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Impact of landscape composition on honey bee pollen contamination by pesticides: A multi-residue analysis

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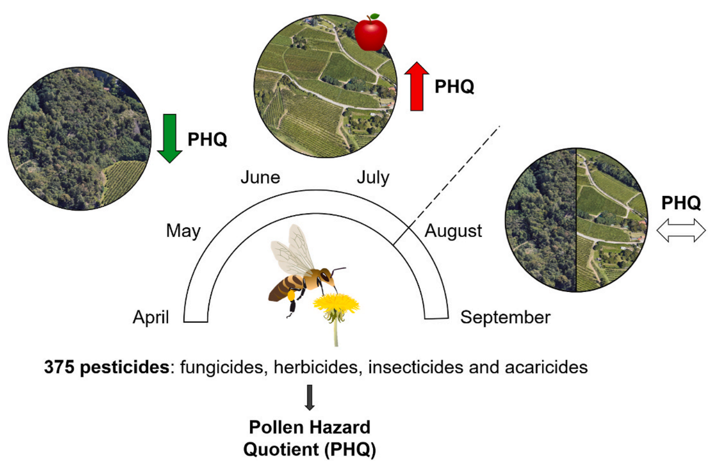
HIGHLIGHTS

- Plant protection products can negatively affect honey bee health.
- We tested the effect of landscape and season on multi-residue pollen contamination.
- Fungicides were the most common compounds, but insecticides were the most toxic ones.
- Semi-natural areas minimized pollen contamination only at the beginning of the season.
- The cover of perennial crops in the landscape increased pollen contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

The honey bee is the most common and important managed pollinator of crops. In recent years, honey bee colonies faced high mortality for multiple causes, including land-use change and the use of plant protection products (hereafter pesticides). This work aimed to explore how contamination by pesticides of pollen collected by honey bees was modulated by landscape composition and seasonality. We placed two honey bee colonies in 13 locations in Northern Italy in contrasting landscapes, from which we collected pollen samples monthly during the whole flowering season in 2019 and 2020. We searched for almost 400 compounds, including fungicides, herbicides, insecticides, and acaricides. We then calculated for each pollen sample the Pollen Hazard Quotient (PHQ), an index that provides a measure of multi-residue toxicity of contaminated pollen. Almost all pollen samples were contaminated by at least one compound. We detected 97 compounds, mainly fungicides, but insecticides and acaricides showed the highest toxicity. Fifteen % of the pollen samples had medium-high or high

Abbreviations: LD_{50}, lethal dose for 50% of a population; PHQ, Pollen Hazard Quotient; RQ, Risk Quotient.

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levels of PHQ, which could pose serious threats to honey bees. Fungicides showed a nearly constant PHQ throughout the season, while herbicides and insecticides and acaricides showed higher PHQ values in spring and early summer. Also, PHQ increased with increasing cover of agricultural and urban areas from April to July, while it was low and independent of landscape composition at the end of the season. The cover of perennial crops, *i.e.*, fruit trees and vineyards, but not of annual crops, increased PHQ of pollen samples. Our work highlighted that the potential toxicity of pollen collected by honey bees was modulated by complex interactions among pesticide category, seasonality, and landscape composition. Due to the large number of compounds detected, our study should be complemented with additional experimental research on the potential interactive effects of multiple compounds on honey bee health.

1. Introduction

The western honey bee, *Apis mellifera* Linnaeus, is the most important managed pollinator species, with an estimated economic value to crop yield of about $6.4 billion in the USA alone (Reilly et al., 2020). Despite a global increase of 85% in the number of managed honey bee colonies since the 1960s, in recent years honey bees have been experiencing high mortality, especially in North America and Europe (Osterman et al., 2021). This syndrome is often referred to as *Colony Collapse Disorder* (vanEngelsdorp et al., 2009) and it is related to several causes (Goulson et al., 2015). Among these causes, the most relevant ones seem to be the spread of parasites and pathogens, such as the parasitic mite *Varroa destructor* Anderson & Trueman and the fungus *Nosema ceranae* (Rosenkranz et al., 2010; Le Conte et al., 2010; Geffre et al., 2020), and nutritional stress related to a restricted diet due to limited availability of floral resources, often caused by semi-natural habitat loss (Naug 2009; Branchiccela et al., 2019). Moreover, the use of plant protection products (hereafter, pesticides) that could contaminate pollen and nectar can also play a key role in the decline of these pollinators (Henry et al., 2012; Sánchez-Bayo et al., 2016; Tsvetkov et al., 2017; Woodcock et al., 2017).

Agriculture has increased by c. 40% globally in the last 50 years (Aizen et al., 2019), and consequently the use of pesticides and their potential impact on bees (DiBartolomeis et al., 2019). Fungicides, herbicides, insecticides, and acaricides are commonly used for crop protection (Zioga et al., 2020). Insecticides and acaricides include the compounds that pose major threats to arthropods, since they are designed to directly affect them, and for this reason they have been extensively studied (Fairbrother et al., 2014; Lundin et al., 2015; Tsvetkov et al., 2017; Woodcock et al., 2017; Wood and Goulson, 2017; Holder et al., 2018). On the other hand, few works have investigated the effects of fungicides and herbicides on honey bees and pollinators in general, despite being the most widely used compounds in terms of applied tonnes (Tamburini et al., 2021, EUROSTAT, 2020). However, effects such as the reduction of bee foraging efficiency, longevity and survival rate, and changes in gut microbiota have been reported, with a large variability among compounds (Cullen et al., 2019; Rondeau and Raine, 2022).

Since most pesticides are applied in crops, bees foraging in landscapes dominated by intensive farming should be more exposed to these compounds (David et al., 2016; Böhme et al., 2018). A high cover of semi-natural habitats could help dilute pollen contamination since honey bees could collect pollen from uncontaminated floral resources. The amount of crops in the landscape is known to potentially boost insecticide concentration in pollen, especially for some highly toxic neonicotinoids, such as thiamethoxam and imidacloprid, and organophosphates, such as chlorpyrifos (Calatayud-Vernich et al., 2018; Wood et al., 2019). Also, the cover of specific crop categories in the landscape, such as apple and cherry orchards and berry plantations, could predict pesticide residue concentration in pollen (McArt et al., 2017; Graham et al., 2021, 2022). However, pesticide drift from focal crops could lead to high contamination also in surrounding areas and result, for example, in a high number of pesticides detected in pollen collected by bees in semi-natural habitats (Lambert et al., 2013; Calatayud-Vernich et al., 2018).

The use of pesticides is not continuous throughout the year. Therefore, seasonality can play a strong role in increasing or reducing the level of contamination by pesticides in pollen collected by honey bees, even because mechanisms of exposure to pesticides of honey bees might change throughout the season (Krupke et al., 2012). The highest concentration of pesticide residues in pollen collected by honey bees is usually observed in April and May since a large part of pesticide applications is made in spring (Lambert et al., 2013; Tong et al., 2018; Liu et al., 2022). However, some studies reported contamination peaks in mid-season or even later, *e.g.*, between July and September (Long and Krupke, 2016; Tosi et al., 2018). Moreover, previous works reported a reduction in pesticide concentration after the blooming of focal crop species, which might be related to different foraging preferences, but also to the biodegradation of pesticides with increasing temperatures (David et al., 2016). Most of these studies, however, focused on specific pesticide categories such as insecticides, or a limited range of compounds, while multi-residue analyses on temporal and spatial variability of pollen contamination are largely still missing. This approach can provide a comprehensive picture of the importance of single crops and associated pesticides across heterogeneous agricultural landscapes.

In this work, we explored how pesticide residues in pollen collected by honey bees were affected by pesticide category, landscape composition, and seasonality, . We selected 13 sampling locations in Northern Italy from which we collected pollen samples monthly for two consecutive years. For each pollen sample, we used liquid chromatography-tandem mass spectrometry and gas chromatography-tandem mass spectrometry to search for 375 compounds, including insecticides, acaricides, fungicides, and herbicides. Then, we calculated the Pollen Hazard Quotient (PHQ) for each pollen sample, a measure of potential pollen toxicity for honey bees. We expected that insecticide would have a major impact on the potential toxicity of pollen, especially for some categories, such as neonicotinoids. We also expected higher pollen contamination at the beginning of the season, especially in areas with a high cover of crops and fruit orchards in particular.

2. Materials and methods

2.1. Study area and site selection

The study was carried out in Northern Italy, in the Trentino-Alto Adige and Veneto regions (NE Italy), where we selected 13 sampling locations characterized by contrasting landscapes. In 3 km radius buffers around the sampling locations, the cover of semi-natural areas ranged from 1 to 92% (mean = 50%), the cover of agricultural areas ranged from 8 to 87% (mean = 38%), and the cover of urban areas ranged from 0 to 30% (mean = 12%) (Table S1, Fig. S1). Site elevation ranged between 91 and 1481 m a.s.l. (mean = 535 m a.s.l.). As a result, the climate in the sampling areas was highly variable: the mean annual temperature ranged between 6.8 °C and 13.5 °C (mean = 10.8 °C), while the total precipitation ranged between c. 1100 and 1700 mm/year (mean = 1260 mm/year).

In 2019 and 2020, we placed two honey bee colonies at each location. Activating pollen traps at the hive entrance for 48 h, we collected pollen samples monthly from April to September, for a total of six samples per year per location. Due to adverse climatic conditions, we were not able to collect pollen samples each month at a few locations: in 2019 we collected only five samples at two locations and three at one, while in 2020 we collected only five samples at three locations. Pollen samples were then stored at −20 °C.

2.2. Landscape composition

We extracted the cover of the main habitat types at each sampling location using the regional land-use map (© European Union, Copernicus Land Monitoring Service, 2018; European Environment Agency) at two scales considering the foraging distance of honey bees, *i.e.*, 3 km and 5 km radius buffers around the sampling locations (Table S2). Since most of the 15 land-use classes were correlated with each other, we performed a Principal Component Analysis (PCA) to extract the landscape composition at each sampling location. We extracted the first three eigenvalues, PC1, PC2, and PC3, which respectively explained 25.9%, 18.9%, and 14.21% of the total landscape variability at 3 km radius buffers (Fig. S2a), and 31.2%, 20.6%, and 12.75% at 5 km radius buffers (Fig. S2b). All statistical analyses were performed using the *R* software version 3.6.1 (R Core Team, 2019).

2.3. Pesticide analysis

We searched for 375 compounds in pollen samples, including insecticides/acaricides (N = 169), fungicides (N = 117), and herbicides (N = 89) (Table S3, Fig. S3a). For the chemical analyses, pollen was grounded using a mill in liquid nitrogen. From each sample, we extracted 2 g of pollen according to the QuEChERS method (EN 15662:2018) (European Standard EN 15662:2018, 2018). The extracts were then analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) (Tables S4 and S5).

LC-MS/MS analyses were performed using an Acquity UPLC coupled with a XEVO TQ mass spectrometer equipped with an electrospray ion source (Waters Corporation, Milford, USA) and operating in MRM mode recording two specific transitions for each pesticide. The column used was an Acquity UPLC BEH C18 (1.7 μm particle size, 100 × 2.1 mm), and the mobile phases were A (water with 0.1% formic acid) and B (methanol with 0.1% formic acid). The gradient conditions were as follows, based on times (t): t_1 = 0–0.25 min, hold 95% A, 5% B; t_2 = 0.25–6 min, ramp linearly to 70% B; t_3 = 6–7.5 min, hold 70% B; t_4 = 7.5–9.5 min, ramp linearly to 100% B; t_5 = 9.5–12 min, hold 100% B.

GC-MS/MS analyses were performed by Agilent 8890 gas chromatograph coupled to a TQ 7010B mass spectrometer (Agilent Technologies Inc., USA) equipped with an electron impact ion source (ionization energy = 70 Ev EI). GC analysis was conducted on a Restek Rxi-5Sil MS capillary column (20 m × 0.18 mm internal diameter × 0.18 μm) (Restek, USA) and the following conditions were used: He constant flow 1 mL/min, inlet temperature 260 °C, injection volume 1 μL (split, 1:10), MS transfer line temperature 280 °C, temperature program: 60 °C for 1 min, then 60 °C/min ramped to 170 °C, followed by 20 °C/min ramped to 320 °C (held for 1 min). The acquisition, as well as for the LC/MS system, was carried out in MRM mode.

Glyphosate was quantified following the QuPPe-PO-Method (M1.9, Version 12) (Anastassiades et al., 2020) which involves the use of an LC-MS/MS (Acquity UPLC coupled with a XEVO TQ mass spectrometer) system equipped with a Raptor Polar X column.

2.4. Validation method

Analytical parameters of the pollen multi-residue method such as matrix effect, limits of quantification (LOQs), limits of detection (LODs), linearity, precision and trueness were evaluated according to SANTE guidelines (SANTE/12682/2019; European Commission, 2020) (Tables S6 and S7). All pesticide parameters were quantified using five-point matrix-matched calibration curves ($R^2 > 0.98$) and triphenyl phosphate as internal standard. Matrix effects were evaluated by comparing the slope of the calibration curve done in solvent and the slope of that prepared in the extract of the pollen matrix. To verify the recovery (Rec%) and the repeatability (RSD%) of the method, a blank pollen matrix (no pesticide contamination) was used. Pesticides were added to the matrix at three concentration levels: 10, 50, and 200 μg/kg, and each added concentration level was analysed sixfold. Average values of Rec% and RSD% over three concentration levels complied with the SANTE guidelines (Rec % 70–120% and RSD% < 20%) (European Commission, 2020). The sensitivity of the method was estimated by establishing the LOQs according to SANTE guidelines, and LODs were estimated as one-third of the quantification limit. According to the SANTE guidelines, all obtained pesticide data were not corrected by the recovery since it was found to be between 80% and 120%.

2.5. Pesticide risk assessment

After determining the concentration in ppm, we calculated the Pollen Hazard Quotient (PHQ) (Stoner and Eitzer, 2013) for each compound in each pollen sample. PHQ is a measure of hazard from pesticide residues in pollen in relation to acute toxicity to honey bees, and it is calculated as the ratio between the compound concentration in ppb (μg/kg) and the oral or contact LD_{50} for honey bees. We retrieved oral LD_{50} from the University of Hertfordshire Pesticide Properties DataBase (Lewis et al., 2016). However, we used contact LD_{50} for five compounds, for which we could not obtain oral LD_{50}. Then, we determined the total PHQ of each pesticide category (fungicides, herbicides, and insecticides/acaricides) in each pollen sample by summing PHQ values of each category in each sample, and the total PHQ for each pollen sample by summing PHQ values of all compounds in each sample. We assumed additive toxic effects of multiple pesticides due to the lack of information on possible synergistic or antagonistic effects.

In addition, we calculated the acute risk quotient (RQ) for honey bees for each compound in each pollen sample using the US Environmental Protection Agency BeeREX model. While PHQ is ideal for evaluating the effect of specific drivers on multi-residue contamination of pollen, it does not take into account the amount of pollen consumed by honey bees, as opposed to the BeeREX model. First, we calculated the total dose of each compound consumed by each bee as the product between the concentration of the compound in μg/mg and the dose of pollen consumed by the honey bee in mg/day. Since we were not interested in testing how pesticide toxicity varied for different bee castes, we considered the highest consumed dose, which is 9.6 mg/day for nurse workers. Second, we calculated the acute RQ as the ratio between the total dose of pollen consumed by each bee and the oral LD_{50} for the compound. An acute RQ > 0.4 exceeds the concern threshold and indicates high toxicity of the compound for honey bees.

2.6. Statistical analyses

In order to determine the effect of pesticide category, landscape composition, and seasonality on PHQ of each pollen sample, we used linear mixed-effects models. We included the total PHQ of each pesticide category (fungicide, herbicide, and insecticide/acaricide) as response variable (ln-transformed), while selected explanatory variables were the year and the interaction between the sampling month and pesticide category, between the sampling month and landscape PC1, between the sampling month and landscape PC2, and between the sampling month and landscape PC3. Landscape PC1, PC2 and PC3 were calculated at both spatial scales, *i.e.*, 3 km and 5 km radius buffers around the sampling locations. To account for the repeated measures, we included the sample ID nested within the location ID as random factor. Then, starting from the full model, we used a backward deletion procedure, removing one-by-one interactions with *p*-value >0.05, and re-ran the model to correctly interpret the main effects. We tested whether model residuals were spatially auto-correlated using Moran's I in the R package *ape* (Paradis and Schliep, 2019) and we detected no spatial autocorrelation (global test, p-value = 0.859).

Then, we focused on the effect of specific crop categories on PHQ of pollen samples. We built two linear mixed-effects models using the total PHQ of pollen samples as response variable, and the cover of annual crops (including non-irrigated arable land, complex cultivation patterns, and agriculture with significant areas of natural vegetation) and perennial crops (including fruit trees, berry plantations, and vineyards) in the landscape at 3 km and 5 km radius buffers around the sampling locations as explanatory variables. We also included the location ID as random factor. Since the results of the models at the two spatial scales were similar, we presented in the main text only the results of the models at the 3-km radius scale.

3. Results

Out of the total 147 samples, only 4% were free of pesticide residues. We detected a total of 97 compounds in pollen samples, mostly fungicides (N = 48), followed by insecticides (N = 32) and only a few herbicides (N = 17) (Fig. S3a). The proportion of the detected compounds was similar throughout the season (Fig. S3b). On average, we detected 11 compounds in each pollen sample.

The concentration of detected pesticides was significantly higher for fungicides than for insecticides/acaricides and herbicides (Fig. 1a). The most abundant compounds were all fungicides, *i.e.*, captan, detected in 30% of samples with a total concentration (summed across all pollen samples) of 320.135 ppm (max = 142 ppm, mean = 2.178 ppm); folpet, detected in 10% of samples with a total concentration of 28.409 ppm (max = 15.6 ppm, mean = 0.193 ppm); and zoxamide, which was the most common compound, detected in 80% of samples, with a total concentration of 16.955 ppm (max = 3.99 ppm, mean = 0.115 ppm) (Table S3). The fungicides spiroxamine and penconazole were also commonly detected in our samples, respectively in 62% and 50% of samples (Table S3).

However, the overall toxicity of fungicides was low, as these compounds are mostly characterized by high LD_{50} values. The highest contribution to the total PHQ of pollen samples was made by insecticides/acaricides, in particular neonicotinoids and organophosphates (Fig. 1b). The compounds with the highest total PHQ (summed across all pollen samples) were all insecticides, *i.e.*, dimethoate, imidacloprid, and indoxacarb (Table S3). Dimethoate showed a total PHQ of 31,870 and, despite its toxicity, it was very common, being detected in 23% of samples, with a total concentration of 3.187 ppm (max = 1.370 ppm, mean = 0.022 ppm). Three pollen samples showed particularly high dimethoate concentrations, which led to PHQ values for the compound of 13,700, 7,470, and 4,840, corresponding to 137%, 75%, and 48% of the oral LD_{50}, respectively. Dimethoate in these three pollen samples exceeded the concern threshold for acute RQ, with acute RQ values of 0.132, 0.072, and 0.046, respectively. Imidacloprid showed a total PHQ of 25,405, and it was also common, being detected in 20% of samples, with a total concentration of 0.094 ppm (max = 0.038 ppm, mean = 0.001 ppm). One pollen sample showed a peak of imidacloprid concentration, which led to a PHQ value of 10,270, corresponding to 102% of the oral LD_{50}, and an acute RQ value of 0.099, beyond the concern threshold. Indoxacarb showed a total PHQ of 4821 and was less common than dimethoate and imidacloprid, being detected in 7% of samples with a total concentration of 1.119 ppm (max = 0.812 ppm, mean = 0.008 ppm). One sample showed a peak of indoxacarb concentration, which led to a PHQ value of 3,500, corresponding to 35% of the oral LD_{50}, which however did not exceed the concern threshold for acute RQ.

Although the toxicity of single compounds in terms of acute RQ was relatively low, total PHQ values of pollen samples were high (total PHQ >1000) in 8% of samples, medium-high (500 < total PHQ <1000) in 7% of samples, medium (50 < total PHQ <500) in 22% of samples, and low (total PHQ <50) in 58% of samples. Total PHQ was influenced by the interactions among pesticide category, landscape composition, and seasonality at both 3 km and 5 km radius buffers around the sampling locations (Table 1, S8). PHQ of pollen samples changed throughout the

Table 1
Results of the linear mixed-effects model testing the effect of the interaction between the sampling month and pesticide category, the interaction between the sampling month and landscape PC1, the interaction between the sampling month and landscape PC2, and the sampling year on PHQ of pollen samples (ln-transformed). Landscape PC1, PC2 and PC3 were calculated using the regional land-use map categories in 3 km radius buffers around the sampling locations. Values in bold indicate significant effects (p-value <0.05). Only significant results after a backward stepwise model selection procedure are reported.

	χ2	df	p-value
Intercept	0.001	1	0.980
Month	14.108	5	**0.015**
Pesticide category	44.770	2	**<0.001**
Landscape PC1 (3 km)	15.363	1	**<0.001**
Landscape PC2 (3 km)	0.137	1	0.711
Landscape PC3 (3 km)	0.103	1	0.749
Year	0.787	1	0.375
Month x Pesticide category	34.736	10	**<0.001**
Month x Landscape PC1 (3 km)	23.513	5	**<0.001**

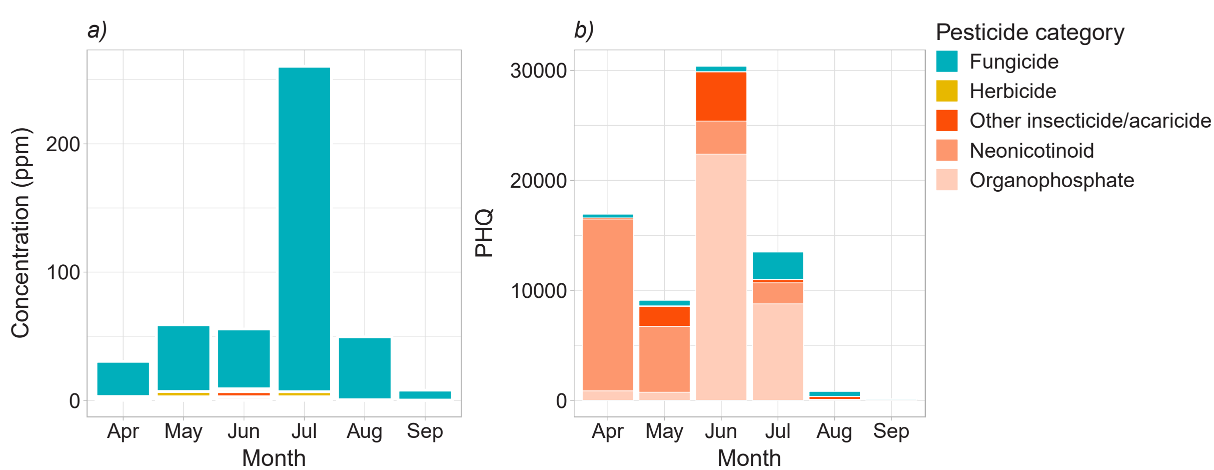

Fig. 1. Barplots showing *a)* total concentrations in ppm of pesticide categories for each sampling month and *b)* total PHQ of pesticide categories for each sampling month.

season based on the pesticide category. The peak of PHQ of fungicides was in July, but PHQ was approximately constant in all months. On the other hand, PHQ of herbicides and insecticides/acaricides was higher from April to June, and it decreased, especially for insecticides/acaricides, at the end of the season (Fig. 2). PHQ was also influenced by the interaction between the sampling month and landscape PC1 calculated at both 3 km and 5 km radius buffers around the sampling locations. PHQ increased with increasing landscape PC1 from April to July, while in August and September, landscape composition did not affect PHQ of pollen samples (Fig. 3, S4). Low values of landscape PC1 were related to semi-natural areas, in particular coniferous forests, natural grasslands, and sparsely vegetated areas, while high values were related to both agricultural and urban areas (Figs. S2a and b).

Total PHQ of pollen samples increased with increasing cover of perennial crops, *i.e.*, fruit trees and berries plantations and vineyards, at both 3 km and 5 km radius buffers around the sampling locations (Tables 2, S9, Fig. 4, S5). However, total PHQ was only marginally affected by the cover of annual crops in 3 km radius buffers around the sampling locations (Table 2), and the effect was not significant at a larger scale (Table S9).

4. Discussion

Contamination by pesticides of pollen collected by honey bees can seriously threaten the health of honey bees and their colonies. Here, we performed a multi-residue analysis, testing for almost 400 compounds, and explored how the potential toxicity of pollen changed based on the pesticide category and how it could be modulated by landscape context and seasonality. For the first time, we demonstrated the complex interactive effects of these three variables on the potential toxicity of pollen collected by honey bees. In particular, we found that the peak of potential toxicity of pollen for honey bees changed for fungicides, herbicides, and insecticides and acaricides. Moreover, the effect of landscape composition, in particular of agricultural and urban cover, was modulated by the sampling month. Also, a high cover of perennial crops in the landscape, but not of annual crops, was associated with a higher potential toxicity.

We detected at least one compound in 96% of analysed pollen samples. The few pesticide-free pollen samples were all collected in the areas with the highest cover of semi-natural areas. Similar works carried out in Italy found a lower rate of pollen sample contamination, with 50–62% of samples contaminated by pesticides (Tosi et al., 2018; Martinello et al., 2019), however, a study conducted in our same study region found no samples free of pesticides (Favaro et al., 2019). Out of the

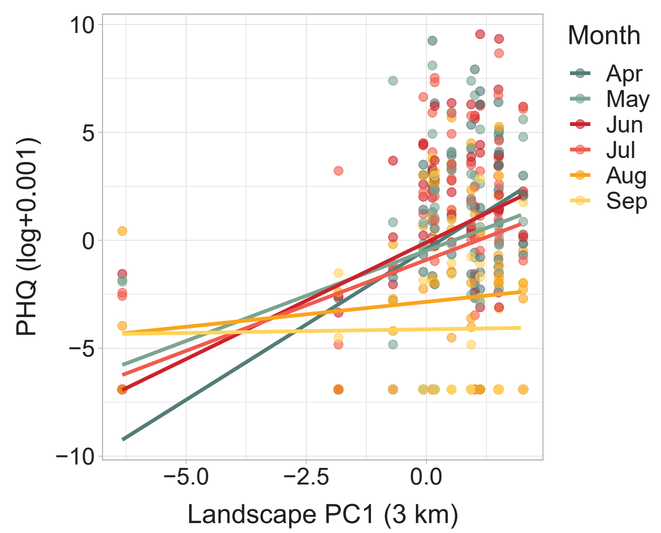

Fig. 3. Plot showing the effect of the interaction between the sampling period and landscape PC1 on PHQ of pollen samples (ln-transformed). Landscape PC1 was calculated using the regional land-use map categories in 3 km radius buffers around the sampling locations. Points represent raw data points and lines represent model estimates.

375 compounds searched in pollen samples, we identified 97 compounds (26%), a percentage similar to the one reported by Böhme et al. (2018) but much higher than Favaro et al. (2019), who only detected 13% of the searched compounds. Our surveys were done halfway through each month, but we had no information on whether pesticide treatments were applied in the surrounding areas before our surveys due to the high number of farmers and fields. Although the investigated areas were mainly mountainous and characterized by a relatively high cover of semi-natural areas, the agricultural and urban areas around the hives boosted the presence of pesticides in pollen.

Herbicides were rare in our pollen samples and comprised the least toxic compounds, despite their toxicity being considered moderate for honey bees (Iwasaki and Hogendoorn, 2021). By far, the most common and abundant pesticides detected in pollen samples were fungicides, as also shown by other works (Mullin et al., 2010; Friedle et al., 2021). The most commonly detected were zoxamide, which affects cytoskeleton and motor proteins, and spiroxamine and penconazole, which affect sterol biosynthesis in membranes. These fungicides are used for the

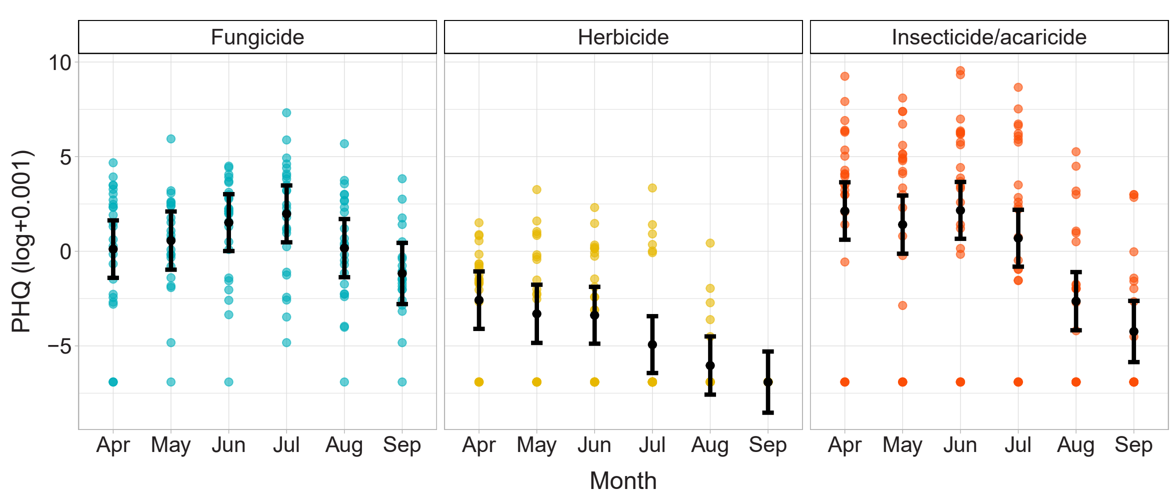

Fig. 2. Plots showing the effect of the interaction between the sampling period and the pesticide category on PHQ of pollen samples (ln-transformed). Small coloured points represent raw data points, large black points represent model estimates, and bars represent the 95% confidence intervals.

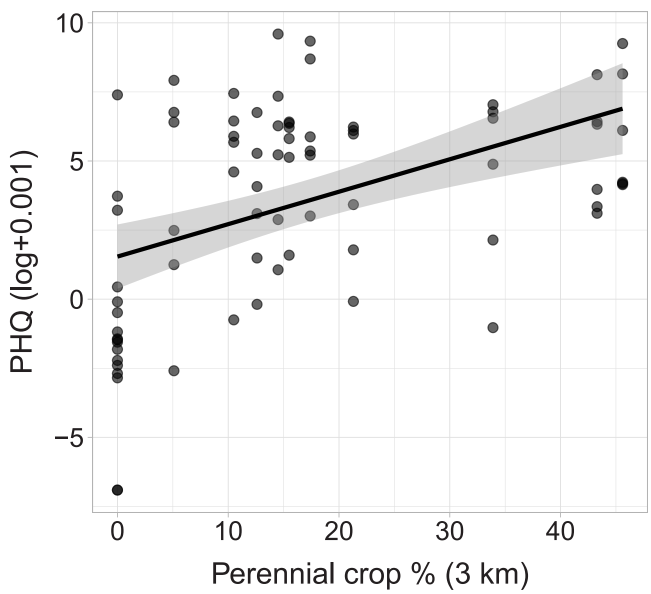

Fig. 4. Plot showing the effect of the cover of perennial crops (fruit trees and vineyards) in 3 km radius buffers around the sampling locations on PHQ of pollen samples (ln-transformed). Points represent raw data points, the line represents the model estimate, and the shaded area represents the 95% confidence interval.

Table 2
Results of the linear mixed-effects model testing the effect of the cover of annual and perennial crops in 3 km buffers around the sampling locations on PHQ of pollen samples (ln-transformed). Values in bold indicate significant effects (*p*-value <0.05).

	value	SE	df	*t*-value	*p*-value
Intercept	0.264	1.012	64	0.261	0.795
Annual crop % (3 km)	5.865	2.892	10	2.028	0.070
Perennial crop % (3 km)	13.108	3.646	10	3.595	**0.005**

control of fungal pathogens in a variety of crops, in particular vineyards, fruit orchards and cereal fields. The toxicity of fungicides for honey bees is generally low since they do not directly impact insects, and therefore their potential negative effects on honey bees are still debated (Iwasaki and Hogendoorn, 2021). For example, Tamburini et al. (2021) highlighted no effect of azoxystrobin, a systemic broad-spectrum fungicide, on honey bee colonies at field-realistic exposure, while Al Naggar et al. (2022) showed that the same compound could have detrimental effects on the gut microbiota of bees. However, the major threat of fungicides is related to their interaction with other compounds (Iwasaki and Hogendoorn, 2021; Ward et al., 2022): for example, the acute toxicity of some insecticides dramatically increases in the presence of fungicides (Tsvetkov et al., 2017). Moreover, even low doses of pesticide mixtures, considered not harmful for honey bees, may reduce the efficiency of insects exposed in early development stages (Prado et al., 2019). While laboratory experiments are fundamental to test the effect of single pesticides, it is crucial to also investigate the effect of multiple pesticides to which bees could be exposed in nature.

In general, only a few single compounds exceeded the concern threshold for the acute RQ. However, multi-residue analysis showed that 15% of analysed pollen samples had medium or high levels of PHQ, which could pose serious threats to honey bees. Insecticides and acaricides were less common than fungicides, however, they contributed most to the total PHQ of pollen (Friedle et al., 2021; Knapp et al., 2023). This result was expected since insecticides and acaricides are specifically formulated to negatively affect arthropods, therefore including non-target species. The most toxic insecticide categories were neonicotinoids, especially abundant in April and May, and organophosphates, which boosted PHQ of pollen in June and July. Neonicotinoids are highly efficient in controlling target species, but consequently also highly toxic to bees. These insecticides are known to strongly negatively impact bee survival, also during overwintering, and bee general health, especially their immune and reproductive systems (Tsvetkov et al., 2017; Woodcock et al., 2017). Since the use of neonicotinoids has led to higher risks to bees in the last decades (Goulson et al., 2018), strict regulations have been imposed. In the analysed pollen samples, imidacloprid was the neonicotinoid with the highest PHQ, as also highlighted by Tosi et al. (2018). Imidacloprid is an insecticide with immunosuppressive activity, that also showed detrimental effects on bee memory (Williamson and Wright, 2013; Di Prisco et al., 2013; Delkash-Roudsari et al., 2022, but see Dai et al., 2019). Despite a shift towards neonicotinoids in the last few years (DiBartolomeis et al., 2019), organophosphates are still commonly used in Italy (Porrini et al., 2016), as demonstrated by the widespread use of the highly toxic dimethoate.

4.1. Banned pesticides

We detected a few compounds that were banned for use in the EU because of health and environmental concerns, as already reported for Italy (Perugini et al., 2018). We identified the fungicide carbendazim, which was banned in the EU since 2014, at 6 locations and in 14 samples, 7 from 2019 to 7 from 2020, at higher concentrations in 2020 (mean = 0.002 ppm) than in 2019 (mean = 0.0003 ppm). The use of carbendazim is still widespread in the EU (Pesticide Action Network Europe, 2020), and it was also detected in food produced in Italy (EFSA, 2022). However, carbendazim is also a metabolite of thiophanate-methyl, a fungicide which was still legal to use until 2021 and was also detected in our samples. Therefore, the contamination of some of our pollen samples by carbendazim could be related to thiophanate-methyl conversion - although carbendazim and thiophanate-methyl were not detected in association in two pollen samples, pointing out the need for stricter controls on pesticide use in Europe. The fungicide quinoxifen and the insecticide chlorpyrifos were banned in Italy in March and April 2020 respectively, and after these dates, we detected them in 2 samples from 2 locations and 4 samples from 4 locations respectively. Some highly toxic compounds were banned in Italy after our surveys, *e.g.*, the neonicotinoid insecticides thiamethoxam and imidacloprid, and the oxadiazine insecticide indoxacarb, which was one of the compounds with the highest total PHQ. Lastly, in June 2020, the use of dimethoate was banned in Italy following the EU Regulation 2019/1090, however, its use was allowed in olive orchards until October 2020.

4.2. Interactive effect of pesticide category and seasonality on pollen toxicity

Seasonality differently affected PHQ of the three pesticide categories. For herbicides and insecticides and acaricides, PHQ was higher in spring and early summer and started to decrease in July. While herbicide contamination was generally low, the result for insecticides is probably largely related to applications in apple orchards, which in Italy are mainly made in spring and early summer (Garthwaite et al., 2015) and may have boosted pollen contamination. Similarly, other works found that pollen contamination by insecticides decreased at the end of summer (Tong et al., 2018; Friedle et al., 2021; Murcia-Morales et al., 2021). Some studies highlighted a peak of acaricide PHQ at the end of the season caused by treatments against *Varroa* mites (Murcia Morales et al., 2020), which we did not observe since our honey bee colonies were only treated with oxalic acid.

On the other hand, PHQ of fungicides slightly decreased in August and September, but it was more uniform throughout the season. The presence of fungicides in pollen samples was probably related to both apple orchards and vineyards in the landscapes. In apple orchards, fungicides are mainly applied to control diseases such as powdery mildews, apple scab, and cankers, while in vineyards they are used to prevent downy mildew, powdery mildew, and grey mould. These treatments are usually applied throughout the season, from the beginning of the vegetative growth to post-flowering of crops, and therefore caused little seasonal variation in fungicide PHQ.

4.3. Interactive effect of landscape composition and seasonality on pollen toxicity

The effect of landscape composition on PHQ of pollen collected by honey bees was modulated by the sampling month. PHQ increased with increasing cover of agricultural and urban areas from April to July, while it was lower in August and September and unrelated to landscape composition. It is well known that seasonality can have a strong effect on the detection of pollen contaminations (Koech et al., 2023), with fewer pesticides detected at the end of the season according to plant protection practices (Murcia-Morales et al., 2021). On the other hand, the effect of landscape context on honey bee pollen contamination is still debated. For example, some works highlighted that pesticide contamination was independent of landscape composition (Raimets et al., 2020; Koech et al., 2023; Knapp et al., 2023), while others showed that samples collected from hives placed in agricultural areas exhibited a higher concentration of pesticides (David et al., 2016; Meikle et al., 2020; Zaller et al., 2022). Our honey bee hives were not placed in intensive agricultural landscapes, since all selected landscapes were characterized by a certain cover of semi-natural areas (mean cover = 50%). Nevertheless, pollen contamination was high in areas with a higher cover of urban and agricultural areas, emphasizing that even a small amount of these areas may seriously threaten bees (Main et al., 2020).

An additional factor that should be considered when exploring the effect of landscape composition on pollen contamination is pesticide drift, which could increase risks to honey bees in agricultural-dominated landscapes. Pesticide residues could drift from focal crops to surrounding areas, contaminating pollen and nectar of wildflowers at field margins (Ward et al., 2022). Since a high diversity of floral resources could be related to a higher contamination risk of pollen collected by bees (Bednarska et al., 2022), the lower diversity of floral resources typical of the end of the season could have also minimized pollen contamination by pesticides.

Unexpectedly, urban areas emerged as key pathways of pollen contamination by pesticides. Contamination is usually lower in urban areas compared to rural areas (David et al., 2016; Siviter et al., 2023). However, recent studies underlined that urban habitats could be associated with high pollen contamination by pesticides, which may exceed that of agricultural habitats in some sampling periods (Benner et al., 2023), in particular for specific compounds such as neonicotinoid insecticides (Botías et al., 2017; Kavanagh et al., 2021). Moreover, urban areas pose additional risks to honey bee health due to air pollution, which can negatively affect odour learning and memory (Leonard et al., 2019). The impacts of pesticide applications and pollution in general on pollinators in urban areas are still largely unexplored, despite the widespread use of pesticides in public and private gardens and the growing interest in urban beekeeping in most cities (Matsuzawa and Kohsaka, 2021), underscoring the need for further studies on this topic.

4.4. Effect of annual and perennial crops on pollen toxicity

The cover of annual crops, which in the study area mostly included maize, did not affect pollen contamination, as also observed by Wintermantel et al. (2020). On the other hand, pollen collected in landscapes with a high cover of perennial crops, *i.e.*, fruit orchards and vineyards, was characterized by a higher PHQ. Böhme et al. (2018) analysed pesticide residues in pollen collected by honey bees in different habitats, highlighting the lowest pollen contamination in semi-natural habitats, intermediate contamination in grain fields, and the highest contamination in fruit orchards, similar to what we observed. Most of the perennial crops in our landscapes were apple orchards, which are crucial for the economy of our study area, and covered a large portion of our landscapes, ranging between 0 and 45% (mean = 9%). Apple is one of the most sprayed crops, with an average of 25 pesticide applications per year (Garthwaite et al., 2015), and therefore pollen collected in apple orchards often shows high levels of pesticide contamination (Knapp et al., 2023). However, it is also important to emphasize that most pesticide applications, especially for insecticides, are not permitted during blooming to safeguard pollinators. The high PHQ observed during apple blooming could be therefore related to pre-blooming pesticide applications, since most treatments in apple orchards in the study region are made between mid-March and the end of May (Garthwaite et al., 2015), but also to contamination at non-focal crops, as also highlighted by McArt et al. (2017).

4.5. Study limitations

Like most of the works performed under field conditions, we were not able to account for the potential interactive effects among pesticides, thus considering only additive effects when estimating multi-residue pollen toxicity, and potentially underestimating the negative effects on bee health. In fact, laboratory trials showed that the toxicity of pesticide mixtures can increase synergistically and also lead to an amplification of the sub-lethal effects of the least-toxic compounds, resulting in detrimental effects on bee health and colony longevity (Di Prisco et al., 2013). Therefore, future studies should also consider the possible synergies among pesticides, in order to have an accurate and realistic assessment of the impacts of pesticides on honey bees.

5. Conclusions

Honey bees are key pollinators and are seriously threatened by pesticide applications. Here, we showed that the potential toxicity of pollen collected by honey bees was influenced by the interaction of multiple factors, *i.e.*, the pesticide category, landscape composition, and seasonality. We highlighted that contamination was generally higher in spring and early summer, and that semi-natural areas can contribute to decreasing pollen contamination. We also found that pesticide applications in urban and agricultural areas, especially in perennial crops, were probably responsible for a high contamination of pollen. To ensure the well-being not only of pollinators but also of humans, without overlooking crop protection, specific actions can be implemented. For example, farmers should decrease their dependency on pesticides, moving towards more sustainable management practices such as the use of pheromones and biopesticides (Pretty, 2018; Baker et al., 2020). Agrochemical companies should formulate compounds that are more selective and less toxic to non-target organisms, focusing on new technologies such as controlled release systems (Singh et al., 2020). Lastly, beekeepers should always carefully assess where to place honey bee hives, preferring whenever possible areas surrounded by semi-natural habitats, in order to provide potentially uncontaminated resources for bees.

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CRediT authorship contribution statement

Andree Cappellari: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Visualization. **Valeria**

Malagnini: Conceptualization, Data curation, Investigation, Methodology, Validation, Project administration, Writing – review & editing. **Paolo Fontana:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing – review & editing, Project administration, Resources. **Livia Zanotelli:** Investigation. **Loris Tonidandel:** Investigation, Validation. **Gino Angeli:** Conceptualization, Methodology. **Claudio Ioriatti:** Conceptualization, Methodology, Writing – review & editing. **Lorenzo Marini:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lorenzo Marini reports financial support was provided by Horizon Europe. Valeria Malagnini, Paolo Fontana, and Gino Angeli report financial support was provided by Associazione Produttori Ortofrutticoli Trentini.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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