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Impact of Seed Treatment with Imidacloprid, Clothianidin and Thiamethoxam on Soil, Plants, Bees and Hive Products

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Abstract: This paper presents studies performed for the monitoring of imidacloprid, clothianidin and thiamethoxam residues applied as seed treatment in rapeseed (*Brassica napus* ssp. *oleifera*), maize (*Zea mays*) and sunflower crops (*Helianthus annuus)*. The experiments were located in representative areas of the mentioned crops. Residue levels were determined in plant samples at different phenological development stages, including flowers, as well as in bees and hive products (pollen, honeycomb, honey) by liquid chromatography/tandem mass spectroscopy (LC-MS/MS). The analyses were performed in ISO 17025-accredited laboratories, referring to the limit of quantification (LOQ), characteristic of the method used to determine the residues. In 2019, the percentage of samples that contained residues of the three substances, applied to the seed, was 16.39%, representing 20 samples out of the total of 122 analyzed samples. In 2020, 10 samples contained neonicotinoid residues above the LOQ, including 5 soil samples and 5 plant samples, representing 6.17% of the total samples. In 2021, from 149 samples with neonicotinoid applied as seed treatment, residues were identified in 12 soil samples and 11 plant samples, representing 15.43% of the total number of samples. In 2022, only 12 soil samples and 1 pasture sample contained residues above the LOQ. The results show that the highest percentage of samples with residues above the LOQ was recorded by the soil samples, while the flower and bee samples had the lowest percentages of samples with residues above the LOQ; no residues of the three neonicotinoid substances were identified in the honey samples.

Keywords: monitoring; neonicotinoids; residues; oilseed rape; maize and sunflower; hive products

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1. Introduction

It is generally accepted that pesticides play an important role in agricultural development because they can reduce the losses of agricultural products and improve the affordable yield and quality of food [1–4].

Worldwide pesticide production increased at a rate of about 11% per year, from 0.2 million tons in the 1950s to more than 5 million tons by 2000 [5]. Three billion kilograms of pesticides are used worldwide every year [6], while only 1% of total pesticides are effectively used to control insect pests on target plants [7]. The farmed crops often suffer from pests, weeds and diseases, which could result in a considerable loss in crop yield. Without pesticide usage, the loss of fruits, vegetables and cereals due to pests and diseases would be as much as 78%, 54% and 32%, respectively [6,8].

In modern agriculture, scholars are trying to develop genetically engineered crops designed to produce their own insecticides or exhibit resistance to broad-spectrum herbicide

products or pests. This new pest management could reduce chemical use and its negative impacts on the environment [7].

About one-third of agricultural products are produced using pesticides. Without the use of pesticides, there would be a 78% loss of fruit production, a 54% loss of vegetable production and a 32% loss of cereal production [9]. Therefore, pesticides play a critical role in reducing diseases and improving the increase in crop yields worldwide. Thus, they have made a significant contribution to alleviating hunger and providing access to an abundant supply of high-quality food.

Insecticides can provide a variety of benefits, the most important of which are financial to farmers, derived from protecting crop quality and production, as well as reducing other expensive inputs such as labor and fuel [10].

Neonicotinoids are a relatively new class of chemically neuroactive insecticides similar to nicotine. The neonicotinoid class includes the following: acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid and thiamethoxam, with imidacloprid being the most widely used insecticide worldwide [11–14].

In the last few decades, they have been the most frequently used insecticides in crop protection due to the broad spectrum of pest control, the relatively low risk to non-target organisms and the environment, and the specificity to target organisms, as well as the versatility of application methods. They are registered in over 120 countries, with a worldwide turnover of EUR 1.5 billion, consisting of 24% of the global insecticide market in 2008 [14–16].

The use of neonicotinoids has been associated in several studies with potential adverse ecological effects, including bee colony collapse disorder (CCD) and bird loss due to reduced insect populations; however, the results of the studies were conflicting and thus controversial, since the cause of the occurrence of this phenomenon (CCD) has not yet been elucidated [17–24].

In 2013, the European Union and some non-EU countries restricted the use of certain neonicotinoids, through Regulation (EU) No 485/2013, banning the use and sale of seeds treated with clothianidin, thiamethoxam or imidacloprid. As a result, the European Food Safety Authority (EFSA) requested additional studies, the deadline for its completion being February 2018. These studies confirmed the already identified risks for the field use of the three substances. As a result, the Commission and most member states prohibit their use under field conditions (The Official Journal of the European Union on 30 May 2018; Regulation (EU) 2018/783, 784, 785), with only their use in permanent greenhouses being allowed [25,26].

This decision strongly affected Romanian agriculture, and, as a consequence, the associations of agricultural producers united their efforts; with the support of the Ministry of Agriculture and Rural Development, they obtained exemptions from the Commission's decision, thus saving the following agricultural years' productions. Even if an insecticide based on cyantraniliprole is approved for rapeseed treatment, through mutual recognition, there are still no alternatives for maize and sunflower crops for the control of soil pests, such as *Tanymecus dilaticollis*, *Agriotes* spp. and *Opatrum sabulosum* [27].

Although alternative insecticides (chlorantraniliprole, neem) are shown to have certain effects on particular pests when applied as seed treatment, it is not likely that any insecticide will be identified as a good candidate for neonicotinoids' substitution in the near future.

Romanian farmers have been confronted with attacks from different species of insects, whose populations exceed the economic damage thresholds, thus requiring the use of active substances with a long period of action, which does not happen in the case of products based on synthetic pyrethroids, which are desired to be an effective alternative following the ban on neonicotinoids. Romanian researchers have demonstrated in their studies that, for an appropriate control of soil pests in rapeseed, maize and sunflower crops, the most recommended are systemic products, which can ensure the protection of plants during the first vegetation phases [28–30].

The aim of this research is to evaluate the residue levels (LC-MS/MS method) of imidacloprid, thiamethoxam and chlothianidin applied as seed dressings in oilseed rape, maize and sunflower plants during five growing seasons in fields located in different agro-climatic regions in Romania and also to establish the impact of these neonicotinoid active substances on *Apis melifera*—common bee and hive products.

2. Materials and Methods

2.1. Experimental Design

Studies regarding the residues of neonicotinoid insecticides (imidacloprid, clothianidin and thiamethoxam) applied to rapeseed, corn and sunflower seeds and their impact on bees and hive products were conducted between 2018 and 2022, being financed by the Ministry of Agriculture and Rural Development, through the ADER Program, as well as from private funds provided by the "Patrimoniu ASAS" Foundation. The projects in which the studies were carried out were of a partnership type, with the participation of researchers from the Research-Development Institute for Plant Protection Bucharest as project leaders and from the Secuieni and Pitești Agricultural Research and Development Stations and the National Agricultural Research Institute Fundulea as projects partners, as well as a group of specialists from the Research Institute for Beekeeping.

2.1.1. Field Site

The studies of five years were conducted between 2018 and 2022 on three different locations. The field experiments were conducted in three pedoclimatic representative areas of Romania for rapeseed, maize and sunflower crops: the Moldavian Plateau (Secuieni Agricultural Research and Development Station—ARDS Secuieni), Subcarpathian Hills (Pitesti Agricultural Research and Development Stations—ARDS Pitesti) and the south of the country (National Agricultural Research Institute Fundulea—NARDI Fundulea), respectively.

2.1.2. Design of Experiments

The experiment included three variants treated with neonicotinoids and an untreated control. The field experiments were arranged in linear blocks with 4 randomized repetitions per variant. At each site, maize (*Zea mays*), sun flower (*Helianthus annuus*) and rape (*Brassica napus* ssp. *oleifera*) were sown as follows:

Rape: There were three treatments, one of which was untreated seed (0 mg a.i./seed), the second of which was rape seed treated with imidacloprid (0.36 mg a.i./seed) and the third of which was rape seed treated with a mixture of clotianidin + beta-ciflutrin (0.05 µg chlotianidin/seed) These treatments were used in 2018 and 2019, since 2020 neonicotinoids were replaced with cyantraniliprol. The rape was sown every year: in the first decade of September, each treatment was sown on 2000 sm, in four replicates of 50 × 10 m, and the distance between rows was 0.125 m with 0.02 m between seeds, at a depth of 3–5 cm, using 500,000 germinal units per ha.

Maize and sunflower: There were three treatments, one of which was untreated seed (0 mg a.i./seed), the second of which was maize seed treated with imidacloprid (2.16 mg a.i./seed) and the third of which was maize seed treated with chlothianidin (2.16 mg/seed). Both crops were sown in 2018, 2019 and 2020 in the second decade of April; in 2021 and 2022, they were sown in the first decade of May. Each treatment was sown on 2000 sm, in four replicates of 50 × 10 m, and the distance between rows was 0.75 m with 0.2 m between seeds, at a depth of 5–7 cm, using 67,000 germinal units per ha.

The experimental variants of applying neonicotinoids to the seed depending on the crop are shown in Tables 1–3:

Table 1. Rapeseed experimental variants.

ARDS Secuieni					ARDS Pitesti					NARDI Fundulea				
2018	**2019**	**2020**	**2021**	**2022**	**2018**	**2019**	**2020**	**2021**	**2022**	**2018**	**2019**	**2020**	**2021**	**2022**
Imidacloprid (Nuprid Al)	Imidacloprid (Nuprid 600 FS)	Cyantraniliprole (Lumiposa 625 FS)			Imidacloprid (Nuprid 600 FS)	Imidacloprid (Nuprid 600 FS)	Cyantraniliprole (Lumiposa 625 FS)			Imidacloprid (Nuprid 600 FS)	Imidacloprid (Nuprid 600 FS)	Cyantraniliprole (Lumiposa 625 FS)		
Clotihanidin + beta-cyfluthrin (Modesto 480 FS)	Clotihanidin + beta-cyfluthrin (Modesto 480 FS)				Clotihanidin + beta-cyfluthrin (Modesto 480 FS)	Clotihanidin + beta-cyfluthrin (Modesto 480 FS)				Clotihanidin + beta-cyfluthrin (Modesto 480 FS)	Clotihanidin + beta-cyfluthrin (Modesto 480 FS)			
Thiamethoxam (Cruiser 350 FS)					Thiamethoxam (Cruiser 350 FS)									

Table 2. Maize experimental variants.

ARDS Secuieni					ARDS Pitesti					NARDI Fundulea				
2018	**2019**	**2020**	**2021**	**2022**	**2018**	**2019**	**2020**	**2021**	**2022**	**2018**	**2019**	**2020**	**2021**	**2022**
Imidacloprid (Nuprid Al)	Imidacloprid (Nuprid Al)	Imidacloprid (Nuprid Al)			Thiamethoxam (Cruiser 350 FS)	Imidacloprid (Nuprid 600 FS)	Thiamethoxam (Cruiser 350 FS)	Imidacloprid (Nuprid Al)		Imidacloprid (Nuprid 600 FS)	Imidacloprid (Nuprid 600 FS)	Imidacloprid (Nuprid 600 FS)	Imidacloprid (Nuprid 600 FS)	
Clotihanidin + beta-cyfluthrin (Modesto 480 FS)	Clothianidin (Poncho 600 FS)	Cypermethrin (Langis)			Imidacloprid (Nuprid 600 FS)	Clothianidin (Poncho 600 FS)	Cypermethrin (Langis)	Cypermethrin (Langis)		Clothianidin (Poncho 600 FS)	Clothianidin (Poncho 600 FS)	Thiamethoxam (Cruiser 350 FS)	Cypermethrin (Langis)	
Thiamethoxam (Cruiser 350 FS)					Clothianidin (Poncho 600 FS)	Thiamethoxam (Cruiser 350 FS)	Imidacloprid (Nuprid Al)			Thiamethoxam (Cruiser 350 FS)	Thiamethoxam (Cruiser 350 FS)	Cypermethrin (Langis)		

Table 3. Sunflower experimental variants.

ARDS Secuieni					ARDS Pitesti				NARDI Fundulea				
2018	**2019**	**2020**	**2021**	**2022**	**2018**	**2019**	**2020**	**2021**	**2018**	**2019**	**2020**	**2021**	**2022**
Imidacloprid (Nuprid Al)	Imidacloprid (Nuprid Al)	Cypermethrin (Langis)		Imidacloprid (Nuprid 600 FS)	Thiamethoxam (Cruiser 350 FS)	Imidacloprid (Nuprid 600 FS)	Cypermethrin (Langis)		Imidacloprid (Nuprid 600 FS)	Imidacloprid (Nuprid 600 FS0029	Cypermethrin (Langis)		Imidacloprid (Nuprid 600 FS)
Clotihanidin + beta-cyfluthrin (Modesto 480 FS)	Clothianidin (Poncho 600 FS)			Cypermethrin (Langis)	Imidacloprid (Nuprid 600 FS)	Clothianidin (Poncho 600 FS)			Clothianidin (Poncho 600 FS)	Clothianidin (Poncho 600 FS)			Cypermethrin (Langis)
Thiamethoxam (Cruiser 350 FS)					Clothianidin (Poncho 600 FS)	Thiamethoxam (Cruiser 350 FS)			Thiamethoxam (Cruiser 350 FS)	Thiamethoxam (Cruiser 350 FS)			

2.2. Sampling

A total of 635 samples were analyzed during the five years of the conducted study. We mention that the results refer, exclusively, to the levels of the residues of the three neonicotinoid substances (imidacloprid, clothianidin and thiamethoxam) applied to rapeseed, maize and sunflower seeds.

In 2018, 105 samples were collected, of which 53 were plant samples in different phenological development stages, of which 36 samples were of flowers in the full bloom stage, namely rapeseed inflorescences, calathids and panicles. In addition, 10 bee samples, 26 pollen samples and 16 honey samples were also collected. During our first year of survey, no soil samples were collected.

In 2019, 122 samples were collected, consisting of 27 soil samples; 74 plant samples, including flowers, of which 35 were flower samples in full bloom; and 21 samples of hive products. The beehive product samples consisted of the following: 5 samples of bees, 4 samples of pollen + honeycomb and 12 samples of honey, collected from the beehives placed in the experimental fields in the 3 experimental locations.

In 2020, the total number of samples was 162, of which 53 were soil; 85 were plants, of which 40 were flower samples; and 6 samples each were of bees, pollen, honeycomb with brood and honey.

In 2021, 149 samples were collected, of which 44 were soil samples; 81 were plant samples, of which 46 were flower samples; and 24 were hive product samples, consisting of, respectively, 6 of bees, 6 of pollen, 6 of honey comb with brood and 6 of honey.

In 2022, there were 97 samples that were subjected to analysis for neonicotinoids residues, of which 38 were soil samples, 35 were plant samples and 24 were hive products.

2.2.1. Sampling of Soil

The collection of soil samples was carried out according to a specific procedure, taking into account the following steps:

Establishing a sampling plan and delimiting the soil surfaces from which the samples are to be taken, so that they do not present significant differences between them, regarding the slope, the preceding plant, etc. The average samples are obtained by combining 4 partial samples.

The time of sampling is determined according to the purpose of the studies. To determine the residues of neonicotinoids applied as seed treatment, soil samples were taken after sowing, at approximately 5–7 days, avoiding sampling immediately after rain, when the soil moisture is high, for reasons of the handling and homogenization of the soil. The actual sampling was carried out with the help of a special probe, from four points, located on the diagonal of the experimental plot, from a depth of 10 cm, making sure that the sampling area is free of organic debris.

Labeling is conducted after combining the partial samples in average samples. The samples are distributed in plastic bags and sealed; for the accurate identification of the samples, the sample location, date of sampling and the treatment/treatments applied in the experimental plot are specified on the label. Labeled samples are either immediately sent for analysis or kept in the refrigerator until shipment.

2.2.2. Sampling of Rapeseed, Maize and Sunflower Plants

For our studies there were also collected samples of plants, including flowers, in different phenophases, namely, 2–3 leaves, 14–16 leaves and at full bloom. They were harvested from the diagonal of the experimental plots, eliminating the edges. Each sample was labeled, stating its date and location. The samples were kept in the freezer, at a temperature of −20 °C, until shipment to the laboratory, to determine the level of residues.

In order to collect samples of bees and hive products, bees' families were placed in rapeseed, maize and sunflower experimental plots from each of the three locations, ARDS Secuieni, ARDS Pitesti and NARDI Fundulea, respectively

The collected samples were inventoried and maintained at −20 °C until they were sent to the analysis laboratories, depending on the minimum requirements (quantity, packaging method and shipment). The evaluation, preparation, labeling, sample registration and completion of the shipment sheets all represent very important stages for ensuring the quality of the analyses.

2.3. Sample Analysis

2.3.1. Neonicotinoid Residues Analysis in Plants and Soil

The determination of the residue levels of imidacloprid, clothianidin and thiamethoxam was performed in ISO 17025-accredited laboratories; the main international standard with general requirements for the competence of testing and calibration laboratories is ISO/IEC 17025: 2017 (SR EN ISO 17025:2018), the latest version of ISO 17025.

The samples collected in 2018 were analyzed in three laboratories, namely the reference laboratory of the EU—ANSES (France), the Quality Services International GmbH—QSI laboratory (Germany) and the PRIMORIS company laboratory, based in Plovdiv, Bulgaria; those from the following years were only analyzed in the laboratory of the Primoris Company. The standard method used for all types of samples was "Multi-residue method with LC-MS/MS for compounds, isomers and degradation products—quantification of pesticides". The determination of neonicotinoid residues in plants and soil was performed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using acetonitrile extraction and the QuEChERS method (EN 15662: 2008). The limit of quantification (LOQ) for this method is 0.01 mg/kg. The neonicotinoids were extracted from the homogenized sample with acetonitrile. The levels of the neonicotinoids, imidacloprid, thiamethoxam and clothianidin, were determined using the LC-MS/MS technique applied to the filtered extract with the Agilent Technologies 6460 TripleQuad LC/MS apparatus.

2.3.2. Statistical Analysis

The data obtained in the 5 years were processed with the GraphPad statistics program—the ANOVA one-way non-parametric test.

3. Results and Discussions

The maximum residue limits allowed considering Regulation (EC) no. 396/2005 of the European Parliament and of the Council of 23 February 2005, regarding the maximum levels applicable to pesticide residues in or on food products and feed of vegetable and animal origin, are 0.01 mg/kg in maize grains, rapeseed and sunflower and 0.05 mg/kg for honey and other hive products, following the implementation of Regulation 1881/2021. By Regulation 671/2017, the European Commission imposed maximum residue limits for thiamethoxam in maize, honey and hive products of 0.05 mg/kg and for rapeseed and sunflower of 0.02 mg/kg. The same Regulation specifies the EU residues limits for clothianidin of 0.02 mg/kg in maize, rapeseed and sunflower and of 0.05 mg/kg in honey and hive products.

According to the presented data in Table 4, it is highlighted that the highest percentage of samples with residues above the limit of quantification was recorded in the soil samples, which is absolutely normal, since the three neonicotinoid substances were applied in the form of seed treatment.

Gasparic et al., [31] also stated that high concentrations of neonicotinoids in soil are to be expected in case of dry conditions, leaching incapacity or irregular flushing (bottom of the container) into groundwater meaning that they can present a potential risk for the succeeding crops.

Moreover, Schaafsma et al. [32] found that the average neonicotinoid concentration in the soil sampled to the 5 cm depth in the corn fields after planting seeds treated with neonicotinoids was approximately 2.5 times higher than that found in soil sampled before planting.

Thus, out of the 27 soil samples collected in 2019, from 3 three areas where the experimental fields were located, 44.44% contained neonicotinoid residues. In 2020, out of the 53 samples, the percentage of samples with residues higher than the LOQ, was 9.43%. In 2021, of the 44 samples analyzed, 27.27% contained residues of one of the neonicotinoid substances applied to the seed. In 2022, from 38 soil samples sent to analysis, only 31.57%, representing 12 samples, registered neonicotinoid residues above the LOQ.

Our results regarding the percentage of soil samples containing neonicotinoid residues are in accordance with authors [33] whom concluded that the high water solubility of neonicotinoids from seed treatment makes it unlikely that they will remain near the relatively confined rhizosphere of the target plant long enough to be absorbed by the plant when not on the seed. Obviously, the degradation of neonicotinoids is an important issue, which has been addressed in numerous experimental and theoretical studies: the loss of neonicotinoids from agricultural soils is thought to occur through degradation or leaching in soil water [34,35].

Table 4. Summary of the results regarding the level of residues of imidacloprid, clothianidin and thiamethoxam during the 5 years of studies.

Sample Type		2018		2019		2020		2021		2022	
		Samples Total No.	% Samples > LOQ	Samples Total No.	% Samples > LOQ	Samples Total No.	% Samples > LOQ	Samples Total No.	% Samples > LOQ	Samples Total No.	% Samples > LOQ
SOIL		-	-	27	44.44 (12 spl)	53	9.43 (5 spl)	44	27.27 (12 spl)	38	31.57 (12 spl)
PLANT (including flowers)		53	20,75 (11 spl)	74	10,81 (8 spl)	85	5.88 (5 spl)	81	13.58 (11 spl)	-	-
FLOWERS		36	16.66 (6 spl)	35	0	40	0	46	0	35	0
HIVE products	Bees	10	20 (2 spl)	5	0	6	0	6	6	6	0
	Pollen	26	26.92 (7 spl)	4 (pollen + honey comb)	0	6	0	6	6	6	16.66 (1 sample pasture)
	Honeycomb with brood	-	-	-	-	6	0	6	6	6	0
	Honey	16	0	12	0	6	0	6	6	6	0
TOTAL		105	19.04 (20spl)	122	16.39 (20 spl)	162	6.17 (10 pl)	149	15.43 (23 spl)	97	13.41 (13 spl)

TOTAL ANALYZED SAMPLES: 635 from which 13.55% (86 probe) > LOQ
LOQ (quantification limit) = 0.01 mg/kg sample; MRL honey and hive products = 0.05 mg/kg sample

The EFSA's risk assessment did not take into account the results of the low probability of residues of neonicotinoids remaining in soil for a longer period of time. Their findings, together with those of the recycling of neonicotinoid insecticides from contaminated groundwater back to crops, point to the possible risk scenario of irrigation.

Monitoring the residue level in soil is very important, since large amounts of pesticides reach the soil, either through direct application or through the fall of vapors on the soils' surface, following spraying, through rain, dust, plant or animal remains, which are later incorporated in the soil. The presence of pesticide residues is a cause for concern, as many compounds give serious adverse effects. The extensive use of pesticides of any kind has created serious problems related to their residues in soil, the pests' resistance and the health problems due to them [36,37].

Regarding the plant samples, in different phenological development stages, including flowers, it is observed that the percentage of positive samples decreased compared to that of the soil samples (Figure 1). Thus, in 2018, 20.75% of the 53 samples had neonicotinoid residues higher than the quantification limit; of the 36 flower samples, only 6 samples, representing 16.66%, contained residues above the LOQ.

In 2019, the percentage of samples with residues above the LOQ decreased to 10.81%; in flowers—rapeseed inflorescences, sunflower calathids and corn panicles—no neonicotinoid residues were identified. In 2020, out of the 85 samples from plants, including flower samples, only 5 samples contained residues, and no residues of the 3 neonicotinoid substances were identified in flowers samples. In 2021, the percentage of plant samples with a residue content above the limit of quantification was 13.58%, and of the 46 flower samples, none contained neonicotinoid residues. In addition, in 2022, from 35 flowers samples, none of them had residues of neonicotinoid substances.

In all positive soil samples (Figure 1, Table 5), values were registered for imidacloprid of 0.01 mg/kg up to 0.18 mg/kg, for clothianidin between 0.01 mg/kg and 0.044 mg/kg, and for one positive sample containing thiamethoxam of 0.013.

Among all plant and flowers samples from all three crops, values of neonicotinoid active substances determined above the LOQ were between 0.012 mg/kg and 0.71 mg/kg for imidacloprid; 0.012 mg/kg and 0.24 mg/kg for clothianidin; and between 0.013 mg/kg and 0.35 mg/kg for thiamethoxam, in plants during early vegetation stages.

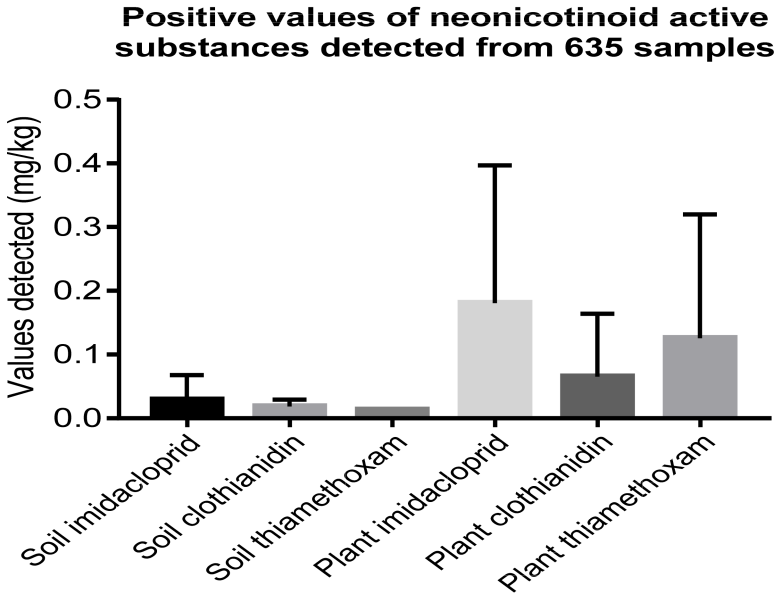


Figure 1. Values of neonicotinoids above the LOQ detected in analyzed samples.

Table 5. Descriptive statistical analysis of neonicotinoids values detected above LOQ.

Descriptive Statistics	Soil Imidacloprid	Soil Clothianidin	Soil Thiamethoxam	Plant Imidacloprid	Plant Clothianidin	Plant Thiamethoxam
Number of values	39	9	1	18	5	3
Minimum	0.01	0.01	0.013	0.012	0.012	0.013
25% percentile	0.012	0.0115	0.013	0.029	0.0125	0.013
Median	0.015	0.012	0.013	0.0885	0.018	0.014
75% percentile	0.026	0.023	0.013	0.235	0.142	0.35
Maximum	0.18	0.044	0.013	0.71	0.24	0.35
Mean	0.02913	0.01844	0.013	0.1807	0.0654	0.1257
Std. deviation	0.03849	0.01092	0	0.2161	0.09847	0.1943
Lower 95% CI of mean	0.01665	0.01005		0.0732	−0.05687	−0.3569
Upper 95% CI of mean	0.0416	0.02684		0.2881	0.1877	0.6083
Coefficient of variation	132.13%	59.21%	0.00%	119.62%	150.57%	154.60%

According to the concentrations of neonicotinoids detected (Figure 1) and the percentage of positive samples identified (Figure 2), it can be stated that these substances are found more often in soil samples than in plant samples, but at lower concentrations. This fact demonstrates that the substance applied to the seeds migrates from the soil to the aerial organs of the plants, where it provides protection against pests.

The hive product samples consisted of pollen, comb with brood and honey. The 10 bee samples collected from the 3 experimental fields in 2018 were the only ones in which the presence of 1 of the 3 neonicotinoid substances was identified, above the limit of quantification, in a percentage of 20% (2 samples). In the other years, 5 samples were collected in 2019 and 6 samples in each of the following years, and none of these samples contained neonicotinoid residues. In the case of pollen samples, residues were identified only in 2018: out of the 26 samples, 7 samples contained residues above the quantification limit (26.92%). In the other years, no residues were identified in any of the 22 samples (4 samples in 2019 and 6 samples each in 2020, 2021 and 2022). In the 18 samples of honeycomb with brood, harvested between 2020 and 2022, no neonicotinoid residues were

identified. In the 46 honey samples collected during the 5-year survey, no residues above the limit of quantification were identified.

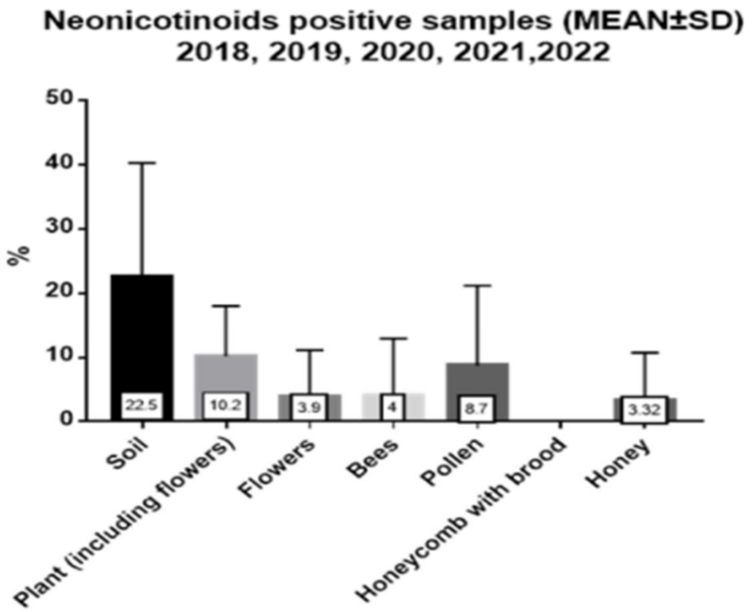


Figure 2. Percentage of NNI-positive samples.

Over 216 studies have been conducted in Europe and North America [38] regarding the influence of the neonicotinoid imidacloprid on *Apis mellifera*. The studies were mostly carried out in corn, canola and sunflower crops, with neonicotinoids being applied to the seed. The use of neonicotinoids is considered a contributing factor to the decline of bee populations. Some of these substances have been shown to be toxic to bees in very small amounts, but, from the estimates made so far, the exposure of bees to neonicotinoids is generally substantially lower than the level at which they cause acute mortality. Neonicotinoid residues can be found in pollen and nectar, the main food sources for bees. Research conducted under field conditions identified the presence of neonicotinoid residues below the limit causing acute mortality. However, these amounts can cause non-lethal effects, which are very difficult to establish.

Statistical analysis demonstrates that there are significant differences between the amounts of insecticides determined. Highlighting a higher value of neonicotinoids in pollen could indicate bioaccumulation phenomena in biological structures with a high lipid content, which could impose the need to analyze the oily extracts from plants, as a potential indicator of the degree of contamination.

The analysis of the variation coefficient demonstrates that the density of neonicotinoid-positive samples decreases with increases in the distance from the application site or with the dilution induced by the accumulation of plant biomass. However, the soil value of the variation coefficient alone (78.84%) could indicate accumulation phenomena or a slow rate of degradation of the active substance in the soil (Table 6).

Regarding the quantities of insecticides determined from the soil samples that exceeded the minimum residue threshold, it can be highlighted that the standard deviation is higher in the case of imidacloprid: this fact could be caused by the accumulation of the substance in the soil from the previous crops according to an approximation of 234 g of S.A/ha in the 0–10 cm layer. In the case of corn, a maximum of 150 g/ha is administered in the treatment of seeds. The mean and standard deviation graphic representation reflects the high variability of the analyzed samples.

Table 6. Descriptive statistical analyses of NNI-positive samples.

Column Statistics	Soil	Plant (Including Flowers)	Flowers	Bees	Pollen	Honeycomb with Brood	Honey
Number of values	5	5	5	5	5	5	5
Minimum	0	0	0	0	0	0	0
25% percentile	4.715	2.94	0	0	0	0	0
Median	27.27	10.81	0	0	0	0	0
75% percentile	38.01	17.17	9.755	10	21.79	0	8.3
Maximum	44.44	20.75	16.66	20	26.92	0	16.6
Mean	22.54	10.2	3.902	4	8.716	0	3.32
Std. deviation	17.77	7.839	7.238	8.944	12.47	0	7.424
Std. Error of mean	7.948	3.506	3.237	4	5.579	0	3.32
Lower 95% CI of mean	0.4745	0.4706	−5.085	−7.106	−6.772	0	−5.898
Upper 95% CI of mean	44.61	19.94	12.89	15.11	24.2	0	12.54
Coefficient of variation	78.84%	76.82%	185.49%	223.61%	143.12%	+infinity%	223.61%

In 2018, the "Science" Journal [39] published an article that had as its subject a new field study on neonicotinoid insecticides, carried out at a pan-European level by the Centre for Ecology and Hydrology of Great Britain (CEH). The study was carried out in Germany, Great Britain and Hungary and aimed to determine the potential impact of the use of plant protection products based on neonicotinoid active substances clothianidin and thiamethoxam, in rapeseed, on bee colonies, under real conditions of exposure. The study indicated that treating canola seeds with the neonicotinoids clothianidin and thiamethoxam would not have a negative effect on honeybees or solitary bees in the three countries. The research results showed that the specific differences between countries in terms of impact indicate that "the effects of neonicotinoids are the result of a combination of factors". Moreover, further analyses showed that the initial size of the swarm is a key indicator, which can explain the subsequent changes it underwent. When colony size differences are properly assessed at the outset, the study data show no difference between colonies that pollinated treated and untreated canola crops.

Schmuck and Lewis, [40], present, in a synthesis article, studies carried out on 402 bee colonies, following the influence of dust resulting from the sowing of seeds treated with neonicotinoids. In most cases, the risk to bees was classified as "minor", and it is necessary to take into account the risk of using other pesticides, which can often be even higher, compared to that of neonicotinoids.

More recent studies [41,42] present research carried out for 3 years, regarding the risk to which bees (*Apis mellifera*) are exposed in a sunflower crop, the seeds being treated with thiamethoxam and clothianidin. The studies were carried out with 180 bee colonies, the health and development of the colonies being evaluated by monitoring the adult population, the development of the brood, the state of the queen, the food reserve and the percentage of survival. After three years of studies, no significant effects of neonicotinoid substances applied to the seed were found, with bee colonies being the main factor for the variability of the results.

It is considered that seed treatment technology with plant protection products based on active substances from the neonicotinoid class has no negative effects, in the short or long term, on bees, when the products are used responsibly and properly, in accordance with the instructions of on the label. We believe that this technological component is essential in supporting sustainable intensive agriculture with a minimal impact on the environment and the biodiversity of beneficial insects and represents an essential and effective crop protection tool for farmers.

The results of our study provide additional arguments for a possible risk assessment for seed treatment with neonicotinoids in the succeeding crop and irrigation scenarios and provide further guidance for the assessment and/or reassessment of the use of neonicoti-

noids in OSR, maize and sunflower production. However, further investigation is needed to assess the possible neonicotinoids uptake by succeeding crops.

The scientific community from Romania is constantly facing the challenge of finding effective alternatives to control the *T. dilaticollis* species, but, for now, for certain areas of the country, treating seeds with neonicotinoids is the only alternative for maize and sunflower producers.

We believe that research for establishing the effects of neonicotinoids on bees in Romania requires further complex investigations, especially through case studies, in the real conditions of the depopulation and mortality of bee families in the targeted crops, to quantify the residues in the conditions of depopulation and actual deaths.

4. Conclusions

Among the analyzed soil samples, only 25.3% had neonicotinoid residues above the limit of quantification. From the plant samples, 11.94% had residues above the LOQ. From the flower samples, no more 3.12% had residues that exceeded the limit of quantification. Regarding bee samples, 1.65% had neonicotinoid residues above the LOQ, at 16.66%. Of the pollen samples, the residues exceeded the limit of quantification. In samples of honeycomb with brood and honey samples, no residues of any of the three substances were identified.

The residue levels in all plants studied were highly dependent on weather conditions, in particular, rainfall. The results of this study highlighted that the seed treatments leave minimal traces in plants because of their complete degradation by the end of the growing season while higher residue concentrations in soil show that there may be a risk in dry climates or after a dry period.

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