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Effect of single and mixture of insecticides on earthworms: results from field and laboratory experiments

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Abstract

The aim of this study was to examine the effect of insecticides Talstar 10 EC (pyrethroid bifenthrin) and Nurelle D (mixture of organophosphorus and pyrethroid: chlorpyrifos and cypermethrin) on the life-cycle parameters (survival, growth, cocoons production, and hatching juveniles) of the non-target organism, earthworm *Eisenia fetida* (Savigny, 1826), in the laboratory and their impact on the natural earthworm population in agricultural field. The experiment was conducted from 2017 to 2019. The field experiment was performed at the Rimski Šančevi site, near Novi Sad, Serbia. The laboratory experiment was conducted according to the OECD guidelines. The earthworms were exposed to the artificial soil supplemented with different concentrations of the tested insecticides according to the recommended agricultural doses (RAD). The mixture of pesticides (Nurelle D) was found ecologically more dangerous to *E. fetida* than Talstar 10 EC. The 50% lethal concentration (LC_{50}) values of both insecticides were higher than the highest concentration used in the experiment. Talstar 10 EC did not affect weight, but affected the production of cocoons and hatching juveniles, even at the RAD. The mixture of pesticides has shown toxicity after a long-time exposure, and it had an impact on the parameters of the life cycle. The results of the field experiment demonstrated that mixtures of pesticides are more toxic than single-component ones. This points out that the chemicals must be used with the maximum responsibility. Information about long-term effects can be important for understanding the field experiment data, which typically show a high variability and, therefore, are difficult to interpret.

Keywords: bifenthrin, chlorpyrifos, cypermethrin, agricultural field, rapeseed.

Introduction

In recent years, the matter of renewable energy has become more important, and the production of biodiesel from rapeseed (*Brassica napus* L.) oil has an increasing importance in it. Rapeseed is among the four most important oil crops. It can be used as an alternative plant to sunflower to increase oil production. An increase in productivity is of major importance for the further enlargement of the field area cultivated by rapeseed (Marjanović-Jeromela et al., 2014).

Analysing the structure of rapeseed plant, the number of plants per hectare, the vegetation period, standard agriculture measures, the absence between row ploughing and minimised other agrotechnical practices, it can be concluded that earthworms are very positive in rapeseed crops. With the increase in the area of rapeseed, the protection of this crop from pests has become more demanding, which usually means more pesticide usage. The primary purpose of insecticide use is the control

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of insects with less or even without attention to other organisms that may be affected. Nevertheless, a large proportion of pesticides used in agriculture ends up as residues in the soil making soil-dwelling organisms more vulnerable to pesticide intoxication (Sekulić et al., 2022).

A widespread and abundant group of invertebrates with one of the most important functions and roles in terrestrial ecosystems are earthworms of the Lumbricidae family (Blouin et al., 2013; Skuodienė et al., 2019). Earthworms could bioaccumulate pesticides, heavy metals, and other chemicals, because unlike many organisms, have a thin cuticle (Schnug et al., 2015). Due to their influence in the soil, ecological importance, sensitivity, and a high biomass per unit area, they are used as test organisms in ecotoxicological studies, especially *Eisenia fetida* (Savigny, 1826). Therefore, this species has been recognised by international and national agencies to diagnose soil ecosystem health (Milanović et al., 2014).

Due to the extensive use of pesticides, pests have become resistant, and mixtures of pesticides are increasingly used (Akhtar, 2020). A mixture of the pesticides can usually produce significant synergistic toxicity effects on target species compared to a single pesticide (Schnug et al., 2013), but also to non-target organisms, mainly due to their physiological similarities (Milanović et al., 2014). However, little is known about the performance of pesticide mixtures (Schnug et al., 2013), especially if the mixtures are known to be often used under agricultural production conditions.

Synthetic pyrethroids are analogues of pyrethrins, which can be found in the plants of *Chrysanthemum cinerariaefolium* (Aebeed, Amer, 2018). Pyrethroids are known by the knock-down effect and expressed initial efficacy. Organophosphorus insecticides do not show such rapid activity, but they are very persistent, which is why they are banned in most countries. Organophosphorus and pyrethroids have become the most prevalent insecticides today (López-Dávila et al., 2020) used in agriculture, veterinary, and domestic applications.

Field studies are relatively expensive, and the results are often affected by natural variations and the influence of changing environmental conditions. It is practically impossible to test all chemicals in field conditions, therefore, laboratory experiments are needed to understand the potential risk of chemicals to ecosystems (Velki et al., 2014). In our opinion, this is the reason why little attention is paid to linking such studies and why this topic is not widely spread among the scientific community. Having this in mind, the action of one typical pyrethroid (a.i. bifenthrin) and the mixture of organophosphorus and pyrethroid (a.i. chlorpyrifos and cypermethrin) was investigated. At the same time, these two types of insecticides were commonly used to control pollen beetles (*Brassicogethes aeneus* F. 1775) and some other rapeseed pests (Milovanović et al., 2019).

Thus, the aims of the study were (i) to evaluate the influence of insecticides on the population of earthworms in real time conditions, (ii) to assess the toxicity of these insecticides under laboratory conditions monitored with traditional parameters such as mortality, weight, and reproduction, and (iii) to compare the results obtained in field and laboratory conditions.

Material and methods

Laboratory design. Tested chemicals. The insecticides being tested in the experiment were insecticides belonging to different chemical groups, the characteristics of which are shown in Table 1. In the laboratory experiment, different concentrations of the insecticides based on their recommended agricultural doses (RAD) were used: ¼RAD, ½RAD, 1RAD, 2RAD, and 4RAD. The amount of insecticide required was determined by the total area of the experimental box (100 cm^2). For the control treatment, distilled water was used. Each concentration and the control were tested in four replicates.

Table 1. Characteristics of applied insecticides with used concentrations (mg kg^{-1})

<table>
<tr><th>Insecticide</th><th>Active ingredient (a.i.)</th><th>Amount L ha^{-1}</th><th>Chemical group</th><th>¼RAD</th><th>½RAD</th><th>1RAD</th><th>2RAD</th><th>4RAD</th></tr>
<tr><td>Nurelle D</td><td>chlorpyrifos and cypermethrin</td><td>1</td><td>organophosphorus and pyrethroid</td><td rowspan="2">0.125</td><td rowspan="2">0.25</td><td rowspan="2">0.5</td><td rowspan="2">1</td><td rowspan="2">2</td></tr>
<tr><td>Talstar 10 EC</td><td>bifenthrin</td><td>0.2</td><td>pyrethroid</td></tr>
</table>

RAD – recommended agricultural dose

Tested organisms. The earthworms of the species *Eisenia fetida* (Savigny, 1826) were taken from the registered farm in Dimitrovgrad, Serbia and cultured in the laboratory in a medium according to the OECD (2016) guidelines. The earthworms selected for the experiment were acclimatised in the soil under the test conditions for at least 24 h before use. The earthworms used in the experiment were adults with well-developed clitella and weighed between 300 and 400 mg.

Artificial tested soil was prepared according to OECD (2016) guidelines. The soil consisted of 70% quartz sand, 20% kaolin clay, and 10% sphagnum peat and calcium carbonate with a pH of 6.0 ± 0.5. The dry components of the artificial soil were thoroughly mixed before adding distilled water to achieve the desired moisture content of approximately 35% dry weight. The experimental soil was then prepared by adding different concentrations of pesticides (on a dry weight basis).

Test performance. The earthworms were washed, dried on filter paper, weighed, and then ten earthworms were placed in a plastic container (10.5 × 9.6 × 7 cm) on the soil surface. The container was covered with perforated plastic lid and kept at a temperature of 20 ± 2°C. The experiment was carried out under light-dark cycles (16:8 h). The earthworms were fed 5 g of ground cattle manure. The soil moisture was tested once a week. The mortality and weight of earthworms were monitored weekly. They were considered dead when they did not respond to a gentle mechanical prodding on the anterior part of the body. Before weighing, all earthworms were sorted, washed with tap water and blotted with filter paper. Then the earthworms were weighed using an electric scale and then returned to the soil.

Field design. Experimental site and insecticide application. The field experiment was conducted at the Rimski Šančevi site (45°19′ N, 19°50′ E) near Novi Sad, Serbia from 2017 to 2019. The main plot size was 24 m^2, with four replications in a randomised block design. Winter rapeseed (*Brassica napus* L.) cultivar 'Banaćanka' registered at the Institute of Field and Vegetable Crops, Novi Sad, Serbia was used. The soil of the experimental plot was chernozem (WRB, 2022). Data of soil analysis and previous crops are given in Table 2. Meteorological conditions before soil sampling had no significant effect on the presence of earthworms.

In the experimental field, pests were controlled with insecticides. Nurelle D is a non-systemic insecticide consisting of two active ingredients: chlorpyrifos (organophosphate group, cholinesterase inhibitor) and cypermethrin (pyrethroid group, which disrupts the flow of Na^+ through the nerve membrane). It has the contact and inhalation effect. Talstar 10 EC (a.i. bifenthrin) is a contact and digestive insecticide from the group of pyrethroids. This compound acts on the central and peripheral nervous system of insects thus causing disturbances in the flow of nerve impulses and paralysis.

Table 2. Soil analysis data and meteorological conditions during the experiment (daily average temperatures and precipitations are presented for eight days before soil sampling)

Soil properties									
Year	Depth cm	pH in KCl	pH in H_2O	$CaCo_3$ %	humus %	total N %	Al-P_2O_5 mg 100 g^{-1}	Al-K_2O mg 100 g^{-1}	Previous crop
2017	0–30	7.58	8.45	8.34	2.37	0.176	32.5	26.4	winter forage pea
	30–60	7.65	8.56	14.59	1.85	0.159	16.6	16.4	
2018	0–30	7.20	8.10	0.84	2.70	0.201	29.6	26.8	pea
	30–60	7.34	8.22	4.64	2.66	0.197	15.3	23.2	
2019	0–30	7.24	8.00	2.46	2.75	0.20	30.2	32.3	winter triticale
	30–60	–	–	–	–	–	–	–	

Meteorological conditions									
Days before soil sampling		8	7	6	5	4	3	2	1
Spring 2017	temperature	18.9	17.4	7.7	10.7	15.2	18	18.1	16.4
	precipitation	–	–	3	2.5	7.5	–	–	–
Autumn 2017	temperature	10.3	11.3	9.6	10.5	6.6	4.3	7.1	9.9
	precipitation	–	–	3.7	–	2.7	–	–	–
Spring 2018	temperature	18.1	17.6	16.7	20.4	17	18.4	19.5	16.3
	precipitation	–	–	–	–	0.9	–	–	–
Autumn 2018	temperature	13.3	17.5	18.2	17.7	14.8	14	12.6	13
	precipitation	–	–	–	–	–	–	–	–
Spring 2019	temperature	8.5	6.6	2.5	2.4	3.8	5.2	6.6	6.5
	precipitation	28.5	8.4	5	3.1	0.7	0.2	–	–
Autumn 2019	temperature	14.4	12.3	14.7	12.4	11.3	3.8	1.9	2.5
	precipitation	–	–	–	–	–	–	8	0.2

The soil was ploughed every year. Cultivation technology was applied according to the local agricultural practices. The use of insecticides and the set-up of the whole experiment were carried out according to the EPPO (2004) standards. Foliar application was done with a backpack sprayer at registered doses of insecticides. The amount of water used was 350 L ha^{-1}.

Earthworm sampling. The presence of earthworms was assessed twice a year, once in spring and once in autumn. Assessments were made two to three weeks after the foliar application of insecticides and usually seven to ten days after rain. The presence of precipitation is very important, because it is expected that some of the insecticides will fall from the surface of the plant into the soil in the earthworm-inhabited zone. Soil samples were collected first by removing rapeseed plants from the experimental area and then by digging soil with a quadric frame with a side length of 40 cm and a depth of 30 cm. The field experiment was conducted and soil invertebrates were sampled according to the ISO 23611:2018 (Soil quality – Sampling of soil invertebrates – Part 1: Hand-sorting and formalin extraction of earthworms). Earthworm samples were hand-sorted and fixed in 90% ethanol. The development stage of each earthworm was determined. Adults were identified according to the identification keys of Mršić (1991).

Calculated indices. Form the obtained data, the following calculations were made: the number of species (S), the Shannon-Weaver diversity index (H), and the Pielou evenness index (H') (Rota et al., 2014). The Shannon-Weaver index (H) was calculated according to the formula:

$$H = \frac{N}{S} \ln \frac{N}{S},$$

where N is the total number of individuals of one species, and S is the total number of individuals of all species.

The Pielou evenness index (H') was calculated according to the formula:

$$H' = H / \ln(S),$$

where H is the Shannon-Weaver diversity index, and S is the total number of individuals of all species.

Statistical analysis. The concentration that is lethal to 50% of individuals (LC_{50}) at the 95% confidence interval was calculated with the program CalcuSyn, version 2.0 (Biosoft, UK). Statistical analysis was performed using the software SPSS, version 16.0 for Windows. The Shapiro-Wilk test was used to ensure the assumption of normality. The one-way analysis of variance (ANOVA) ($p < 0.05$) and Kruskal-Wallis test (H-test) were used to assess the effect of contaminants on growth. After the Post hoc analysis, in comparison of means (growth), the Dunnett's test was applied. Data are presented as the mean ± standard deviation (SD).

Results

Results of the laboratory experiment. Each of the insecticides used in laboratory conditions has shown a different degree of toxicity to the *E. fetida* earthworms. In the control treatment, 100% survival of earthworms was recorded at the end of the experiment. Nurelle D did not show a significant reduction in the number of earthworms, so after the 3rd week of exposure, no mortality was recorded at any of the concentrations. After the 7-day exposure, mortality from Talstar 10 EC was recorded at any concentration, and the situation did not change significantly until the end of the experiment. After 28 days of exposure, LC_{50} values for Nurelle D and Talstar 10 EC were 4.88 and 8.83 mg kg^{-1}, respectively. The values are higher than the highest concentration, which was used in the experiment.

The results of earthworm weight are shown in Table 3. It was observed that the earthworms gained slightly weight in the control treatment. In the case of Talstar 10 EC, the weight ranged from 375 to 283 mg.

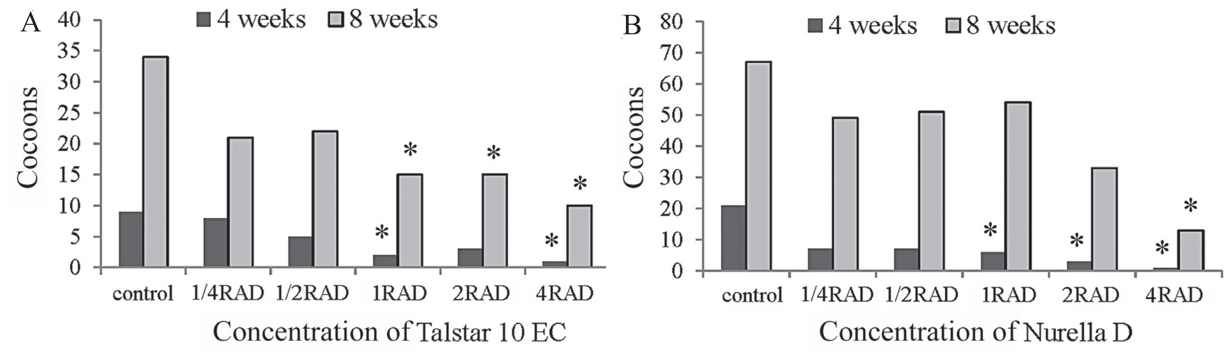
Table 3. Eight-week growth of the *Eisenia fetida* earthworms exposed to insecticides

Insecticides	Average weight per earthworm mg				
	0 week	2 weeks	4 weeks	6 weeks	8 weeks
Nurelle D					
Control (H_2O)	395 ± 21	423 ± 21	423 ± 22	363 ± 33	325 ± 17
¼RAD	358 ± 52	375 ± 42	383 ± 39	335 ± 45	268 ± 21
½RAD	393 ± 32	383 ± 33	388 ± 17	365 ± 21	313 ± 36
1RAD	408 ± 54	398 ± 17	413 ± 22	368 ± 28	285 ± 45
2RAD	378 ± 38	330 ± 12*	333 ± 19*	305 ± 24*	265 ± 19*
4RAD	370 ± 63	373 ± 33*	333 ± 43*	313 ± 40*	268 ± 17*
Talstar 10 EC					
Control (H_2O)	330 ± 32	330 ± 35	300 ± 24	295 ± 17	305 ± 21
¼RAD	320 ± 14	333 ± 17	315 ± 13	293 ± 22	298 ± 15
½RAD	343 ± 29	325 ± 65	293 ± 39	315 ± 24	288 ± 51
1RAD	320 ± 29	325 ± 13	300 ± 8	303 ± 17	283 ± 38
2RAD	308 ± 25	325 ± 21	298 ± 22	293 ± 13	305 ± 38
4RAD	375 ± 64	333 ± 22	310 ± 26	308 ± 40*	310 ± 28*

Note. RAD – recommended agricultural dose; * – significant differences ($p < 0.05$) between the insecticide and control treatments are indicated for each week.

The only significance ($p < 0.05$) was found at the highest concentration at 6- and 8-week assessments. The earthworms treated with Nurelle D ¼RAD gained slightly weight in four weeks. Following the Nurelle D application, the mean biomass ranged from 398 to 330 mg in the 2nd week of assessment. The statistical analysis showed a significant difference between 2RAD and 4RAD concentrations. The earthworms treated with Nurelle D had the average biomass between 413 and 333 mg after the 4-week assessment and between 368 and 305 mg after the 6-week assessment. The effect was significant ($p < 0.05$) for 2RAD and 4RAD compared with the control. After 8 weeks, there was also a significant difference ($p < 0.05$) between these doses and the control with the mean biomass between 313 and 265 mg.

After the application of Talstar 10 EC, cocoon production was significantly lower ($p < 0.05$) than the control at the 1RAD and 4RAD after 4 weeks and 1RAD, 2RAD, and 4RAD after 8 weeks (Figure 1A). It was found that the number of cocoons decreased with increasing the concentration and time. In addition, Nurelle D showed a significant difference ($p < 0.05$) of cocoon production between the control and 1RAD, 2RAD, and 4RAD after 4 weeks and 4RAD after 8 weeks (Figure 1B).



* – significant difference

Figure 1. Cocoon production of the *Eisenia fetida* earthworms after exposure to different concentrations of Talstar 10 EC (A) and Nurelle D (B)

As for the hatching juveniles, Nurelle D showed a greater effect compared to Talstar 10 EC. Significant differences were found between the control and 1RAD, 2RAD, and 4RAD for Nurelle D and between the control and 1RAD and 2RAD for Talstar 10 EC.

Results of the field experiment. The total number of earthworms collected in all samples was 895, of which 44.58% were adults and 55.42% were juveniles. All the adult earthworms belonged to four different species of the family Lumbricidae: *Allolobophora chlorotica* (Savigny, 1826), *Aporrectodea trapezoides* (Duges, 1828), *Aporrectodea rosea* (Savigny, 1826), and *E. fetida*. According to ecological forms, all species were endogeic, except *E. fetida*, which was epigeic. *E. fetida* appeared only in the field treated with Talstar 10 EC in spring 2019. In autumn 2019, *Ap. trapezoides* did not occur at all, while in the spring of that year it was found only in the control treatment. Conversely, *Ap. rosea* did not appear in 2019, while in 2018 it appeared only in the field treated with Nurelle D. The density of species and juvenile earthworms in the insecticide and control treatments are shown in Figure 2.

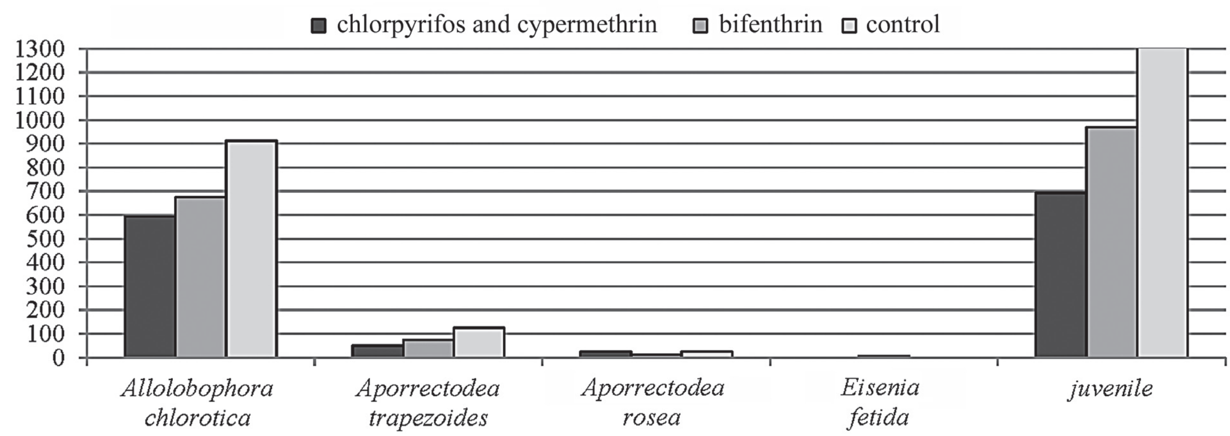


Figure 2. Structure of the earthworms of the family Lumbricidae in the insecticide and control treatments

The age of the earthworm population varied. Until the autumn of 2018, there were more adults than juveniles, and then the situation changed. The density of adults, juveniles, and total earthworms are presented in Table 4. The highest density of earthworms was in the control treatment, then in the field treated with Talstar 10 EC and finally in the field treated with Nurelle D (Figure 2). The decreasing order of the most dominant species was as follows: *All. chlorotica* > *Ap. trapezoides* > *Ap. rosea* > *E. fetida*, without changes in their dominance during the experiment. *All. chlorotica* was the most dominant species with the dominance from 96.55% in the Nurelle D treatment in the spring of 2018 to 57.14% in the control in the spring of 2019. This was followed by *Ap. trapezoides* with the dominance from 42.86% in the control in the spring of 2019 to 3.45% in the Nurelle D treatment in the spring of 2018. At the same time, the dominant species was a species that also had the highest frequency.

Table 4. Density of the adult and juvenile *Eisenia fetida* earthworms during the experiment

Year	Season	Treatment	Abundance			Index		
			adults	juveniles	total	*S*	*H**	*H′*
2017	spring	control (H_2O)	300	456.25	756.25	2	0.23	0.33
		Talstar 10 EC	187.5	156.25	343.75	3	0.58	0.53
		Nurelle D	175	118.75	293.75	3	0.33	0.30
	autumn	control (H_2O)	137.5	68.75	206.25	3	0.96	0.87
		Talstar 10 EC	75	43.75	118.75	3	0.57	0.52
		Nurelle D	68.75	31.25	100	3	0.91	0.83
2018	spring	control (H_2O)	318.75	187.5	506.25	2	0.37	0.54
		Talstar 10 EC	275	200	475	2	0.31	0.45
		Nurelle D	181.25	125	306.25	2	0.13	0.19
	autumn	control (H_2O)	256.25	450	706.25	2	0.25	0.36
		Talstar 10 EC	218.75	268.75	487.5	2	0.23	0.33
		Nurelle D	206.25	362.5	568.75	2	0.23	0.33
2019	spring	control (H_2O)	43.75	243.75	287.5	2	0.68	0.99
		Talstar 10 EC	25	193.75	218.75	2	0.57	0.83
		Nurelle D	25	143.75	168.75	1	–	–
	autumn	control (H_2O)	6.25	25	31.25	1	–	–
		Talstar 10 EC	–	12.5	12.5	–	–	–
		Nurelle D	–	6.25	6.25	–	–	–

S – total number of individuals of all species; *H* – Shannon-Weaver diversity index; *H′* – Pielou evenness index; * – significantly lower Shannon-Weaver index ($p < 0.05$) in 2019 compared to 2017

The Shannon's diversity index and evenness of the values were shown in Table 4. The Shannon's diversity index was significantly lower ($p < 0.05$) in 2019 compared to 2017. In all other cases, similar values were found.

Discussion

Results of the laboratory experiment. Mixtures of insecticides in agricultural practices are becoming increasingly popular due to their high efficiency and rapid action (Vuković et al., 2014). In the present study, the insecticide with two active ingredients (Nurelle D) showed a greater effect compared to the insecticide with one component (Talstar 10 EC). The Nurelle D demonstrated toxicity even at low doses, although the LC_{50} value was much higher than the highest concentration used in the experiment. This insecticide influenced the growth, cocoon production, and hatching of juveniles. This indicates that they do not act quickly or are very persistent and have a long period of decomposition in the soil.

Other authors have demonstrated the toxicity of the individual components of this insecticide at much higher concentrations than in the current experiment. Mostert et al. (2000) concluded that chlorpyrifos had a greater negative effect on earthworms than other insecticides (cyfluthrin, carbaryl, fipronil, and imidacloprid) in terms of mortality and biomass. Booth and O'Halloran (2001) observed a decrease in the growth rate of the earthworms *Aporrectodea caliginosa*. The use of chlorpyrifos at the recommended agricultural doses may cause a delay in juvenile growth and a decrease in the cocoon production of *Ap. caliginosa* (Booth, O'Halloran, 2001; Alshawish et al., 2004). Chlorpyrifos and cypermethrin have also been shown to be toxic to the tropical earthworms *Perionyx excavates* (De Silva et al., 2010). Wang et al. (2012) showed that cypermethrin had the lowest toxicity to *E. fetida* in artificial soil tests and was very toxic to *E. fetida* in contact toxicity tests. This phenomenon was explained by the fact that pyrethroids are more easily absorbed through the skin than through the intestines. Zhou et al. (2011) concluded that the mixture of chlorpyrifos and cypermethrin had a greater effect on mortality, growth, and reproduction than pyrethroid (cypermethrin) and an organophosphate insecticide (chlorpyrifos) individually. The mixture of these pesticides was more toxic, as shown by the LC_{50} values for 14 days which were significantly higher compared to those obtained using the pesticides separately. Also, these authors concluded that the effective dose of mixed pesticides disturbed normal growth and reproduction processes at lower concentrations than with single pesticides. According to Vuković et al. (2014), mixtures with different modes of action are significant in several aspects. In addition to being more effective and faster on target organisms, they reduce the amount of active ingredients in the mixture and prevent pest resistance. Although the mixture of chlorpyrifos and cypermethrin (Nurelle D) was registered almost 30 years ago, it is still very effective in pest control (Vuković et al., 2014). However, when chlorpyrifos was included in the Directive (2008) of the European Parliament, it was proposed to reduce its use per hectare or to replace it with less harmful substances, and finally its use was banned in the EU, like bifenthrin.

In the present experiment, the mortality of earthworms at all insecticide concentrations was recorded already in the 1st week, which corresponds to the main property of pyrethroids to act quickly (Gajendiran, Abraham, 2018). Talstar 10 EC had no significant effect on changes in earthworm weight, except at the highest concentrations in the 6th and 8th week. Nevertheless, Talstar 10 EC had an impact on the production of cocoons and hatching juveniles even at the recommended agricultural doses.

Reproduction can be inhibited or stopped when contaminant concentrations are well below lethal (LC_{50}) concentration (Sekulić et al., 2020). According to Zhou et al. (2007), reproduction parameters are clearly more sensitive tests of change than other risk assessment ones. Stäbler (2002) showed that NOEC (No Observed Effect Concentration) for reproduction on samples of *E. fetida* under test conditions was equal to 2.13 mg kg^{-1}. Pyrethroids are less toxic to mammals compared to invertebrates due to the higher body temperature, larger body size, and lower sensitivity of ion channels of mammals (Gajendiran, Abraham, 2018). Many studies have shown the effect of bifenthrin on invertebrate species. It was found to be highly toxic to honeybee (*Apis mellifera ligustica*) (Dai et al., 2010) and water flea (*Daphnia magna*) (Wang et al., 2009).

Results of the field experiment. Monitoring the population of earthworms is considered an important and useful indicator of soil quality in the environmental assessment of agroecosystems (Andriuzzi et al., 2017). The earthworms tested in the experiment (*All. chlorotica*, *Ap. trapezoides*, *Ap. rosea*, and *E. fetida*) are the most common species in the European soils. Endogeic species were dominant, and three of the four species belonged to this group. This corresponds with the finding that endogeic earthworms are dominant in fields (Frazão et al., 2017). Pink earthworms (*Ap. rosea*, endogeic) are species living mainly in the topsoil, feeding in soil and comminated soil organic matter. The field earthworms (*Ap. trapezoides*, endogeic) are known to be more sensitive to chemicals than the compost earthworm (*E. fetida*) (Schnug et al., 2015). *E. fetida* (epigeic) is not typical in agricultural fields but is often found in areas rich in organic matter. *All. chlorotica* is a typical synanthropic species that can be found in pastures, gardens, and forests. It has almost no preference for soil type, but usually they are more abundant in moist, highly organic soils. According to the ecological characteristic, *All. chlorotica* also belongs to the epi-endogeic group, living and feeding in the mineral soil layer.

Species of the genus *Apporectodea*, unlike other endogenic species, can penetrate deeper into the soil and thus avoid the effect of conventional agricultural practices (Frazão et al., 2019). In a conventional winter wheat monocropping system and a low-input intercropping system, Schmidt et al. (2001) found, among others, *All. chlorotica*, *Ap. caliginosa*, and *Ap. rosea*. A high abundance of species in this group reflects their reproductive capacity and the potential for adaptation and colonisation (Sekulić et al., 2022).

The results of the current study showed that the application of Talstar 10 EC had lower effect on the abundance of earthworms in comparison with Nurelle D (Table 4). Shannon's diversity index was significantly lower in 2019 compared to 2017 indicating that diversity has been reducing over time. In the control field and sometimes in a field treated with Talstar 10 EC, the number of juvenile earthworms was higher. The findings of the present experiment agree with the explanation given in the study of Sekulić et al. (2022) that juveniles prefer the upper layers of soil and are unable to penetrate in the deeper layers, so they are more exposed to pesticides than adults. On the other hand, the reproductive capacity of the earthworms has decreased due to the use of pesticides (Pelosi et al., 2013). Additionally, the use of pesticides indirectly changes living conditions and the chemical properties of the soil (Aebeed, Amer, 2018).

Comparison of the results of field and laboratory experiments. Laboratory tests are required to determine the potential risk of certain chemicals on the environment. These tests are conducted under controlled conditions, unlike field conditions that are very variable (Velki et al., 2014). The impact of pesticides on earthworms in agroecosystems depends on several factors that are not present in laboratory conditions. These are primarily the depth of penetration of pesticides into the soil and the vertical distribution of earthworms. According to some authors (Pelosi et al., 2013), earthworms feed on or near the surface of the soil, which is more affected by pesticides. On the other hand, in nature, animals can avoid pesticides by moving into deeper layers, unlike laboratory ones. In laboratory tests, the repeated use of the same or different pesticides is rare, but this is common in agricultural fields. Testing a mixture of active ingredients is only the initial step in determining of long-term effects of pesticides in the field conditions.

There are many differences between laboratory and field experiments, but the laboratory results of the present study confirm the results obtained in the field research. The greatest effect on the earthworms was shown by the pesticide with two active ingredients followed by the pesticide with one active ingredient. The results of the present field and laboratory experiment confirm the previous findings (Schnug et al., 2015). However, laboratory tests not only predict the situation in the field but also supplement the existing knowledge about the impact of pesticides on agroecosystems. They are also useful in helping to interpret the results of field experiments, as many ecological factors are highly variable making the results obtained difficult to interpret.

Further comprehensive studies with other pesticides are needed to develop laboratory and field research.

Conclusions

1. Insecticide Nurelle D (mixture of organophosphorus and pyrethroid: chlorpyrifos and cypermethrin) did not show a significant reduction in the number of earthworms, so no mortality was recorded at any concentration after the 3rd week of exposure. The Nurelle D was found more ecologically dangerous to *Eisenia fetida* than the single-component insecticide Talstar 10 EC (pyrethroid bifenthrin). Both insecticides had 50% lethal concentration (LC_{50}) values higher than the highest concentration used in the experiment.

2. Talstar 10 EC had no effect on weight of earthworms, and the only significance was found at the highest concentration at the 6- and 8-week assessments. At the application of Nurelle D, the earthworms had gained weight. The significance was determined at 2RAD and 4RAD concentrations from the 2nd week to the end of the experiment.

3. Talstar 10 EC affected the production of cocoons and hatching juveniles at the highest concentration (4RAD) at 6 and 8 weeks, even at the 1RAD. The mixture of insecticides Nurelle D showed toxicity after a long-time of exposure and affected the parameters of the life cycle of cocoon production and hatching of juveniles. Regarding the hatching juveniles, Nurelle D showed a greater effect compared to Talstar 10 EC, even at the 1RAD.

4. The results showed that the tested insecticides were toxic to natural populations of earthworms. The results of the study showed that the application of Talstar 10 EC had lower effect on the abundance of earthworms in comparison with Nurelle D. Due to the high degree of reproduction (parthenogenesis) and the ability to tolerate poor-quality soil, the species *Allolobophora chlorotica* dominated in the field experiment. Shannon's diversity and Pielou evenness indices had lower values in the studied habitats due to the anthropogenic impact.

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