albidus* after exposure to azoxystrobin as the pure active ingredient (AZO_AI) and formulated product (AZO_FP). Energy reserves are expressed as average values ± SD.

2014; Xu et al., 2021). However, while SOD activity in earthworms increased with exposure time, SOD activity in enchytraeids was induced only after seven days of exposure. This response suggests the high efficiency of this enzyme in enchytraeids, which successfully convert O^{2-} after a short exposure time. Although the research suggests different importance and CAT responses in *E. fetida* (Han et al., 2014; Xu et al., 2021), the results obtained indicate that its activity plays a vital role in enchytraeids. Like SOD, CAT showed high efficacy, and after prolonged exposure due to substrate deficiency, its activity returned to the levels observed in control. GST is a crucial second-phase detoxifying enzyme that scavenges lipid hydroperoxides to reduce oxidative damage. GST was the most important detoxification enzyme in earthworms after AZO_AI treatment (Han et al., 2014; Xu et al., 2021). Namely, GST activity increased with increasing concentration and exposure time. Although AZO_AI induced GST activity in enchytraeids after seven days of exposure, after 21 days, induction was recorded only at the highest concentration tested. Again, this response suggests the success of the detoxification system in enchytraeids and the lack of substrate for GST after 21 days of exposure. On the contrary, AZO_CP caused GST induction only after 21 days of exposure. Namely, the substrate concentration formed after seven days of exposure was not sufficient for GST induction. However, the higher induction of GST after 21 days of exposure suggests a high substrate production and thus stronger oxidative stress than observed in the AZO_AI treatments. When ROS are formed in significant amounts, a saturation of antioxidant defence occurs. As a consequence, damage to lipids and other vital molecules may occur. Most commonly, the degree of lipid peroxidation (LPO) is assessed by measuring the MDA content as a biomarker of oxidative damage (Duryee et al., 2010). ROS production after seven days was insufficient to promote LPO and increase MDA content in *E. fetida* exposed to AZO_AI (Han et al., 2014; Xu et al., 2021). However, after prolonged exposure, LPO was observed. The same pattern was observed in enchytraeids. Moreover, the level of LPO again suggests a higher impact of AZO_CP than AZO_AI.

The impact of AZO on mitochondrial metabolism and induction of oxidative stress suggests a possible imbalance in the energy metabolism of *E. albidus*. Therefore, total protein, lipid, and carbohydrate content were measured to determine the level of available energy reserves. AZO_AI and AZO_FP affected the energy reserves of *E. albidus* differently. Although no changes in total available energy reserves were observed after exposure to AZO_AI, AZO_FP caused an increase in available energy reserves at the highest tested concentration after seven and 21 days of exposure. Moreover, both AZO forms induced a change in the proportion of energy fractions. In control organisms, the proportion of energy reserve fractions was similar to previous studies (50 (proteins): 40 (lipids): 10 (carbohydrates)) (Amorim et al., 2012; Novais et al., 2013). However, after seven days of exposure to AZO_AI, protein and carbohydrate content were significantly reduced, due to an intense defence against oxidative stress and increased energy demand. Carbohydrates are commonly used as a primary energy source under stress conditions (Moolman et al., 2007). A reduction in carbohydrate content is a common response in enchytraeids after exposure to pesticides (Novais and Amorim, 2013). On the contrary, lipid content was increased after seven days of exposure to AZO_AI, indicating inflammatory stress (Gomes et al., 2015). After 21-days, proteins and carbohydrates were equivalent to the control, while lipid content was significantly reduced. The reduction in lipid content is associated with increased LPO, which may lead to a reduction in available energy. Exposure to AZO_FP had a different effect on the time course of the energy reserves. After seven days, the carbohydrate content was reduced and lipids increased. However, a significant increase in protein, carbohydrate, and lipid content was observed only at the highest concentration after 21 days of exposure. This increase in protein synthesis, carbohydrate, and lipid accumulation can be explained as a stress response. Namely, Tripathi et al. (2010) reported that an increase in protein content observed in earthworms indicates the possibility of increased synthesis of stress proteins.

After application, formulated products can be absorbed by the plant, deposited on the surface of the soil, or adsorb onto organic matter or clay in the soil (Hildebrandt et al., 2007). Azoxystrobin is mainly applied as a foliar fungicide, and if the treatment is carried out too early, >50% of the applied fungicide can end up directly on the soil (Jensen and Spliid, 2003). According to Flury (1996), the active ingredient is released from the formulation after application. However, the release rate depends on various factors such as the type of formulation and environmental conditions. The observed differences between the response of *E. albidus* to AZO_FP and AZO_AI indicate a slow release of azoxystrobin from the tested formulation. Furthermore, low mobility of different forms of azoxystrobin and a higher residue level were observed in the upper layers of the soil (0–20 cm) (Herrero-Hernández et al., 2015; Ghosh and Singh, 2009). Considering that enchytraeids are found in the surface layers of the soil, their exposure to azoxystrobin becomes almost inevitable. Moreover, the oxidative stress observed at the recommended application dose of AZO_CP (0.17 $mg_{a.i.}/kg_{soil}$) indicates a possible risk to enchytraeids after field application of the formulated products. Although enchytraeids successfully eliminated oxidative stress and reproduced unhindered, higher concentrations may affect embryonic development and reproduction. The leaching behaviour of fungicides plays a major role in the accumulation and impact of fungicides on soil organisms. Khan and Brown (2016) observed differences in leaching between the active ingredient and the formulated products. However, the biggest difference was observed between the emulsifiable concentrate (EC) and suspension concentrate (SC) formulation, indicating a high impact of adjuvants on the leaching of the active ingredient. EC formulations contains emulsifying agents in a water-insoluble organic solvent, which is designed to form an oil-in-water emulsion upon dilution that affects the behaviour of pesticide activity. They can restrict the pesticide molecule from dissolving in water or may retard processes controlling sorption to soil with the oily organic solvents surrounding the pesticide molecule, thus increasing pesticide leaching. On the other hand, SC usually contains suspension agents, wetting agents, and thickeners that may reduce leaching (Khan and Brown, 2016). Hence, adjuvants can affect not only the leaching of fungicides but also the initial and total availability of fungicide. Current maximum concentrations of AZO in European soils reach up to 0.25 $mg_{a.i.}/kg_{soil}$ (Silva et al., 2019), but climate change and agricultural intensification lead to a higher demand for plant protection products. Consequently, this can result in higher concentrations of AZO in the soil, as is already the case in China (>9 $mg_{a.i.}/kg_{soil}$, Xu et al., 2021). Such high concentrations may significantly impact enchytraeid reproduction, and thus the stability of their populations.

5. Conclusion

A detailed insight into the changes in the activity of the measured biomarkers in enchytraeids allows a better assessment of the potential toxicity and the difference between AZO_AI and AZO_FP. The opposite response of the MXR system activity indicates an increased availability of the active substance upon exposure to AZO_FP compared to AZO_AI. Although AZO_AI impairs enchytraeid reproduction only at the highest tested concentration, the changes in enzyme activity indicate the occurrence of oxidative stress. The activities of SOD, CAT, and GST were induced during exposure to AZO_AI and AZO_FP, suggesting their involvement in reducing oxidative damage and detoxification. Furthermore, changes in MDA content after 21 days of exposure showed the occurrence of LPO. Therefore, assessment of multiple endpoints is recommended to more accurately predict potential adverse effects and avoid the possibility of underestimating the effects. Since the toxicity of a formulated product may be higher than that of the active ingredient itself, testing both is crucial to reveal differences in toxicity. Furthermore, the differences obtained between the AZO forms imply the importance of evaluating various formulated products. Although the

recommended application dose has not shown an effect on survival and reproduction, such concentrations can cause oxidative stress and affect enchytraeid populations after multiple or multigenerational exposures. Therefore, under the conditions of imminent climate change, it is necessary to determine the most suitable formulated products to establish successful crop protection and ensure the stability of soil communities.

Acknowledgement

This research was conducted in the framework of the DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) funded by the Croatian Science Foundation (HrZZ) under the contract number: IP-09-2014-4459 and "Young Researchers' Career Development Project–Training of Doctoral Students" funded by the Croatian Science Foundation (HrZZ) (DOK-2018-01-4257).

References

Amorim, M.J.B., Gomes, S.I.L., Soares, S.M.V.M., Scott-Fordsmand, J.J., 2012. Energy basal levels and allocation among lipids, proteins, and carbohydrates in *Enchytraeus albidus*: changes related to exposure to cu salt and Cu nanoparticles. Water Air Soil Pollut. 223, 477–482.

Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., Parr-Dobrzanski, B., 2002. The strobilurin fungicides. Pest Manag. Sci. 58, 649–662. https://doi.org/10.1002/ps.520.

Bradford, MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Briones, M.J.I., 2014. Soil fauna and soil functions: a jigsaw puzzle. Front. Environ.Sci. 2. https://doi.org/10.3389/fenvs.2014.00007.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., Amorim, M.J.B., 2012. *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87, 1222–1227.

Christensen, B., Dózsa-Farkas, K., 2006. Invasion of terrestrial enchytraeids into two postglacial tundras: North-eastern Greenland and the Arctic Archipelago of Canada (Enchytraeidae, Oligochaeta). Polar Biol. 29 (454–466), 444.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods of Oxygen Radical Research, pp. 283–284.

De Coen, W., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress. Recover. 6, 43–55.

De Coen, W., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632–1641.

Didden, W., Römbke, J., 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicol. Environ. Saf. 50, 25e43. https://doi.org/10.1006/eesa.2001.2075.

Duryee, M.J., Klassen, L.W., Schaffert, C.S., Tuma, D.J., Hunter, C.D., Garvin, R.P., Anderson, D.R., Thiele, G.M., 2010. Malondialdehyde-acetaldehyde adduct is the dominant epitope after MDA modification of proteins in atherosclerosis. Free Radic. Biol. Med. 49 (10), 1480–1486. https://doi.org/10.1016/j.freeradbiomed.2010.08.001.

EPA (the United States Environmental Protection Agency), 2008. Reregistration Eligibility Decision for Naphthalene. https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/red_PC-055801_12-Sep-08.pdf.

EPA (the United States Environmental Protection Agency), 2021. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/databases-related-pesticide-risk-assessment.

Epel, D., Luckenbach, T., Stevenson, C.N., MacManus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: newly appreciated roles in protection against pollutants. Environ. Sci. Technol. 42, 3914–3920. https://doi.org/10.1021/es087187v.

EU regulation No 528/2012, 2022. https://echa.europa.eu/documents/10162/5a180829-e031-777e-db83-477195663762.

Flury, M., 1996. Experimental evidence of transport of pesticides through field soils – a review. J. Environ. Qual. 25, 24–45.

Frings, C.S., Fendley, T.W., Dunn, R.T., Queen, C.A., 1972. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin. Chem. 18 (7), 673–674.

Gagne, F., 2014. Biochemical ecotoxicology: principles and methods. In: Oxidative Stress. Academic Press, New York, pp. 110–111.

Ghosh, R.K., Singh, N., 2009. Leaching behaviour of azoxystrobin and metabolites in soil columns. Pest Manag. Sci. 65 (9), 1009–1014.

Gomes, S.I., Scott-Fordsmand, J.J., Amorim, M.J., 2015. Cellular energy allocation to assess the impact of nanomaterials on soil invertebrates (Enchytraeids): the effect of Cu and Ag. Int. J. Environ. Res. Public Health 12 (6), 6858–6878.

Gomes, S.I.L., Ammendola, A., Casini, S., Amorim, M.J.B., 2021. Toxicity of fungicides to terrestrial non-target fauna – formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – a case study with *Enchytraeus crypticus* (Oligochaeta). Sci. Total Environ. 754, 142098 https://doi.org/10.1016/j.scitotenv.2020.142098.

Habig, W.H., Pabst, M.J., Jakobi, W., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Han, Y., Zhu, L., Wang, Jinhua, Wang, Jun, Xie, H., Zhang, S., 2014. Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin. Ecotoxicol. Environ. Saf. 107, 214–219. https://doi.org/10.1016/j.ecoenv.2014.06.006.

Herrero-Hernández, E., Marín-Benito, J.M., Andrades, M.S., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S., 2015. Field versus laboratory experiments to evaluate the fate of azoxystrobin in an amended vineyard soil. J. Environ. Manag. 163, 78–86.

Hildebrandt, A., Lacorte, S., Barceló, D., 2007. Assessment of priority pesticides, degradation products, and pesticide adjuvants in groundwaters and top soils from agricultural areas of the Ebro river basin. Anal. Bioanal. Chem. 387 (4), 1459–1468.

Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54, 287–293. https://doi.org/10.1016/j.ajme.2017.09.001.

IMAP, 2020. Accelerated Assessment of Industrial Chemicals in Australia. https://www.industrialchemicals.gov.au/sites/default/files/1%2C2-Benzisothiazolinone%20preservatives_%20Environment%20tier%20II%20assessment.pdf.

International Organization for Standardization (ISO), 2004. Soil Quality—Effects of Pollutants on Enchytraeidae (*Enchytraeus* sp.) - Determination of Effects on Reproduction and Survival ISO 16387:2004.

International Organization for Standardization (ISO), 2012. Water Quality Determination of the Inhibition of the Mobility of Daphnia magna Straus (Cladocera, Crustacea) - Acute Toxicity Test. ISO 6341, p. 2012.

Jensen, P.K., Spliid, N.H., 2003. Deposition of spray liquid on the soil below cereal crops after applications during the growing season. Weed Res. 43 (5), 362–370.

Jermyn, M.A., 1975. Increasing the sensitivity of the anthrone method for carbohydrate. Anal. Biochem. 68 (1), 332–335.

Khan, M.A., Brown, C.D., 2016. Influence of commercial formulation on leaching of four pesticides through soil. Sci. Total Environ. 573, 1573–1579.

Kovačević, M., Hackenberger, D.K., Hackenberger, B.K., 2021a. Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifloxystrobin) on survival, reproduction and hatching success of *Enchytraeus crypticus*. Sci. Total Environ. 790 https://doi.org/10.1016/j.scitotenv.2021.148143.

Kovačević, M., Hackenberger, D.K., Lončarić, Ž., Hackenberger, B.K., 2021b. Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology. Chemosphere 279. https://doi.org/10.1016/j.chemosphere.2021.130549.

Kurelec, B., 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22, 23–43.

Lam, P.K.S., Gray, J.S., 2003. The use of biomarkers in environmental monitoring programmes. Mar. Pollut. Bull. 46 (2), 182–186.

Leitão, S., Cerejeira, M.J., Van den Brink, P.J., Sousa, J.P., 2014. Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. Appl. Soil Ecol. 76, 124–131. https://doi.org/10.1016/j.apsoil.2013.12.013.

Maraldo, K., Christensen, B., Holmstrup, M., 2011. The excretion of ammonium byenchytraeids (*Cognettia sphagnetorum*). Soil Biol. Biochem. 43, 991–996.

Marques, C., Pereira, R., Gonçalves, F., 2009. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. J. Soils Sediments 9, 137–147. https://doi.org/10.1007/s11368-009-0058-0.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244 (22), 6049–6055.

Mesnage, R., Antoniou, M.N., 2018. Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Public Health Front. 5, 361. https://doi.org/10.3389/fpubh.2017.00361.

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.-E., 2014. Major pesticides are more toxic to human cells than their declared active principles. Biomed. Res. Int. 2014, 1–8. https://doi.org/10.1155/2014/179691.

Moolman, L., Van Vuren, J.H., Wepener, V., 2007. Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. Ecotoxicol. Environ. Saf. 68, 443–450. https://doi.org/10.1016/j.ecoenv.2006.12.017.

Novais, S.C., Amorim, M.J.B., 2013. Changes in cellular energy allocation in Enchytraeus albidus when exposed to dimethoate, atrazine, and carbendazim. Environ. Toxicol. Chem. 32, 2800–2807. https://doi.org/10.1002/etc.2368.

Novais, S.C., Soares, A.M.V.M., De Coen, W., Amorim, M.J.B., 2013. Exposure of *Enchytraeus albidus* to cd and Zn - changes in cellular energy allocation (CEA) and linkage to transcriptional, enzymatic and reproductive effects. Chemosphere 90, 1305–1309. https://doi.org/10.1016/j.chemosphere.2012.09.030.

Organisation for Economic Cooperation and Development (OECD), 2016. Test no. 220: enchytraeid reproduction test. In: OECD Guidelines for Testing of Chemicals. OECD Publishing, Paris France.

Pelosi, C., Römbke, J., 2016. Are Enchytraeidae (Oligochaeta, Annelida) good indicators of agricultural management practices? Soil Biol. Biochem. 100, 255–263.

Pereira, J.L., Antunes, S.C., Castro, B.B., Marques, C.R., Gonçalves, A.M.M., Gonçalves, F., Pereira, R., 2009. Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. Ecotoxicology 18, 455–463. https://doi.org/10.1007/s10646-009-0300-y.

Pokarzhevskii, A., Filimonova, Z.V., Goryachev, O.A., 2003. Life-cycle length determines the differences in sensitivity to toxicants between enchytraeid species. Dokl. Biol. Sci. 390, 256–258. https://doi.org/10.1023/a:1024469919915.

R Studio Team, 2021. RStudio: Integrated Development for R. RStudio, Inc, Boston, MA. http://www.rstudio.com.

RCore Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLoS One 598 (10), e0146021.

Rodrigues, E.T., Lopes, I., Pardal, M.Â., 2013. Occurrence, fate and effects of azoxystrobin in aquatic ecosystems: a review. Environ. Int. 53, 18–28. https://doi.org/10.1016/j.envint.2012.12.005.

Römbke, J., Moser, T., 1999. Organisation and Performance of an International Ring Test for the Validation of the Enchytraeid Reproduction Test. Vols. I and II.; BA-πexte4/99.

Silva, V., Mol, H.G.J., Zomer, P., Tienstra, M., Ritsema, C.J., Geissen, V., 2019. Pesticide residues in European agricultural soils – a hidden reality unfolded. Sci. Total Environ. 653, 1532–1545. https://doi.org/10.1016/j.scitotenv.2018.10.441.

Tripathi, G., Kachhwaha, N., Dabi, I., 2010. Comparative studies on carbofuran-induced changes in some cytoplasmic and mitochondrial enzymes and proteins of epigeic, anecic and endogeic earthworms. Pestic. Biochem. Physiol. 96, 30–35. https://doi.org/10.1016/j.pestbp.2009.08.012.

Velki, M., Hackenberger, B.K., 2013. Biomarker responses in earthworm *Eisenia andrei* exposed to pirimiphos-methyl and deltamethrin using different toxicity tests. Chemosphere 90, 1216–1226. https://doi.org/10.1016/j.chemosphere.2012.09.051.

Wang, Y., Wu, S., Chen, L., Wu, C., Yu, R., Wang, Q., Zhao, X., 2012. Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. Chemosphere 88, 484–491. https://doi.org/10.1016/j.chemosphere.2012.02.086.

Wu, R., Zhou, T., Wang, J., Wang, J., Du, Z., Li, B., Juhasz, A., Zhu, L., 2021. Oxidative stress and DNA damage induced by trifloxystrobin on earthworms (*Eisenia fetida*) in two soils. Sci. Total Environ. 797, 149004 https://doi.org/10.1016/j.scitotenv.2021.149004.

Xu, Y., Li, B., Hou, K., Du, Z., Allen, S.C., Zhu, Lusheng, Li, W., Zhu, Lei, Wang, Jinhua, Wang, Jun 2021. Ecotoxicity evaluation of azoxystrobin on *Eisenia fetida* in different soils. Environ. Res. 194, 110705 https://doi.org/10.1016/j.envres.2020.110705.

Yang, L., Huang, T., Li, R., Souders II, C.L., Rheingold, S., Tischuk, C., Li, N., Zhou, B., Martyniuk, C.J., 2021. Evaluation and comparison of the mitochondrial and developmental toxicity of three strobilurins in zebrafish embryo/larvae. Environ. Pollut. 270, 116277.

Zhang, C., Zhou, T., Xu, Y., Du, Z., Li, B., Wang, Jinhua, Wang, Jun, Zhu, L., 2020. Ecotoxicology of strobilurin fungicides. Sci. Total Environ. 742, 140611 https://doi.org/10.1016/j.scitotenv.2020.140611.

Zhang, C., Zhu, L., Wang, Jun, Wang, Jinhua, Du, Z., Li, B., Zhou, T., Cheng, C., Wang, Z., 2018. Evaluating subchronic toxicity of fluoxastrobin using earthworms (*Eisenia fetida*). Sci. Total Environ. 642, 567–573. https://doi.org/10.1016/j.scitotenv.2018.06.091.