Ecotoxicology (2022) 31:1554–1564
https://doi.org/10.1007/s10646-022-02609-4

Comprehensive study of the effects of strobilurin-based fungicide formulations on *Enchytraeus albidus*

Marija Kovačević[1] · Nikolina Stjepanović[1] · Davorka K. Hackenberger[1] · Željka Lončarić[1] · Branimir K. Hackenberger[1]

Accepted: 28 November 2022 / Published online: 3 December 2022

Abstract

Excessive application of fungicides in crop fields can cause adverse effects on soil organisms and consequently affect soil properties. Existing knowledge on the effects of strobilurin fungicides has been primarily based on toxicity tests with active ingredients, while the effects of fungicide formulations remain unclear. Therefore, this work aims to provide new data on the effects of three commercial formulations of strobilurin fungicides on the soil organism *Enchytraeus albidus*. The tested fungicide formulations were Retengo® (pyraclostrobin—PYR), Zato WG 50® (trifloxystrobin—TRI) and Stroby WG® (kresoxim-methyl—KM). In laboratory experiments, multiple endpoints were considered at different time points. The results showed that PYR had the greatest impact on survival and reproduction ($LC_{50} = 7.57\ mg_{a.i.}kg_{soil}{}^{-1}$, $EC_{50} = 0.98\ mg_{a.i.}kg_{soil}{}^{-1}$), followed by TRI ($LC_{50} = 72.98\ mg_{a.i.}kg_{soil}{}^{-1}$, $EC_{50} = 16.93\ mg_{a.i.}kg_{soil}{}^{-1}$) and KM ($LC_{50} = 73.12\ mg_{a.i.}kg_{soil}{}^{-1}$, $EC_{50} \geq 30\ mg_{a.i.}kg_{soil}{}^{-1}$). After 7 days of exposure, MXR activity was inhibited at the highest concentration of all fungicides tested ($6\ mg_{PYR}kg_{soil}{}^{-1}$, $15\ mg_{TRI}kg_{soil}{}^{-1}$ and $30\ mg_{KM}kg_{soil}{}^{-1}$). Furthermore, oxidative stress (induction of SOD, CAT and GST) and lipid peroxidation (increase in MDA) were also observed. In addition, there was a decrease in total available energy after exposure to PYR and KM. Exposure to fungicides resulted in a shift in the proportions of carbohydrates, lipids, and proteins affecting the amount of available energy. In addition to the initial findings on the effects of strobilurin formulations on enchytraeids, the observed results suggest that multiple and long-term exposure to strobilurin formulations in the field could have negative consequences on enchytraeid populations.

Keywords Strobilurin fungicides · Toxicity · Enchytraeus · Oxidative stress · Energy available

Introduction

The use of pesticides in excessive quantities is of great concern for the environment. According to Nguyen et al. (2016), <0.1% of the pesticides reach their specific targets, while the rest go into ecosystems. Among pesticides, fungicides account for 40% of sales. In the last two decades, strobilurin fungicides have become an indispensable part of agricultural production (Bartlett et al. 2002; Zhang et al. 2020). According to Eurostat data, sales of strobilurin fungicides in Europe almost doubled from 2011 to 2020, reaching ~3 million kg per year (Eurostat). Besides azoxystrobin (AZO), pyraclostrobin (PYR), trifloxystrobin (TRI) and kresoxim-methyl (KRE) are the most commonly used strobilurin fungicides (Zhang et al. 2020; Xu et al. 2021; Wu et al. 2021). PYR, TRI, and KRE are used on various cereal, oil, vegetable, fruit and other crops worldwide (Zhang et al. 2020). Although strobilurin fungicides are expected to be rapidly degraded, other trends have been observed. Namely, the average half-life (DT_{50}) reported for PYR is 28 days, but can extend up to 63 days (Fulcher et al. 2014). In contrast to PYR, the DT_{50} reported for TRI and KM is shorter. However, their metabolites are formed, which may have adverse effects. Although the DT_{50} for TRI ranged from 5 to 25 days, the DT_{50} for the metabolites of TRI ranged from 138 to 231 days (Wang et al. 2015). In addition, KM rapidly dissipates into its acidic metabolites in soil (Khandelwal et al. 2014). The mean DT_{50} values

---

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10646-022-02609-4.

✉ Davorka K. Hackenberger
davorka@biologija.unios.hr

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

calculated for KM were ~3 days, while its metabolites are present in the soil for more than 90 days. The high-water solubility of strobilurin fungicides can lead to their accumulation in water and soil (Zhang et al. 2020). Previous research has focused mainly on the effects of active ingredients on survival, reproduction, and the occurrence of oxidative stress in aquatic organisms (Li et al. 2016; Jiang et al. 2019; Li et al. 2018; Kumar et al. 2020; Mao et al. 2020; Kim et al. 2021; Li et al. 2021; Yang et al. 2021). However, there are limited data on the impact of strobilurin fungicides on soil ecosystems.

The widespread and extensive application of strobilurin fungicides, where soil ecosystems are the main sink, leads to potential risks for soil organisms and ecosystems (Zhang et al. 2020). Soil processes are mainly influenced by the indispensable part of this ecosystem—the soil fauna (Brussaard 1998). The decline in soil biodiversity and biological activity has been linked to the excessive application of fungicides in agriculture (Pelosi et al. 2014). Enchytraeids are considered indicators of soil quality (Didden and Römbke 2001; Pelosi and Römbke 2016) and perform similar functional role as earthworms, but in a lesser extent (Marinissen and Didden 1997). Namely, they affect the decomposition of organic matter and the circulation of nutrients in the soil (Briones et al. 1998; Maraldo et al. 2011). In this way, they create a favourable microhabitat for the growth and development of microorganisms (Bardgett 2005). Moreover, under tillage pressure in cultivated soils, a decrease in earthworm populations was observed, while enchytraeids activity increased (Topoliantz et al. 2000). Enchytraeids affect soil structure and porosity in such soils, thus improving their quality. Their role in decomposition and bioturbation together with living close to the surface layers of the soil brings them into direct contact with pollutants and makes them easy targets of environmental pollution.

Enchytraeus albidus is a model organism in soil ecotoxicology (OECD 2016; ISO 2014). However, information on the effects of strobilurin fungicides on this species is insufficient. Apart from azoxystrobin, which has been extensively studied (Zhang et al. 2020; Xu et al. 2021; Wu et al. 2021), pyraclostrobin (PYR), trifloxystrobin (TRI) and kresoxim-methyl (KM) are the most widely sold fungicides in this group. PYR and TRI in the form of a pure active ingredient have been shown to inhibit growth and induce oxidative stress and DNA damage in the earthworm *Eisenia fetida* (Ma et al. 2019; Liu et al. 2020; Wu et al. 2021). Furthermore, exposure to formulations containing the active ingredient PYR and TRI impaired the survival and reproduction of *E. crypticus* (Kovačević et al. 2021a), while the effects on other endpoints at the suborganismal level are unknown. To our knowledge, KM has not yet been studied in soil organisms, apart from regulatory risk assessments in which the risk to soil organisms was considered low (EFSA 2010). However, although the risk to aquatic organisms has been classified as low, both KM and its main metabolite BF-490-1 (acid of kresoxim-methyl) are toxic to non-target aquatic organisms (Li et al. 2021). In addition, strobilurin fungicides have been repeatedly detected in aquatic and terrestrial environments at concentrations above the Regulatory Acceptable Concentration (RAC) and could threaten non-target organisms (Feng et al. 2020; Li et al. 2021).

The ecotoxicological effects of formulations may differ significantly from those of a pure active ingredient (Mesnage et al. 2014; Gomes et al. 2021). Therefore, the need for a detailed assessment of the impacts of formulations has been highlighted previously (Marques et al. 2009). However, a comprehensive analysis of different endpoints over different time intervals is needed for a better understanding of the mechanisms of action that will allow a more accurate prediction of population-level consequences. Standardised tests focus primarily on endpoints such as survival and reproduction and ignore effects on crucial physiological processes that are important for organisms to adapt to stressful conditions. Therefore, it is important to link subindividual and population biomarkers to elucidate the mechanisms that are important for the survival of organisms and their populations.

This study aimed to comprehensively evaluate the effects of three commercial formulations containing strobilurin active ingredients (pyraclostrobin, trifloxystrobin, and kresoxim-methyl) on *E. albidus* at different time points. In addition to survival and reproduction, the effects on MXR activity, oxidative status, and available energy reserves were also investigated. The results obtained will provide valuable information on the effects of different commercial strobilurin fungicides on enchytraeids.

Material and methods

Test organism and soil

Enchytraeus albidus (Oligochaeta: Enchytraeidae) was used as the test species. Cultures were maintained in moist soil under controlled conditions according to the prescribed guidelines (OECD 220 2016; ISO 16387 2014) at the University of Osijek (Croatia).

All experiments were conducted in the standard artificial soil (AS), which consists of 70% air-dried quartz sand, 20% kaolin clay, and 10% sphagnum peat. The AS was prepared according to OECD guidelines (2016).

Test materials and spiking procedures

The strobilurin fungicides used were the formulations Retengo® (BASF, 200 gL^{-1} pyraclostrobin—PYR), Zato

WG 50® (Bayer, 500 $g kg^{-1}$ trifloxystrobin—TRI), and Stroby WG® (BASF SE, 500 $g kg^{-1}$ kresoxim-methyl—KM).

The concentrations selected for the range-finding test were 0, 1.87, 3.75, 7.5, 15 and 30 $mg_{a.i.}kg_{soil}{}^{-1}$ for PYR and 0, 9.38, 18.75, 37.5, 75 and 150 $mg_{a.i.}kg_{soil}{}^{-1}$ TRI and KM. Based on the recommended application doses (RD) for formulations ($RD_{PYR} = 0.16\ mg_{a.i.}kg_{soil}{}^{-1}$; $RD_{TRI} = 0.1\ mg_{a.i.}kg_{soil}{}^{-1}$; $RD_{KM} = 0.1\ mg_{a.i.}kg_{soil}{}^{-1}$) and the results of the range-finding tests, the final test concentrations were selected. The concentrations selected were 0, 0.08, 0.16, 1.107, 2.053, 3 and 6 $mg_{a.i.}kg_{soil}{}^{-1}$ for PYR, 0, 0.05, 0.1, 2.57, 5.03, 7.5 and 15 $mg_{a.i.}kg_{soil}{}^{-1}$ for TRI and 0, 0.05, 0.1, 5.07, 10, 15 and 30 $mg_{a.i.}kg_{soil}{}^{-1}$ for KM. The fungicides were dissolved in distilled water to obtain the desired concentrations. The solutions were prepared to contain the volume required for 60% of the water holding capacity. After the addition of the pesticide, the soil was homogeneously mixed and allowed to equilibrate 24 h before the start of the experiment.

Test procedures

As no sufficient information on the effects of PYR, TRI, and KM on *E. albidus* is available, the range-finding test was performed according to the recommended guidelines (OECD 2016) followed by the enchytraeid reproduction test (ERT) (ISO 2014; OECD 2016). Ten adult organisms with well-developed clitellum were used per replicate. Each test vessel contained 20 g of moist soil and a food supply. For the range-finding test, the organisms were exposed for 7 days and only survival was assessed. For the final experiment twenty replicates per test concentration were used. In the ERT five replicates were used and adults were exposed for 21 day, with an additional twenty-one day for hatching and growth of juveniles. Five replicates were used for each tested concentration. Simultaneously with the ERT, an additional five replicates per tested concentration were set up for seven-day exposure. Each of the test vessels contained 20 g of soil and ten adult organisms with well-developed clitellum. The 7-day exposure was added to allow assessment at an additional time point. Moreover, to determine changes in multixenobiotic resistance mechanism activity (MXR), additional ten replicates were set up, five to assess effects after 7 days of exposure, and five to assess MXR activity after 21 day. During exposure, a constant temperature of 20 ± 1 °C and a photoperiod of 16 h of light and 8 h of darkness were maintained. Organisms were fed once per week with autoclaved oatmeal, while water was replenished weekly, based on weight loss.

After exposure, adults were carefully removed from the soil, counted, pooled per replicate, and weighed. Organisms were homogenised using the IKA RW20 homogeniser in cold 0.1 M potassium phosphate buffer (pH 7.4) at a ratio of 1:15 (w:v). The post-mitochondrial fraction (S9) was obtained after centrifugation of the homogenate (30 min at 9000 *g* and 4 °C). The post-mitochondrial fraction and homogenates were stored at −80 °C before analysis.

Juveniles were counted 21 day after extraction of the adults. To enable counting, juveniles were fixed with ethanol and stained with Bengal rose (1% in ethanol). The soil samples were sieved through the mesh (63 µm) to prevent blurring and to facilitate counting with a stereomicroscope.

Organisms used for the MXR system analyses were isolated, washed, and transferred to a 5 mM solution of Rh123 in ISO water. Exposure to the fluorescent substrate took place in the dark at 20 °C and lasted for 1 h. MXR activity was measured according to Kovačević et al. (2021b). The concentration of Rh123 in the organisms was expressed as µM of Rh123 per g of wet tissue and normalised to the control.

To determine the oxidative status of the organisms, the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) were measured in the S9 fraction. The method described by McCord and Fidovich (1969) was used to measure SOD activity. The CAT activity was measured according to Claiborne (1985) and the GST according to Habig et al. (1974). Malondialdehyde (MDA) content was measured as described by Gagne (2014) and used to determine LPO. Enzyme activities and LPO were calculated per protein content and expressed as relative values compared to the control. Lipid and carbohydrate content were measured in the homogenate using the method described by Frings et al. (1972) and Jermyn (1975). The protein content in the S9 fraction was determined according to the method of Bradford (1976). To allow calculation of available energy reserves, the determined content of carbohydrates, lipids, and proteins was expressed in energetic equivalents using the enthalpy of combustion (17.5 kJg^{-1} carbohydrates, 39.5 kJg^{-1} lipids, and 24 kJg^{-1} protein) as described in De Coen and Janssen (1997); De Coen and Janssen (2003).

Statistical analysis

Data analysis was performed using R software version 4.3.0 (R Development Core Team 2022) and RStudio (RStudio Team 2022). The Shapiro–Wilk test was used to test the distribution of data and Bartlett's test was used to test the homogeneity of variances. As the data did not deviate from the normal distribution, one-way ANOVA followed by Dunnett's post hoc test ($p \leq 0.05$) was used to determine the difference between the control group and the tested concentrations. The package drc (Ritz et al. 2015) was used to calculate the lethal (LC_X) and effect concentrations (EC_X).

Results & discussion

Survival and reproduction

Our results showed a higher impact of PYR ($LC_{50} = 7.6\ mg_{a.i.}kg_{soil}^{-1}$) on the survival of *E. albidus* compared to TRI ($LC_{50} = 73\ mg_{a.i.}kg_{soil}^{-1}$) and KM ($LC_{50} = 73\ mg_{a.i.}kg_{soil}^{-1}$) (Table 1). Reported LC_{50} for *E. crypticus* exposed to PYR ($LC_{50} = 4.26\ mg_{a.i.}kg_{soil}^{-1}$) and TRI ($LC_{50} = 2.34\ mg_{a.i.}kg_{soil}^{-1}$) suggest a higher resistance of *E. albidus* to PYR and, in particular, to TRI (Kovačević et al. 2021a).

Consistent with the effects on survival, PYR was most detrimental to reproduction (Table 1). While PYR showed the strongest effect on reproduction, the effects of TRI ($EC_{50} = 17\ mg_{a.i.}kg_{soil}^{-1}$) and KM ($EC_{50} \geq 30\ mg_{a.i.}kg_{soil}^{-1}$) were lower (Fig. 1). Unlike the other two strobilurins, KM significantly impaired reproduction only at the highest concentration tested. Although *E. albidus* showed the ability to survive exposure to higher concentrations of PYR than *E. crypticus*, the effect on reproduction was the opposite. With an EC_{50} of $0.98\ mg_{a.i.}kg_{soil}^{-1}$, *E. albidus* appears to be more sensitive to PYR than *E. crypticus* ($EC_{50} = 1.85\ mg_{a.i.}kg_{soil}^{-1}$). The recommended application dose prescribed for PYR exceeds the EC_{50} value calculated for *E. albidus*. Additionally, soil analysis of some agricultural land revealed the presence of PYR in the range of $0.0128–1.5\ mg_{a.i.}kg_{soil}^{-1}$ (Zhao et al. 2020), which also exceeds the EC_{50} value. Therefore, the concentrations of PYR present in the environment may pose a threat to the stability of enchytraeid populations and the function of soil ecosystems.

MXR

The negative effects of strobilurin fungicides were also evident at the sub-organismal level, causing impairment of MXR and induction of oxidative stress. A significant inhibition (ANOVA, $p < 0.001$, $F = 44.675$) of the MXR system was observed after exposure to higher concentrations of the three fungicides (Fig. 2). The strongest inhibition of the MXR system (up to 7-fold) was observed after a seven-day exposure to PYR ($6\ mg_{a.i.}kg_{soil}^{-1}$), followed by a 4-fold increase at the same concentration after twenty-one day of exposure. TRI caused a significant dose-dependent increase in Rh123 levels after 21 day of exposure. As with reproductive success, exposure to KM inhibited the MXR system only at the highest concentration ($30\ mg_{a.i.}kg_{soil}^{-1}$) after 7 and 21 day. The dye efflux assay is a commonly used method to quantify the effects of environmental contaminants on MXR activity (Luckenbach et al. 2014). Few studies have reported the effects of fungicides on the MXR system of enchytraeids (Kovačević et al. 2021b, Kovačević et al. 2022), but such a response has also been observed in earthworms (Velki et al. 2018). The MXR mechanism removes harmful substances from cells immediately after exposure and is therefore considered the first line of defence (Ferreira et al. 2014). Furthermore, changes in MXR activity are used as biomarkers of exposure and can provide additional information on the effects and toxicity of fungicides. Indeed, inhibition of MXR activity may lead to a longer residence time of fungicides in cells, which consequently influences their toxicity (Hackenberger et al. 2012). Although there is no available information on the accumulation of PYR in soil organisms, studies with zebrafish (*Danio rerio*) show that most of PYR enters the body through the gills and intestines - epithelial tissues rich in MXR transporters (Huang et al., 2021). Furthermore, the toxicity of PYR in zebrafish was related to its accumulation in the organism. Therefore, the higher toxicity of PYR could be related to the strong inhibition of the MXR system, which consequently led to the accumulation of fungicides in the organism. Furthermore, the accumulation of fungicides in cells can lead to oxidative stress, as antioxidant defence may be overwhelmed.

Table 1 Lethal (LC_x) and effect (EC_x) concentrations calculated for *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI), and kresoxym-metil (KM)

		LC_{10}	LC_{50}	LC_{90}
Survival	PYR	4 (3–5)	7.6 (6–8)	10 (9–11)
	TRI	15 (9–20)	73 (67–81)	130 (110–152)
	KM	21 (19–24)	73 (47–91)	98 (90–109)
		EC_{10}	EC_{50}	EC_{90}
Reproduction	PYR	0.12 (0.025–0.26)	0.98 (0.51–1.5)	8 (7–12)
	TRI	3 (1.8–4.5)	17 (12–18)	31 (28–>30)
	KM	17 (10–24)	>30	>30

Concentrations are presented as $mg_{a.i.}kg_{soil}^{-1}$ and show LC/EC and the 95% confidence intervals

Oxidative stress

Changes in the activity of enzymes of the antioxidant system (SOD, CAT, GST) and the observed LPO indicate the appearance of oxidative stress. Although data on antioxidant enzymes in enchytraeids after exposure to strobilurin fungicides are lacking, there is information on the effect of the pure active ingredient on the earthworm *E. fetida* (Ma et al. 2019; Liu et al. 2020; Wu et al. 2021). Listed studies have shown that strobilurin fungicides in the form of pure active ingredients cause oxidative stress, suggesting similar effects in other non-target soil organisms. One of the enzymes that respond first to the onset of oxidative stress is SOD. As part of the primary antioxidant defence mechanism, SOD converts the superoxide anion radical (O_2^{-}) into hydrogen peroxide (H_2O_2) (Ighodaro and Akinloye 2018). In this study, there was a significant induction of SOD activity after a seven-day exposure to lower concentrations of PYR and almost all

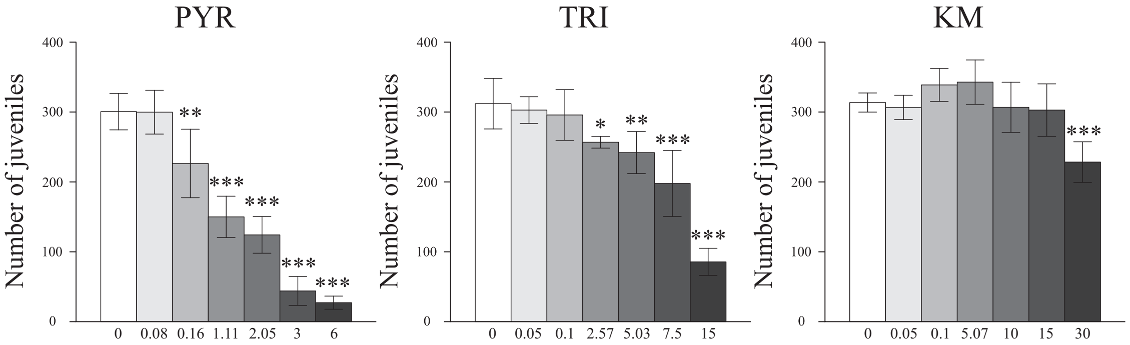

Fig. 1 Reproduction of *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI), and kresoxym-metil (KM) presented as the number of juveniles. Results express average values ± SD. Significant differences compared to control are labeled with *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$)

concentrations of TRI (Fig. 2). The induction of SOD indicates the presence of O_2^-, which the enzyme attempts to remove. SOD induction has been observed after 7 days of exposure to PYR ($0.08\ mg_{a.i.}kg_{soil}^{-1}$ and $16\ mg_{a.i.}kg_{soil}^{-1}$) and TRI ($0.1\ mg_{a.i.}kg_{soil}^{-1}$ to $15\ mg_{a.i.}kg_{soil}^{-1}$). The activity of SOD remained induced at lower concentrations of PYR ($0.16\ mg_{a.i.}kg_{soil}^{-1}$ and $1.107\ mg_{a.i.}kg_{soil}^{-1}$) and was induced at the three highest concentrations of KM after 21 days of exposure ($p < 0.05$). However, at the highest tested concentrations of PYR ($6\ mg_{a.i.}kg_{soil}^{-1}$) and TRI ($7.5\ mg_{a.i.}kg_{soil}^{-1}$, $15\ mg_{a.i.}kg_{soil}^{-1}$), the amount of substrate exceeds the enzyme capacity, and its depletion and degradation occur. Since no new enzyme is synthesised, this reaction leads to a significant reduction in the amount of enzyme. A similar response to PYR and TRI has been observed in the earthworm *E. fetida*. Namely, Ma et al. (2019) reported induction of SOD after 7 and 14 days of exposure to PYR, while inhibition was observed after 21 and 28 days. Furthermore, Liu et al. (2020) observed induction only at the highest TRI concentration tested ($10\ mg_{a.i.}kg_{soil}^{-1}$), while Wu et al. (2021) observed induction of SOD activity after 7 and inhibition after 28 and 56 days. SOD scavenges excess O_2^- and converts it to H_2O_2 (Shao et al. 2019). As H_2O_2 is a substrate for CAT, its activity was induced in all treatments after 7 days (ANOVA, $p = 0.0058$, $F = 9.4$) (Fig. 2). However, after 21 day, the activity of CAT returned to the control level, indicating that the elimination of H_2O_2 was successful at lower concentrations. Only at the highest concentrations of PYR and KM, the activity of CAT was significantly inhibited (ANOVA, $p = 0.0092$, $F = 3.585$), indicating that the enzyme capacity was exceeded and degraded. Similarly, in *E. fetida*, induction of CAT activity was observed after shorter exposures (7, 14, and 21 day) to PYR and TRI (7 and 28 days) and without change or inhibition after longer exposures (Ma et al. 2019; Wu et al. 2021). GST is a crucial enzyme in the second biotransformation phase. Unlike SOD and CAT, which are activated immediately after exposure to free radicals, the effect of GST is most pronounced after prolonged exposure. Indeed, in this study, no significant changes in GST activity were observed after a seven-day exposure (Fig. 2). However, after twenty-one day of exposure to TRI and KM, there was a dose-dependent induction (ANOVA, $p < 0.0001$, $F = 27.46$). GST was also induced at two lower concentrations of PYR, but at higher concentrations, its activity was significantly inhibited. This result suggests a higher ROS production caused by exposure to PYR and/or glutathione depletion (a substrate for GST). Earthworms appeared to cope better with PYR since only induction was detected (Ma et al. 2019), although the duration of exposure may not have been long enough to detect GST inhibition. Indeed, exposure to TRI induced GST in earthworms after 7 days but inhibited it after 56 days (Liu et al. 2020; Wu et al. 2021). This also suggests a prolonged effect of strobilurins on soil invertebrates and urges caution, as multiple and mixed applications of pesticides regularly occur in this time frame under the given environmental conditions. Excessive long-term ROS production can cause LPO. LPO is a sign of severe oxidative stress and the inability of an organism to defend itself. Measurement of MDA content is often used to determine the extent of LPO (Gaweł et al. 2004). Normally, LPO does not occur after a short exposure. However, after only 7 days, the MDA content increased significantly at the highest concentrations of PYR ($6\ mg_{a.i.}kg_{soil}^{-1}$) and TRI ($30\ mg_{a.i.}kg_{soil}^{-1}$) (ANOVA, $p = 0.002$, $F = 4514$) (Fig. 2). After 21 day of exposure to the three fungicides, a significant increase in MDA was observed at higher concentrations. These results are consistent with the previously reported increase in MDA after exposure of *E. fetida* to PYR and TRI (Ma et al. 2019; Liu et al. 2020; Wu et al. 2021).

Available energy reserves

Detoxification processes are energetically costly for organisms, which can lead to a reduction in the energy used for basic processes such as growth and reproduction (Świątek and Bednarska 2019). The total available energy of the

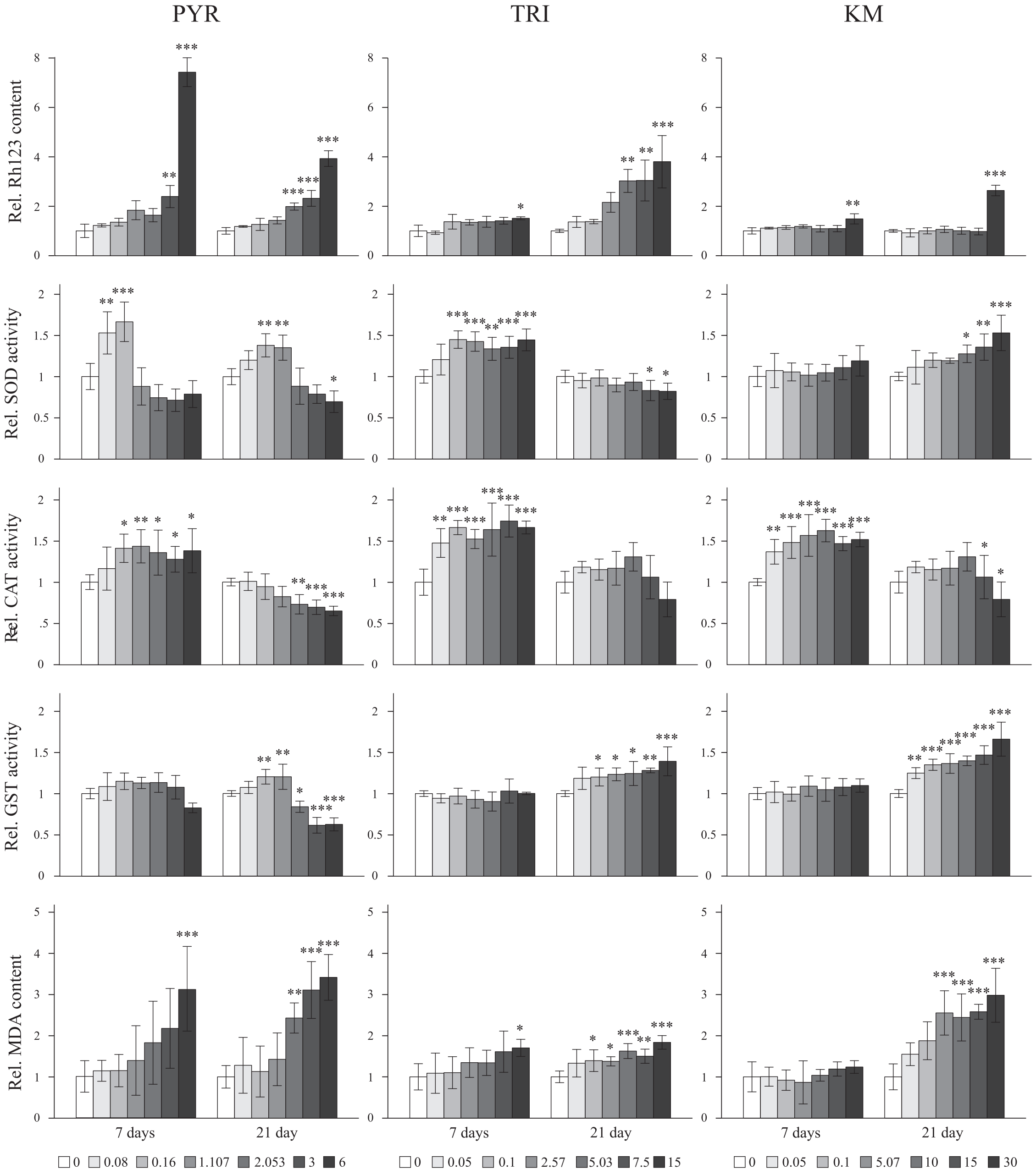

Fig. 2 Responses of different endpoints (MXR (Rh123 content), SOD, CAT, and GST activity, and lipid peroxidation (MDA content)) in *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI) and kresoxym-metil (KM). Results are expressed as average ± SD and relative to the corresponding control. Significant differences compared to control are labeled with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

organisms in the control groups was approximately 1700 $\mathrm{mJmg_{wt}}^{-1}$ while the ratio of energy reserves was about 10:35:55 (carbohydrates:lipids:proteins). These results agree with previous studies on enchytraeids (Amorim et al. 2012; Novais et al. 2013).

Exposure to commercial strobilurin fungicides resulted in the available energy changes in *E. albidus* (Table 2). A significant decrease in carbohydrate fraction was observed after a seven-day exposure to the two highest concentrations of PYR and after a twenty-one day exposure to all fungicides tested. The decrease in the carbohydrate fraction is a result of oxidative stress, a condition in which carbohydrates are used as the primary source of energy (Moolman et al. 2007). The reduction of carbohydrate content has been observed after exposure of enchytraeids to the pesticides dimethoate, anthrazine, and carbendazim (Novais and Amorim 2013). Since lipids are extremely efficient as energy storage, they are usually mobilised simultaneously

Table 2 Total energy available and proportions of each energy reserve (carbohydrates, lipids, and proteins) in *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI), and kresoxym-metil (KM)

	PYR				TRY				KM			
	Energy available	Proportion of energy reserve			Energy available	Proportion of energy reserve			Energy available	Proportion of energy reserve		
		CH	Lipid	Protein		CH	Lipid	Protein		CH	Lipid	Protein
7-day exposure												
Ctrl.	1697 ± 88	12 ± 2.1	37 ± 3.7	50 ± 2.8	1673 ± 181	11 ± 1.9	34 ± 5	54 ± 3.8	1735 ± 98	11 ± 1.4	35 ± 3.7	54 ± 2.7
C1	1692 ± 112	12 ± 1.4	38 ± 1.8	50 ± 2.7	1591 ± 128	10 ± 0.9	32 ± 4.9	58 ± 4.1	1825 ± 114	11 ± 0.3	35 ± 3.5	53 ± 3.4
C2	1750 ± 23	12 ± 2.5	42 ± 5.7	46 ± 3.8	1755 ± 87	11 ± 1.4	39 ± 2.5	50 ± 1.5	1905 ± 82	10 ± 1.4	37 ± 1.9	53 ± 2.8
C3	1782 ± 82	12 ± 1	41 ± 2.9	47 ± 2.8	1796 ± 139	13 ± 2.3	36 ± 5.5	50 ± 3.4	$1977 \pm 168^{**}$	10 ± 1.4	$45 \pm 3^{***}$	45 ± 4
C4	1696 ± 15	9.3 ± 2	40 ± 6.4	50 ± 6.3	1863 ± 178	12 ± 1.9	40 ± 5.1	48 ± 5.3	$1997 \pm 48^{**}$	11 ± 2	$45 \pm 1.8^{***}$	43 ± 2.2
C5	1719 ± 86	$5.2 \pm 1.3^{***}$	44 ± 2.8	50 ± 2.4	1795 ± 143	9 ± 2.5	41 ± 2.4	50 ± 1.3	$1957 \pm 65^{*}$	10 ± 1.8	$41 \pm 2.9^{**}$	49 ± 2.7
C6	1624 ± 170	$3 \pm 0.66^{***}$	44 ± 4.2	51 ± 4.7	1796 ± 43	10 ± 1.3	41 ± 2.7	48 ± 2	1864 ± 111	10 ± 1.5	$43 \pm 3^{**}$	47 ± 2.4
21-day exposure												
Ctrl.	1632 ± 99	12 ± 1.6	35 ± 1.7	52 ± 1.9	1613 ± 62	10 ± 1.4	36 ± 2.5	53 ± 3.6	1753 ± 166	10 ± 0.9	35 ± 2.3	54 ± 1.7
C1	1622 ± 80	12 ± 2.8	30 ± 1.8	58 ± 4.4	1335 ± 38	$7 \pm 1.5^{***}$	$30 \pm 3.6^{***}$	63 ± 3	1737 ± 120	$8 \pm 0.9^{**}$	35 ± 1.1	57 ± 0.8
C2	1553 ± 51	10 ± 3.1	$28 \pm 1.4^{*}$	$62 \pm 2.6^{*}$	1507 ± 130	$6 \pm 1^{***}$	$27 \pm 1.2^{***}$	$67 \pm 2^{*}$	1706 ± 73	$8 \pm 1.9^{**}$	33 ± 3.5	59 ± 2.9
C3	1540 ± 38	$8 \pm 3^{**}$	$27 \pm 4^{***}$	$65 \pm 1.8^{**}$	1784 ± 103	$6 \pm 0.35^{***}$	$24 \pm 1.9^{***}$	$70 \pm 2^{***}$	1599 ± 86	$8 \pm 1^{*}$	$31 \pm 2.3^{*}$	61 ± 2.2
C4	$1423 \pm 99^{**}$	$6 \pm 1.6^{***}$	$22 \pm 4.5^{***}$	$71 \pm 4.9^{**}$	1634 ± 160	$6 \pm 0.8^{***}$	$22 \pm 4.6^{***}$	$72 \pm 4.6^{***}$	1582 ± 45	$8 \pm 1.7^{**}$	$30 \pm 2.3^{**}$	62 ± 1.1
C5	$1343 \pm 100^{***}$	$5 \pm 0.75^{***}$	$18 \pm 4^{***}$	$77 \pm 3.3^{**}$	1741 ± 56	$7 \pm 1.5^{*}$	$22 \pm 2^{***}$	$71 \pm 3.1^{***}$	$1546 \pm 75^{*}$	$8 \pm 1.5^{**}$	$29 \pm 3.7^{**}$	63 ± 4.1
C6	$1263 \pm 58^{***}$	$3 \pm 0.78^{***}$	$17 \pm 1.4^{***}$	$80 \pm 2^{**}$	1789 ± 91	$6 \pm 0.76^{***}$	$24 \pm 1.8^{***}$	$70 \pm 1.3^{***}$	$1528 \pm 120^{*}$	$7 \pm 1.2^{***}$	$25 \pm 2.9^{***}$	67 ± 4

Results are expressed as average ± standard deviation. Significant differences compared to control are labeled with *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$)

with carbohydrates under stress conditions (Smolders et al., 2003). However, after a 7-day exposure to KM (2.57, 10, 15 and 30 $mg_{a.i.}kg_{soil}{}^{-1}$), lipid content increased. An increase in lipid content has previously been associated with inflammatory stress (Gomes et al. 2015). The tendency to accumulate lipids was also observed after 8 days of exposure of *E. albidus* to the fungicide carbendazim (Novais and Amorim 2013). The lipid content decreased after 21 day of exposure to all tested fungicides. Lipid depletion could be a consequence of severe oxidative stress and damage to the cell membrane. Increased levels of MDA and changes in activities of SOD, CAT, MXR, and GST were measured in all treatments in our studies. Similar changes in lipids have been reported after exposure of *E. albidus* to Cd and Zn (Novais et al. 2011; Novais et al. 2013). As anabolic components, proteins are only used as an energy source during extreme energy deficiency (Świątek and Bednarska 2019). Sokolova et al. (2012) reported that an increase in protein levels under intermediate stress indicates a higher expression of stress response proteins. In our research proteins increased significantly after 21 day of exposure to higher concentrations of PYR and TRI (Table 2) and no decrease was observed. These shifts in carbohydrate, lipid, and protein content affected total available energy, which increased after 7 days of exposure to KM and significantly decreased after 21 day of exposure to higher concentrations of PYR and KM. Exposure to TRI did not affect the total available energy. Since an increase in lipid content strongly influences total available energy, an increase in lipid content after exposure to different pesticides led to an increase in available energy (Novais and Amorim 2013). According to the general prediction of metabolic models, the metabolic rate should increase with intoxication (Calow and Sibly 1990), which may be related to the results of this research. Although in KM treatments, the decrease in available energy may be a consequence of excessive energy consumption as a result of the simultaneous defence against oxidative stress and reproduction, in the case of PYR, it was due to the higher toxicity and the need for a more intense defence against stress. Furthermore, although oxidative stress was observed after TRI treatment, the lack of reproduction compensated for the loss of energy, and the decrease in total available energy, was not observed.

Strobilurin fungicides are known for their ability to act on the mitochondrial respiratory chain and consequently reduce the available energy of organisms. Furthermore, the breakdown of the electron transfer chain and mitochondrial membrane protein leads to the formation of ROS and, consequently, oxidative stress. Although changes in apical endpoints, such as growth and behaviour, may not be observed, even extremely low concentrations of strobilurin fungicides can cause oxidative stress in aquatic organisms (Wang et al. 2021). Consequently, although exposure to some formulations did not affect survival and reproduction, changes in CAT and GST activity when exposed to the recommended application doses indicate a negative effect. In addition to oxidative stress, the reduction in available energy observed after prolonged exposure suggests a negative effect on mitochondrial respiration. Similar conclusions about the strobilurin mechanism were made for different non-target organisms (Wang et al. 2021). Furthermore, a high concentration of strobilurin fungicides suppresses mitochondrial respiration, and consequently, cell apoptosis occurs (Rodrigues et al. 2015). Therefore, inhibition of the MXR system and accumulation of fungicides within the cell, observed in PYR treatments, increase the possibility of fungicide binding to the mitochondria, causing apoptosis and enchytraeid mortality.

Most laboratory studies with suborganismic biomarkers are based on short-term exposure to high concentrations of pollutants (Rodríguez-Castellanos and Sanchez-Hernandez, 2007). However, in the environment, with soil as the main sink, long-term exposure to low concentrations is a more common scenario. Although environmentally relevant concentrations did not show effects on enchytraeid reproduction, the occurrence of oxidative stress and reduction in available energy showed that the adverse impact of strobilurin formulations begins much earlier than is evident from population biomarkers. The inclusion of the suborganismic biomarker approach showed a marked adaptability of enchytraeids to exposure. Although activation of the antioxidative system has been shown as a successful defence method, changes in the amount of available energy suggest that this strategy is not sustainable in long-term exposure. Namely, in such situations, there is a certain reduced rate of reproduction or its absence, which can ultimately lead to the collapse of populations. Moreover, formulated products often exhibited stronger adverse effects on non-target organisms than a pure active ingredient commonly used in research (Marques et al. 2009; Mesnage et al. 2014; Gomes et al. 2021). Therefore, research carried out with formulated products provides results more relevant to environmental conditions. Adjuvants present in commercial products increase the absorption rate and stability of the active ingredient, consequently affecting its bioavailability and causing higher toxicity to non-target organisms (Pereira et al. 2009; Mesnage and Antoniou 2018). PYR applied in the form of suspension concentrate (SC) showed the strongest effect on enchytraeids compared to TRI and KM applied in the form of water-dispersible granules (WG). SC formulations contain small particles of the active substance dissolved in a liquid medium, usually an aqueous solution with various additives. Among the additives are various suspension agents, wetting agents, and thickeners that increase the availability of the active substance and can affect the behaviour of the formulation in the environment

(Khan and Brown, 2016). Compared to other formulations, SC formulations have reduced leaching, which results in increased persistence and accumulation in the environment, which can result in a stronger effect on non-target organisms compared to other forms of formulations.

Conclusion

This study highlights the importance of assessing effects on multiple endpoints over different periods. Therefore, comprehensively considering the changes in multiple endpoints over different periods provided better insight into the impact of strobilurin fungicides. The results obtained indicate differences in the response of *E. albidus* to three commercial formulations of strobilurin fungicides. Overall, when all measured endpoints, are assessed PYR was the most toxic, followed by TRI and KM. Although with different intensities, all three strobilurins affected reproduction and induced oxidative stress in *E. albidus*. The analyses of available energy showed that the exposure also represented a high energetic cost for the organisms. While these costs after exposure to PYR and TRI were mainly related to antioxidant defence, the energetic costs after KM exposure, which was the least toxic, were associated with both costly defence and reproduction processes. Research like this is important to determine the sublethal toxicity of these fungicides because the frequent detection of strobilurins in the terrestrial environment suggests the potentiality for adverse effects on soil-dwelling organisms. Furthermore, like other pesticides, strobilurins are used indiscriminately in agriculture and predicted climate changes indicate an increase in fungicide use. The EC_{50} for PYR determined in this study is already within the range of concentrations observed in the environment. Future research should consider several aspects: exposure to formulations under realistic conditions in a native soil, exposure to pesticide mixtures or formulations with different combinations of strobilurins, which are commonly used, and multigenerational studies in which the consequences of oxidative stress and higher energy expenditure could be investigated at the population level.

Acknowledgements 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).

Author contributions MK—Conceptualization, investigation, data curation and interpretation, processing of figures and tables in the paper, original draft writing, revising the draft; NS—Investigation, review and editing of draft paper; ŽL—Investigation, review and editing of draft paper; DKHr—Conceptualization, supervision, review and editing of draft paper; BKH—Conceptualization, supervision, funding acquisition, review and editing of draft paper.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

References

Amorim MJB, Gomes SIL, Soares AMVM, Scott-Fordsmand JJ (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. https://doi.org/10.1007/s11270-011-0867-9

Bardgett RD (2005) The Biology of Soil. Oxford University Press Inc., New York

Bartlett DW, Clough JM, Godwin JR, Hall AA, 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

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

RCore Team (2022) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/

R Studio Team (2022) RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com

Briones MJI, Carreira J, Ineson P (1998) *Cognettia sphagnetorum* (Enchytraeidae) and nutrient cycling in organic soils: a microcosm experiment. Appl Soil Ecol 9:289–294

Brussaard L (1998) Soil fauna, guilds, functional groups, and ecosystem processes. Appl Soil Ecol 9(1–3):123–135

Calow P, Sibly RM (1990) A physiological basis of population processes: ecotoxicological implications. Funct Ecol 4:283–288

Claiborne A (1985) Catalase activity. In: Greenwald RA, Ed., CRC Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, 283-284.

De Coen W, Janssen CR (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 Recovery 6:43–55

De Coen W, Janssen CR (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 50:25–43

European Food Safety Authority, 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance kresoxim-methyl. EFSA J 8(11):1891. https://doi.org/10.2903/j.efsa.2010.1891. Available online: www.efsa.europa.eu/efsajournal.htm

Feng Y, Huang Y, Zhan H, Bhatt P, Chen S (2020) An overview of strobilurin fungicide degradation: current status and future perspective. Front Microbiol 11:389

Ferreira M, Costa J, Reis-Henriques MA (2014) ABC transporters in fish species: a review. Front Physiol 5:266. https://doi.org/10.3389/fphys.2014.00266

Frings CS, Fendley TW, Dunn RT, Queen CA (1972) Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin Chem 18(7):673–674

Fulcher JM, Wayment DG, White Jr PM, Webber CL (2014) Pyraclostrobin wash-off from sugarcane leaves and aerobic dissipation in agricultural soil. J Agric Food Chem 62(10):2141–2146. https://doi.org/10.1021/jf405506p

Gagne F (2014) Biochemical Ecotoxicology: Principles and Methods. Oxidative stress Academic Press, New York, p110e111

Gaweł S, Wardas M, Niedworok E, Wardas P (2004) Malondialdehyde (MDA) as a lipid peroxidation marker. Wiadomosci lekarskie (Warsaw, Poland: 1960) 57(9–10):453–455

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

Gomes SIL, Ammendola A, Casini S, Amorim MJB (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 WH, Pabst MJ, Jakobi W (1974) Glutathione S-Transferases the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Hackenberger BK, Velki M, Stepić S, Hackenberger DK (2012) First evidence for the presence of efflux pump in the earthworm *Eisenia andrei*. Ecotoxicol Environ Saf 75:40–45. https://doi.org/10.1016/j.ecoenv.2011.06.024

Huang T, Souders CL, Wang S, Ganter J, He J, Zhao YH, Cheng H, Martyniuk CJ (2021) Behavioral and developmental toxicity assessment of the strobilurin fungicide fenamidone in zebrafish embryos/larvae (Danio rerio). Ecotox Environ Safety 228112966-S0147651321010782:112966. https://doi.org/10.1016/j.ecoenv.2021.112966

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

International Organization for Standardization (ISO) (2014) Soil quality—effects of pollutants on Enchytraeidae (*Enchytraeus* sp.) - determination of effects on reproduction and survival. ISO 16387, Switzerland, Geneva

Jermyn MA (1975) Increasing the sensitivity of the anthrone method for carbohydrates. Analytical Biochem 68(1):332–335

Jiang J, Lv L, Wu S, An X, Wang F, Liu X, Zhao X (2019) Developmental toxicity of kresoxim-methyl during zebrafish (*Danio rerio*) larval development. Chemosphere 219:517–525. https://doi.org/10.1016/j.chemosphere.2018.12.061

Khan MA, Brown CD (2016) Influence of commercial formulation on leaching of four pesticides through soil. Sci Total Environ 5731573-1579S0048969716319957. https://doi.org/10.1016/j.scitotenv.2016.09.076

Khandelwal A, Gupta S, Gajbhiye VT, Varghese E (2014) Degradation of kresoxim-methyl in soil: Impact of varying moisture, organic matter, soil sterilization, soil type, light and atmospheric CO_2 level. Chemosphere 111:209–217. https://doi.org/10.1016/j.chemosphere.2014.03.044

Kim C, Choe H, Park J, Kim G, Kim K, Jeon HJ, Moon JK, Kim MJ, Lee SE (2021) Molecular mechanisms of developmental toxicities of azoxystrobin and pyraclostrobin toward zebrafish (*Danio rerio*) embryos: Visualization of abnormal development using two transgenic lines. Environ Pollut. 270. https://doi.org/10.1016/j.envpol.2020.116087

Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product. Pestic Biochem Physiol 187(454–466):105198. https://doi.org/10.1016/j.pestbp.2022.105198

Kovačević M, Hackenberger DK, Hackenberger BK (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 DK, Lončarić Ž, Hackenberger BK (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

Kumar N, Willis A, Satbhai K, Ramalingam L, Schmitt C, Moustaid-Moussa N, Crago J (2020) Developmental toxicity in embryo-larval zebrafish (*Danio rerio*) exposed to strobilurin fungicides (azoxystrobin and pyraclostrobin). Chemosphere. 241:124980. https://doi.org/10.1016/j.chemosphere.2019.124980

Li D, Liu M, Yang Y, Shi H, Zhou J, He D (2016) Strong lethality and teratogenicity of strobilurins on *Xenopus tropicalis* embryos: Basing on ten agricultural fungicides. Environ Pollut 208:868e873. https://doi.org/10.1016/j.envpol.2015.11.010

Li H, Cao F, Zhao F, Yang Y, Teng M, Wang C, Qiu L (2018) Developmental toxicity, oxidative stress, and immunotoxicity are induced by three strobilurins (pyraclostrobin, trifloxystrobin, and picoxystrobin) in zebrafish embryos. Chemosphere 207:781–790. https://doi.org/10.1016/j.chemosphere.2018.05.146

Li H, Jing T, Li T, Huang X, Gao Y, Zhu J, Lin J, Zhang P, Li B, Mu W (2021) Ecotoxicological effects of pyraclostrobin on tilapia (*Oreochromis niloticus*) via various exposure routes. Environ Pollut 285:117188. https://doi.org/10.1016/j.envpol.2021.117188

Li XY, Qin YJ, Wang Y, Huang T, Zhao YH, Wang XH, Martyniuk CJ, Yan B (2021) Relative comparison of strobilurin fungicides at environmental levels: Focus on mitochondrial function and larval activity in early staged zebrafish (*Danio rerio*). Toxicology. 452:152706

Liu T, Liu Y, Fang K, Zhang X, Wang X (2020) Transcriptome, bioaccumulation, and toxicity analyses of earthworms (*Eisenia fetida*) affected by trifloxystrobin and trifloxystrobin acid. Environ Pollut 265:115100. https://doi.org/10.1016/j.envpol.2020.115100

Luckenbach T, Fischer S, Sturm A (2014) Current advances on ABC drug transporters in fish. Comp Biochem Physiol C Toxicol Pharmacol 165:28e52. https://doi.org/10.1016/j.cbpc.2014.05.002

Ma J, Cheng C, Du Z, Li B, Wang J, Wang J, Wang Z, Zhu L (2019) Toxicological effects of pyraclostrobin on the antioxidant defense system and DNA damage in earthworms (*Eisenia fetida*). Ecol Indic 101:111–116. https://doi.org/10.1016/j.ecolind.2019.01.015

Mao L, Jia W, Zhang L, Zhang Y, Zhu L, Sial MU, Jiang H (2020) Embryonic development and oxidative stress effects in the larvae and adult fish livers of zebrafish (*Danio rerio*) exposed to the strobilurin fungicides, kresoxim-methyl, and pyraclostrobin. Sci Total Environ 729:139031. https://doi.org/10.1016/j.scitotenv.2020.139031

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

Marinissen JCY, Didden WAM (1997) Influence of the Enchytraeid worm *Buchholzia appendiculate* aggregate formation and organic matter decomposition. Soil Biol Biochem 29:387–390

Marques C, Pereira R, Goncalves F (2009) Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. J. Soils Sediment 9:137–147

Marques C, Pereira R, Gonçalves F (2009) Using earthworm avoidance behaviour to assess the toxcity 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 JM, Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244(22):6049–6055

Mesnage R, Antoniou MN (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 GE (2014) Major pesticides are more toxic to human cells than their declared active principles. Biomed Res Int 2014:179691. https://doi.org/10.1155/2014/179691

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

Moolman L, Van Vuren JH, 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

Nguyen DB, Rose MT, Rose TJ, Morris SG, Van Zwieten L (2016) Impact of glyphosate on soil microbial biomass and respiration: a meta-analysis. Soil Biol Biochem 92:50e57. https://doi.org/10.1016/j.soilbio.2015.09.014

Novais SC, Amorim MJB (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 SC, Soares AMVM, de Coen W, Amorim MJB (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

Novais SC, Gomes SIL, Gravato C, Guilhermino L, de Coen W, Soares AMVM, Amorim MJB (2011) Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ Pollut 159:1836–1843

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

Pelosi C, Barot S, Capowiez Y, Hedde M, Vandenbulcke F (2014) Pesticides and earthworms. A review. Agron Sustain Dev 34:199–228

Pereira JL, Antunes SC, Castro BB, Marques CR, Gonçalves AMM, 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

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLOs ONE 598(10):e0146021

Rodrigues ET, Pardal MA, Laize V, Cancela ML, Oliveira PJ, Serafim TL (2015) Cardiomyocyte H9c2 cells present a valuable alternative to fish lethal testing for azoxystrobin. Environ Pollut 206:619e626

Rodríguez-Castellanos L, Sanchez-Hernandez JC (2007) Earthworm biomarkers of pesticide contamination: Current status and perspectives. J Pest Sci 32(4):360–371. https://doi.org/10.1584/jpestics.R07-14

Shao Y, Hou K, Du Z, Li B, Wang J, Juhasz AL, Zhu L (2019) Evaluation of the toxicity of 1-butyl-3-methyl imidazolium tetrafluoroborate using earthworms (*Eisenia fetida*) in two soils. Sci Total Environ 686:946e958

Smolders R, De Boeck G, Blust R (2003) Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). Environ Toxicol Chem 22(4):890–899. https://doi.org/10.1002/etc.5620220429

Sokolova IM, Frederich M, Bagwe R, Lannig G, Sukhotin AA (2012) Energy homeostasis as an integrative tool for assessing the limits of environmental stress tolerance in aquatic invertebrates. Environ Res 79:1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Świątek ZM, Bednarska AJ (2019) Energy reserves and respiration rate in the earthworm Eisenia andrei after exposure to zinc in nanoparticle or ionic form. Environ Sci Pollut Res 26:24933–24945. https://doi.org/10.1007/s11356-019-05753-3

Topoliantz S, Ponge JF, Viaux P (2000) Earthworm and enchytraeid activity underdifferentarable farming systems, as exemplified by biogenic structures. Plant Soil 225:39–51

Velki M, Weltmeyer A, Seiler TB, Hollert H (2018) Acute toxicities and effects on multixenobiotic resistance activity of eight pesticides to the earthworm *Eisenia andrei*. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-018-3959-x

Wang C, Wu J, Zhang Y, Wang K, Zhang H (2015) Field dissipation of trifloxystrobin and its metabolite trifloxystrobin acid in soil and apples. Environ Monit Assess 187:4100. https://doi.org/10.1007/s10661-014-4100-3

Wang Y, Qin Y, Bing Y, Martyniuk CJ (2021) A comprehensive review of strobilurin fungicide toxicity in aquatic species: Emphasis on mode of action from the zebrafish model. Environ Poll 275116671-S0269749121002499:116671. https://doi.org/10.1016/j.envpol.2021.116671

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 SC, Zhu L, Li W, Zhu L, Wang J, Wang J (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, Houang T, Li R, Souders CL, Rheingold S, Tischuk C, Zhou B, Martyniuk CJ (2021) Evaluation and comparison of the mitochondrial and developmental toxicity of three strobilurins in zebrafish embryo/larvae. Environ Pollut 270:116277. https://doi.org/10.1016/j.envpol.2020.116277

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

Zhao L, Li Y, Ren W, Huang Y, Wang X, Fu Z, Ma W, Teng Y, Luo Y (2020) Pesticide residues in soils planted with *Panax notoginseng* in south China, and their relationships in *Panax notoginseng* and soil. Ecotoxicol Environ Saf. 201. https://doi.org/10.1016/j.ecoenv.2020.110783