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Findings of herbicide and fungicide residues in bee bread

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Abstract: The honey bee is one of the insects that is significantly endangered by the application of pesticides in the cultivation of crops. Not only is acute toxicity dangerous, but the importance of chronic poisoning by low doses of pesticides in hives is growing. The behavior of bees can be affected not only by insecticide residues but also by herbicide and fungicide residues. In 2016–2018, samples of bee bread were analysed for pesticide content at 25 different localities from intensive agricultural production areas of the Czech Republic. Substances were extracted by QuEChERS and determined by liquid chromatography, together with mass spectrometric detection. We detected up to 18 pesticides in one sample. In total, during 2016–2018, we identified 53 active substances. Fifteen substances (31%) were herbicidal, 23 substances (47%) of fungicidal nature and 6 substances (12%) of insecticidal nature. The coefficient of variation showed large differences in the frequency of revealed pesticides between years. For substances sprayed outside period attractive for pollinators (mainly herbicides and some fungicides), the usual methodology cannot reliably determine the degree of contamination, and thus the actual contamination with these substances may be even higher than demonstrated in this study.

Keywords: pollen; chemical analyses; plant protection; *Apis mellifera* L.; chronic toxicity

The application of plant protection products has become an essential part of intensive agricultural production. Pesticides are biologically very active substances and also affect many non-target organisms. Adverse effects of pesticides do not only occur shortly after application, but pesticide residues pollute the environment virtually perpetually. Considering all of the non-target organisms, pollinators are perhaps at the most significant risk from acute as well as chronic effects of pesticide usage. It is this group of insects that is essential for nature and humanity. The use of pesticides in agriculture is one of the main factors contributing to the decline of pollinators (Wu et al. 2011). Many cases of bee extinction are confirmed worldwide due to the application of pesticides to neighboring habitats (Henry et al. 2012). In Europe, four-fifths of wild plants and crops depend on insect-assisted pollination. Pollinators contribute $15 billion to European agriculture (Potts et al. 2015).

The main pollinator in Europe is the honey bee (*Apis mellifera* L.) (Klein et al. 2007). Bees are of agronomic, environmental, and economic importance. In addition to pollination, they are also important in the commercial production of wax, pollen, propolis, royal jelly, and honey (Brown and Paxton 2009). However, bees live in the agricultural landscape and

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encounter applied pesticides that they carry into hives (Mitchell et al. 2017). Insecticides sprayed on plants can be toxic to bees when they come into contact with treated plants or when they fly over a contaminated area and absorb toxic particles in the air. They can also poison the entire colony with contaminated pollen and nectar (Dively and Kamel 2012).

However, pesticides in hives may not only come from ongoing applications. Contamination could come from previous years (Karise et al. 2017). Pesticide residues can also reach beehives from non-agricultural areas. According to the Czech Hydrometeorological Institute, pesticide residues occur in the Czech Republic both in groundwater and surface waters. Therefore, bees can also bring contaminated water to the hive with these substances. In 2017, we found up to 22 substances in surface water samples. In one sample, there were up to 8 substances, and some exceeded the allowed limit. Active substances such as azoxystrobin and tebuconazole were found. Both above-recommended limits and chlorpyrifos were found but below recommended limit. We found these active substances of a fungicidal and insecticidal nature in surface waters similar to those in the bee products (Kodeš V. 2018).

Pesticides can also contaminate bee products, which are part of human nutrition. Monitoring of pesticide residues can provide information on the use of pesticides in the vicinity of hives. We can use bee products, especially pollen, as a bioindicator to assess the environmental impact of pesticide applications.

The bees of one colony consume several tens of kilograms of honey and pollen during the year. Pollen is consumed in large quantities, in particular by young bees feeding larvae (Lipinski 2018). After the application of pesticides, their residues were found in pollen in May. A compilation of published data from 1968 to 2019 (Zioga et al. 2020) summarised that 17 fungicides and 14 insecticides were detected directly in flowered pollen.

More often than pollen sampled directly from plants, pollen in beehives is analysed for pesticide residues. In recent decades, there has been regular evidence from around the world that pesticide residues are present in the pollen samples. In the Pilling and Jepson (1993) study, it was found that of 73 samples of bee bread (ambrosia) analysed, only 9 samples were free of pesticide residues. Colwell et al. (2017) collected bee bread from Canada and France, where at least one pesticide was detected in 83% of samples. However, there is still little information on changes in the number of pesticide residues in hives. There is no knowledge of how pesticides are preserved or metabolised in bee products.

Due to the high content of monosaccharides, stocks of honey and bee bread present a reducing environment to chemical residues. Since honey contains low total water content (15–20%) and bee bread also enzymatically consumes oxygen from its surroundings, it stabilises the bee products but also stabilises residues of foreign substances, including neonicotinoids (Titěra and Kamler 2013).

Monitoring the presence of pesticides in pollen can provide information on the use of pesticides around hives, and bee pollen analyses can become a bioindicator for assessing the number and quantity of pesticide residues in the environment (Martel and Lair 2011, Tette et al. 2016). On average, a large beehive per year has a pollen clutch of about 30 kg (Tautz 2016). Bioallethrin and pendimethalin in Brazil were not detected in the wild, but analysis of pollen samples from commercial apiaries was positive in 33%. These results demonstrated the potential of bee pollen as a bioindicator of environmental contamination by pesticides (Garbuzov et al. 2015)

In addition to the commonly observed insecticides, fungicides and herbicides, other compounds may also appear in pollen samples. Stoner and Eitzer (2013) detected 78 pesticides and their metabolites in pollen in the USA, of which 20 were fungicides and 15 herbicides. Analyses from Canada and France found 11 fungicides, 5 acaricides, and 2 herbicides (Colwell et al. 2017).

Because herbicides and fungicides are not targeted against insects, it is not known exactly whether they pose a risk to bees and other pollinators. However, emerging evidence suggests that herbicides may affect bee navigation, learning, and larval development, while fungicides may affect food consumption, metabolism and the bee's immune response if exposed to these compounds directly by contact exposure during or after application or oral exposure through contaminated nectar and pollen (Cullen et al. 2019). To minimise the impact on non-target, pollinating insects such as bees, it is important to understand any possible effects that these compounds may have, to identify the risks they pose; and, to mitigate them. Bees can come into contact with herbicides and fungicides in the environment in two main ways; contact or local exposure when the bee is directly sprayed or comes into physical contact with the sprayed part of the plant, or oral exposure through foraging for

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nectar and pollen on a plant treated with a herbicide or fungicide (Böhme et al. 2018).

There is also evidence that the use of herbicides could affect the intestinal microbiota. Fungicides and herbicides may also potentiate the effects of insecticides on bees when applied together. The insecticidal active ingredients acetamiprid or thiacloprid, when mixed with fungicides that inhibit the demethylation of myclobutanil or tebuconazole, may increase toxicity to bees (Moreno-Gondález et al. 2020).

Traditionally, only the effects of direct poisoning are taken into account when preparing legislative restrictions on the use of pesticides. However, damage caused by acute toxicity is not the only threat bees face. Therefore, sublethal effects on bees, such as paralysis, disorientation, or behavioral changes, both short-term and long-term, are increasingly being investigated (Rortais et al. 2005). Thus, sublethal doses pose a threat to the survival of the entire bee colony (Wu-Smart and Spivak 2016), with detrimental effects having reduced sensitivity to sucrose stimulation (Aliouane et al. 2009), hive contamination (Rortais et al. 2005) and, reduced ability to pollinate plants (Gill et al. 2012).

The study in this article from 2016–2018 was focused on the comparison of the content of fungicide and herbicide residues with the content of insecticide residues in pollen. The study aimed to point out that even substances that are not classified on safety to bees often appear in hives. Subsequently, it is anticipated that additional research will clarify the effect of these residues on bee colonies.

MATERIAL AND METHODS

In the project, we focused on a detailed analysis of bee bread from hives at 25, intensively farmed localities in the Czech Republic, where oilseed rape is significantly represented in the range of hives. In 2016–2018, bee bread samples were taken from the hives (beginning of June). We used new pesticides-free frames previously inserted at the beginning of winter oilseed rape vegetative growth in early spring.

Due to unsuitable climatic conditions for bee pollen collection, it was not possible to evaluate all localities in each year. In 2016, 19 samples were evaluated; in 2017, there were 24; and in 2018, 14 samples were evaluated. The results were also evaluated by the coefficient of variation, which showed the variability of the occurrence of active substances between years.

Pollen samples were also taken in 2019, similarly to previous years, but only from 1 locality from neighboring beehives. This cohort consisted of 20 samples.

Chemical analysis. Pollen samples were analysed for pesticide residues at the Department of Food and Nutrition Analysis at the University of Chemistry and Technology (UCT) in Prague.

Targets were extracted by QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) and determined by liquid chromatography along with mass spectrometric detection using a Waters Acquity UPLC liquid chromatography with a Waters Xevo TQ-S mass detector (Agilent Technologies, Santa Clara, USA).

We detected a total of 330 active substances of pesticides in the samples, registered in the past or present in the Czech Republic.

RESULTS AND DISCUSSION

Residues of different plant protection products were found in individual pollen samples. Pollen samples from one hive often differed significantly in the number of active substances detected. There were also differences between years (Table 1).

In 2016, 41 active substances were found in all pollen samples; in 2017, there were 27; and in 2018, 23. In individual samples, 5–12 active substances of pesticides were most often found. Figure 1 shows the percentage of substances found in each sample for all years of observation. In the sum of all three years, one active substance was found in one pollen sample as the smallest whereas, the highest number found in a single sample was 18 active substances.

However, the values indicated in Figure 1 and Table 1 do not show the number of residues of the individual active substances.

Of the three main groups of pesticides, insecticides are usually considered to be the most dangerous group on bees because they are intended to kill insects and therefore pose the greatest risk. However, pollen analyses show that bees encounter many other types of chemicals in their search for food (Table 1).

Figure 2 shows that fungicide residues predominate in all years during the 3-years of sampling, followed by herbicides residues. Relatively few samples contained insecticide residues, and there were minimal differences between years.

In a total of three years, 2016–2018, out of 49 identified active substances, 15 substances (31%) were herbicidal, 23 substances (47%) of fungicidal nature, 6 substances (12%) of insecticidal nature. Acaricides, drugs, and repellents also occurred in

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Table 1. Active substances found in pollen samples in 2016, 2017 and 2018 and area treated in the Czech Republic by them

Active substance (AS)	Biological function	Treated area (thousand ha)			Number of samples with confirmed AS (%) pollen			Coefficient of variation
		2016*	2017**	2018***	2016 (n = 19)	2017 (n = 25)	2018 (n = 14)	
2,4,6-Trichlorophenol	herbicide				0	0	7	1.4
2-Phenylphenol	preservative				11	16	36	0.5
Acetamiprid	insecticide	19	21	28	18	35	21	0.3
Acrinathrin	bee medicine				6	5	0	0.7
Azoxystrobin	fungicide	49	48	41	71	80	36	0.3
Bixafen	fungicide	3	4	3	6	0	0	1.4
Boscalid	fungicide	31	39	38	41	60	43	0.2
Carbendazim	fungicide				5	4	7	0.2
Clomazone	herbicide	88	92	73	0	0	7	1.4
Cyproconazole	fungicide	22	12	10	47	35	7	0.6
N,N-Diethyl-m-toluamide	repelent				0	0	14	1.4
Deltamethrin	insecticide	3	3	4	6	0	0	1.4
Dimoxystrobin	fungicide	16	20	20	29	45	29	0.2
Dodine	fungicide	3	0,7		6	0	0	1.4
Ethofumesate	herbicide	27	28	27	6	5	0	0.7
Phenmedipham	herbicide	28	29	33	6	5	0	0.7
Fenpropidin	fungicide	70	64	57	0	0	7	1.4
Fenpropimorph	fungicide	50	38	35	0	5	0	1.4
Fluazifop	herbicide (graminicide)	3	3	1	18	25	7	0.4
Fluopyram	fungicide	3			29	35	14	0.3
Haloxyfop	herbicide (graminicide)	6	5	5	6	50	7	1.0
Hexachlorobenzene	fungicide				6	0	0	1.4
Hexythiazox	akaricide	19	22	23	6	0	0	1.4
Chlorotoluron	herbicide	73	91	118	0	0	7	1.4
Chlorpyrifos	insecticide	311	259	273	53	40	43	0.1
Iprovalicarb	fungicide	0.6	0.5	0.5	6	0	0	1.4
Clopyralid	herbicide	27	28	19	12	0	0	1.4
Lenacil	herbicide	21			6	5	0	0.7
Metamitron	herbicide	15	16	16	12	5	0	0.9
Metoxyfenozide	insecticide	8			6	0	0	1.4
Metolachlor	herbicide (graminicide)				12	0	0	1.4
Myclobutanil	fungicide	0.7	0.6	1	6	0	0	1.4
Pendimethalin	herbicide	20	18	22	18	35	0	0.8
Permethrin	repelent				12	0	0	1.4
Picloram	herbicide	13	13	16	12	0	0	1.4
Picoxystrobin	fungicide	12	12	6	12	10	0	0.7
Prochloraz	fungicide	134	140	101	24	20	0	0.7
Propiconazole	fungicide	56	37	30	24	15	0	0.8
Prothioconazole	fungicide	40	43	40	29	35	0	0.7
Quizalofop	herbicide (graminicide)				24	35	7	0.5
Spiroxamine	fungicide	94	103	93	6	30	7	0.8
Tau-fluvalinate	insecticide, bee medicine	4	4	6	53	50	21	0.3
Tebuconazole	fungicide	165	171	145	59	70	14	0.5
Terbuthylazine	herbicide	39			12	5	0	0.9
Tetraconazole	fungicide	3	3	3	6	0	0	1.4
Thiabendazole	fungicide				0	0	7	1.4
Thiacloprid	insecticide	86	97	118	88	95	50	0.3
Thiophanate-methyl	fungicide	46	65	79	18	5	0	1.0
Trifloxystrobin	fungicide	50	45	35	0	0	7	1.4

Empty cell – not published data; 0 – found below the LOQ limit; *CISTA (2016); **CISTA (2017); ***CISTA (2018)

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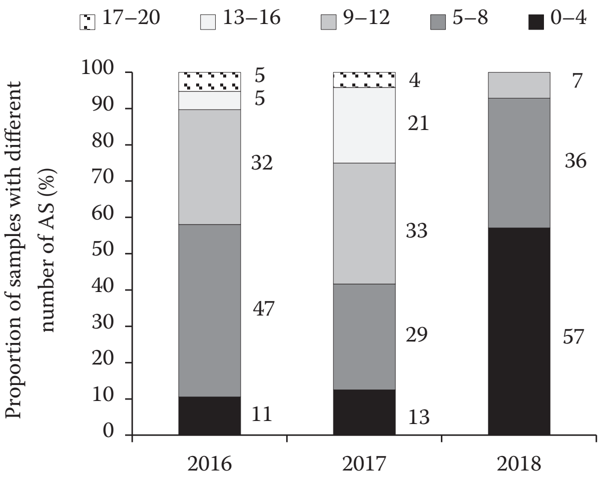

Figure 1. The proportion of samples with different number of active substances in 2016–2018

small numbers and even a dangerous food preservative (8% in total).

Similar results were reported by Pohorecka et al. (2017), which analysed a total of 123 samples of bee bread, of which 60.2% were contaminated. In that study, the most frequently revealed residues came from fungicides (45.3% of samples), insecticides (32.0%) and herbicides (24.5%). Twenty-two separate pesticides were recorded in positive pollen samples: ten fungicides, eight insecticides, and four herbicides.

Most of the detected pesticide residues were revealed in diminutive values – hundredths and sometimes in thousands of mg/kg of pollen. We found clopyralid, thiacloprid, boscalid, dodine, tebuconazole, quiazalofop, chlorpyrifos, prochloraz, dimoxystrobine, haloxyfop, and acetamiprid in pollen in larger amounts (tenths of mg/kg). In one case, we detected in pollen a quantity of 1 568 mg/kg of the fungicidal active ingredient tebuconazole. Thus, we revealed fungicidal substances in the highest amounts, followed by insecticidal and herbicidal substances predominately, graminicides.

Of all groups of plant protection products, fungicides appear in pollen most frequently. Detections varied from 48% to 69% from year to year (Table 1). In agricultural practice, fungicidally active substances from the groups of azoles and strobilurins are applied first to cereals and oilseed rape in the spring. These two crops are grown on approximately 65% of arable land in the Czech Republic. Usually, more than 95% of the areas of these crops are treated. Not all of these fungicide substances are classified as bee threatening and can be used in all crops without restriction. In oilseed rape, they are commonly applied to pre-flowering vegetation (CISTA 2020). We assume in our conditions the same high content of residual fungicides in oilseed rape pollen, as discovered by David et al. (2016). The author states that oilseed rape pollen was heavily contaminated with a whole spectrum of pesticides, as well as the pollen of wildflowers growing near treated areas with metconazole in amounts up to 0.07 mg/kg (David et al. 2015). He also detected the fungicides carbendazim, boscalid, tebuconazole, flusilazole, and metconazole in concentrations from 0.0008 mg/kg to 0.03 mg/kg in pollen from some hives.

In Germany, Böhme et al. (2018) also analysed pollen for pesticide residues. They found the most common substances were of a fungicidal nature in all years and all habitats, occurring in more than 50% of samples. Most alarming were the values found for boscalid at 1.49 mg/kg (MLR = 0.5 mg/kg) and dimoxystrobin 0.57 mg/kg sample (MLR = 0.5 mg/kg).

According to the authors Sanches-Bayo and Goka (2014), there were 14 fungicides and herbicides among the pesticides found in pollen, which are generally less toxic to bees than insecticides. Five substances (cardetamine, boscalid, pendimethalin, tebuconazole, and thiophanate-methyl) occurred more frequently (10–30% of samples).

Herbicides effective against dicotyledonous weeds were abundant in bee bread. The most frequently found active substance in 2016 was pendimethalin in 18% of samples. In 2017 the same substance was found in 35% of samples. Bee bread contaminants found in the hives were mainly herbicides, which were applied in the spring, especially to sugarbeet to control emerging dicotyledonous plants from uterine

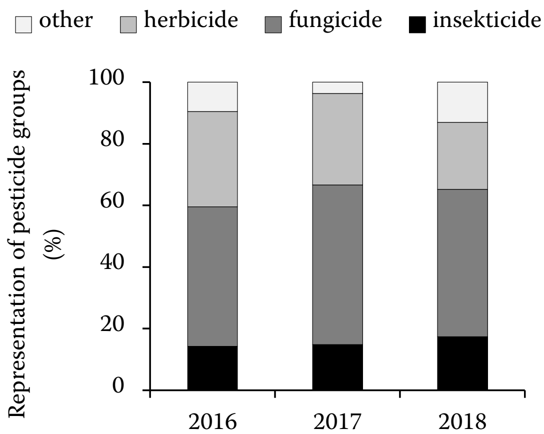

Figure 2. Residues found in pollen samples by groups of pesticides in 2016–2018

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leaves to 3–4 existing leaves. The active ingredients of herbicides applied against monocotyledonous weeds (graminicides) were often also present in the samples. In 2016, the most frequently found active substance was quizalofop in 24% of samples, and in 2017, haloxyfop was found in 50% of samples. The frequent occurrence of pendimethalin in bee bread has also been proven abroad (Garbuzov et al. 2015).

Herbicides against dicotyledonous weeds, as well as, graminicides have long residual effects. They are selective herbicides, and bees can be easily contaminated from foraging on resistant flowering weeds in treated areas. Another possibility of contamination is the spray drift of these herbicides to flowering perennial herbs, shrubs, or trees in the vicinity. These mature plants may not show a visible herbicidal effect, but the bees can again be easily contaminated. Not all of these herbicides are classified as hazardous to bees and can therefore be applied by growers without restriction. Evidence of the presence of herbicide residues in plant pollen is still lacking. In an extensive review of pesticide contamination of flowers with pesticides, the authors analysed 4 360 papers from 1968 to 2019 and did not find any data on the occurrence of herbicides in plant pollen (Zioga et al. 2020).

From the group of insecticides, only six insecticides appeared in bee bread, which is the least of all important groups of plant protection products. This can be explained by the fact that most insecticides are currently classified as hazardous, or particularly hazardous, to bees, and their application is thus significantly limited to flowering stands visited by pollinators.

Residues of pyrethroids are minimally present in the samples because they are relatively rapidly degradable in nature – at current higher temperatures. Also, pyrethroids have not been used so much in the last 5–8 years due to high levels of pyrethroid resistance in pollen beetle, *Brassicogethes* (Meligethes) *aeneus* (Stará and Kocourek 2018).

Presently, they are mostly classified as hazardous, or particularly hazardous, for bees and are therefore applied to a limited extent by growers. There is also a risk of contamination from non-professional users, as they are often sold in retail packaging in the regular sales network.

The active substance chlorpyrifos is present in large numbers of pollen samples (up to 65%). The amount of chlorpyrifos in pollen varied widely across samples but was found at mostly low levels. Only in 3 samples during the four years of our study did we detect chlorpyrifos residues higher than 0.1 mg/kg of pollen. This substance is classified as particularly hazardous for bees, so it must not be applied to flowering stands and is subject to notification to beekeepers. Since 2020, the use of chlorpyrifos in the European Union has been banned. It was most often applied in the spring to oilseed rape against spring pests, up to 80% of the sown area. The product is not systemic but has long residual effects. Stanley et al. (2015) state that chlorpyrifos is highly toxic to bees. Its rapid action can prevent bees from reaching the hive. The finding of residues can be explained by its long residual efficiency, frequent use on large areas (Table 1), or violation of prescribed conditions for application.

Residues of neonicotinoids: thiacloprid and acetamiprid are very problematic. Thiacloprid will no longer be allowed in EU countries from 2021. In particular, thiacloprid was present in up to 95% of pollen samples with relatively large residue loads, ranging between 0.1–0.2 mg/kg of pollen in many samples. Acetamiprid was significantly less frequently detected with only 3 samples in all years; they exceeded 0.1 mg/kg. These substances have been applied by growers to flowering stands of all crops especially, oilseed rape, because these two neonicotinoids are not classified in terms of toxicity to bees, and there is practically no substitute for them. In addition, they are very often used in non-professional applications because they are readily available in retail packaging. In general, these substances are not directly toxic to bees, but there is an increasing number of studies showing the adverse effects of their residues on all developmental stages of bees in the hive. Sublethal side effects of neonicotinoids on bees have been described in laboratory studies (Aliouane et al. 2009, Laurino et al. 2011). Global monitoring of neonicotinoid residues has also shown acetamiprid in a large number of honey samples from Europe, Asia, and South America (Mitchell et al. 2017). The presence of oxygen and water plays a major role in the breakdown of residues, and instability in light is crucial (in light, the half-life is in a matter of hours, in the dark up to a matter of years). As a result, neonicotinoid residues may persist in the bee's natural food supply. Due to the high content of monosaccharides, stocks of honey and bee bread are a reducing environment. Since honey contains a very low total water content (15–20%) and bee bread also enzymatically consumes oxygen from its surroundings, it stabilises the honey

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but also stabilises residues of foreign substances, including neonicotinoids as well (Titěra and Kamler 2013).

The active substance tau-fluvalinate achieved a high proportion of detections in pollen (up to 41% of samples) but usually in small amounts. Its use as an insecticide in field conditions does not correspond to this proportion (Table 2), but this acaricide is also used as an in-hive medicine for bees against the *Varroa destructor* mite. Other acaricides used as bee medicines also appeared in small amounts of pollen in 2016. Determining the proportion of acaricides used, including tau-fluvalinate in pollen from the intentional application as a drug or as an insecticide used in the protection of flowering crops, is difficult but important. Laboratories try to determine differences based on the analysis of specific bee enzymes degrading toxic substances (Mao et al. 2011).

When determining the pesticide residue content of bee products, the aim is to take samples from many habitats to obtain objective information corresponding to a large area. Since laboratory determination of pesticide residues is economically demanding, there is an effort to limit the number of analysed samples at one site. To verify the suitability of this procedure, a coefficient of variation was calculated for each active substance.

The values of the coefficient of variation of the frequencies of active substances found in pollen in the years 2016–2018 show that large differences were found in the regularity of the occurrence of individual active substances in individual years. The coefficient of variation ranged from 0.1 to 1.4. The low coefficient of variation draws attention to substances that contaminate pollen sources for pollinators in a large area every year. A low coefficient of variation of 0.1–0.4 was found for insecticides (acetamiprid, chlorpyrifos, and thiacloprid) as well as for active fungicidal substances (azoxystrobin, fluopyram, boscalid, and dimoxystrobin). All these substances are applied to large areas during the flowering period or just before the flowering of rape and sunflower. These crops are, of course, frequently visited by bees and other pollinators. This finding is certainly not surprising, but it is worth noting that a low coefficient of variation was also found for the herbicidal active substance (graminicide) – fluazifop. Only a slightly higher coefficient of variation was found for another herbicide (graminicide), quizalofop. Mean values of the coefficient of variation were also found for the herbicides ethofumesate and enmedipham. These

Table 2. Found amount of pesticide residues (mg/kg) in 2019 from one locality

AS/sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Acetamiprid	0.02	0.008		0.007	0.007	0.006	0.005	0.015	0.002	0.006	0.018	0.005	0.002	0.01	0.002		0.003		0.024		80
Azoxystrobin					0.012		0.004			0.039	0.002						0.004	0.006			30
Boscalid	0.042				0.002			0.003			0.006								0.002	0.002	30
Difeconazole							0.002														5
Fluazifop	0.01							0.006	0.004		0.003										20
Fluopyram	0.046	0.016		0.012	0.018		0.006	0.017		0.018	0.04	0.021		0.004	0.003	0.004	0.009		0.023		70
Chlorpyrifos	0.005	0.009	0.007	0.009	0.005	0.028	0.006	0.018	0.006	0.004	0.006	0.007	0.007	0.012	0.006	0.005	0.004	0.005	0.014	0.004	100
Metolachlor												0.002									5
Prothioconazole	0.011	0.003		0.003	0.004					0.003	0.01	0.004							0.006		40
Tebuconazole						0.002		0.003													10
Tetraconazole																		0.008			5
Thiacloprid	0.073	0.089	0.004	0.055	0.037	0.052	0.055	0.11	0.014	0.053	0.066	0.036	0.016	0.24	0.02	0.015	0.009	0.011	0.02	0.01	100
Thiophanete- methyl	0.15																				5
Number of AS in sample	8	5	2	5	7	4	6	7	4	6	8	6	3	4	4	3	5	4	6	3	

*LOQ for all active substance (AS) is 0.002 mg/kg

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herbicides are effective against annual weeds and are usually applied to small plants. In both cases, many species of dicotyledonous weeds on which the herbicides do not act can bloom on the treated area. These weeds are regularly visited by bees. These findings confirm our assumption that much more attention needs to be paid to herbicide residues to protect pollinators.

Substances with a coefficient of variation above 1 are rarely and randomly detected in hives. Examples are chlorotoluron or clopyralid. These substances are broad-spectrum and control a wide range of flowering dicotyledonous weeds in cereals or during the leaf rosette period of rape or poppy. Bees do not visit crops treated with these substances as they are not the usual source of food and the cultivated plant is in a non-flowering stage of development, and most dicotyledonous weeds are extinct from the herbicide usage.

Given the fact that 50% of active substances with a commonly used pollen collection methodology in 2016–2018 were found to have a coefficient of variation of 1 or higher, it was necessary to determine how often these substances could occur at one site when collecting pollen from multiple hives. Ghosh et al. (2020) found in their study that bee colonies in different habitats collect different amounts of pollen, and, in individual colonies, pollen from different plant species was determined in different amounts. The differences may be related to the number of active fliers.

Pollen in 2019 was collected according to a modified methodology, the aim of which was to show possible differences in the content of pesticides in the pollen in different hives at one site. Pollen from 20 hives of the same origin and comparable strength was tested at the same spot. The results are summarised in Table 2.

The results in Table 2 are similar to the data obtained in previous years at sites quite distant in the sense that a similar spectrum of substances was detected (Table 1), of which chlorpyrifos and thiacloprid were predominant. However, the spectrum of detected pesticides is narrower. This, of course, correlates with a much smaller area for obtaining food and a limited amount of pesticides applied to a given area. Table 2 shows that there are large differences in the pesticide residue content between the hives, even though they were in the same habitat and theoretically within reach of the same food source. However, bees may prefer some sources for various reasons (Saifuddin and Jha 2014, Ghosh et al. 2020). Thus, the toxic load of the landscape is better monitored by a set of pollen samples from more hives on the site than by a pollen sample from one hive. In pesticides, where a low coefficient of variation (0.1–0.4) was found (thiacloprid, chlorpyrifos, acetamiprid), the occurrence was detected in most hives. However, large differences in the amount were found – chlorpyrifos up to 700%, thiacloprid 6 000%, and acetamiprid 1 200% in individual samples. Less frequently used pesticides (coefficient of variation 1 and higher) were detected only in a part of hives – difeconazole, metolachlor, tetraconazole, and thiophanete-methyl only in one of the 20 hives monitored, i.e., in 5%.

With a low value of the coefficient of variation of 0.1–0.4 and partly also with medium values (0.5–0.9), there is a relatively high probability that these substances will be detected in a set of hives in individual years, even when using only a limited number of samples from one location. In the case of a high value of the coefficient of variation (1 and higher), the probability of the active substance in beehives is lower, and when taken from a limited number of hives at one site, the risk increases that the substance will not be detected in the whole group. However, with a larger set of samples from one site, the probability of detection increases significantly. In our study, these are often some fungicidal or herbicidal active ingredients.

The mentioned pollen sampling methodology (2016–2018) will make it possible to reliably determine the rate of occurrence of pesticides applied in the Czech Republic on large areas in crops in the period when they serve pollinators as a food source. The date of application is a significantly more important factor than the size of the treated area. The herbicide chlorotoluron used in the Czech Republic on a significantly higher area than acetamiprid, thiacloprid, azoxystrobin, fluopyram, boscalid, or dimoxystrobin (var. coefficient 0–0.4), and its coefficient of variation is 1.4. However, this substance is used in cereals and non-flowering poppy plants, which are unattractive to pollinators. In our study, fungicides and herbicides are often present in pollen in addition to insecticides. Although fungicides and herbicides are detected relatively frequently in the current methodology for collecting pollen from hives, their frequency of incidence in field-collected pollen is probably even higher. Using the current methodology of sampling pollen from one colony

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on a site suggests that detection of some substances cannot be reliably demonstrated.

As fungicides and herbicides are not classified in the categories of particularly dangerous or dangerous pesticides, there are no restrictions on their application to flowering plants. Bees frequently encounter fungicide and herbicide residues and take them to hives because they are not acutely toxic. Zioga et al. (2020) report that there is very little information on the chronic toxicity, metabolites, and degradation of fungicides and herbicides in bee products and on the influence of bee behavior. According to them, it is therefore important to continue this research.

The obtained results of pesticide residue content must be interpreted with the knowledge of high variability, which is a combination of intrinsic uncertainty of the method of determination by HPLC method (many results are at the LOQ limit) and matrix variability. Our results show a coefficient of variation of 10–140%. Compared to the combined expanded uncertainty of the analytical determination, which is in the order of percentage units, it is clear that variability between hives is the main component of the uncertainty of the result. Thus, experiments testing the level of pesticide residues should not be based on sampling models No. 1 from a single locality but on a representative group of hives at each test site.

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