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Article

Are intercropping cover crops a potential threat for pollinators due to neonicotinoid residues in floral resources?

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Simple Summary: Neonicotinoids, a class of pesticides, are largely used to protect cultures from pests such as aphids. Due to their detrimental effects on pollinators, these pesticides have been forbidden a few years ago in Europe, whereas derogations are still delivered in several countries, particularly for prophylactic seed coating. On the other hand, intercropping cover crops are encouraged to protect water resources from nitrogen leaching and to offer floral resources to pollinators in late season. We tested whether traces of clothianidin, a neonicotinoid insecticide, were still present in the soil two years after sugar beet cultivation. We also tested whether clothianidin was found among the pesticides accumulated in plants, flowers, and floral resources of the intercropping insect-pollinated cover crops. Our results showed that clothianidin was still present in the soil and highlighted the potential accumulation in plants and their transfer to pollen and nectar. The risk for pollinators exists and alternatives to neonicotinoids should be developed.

Abstract: Intercropping cover crops have become mandatory in areas at risk of nitrogen leaching to groundwater. These covers include several attractive late-flowering entomophilous species. They can therefore represent crucial floral resources (pollen and nectar) for pollinating insects in early autumn. Pesticides used in previous crops, however, represent a potential risk for pollinators when they are transferred to the intercropping cover plants and their floral resources. We studied the potential transfer of clothianidin (a neonicotinoid insecticide), applied two years earlier in a beet cultivation, from soil to plants and to the floral resources of three common cover species: *Phacelia tanacetifolia*, *Sinapis alba*, and *Vicia faba*. Soils, entire plants, flowers, and nectar were collected from plants grew in greenhouses, and soils and pollen were collected on a treated field. Our results showed that clothianidin was still present in soils (4.5 ng g^{-1}). The residues accumulated in plants (5-15 times higher concentrations than in soils) and were present in pollen of both *Vicia faba* (0.07 ng g^{-1}) and *Sinapis alba* (1.7 ng g^{-1}) and in nectar of both *Sinapis alba* and *Phacelia tanacetifolia*.

Keywords: clothianidin, sugar beet, soil, bees, pollen, *Phacelia tanacetifolia*, *Sinapis alba*, *Vicia faba*

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1. Introduction

The European Union (EU) is the world leading producer of beet sugar, with around 50% of the total amount produced. In 2018, the EU produced 119.6 million tonnes of sugar beets on 1.5 million hectares [1]. European most producing areas are in northern France, Germany, the Netherlands, Belgium, and Poland [2]. As other intensively cultivated crops, sugar beet is damaged by a range of pests and pathogens, and among the most damageable diseases, the *Beet Yellows Virus* (BYV), transmitted by aphids, has a particularly strong effect on yield [3,4]. Prophylactic control methods are employed which typically involve use of insecticidal seed treatments, such as neonicotinoids [3,5]. Neonicotinoid seed treatments are commonly used by producers worldwide (>120 countries) on a large number of crops [5]. The neuroactive pesticides are used in seed coatings to control herbivorous insect pests in a variety of crops such as cereals, oilseed rape and sugar beets. These chemicals account for more than one fifth of the world insecticide market, and this widespread use requires that their effects on non-pest organisms are investigated. It has recently been shown that neonicotinoid imidacloprid was found as pesticide residues above the limit of quantification of 0.01 mg kg^{-1} in 7% of European agricultural soils, and up to 0.06 mg kg^{-1} [6]. Neonicotinoids are highly water soluble promoting their root absorption, and eventually becoming systemic throughout all tissues [7]. The aim of systemically treating crops with insecticides is to kill herbivorous pests while limiting insecticide contact to non-pests. However, there are numerous cases describing detrimental effects of neonicotinoid seed treatments on non-target invertebrate species [8,9]. A particular concern is the effect of neonicotinoids on pollinators, mainly bees, because of their role in pollinating crops and global declines in pollinator diversity and abundance [8,10]. For bees, pesticide exposure routes include atmospheric particles (dust and spray), soil, plant surfaces, and flowers [8]. Indeed, neonicotinoids were detected in several plant species and their floral resources, nectar and pollen [7,10–15]. Dietary exposure through consumption of pollen and nectar is therefore assumed to be a significant exposure route, posing highest risk across all bee species [10,16]. Non-target pollinating insects can experience direct mortality when lethal or sub-lethal concentrations are encountered [17]. Neonicotinoids affect individual mobility, nutritional intake, learning, memory, taste and odour perception, or nutrient intake and decreased lifespan. Negative effects of neonicotinoids on social bees include poor pollen efficacy, reduced brood and colony development, decreased offspring and queen production, or social and spatial behaviour disruptions [18–21]. The effects differ according to the exposure time and mode (contact or oral), the insect species considered, and the neonicotinoid concentration used [22–28]. Importantly, treated seeds may not only contain neonicotinoids, but also contain other active pesticides, which could lead to interactions between chemicals [10].

These concerns have led to an EU restriction from 1 December 2013 on the use of three neonicotinoids, clothianidin, imidacloprid, and thiamethoxam, as seed coating. The use of these neonicotinoids has been further strongly restricted in 2018 (CE 1107/2009/EU 2018/784). The derogations have, however, been granted for particular use limited to the coating of beet seeds. For

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example, Belgium has granted such derogations in 2019 and 2020 and France is preparing to grant derogations for several years [29]. These derogations specify that no flowering of entomophilous species will be allowed during the five years following sugar beet cultivation. In Belgium, however, the crop succession after sugar beet is mainly winter wheat, followed by a intercropping cover crop when the crop is sown after winter, which is usually the case. The destruction of this cover is strictly regulated which would be contrary to the derogation. In addition to the limitation of nitrogen leaching, intercropping cover crops present other advantages. Cover crops control weed development, reduce soil erosion, and improve soil fertility. In contrast to permanent wildflower strips, annual cover crops are often inexpensive and quick to flower, and are therefore ideal to use as a temporary planting on rotated land [30]. Their late-flowering dense floral displays attract diverse pollinators and may counteract floral resource gaps for late foraging insects. They include several highly attractive entomophilous species mixing mustard (*Sinapis alba*), phacelia (*Phacelia tanacetifolia*), faba bean (*Vicia faba*), radish (*Raphanus sativus*), and sunflower (*Helianthus annuus*) [30,31].

These mass-flowering cover crops are valuable food resources for pollinating insects but may act as ecological traps if foraging insects are exposed to detrimental pesticides such as neonicotinoids. We chose to trace clothianidin, an active substance and one of the breakdown products of thiamethoxam, both under field and greenhouse conditions. Traces of clothianidin were searched for in soils, flowers, pollen, and nectar for three main entomophilous cover crops, phacelia (*P. tanacetifolia*), mustard (*S. alba*), and faba bean (*V. faba*).

Our research questions are as follows: (i) Do pollinating insects visit the cover crops in late season (September-October)? (ii) Can we detect clothianidin residues in the soil 2 years after sugar beet cultivation? (iii) Can we detect clothianidin residues in the target cover crop vegetative parts, flowers, and pollen and nectar floral resources?

2. Materials and Methods

2.1. Plant material

Three species were considered in this study. Phacelia, *Phacelia tanacetifolia* (Boraginaceae), native from North America, is a highly attractive species offering high (up to 5000) number of flowers per plant with copious nectar resources. Nectar production averages 1.02–1.62 mg total sugars per flower per day [32]. Its main pollinators are Apidae (Hymenoptera), mainly honeybees (*Apis mellifera*), followed by bumblebees (*Bombus* spp.) [33]. White mustard, *Sinapis alba* (Brassicaceae), native to Europe, is also highly attractive. Nectar production averages 1.34 mg total sugars per flower per day [34]. The main bee pollinators comprise Apidae (Hymenoptera), mainly honeybees (*Apis mellifera*), followed by Andrenidae and Halictidae species. Diptera (mainly Syrphidae) are considered as co-pollinators whilst insects from other orders such as Coleoptera are considered visitors [35]. The faba bean, *Vicia faba* (Fabaceae), presents 50–80 typical bee-pollinated flowers per plant, with 3.44 µL nectar per flower and per day [36–38]. The visiting bees include honeybees (*Apis mellifera*),

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bumblebees (*Bombus*), and several species belonging to Anthophoridae and Halictidae families [39,40].

2.2. Field experiment

The field experiment was located at the experimental farm of the University campus, central Belgium (Centre Alphonse de Marbaix, Corroy-le-Grand, 50°40′05″ N; 4°38′20″ E). The field was sown in October 2018 with sugar beet seeds (Lisanna KWS®) coated with neonicotinoid clothianidin (Poncho beta®). The sugar beet crop was followed by winter wheat. Intercropping cover crops, comprising *P. tanacetifolia* (Balo®), *S. alba* (Severka®), and *V. faba* (Julia®), were sown in October 2019.

Soil sampling was performed in September 2019 and June 2020. The composite samples comprised 20 random samples (20 cm soil core) for a total weight of about 1 kg.

In March 2020, we collected pollen from the flowering *S. alba* and *V. faba* (*P. tanacetifolia* did not flower). The inflorescences were shaken above a container and pollen was collected with a brush. Air dried pollen was sieved successively at 180, 125, and 90 µm (Gilson company) to separate pollen from stamen tissues. Two pollen samples of 0.3 g were constituted per species.

2.3. Greenhouse experiment

Experiments were carried out in a greenhouse at the University campus, central Belgium (SEFY platform, Louvain-la-Neuve, 50°39′58″ N; 4°37′9″ E). Two trials were achieved in order to collect the required quantities for chemical analyses (September 2019–February 2020 and January 2020–April 2020).

Sugar beet seeds (Lisanna KWS®) were sown in 2 L pots filled with a 1:1 (v/v) mix of sand (size 0/5, M PRO, Netherlands) and universal peat compost (DCM, Amsterdam, Netherlands). Beet seedlings were cut 10–15 days after emerging. Three entomophilous cover plants were grown in 2 L pots treated with three levels of clothianidin: untreated (UT, control), single dose (SD, 1 beet seed per pot), and double dose (DD, 2 beet seeds per pot). Due to the size of the pots used, one beet seed represents four times the dose usually applied in the field.

Seeds of *P. tanacetifolia* (Balo®), *S. alba* (Severka®), and *V. faba* (Julia®) were placed in germination plates in a germination chamber (Economic Delux model ECD01E; Snijders Scientific, Tilburg, Netherlands) under 20 °C/20 °C day/night temperature and a 12 h light (L): 12 h dark (D) photoperiod. Seedlings at the three-leaf stage were transplanted into "pretreated" pots in the greenhouse under 24 °C/22 °C day/night temperature and approximately 60% of relative humidity. They were watered every two days with rainwater.

During flowering, nectar was extracted with 5 or 10 µL glass capillary tubes (Hirschmann Laborgeräte, Eberstadt, Germany). We collected nectar for the three modalities on *S. alba* and *P. tanacetifolia* flowers (*V. faba* plants did not flower). After the nectar collection, the entire flowers were sampled for *S. alba* and for *P. tanacetifolia.* Only the entire plants of *V. faba* were analysed. After 150 days cultivation in the greenhouse, soil samples of soils, and all vegetative parts were collected. Due

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to thrips infestations, methiocarb (Mesurol®) and spinosad (Tracer®) treatments were applied during cultivation.

2.4. Chemical analysis

Clothianidin was quantified in bulk samples of soils, plants, and flowers by Primoris Belgium lab (Gent, Belgium). Limits of quantification reported were of 10 ng g^{-1}. Because the analysis was a multi-residue method, we also measured for other chemicals [44]. Further samples of soil and pollen were analysed for clothianidin residues by the method which is a modification of QuEChERS protocol already described in Kiljanek et al. (2016) [45]. Soils were thawed and air dried at room temperature the day before analysis and stirred until five aliquots of homogenous samples were obtained, according to described protocol [6]. Samples of 3 g of soil and 0.3 g of pollen were used for analysis. Isotopically labeled internal standard of clothianidin-D3 were added to each sample before analysis. Briefly, samples were extracted with acetonitrile containing 1% acetic acid and then subjected to clean up by dispersive solid phase extraction (dSPE) using QuE Verde mixture of sorbents (Z-Sep+, PSA, ENVI-Carb Y and MgSO4) obtained from Sigma Aldrich (Bellefonte, PA, USA). In case of pollen samples additional dSPE clean-up step with QuE Verde sorbents were applied. After clean-up supernatant was evaporated to near dryness under a gentle stream of nitrogen and reconstituted with 4-times lower volume of acetonitrile and filtered (0.2 µm PTFE). Final extract was transferred into injection vial for LC–MS/MS analysis carried out using Agilent 1260 HPLC system (Waldronn, Germany) equipped with Phenomenex Luna Phenyl-Hexyl column (3 µm, 150 × 2.0 mm) thermostated at 50 °C, with the gradient mobile phase consisted of acetonitrile and water with 5 mM ammonium formate (acidified with formic acid to pH 6.0). The injection volume was 5 µL. AB Sciex QTRAP 6500 LC–MS/MS system (Framingham, MA, USA) in a scheduled MRM advanced mode with Turbo Spray Ion Drive in positive ionisation was used for the mass spectrometric analysis. LC–MS/MS system was controlled by Analyst software version 1.6.2. Quantitative and qualitative analysis was done with MultiQuant software version 3.0 based on two most intensive precursor ion-> product ion MRM transitions (250>169; 250>132). Multi-level external calibration curve with standard solutions in acetonitrile at 8 concentration levels and internal standard corresponding to concentrations expected in final extracts of samples spiked with 0.005 to 10 ng g^{-1} clothianidin were used for quantification. The limit of quantification was 0.01 ng g^{-1}. Recovery checks were done in each batch of analyses for quality control purpose. Method is accredited according to ISO/IEC 17025:2018 standard [45].

The nectar samples were collected from flowers by capillary action using glass capillaries packaged in Eppendorf tubes for each modality. The total volume of nectar collected on *S. alba* was 27.0 µL of untreated plants, 57.5 µL for SD plants, and 26.5 µL for DD plants. The total volume of nectar collected on phacelia was 18.0 µL of untreated plants, 22.5 µL for SD plants, and 64.5 µL for DD plants. The glass capillaries containing the nectar samples were stored at −20 °C until sent to the laboratory for analysis. Samples were analysed by ANSES Sophia Antipolis. Due to the crystallization

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of the samples of nectar in the glass capillaries, not all of the nectar sampled could be extracted from the glass capillaries and collected in the respective Eppendorf tubes. Chemical analyses of nectar required a minimum of 10 µL nectar. To dilute and extract the crystallized nectar samples from the glass capillaries, 50 µL of ultra-pure water was added in the Eppendorf tubes. After agitation of the tubes on vortex and centrifugation at 10000 rpm for 5 min, liquid extracts of nectar were obtained. Only qualitative analysis could be done on these diluted samples of nectar (50 µL of samples for each modality) because the initial quantity of nectar recovered in each Eppendorf tube was not exactly known. Samples (10 µL of extracts of nectar) were analysed by LC-ESI-MS/MS [46]. High performance liquid chromatography (HPLC) was performed with an autosampler and a column compartment thermostatted (UltiMate 3000, Thermo Fisher Scientific) coupled with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific) equipped with HESI-II probe (Heated Electrospray Ionization Source). The acquisition mode used was the SRM mode (Selected Reaction Monitoring). Chromatographic separation was carried out on a Pursuit PFP (pentafluorophenyl) analytical column 100 × 3 mm (3 µm) (Agilent). The mobile phase consisted of ultra-pure water (A) and methanol (B), each solution (A and B) being acidified with 0.02% of formic acid. The neonicotinoid residues (clothianidin, thiamethoxam, imidacloprid, thiacloprid, and acetamiprid) were separated by gradient elution. The column and autosampler temperature were 25 °C, the flow rate was 0.4 mL min^{-1} and the injection volume of the sample was 15 µL. The limit of quantification was 0.3 pg μL^{-1} for all neonicotinoid residues analysed simultaneously [46].

3. Results

3.1. Residues in soils

No residue was detected in the first soil sample in September 2019, probably due to the quantification level in the Primoris protocol (10 ng g^{-1}). The second soil sample collected in the field (June 2020) contained 4.5 ng g^{-1} of clothianidin.

Soil samples of the experimental pots placed in the greenhouse contained clothianidin residues at 240 ng g^{-1} for the single dose (SD) and 500 ng g^{-1} for the double dose (DD) treatments (Figure 1).

3.2. Residues in plants

Under field conditions, clothianidin residues were detected in pollen samples with concentrations of 0.07 and 1.7 ng g^{-1} in *V. faba* and *S. alba*, respectively.

Under greenhouse conditions, in whole plant samples of *P. tanacetifolia*, *S. alba*, and *V. faba*, clothianidin was detected in all modalities, the maximum was reached for *P. tanacetifolia* with concentrations of 3600 and 6700 ng g^{-1} for SD and DD, respectively (Figure 1). Clothianidin concentrations measured in flowers reached: 200 ng g^{-1} (SD) and 380 ng g^{-1} (DD) for *P. tanacetifolia* and 420 ng g^{-1} (SD) and 510 ng g^{-1} (DD) for *S. alba*. Clothianidin mass per flower can thus be extrapolated to 4 ng per flower of *P. tanacetifolia* and 10 ng per flower of *S. alba*.

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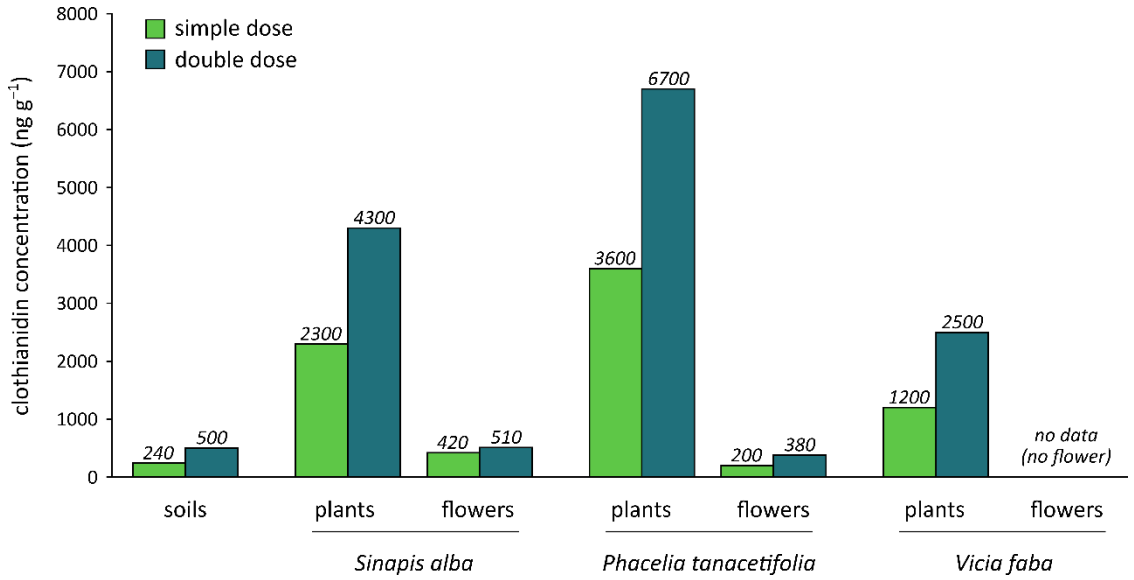


Figure 1 Clothianidin residues concentrations in soils, plants, and flowers during the greenhouse experiment for both simple and double doses. The numbers in italics indicate the concentrations reached.

For the nectar qualitative analyses, clothianidin residues were detected in all samples, except in nectar collected on the *P. tanacetifolia* untreated, and very low clothianidin concentration was detected in nectar collected on *S. alba* untreated. The qualitative results obtained from the 50 µL nectar extracts showed double clothianidin concentrations in nectar collected on plants from the DD modality compared to the SD modality, for both *P. tanacetifolia* and *S. alba*. Concentrations of clothianidin residues detected were twice as high in nectar samples collected on *S. alba* than in nectar samples collected on *P. tanacetifolia*. Residues of other neonicotinoid pesticides were also detected (data not shown): imidacloprid for all nectar samples (concentrations much lower than those of clothianidin), with the exception of nectar sampled from untreated *P. tanacetifolia*, and thiacloprid in all nectar samples, except for nectar sampled from untreated *P. tanacetifolia* and *S. alba*. No residue of thiamethoxam neither acetamiprid was detected in the six samples of nectar analysed.

4. Discussion

4.1. Pesticide residues

Results showed that clothianidin was present in the full soil–plant–floral resource continuum. Under field conditions, the soil still contained 4.5 ng g^{-1} of clothianidin two years after sugar beet cultivation. Other studies reported field concentrations of neonicotinoid residues <6 ng g^{-1} after 3 to 4 years when treated seeds are used in corn crops [47]. The residue particularly accumulated in the entire plants of the studied species. Our results showed that clothianidin was between 5- to up to 15-times more concentrated in the vegetative parts of plants than in the soils, indicating the important bioconcentration of this pesticide into plants. The bioconcentration factor varied according to the considered species: 5-times for *V. faba*, 9–10-times for *S. alba*, and up to 15-times for *P. tanacetifola*.

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Despite lower clothianidin concentrations in flowers compared to vegetative parts, we extrapolated that one flower of *S. alba* accumulated around 10 ng of clothianidin, and one flower of *P. tanacetifola* about 4 ng. Residues of clothianidin were also detected in pollen and nectar samples collected on plants grown on contaminated soils.

There are few direct measurements of pesticides in pollen or nectar collected by bee colonies and even fewer measures from pollen and nectar directly collected on flowers due to the high material quantities required for chemical analyses [10]. According to recent studies, wild plants growing in close proximity to agricultural fields can be a source of higher and more prolonged pesticide exposure for insects than the crops themselves [10, 55]. Most of the studied plants had higher residues in pollen than in nectar, potentially due to pollen physical characteristics [10]. Thiamethoxam, chemically close to clothianidin, was found in all pollen stored by bumblebees in rural areas and a surprisingly high concentration (mean 18 ng g^{-1}) in pollen from wildflowers than in oilseed rape pollen [15]. Clothianidin was also found (5 ng g^{-1}) in bumblebee nectar near the fields [18].

4.2. Risk assessment

Pesticide risk assessments require identifying pesticide concentrations causing adverse effects on species survival and reproduction, knowing that the effects are variable according to the pollinator species in contact with the pesticide.

The toxicity effects of contaminated pollen consumption by honeybees, bumblebees, or solitary bees have been assessed based on pollen contamination and pollen consumption, in comparison with the toxicity endpoint such as chronic oral 10-day median lethal dietary doses (LDD_{50}) [50,51]. Accounting that a honeybee nurse consumes 12 mg of pollen per day, it can be exposed to 0.00084–0.0204 ng of clothianidin per day according to our results. This contamination represents 0.09–2.15% of 10-day LDD_{50}, stated as 0.95 ng per honeybee and per day. LDD_{50} values for bumblebees and solitary bees were established based on extrapolation of experimental value for honeybees, by dividing it by a factor of 10 [51]. For an adult bumblebee, which consumes 30.3 mg of pollen per day, it is exposed to 0.00212–0.05151 ng of clothianidin. This contamination represents 2.23–54.2% of 10-day LDD_{50}, stated as 0.095 ng per bee and per day. For an adult solitary bee, consuming 10.2 mg of pollen per day and thus exposed to 0.00071–0.01734 ng of clothianidin, the contamination represents 0.75–18.25% of 10-day LDD_{50} [51]. This chronic toxicity exceeds 10% of 10 days for solitary bees and bumblebees and is considered an unacceptable risk for bee species. Because of the lack of information on the mixture of treated pollen in bee diet, we assumed that bees consume only one pollen type from cover crop flowers for 10 days. This is somehow realistic in late season when cover crops offer large floral resources to insects contrarily to other common entomophilous species. Moreover, as in other studies, the observed honeybee individuals exhibited high floral fidelity, as only pollen from the cover crop visited species (*P. tanacetifolia* or *S. alba*) was detected in pollen loads [52–54]. Despite the extrapolated variable exposure from 2.15% up to 54.2% of the chronic median lethal dietary dose

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LDD_{50}, our results clearly showed a potential threat for pollinators due to neonicotinoid residues in the floral resources of cover crops.

Bees could be exposed to a high percentage of a toxic dose of several pesticides and the poisonous cocktail of pesticides is more detrimental than each separate compound. For example, a nectar foraging honeybee over a single foraging bout is likely to be exposed to values that exceed the honeybee LD_{50} for clothianidin [10]. However, very few studies examined the long-term or chronic effect of pesticides [56, 57]. Finally, other chemicals such as herbicides and fungicides residues might display synergetic effects with neonicotinoids. Further studies require the standardization of protocols for multi-residue analyses [10].

5. Conclusions

The results of this research prove that two years after the use of treated sugar beet seeds, the clothianidin is still present in soils. The intercropping cover crops accumulate the pesticide from soils to plants and to floral resources of the plants. The greenhouse experiment showed that plant species commonly used in intercropping cover crops (*Sinapis alba, Phacelia tanacetifolia*, and *Vicia faba*) can accumulate a high quantity of clothianidin in various part of plants. Uptake and persistence of clothianidin by cover crops pose a risk to pollinators attracted to the floral resources of the late-flowering attractive species. While current mitigation strategies are mainly oriented to provide additional floral resources to pollinators, such practices are not convenient and reinforce the necessity of a drastic reduction or a total ban of pesticides. New pesticides with similar modes of action are already emerging, which raises the alarm. Reduction of the use of phytochemicals, as well as quick complete risk assessments, are essential to reach the identified protection goal to maintain pollination ecosystem service, and the biodiversity and abundance of pollinators.

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