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Neonicotinoid Residues in Earthworms and Ground Beetles under Intensive Sugar Beet Production: Preliminary Study in Croatia

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Abstract: Neonicotinoids are pesticides widely used for pest control in agriculture with undesirable effects on pollinators. However, other beneficial insects are exposed to insecticides that are not lethal to them but may accumulate and affect their vital characteristics. The objective of this study was to determine neonicotinoid residues in two types of beneficial soil organisms. The first group includes ground beetles (family: Carabidae, order: Coleoptera). They are important in the food web within existing ecosystems, especially in agricultural areas. The second group includes earthworms (family: Lumbricidae, order: Opisthopora) as humifiers, important members of the soil fauna. Fauna was collected at two sugar beet growing areas in Croatia under intensive sugar beet management. Ground beetles were collected from six plots of sugar beet fields treated with imidacloprid and thiamethoxam or left untreated with neonicotinoids. Earthworms were collected from the eight fields involved in four-year sugar beet crop rotation (sugar beet, maize, soybean, oilseed rape). Detection of neonicotinoid residues was performed by LC-MS/MS, SPE-QuEChERS method. The limit of quantification (LOQ) was 0.001 mg/kg. In ground beetles, the highest concentration of imidacloprid was detected at 0.027 mg/kg, while the residues of thiamethoxam and clothianidin were below LOQ. The highest concentration of imidacloprid in earthworms was 0.2141 mg/kg, while residues of thiamethoxam did not exceed 0.0008 mg/kg. This is the first study of this kind on Croatian territory and provides a valuable first insight into the ecotoxicological status of beneficial soil fauna. More comprehensive studies are needed to assess the extent of accumulation in and to take further steps regarding conservation programs for beneficial soil organisms.

Keywords: beneficial organisms; imidacloprid; liquid chromatography–mass spectrometry; neonicotinoid; pitfall; thiamethoxam

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1. Introduction

Intensification and modernization of agricultural production has led to a decline in the number of individuals or species due to the negative impact of various factors [1]. Pest control exposes non-target organisms to insecticides that can affect their development, physiology, behavior, and communication [1]. Special concern is put on beneficial fauna. Beneficial fauna is a group of organisms that indirectly have a positive effect on crops by increasing soil fertility, regulating the water–air ratio, or feeding on pests and reducing their numbers. The beneficial soil fauna of agricultural lands includes insects, earthworms, nematodes, mites, and spiders. Insects that are part of the beneficial soil fauna and are important as indicators of habitat biological stability include ground beetles (family: Carabidae, order: Coleoptera) [2]. and earthworms (family: Lumbricidae, order: Opisthopora) [3] Intensive agriculture with high use of pesticides and fertilizers poses a threat to beneficial insects and leads to a loss of biodiversity [4].

Ground beetles are important predators of numerous pests, and they also feed on weed seeds and are a food source for animals at a higher trophic level [5,6]. The decline in

ground beetle populations is explained by the higher use of agrochemicals, loss of grassland for foraging, and increasing average field size, the negative effect of which is even stronger than the effect of intensive cultivation [4–8].

Earthworms are important members of the fauna of agricultural soils, where they account for up to 80% of the total animal biomass [9]. They play a key role in the development and maintenance of physical, chemical, and biological soil properties [10]. In cultivated fields, earthworms are exposed to frequent and varied pesticide applications [7]. The seriousness of the problem of earthworms with pesticides is shown by the results of a study conducted in France. At least one pesticide was detected in 92% of the earthworms studied, both in treated crops and untreated habitats [11].

Neonicotinoids are highly toxic to most arthropods and have been widely used for pest control in agriculture and horticulture [12]. Although neonicotinoids are banned in Europe and the UK, they are still used for crop protection under special permits [13]. The top ten destinations for banned neonicotinoid exports from the EU, by weight of active ingredients, are Brazil, Russia, Ukraine, Argentina, Iran, South Africa, Singapore, Indonesia, Ghana, and Mali [14]. One of the most important reasons for the ban of neonicotinoids was the use of treated seeds and the use of pneumatic seeders, which create dust during sowing that gets onto the surrounding flowering plants and is carried by bees into the hive along with pollen [15,16]. For example, Krupke et al. [17] detected residues of thiamethoxam (68 to 13.240 mg/kg) and clothianidin (3.400–15.030 mg/kg) in dust from treated maize seeds. The undesirable effect on pollinators during foliar application, on treated areas and outside treated areas has adverse effects as well [16]. Exposure of beneficial or non-target organisms to insecticides need not be lethal to them, but can seriously affect their development, physiology, behavior, and communication [4].

The objective of this study was to determine pesticide residues in: (1) ground beetles (Carabidae) collected from sugar beet fields whose seeds had been treated with imidacloprid and thiamethoxam and from field without insecticide seed treatment, and (2) earthworms (Lumbricidae) collected from the fields involved in four-year sugar beet crop rotation to assess accumulation of neonicotinoids in the tested organisms.

Sugar beet was selected as a high-yielding crop that was frequently treated with neonicotinoids in the last decade and for which European Food Safety Authority (EFSA) assessed and permitted emergency neonicotinoid uses after general banning.

2. Materials and Methods

The investigation was conducted in the northern Croatia location Lukač (45.8739° N, 17.4191° E) and in the eastern Croatia location Tovarnik (45.1649° N, 19.1522° E). Average air and soil temperatures were higher in Tovarnik, while the amount of precipitation was higher in Lukač. The soils in Tovarnik have a higher content of soil organic matter, but on both locations, soils are classified as silty clay according to the soil particle size fractions [18]. On both locations' sugar beet fields, over 5 ha were chosen for setting up the experiment. Sowing of sugar beet included the untreated plot, a plot sown with seeds treated with imidacloprid at a dosage of 0.00091 a.i./seed, and a plot treated with a combination of 0.00036 thiamethoxam and 0.000036 a.i./seed tefluthrin, each sown on 1000 m^2.

All agrotechnical measures taken at both locations were standard for each investigated area, including the application of different plant protection products and fertilizers.

Samples of ground beetles were collected using 40 pitfall traps set in the form of a net (12 per plot + two indicative per location were initially sent to analysis to confirm if it is possible to detect residues in animal samples). Samples were collected three times during the growing season over a period of seven days in May (20.05), July (01.07), and September (22.09). In the meantime, the traps were closed with plastic covers. Ground beetle samples were deep frozen until analysis. Other organisms collected in the traps were not subjects of the study and were not considered for analysis.

Earthworm samples were collected at the same sugar beet fields and additionally, from the fields included in the sugar beet four-year crop rotation system (details on sugar beet

crop rotation are in Table 1). Samples were collected three times on each field (autumn, spring, autumn) using the standard ISO method [19] that includes digging 60 × 60 cm holes filled with water and formalin. Per each field, four holes on randomly selected places were dug, and samples were handpicked. All collected samples per extraction hole presented a repetition in the experiment. All samples were deep frozen until analysis.

Table 1. Historical crop rotation at the locations included in the research.

Locality		Four Years Sugar Beet Crop Rotation System				
	Field	**I**	**II**	**III**	**IV**	**V**
Tovarnik	1.	maize *	**sugar beet**	soybean	wheat	**sugar beet**
	2.	wheat	maize	**sugar beet**	wheat	sunflower
	3.	**sugar beet**	wheat	sunflower	**sugar beet**	wheat
	4.	soybean	wheat	sunflower	barley	sugar beet
Lukač	1.	wheat	**sugar beet**	wheat	sunflower	maize
	2.	**sugar beet**	wheat	**sugar beet**	maize	maize
	3.	soybean	maize	wheat	**sugar beet**	bare soil
	4.	maize	oilseed rape	wheat	sunflower	**sugar beet**

* Fields marked with dark grey were under sampling during spring and autumn, I—sugar beet sown in testing year, fields marked with light gray were under sampling during autumn previous year, II—sugar beet sown one year ago. III—sugar beet grown before two years ago; IV—sugar beet sown three years ago; V—sugar beet sown four years ago.

Neonicotinoid residue analysis was done by certified laboratory Euroinspekt Croatiakontrola Ltd. for Control of Goods and Engineering, Zagreb, Croatia, using a multiresidue method for the determination of pesticide residues by gas and liquid chromatography after extraction with acetonitrile and purification by solid-phase dispersive extraction (SPE)—Modular method QuEChERS (EN 15662:2018). The method is standardized for the analysis of foods of plant origin. However, since it covers a wide range of matrices in terms of chemical composition, including samples with a high protein and/or fat content, it is validated for samples of animal origin as well [20]. The limit of residue quantification, that is, the amount of active substance that could be detected by this method, was 0.001 mg/kg or ppm.

According to HRN EN 15662:2018 for multiresidue pesticide analysis, procedure includes homogenization of samples, which should not weigh less than 5 g each. Data on neonicotinoid residues were processed with ANOVA using ARM 9® GDM Software, Revision 2019.4; (B = 25105), SD, USA, [21] to determine the differences between sampling periods on both locations and crops involved in the research.

3. Results

A total of number of collected ground beetles in sugar beet fields was 1.131 in Vukovar-Syrmia County and 1.250 Virovitica-Podravina County. On both locations, the species *Poecilus cupreus cupreus* Linnaeus, *Harpalus rufipes* De Greer, *Pterostihus melanarius melanarius* Illiger and *Pterostihus melas melas* Creutzer accounted for more than 80% of the individuals captured, while the remaining species were sporadic. A total of 14 homogenized ground beetle samples were analytically prepared for multiresidue analysis. Each sample contained an average of 150 beetles.

During total of 96 samplings, 419 earthworms were collected in Vukovar-Syrmia County and 650 in Virovitica-Podravina County. Distinguished species included *Allolobophora caliginosa* Savigny and *Lumbricus terrestris* Linnaeus. A total of 58 homogenized earthworm samples were analytically prepared for multiresidue analysis. Each sample contained an average of 30 earthworms.

The multiresidue method described above was used to determine the residues of 300 different active ingredients of plant protection products, but only the results of neonicotinoids are considered (Tables 2–4).

Table 2. Determined residues of neonicotinoids (in mg/kg) in ground beetle samples from different variants collected from sugar beet fields in Lukač and Tovarnik.

Lukač									
	imidacloprid residues			thiamethoxam residues			clothianidin residues		
Variant	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
V_1	0.004	0.002	0,011	0.002	<0.001	<0.001	0.001	<0.001	<0.001
V_2	0.004	0.004	0,027	0.002	<0.001	<0.001	0.001	<0.001	<0.001
V_3	0.004	0.006	0.008	0.002	<0.001	<0.001	0.001	<0.001	0.001
Tovarnik									
	imidacloprid residues			thiamethoxam residues			clothianidin residues		
	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
V_1	<0.001	0.002	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
V_2	0.001	0.008	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
V_3	<0.001	0.001	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

V_1—untreated sugar beet seeds; V_2—sugar beet seeds treated with imidacloprid; V_3—sugar beet seeds treated with a combination of thiamethoxam and tefluthrin, S_1—sampling in spring; S_2—sampling in summer, S_3—sampling in autumn.

Table 3. Determined residues of neonicotinoids (in mg/kg) in earthworm samples from fields with different crop rotations in Lukač.

Active Ingredient	**Sample Collection Period**					**LSD P = 0.05** [1]
	Crop Rotation	**S_1**	**Crop Rotation**	**S_2**	**S_3**	
Imidacloprid	sugar beet	0.0321	wheat	0.0184	0.0800	ns [2]
	oilseed rape	0.0044 b	maize	0.0049 b	0.0166 a	0.00495
	wheat	0.0493 a	sugar beet	0.0107 b	0.0663 a	0.01929
	maize	0.0334 a	soybean	0.0067 b	0.035 a	0.01220
Thiamethoxam	sugar beet	0.0005	wheat	0.0002	0.0001	ns [2]
	oilseed rape	0.001 a	maize	0.0001 b	0.0001 b	0.00007
	wheat	0.0001	sugar beet	0.0003	0.0001	ns
	maize	0.0001	soybean	0.0001	0.0001	ns
Clothianidin	sugar beet	0.0084	wheat	0.0037	0.0001	ns [2]
	oilseed rape	0.0077 a	maize	0.003 b	0.003 b	0.00207
	wheat	0.0054	sugar beet	0.0218	0.0105	ns
	maize	0.0133	soybean	0.0235	0.0070	ns

[1] Analysis of differences between fields in different rotations with respect to the time of sampling: values marked with the same lowercase letter belong to the same rank; [2] difference is not statistically significant (ns—no significant); S_1—sampling in autumn previous year; S_2—sampling in spring, S_3—sampling in autumn.

Residues of imidacloprid are present in all samples. The highest detected imidacloprid concentration was 0.027 mg/kg in Lukač during autumn sampling (Table 2). In most cases, the residues of thiamethoxam and clothianidin in the ground beetle samples were below LOQ. It can be observed that thiamethoxam is degraded faster than imidacloprid.

Residues of imidacloprid in earthworms changed depending on the sampling period and their degradation dynamics depended on crop rotation. At location Lukač (Table 3), imidacloprid residues increased, especially towards the end of the growing season, even when no additional treatments were applied in the same vegetation. The highest concentration of imidacloprid measured in earthworm samples was 0.2141 mg/kg in Tovarnik during the final sampling in autumn (Table 4). Thiamethoxam and clothianidin residues are usually observed together because thiamethoxam is metabolized in the soil to clothianidin. This explains the fact that clothianidin was not used at seeding, but residues of clothianidin are still found in samples. At both locations (Tables 3 and 4) residues of thiamethoxam are not above 0.0008 mg/kg, and in most cases, results do not significantly differ between crops or sampling periods on both locations. Same as above, residues of clothianidin were somewhat elevated, but were still far below lethal doses.

Table 4. Determined residues of neonicotinoids (in mg/kg) in earthworm samples from fields with different crop rotations in Tovanik.

Active Ingredient	Sample collection period					LSD P = 0.05 [1]
	Crop Rotation	S_1	Crop Rotation	S_2	S_3	
Imidacloprid	sugar beet	0.057 a	maize	0.0234 b	0.05 b	0.01609
	maize	0.0128 b	wheat	0.0958 a	0.1144 a	0.0289
	wheat	0.0058 c	sugar beet	0.0295 b	0.2141 a	0.00557
	wheat	-	soybean	0.0275	0.1191	ns
Thiamethoxam	sugar beet	0.0008	maize	0.0004	0	ns
	maize	0.0001	wheat	0.001	0.0001	ns
	wheat	0.0001	sugar beet	0.0001	0.0001	ns
	wheat	-	soybean	0.0001	0.0001	ns
Clothianidin	sugar beet	0.0073	maize	0.005	0.001	ns
	maize	0.0008	wheat	0.0048	0.0048	ns
	wheat	0.0053 b	sugar beet	0.018 a	0.0013 c	0.00285
	wheat	0.057 a	soybean	0.0182	0.0347	ns

[1] Analysis of differences between fields in different rotations with respect to the time of sampling: values marked with the same lowercase letter belong to the same rank; S_1—sampling in autumn previous year; S_2—sampling in spring, S_3—sampling in autumn; ns—no significant.

4. Discussion

Residues of imidacloprid were detected in all samples in our study, including those from the untreated plot. The reason for this is that ground beetles are very mobile insects, and individuals from one plot can easily be present in samples from the other plot or even neighboring fields. Within our study, the highest concentration of imidacloprid was 0.027 mg/kg in Lukač during the autumn sampling, while residues of thiamethoxam and clothianidin between <0.001–0.002 are negligible in all variants. In a study by Mullin et al. [22], almost 100% mortality of 18 ground beetle species and extreme sensitivity of ground beetle (*Poecilus cupreus* L.) larvae exposed to commercial corn seed treated with neonicotinoids at a dose of 700 g/kg were observed.

In the case of earthworms, toxicological studies show the risk of mortality of individuals of all known species when they ingest soil or organic material containing neonicotinoid residues at a concentration $\geq$ 1 mg/kg [3]. According to Gomez-Eyles et al. [23], imidacloprid can negatively affect the reproduction and growth of earthworms at 1.91 mg/kg. At a concentration of 3 mg/kg, 50% mortality of earthworms is expected [3]. Within our study, the highest detected residues of imidacloprid were far below the value of acute and chronic toxicity of the same pesticide (LC_{50} = 10.7 mg/kg). Increase of imidacloprid residues in earthworms at the end of sugar beet vegetation can be explained by their more active period toward the end of the vegetation season [10]. According to PPDB [24], imidacloprid is moderately toxic to earthworms with a low risk of bioaccumulating.

The use of neonicotinoids has become a major controversy because of their negative effects on pollinators. Studies by EFSA [25–27] have shown that neonicotinoids have negative effects on bees, other pollinators, and possibly other non-target organisms. EFSA was requested by the European Commission (EC) to provide technical assistance under Article 53(2) of Regulation (EC) No. 1107/2009 [25] to review the emergency authorizations in Croatia for pesticides containing the neonicotinoids (clothianidin, imidacloprid, or thiamethoxam) banned in May 2018 for use on sugar beets. EFSA was asked to evaluate whether the granting of this emergency authorization was necessary due to a hazard that could not be contained by other appropriate means. EFSA collected and evaluated the information related to the emergency authorization of neonicotinoids (thiamethoxam) in Croatia. The evaluation concluded that there are currently no sufficient alternatives for the tested sugar beet pests *Agriotes* sp., *Atomaria linearis* Stephens, *Bothynoderes punctiventris* Germar and *Chaetocnema* sp.

While clear results have been published on sugar beet pests, no relevant data were available on neonicotinoid influence on beneficial soil fauna on fields under intensive sugar beet production. According to EFSA, the treatment of sugar beet seeds with neonicotinoids poses a risk for the succeeding crop scenario where residue remains in the soil and can be absorbed [25–27]. High concentrations of neonicotinoids in soil are especially expected in cases of dry conditions, leaching incapacity, or irregular flushing into ground water [18]. Ground beetles feed on various economically damaging species [28] that have fed on the treated crop or through the treated surface on which they move [29–33] so they can easily be exposed to the elevated neonicotinoid residues. Earthworms, as organisms mostly living below the soil, have a specific way of feeding, leading them to ingest contaminated soil and organic particles [34] At higher neonicotinoid concentrations used to protect agricultural crops, the same neural pathways through which neonicotinoids affect invertebrates [35] may also affect those of earthworms [36].

5. Conclusions

In the two beneficial soil organisms studied, ground beetles and earthworms, the neonicotinoid residues were below concentrations reported as lethal. If the elevated concentrations of neonicotinoids remain in the soil after the growing season, residues in soil fauna can be expected. Considering the data presented in this preliminary study, approved seed treatments can be continued, but only under strict controls to minimize risks to the environment while providing effective and appropriate crop protection for key pests. The results of our study provide an important contribution and additional arguments for this and future assessment as well as conservation programs. More comprehensive studies are needed to assess the extent of accumulation in beneficial soil organisms.

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