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Effect of combined fungicide treatments on fatty acid content in *Eisenia fetida* earthworms

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Abstract

Aim of study: To study the toxic effects of a combined fungicide in *Eisenia fetida* earthworms and in its lipid fatty acid composition.

Material and methods: We investigated the acute toxicity (LC_{50}, 14 days) of a combined fungicide (active ingredients: carbendazim + cyproconazole) in *Eisenia fetida* using artificial substrate. The quantitative content of fatty acids (FAs) of body lipids was determined by the method of high-sensitivity gas chromatography.

Main results: Moderate toxicity of the combined fungicide was determined for *Eisenia fetida* earthworms. Changes in behavioral response, biomass loss, and mortality of test objects were detected. A decrease in the content of saturated FAs was found. At the same time, an increase of long-chain polyunsaturated FAs content of ω6 and ω3 families, which are involved in the regulation of a wide range of physiological processes, was revealed. Modulation of the lipid FAs profile in *Eisenia fetida* worms is explained by the participation of the FAs in the restructuring of the organism reactivity system under fungicidal load.

Research highlights: The reorganization of the FA profile can be considered as an early criterion of metabolic perturbations in earthworms under the action of fungicides. Understanding the toxic potential of fungicides to organisms in soil ecosystems is essential for practical risk assessment in response to pesticide application.

Additional keywords: pesticides; toxicity; soil biota.

Abbreviations used: a.i. **(**active ingredients); BAF (bioaccumulation factor); FA (fatty acid); MUFA (monounsaturated fatty acid); PUFA (polyunsaturated fatty acid); SFA (saturated fatty acid); UFA (unsaturated fatty acid).

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Introduction

Widespread use of pesticides in agriculture creates an increasing threat of soil contamination by toxic substances and can lead to irreversible damage to the structure and function of the soil ecosystem (Chagnon *et al.,* 2015). Soil organisms living on agricultural land, pastures and meadows can be exposed to pesticides. In particular, it concerns earthworms, which play a crucial role in soil-forming processes, for which soil is an environment for existence, growth and development (Katagi & Ose, 2015).

Earthworms are sensitive indicators of changes in the ecological status of the habitat considering their environmental importance, high soil biomass and sensitivity to relatively low concentrations of pollutants (Dureja & Tanwar, 2012). It is known that insecticides and fungicides exert a toxic effect on earthworms (Katagi & Ose, 2015). It should be considered that modern commercial pesticide formulations contain several active ingredients, so earthworms can be subjected to the combined action of pesticides, the cocktail effect. Numerous studies have been conducted on the toxic effects of pesticides on non-target organisms, but it is difficult to predict the cumulative effects of different pesticides through complex synergistic and antagonistic responses (Wang *et al.,* 2012; Yang *et al.,* 2017). Earthworms, whose physiology is well understood, are well suited for the study of pesticide toxicity considering pesticide exposure through the skin or soil swallowing. Standardized worm toxicity test protocols are also available.

An important indicator that can characterize the influence of exogenous factors on the body is the content of lipids and their fatty acids, which is dynamically associated with the functional state – the course of physiological and biochemical processes in the body of animals. Understanding the effects of complex mixtures of pesticides on biota remains a major challenge. Methodological approaches were consistent with those previously used (Khyzhnyak *et al.,* 2020).

The aim of the current research was to study the acute toxicity of the combined fungicide (carbendazim + cyproconazole) to *Eisenia fetida* earthworms and its effect on lipid fatty acid composition of these organisms.

Material and methods

In our research we studied a combined fungicide with commercial name "Carbenasol" used on crops in Ukraine, the stock solutions of which contain the active ingredients (a.i.): carbendazim, 300 g/dm^3 + cyproconazole, 66 g/dm^3 in the formulation of a suspension concentrate with a maximum agronomic dose of 1.0 dm^3/ha.

In acute test conditions fungicide toxicity was determined by biotesting using an artificial substrate (soil) according to OECD (1984). The test object was a culture of sexually mature adult *E. fetida* earthworms (Savigny, 1826) weighing between 300 and 500 mg each. The artificial substrate had the following composition (content by dry weight): 10% finely ground sphagnum peat, 20% kaolin clay and 70% quartz sand. To determine the half-lethal concentration (LC_{50}) a fungicide solution was added in amounts of 1, 10, 100, 200, 400, 600, 800, 1000 mg/kg artificial substrate. The study was conducted under standard conditions according to OECD (1984). The percentage of mortality, changes in weight and behavioral responses of test objects were determined. The LC_{50} of the fungicide was determined by probit-analysis (LC_{50}, 14 days).

The identification and determination of the active ingredient content in the bioobjects and artificial soil samples were carried out after extraction with organic solvent, extract purification and subsequent detection by gas chromatography using an Agilent Technologies 7900-MSD 5975C chromatographic mass spectrometer with HP-5 MS 15 m × 0.25 mm ID × 0.25 µm column according to EVS-EN 15662:2008.

The accumulation of fungicide in worms was assessed by the predominant active ingredient in the formulation, carbendazim.

To characterize the fungicide accumulation by worms the bioaccumulation factor (BAF) (Gobas & Morrison, 2000) was determined as an indicator of chemical exchange between the environment and the organism, and calculated as ratio of the carbendazim amount in the earthworm organism (C_w, mg/kg dry weight) to its content in the soil (C_s, mg/kg substrate).

The fatty acids (FAs) content of earthworm lipids was determined after homogenization and subsequent lipid extraction using a chloroform-methanol mixture. Lipid hydrolysis and FAs methylation were performed according to ISO 12966-2:2017. FAs methyl esters were analyzed on a Trace GC Ultra gas chromatograph (USA) using a flame ionization detector. Separation was performed on a high-polar capillary chromatographic column SPTM-2560 (Supelco, USA). For the quantitative assessment of individual FAs the method of internal normalization was used and the relative content of FAs was represented as a percentage of their total amount. The FAs composition in *E. fetida* was determined in groups of test objects treated with fungicide in the amount of 200 (Experiment 1) and 400 (Experiment 2) mg/kg substrate. The following combinations of the FAs were calculated: ω3 FAs, ω6 FAs, total saturated fatty acids (SFAs), total unsaturated fatty acids (UFAs), total monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), SFAs/UFAs ratio and ω3/ω6 ratio.

The research data were processed using Origin 6.0 and Excel (Microsoft, USA) computer software using Student's t-test. Differences were considered significant when $p < 0.05$.

Results and discussion

With the increase in the amount of carbendazim in the artificial substrate, its accumulation in the body of worms inhabiting this substrate also increased (Table 1). The calculated BAF value indicated a low level of carbendazim accumulation in the worms (Table 1). This is consistent with other studies that indicate a low bioaccumulation of carbendazim and cyproconazole (Katagi & Ose, 2015). However, to understand the toxic potential of a fungicide, it is necessary to consider not only its accumulation in the body, but also further involvement in metabolism (Ye *et al.,* 2016).

In acute toxicity experiments (LC_{50}, 14 days) of fungicide (a.i.: carbendazim, 300 g/dm^3 + cyproconazole, 66 g/dm^3) the behavioral response and total biomass of *E. fetida* worms were characterized, depending on the amount of agent in the substrate. During the experiment no behavioral changes were observed compared to control at fungicide amount of 100 mg/kg substrate after 14 days of exposure. The worms were mobile and responded equally to light and mechanical irritation. At 200 mg/kg substrate or above, reduced mobility and reduced response to light and mechanical irritation of living worms were detected compared to control at the end of the experimental period (14 days).

The half-lethal concentration (LC_{50}, 14 day period) of the tested fungicide for *E. fetida* was estimated to be 250 mg/kg. According to the IUPAC classification the studied fungicides exhibit moderate toxicity (Lewis *et al.,* 2016). Our results indicate a moderate toxicity of the combined fungicide (carbendazim + cyproconazole) for *E. fetida* earthworms, characterized by changes in behavioral response, biomass loss and mortality of test organisms. Nevertheless, carbendazim is known to be highly toxic (LC_{50} = 5.4 mg/kg) and cyproconazole is moderately toxic (LC_{50} = 165 mg/kg) to *E. fetida* (Lewis *et al.,* 2016).

The toxic effect of the fungicide associated with the processes of absorption and metabolism of substances in earthworms have been evaluated in the study of FA profile of *E. fetida* lipids. The results are shown in Table 2. The 18 FAs of *E. fetida* lipids were identified and quantified in the control and experimental groups. SFAs are predominantly represented by palmitic (C16:0) and stearic (C18:0) acids. The pool of UFAs is represented by MUFAs, in particular oleic (C18:1ω9) and polyenoic acids like linoleic (C18:2ω6), linolenic (C18:3ω3) and arachidonic (C20:4ω6) acids. Under these experimental conditions short-chain SFAs were not found in the organism of *E. fetida* worms. Besides, the proportion of docosahexaenoic (C22:6ω3) acid in *E. fetida* was lower than 1% of the FAs. The observed FA composition of *E. fetida* has also been confirmed by Gunya *et al.* (2016).

As shown in Table 2 (Experiments 1 and 2), the effect of the fungicide leads to a decrease in the total content of SFAs by 24% and 44% in comparison with control ($p < 0.05$), respectively. The decrease in the content of such abundant FAs as palmitic (C16:0) by 15% and 32%, and stearic (C18:0) acids by 23% and 51%, respectively, may be of particular importance and indicate a lower level of energy supply and structural reserve in organisms. In this case, the saturation rate (SFAs/UFAs) in Experiments 1 and 2 was 0.47 and 0.31, respectively, compared to 0.72 in the control. Under these conditions the content of MUFAs did not change significantly and the content of PUFAs increased by 25% and 43% compared to the control value. Among long-chain FAs the arachidonic (C20:4ω6) acid predominated and its content increased by 35% and 57% relative to the control.

Accumulation of arachidonic acid in the worm body, which is involved in the regulation of synthesis of substances of lipid nature in a wide range of physiological processes, can lead to an increase in the content of prostaglandins in cells (Canbay *et al.,* 2007). As well, changes in docosahexaenoic acid content (C22:6ω3), which is capable to attenuate cyclooxygenase, are considered an adaptive mechanism, but its content was relatively low both in the control and experimental conditions. As to fatty acids of the ω3 family in general, the increase of their total content may correspond to the energy requirements of the organism in extreme conditions.

Table 1. Indicators of carbendazim accumulation in *Eisenia fetida* (in terms of dry weight) after 14 days of exposure.

Comb [1] (mg/kg)	C_s [2] (mg/kg)	C_w [3] (mg/kg)	BAF [4] C_w/C_s
200	53.0±0.5	11.33±0.45	0.21
400	106.0±0.6	18.41±0.51	0.17
600	159.0±0.8	25.45±0.66	0.16

[1] Comb: content of the combined fungicide. [2] C_s: content of carbendazim in the substrate. [3] C_w: content of carbendazim in the body. [4] BAF: bioaccumulation factor of carbendazim.

It is significant that the content of ω6 prevailed over ω3 FAs family in earthworms, consistent with research data (Grdisa *et al.,* 2013). It is known that cell membranes enriched with ω6 FAs are more resistant to exogenous factors (Simopoulos, 2003). Among PUFAs the families ω3 and ω6 are precursors of biologically active substances synthesized in many animal tissues in response to exogenous influences. As follows from Table 2, under the action of the combined fungicide the total content of ω3 and ω6 FAs increases in *E. fetida*.

An important indicator characterizing the intensity of lipid metabolism is the ratio of palmitic to oleic acid (C16:0/C18:1ω9). According to Table 2 (Experiments 1 and 2), this value was reduced by 17% and 40% *vs* control, respectively. The obtained results indicate the inhibition of the FAs metabolism of the worm lipids in the dynamics of pesticide exposure. It is important to emphasize the presence of dose-dependent quantitative changes of lipid FAs under the influence of combined fungicide on earthworms *E. fetida* (Table 2). The results show that the metabolism of lipid FAs in earthworms is sensitive to the studied combined fungicide.

Thus, in the laboratory experiments (during 14 days of exposure) moderate toxicity of the combined fungicide for *E. fetida* with LC_{50} = 250 mg/kg of substrate was found, accompanied by changes in behavioral response and loss of worm biomass. Demonstration of chemical avoidance response and loss of biomass in the presence of carbendazim in soil has also been reported for earthworms (Rico *et al.,* 2016). In tests on direct application (using natural soil, pH 6.6) the avoidance response of worms to carbendazim (EC_{50}) has been detected in a wide range, from 7.1 to 127.4 mg/kg for 48 h (Lewis *et al.,* 2016).

The study of the biochemical response of *E. fetida* to the toxic effect of the combined fungicide was based on the determination of the FAs profile of earthworm lipids. It is known that FAs, as important structural and energy components of cells, play a substantial role not only in

Table 2. Fatty acid content (%) in *Eisenia fetida* after 14 days of exposure to the combined fungicide.

Fatty acids, %	Control group	Experiment 1	Experiment 2
C14:0	3.25 ± 0.08	1.22 ± 0.06*	1.19 ± 0.05*
C15:0	1.11 ± 0.04	0.67 ± 0.01*	0.64 ± 0.02*
C16:0	15.80 ± 0.27	13.42 ± 0.24*	10.78 ± 0.16*,**
C16:1	0.08 ± 0.01	0.07 ± 0.01	0.05 ± 0.01*,**
C17:0	0.48 ± 0.03	0.40 ± 0.03	0.42 ± 0.03
C18:0	19.2 ± 0.12	14.71 ± 0.37*	9.50 ± 0.26*,**
C18:1ω9	19.53 ± 0.30	20.01 ± 0.39	22.22 ± 0.34*
C18:2ω6	20.50 ± 0.41	23.5 ± 0.44*	25.28 ± 0.22*
C20:0	0.21 ± 0.02	0.15 ± 0.01*	0.12 ± 0.01*,**
C18:3ω3	0.29 ± 0.03	0.66 ± 0.05*	0.69 ± 0.09*
C20:1ω9	2.43 ± 0.08	1.58 ± 0.05*	1.12 ± 0.04*,**
C21:0	1.26 ± 0.04	0.79 ± 0.04*	0.55 ± 0.03*,**
C20:2ω6	2.07 ± 0.09	2.37 ± 0.05	3.23 ± 0.06*,**
C22:0	0.69 ± 0.03	0.46 ± 0.02*	0.35 ± 0.02*
C20:4ω6	11.45 ± 0.22	15.41 ± 0.22*	18.03 ± 0.21*
C22:2ω6	1.07 ± 0.05	2.32 ± 0.10*	3.31 ± 0.11*,**
C20:5ω3	0.46 ± 0.02	0.89 ± 0.10*	1.31 ± 0.06*,**
C22:6ω3	0.66 ± 0.02	1.37 ± 0.04*	1.21 ± 0.05*,**
Σ SFAs	42.0 ± 0.62	31.82 ± 0.52*	23.55 ± 0.42*,**
Σ UFAs	58.0 ± 0.52	68.18 ± 0.54*	76.45 ± 0.62*
Σ MUFAs	19.86 ± 0.20	21.66 ± 0.21	23.39 ± 0.22
Σ PUFAs	37.21 ± 0.21	46.52 ± 0.32*	53.06 ± 0.33*
Σ ω3	1.41 ± 0.02	2.92 ± 0.06*	3.21 ± 0.05*
Σ ω6	35.09 ± 0.19	43.61 ± 0.21*	49.85 ± 0.21*
SFAs/UFAs	0.72	0.47	0.31
C16:0/C18:1ω9	0.81	0.67	0.49

[Data are presented as mass fraction of fatty acid in % of total fatty acids. Experiment 1: fungicide amount of 200 mg/kg artificial substrate. Experiment 2: fungicide amount of 400 mg/kg artificial substrate. SFAs: saturated fatty acids. UFAs: unsaturated fatty acids. MUFAs: monounsaturated fatty acids. PUFAs: polyunsaturated fatty acids. *: $p < 0.05$ *vs* control, **: $p < 0.05$ *vs* Experiment 1.

the processes of metabolism, but also mediate protective responses of the organism under the action of exogenous factors (Wang *et al.,* 2012).

We detected dose-dependent changes in the content of FAs in the body: a decrease in the content of SFAs and an increase in the content of UFAs, which lead to a decrease in the saturation rate. A decrease in the intensity of lipid metabolism in the worms occurred under fungicide action. Despite the moderate toxicity and low level of accumulation, the combined fungicide affected the FAs profile of earthworm lipids, which was accompanied by a decrease in the content of lipid SFAs (especially palmitic and stearic acids) and an increase of the PUFAs content of the ω3 and ω6 families, indicating the pathways of involvement of FAs in the regulation of vital functions of *E. fetida* under fungicide exposure. Modulation of the FAs profile of biological objects can be considered as a sensitive biomarker of the toxic potential of pesticides, which is essential at their combined use. Other studies have shown that the effect of pesticides (including carbendazim) on *E. fetida* is manifested in changes in the enzymatic activity of cholinesterase, lactate dehydrogenase and alkaline phosphatase and causes histopathological disorders after exposure for 14 days (Rico *et al.,* 2016).

In summary, our findings indicate the involvement of fatty acids of *E. fetida* lipids in the early reactions to combined fungicide exposure in doses close to its half-lethal concentration. The obtained results indicate the importance of studying the biochemical pathways of the non-target organism response to the combined pesticides, especially for the practical risk assessment.

Authors' contributions

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Supervision: S. V. Khyzhnyak.
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