

## Article

# Comparative Analysis of Pesticide Residues in Hive Products from Rapeseed (*Brassica napus* subsp. *napus*) and Sunflower (*Helianthus annuus*) Crops Under Varying Agricultural Practices in Romania During the 2020–2021 Beekeeping Seasons

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## Abstract

Over the past years, increasing attention has been drawn to the adverse effects of agricultural pesticide use on pollinators, with honeybees being especially vulnerable. The aim of this study was to evaluate the levels of residues detectable and/or quantifiable of neonicotinoid pesticides and other pesticides in biological materials (bees, bee brood, etc.) and beehive products (honey, pollen, etc.) applied as seed dressings in rapeseed and sunflower plants in two growing seasons (2020–2021) in fields located in three agro-climatic regions in Romania. The study involved the comparative sampling of hive products (honey, pollen, adult bees, and brood) from experimental and control apiaries, followed by pesticide residue analysis in an accredited laboratory (Primoris) using validated chromatographic techniques (LC-MS/MS and GC-MS). Toxicological analyses of 96 samples, including bees, bee brood, honey, and pollen, confirmed the presence of residues in 46 samples, including 10 bee samples, 10 bee brood samples, 18 honey samples, and 8 pollen bread samples. The mean pesticide residue concentrations detected in hive products were 0.032 mg/kg in honey, 0.061 mg/kg in pollen, 0.167 mg/kg in bees, and 0.371 mg/kg in bee brood. The results highlight the exposure of honeybee colonies to multiple sources of pesticide residue contamination, under conditions where legal recommendations for the controlled application of agricultural treatments are not followed. The study provides relevant evidence for strengthening the risk assessment framework and underscores the need for adopting stricter monitoring and regulatory measures to ensure the protection of honeybee colony health.

**Keywords:** bees; hive products; pesticide residues



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## 1. Introduction

Pesticides are widely recognized as a major driver in the decline of honeybee colonies, acting synergistically with multiple stressors, such as pathogens [1]; parasites, especially *Varroa destructor* [2]; climate change [3]; poor nutrition [4]; habitat loss [5]; and intensified agricultural practices [6]. Given the essential role of *Apis mellifera* in the pollination of crops, declines in bee populations pose a significant threat to ecosystem functioning and food security [7,8]. Overall, honeybees are exposed to multiple stressors resulting from habitat degradation and the increased use of synthetic chemical substances in agriculture [8,9].

The active substances, commonly applied at the seed or soil level, can be absorbed by plants and translocated systemically to all tissues, including floral structures. During the flowering stage, these compounds can be detected in pollen and nectar-essential nutritional sources for honeybees. Once collected by worker bees, these contaminated resources are brought into the hive and used to feed both larvae and the queen. In this way, the initial exposure infiltrates the internal trophic chain of the colony, negatively impacting larval development, altering adult bee behavior, and ultimately compromising the overall stability and functionality of the hive [10,11].

In the European Union, the use of neonicotinoids is prohibited, the ban being in force since 2018, according to Regulations 783/2018, 784/2018, and 785/2018. However, according to Article 53 of Regulation (EC) No. 1107/2009, European legislation allows for the temporary authorization (up to 120 days), only in exceptional circumstances (when no other means can control an imminent danger, such as a rare pest), of plant protection products not approved or restricted by the EU [12]. In March 2019, the European Commission requested the European Food Safety Authority (EFSA) to update its guidance document on the risk assessment of plant protection products for honeybees [13]. In this context, strict criteria were established for the authorization of pesticides, aiming to limit colony exposure and prevent both acute and chronic effects, including those affecting larvae and individual bee behavior [14]. EFSA's recent reviews [15] concluded that colony mortality has significantly increased across Europe, with limited but notable contributions from beekeeping practices [16].

In line with the European Commission's recommendations, four key priorities have been established regarding the effects of pesticides on pollinators: assessment of acute and chronic toxicity on colonies, evaluation of long-term low-dose exposure, analysis of cumulative effects and active substance mixtures, and the adaptation of testing protocols to better reflect real exposure scenarios through nectar and pollen [17]. Evaluation methods developed for *Apis mellifera* include 7–10-day toxicity tests for adults and larvae under laboratory and field conditions [18,19]. However, current protocols remain limited in detecting sublethal, inhalation, or prolonged exposure effects, particularly in larvae. Improved detection, monitoring, and statistical tools are needed to reduce uncertainty in risk assessment [20]. The European Commission's 2019 initiative further emphasized pollination as a critical ecosystem service for biodiversity and agricultural productivity, prompting revisions to EFSA's methodological framework [21].

Chemical intoxication in bees is frequently caused by insecticides and other pesticides that affect the nervous, respiratory, and digestive systems [22,23]. Exposure to sublethal doses may reduce productivity, impair brood development, alter hygienic behavior, and weaken immune responses [24,25]. Pesticide residues can exert negative effects at all stages of the life cycle, both through direct and indirect contact [26–28]. Scientific studies highlight that such effects can influence memory, immunity, and colony efficiency. Moreover, interactions between active substances can enhance toxicity, affecting larval development, survival, and behavior and the longevity of worker bees through mechanisms such as enzyme detoxification inhibition or delayed development [29].

The composition and concentration of pesticide residues depend on the type of crop, the application methods, and the degradation rate of the active substance. Although most research focuses on adult bees, the brood stage represents a critical phase in colony development and stability [26–28]. Chronic exposure to sublethal doses has been associated with significant negative effects on colony health, particularly during the cold season, when stress levels are elevated [30].

Neonicotinoids, introduced in the 1990s, have become one of the most widely used classes of insecticides globally, often applied as seed treatments. Due to their high water solubility, these substances are absorbed by plants and become systemic, being distributed throughout all plant tissues, including nectar and pollen, thereby providing protection against phytophagous insects. In recent years, increasing concerns have been raised regarding the effects of neonicotinoids on non-target organisms. During mechanical sowing, their dispersion in dust form has been linked to mass bee poisoning incidents in Germany and Italy, and residues have been detected in soil as well as in the pollen and nectar of treated plants [31]. These insecticides have been associated with negative ecological impacts, including the emergence of colony collapse disorder (CCD) [32,33].

This research addresses the current data gaps highlighted by experts [17], particularly those concerning sublethal pesticide exposure. While concerns regarding potential physiological and behavioral effects remain relevant, the study does not directly investigate toxicological outcomes. Instead, its primary objective is to identify and quantify pesticide residues in beehive products (honey, pollen, bees, and bee brood) collected during 2020–2021 and obtained from distinct beekeeping contexts. The experiments were conducted in representative agricultural areas from Romania, cultivated with rapeseed (*Brassica napus*) and sunflower (*Helianthus annuus*). A comparative approach was applied, involving an experimental group from fields treated with known active substances and a control group from private apiaries where colonies showed clinical signs of intoxication of unknown etiology. The goal was to assess potential risks to honeybee (*Apis mellifera* L.) health and consumer safety in the context of current intensive agricultural practices as well as to evaluate contamination patterns under different exposure scenarios. This study also serves as a pilot study designed to validate analytical methodologies for future implementation in large-scale monitoring programs across broader agro-climatic regions.

The present study represents a continuation of a previous research [33], which focused on monitoring neonicotinoid residues in soil, plants, honeybees, and hive products. The experiments in that study were conducted on agricultural plots where seeds were treated with accurately dosed active substances, allowing for a controlled assessment of residue transfer within the ecosystem. In contrast, the current study places greater emphasis on honeybee (*Apis mellifera*) hive products and particularly on bee brood, a critical developmental stage within the colony. Moreover, the toxicological scope has been expanded to include additional classes of pesticides (fungicides, herbicides, acaricides, and insecticides), offering a more complex and realistic depiction of field exposure. While the methodology retains certain similarities, this continuity allows for consistent comparison of results across different years and agro-climatic regions. Analyses were carried out in ISO 17025-accredited laboratories [34,35] by specialists in honeybee pathology, ensuring scientific rigor. Furthermore, the inclusion of new biological samples increases the study's relevance by enabling a more comprehensive evaluation of cumulative risks under real-world agricultural conditions.

## 2. Materials and Methods

### 2.1. Experimental Design

Studies on the residues of neonicotinoid (acetamiprid, clothianidin, imidacloprid, and thiamethoxam) and other pesticides used in two agricultural crops, along with their impact on bees and beehive products, were conducted during 2020–2021.

The study utilized two groups of bee colonies: experimental and control groups. The experimental group consisted of clinically healthy bee colonies (3 colonies for each crop and each location, totaling 18 colonies per year) from the Beekeeping Research and Development Institute Bucharest [36] (Băneasa-București—44°29033" N, 26°04045" E), which did not receive preventive external treatments, while the control group included colonies from private apiaries in the same regions (one apiary for each culture and each county, totaling six apiaries each year), treated preventively with external anti-parasitic products. The main difference between the groups lies in the application of these preventive treatments. Additionally, the methodology and substances used for treating agricultural crops were known for the experimental group, whereas the details of the methodology and active substances used for the agricultural crops in the control group were not known.

Colony selection within each apiary was based on clinical assessment supported by laboratory analysis. In the experimental group, colonies were identified as clinically healthy through field inspection and confirmed by subsequent laboratory testing. In the control group, colonies exhibiting symptoms indicative of pesticide intoxication were selected, and their condition was similarly verified through diagnostic analysis. This targeted selection strategy aimed to provide a meaningful contrast in contamination profiles between groups. The absence of randomization is acknowledged as a methodological limitation.

While referred for comparison, the control group does not represent a true negative control, as it was not established in pesticide-free or geographically isolated areas. Its selection was constrained by practical field access and logistical limitations. Furthermore, pesticide exposure in the foraging environment of the reference colonies was not systematically monitored, making it difficult to determine whether detected residues resulted from background contamination, incidental exposure, or nearby agricultural practices. The intent of the comparison was therefore not to isolate toxicological effects but to assess contamination levels across real-world exposure scenarios. This limitation is acknowledged, and future research will aim to incorporate well-characterized negative control sites to improve experimental rigor.

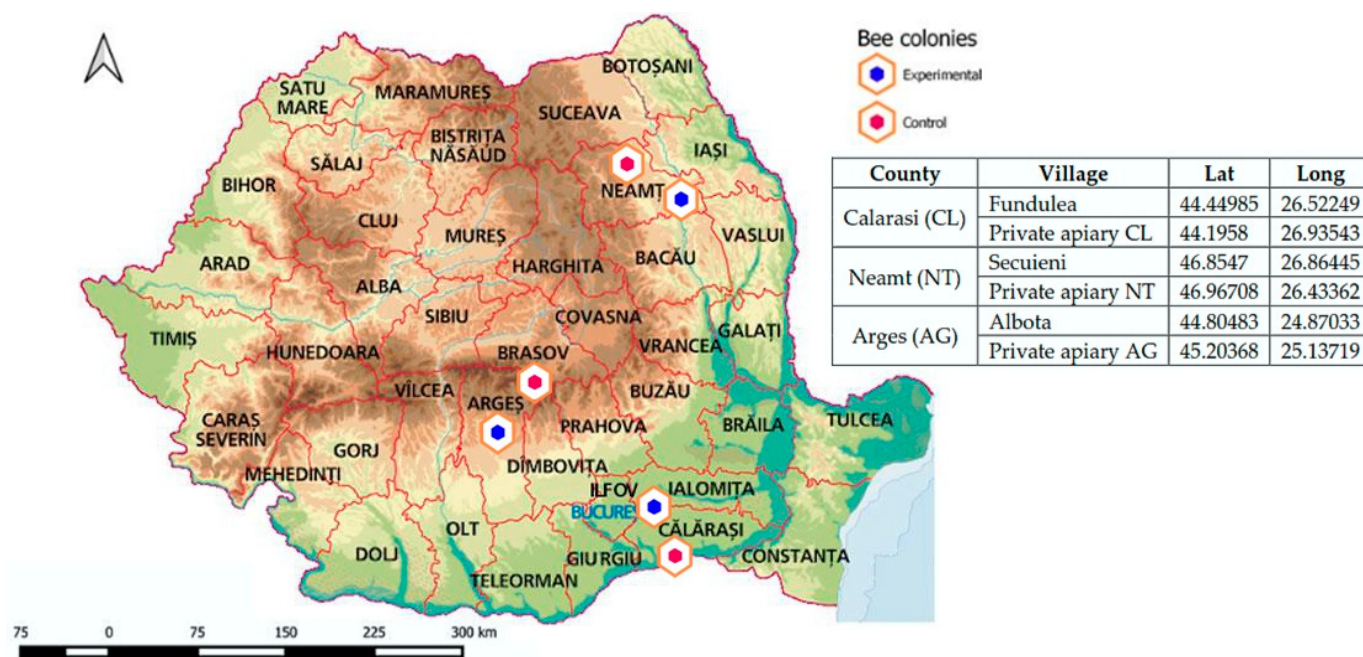
### 2.2. Crop Species

The experiments were conducted in areas cultivated with rapeseed (*Brassica napus*) and sunflower (*Helianthus annuus*). Only two crops were selected, as they are the main honey sources exploited by beekeepers in Romania, playing a central role in the national pastoral calendar. Their choice was based both on agricultural importance and on field observations that signaled recurrent depopulations of colonies immediately after the end of flowering periods, in the absence of clinical manifestations specific to known bee pathologies. Furthermore, the phenology of these crops—flowering in April (rapeseed) and July (sunflower)—overlaps with peak beekeeping periods, which makes them critical contexts for assessing the possible sublethal effects of pesticides on bee colony.

### 2.3. Study Area

The bee colonies of the experimental group were transported to three locations: Fundulea (CL, Călărași County—44°27010" N, 26°30055" E), Secuieni (NT, Neamț County—46°51045" N, 26°49042" E), and Albota (AG, Argeș County—44°46054" N, 24°49031" E) (Figure 1). These locations were situated near oilseed rape and sunflower crops (in close

proximity, the distance between the hives and fields being about 10 m), which were treated according to legal regulations (conventional crops, with a cultivation area of 8–14 hectares, located in a plain area), over two consecutive beekeeping seasons (2020–2021).



**Figure 1.** The locations where the monitored bee colonies were placed.

The experimental groups, which involved the application of neonicotinoids to the seed depending on the crop, are detailed in Table 1.

**Table 1.** Chemical treatments in rapeseed and sunflower used as experimental fields.

| Crops     | Year | Active Substances Used                                                         |                                                                                               |                                                                                               |
|-----------|------|--------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
|           |      | Secuieni Group (NT)                                                            | Albota Group (AG)                                                                             | Fundulea Group (CL)                                                                           |
| Rapeseed  | 2020 | Imidacloprid (Nuprid 600 FS)<br>Clotihanidin + betacyfluthrin (Modesto 480 FS) | Imidacloprid (Nuprid 600 FS)<br>Clotihanidin + betacyfluthrin (Modesto 480 FS)                | Imidacloprid (Nuprid 600 FS)<br>Clotihanidin + betacyfluthrin (Modesto 480 FS)                |
|           | 2021 | Cyantraniliprole (Lumiposa 625 FS)                                             | Cyantraniliprole (Lumiposa 625 FS)                                                            | Cyantraniliprole (Lumiposa 625 FS)                                                            |
| Sunflower | 2020 | Imidacloprid (Nuprid Al)<br>Clotihanidin (Poncho 600 FS)                       | Imidacloprid (Nuprid 600 FS)<br>Clotihanidin (Poncho 600 FS)<br>Thiamethoxam (Cruiser 350 FS) | Imidacloprid (Nuprid 600 FS)<br>Clotihanidin (Poncho 600 FS)<br>Thiamethoxam (Cruiser 350 FS) |
|           | 2021 | Cypermethrin (Langis)                                                          | Cypermethrin (Langis)                                                                         | Cypermethrin (Langis)                                                                         |

The application rates of each active ingredient used as seed treatment, expressed in g a.i./kg seed, are given in Table 2. Pesticides were applied as seed treatments for rapeseed and sunflower prior to sowing, using commercially authorized doses approved for agricultural use in Romania. Application occurred once, with all active substances administered simultaneously, and no additional treatments were performed during crop development. No pesticides were applied during the flowering phase, but residues could be systemically translocated into pollen and nectar, exposing bees during foraging.

**Table 2.** Standard seed treatment doses in oilseed rape and sunflower crops (expressed as g active ingredient/kg seed) \*.

| Crop         | Active Substance              | Commercial Product | Concentration (g/L) | Recommended Dose (mL/kg Seed) | Equivalent Dose (g a.i./kg Seed) |
|--------------|-------------------------------|--------------------|---------------------|-------------------------------|----------------------------------|
| Oilseed rape | Imidacloprid                  | Nuprid 600 FS      | 600                 | 10                            | 6.0                              |
|              | Clotianidin + Beta-cyfluthrin | Modesto 480 FS     | 400 g/L + 80        | 10                            | 4.00 + 0.80 = 4.80               |
|              | Cyantraniliprol               | Lumiposa 625 FS    | 625                 | 10                            | 6.25                             |
| Sunflower    | Imidacloprid                  | Nuprid AI/600 FS   | 600                 | 10                            | 6.0                              |
|              | Clotianidin                   | Poncho 600 FS      | 600                 | 10                            | 6.0                              |
|              | Thiamethoxam                  | Cruiser 350 FS     | 350                 | 10                            | 3.5                              |

\* Application rates are based on label-recommended seed treatment practices approved in Romania. All values are calculated according to the commercial product formulation and application volume per kg of seed.

#### 2.4. Sampling

The experiment was carried out during the flowering period of rapeseed and sunflower crops, representative of agricultural regions in Romania. Honey sample classification was performed at a RENAR-accredited Chemistry Laboratory, using a combination of chemical analysis, organoleptic evaluation (assessing color, odor, taste, and consistency), and microscopic pollen analysis. This integrated approach ensured accurate determination of the botanical origin of the rapeseed and sunflower honey samples included in the study.

Between 2020 and 2021, a total of 96 samples were collected, equally distributed between the experimental group (48 samples) and the control group (48 samples), as shown in Table A1 (Appendix A). The samples were categorized into four types: adult bees, brood, honey, and pollen/bee bread. Each location provided samples from both oilseed rape and sunflower crops, resulting in 48 samples in total (24 per crop): 12 bee samples, 12 brood samples, 12 honey samples, and 12 pollen samples. In parallel, 48 samples were also collected from private apiaries located in the same regions as the experimental sites, representing the control group. These apiaries had been treated with anti-Varroa products. Sampling was carried out at regular intervals using standardized procedures. All samples were stored following accredited Primoris protocols to ensure integrity for residue analysis.

A total of 18 monitoring sheets (detailed records for tracking sample collection, analysis, and results) were prepared, one for each location and crop type: 9 for oilseed rape and 9 for sunflower. For this study, each of the three locations required three monitoring sheets per year, totaling six sheets per location over the two years.

All examination protocols followed the standards set by the World Organisation for Animal Health (OIE) [37]. After conducting a thorough anamnesis—including colony history, environmental conditions, and prior treatments—detailed morpho-clinical assessments were carried out. These involved direct visual inspections to identify abnormal behavior, changes in physical appearance, and altered flight activity in the bees.

Bacterioscopic evaluations using direct microscopy with Gram staining were performed to detect bacterial pathogens. Bacteriological tests included culturing samples on selective and differential media to identify the etiological agents of significant bacterial diseases in bees, such as American foulbrood and European foulbrood. Mycological examinations aimed to identify fungal agents responsible for conditions like *Ascosphaerosis* and *Aspergillosis*, while parasitological tests focused on detecting internal and external parasites, including *Varroa*, *Braula*, *Acarapis*, *Tropilaelaps*, *Nosema*, and *Malpighamoeba*. Detailed methods for laboratory diagnosis of the primary parasitic, bacterial, mycotic diseases, as well as bee poisonings are provided in Table A2 (Appendix A).

### 2.5. Sample Analysis

The samples selected for toxicological analysis were placed in suitable containers, coded, labeled, and conditioned according to the established protocol (Table 3) before being sent to the ISO 17025-accredited laboratory Primoris-Bulgaria for analysis.

**Table 3.** Protocol for the collection, storage, and transport of bee biological material.

| No. | Type of Biological Material | Quantity per Sample                          | No of Samples | Accepted Containers                                                              | Storage and Transport Conditions                              |
|-----|-----------------------------|----------------------------------------------|---------------|----------------------------------------------------------------------------------|---------------------------------------------------------------|
| 1.  | Adult bees                  | Minimum 200 g                                | 24            | Plastic or glass containers, resistant to $-20^{\circ}\text{C}$ , tightly sealed | Frozen, transported within 24 h                               |
| 2.  | Honey                       | 250 g or $15\text{--}20\text{ cm}^2$ of comb | 24            | Food-grade plastic containers                                                    | Stored at room temperature, protected from light and heat     |
| 3.  | Pollen/Bee bread            | 150 g                                        | 24            | Food-grade plastic containers                                                    | Refrigerated ( $4^{\circ}\text{C}$ ), transported within 24 h |
| 4.  | Bee brood (comb section)    | $15\text{--}20\text{ cm}^2$                  | 24            | Plastic containers                                                               | Refrigerated ( $4^{\circ}\text{C}$ ), transported within 24 h |

The standard method employed for analyzing all samples types was “multi-residue method with LMS–LC-MS/MS for compounds, isomers, and degradation products—quantification of pesticides”. According to the protocols used by Primoris Laboratory, pesticides were extracted from the samples using acidified acetonitrile (AOAC-modified QuEChER). Following extraction, samples were diluted and analyzed using LC-MS/MS and GC-MS/MS techniques. The limit of quantification (LOQ) for this method is 0.01 mg/kg. Each sample was initially analyzed once; if the maximum residue levels were exceeded, the sample was re-extracted and re-analyzed to confirm the results. To validate the data obtained, the procedure VAL1\_E—“Validation requirements for pesticides”—was applied (see Supplementary Materials).

### 2.6. Statistical Analysis

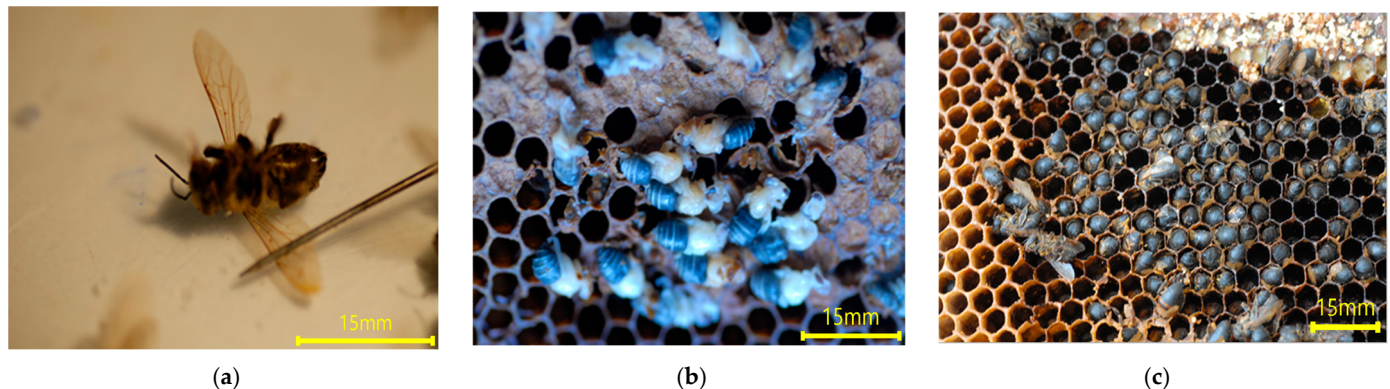
The statistical analysis was performed using IBM SPSS Statistics software (version 19, 2011). The Kruskal–Wallis H-test “one-way ANOVA” was applied to assess differences in variance for variables that did not meet the assumption of normal distribution, while Mann–Whitney U-test was used for pairwise comparisons between groups under the same condition. For variables that followed a normal distribution, the LSD (least significant difference) test was employed. No statistical analysis was performed for parameters with a single value in one of the two groups. If no similar products were identified between the years, no statistical analysis was carried out with the year as a fixed factor. Treatments and products (bee, bee bread, honey, and pollen) were established as fixed factors in the statistical analysis. Parameters that showed values only in one of the two groups were excluded from comparative statistical testing. The selection of these statistical methods was based on the distribution characteristics and variable types, ensuring the validity and relevance of the results obtained when comparing the experimental and control groups.

### 2.7. Clinical and Morphological Findings of Suspected Intoxication in Honey Bees: Evidence from Anamnesis and Laboratory Examinations

To evaluate the suspicion of intoxication in honey bee colonies, an integrated approach was applied, including detailed anamnesis, morpho-clinical examinations, and laboratory testing. These investigations, carried out in a specialized apicultural pathology laboratory,

aimed to exclude alternative causes such as infectious diseases, parasitic infestations, or technological stress factors. Once these potential etiologies were ruled out, a presumptive diagnosis of chemical intoxication was established, in accordance with diagnostic protocols cited in previous studies [38–40].

The clinical and morphological signs supporting this suspicion included darkened bees with extended proboscis, high mortality inside and around the hive, wings held abnormally at 90°, digestive disorders (alternating diarrhea and constipation), abdominal bloating, exposed stingers, reduced activity inside and outside the hive, paresis, paralysis, and ultimately colony decline (Figure 2). These observations are consistent with intoxication symptoms reported in international research [36,41,42].



**Figure 2.** Clinical signs of poisoning in bee, (a); bee brood (b), and foraging bees (c).

The detection of pesticide residues in biological samples and hive products was performed in an accredited laboratory (Primoris), further supporting the hypothesis of toxic exposure.

### 3. Results

#### 3.1. Evaluation of the Experimental Group Results

During 2020, in the experimental group located in the rapeseed crop, acaricides (tau-fluvalinate and bromopropylate) were identified in live bee samples and hive products (honey and pollen) (Table 4). In contrast, during 2021, a neonicotinoid (acetamiprid) and fungicides (prothioconazole-dethio, tebuconazole, and trifloxystrobin) were detected in the pollen.

The main difference between the years lies in the types of pesticides identified: in 2020, a range of insecticides, acaricides, neonicotinoids, and fungicides were found, while in 2021, only fungicides were detected in the pollen. No common pesticides were identified in both analysis reports for the sunflower crop between the two years.

**Table 4.** Positive samples with the presence of pesticide and/or insecticide residues in the oilseed rape and sunflower crops, in the 2020 and 2021 active seasons (Experimental group).

| Year | Group Location | Crop         | Type of Sample | Tau-Fluvalinate (mg/kg)<br>(Limit 0.01 mg/kg) | Other Pesticides (mg/kg)                     |
|------|----------------|--------------|----------------|-----------------------------------------------|----------------------------------------------|
| 2020 | Fundulea (CL)  | Oilseed rape | Honey          | 0.12                                          | Bromopropylate—0.011                         |
|      |                |              | Pollen         | 0.38                                          | -                                            |
|      |                | Sunflower    | Honey          | -                                             | Acetamiprid—0.05<br>dimoxystrobin—0.05       |
|      |                |              | Bees           | -                                             | piperonyl-butoxide—0.01<br>tetramethrin—0.01 |
|      |                |              | Bee brood      | 0.22                                          | -                                            |

Table 4. Cont.

| Year | Group Location | Crop         | Type of Sample | Tau-Fluvalinate (mg/kg)<br>(Limit 0.01 mg/kg) | Other Pesticides (mg/kg)                                                                                          |
|------|----------------|--------------|----------------|-----------------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| 2021 | Fundulea (CL)  | Oilseed rape | Pollen         | -                                             | Prothioconazole-dethio (sum of isomers)—0.016<br>tebuconazole—0.11 and 0.12                                       |
|      | Albota (AG)    |              | Pollen         | -                                             | Acetamiprid—0.010<br>prothioconazole-dethio (sum of isomers)—0.019<br>tebuconazole—0.012<br>trifloxystrobin—0.010 |

### 3.2. Evaluation of the Control Group Results

Out of a total of 48 samples collected during the two years (2020–2021) from six private apiaries, contamination with neonicotinoid residues and other pesticides was confirmed in 24 samples. This contamination was observed in both rapeseed (21 samples) and sunflower crops (18 samples). The distribution of contaminated samples was as follows: bees (9 samples), bee brood (9 samples), honey (16 samples), and pollen (5 samples). Data on positive samples collected from the control group were summarized in Table 5.

**Table 5.** Positive samples of honey, pollen, bees, and bee brood with neonicotinoid residues and/or other pesticides or insecticides in the 2020 and 2021 active seasons, from oilseed rape crop and sunflower crop (control group).

| Year | Crop         | Group Location | Type of Sample | Amitraz (mg/kg) | Tau-Fluvalinate (mg/kg)<br>(Limit 0.01 mg/kg) | Other Pesticides (mg/kg)                                                                                                                                |
|------|--------------|----------------|----------------|-----------------|-----------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2020 | Oilseed rape | NT             | Honey          | -               | 0.12                                          | Bromopropylate—0.011<br>captan—0.57<br>dimethoate—0.12<br>cymoxanil—0.022<br>fluopicolide—0.026<br>hexythiazox—0.010                                    |
|      |              |                | Pollen         | -               | -                                             | metalaxyl and metalaxyl-M—0.031<br>propiconazole (sum of isomers)—0.026<br>spiroxamine (sum of isomers)—0.036<br>tebuconazole—0.094                     |
|      |              |                | Bees           | 0.050           | 0.020                                         | -                                                                                                                                                       |
|      |              |                | Bee brood      | 0.13            | 0.61                                          | -                                                                                                                                                       |
|      |              |                |                | 0.24            | 0.36                                          | -                                                                                                                                                       |
|      |              |                |                |                 |                                               |                                                                                                                                                         |
|      |              | CL             | Pollen         | -               | -                                             | Captan—0.31<br>cymoxanil—0.011<br>azoxystrobin—0.022<br>metalaxyl and metalaxyl-M—0.024<br>pyraclostrobin—0.053<br>tebuconazole—0.068<br>zoxamide—0.025 |
|      |              |                | Bees           | -               | 0.011                                         | -                                                                                                                                                       |
|      |              |                | Bee brood      | -               | 0.26                                          | -                                                                                                                                                       |
|      |              |                | Honey          | -               | 0.15                                          | -                                                                                                                                                       |
|      |              |                |                | -               | 0.30                                          | -                                                                                                                                                       |
|      |              |                | Pollen         | -               | -                                             | Captan—0.069<br>dimethoate—0.023<br>tebuconazole—0.023                                                                                                  |
|      |              |                | Bee brood      | -               | 0.022                                         | -                                                                                                                                                       |
|      |              |                |                | -               | 0.56                                          | -                                                                                                                                                       |

Table 5. Cont.

| Year | Crop         | Group Location | Type of Sample | Amitraz (mg/kg) | Tau-Fluvalinate (mg/kg)<br>(Limit 0.01 mg/kg) | Other Pesticides (mg/kg)                                                                                                                                                                          |
|------|--------------|----------------|----------------|-----------------|-----------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2020 | Sunflower    | NT             | Honey          | -               | -                                             | Acetamiprid—0.034<br>dimoxystrobin—0.013                                                                                                                                                          |
|      |              |                |                | -               | 0.040                                         | -                                                                                                                                                                                                 |
|      |              |                | Bees           | -               | -                                             | piperonyl-butoxide—0.012<br>tetramethrin—0.012                                                                                                                                                    |
|      |              | AG             |                | -               | 58.7                                          | DDT—0.015                                                                                                                                                                                         |
|      |              |                | Bees           | -               | 1.0                                           | -                                                                                                                                                                                                 |
|      | Oilseed rape | NT             |                | -               | 0.021                                         | Coumaphos—0.033                                                                                                                                                                                   |
|      |              |                | Honey          | -               | 0.021                                         | -                                                                                                                                                                                                 |
|      |              | AG             | Bees           | -               | 0.032                                         | -                                                                                                                                                                                                 |
|      |              |                |                | -               | 0.034                                         | -                                                                                                                                                                                                 |
|      |              |                | Bees           | -               | 0.014                                         | Fluazifop-P-butyl (fluazifop acid (free)—0.062                                                                                                                                                    |
| 2021 | Sunflower    | NT             |                | -               | 0.016                                         | -                                                                                                                                                                                                 |
|      |              |                | Honey          | -               | 0.013                                         | -                                                                                                                                                                                                 |
|      |              | AG             | Bees           | -               | -                                             | Thiametoxam—0.46                                                                                                                                                                                  |
|      |              |                |                | -               | 0.014                                         | -                                                                                                                                                                                                 |
|      |              |                | Honey          | -               | 0.018                                         | Boscalid—0.026<br>dimoxystrobin—0.040                                                                                                                                                             |
|      |              | CL             |                | -               | 0.018                                         | AMPA (aminomethylphosphonic acid) limit 0.01 mg/kg<br>glufosinate-ammonium (sum of glufosinate, its salts, MPP, and NAG expressed as glufosinate equivalents) limit 0.01 mg/kg<br>glyphosate—0.01 |
|      |              |                | Pollen         | -               | 0.035                                         | -                                                                                                                                                                                                 |
|      |              |                | Bee brood      | -               | 0.49                                          | -                                                                                                                                                                                                 |
|      |              |                |                | -               | 0.024                                         | -                                                                                                                                                                                                 |
|      |              | AG             | Honey          | -               | 0.18                                          | -                                                                                                                                                                                                 |
|      |              |                | Bees           | -               | -                                             | Fluazifop-P-butyl (fluazifop acid (free)—0.019                                                                                                                                                    |
|      |              | CL             | Bee brood      | -               | 0.57                                          | -                                                                                                                                                                                                 |
|      |              |                |                | -               | 0.039                                         | -                                                                                                                                                                                                 |
|      |              | CL             | Honey          | -               | 0.076                                         | -                                                                                                                                                                                                 |
|      |              |                | Bee brood      | -               | 0.13                                          | -                                                                                                                                                                                                 |

Table 6 presents the mean concentrations and concentration ranges (min–max) for each pesticide detected in honey, pollen, bees, and bee brood.

### 3.2.1. Results Obtained from the Control Group by Sample Types

- Live bee samples:

Based on the data obtained from the years 2020 and 2021, it is evident that the control group of live bees was exposed to a diverse array of pesticides over the two years, with varying profiles and concentrations each year, from both rapeseed and sunflower crops.

In 2020, two acaricides (amitraz and tau-fluvalinate) were identified in live bee samples from the rapeseed crop. In the 2021, the detected pesticides included an acaricide (tau-fluvalinate), an herbicide (fluazifop-P-butyl), and a neonicotinoid (thiamethoxam) (Table 4).

**Table 6.** Summary of pesticide residues detected in hive products (mg/kg).

| Year | Crop         | Type of Sample | Pesticide            | Mean  | Min   | Max   |
|------|--------------|----------------|----------------------|-------|-------|-------|
| 2020 | Oilseed rape | Honey          | Tau-fluvalinate      | 0.190 | 0.120 | 0.300 |
|      |              | Bees           | Amitraz              | 0.050 | 0.050 | 0.050 |
|      |              | Bee brood      | Amitraz              | 0.130 | 0.130 | 0.130 |
|      |              |                | Tau-fluvalinate      | 0.490 | 0.360 | 0.610 |
|      | Sunflower    | Honey          | Acetamiprid          | 0.034 | 0.034 | 0.034 |
|      |              |                | Dimoxystrobin        | 0.013 | 0.013 | 0.013 |
|      |              | Bees           | Piperonyl-butoxide   | 0.012 | 0.012 | 0.012 |
|      |              |                | Tetramethrin         | 0.012 | 0.012 | 0.012 |
|      |              |                | DDT                  | 0.015 | 0.015 | 0.015 |
|      |              |                | Tau-fluvalinate      | 1.000 | 1.000 | 1.000 |
|      |              |                | Coumaphos            | 0.033 | 0.033 | 0.033 |
| 2021 | Oilseed rape | Honey          | Tau-fluvalinate      | 0.027 | 0.013 | 0.034 |
|      |              | Bees           | Fluazifop-P-butyl    | 0.062 | 0.062 | 0.062 |
|      |              |                | Thiamethoxam         | 0.460 | 0.460 | 0.460 |
|      | Sunflower    | Honey          | Boscalid             | 0.026 | 0.026 | 0.026 |
|      |              |                | Dimoxystrobin        | 0.040 | 0.040 | 0.040 |
|      |              |                | Glyphosate           | 0.010 | 0.010 | 0.010 |
|      |              |                | Glufosinate-ammonium | 0.010 | 0.010 | 0.010 |
|      |              |                | AMPA                 | 0.010 | 0.010 | 0.010 |
|      |              | Bees           | Fluazifop-P-butyl    | 0.019 | 0.019 | 0.019 |
|      |              | Bee brood      | Tau-fluvalinate      | 0.385 | 0.130 | 0.570 |

For the sunflower crop, 2020 samples showed the presence of several insecticides (piperonyl butoxide, tetramethrin, and DDT) as well as acaricides like tau-fluvalinate (ranging from 1.0 to 58.7 mg/kg) and coumaphos. In contrast, in 2021, only one herbicide (fluazifop-P-butyl) was identified.

Samples collected from private apiaries during the oilseed rape crop revealed pesticide residues and/or other insecticides in four samples of bees—two from 2020 and two from 2021.

Comparing the presence of pesticides in live bee samples from rapeseed between 2020 and 2021, we observed some differences: in 2020, acaricides (amitraz and tau-fluvalinate) were detected, whereas in 2021, acaricides (tau-fluvalinate), herbicides (fluazifop-P-butyl), and neonicotinoids (thiamethoxam) were found. For sunflower crops, differences were also noted: in 2020, the samples contained insecticides (piperonyl butoxide, tetramethrin, and DDT) and acaricides (tau-fluvalinate and coumaphos), while in 2021, only one herbicide (fluazifop-P-butyl) was detected. These changes highlight a significant variation in the types of pesticides present in sunflower crops between the two years.

- Bee brood samples:

Regarding brood samples, out of the nine positive results identified, five positive samples were detected after the 2020 rapeseed harvest. Of these, two samples from the eastern region were contaminated with acaricides (amitraz and tau-fluvalinate), while the remaining three samples were only with only one acaricide (tau-fluvalinate) (Table 4). No pesticide residue contamination was found in bee brood samples from sunflower crops in 2020.

In 2021, no contamination with pesticide residues was observed in the bee brood samples collected after the oilseed rape harvest. However, four samples of bee brood from sunflower crops were contaminated with one acaricide (tau-fluvalinate).

In 2020, during the oilseed rape crop, acaricides (amitraz and tau-fluvalinate) were identified in bee brood samples. This suggests that bee larvae in future generations were exposed to these pesticides during development, potentially impacting their health and survival. In contrast, no pesticides were detected in the bee brood sample in the oilseed rape crop during 2021.

- Honey samples:

The analysis indicates a decrease in the levels of acaricides in honey from the rape crop in 2021 compared to 2020. Specifically, 2020 samples contained tau-fluvalinate and bro-mopropylate, whereas 2021 samples contained only tau-fluvalinate at lower concentrations, suggesting a potential reduction in exposure (Table 4).

In sunflower honey, the 2020 samples showed the presence of neonicotinoids, fungicides, and acaricides, while 2021, samples showed only acaricides and fungicides, with the addition of glyphosate metabolites.

- Pollen samples:

Analysis of rapeseed pollen in 2020 revealed contamination with a wide range of pesticides: numerous and varied fungicides, one insecticide (dimethoate), and two acaricides. (hexythiazox and tau-fluvalinate) (Table 4). No pesticides were detected in sunflower pollen in 2020, and only tau-fluvalinate was identified in 2021.

### 3.2.2. Results Obtained from the Control Group for Rapeseed and Sunflower Crops

In the control group, the common pesticide identified in 2020–2021 in both rape and sunflower was the acaricide tau-fluvalinate (used by beekeepers to control *Varroa destructor* in bee colonies). The results obtained highlight variations in the types and concentrations of pesticides between rapeseed and sunflower:

- In rape crop (2020), acaricides (amitraz and tau-fluvalinate), an herbicide (fluazifop-P-butyl), and fungicides (captan, tebuconazole, cymoxanil, azoxystrobin, pyraclostrobin, zoxamides, fluopicolide, metalaxyl, propiconazole, and spiroxamine) were identified compared to 2021, when an acaricide (tau-fluvalinate), an herbicide (fluazifop-P-butyl) and a neonicotinoid (thiamethoxam) were detected;
- In sunflower crop (2020), only insecticides (piperonyl-butoxide, tetramethrin, tau-fluvalinate, DDT, and coumaphos) were identified compared to 2021, when only herbicides were identified (fluazifop-P-butyl).

### 3.2.3. Monitoring of Bee Colonies During the Active Season Between 2020 and 2021

The experimental group colonies that contained pesticide residues exhibited reduced survival periods. Typically, bee colonies in Romania should survive for 5 months (October–March), but in this case, the survival period was reduced to 3–4 months during the winter. Consequently, these colonies failed to endure the 2020–2021 winter season. The negative impact on the bees resulted in a reduction in colony strength, with the Albota group falling to 3–4 frames (25%) and the Fundulea and Secuieni groups to 4–5 frames (50%). This reduction in colony strength heightened the risk of non-survival.

Private apiaries monitored in the three counties (Argeş, Călăraşi, and Neamţ) had a total of 825 bee colonies during the 2021 inactive season. Mortality was recorded in 359 colonies (21%). Analysis of the data from apiaries with confirmed pesticide contamination showed a mortality rate of 44% among the colonies from these monitored apiaries (Table 7).

**Table 7.** Apiaries identified with suspicion of poisoning in the active season and monitored in the inactive season 2020–2021 (control group).

| No Private Apiaries Studied | Total Number of Private Bee Colonies Monitored in the Inactive Season (2021) | Total Number of Dead Bee Colonies from Private, Confirmed of Pesticides in the Inactive Season (2021) |
|-----------------------------|------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|
| NT                          | 285                                                                          | 182                                                                                                   |
| AG                          | 233                                                                          | 59                                                                                                    |
| CL                          | 307                                                                          | 118                                                                                                   |
| Total = 6                   | 825                                                                          | 359                                                                                                   |

#### 4. Discussion

Honeybees can be exposed to multiple chemical residues through complex and indirect routes within the colony environment. While this study did not assess exposure pathways directly, mechanisms such as trophallaxis, nurse-to-brood transfer, and diffusion of compounds through wax are recognized contributors to colony-wide contamination. These processes may help explain the distribution of residues observed and highlight the potential for synergistic effects between co-occurring substances [30,43].

The results obtained from the experimental group revealed the presence of pesticide and insecticide residues in samples collected from hives located near oilseed rape and sunflower crops during the 2020 and 2021 active beekeeping seasons. In 2020, at the Fundulea (CL) site, honey from oilseed rape contained tau-fluvalinate at a concentration of 0.12 mg/kg, exceeding the EU maximum residue limit (MRL) of 0.01 mg/kg, along with bromopropylate at 0.011 mg/kg, with these results suggesting that future generations of bees may be exposed to these pesticides. Pollen from the same crop showed a high concentration of tau-fluvalinate at 0.38 mg/kg. In the case of sunflower, honey samples contained acetamiprid and dimoxystrobin (each at 0.05 mg/kg), while adult bee samples tested positive for piperonyl butoxide and tetramethrin (each at 0.01 mg/kg). Additionally, brood samples showed tau-fluvalinate at 0.22 mg/kg. In 2021, pollen collected from oilseed rape at Fundulea (CL) contained prothioconazole-desthio (sum of isomers) at 0.016 mg/kg and tebuconazole at 0.11 mg/kg and 0.12 mg/kg. At the Albota (AG) site, sunflower pollen samples contained acetamiprid (0.010 mg/kg), prothioconazole-desthio (0.019 mg/kg), tebuconazole (0.012 mg/kg), and trifloxystrobin (0.010 mg/kg).

The results obtained in the control group during the 2020 and 2021 beekeeping seasons revealed the presence of numerous pesticide residues—including substances not authorized for use in the European Union—across all sample types analyzed: honey, pollen, adult bees, and brood. In 2020, in oilseed rape crops (NT and CL sites), honey samples contained tau-fluvalinate at concentrations up to 0.15 mg/kg, significantly exceeding the EU maximum residue limit (0.01 mg/kg), and bromopropylate at 0.011 mg/kg. Pollen from the same sites showed complex contamination, including captan (0.57 mg/kg), dimethoate (0.12 mg/kg), cymoxanil (0.022 mg/kg), fluopicolide, hexythiazox, metalaxyl, propiconazole, spiromoxamine, and tebuconazole, with concentrations ranging from 0.010 to 0.094 mg/kg. Biological samples from bees and brood showed amitraz (up to 0.13 mg/kg) and very high levels of tau-fluvalinate, reaching 0.61 mg/kg. For sunflower crops (2020), honey samples contained acetamiprid (0.034 mg/kg) and dimoxystrobin (0.013 mg/kg), while adult bee samples tested positive for piperonyl butoxide and tetramethrin (both at 0.012 mg/kg). Notably, one sample from the NT location showed DDT at 0.015 mg/kg, along with a suspicious value of 58.7 mg/kg, likely due to a transcription error. In Albota (AG), bees were found to contain tau-fluvalinate at 1.0 mg/kg and coumaphos at 0.033 mg/kg, while honey from the CL site showed tau-fluvalinate at 0.021 mg/kg. In 2021, honey samples from oilseed rape showed tau-fluvalinate concentrations ranging from 0.013 to 0.034 mg/kg, and bee

samples contained fluazifop-P-butyl (as free acid) at 0.062 mg/kg. In sunflower crops, honey samples revealed residues of boscalid (0.026 mg/kg), dimoxystrobin (0.040 mg/kg), glyphosate (0.010 mg/kg), as well as AMPA and glufosinate-ammonium (including its salts and metabolites), all within regulated limits (<0.01 mg/kg). Brood samples again showed significant levels of tau-fluvalinate, reaching up to 0.57 mg/kg (AG) and 0.076 mg/kg (CL).

For tau-fluvalinate, Mann–Whitney U-test allowed us to interpret the data, assuming that our data were not normally distributed. In honey products, according to the data distribution of the tau-fluvalinate compound, the Mann–Whitney U-test showed that the null hypothesis was the same across groups and as such must be rejected ( $p < 0.0001$ , Table 8).

**Table 8.** Descriptive statistic for compounds identified in honey, bee, and bee brood products (mg/kg).

| Products  | Compounds (Pesticides) | Control Group | Experimental Group | $p$ -Value * |
|-----------|------------------------|---------------|--------------------|--------------|
| Honey     | Tau-fluvalinate **     | 0.09          | 0.01               | <0.0001      |
|           | Captan                 | 0.04          | 0                  | NS           |
|           | Dimethoate             | 0.01          | 0                  | NS           |
| Bee       | Tau-fluvalinate        | 0.02          | 0                  | NS           |
|           | Piperonyl-butoxide     | 0.01          | 0.01               | NS           |
|           | Amitraz                | 0.05          | 0                  | NS           |
|           | DDT                    | 0.02          | 0                  | NS           |
|           | Coumaphos              | 0.03          | 0                  | NS           |
|           | Tetramethrin           | 0.01          | 0.01               | NS           |
| Bee brood | Tau-fluvalinate **     | 0.34          | 0.01               | NS           |
|           | Amitraz                | 0.04          | 0                  | NS           |

\* NS—non significant differences,  $p > 0.05$ , LSD test. \*\* According to Kruskal–Wallis H-test,  $p = 0.0019$ , the variance was not homogenous for tau-fluvalinate, and as a consequence, we continued analyses with Mann–Whitney U-test, a non-parameter test ( $p < 0.0001$ ), in bee brood.

The Mann–Whitney U-test showed the asymptotic significance  $> 0.05$  for tau-fluvalinate in bee brood, while in bee samples, it was identified only in the control group. The tau-fluvalinate values were not significantly influenced by group in bee brood. According to the Mann–Whitney U-test, the data in Table 8 indicate that the honey in the control group had significantly higher levels ( $F = 6.30$ ,  $df = 1$ ,  $p = 0.0001$ ) of tau-fluvalinate compounds. Even though the control group's levels of tau-fluvalinate compounds were higher in both the bees and the brood, Mann–Whitney U-test showed that there were no significant differences when compared to the experimental group.

Only in the control group of honey products were the captan and dimethoate pesticides detected. Both bees and their brood contained amitraz pesticides but only in the control group. Tetramethrin pesticides were only detected in bees at the same concentration in the experimental and control groups, at 0.01 and 0.01, respectively ( $p > 0.05$ ). Along with other products, it was determined that only the control group contained the compounds piperonyl-butoxide (which was present at the same level in both groups), DDT, and coumaphos ( $p > 0.05$ ).

The present study identified several active substances with known toxicological relevance for honey bees, including neonicotinoids such as thiamethoxam and acetamiprid, as well as fungicides and insecticides no longer approved for use in the EU, such as bromopropylate, captan, cymoxanil, and propiconazole. These findings indicate multi-residue contamination and support the hypothesis of chronic exposure with potential sublethal and cumulative effects on honeybee colony health. In some honey samples, thiamethoxam concentrations reached values close to or exceeding the NOAEL thresholds reported in pre-

vious toxicological assessments [44]. Similarly, acetamiprid residues in pollen samples were within a range that, when accumulated through typical foraging and trophallactic exchange, could contribute to chronic exposure at the colony level. Although the present study did not investigate behavioral or physiological responses directly, the residue concentrations detected are concerning in light of sublethal effects previously documented in the literature, including disorientation, immune suppression, and metabolic disturbances [45–47]. For example, Rondeau et al. (2014) [44] reported significant mortality after chronic exposure to thiamethoxam at doses as low as 0.005 ng/day over 150 days. Given that individual honeybees consume approximately 0.02 g of honey per day [48], even low-level contamination could become biologically significant during prolonged periods such as wintering. These findings highlight the need for continuous monitoring of pesticide residues, especially in light of the detection of substances banned under EU regulations, and emphasize the importance of evaluating cumulative exposure risks in real-world conditions.

A significant outcome of this study was the identification of active substances that are currently prohibited or not approved for use in the European Union, both in bee tissues and in hive products (such as honey and pollen). This raises serious concerns about the enforcement of existing pesticide regulations and suggests the possibility of unauthorized agricultural applications or persistence in the environment beyond legal periods of use. These findings highlight the necessity for enhanced residue surveillance and stricter compliance monitoring in crop protection practices. Furthermore, they reinforce the importance of transitioning towards sustainable and pollinator-safe agricultural strategies not only to safeguard pollinator health and ecosystem resilience but also to ensure the quality and safety of apicultural products intended for human consumption.

The absence of pesticide residues in rapeseed pollen samples in 2021, the presence of glyphosate in honey samples from 2021 but its absence in 2020, and a slight reduction in the maximum values of tau-fluvalinate in 2021 compared to 2020 may reflect changes in agricultural practices or reduced pesticide application. However, further investigation is necessary to confirm these factors. The presence of a single pesticide in sunflower pollen in 2021 is a relatively positive observation, yet continued monitoring of pesticide levels in hive products and the surrounding environment remains crucial for safeguarding bee health and ensuring the long-term viability of pollinators. The observed differences may be attributed to changes in agricultural practices, variations in producers' preferences, or evolving regulations concerning pesticide use across different regions and time periods in Romania.

The number of pesticides detected in samples from the control groups (colonies from private apiaries in the same areas as the experimental group but employing different agricultural and beekeeping practices) was approximately double that found in the experimental groups (colonies subjected to standard treatments in accordance with legal regulations). This discrepancy suggests that some farms may be applying treatments incorrectly. The higher level of residues detected in the control group compared to the experimental group may be explained by indirect exposure mechanisms such as pesticide drift, contamination of shared foraging resources, or environmental persistence of active substances. Undeclared pesticide use near privately managed apiaries also remains a plausible hypothesis. These findings highlight the need for further investigation into landscape-scale pesticide dispersion and honeybee foraging behavior.

In early 2021, spring monitoring was conducted on the bee colonies introduced in the experimental group after the 2020 active beekeeping period. This monitoring revealed progressive declines and weakening of the bee colonies. Despite the survival of the bee colonies at all three experimental locations after the 2021 inactive season, there was a notable decline in the number of worker bees entering the winter. The mortality rate in

2021 in private apiaries with confirmed pesticide contamination (44%) is consistent with the results reported by Taenzler et al. (2023) [49].

The decline of honeybee colonies in the control group observed after the 2020–2021 inactive season may be associated with pesticide use in agricultural crops such as oilseed rape and sunflower. The presence of residues in pollen and honey—essential nutritional resources during overwintering—could have contributed to weakened immune response and reduced colony vitality in the following active season. Although this study did not directly investigate physiological mechanisms, previous research suggests that prolonged exposure to contaminated forage may impair metabolic and immune functions, increasing the risk of winter mortality [43]. Several colonies did not survive the winter or emerged in a severely weakened state, requiring corrective actions such as hive merging to maintain viable populations.

Considering the results obtained, it is evident that there is a significant concern in our country regarding the presence of pesticides, especially neonicotinoids and other harmful chemicals, in bee colonies and bee products. This indicates an urgent need for action to regulate and monitor pesticide use in agriculture. It is also crucial to continue research in this area to fully understand the impact of pesticides on bees and the ecosystems they inhabit. Only through collaboration between researchers, farmers, governments, and other stakeholders can effective solutions be developed to protect bees, ensure food security, and maintain ecosystem health.

The results obtained suggest that some Romanian farmers may not be purchasing products recommended by current legislation and are instead using products that are prohibited or not approved in the EU. Additionally, they may not be using the correct dosage of chemicals for plant protection in a judicious and professional manner. This situation appears to be particularly relevant for agricultural areas with rapeseed and sunflower crops where bee colonies were placed during harvesting (control group).

## 5. Conclusions

Toxicological analyzes of bee samples and hive products (honey and pollen) identified residues of 2 neonicotinoids, 14 fungicides, 5 acaricides, 4 herbicides, and 4 insecticides, with notable variations in the types and concentrations of residues depending on the crop analyzed (rapeseed vs. sunflower).

In the experimental group, samples collected in 2020 indicated multi-residue contamination with pesticides including acaricides (tau-fluvalinate and bromopropylate), neonicotinoids (acetamiprid), pyrethroids, and synergists. By contrast, 2021 samples contained only fungicides in pollen (prothioconazole-desthio, tebuconazole, and trifloxystrobin), along with traces of acetamiprid.

In the control group, the most consistently identified pesticide in both 2020 and 2021, across samples from both rapeseed and sunflower crops, was the acaricide tau-fluvalinate. This active substance is commonly used by beekeepers to control *Varroa destructor* infestations in bee colonies and by farmers to treat mite infestations in agricultural crops.

Data collected from the three sampling locations in Romania show that the presence of neonicotinoid and other pesticide residues—especially in the control group—is consistent with international scientific evidence regarding the lethal and sublethal toxic effects of chronic exposure. These findings highlight significant risks to bee health and apicultural sustainability, underlining the need for stricter regulations on pesticide use to protect essential pollinators and the environment.

The comparison between the two groups indicates a substantially higher contamination risk in the control group, where a wider range and higher concentrations of pesticide residues—including systemic insecticides, fungicides, and herbicides—were detected. In



**Table A2.** Laboratory diagnostic methods of the main bee diseases.

| Disease/<br>Etiological Agent                      | Biological Material                                                                                   | Macroscopic Exam<br>Morphological Characters                                                                                                                                                                                                                                                                                                                                                                                                           | Bacterioscopic<br>Examination | Bacteriological<br>Examination | Specific<br>Methods                          | Serology | Chemical<br>Methods | Other<br>Methods |
|----------------------------------------------------|-------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|--------------------------------|----------------------------------------------|----------|---------------------|------------------|
| External Parasitital Diseases                      |                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                               |                                |                                              |          |                     |                  |
| Varroosis/<br><i>Varroa destructor</i>             | Bees (30–50 individuals/sample) queens, drones, honeycomb with overgrown brood (15 × 10 cm), detritus | Body flat, transversely oval, 1.1 mm long, 1.6 mm wide; the presence of the reddish-brown chitinous coating, covered with bristles; four pairs of legs; mouthpiece for stinging and sucking                                                                                                                                                                                                                                                            | -                             | -                              | Flotation method; washing method             | -        | -                   | -                |
| Acarapiosis/<br><i>Aethina tumida</i>              | Adult bees alive, dying, or dead for 1–2 days                                                         | Adult—5 mm long and 3 mm wide, females being slightly longer than males; dark brown—black color (slightly lighter after copped);<br>Egg—white, reniform (2/3 of the size of the bee egg); Larva—1.2 cm long, whitish, often covered with a thin sticky layer                                                                                                                                                                                           | -                             | -                              | Examining the bottom of the hive using traps | -        | -                   | -                |
| Braulosis/<br><i>Braula coeca</i>                  | Bees, queens, drones, honeycomb with covered honey, and brood                                         | Adult—almost spherical body, reddish brown color, covered entirely by black hairs; head with a pair of antennae, oral apparatus adapted for sucking and licking; three pairs of legs located on the ventral side of the thorax, finished with suction cups with which it clings to the pericarps on the bees' thoraxes;<br>Egg—(0.76/0.37 mm) oval shape, matte white;<br>Larva—(0.8–2 mm) oval shape, transparent white, with two typical mouth hooks | -                             | -                              | Examination of the thoracic muscles          | -        | -                   | -                |
| Tropilaelaps Infestation/ <i>Tropilaelaps</i> spp. | Honeycomb, adult bees                                                                                 | <i>Tropilaelaps clareae</i> (<1 mm in length);<br><i>T. mercedesae</i> (<9 mm in length);<br><i>T. koenigerum</i> (0.7 mm length)                                                                                                                                                                                                                                                                                                                      | -                             | -                              | Examination with the help of sticky foil     | -        | -                   | -                |

Table A2. Cont.

| Disease/<br>Etiological Agent                                                                                                                                          | Biological Material                                                                                                    | Macroscopic Exam<br>Morphological Characters                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Bacterioscopic<br>Examination                                                                                                             | Bacteriological<br>Examination                                                              | Specific<br>Methods                                         | Serology                                                                                                                   | Chemical<br>Methods | Other<br>Methods      |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|---------------------|-----------------------|
| Bacterial Diseases                                                                                                                                                     |                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                                                                                           |                                                                                             |                                                             |                                                                                                                            |                     |                       |
| American Foulbrood<br>(AFB)/ <i>Paenibacillus</i><br><i>larvae</i>                                                                                                     | Honeycomb with copped<br>brood                                                                                         | <i>Bacillus</i> Gram (+), $1.5 - 5 \times 0.5 - 0.6 \mu$ ,<br>with rounded ends, mobile, cilia<br>arranged peritrichously, sporulate, spore<br>arranged centrally or subterminally,<br>deformed, often spores are free                                                                                                                                                                                                                                                                                                                                      | - Smear technique<br>(Gram<br>staining)—spores,<br>vegetative forms;<br>- Carbol fuchsin<br>staining of slides<br>obtained from<br>larvae | Cultures on enriched<br>medium (broth-agar<br>and normal horse<br>serum 15%)                | - The Holst test<br>- modified<br>hanging drop<br>technique | Polymerization<br>chain<br>reaction<br>(PCR)                                                                               | -                   | Dry kits<br>technique |
| European Foulbrood<br>(EFB)/ <i>Melissococcus</i><br><i>plutonius</i> ,<br><i>Enterococcus faecalis</i> ,<br><i>Paenibacillus alvei</i> ,<br><i>Bacterium eurydice</i> | Honeycomb with dead<br>brood, dead and<br>dehydrated larvae, and<br>larvae intestine with clinical<br>signs of disease | <i>Melissococcus plutonius</i> —Gram (+) cocci,<br>typical lanceolate shape, dimensions<br>$0.5 \times 1.0 \mu$ , placed in chains or<br>clusters—sometimes <i>Enterococcus faecalis</i> ;<br><i>Paenibacillus alvei</i> —Gram-labile bacilli,<br>$-5 \mu \times 0.5 - 0.8 \mu$ , sporulated, the<br>ellipsoidal spore located terminally or<br>subterminally, which deforms the<br>bacterial body;<br><i>Brevibacillus laterosporus</i> —Gram (+),<br>sporulate bacilli, the spore is placed<br>laterally, the bacterial body has a typical<br>lance shape | Smear technique<br>(Gram stain)                                                                                                           | Medium Barley<br>Collins;<br>Products: “Bacto”<br>“Oxoid”;<br>Alexandrova<br>seeding medium | -                                                           | Immuno-<br>logical<br>methods:<br>- tube ag-<br>glutination<br>test<br>- polymer-<br>ization<br>chain<br>reaction<br>(PCR) | -                   | Dry kits<br>technique |
| Internal Parasitic Diseases                                                                                                                                            |                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                                                                                           |                                                                                             |                                                             |                                                                                                                            |                     |                       |
| Amoebiasis/<br><i>Malpighamoeba</i><br><i>mellifica</i>                                                                                                                | Dead or dying bees                                                                                                     | Cysts of <i>Malpighamoeba mellifica</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Smear technique                                                                                                                           | -                                                                                           | -                                                           | -                                                                                                                          | -                   | -                     |
| Nosemosis/<br><i>Nosema</i> sp.                                                                                                                                        | Adult bees, droppings and<br>the whole intestine                                                                       | Spores of <i>Nosema apis</i> —completely oval,<br>with a dark edge, well outlined, $5-7 \mu$<br>long and $3-4 \mu$ wide                                                                                                                                                                                                                                                                                                                                                                                                                                     | The midgut smear<br>technique of adult<br>bees                                                                                            | -                                                                                           | Giemsa<br>staining                                          | -                                                                                                                          | -                   | -                     |

Table A2. Cont.

| Disease/<br>Etiological Agent             | Biological Material            | Macroscopic Exam<br>Morphological Characters                                                                                                                                                                                                                                                                                                                                                                                                                             | Bacterioscopic<br>Examination                      | Bacteriological<br>Examination           | Specific<br>Methods | Serology | Chemical<br>Methods         | Other<br>Methods |
|-------------------------------------------|--------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------|------------------------------------------|---------------------|----------|-----------------------------|------------------|
| Acarapiosis/<br><i>Acarapis woodi</i>     | Live or dying bees             | Adult—oval-shaped body, segmented, with dimensions of 123–180/70–100 µm for the female and 85–116/57–85 µm for the male. In the tracheas of bees, the developmental stages of the mite (egg, larva, nymph) can sometimes be observed. Eggs—sizes of 60–120 µ                                                                                                                                                                                                             | Usual histological techniques—methyl blue staining | -                                        | -                   | -        | Enzyme immuno-assay (ELISA) | -                |
| Mycotic Diseases                          |                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                    |                                          |                     |          |                             |                  |
| Chalkbrood/<br><i>Ascosfera apis</i>      | Honeycombs with bee larvae     | Ascochists—45–119 µm, dark green, translucent, inside there is a variable number of small spheres (ascospheres) containing ascospores and mycelial hyphae. Ascospores are ellipsoidal or slightly reniform, refringent, with dimensions of 1–2/2–3.5 µm                                                                                                                                                                                                                  | Direct smear technique                             | Mycological examination—Sabouraud medium | -                   | -        | -                           | -                |
| Aspergillosis/<br><i>Aspergillus</i> spp. | Larvae, nymphs, and adult bees | Septate mycelia from which branch hyphae with a diameter of 2–3 µm. The hyphae form the conidiophore with the aspergillar vesicle on which the sterigmata are found. Several conidia arise from each sterigma, arranged in an oval-shaped chain and measuring 2–3 µm. Mature fungi have different colors and shades, depending on the species: <i>Aspergillus flavus</i> —greenish yellow, <i>Aspergillus fumigatus</i> —greyish green, <i>Aspergillus niger</i> —black. | Direct smear technique                             | Mycological examination—Sabouraud medium | -                   | -        | -                           | -                |

Table A2. Cont.

| Disease/<br>Etiological Agent | Biological Material                                              | Macroscopic Exam<br>Morphological Characters                                                | Bacterioscopic<br>Examination | Bacteriological<br>Examination | Specific<br>Methods | Serology | Chemical<br>Methods                                                                           | Other<br>Methods |
|-------------------------------|------------------------------------------------------------------|---------------------------------------------------------------------------------------------|-------------------------------|--------------------------------|---------------------|----------|-----------------------------------------------------------------------------------------------|------------------|
| Intoxications                 |                                                                  |                                                                                             |                               |                                |                     |          |                                                                                               |                  |
| With drugs                    | Dying or dead bees<br>(150–200 g/sample),<br>honeycomb fragments | Identification of the medicinal substance                                                   | -                             | -                              | -                   | -        | Thin-layer<br>chromatogra-<br>phy and<br>Averoll–Norris<br>(ethyl and<br>methyl<br>parathion) | -                |
| Toxic food poisoning          | The hindgut from 10–12<br>bees                                   | Pollen provenance using a pollen<br>determiner                                              | -                             | -                              | -                   | -        | -                                                                                             | -                |
| With chemical<br>substances   | Dying bees, honeycomb<br>fragments                               | Identification of the type of chemical;<br>rear intestine enlarged and with dark<br>content | -                             | -                              | -                   | -        | Thin-layer<br>chromatogra-<br>phy                                                             | -                |

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