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Pesticide and veterinary drug residues in Belgian beeswax: Occurrence, toxicity, and risk to honey bees

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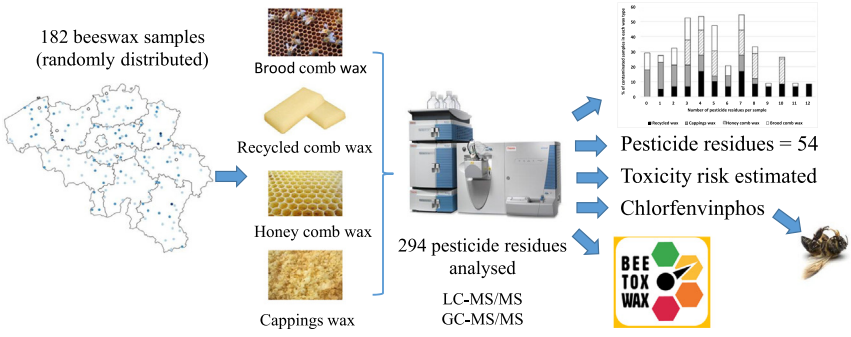
HIGHLIGHTS

- Pesticide levels in brood comb, recycled comb, honey comb, and cappings wax were compared.
- 54 different pesticide and veterinary drug residues were found in the four types of beeswax.
- In-hive applied or high lipophilic residues are more likely to be found in beeswax.
- A statistically significant influence of chlorfenvinphos on bee mortality was found.
- Cappings wax was substantially less contaminated.

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticide and veterinary drug residues are one of the stress factors affecting bee health and mortality. To investigate the occurrence, the concentration and the toxicity risk to bees of pesticide residues in four different types of beeswax (brood comb wax, recycled comb wax, honey comb wax, and cappings wax), 182 samples were collected from apiaries located all over the Belgian territories, during spring 2016 and analysed by LC-MS/MS and GC–MS/MS for the presence of 294 chemical residues. The toxicity risk to bees expressed as the Hazard Quotient (HQ) was calculated for each wax sample, according to two scenarios with different tau-fluvalinate LD_{50} values. Residues showing the highest prevalence were correlated to bee mortality in a multivariate logistic regression model and a risk-based model was used to predict colony bee mortality. Altogether, 54 different pesticide and veterinary drug residues were found in the four types of beeswax. The residues with a higher likelihood to be retained in beeswax are applied in-hive or with a high lipophilic nature. The multivariate logistic regression model showed a statistically significant influence of chlorfenvinphos on bee mortality. All our results indicated that cappings wax was substantially less contaminated. This national survey on beeswax contamination provides guidelines on the re-use of beeswax by beekeepers and shows the necessity to introduce maximum residue levels

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Chlorfenvinphos
Hazard Quotient
Risk assessment
Toxicity
Modelling

for global trade in beeswax. An online tool was developed to enable beekeepers and wax traders to estimate the risk to honey bee health associated with contaminated wax.

1. Introduction

The presence of residues in apicultural matrices reflects anthropogenic activities whether they come from agricultural use or veterinary treatments (Balayiannis and Balayiannis, 2008; Berthoud et al., 2010). Honey bees and other pollinators are at risk from multiple stress factors (Berthoud et al., 2010; Dainat et al., 2012; Goulson et al., 2015; Le Conte et al., 2010; Van Engelsdorp et al., 2008) and pesticide residues play an undeniable role. The contribution of pesticide residues to the global decline of honey bees and other pollinators has lately received much attention from the scientific community (Mitchell et al., 2017; Tsvetkov et al., 2017; Woodcock et al., 2017). Since the arrival of the parasitic mite *Varroa destructor* in Belgium in 1984, the most common means of controlling *Varroa* has been through the use of synthetic acaricides (Bogdanov et al., 1998; Mullin et al., 2010). Treatments are placed in-hive, thus exposing not only the mites to the compound but honey bee eggs, larvae, adults, and beehive products. Residues acute toxicity to honey bees is characterised by the determination of the acute median lethal dose (LD_{50}) values, which is the residue dose that is required to kill half of the tested animals. LD_{50} values may differ based on the route of exposure and species exposed (Haschek et al., 2013).

Regarding adult honey bees, residues associated with acute contact LD_{50} values inferior to 2 μg bee^{-1} are considered as highly toxic, moderately toxic with acute contact LD_{50} values between 2 and 10.99 μg bee^{-1}, slightly toxic with acute contact LD_{50} values between 11 and 100 μg bee^{-1}, and essentially non-toxic with acute contact LD_{50} values higher than 100 μg bee^{-1} (Washington State Department of Agriculture, 2010). The persistence of a residue depends on its physical and chemical properties (partition coefficients, degradation rates, deposition rates) and the characteristics of the contaminated matrix. Honey bees are typically exposed to a cocktail of residues; in-hive (beekeeper applied) acaricides and other veterinary drugs applied over long-time periods and out-of-hive (farmer applied) pesticides encountered in pollen, nectar, and water during their foraging activity (Bogdanov, 2006; Chauzat et al., 2011; Rortais et al., 2005). These pesticide residue mixtures may act alone or in interaction (Carnesecchi et al., 2019), in ways currently difficult to predict, potentially creating a toxic environment for honey bee growth and development (Tomé et al., 2020; Yao et al., 2018; Zhu et al., 2014).

Beeswax is a natural honey bee product. It is secreted in liquid form by special wax glands in the abdomen of younger worker bees (aged between 12 and 18 days) (Bogdanov, 2016) and solidifies into translucent white scales when in contact with air. Wax combs are constructed from these wax scales, molded into shape by honey bee mandibles. In Europe, as wax production is not the aim in beekeeping, beekeepers provide bee colonies with manufactured wax sheets of foundation, which the bees draw out into the full depth comb. The raw materials for wax manufacture are recycled from old brood combs, honey combs and cappings wax. Cappings wax contains almost exclusively pure wax. Beeswax is a complex mixture consisting mainly of esters of higher fatty acids (Aichholz and Lorbeer, 1999; Tulloch, 1980). Due to its high composition in fatty acids, and as most acaricides are fat-soluble and non-volatile (Wallner, 1999), beeswax is a relevant matrix to assess in-hive chemical exposure history for lipophilic compounds (Lozano et al., 2019; Ravoet et al., 2015). Of all beehive products, it has the lowest replacement rate, can remain in the hive for many years and is recycled by the beekeepers into new wax foundations for comb building, thus leading to a greater accumulation of different pesticide residues used in beekeeping and agriculture (Chauzat and Faucon, 2007; Mullin et al., 2010). Beeswax can be considered as a contaminant reservoir (Yáñez et al., 2013) or a final sink (Bommuraj et al., 2019). Even though most residues remain in the wax, residues migration from the wax to beebread, and larvae is a crucial factor that could affect the evolution of the colony (Murcia Morales et al., 2020). A residue accumulation can affect worker honey bee and queen development (Haarmann et al., 2002), bee longevity (Wu et al., 2011), and colony performance (Desneux et al., 2007).

Assessment/registration authorities like e.g. World Health Organisation (WHO), United States Environmental Agency (EPA), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) ensure that each registered pesticide/veterinary drug continues to meet the highest standards of safety to protect human health and the environment. Within this context, older pesticides are being reviewed to ensure that they meet current scientific and regulatory standards. As an example, EPA screening-level assessors re-evaluated in 2005 tau-fluvalinate, one of the acaricides frequently used for *Varroa* control and reset its median acute contact lethal dose (LD_{50}) at 0.2 μg bee^{-1} (EPA, 2005). This classifies tau-fluvalinate as highly toxic to honeybees. Tau-fluvalinate is expected to pose an acute health risk to non-target insects. Nevertheless, in Europe, the acute LD_{50} of tau-fluvalinate is still set at 12 μg bee^{-1} (worst case from 24, 48 and 72-hour values) reported by the University of Hertfordshire Pesticide Properties DataBase (PPDB) (Lewis et al., 2016).

The European legislation on animal by-products (ABPs) defines beeswax as an "apiculture product" used in beekeeping (Regulation (EC) No 1774/2002) and categorises beeswax as an ABP Category 3 material, i.e. not intended for human consumption (Regulation EC No 1069/2009). This categorisation does not prevent the presence of contaminants and/or adulterants. Moreover, it allows the commercialisation of beeswax used in apiculture without previous quality (authenticity) control. In Belgium, the guidelines contained in the advice 18–2018 (Scientific Committee of the FASFC, 2018) set the limits for pesticide and veterinary drug residues at 9 different products and proposed limiting the sale of re-melted beeswax that exceeds these limits.

This first national pilot survey aimed to improve our understanding of the pesticide residues currently present, their rate of occurrence, and their concentration in four types of beeswax. The survey also aimed to assess the exposure risk to honey bees, comparing the toxicity of pesticide residues in the four beeswax types and the potential implications for beekeeping management practices.

The results obtained led us to develop an online tool (BeeToxWax) to empower beekeepers and wax traders to estimate the risk to honey bees associated with contaminated wax based on the residue concentrations reported in a laboratory analysis report and the pesticide residues acute LD_{50}. The tool gives automated real-time recommendations on whether the tested sample can be reused in a colony or should be discarded based on the current scientific literature: contact Hazard Quotient (HQ) value over 250 are considered to have significant toxicity and elevated toxicity is associated with HQ values over 5000 (Traynor et al., 2016). The tool is a web-based calculator of risk associated with contaminated wax; its use could be an important strategy to sanitize beeswax available in the commercial trade stream (https://www.beetools.uliege.be).

2. Materials and methods

2.1. Beeswax and residues

2.1.1. Origin and characterisation of the wax samples

A total of 200 beekeepers were randomly selected from the Federal Agency for the Safety of the Food Chain (FASFC) beekeepers database including 4949 registered beekeepers in 2015. Beeswax wax collected from a single hive out of one apiary per beekeeper during spring 2016. The number of beekeepers was stratified by province. Out of the selected beekeepers ($N = 200$), 91.5% of the beekeepers provided a wax sample of sufficient amount (100 g) for analysis (182 samples). Wax samples were differentiated into four types: brood comb[1] wax ($N = 89$), recycled comb[2] wax ($N = 59$), honey comb[3] wax ($N = 6$), and cappings[4] wax ($N = 28$). The different types of waxes are easily identifiable by colour, shape, and consistency. Brood combs are dark, honey combs are light with no pupal cocoons, cappings wax is cut off comb when extracting honey and melted wax is received as a block or pressed into sheets of foundation. Beekeepers donated less honey comb wax as they reuse these light coloured frames for honey production. The samples were free of beebread, honey or brood, they were kept in hermetic plastic bags and stored at −20 °C until analysis.

2.1.2. Multi-residue analysis

Analysis of beeswax was carried out at an independent laboratory in Germany (Intertek Food Services GmbH) according to the European EN 15662 method (CEN 2008), between October 2016 and January 2017, using a common analytical protocol (QuEChERS) designed for the analysis of food materials and suitably adapted. All residues were analysed using multi-residue GC–MS/MS and LC-MS/MS methods covering 294 different substances with detection limits (LOD) of 0.003 mg/kg and limits of quantification (LOQ) of 0.01 mg/kg in most cases and with recoveries between 70% and 120%. The quality control is done using quality control samples and spiking experiments.

Generally, 10 ml of deionized water (BarnsteadTM, Nanopure DiamondTM, Thermo Scientific) was added to approximately 5 g of beeswax accurately weighed into a 50 ml-Teflon centrifuge tube. 10 ml of acetonitrile (HPLC Gradient Grade, VWR) was added together with an internal standard solution containing isoproturon-d6 for LC-MS/MS analysis, anthracene-d10 for GC–MS/MS analysis and octachlorostyrene for negative chemical ionization GC–MSD analysis.

The whole preparation was mixed using a horizontal shaker for 20–30 min. Then 6.5 g QuEChERS salt mixture was added, consisting of 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate, and the whole was mixed by hand for approximately 1 min, then centrifuged for 13 min at 10,000 Relative Centrifugal Force (RCF; refrigerated centrifuge Rotina 380 R). 7 ml of the supernatant was transferred to a tube containing 1 g of anhydrous calcium chloride and 300 mg of PSA as a sorbent. After briefly shaking by hand, this mixture was centrifuged again for 13 min at 10,000 RCF. 1 mL of the supernatant was then removed for LC-MS/MS analysis. Further two aliquots of 1 ml each were filled into vials and 8 μl of 5% formic acid solution in acetonitrile were added as analyte protectant for GC–MS/MS and GC–MSD analyses, respectively. The addition of 5% formic acid solution in acetonitrile is done to stabilize the analytes in the solution. This is not meant as a classical Analyte Protectants (AP) for GC–MS analysis. An AP-Mix (mixture of 3-Ethoxypropandiol, Shikimic acid, Glucuronolactone and Sorbitol) for GC–MS analysis was used to block free active spaces on the liner to prevent interactions between the liner and the analytes.

LC-MS/MS was performed on a Thermo Scientific system consisting of an Accela 1250 pump and a TSQ Quantum Access mass spectrometer with a Hypersil Gold C8 (150 × 2.1 mm, 5 μm) column. The GC-MS/MS system was a GC 7890 equipped with a HP-5 ms column (30 m × 0.25 mm × 0.25 μm, Varian) combined to a 7000 Triple Quadrupole mass spectrometer (Agilent Technologies). The GC-MSD system consisted of a GC 6890 N with a VF-5 ms column (30 m × 0.25 mm × 0.25 μm, Varian) combined to a 5975 XL inert MS (Agilent Technologies).

2.1.3. Regression modelling of residue per wax type

In a first step, a descriptive analysis was performed to examine data for completeness and validity and to identify the wax type with the least residues. After this validation, a univariate logistic regression model was performed for each residue ($N = 54$) to examine associations between a range of independent variables (i.e. the four wax types, with cappings wax considered as the purest reference wax) and the outcome of interest (each residue). The level of statistical significance was set to $P = 0.05$.

2.1.4. Hazard Quotient and toxicity to bees

To estimate contaminated wax contact toxicity to bees, a mean Hazard Quotient (HQ) was calculated for each of the four wax types. Until now, toxicity for larvae has not been well studied. As chronic median lethal dose data for bees are extremely rare (EFSA, 2012), the acute contact median lethal dose (LD_{50} 48 h for adult bees) was used in the HQ calculation. Per sample then gathered by wax type, HQ was calculated as the sum of the concentration of the residue (mg kg^{-1}) divided by its respective acute contact LD_{50} (μg bee^{-1}). The HQ provides an estimate based on percentages of LD_{50} equivalents present in the wax. For oral contact in pollen instead of beeswax, HQ is considered notable when it is >50 and is considered as elevated when it is >500 (Stoner et al., 2013). In beeswax, pesticide residues are embedded in the matrix and not all residues are in contact with honey bees. Only a fraction of the pesticide load is exposed to the individuals of the colony, so HQ in beeswax samples was considered as notable when >250 (Calatayud-Vernich et al., 2019). Samples with contact HQ beeswax >5000 were considered to have an elevated pesticide load (Traynor et al., 2016).

Acute contact LD_{50} values were retrieved from the Pesticide Properties DataBase (PPDB) and the Veterinary Substances DataBase (VSDB) reported by the University of Hertfordshire (Lewis et al., 2016) or from some additional primary literature (Sanchez-Bayo and Goka, 2014; Stoner et al., 2013) (Table 1). For substances with multiple LD_{50}, the lowest value was considered according to a conservative scenario. For unknown contact LD_{50}, when possible, the LD_{50} of the respective parent compound was used in the HQ calculation acknowledging that some metabolites may have either lower or higher toxicity than the parent compound (Suchail et al., 2001). When the substance was not assimilated to a pesticide (e.g. solvent), a low toxicity value of 200 μg bee^{-1} was assigned. In the case of tau-fluvalinate, both values proposed by the EPA (0.2 μg bee^{-1}) and PPDB (12 μg bee^{-1}) were considered in two toxicity scenarios as an important 60-fold disparity appeared with its toxicity.

Cumulative risk by contact exposure estimate.

To assess the risk to larvae in contact with contaminated wax topical contact during their development, it is necessary to consider the frequency of detection of each pesticide residue in this matrix, because prevalence indicates the probability of exposure to the contaminants. We used the method suggested by Sanchez-Bayo and Goka (Sanchez-Bayo and Goka, 2014) that takes into consideration the cell weight (0.0232 g) (El Agrebi et al., 2019) and the development time (21 days) of bee larvae (Eq. (1)).

[1] Wax comb in which the brood was reared.
[2] Melted old brood and/or honey wax comb to be reused.
[3] Wax comb in which honey was stored.
[4] Virgin wax covering on sealed honey combs rendered by beekeepers.

Table 1
Residue levels of pesticides found in the four types of beeswax in Belgian apiaries. The type of each active substance, the contact acute median lethal dose and number of positive samples found are reported.

Active ingredient	Active ingredient type				Contact acute 48 h LD50 (μg bee-1)			Brood comb wax ($n = 89$)					Recycled comb wax ($n = 59$)
	Insecticide	Fungicide	Acaricide	Other	PPDB/VSDB	Stoner et al., 2013	Sanchez-Bayo and Goka, 2014	# positive samples	Frequency (%)	Mean (mg/kg)	Min (mg/kg)	Max (mg/kg)	Frequency (%)
Acrinathrin	X		X		0.084		0.17	1	1.1%	0.014	0.014	0.014	1.7
Amitraz (incl. Metabolites)	X		X	Antiparasite	50			25	28.1	0.740	0.010	16.7	16.9
Azoxystrobin		X			200	200		3	3.4	0.047	0.011	0.117	1.7
Biphenyl	X	X	X		/								1.7
Boscalid		X			200	200		5	5.6	0.121	0.038	0.310	11.9
Bromopropylate			X		/			22	24.7	0.024	0.010	0.058	39.0
Captan		X		Bactericide	200			3	3.4	0.646	0.014	1.837	8.5
Carbendazim		X		Metabolite	50	50		6	6.7	0.040	0.014	0.098	
Chlorfenvinphos	X		X	Sheep dip	/		4.1	20	22.5	0.036	0.012	0.084	32.2
Chloropropylate	X		X		/			2	2.2	0.024	0.011	0.036	5.1
Chlorothalonil		X			101		135.32	1	1.1	0.066	0.066	0.066	
Chlorpropham				Herbicide	86			3	3.4	0.034	0.025	0.053	25.4
Chlorpyrifos (-ethyl)	X				0.059	0.01	0.07	12	13.5	0.025	0.011	0.041	11.9
Coumaphos	X		X	Antiparasite	/	24	20.29	72	80.9	0.150	0.010	2.257	89.8
Cypermethrin	X			Sheep dip	0.02		0.03	4	4.5	2.34	0.023	9.300	
Cyprodinil		X			784	784		2	2.2	0.063	0.062	0.063	10.2
p,p′-DDE (Dichlorodiphenyldichloroethylene)				Metabolite	/								1.7
DDT (Sum, expressed as DDT)	X				0.54								1.7
o,p′-DDT				Isomer	0.54								3.4
p,p′-DDT (Chlorophenothane)	X				0.54			1	1.1	0.010	0.010	0.010	8.5
DEET (diethyltoluamid)	X			Repellent	/			23	25.8	0.102	0.010	0.707	52.5
Deltamethrin	X			Metabolite	0.0015		0.02						1.7
Diazinon	X		X	Repellent	0.13	0.22	0.38						3.4

Dibromobenzophenone				Metabolite	Not listed			3	3.4	0.013	0.010	0.015	1.7
Dichlofluanid		X			16			3	3.4	0.174	0.012	0.494	11.9
Dichlorobenzophenone				Metabolite	Not listed								
Dimethomorph		X			102	10		2	2.2	0.285	0.046	0.523	
Dimoxystrobin		X			100			1	1.1	0.022	0.022	0.022	
Etridiazole		X			/								1.7
Fenpyroximate			X		15.8			8	9.0	0.029	0.010	0.064	6.8
tau-Fluvalinate	X		X		12	0.2	8.66	82	92.1	0.530	0.010	6.460	94.9
Hexythiazox			X		200			9	10.1	0.015	0.010	0.030	3.4
Iprodione		X			200			10	11.2	0.058	0.010	0.130	22.0
Lindane (γ-HCH)	X		X		0.23			1	1.1	0.023	0.023	0.023	8.5
Metalaxyl		X			200	100		1	1.1	0.015	0.015	0.015	
Methoxychlor	X				23.6								3.4
Parathion	X		X		/			1	1.1	0.016	0.016	0.016	
Pendimethalin				Herbicide	100	49.8		5	5.6	0.017	0.012	0.030	
Pentachloroanisole				Metabolite Pentachlorophenol	48			6	6.7	0.026	0.010	0.065	23.7
Permethrin (Sum all Isomere)	X			Antiparasite	0.29		0.06	12	13.5	0.077	0.011	0.311	27.1
2-phenylphenol		X		Other substance	/			17	19.1	0.022	0.010	0.074	8.5
Piperonyl butoxide				Performance enhancer	294			20	22.5	0.055	0.010	0.376	40.7
Pirimicarb	X				53.1	12.56		2	2.2	0.014	0.011	0.016	
Propamocarb		X			100			3	3.4	0.018	0.010	0.027	1.7
Propargite			X		47.9			51	57.3	0.124	0.011	0.375	57.6
Propiconazole		X			100	25		1	1.1	0.378	0.378	0.378	3.4
Pyridaben	X		X		0.024		0.05	1	1.1	0.010	0.010	0.010	
Pyrimethanil		X			100	100		3	3.4	0.048	0.012	0.080	11.9
Tebuconazole		X		Plant growth regulator	200								1.7
Tetradifon	X		X		11								5.1
Tetramethrin	X				/								1.7
Thiacloprid	X			Molluscicide	38.82	37.83		2	2.2	0.030	0.014	0.046	1.7
Trifloxystrobin		X			100	200		1	1.1	0.025	0.025	0.025	
Vinclozolin		X			/								1.7

Legend: Amitraz, including the metabolites DMPF, DMF and the 2,4 -dimethylaniline moiety; PPDB/VSDB, data was retrieved from Pesticide Properties DataBase and Veterinary Substances DataBase (Lewis et al., 2016).

$$\text{Risk}\ \% = \left(\frac{\text{Frequency\%x Residue concentration [µg/g]}}{\text{LD}_{50}\text{acute contact}\left[\text{µg bee}^{-1}\right]}\right) \times 0,023\ [\text{g}] \times 21\ [\text{days}] \quad (1)$$

$$\text{Cumulative risk}_{P1-P54}\ \% = \sum_{P=1}^{54}\left(\frac{\text{Frequency\%x Residue concentration [µg/g]}}{\text{LD}_{50}\text{acute contact}\left[\text{µg bee}^{-1}\right]}\right) \times 0,023\ [\text{g}] \times 21\ [\text{days}] \quad (2)$$

Eq. (1) indicates the percentage of risk (i.e. likelihood of causing 50% mortality) caused by a given pesticide residue on honey bee larvae that come into contact with contaminated wax during their development. For each wax type, a cumulative risk by contact exposure was calculated as the summation of the risk caused by each pesticide in the sample (Eq. (2)). The cumulative risk expresses the risk that larvae would be exposed to during their development to a higher pesticide dose than the contact lethal dose (LD_{50}).

2.2. Pesticide and veterinary drug residues and honey bee mortality

2.2.1. Data on bee mortality

Sampling was conducted jointly with a questionnaire to record colony losses and management practices. The total loss rate was calculated by dividing the total number of colonies lost between September 2015 and April 2016 (winter and seasonal) by the number of colonies in September 2015 multiplied by 100 (Clermont et al., 2014) excluding removed, sold and purchased colonies. Bee mortality rate in function of the presence or the absence of a specific pesticide residue was tested with a two-sample Wilcoxon rank-sum (Mann-Whitney) test for significance. The limit of statistical significance of the test was defined as 0.05.

2.2.2. Logistic regression model

A univariate logistic regression model was used to explain colony mortality expressed as a binary dependent variable, taking into account the acceptable level of mortality (0 for colony mortality rates ≤10%; 1 for colony mortality rates >10% (Morgenthaler, 1968)) associated with residues. Then, a multivariate logistic regression was performed using the most significant variables ($P < 0.1$) out of the univariate model. Finally, in a backward stepwise multivariate model, the least significant variable (with the highest P value) were eliminated in a step-by-step approach. At each stage, a likelihood ratio test was used to compare the complex and simplified models. When there was no significant difference between them (using value of $P > 0.10$), the simplified model was used. The interaction between variables in the multivariate final retained model was tested. All models and tests were performed using Stata SE 14.1® (StataCorp LP, College Station, TX, USA) and the limit of statistical significance of performed tests was defined as 0.05.

2.2.3. Development of a risk-based model

To predict the colony mortality expressed as a binary variable (0 for colony mortality rates ≤10%; 1 for colony mortality rates >10%) in function of the different combinations of pesticide residues present in each beeswax sample, a Receiver Operating Characteristic (ROC) curve was established. For this, only the 10 residues with a P-value <0.20 in the previous univariate logistic regression analysis were retained. Next, ten different receivers operating characteristic (ROC) curves were established (i.e. with the first, the two first, the three first, until the ten-first pesticide residues retained). The ROC is a probability curve that plotted with true-positive results (Y-Axis) against the false-positive results (X-Axis). Each point of the curve is determined by a specific threshold = cut-off (i.e. a certain combination of pesticide residues). The area under the ROC curve (AUC) is the performance measurement for the classification test at various threshold settings. Higher the AUC, better the test can distinguish between colony mortality status (0 for colony mortality rates ≤10%; 1 for colony mortality rates >10%). Indeed, the ROC curve with the higher AUC (among the ten tested) was retained to determine the best cut-off (i.e. optimal number of residues in combination) related to the bee colony mortality.

2.2.4. The number of residues per wax type

The relationship between the number of residues and the type of beeswax was assessed using a negative binomial regression due to the over-dispersion of the variable outcome. Possible residue synergies were looked for in residue combinations.

3. Results

3.1. Beeswax, pesticides and veterinary drug residues

Descriptive data of the residues found in Belgian beeswax are presented in Table 1. The analysed samples revealed a contamination prevalence of 97.3% and the presence of 54 different compounds for all wax types jointly. Per sample, the number of different residues ranged from 1 to 12 with a median value of 5. Ten different residues were commonly found in the four wax types. Acaricides (i.e. tau-fluvalinate and coumaphos) have the highest prevalence in all wax types (respectively 89.6% and 78.6%), followed by propargite, chlorfenvinphos, bromopropylate (including metabolite 4,4′-Dibromo-benzophenone). Also, the insecticide permethrin, the repellent DEET (diethyltoluamide), the fungicide pentachloroanisole, and its metabolite pentachlorophenol, as well as the performance enhancer substance piperonyl butoxide were frequently found in the wax samples. The frequency of occurrence of each residue per wax type is shown in Table 1. The percentage of contaminated samples as a function of the number of residues per wax type is shown in Fig. 1. This percentage is significantly higher in cappings wax for lower residue numbers than in the other wax types (Negative binomial regression; $P < 0.001$).

3.2. Frequency of pesticide and veterinary drug residues per wax type

3.2.1. Brood comb wax

A total of 41 different residues were found in brood comb wax ($N = 89$). The median number of different residues per sample was 5 (min-max, 1–12). Residues with the highest prevalence were tau-fluvalinate (92.1%), coumaphos (80.9%), propargite (57.3%), amitraz (28.1%), and DEET (25.8%). The highest maximum concentrations were observed for amitraz (including the metabolites containing the 2,4-dimethylaniline expressed as amitraz) with 16.7 mg kg^{-1} followed by cypermethrin (9.3 mg kg^{-1}), and by tau-fluvalinate (6.46 mg kg^{-1}). Seven highly toxic residues to bees (with DL_{50}<2 µg bee^{-1}) were found: chlorpyrifos (-ethyl) and permethrin, both in 13.5% of the samples, cypermethrin in 4.5% of the samples, acrinathrin, lindane, p,p′-DDT, and pyridaben in 1.1% of the samples. The neonicotinoid thiacloprid was detected in 2.2% of the sample with a maximum concentration of 0.046 mg kg^{-1} (Table 1).

Increased odds of tau-fluvalinate were observed in brood comb wax compared with reference cappings wax (OR = 5.36 with 95% CI: 1.82–15.73; $P = 0.002$) (Table 2).

3.2.2. Recycled comb wax

In recycled comb wax ($N = 59$), 42 different residues were quantified. The median number of different residues per sample was 7 (min-max: 1–12). Residues with the highest prevalence were tau-fluvalinate (94.4%), coumaphos (89.8%), propargite (57.6%), DEET (52.5%), Piperonyl butoxide (40.7%), bromopropylate (39%), chlorfenvinphos (32.2%), permethrin (27.1%), chlorpropham (25.4%) and pentachloroanisole (23.7%). Tau-fluvalinate had the highest concentration with 8.68 mg kg^{-1} followed by coumaphos with 7.41 mg kg^{-1} and chlorpyrifos (-ethyl) with 4.38 mg kg^{-1} (Table 1). Highly toxic

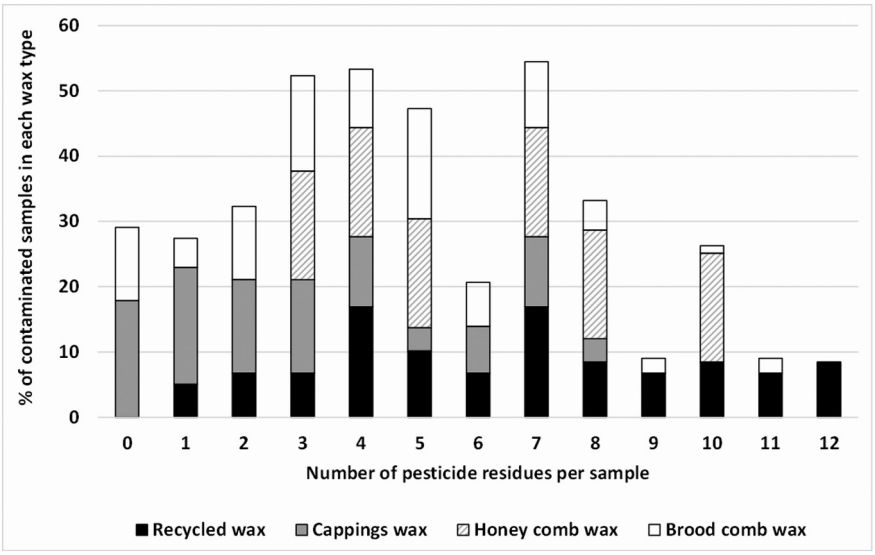

Fig. 1. Percentage of contaminated samples with to 0 to 12 pesticides in the four wax types, brood comb wax ($N = 89$), recycled comb wax ($N = 59$), honey comb wax ($N = 6$) and cappings wax ($N = 28$).

residues to bees (with DL_{50}<2 μg bee^{-1}) were found: permethrin in 27.1% of the samples, chlorpyrifos (-ethyl) in 11.9%, p,p′-DDT and lindane in 8.5%, dianizon in 3.4%, cypermethrin, acrinathrin, deltamethrin, DDT and tetramethrin in 1.7% of the samples. The neonicotinoid thiacloprid was detected in one sample with a maximum concentration of 0.014 mg kg^{-1}.

Increased odds of coumaphos were observed in recycled comb wax compared with reference cappings wax (OR = 10.19 with 95% CI: 3.31–31.37; $p = 0.000$) (Table 2).

3.2.3. Honey comb wax

The results interpretations for this wax type are only indicative as they are derived from a comparatively smaller sample size. Honey comb wax ($N = 6$) contained 13 different pesticide residues, the median number of different residues per sample was 6 (min-max: 3–10). Tau-fluvalinate was detected in 100% of the samples, coumaphos, and propargite in 83.3% of the samples, piperonyl butoxide in 66.7%, and fenpyroximate in 50% of the samples. Six molecules were found in 33.3% of the analysed samples, i.e. bromopropylate (and its metabolite 4,4′-Dibromo-benzophenone), chlorfenvinphos, chlorpyrifos (-ethyl), pentachloranisole, and permethrin (Table 1). In honey comb, two insecticides considered as highly toxic to bees (<2 μg bee^{-1}) were detected: permethrin and chlorpyrifos (-ethyl).

Table 2
Univariate logistic regression model outputs for residues detection rate in brood comb wax, in recycled comb wax and in honey comb wax with cappings wax as reference.

Residue	Wax type	Odds ratio	95% confidence interval	P-value
Brompropylat*	Recycled	3.51	(1.13–10.86)	0.03
Chlorpyrifos (-ethyl)	Honey comb	31.67	(1.29–772.98)	0.034
Coumaphos	Brood comb	4.89	(1.96–12.15)	0.001
	Recycled	10.19	(3.31–31.37)	0.000
Fenpyroximate	Honey comb	57.00	(2.40–1349.32)	0.012
Pentachloranisole	Recycled	8.40	(1.04–67.51)	0.045
Piperonylbutoxide	Recycled	2.95	(1.02–8.52)	0.046
	Honey comb	7.69	(1.26–46.68)	0.027
Propargite	Brood comb	4.03	(1.55–10.44)	0.004
	Recycled	4.08	(1.50–11.08)	0.006
	Honey comb	15.00	(1.48–151.28)	0.022
tau-Fluvalinate	Brood comb	5.36	(1.82–15.73)	0.002
	Recycled	7.86	(2.08–29.71)	0.002

Legend: *An example of interpretation is presented: significant more detection of Brompropylat was found in recycled comb in comparison with the cappings wax as reference. Other beeswax types are not different from the reference.

The highest maximum concentrations were observed for tau-fluvalinate with 0.91 mg kg^{-1} followed by DEET with 0.78 mg kg^{-1} and coumaphos with 0.45 mg kg^{-1}. Two highly toxic residues to bees (with DL_{50} < 2 μg bee^{-1}) were detected: permethrin and chlorpyrifos (-ethyl). No trace of thiacloprid (neonicotinoids) was detected in honey comb wax.

Increased odds of fenpyroximate were observed in honey comb wax compared with reference cappings wax (OR = 57 with 95% CI: 2.40–1349.32 [wide range due to small sample size]; $P = 0.012$) (Table 2).

3.2.4. Cappings wax

In cappings wax ($N = 28$), 18 different residues were detected. The median number of different residues per sample was 3 (min-max: 0–8) (Table 1). Tau-fluvalinate (65.5%), coumaphos (44.48%), DEET (37.93%), propargite (24.1%) and piperonyl butoxide (17.2%) were the most frequently detected residues in cappings wax. The highest maximum concentrations were observed for coumaphos with 0.93 mg kg^{-1} followed by tau-fluvalinate with 0.53 mg kg^{-1} and propargite with 0.45 mg kg^{-1}. Permethrin (13.8%) was the only substance found with high toxicity to bees. No trace of thiacloprid (neonicotinoids) was detected in cappings wax.

3.3. Wax Hazard Quotient and toxicity to bees

Overall, in the first scenario (tau-fluvalinate $DL_{50} = 12$ μg bee^{-1}), out of $N = 182$, 123 samples of the samples (67.5%) had a low HQ value (<250), 55 samples (30.2%) had significant toxicity (250 < HQ < 5000) and 4 samples (2.2%) of the total number of samples had elevated toxicity to bees (HQ > 5000) (Fig. 2). At the territorial level, the samples with the highest HQ ($N = 4$) were reported in the province of Luxembourg (max = 466,246), in Limburg (max = 5242 and 74,208) and East Flanders (max = 17,536) (Table 3). Detailed results per wax type are shown in Table 4. With the second toxicity scenario (tau-fluvalinate $DL_{50} = 0.2$ μg bee^{-1}), the HQ levels approach alarming levels and the number of samples exceeding the threshold values increases (Table 4).

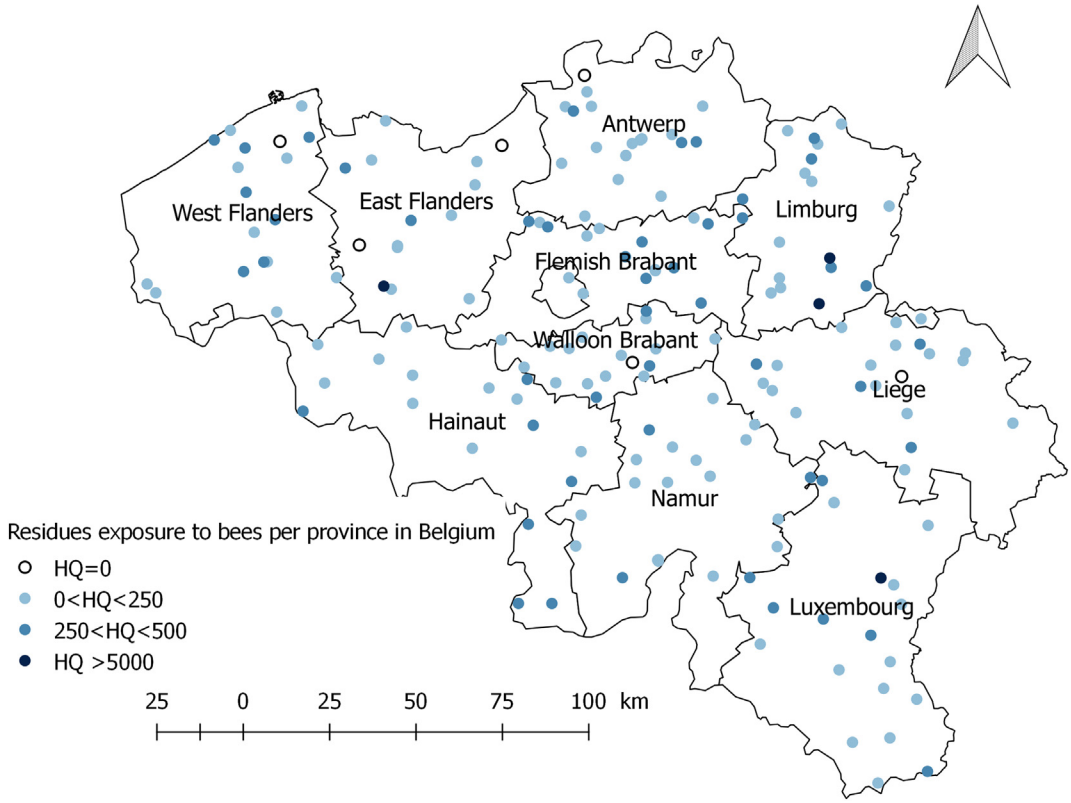

Fig. 2. Residues exposure risk to bees per province in Belgium ($N = 182$).

3.4. Cumulative risk by contact exposure to bee larvae

In the scenario where tau-fluvalinate mean $DL_{50} = 12$ μg bee^{-1} and considering the Eq. (2), the risk posed to bee larvae by the presence of residues in brood comb, recycled comb, honey comb, and cappings waxes is respectively of 15.12%, 12.3%, 4.79%, and 0.73%. With the second scenario (tau-fluvalinate $DL_{50} = 0.2$ μg bee^{-1}) the cumulative risk for bee larvae in the four wax types are respectively of 122%, 119%, 104%, and 16% (Table 4).

3.5. Pesticide and veterinary drug residues and honey bee mortality

3.5.1. Logistic regression model

An individual residue's possible correlation with mortality rates was tested using a univariate logistic regression model (Table 5). After multivariate logistic regression analysis, only chlorfenvinphos exhibited a significant correlation with bee mortality (OR = 2.15; 95% CI: 1.04–4.44; $P = 0.038$). Moreover, no interaction between chlorfenvinphos and permethrin was found in the final multivariate logistic regression model. In addition, bee mortality rate was significantly higher in samples contained Chlorenvinfos (two-sample Wilcoxon rank-sum test; $P = 0.026$).

3.5.2. Development of a risk-based model

The area under the ROC curve (AUC) was estimated for 10 different ROC curves, i.e. with the first, the first two, the first three, until it incorporated the first ten most commonly found residues presented in Table 5. The two ROC curves with close higher AUC were retained for future fitting of the binomial model (Fig. 3). The final model retained and presented in Fig. 4 corresponds to the ROC curve fitted with the higher AUC, i.e. the ROC curve fitted with the three first pesticide residues related to the colony bee mortality (i.e. bromopropylate, chlorfenvinphos and chlorpyrifos-(ethyl)). For this final ROC curve, the AUC = 0.6128 (Fig. 4). Considering this final ROC curve, the best cut-off related to the prediction of the colony bee mortality corresponds to at least one of these three residues (Fig. 4).

Table 3
Hazard quotient values per province in Belgium.

Provinces	Mean HQ	S.D.	Median HQ	Min HQ	Max HQ
Antwerp	79	150	14	0,0	533
Eastern Flanders	1494	4515	10	0,0	17,536
Flemish Brabant	369	273	2	0,6	837
Hainaut	482	769	189	3,0	2408
Liège	251	545	28	1,4	2295
Limburg	4996	17,896	131	4,1	74,208
Luxemburg	24,738	106,917	36	1,9	466,249
Namur	374	1085	22	0,1	4561
Walloon Brabant	108	157	44	0,2	501
Western Flanders	266	337	86	0,0	1081

Legend: S.D., standard deviation; HQ, Hazard quotient, Min, minimum; Max, maximum.

3.6. Potential interactions of residues

We looked for the most prevalent pesticide combination in all wax types combined ($N = 182$). The most frequent combination was tau-fluvalinate together with coumaphos ($N = 142$), tau-fluvalinate together with coumaphos, and propargite ($N = 94$), tau-fluvalinate together with DEET and coumaphos ($N = 56$), coumaphos together with propargite and tau-fluvalinate ($N = 48$), coumaphos together with chlorfenvinphos ($N = 44$). Other relevant combinations with proven synergies we detected were; amitraz together with tau-fluvalinate ($N = 37$), piperonyl butoxide together with fenpyroximate (N = 9), chlorothalonil together with coumaphos (N = 1), and tau-fluvalinate (N = 1).

Table 4
Risk to bees expressed in percentage, Hazard Quotient (HQ) and HQ values exceeding threshold toxicity in beeswax for the four wax types (brood comb wax, recycled comb wax, cappings wax and honey comb wax) for two different tau-fluvalinate LD_{50} values.

Tau-fluvalinate DL_{50} (μg bee^{-1})		HQ value	Brood comb wax (N = 89)	Recycled wax (N = 59)	Cappings wax (N = 28)	Honey comb wax (N = 6)
12 (Lewis et al.,2016)	HQ_1	Mean	5562	1901	54	213
		SD	49,395	9855	116	193
		Median	27	136	4	169
		Min	0	0	0	6
		Max	466,249	74,208	507	452
		250 > value > 5000	24	26	2	3
		Value > 5000	2	2	0	0
	Risk %	Mean	0.151	0.123	0.007	0.048
		SD	0.010	2.399	0.011	0.044
		Median	1.079	0.553	0.001	0.034
		Min	0	0	0	0
		Max	10.193	4.264	0.039	0.090
0.2 (EPA, 2008)	HQ_2	Mean	7961	4238	533	2262
		SD	49,745	11,341	744	1581
		Median	753	1330	184	2466
		Min	0	0	0	75
		Max	468,324	75,476	2677	4584
		250 > value > 5000	54	42	11	5
		Value > 5000	10	9	0	0
	Risk %	Mean	1.219	1.194	0.160	1.037
		SD	2.924	2.706	0.053	1.064
		Median	0.283	0.507	0.240	0.771
		Min	0	0	0	0
		Max	14.376	19.958	0.871	2.213

Legend: LD_{50}, acute median lethal dose; HQ_1 and HQ_2, Hazard Quotient calculated 2 different tau-fluvalinate LD_{50} values.

4. Discussion

4.1. Validation of analytical method

The QuEChERS extraction method followed by LC-MS/MS is well established to assess pesticide residues in beeswax (Herrera López et al., 2016; Niell et al., 2014; Svečnjak et al., 2019). From an analytical point of view, sample preparation should guarantee the representativeness and complete extraction of the residues for a high recovery (Niell et al., 2014). As pesticide residues in beeswax samples are not evenly distributed, beeswax wax was grounded and homogenised using liquid nitrogen. This method allows limits of quantification (LOQs) of 0.01 mg/kg and limits of detection (LODs) of 0.003 mg/kg for most residues, these limits were considered as the lowest successfully validated levels, that is, the levels at which acceptable recoveries (70–120%) were achieved.

Pesticides and veterinary drug residues in beeswax.

The results confirmed our first hypothesis; residues of pesticides applied in agriculture and as veterinary drugs in-hive are ubiquitous contaminants in beeswax. In 2012, Ravoet et al. (2015) already reported the presence of 18 pesticide residues in a restricted area in Flanders with a similar median number of residues per wax sample. Simon-Delso et al. (2014) analysed 54 wax samples for 99 different residues, detecting 15 different active ingredients overall. Worldwide, numerous studies (Boi et al., 2016; Calatayud-Vernich et al., 2017; Chauzat and Faucon, 2007; Fulton et al., 2019; Harriet et al., 2017; Lozano et al., 2019; Serra-Bonvehí and Orantes-Bermejo, 2010; Shimshoni et al., 2019; Zawislak et al., 2019) acknowledge that beeswax is a major contamination sink for pesticide residues, thereby constituting hazardous health implications for bees and potentially for humans.

Overall in our study, typical residues of beekeeper-applied veterinary treatments such as tau-fluvalinate (Apistan®) and coumaphos (Checkmite®) had the highest contamination prevalence and concentrations. These products, by design, have low toxicity relative to the dose required for adverse effects. Pesticide residues from agricultural were found with lower prevalence and concentrations, nevertheless, these products have higher toxicity to bees and are known to have synergistic effects with other pesticides, which increase the toxicity of one or more of the compounds (Johnson et al., 2013; Thompson and Wilkins, 2003).

Table 5
Univariate logistic regression model outcome, pesticides with possible correlation to bee mortality (only pesticides with *P* value <0.2 are presented).

Pesticides with *P* value <0.20	Odds ratio	95% CI	*P* value
Bromopropylate	1.68	0.86–3.27	0.124
Chlorfenvinphos	2.24	1.09–4.58	0.028*
Chlorpyrifos (-ethyl)	2.38	0.88–6.44	0.088
Diclofluanid	2.43	0.62–9.46	0.2
Pendimethalin	0.21	0.02–1.90	0.16
Permethrin	2.06	0.93–4.53	0.072
Piperonylbutoxid	1.58	0.82–3.04	0.175
p,p′-DDT	4.51	0.52–39.4	0.17
Propargite	1.80	0.99–3.25	0.051
Thiacloprid	0.12	0.007–2.34	0.16

Legend: CI, confidence interval; * *P* value <0.05.

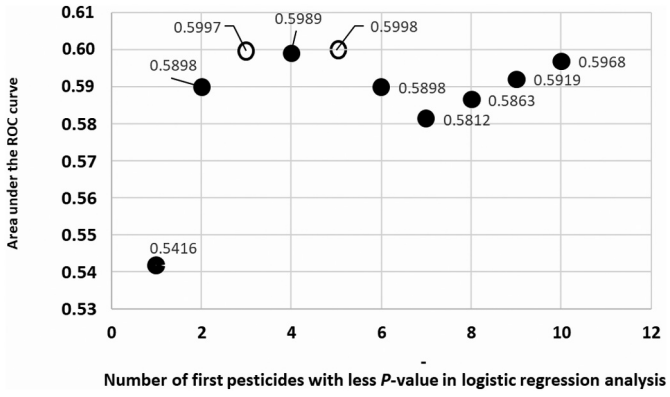

Fig. 3. Area under the curve estimated for each of the ten receiver operating characteristic (ROC) curves tested. Legend: Circle, area under the curve for each of the ten different receiver operating characteristic (ROC) curves tested; black circle, ROC curve not retained; circle with white centre, two best ROC curves retained.

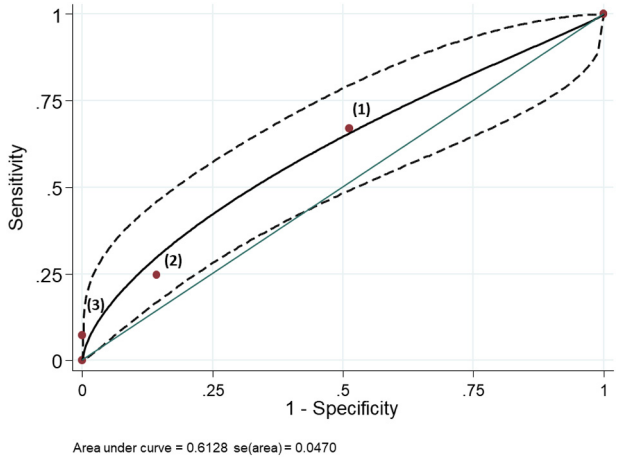

Fig. 4. Best predicted receiver operating characteristic (ROC) curve. Legend: ROC, Receiver Operating Characteristic; solid line, fitted ROC curve (fitting binormal model); dashed lines, 95% confidence interval of the fitted ROC curve; cut-off (= number of pesticides in the combination of the three pesticide residues considered) was noted as a number in brackets. The best cut-off corresponds to at least one pesticide residue(s) of the three considered.

Tau-fluvalinate and coumaphos are currently not approved in Belgium but are permitted in at least one of the other Member States of the European Union. Through the "cascade system[5]" (El Agrebi et al., 2019), they can, therefore, be used in Belgium, under certain conditions and the responsibility of a veterinarian. Their frequent use over the past few years resulted in substantial residue levels in beeswax. These *Varroa*-treatments are well known and have previously been reported worldwide as prevalent contaminants in honey bee colonies (Bommuraj et al., 2019; Chauzat and Faucon, 2007; Harriet et al., 2017; Herrera López et al., 2016; Johnson et al., 2010; Mullin et al., 2010; Perugini et al., 2018; Van Engelsdorp et al., 2010). The high chemical stability and the low migration rate of these highly lipophilic acaricides drive them to accumulate in wax to concentrations up to the mg kg $^{-1}$ (Lozano et al., 2019). This phenomenon seems to occur especially with coumaphos, whose concentration levels vary significantly from 0.01 mg kg $^{-1}$ up to 7.41 mg kg $^{-1}$, probably due to different application events, but also to its high beeswax persistence (half-life of 115–356 days) (Martel et al., 2007; Zhu et al., 2014) and the extensive recycling of old beeswax into new foundations.

In contrast amitraz (Apivar®), an approved acaricide that is frequently used in Belgium is rarely detected in beeswax samples, due to its short half-life, requiring its quantification indirectly through its metabolites (Shimshoni et al., 2019). Amitraz is reported to degrade within 1 day in beeswax and within 10 days in honey (Korta et al., 2001). In our study, one very high amitraz detection (16.7 mg kg^{-1}) was registered in a comb wax; probably due to a massive recent application. No other value exceeded 0.54 mg kg^{-1}.

Other acaricides were also found with a high prevalence (28%) such as bromopropylate (and its metabolite dibromo-benzophenone). This acaricide was used in the early years of *Varroa*-treatments (e.g. Folbex VA®), in addition to its agricultural use against other mites. Bromopropylate shows high lipophilic properties (log $P = 5.4$) and high persistence, therefore its use in agriculture was banned in Europe in 2003 and Belgium in 2007 (Commission Regulation (EC) No 2076/2002). Nowadays, its use in beekeeping is no longer approved. The acaricide propargite was detected with a high prevalence (53.3%) as well. This residue comes from agricultural applications and not from *Varroa* control. Its accumulation in beeswax came from external contaminants brought back to the hive via foraging. Its use is no longer authorized by the European regulation (EC) 1107/2009.

[5] The cascade system was introduced to solve the general problem of availability of veterinary medicinal products for minor species and for minor uses.

Agricultural pesticides such as piperonyl butoxide were found with a prevalence of 29.1%, it is a classic P450 inhibitor that has been reported to increase the toxicity of thiacloprid to honey bee (Iwasa et al., 2004) and to affect the bee's ability to detoxify, contributing significantly to honey bee intolerance of pyrethroid insecticides (Johnson et al., 2006). Diethyltoluamide (DEET) an insect repellent, was found with a prevalence of 36.3%, confirming its presence in Belgian beeswax (Ravoet et al., 2015). DEET has relatively high lipophilic properties (Log $P =$ 2.1), which could explain its accumulation. Nevertheless, DEET contamination source could not be determined.

We analysed the samples for six neonicotinoids insecticides. Similarly to the study of Simon-Delso et al., (2014), only residues of thiacloprid were detected in 3 wax samples. In the past years, neonicotinoids have been under particular surveillance for their implication in honey bee losses, and their use as seed treatments has been partially restricted in the European Union (European Commission, 2013).

More alarming was the detection of highly toxic to bees and EU banned molecules such as lindane (gamma-HCH) (prevalence of 3.29%) and dichlorodiphenyltrichloroethane (DDT) including its breakdown product dichlorodiphenyldichloroethylene (prevalence of 0.54%). Since 2008, all uses of lindane are banned in the EU. In 2009, lindane and two other HCH-isomers were included in the Stockholm Convention (ECE/EB.AIR/104) on Persistent Organic Pollutants (POPs) to achieve the global elimination of these substances (Vega et al., 2016). DDT's use has also been banned in Europe since 2009 (Regulation (EC) No 1107/2009). Our results confirm previous ones showing that pesticides can continue to contaminate the environment long after their ban (Tosi et al., 2018).

4.2. Pesticide and veterinary drug residues and honey bee mortality

In the multivariate logistic regression analysis, only chlorfenvinphos appeared to have a significant correlation with bee mortality (OR = 2.15; 95% CI: 1.04–4.44; $P = 0.038$), in the risk-based model, this compound was also targeted. The Honey bee mortality data used should be interpreted with caution as the underlying factors responsible for bee mortality are generally multi-factorial (Potts et al., 2010).

Chlorfenvinphos use is no longer authorized for agricultural use in the EU (Commission Regulation (EC) No 2076/2002) and is not approved as veterinary treatment for controlling this, the molecule was found in 24.7% of all wax samples. As no maximum residue level (MRL) was defined for the substance, a default value of 0.01 mg kg^{-1} is applied as MRL for honey following Reg. (EC) No 396/2005. The mean concentration in beeswax of the positive samples to chlorfenvinphos (all wax types together) is 0.033 mg kg^{-1} (min-max: 0.01–0.15 mg kg^{-1}), thus exceeding the MRL set for the honey of 0.01 mg kg^{-1}. Chlorfenvinphos presence has already been reported in a previous Belgian survey, with 50% occurrence ($N = 10$) and a concentration fluctuating between 0.008 and 0.015 mg kg^{-1} (Ravoet et al., 2015) as well as in a German study in 8.6% of the analysed samples ($N = 288$), with concentrations ranging from 0.001 to 6.4 mg kg^{-1} (Shimshoni et al., 2019). In Italy, 34.5% of the analysed wax samples ($N = 178$) were positive to chlorfenvinphos with concentrations reported of 0.01 to 0.63 mg kg^{-1} (Perugini et al., 2018). Pollen was as well continuously contaminated over months and years (Tosi et al., 2018). In Spain, 88.5% of the samples were found positive for chlorfenvinphos, with concentrations up to 10.64 mg kg^{-1} during a survey between 1996 and 2006 (Orantes-Bermejo et al., 2010). In another Spanish study, differentiating wax types, Calatayud-Vernich et al. (2019) reported a 100% prevalence and concentrations ranging from 0.21 to 0.79 mg kg^{-1} in old comb wax, 33.3% prevalence and concentrations ranging from 0.005 to 0.05 mg kg^{-1} in cappings wax.

Studies on the effects of chlorfenvinphos on honey bee larvae health are not yet available. However, like coumaphos, it is an organophosphorus insecticide, whose adverse effects on adult worker bees have been studied at different levels (Fell and Tignor, 2001; Haarmann et al., 2002;

Pettis et al., 2004). The origin of chlorfenvinphos in Belgian waxes is uncertain. Chlorfenvinphos illegal use as acaricide has been suspected in Spain, Portugal, France, and Italy (Orantes-Bermejo et al., 2010), where unauthorized chemicals are used as an alternative to the limited efficacy of some authorized treatments. The residues may also have been taken up by honey bees during the collection of nectar and/or pollen in the environment around the hive when the pesticide was illegally applied on flowering crops (Lozano et al., 2019). Chlorfenvinphos could also originate from river pollution, the substance has been identified among 45 other as a priority substances to be monitored in the European Union (Directive 2013/39/EU) (Pistocchi et al., 2019). Another possible route of wax contamination is the use of legally traded wax from non-EU countries. Chlorfenvinphos concentrations in Belgian beeswax appear to be low and therefore does not seem to be the result of illegal use in-hive as veterinary treatment.

4.3. Wax Hazard Quotient and toxicity to honey bees

Overall, in the first scenario (tau-fluvalinate DL_{50} = 12 μg bee^{-1}), the majority of the analysed samples (68%) had a low HQ value (<250) and should not represent a danger for honey bees, nevertheless, 30.2% have significant HQ.

The mean HQ value for brood comb (N = 89) showed the highest toxicity to bees (μ = 5562; σ = 49,395: min-max: 0–466,249), this value is due to one sample with an extremely high HQ value (466,249) elevating the HQ mean significantly from 326.7 to 5562. The sample contaminated with a high concentration of cypermethrin (9.3 mg kg^{-1}) was recorded in the province of Luxembourg, where agricultural land is essentially devoted to dairy and, above all, meat cattle farming. Cypermethrin is used massively in livestock worldwide for topical administration, either as concentrates for dipping or spraying or in ready-to-use products such as pour-on, dressings, ear-tags. In recycled wax (N = 59) (μ = 1901; σ = 9855; min-max: 0–74,208) mean HQ value is significantly high (250 < HQ < 5000) but again, was due to 2 samples with extremely elevated toxicity values (17,536 and 74,208). These values elevated the mean HQ value from 358.2 to 1901. Two contamination (HQ = 74,208 and 5242) were located in the province of Limburg, in a region devoted to horticulture, the other in East Flanders (HQ = 17,536). The contaminations in Limburg are due to permethrin (0.31 mg kg^{-1}) and chlorpyrifos (-ethyl) (4.38 mg kg^{-1}) both used over a long period respectively to control Lepidoptera and Coleoptera in ornamental, fruit and vegetable crops and a wide range of foliar pests. In East Flanders (cattle farming), the contamination was due to the presence of deltamethrin (0.026 mg kg^{-1}) and lindane (0.021 mg kg^{-1}). Deltamethrin is a pyrethroid insecticide used to eradicate external parasites on animal farms, lindane an obsolete topical substance that was used to treat parasites. Honey comb (N = 6) (μ = 213; σ = 193: min-max: 6–452), had three samples with significant toxicity. The limited number of honey comb wax samples does not allow us to draw clear conclusions about this wax type.

Cappings wax (N = 28) had the lowest mean (μ = 53; σ = 114; min-max: 0–507), this maximum value (507) is due to permethrin contamination in a single sample. Two samples were found with significant toxicity (250 < HQ < 5000). Cappings wax and honey comb wax toxicity can be considered as low or non-toxic to bees compared to recycled and brood comb wax.

The results of our study are very much in line with the findings of Calatayud-Vernich et al., 2017; where pyrethroids together with organophosphate chlorpyrifos were the main contributors to the HQ scores. This is due to their great toxicity through contact for honey bees and/or significant concentrations in the samples. Furthermore, cappings wax were also substantially less contaminated than foundations (made out of recycled wax) (Calatayud-Vernich et al., 2017; Harriet et al., 2017) and old combs beeswax.

With the second toxicity scenario (tau-fluvalinate DL_{50} = 0.2 μg bee^{-1}), the HQ levels near alarming levels, and the number of samples exceeding threshold values increases. A revision is needed to clarify Tau-fluvalinate DL_{50} value.

The HQ model used in this study is simplistic as it considers toxic effects as cumulative and additive but does not take into account any synergistic or antagonistic effects, as these are not yet well documented and thus not yet integrated into the used equation. Better models for estimation of potential adverse effects of residue cocktails with greater reliability than those already existing are needed to assess more properly the potential risks of residues.

Cumulative risk by contact exposure to honey bee larvae.

The highest risk was posed by brood comb wax where 15% of larvae were exposed to pesticide doses higher than the lethal dose, followed by the recycled comb, and honey comb wax. Our results point out that cappings wax was substantially less contaminated than the 3 other wax types and presented a very limited risk (0.7%). In the scenario were tau-fluvalinate DL_{50}= 0.2 μg bee^{-1}, the cumulative by contact exposure risk increased considerably to exceed 100% except for cappings wax (16%) but is still high. However, it may not be appropriate to assess risk by acute DL_{50} values for adult honey bees when it is the larvae that develop in wax for a specific time, hence the cumulative risk value estimated using the current calculation represents an inaccurate scenario, but to date, the necessary toxicity values for larvae is not sufficiently documented. Other studies already estimated contact exposure risk to worker bees or bee larvae of single pesticides (Harriet et al., 2017; Sanchez-Bayo and Goka, 2014). Using a slightly different equation, Harriet et al., 2017, found Chlorpyrifos-ethyl (198%) and coumaphos (21%) to have the highest risk to bee larvae.

4.4. Potential synergies and interactions of residues

With up to 12 different residues detected in a single wax sample, it is very difficult to elucidate the potential interactions of products. The risk assessments may thus underestimate the true risk to bees, as the more residues in a given sample, the greater potential for unexpected synergistic interactions. The most prevalent combinations included acaricides for *Varroa* treatments as they are directly applied in the hive. Pesticide residues synergies have scarcely been evaluated, nevertheless, the current pesticide combinations would probably damage colony health, because synergistic effects have been identified for combinations such as piperonyl butoxide that seems to increases the toxicity of fenpyroximate, while amitraz seems to increase the toxicity of tau-fluvalinate (Johnson et al., 2013).

5. Conclusion

Bees are at risk from many stress factors, which occur individually but most commonly in combinations, affecting bee health and mortality. Pesticides are one of the factors impacting colony health. Our study highlights the ubiquitous presence of pesticides in all wax types, besides veterinary drug residues have the highest concentration and prevalence but the lowest toxicity compared to agricultural pesticides that have a lower prevalence but higher toxicity to bees and can have synergistic effects with other pesticides. Significantly lower residue diversity and concentrations were found in cappings wax compared to the other three types. Brood comb wax exhibited the highest rates of contamination. In light of these results, beekeepers should replace brood comb wax more frequently than recommended (1/4 to 1/3 of than old brood frames (ITSAP, 2017)) rather than recycling them back into the wax stream, where they will continue to potentially impact colony health. We highly recommend the use of greater amounts of cappings wax in the manufacturing process of foundation, the substrate beekeepers purchase to aid their bees in building comb, as well as using organic wax sources to gradually decrease residues in the colony matrix. Furthermore, the marketing and the recommendation regarding the use of plant protection products and as well as veterinary

treatments should take into account that compounds with highly lipophilic properties accumulate in wax. Given the large number of residues found in beeswax and the amount of potential synergistic effects among the different residues detected, we recommend testing commonly found combinations in field experiments to determine the potential synergetic effects on colony health. The use of alternative veterinary substances (e.g. acids) should be encouraged. An educational campaign for users of pesticides or veterinary drugs is needed to increase awareness and good practices. The use of the BeeToxWax tool designed to estimate the risk associated with contaminated beeswax is recommended when pesticide analyses are available (Appendix 2). It is crucial to introduce maximum residue limits for beeswax trade, taking into account residue toxicity for bees and, ideally, for their larvae. Furthermore, EPA and PPDB toxicity values for tau-fluvalinate should be scientifically re-examined in depth.

Abbreviations used

ABP	animal by-products
AUC	Area under the curve
DEET	N,N-Diéthyl-3-méthylbenzamide
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Unites States Environmental protection Agency
FASFC	Federal Agency for the Safety of the Food Chain
HQ	Hazard Quotient
LC/MS-MS	liquid chromatography/tandem mass spectroscopy
GC/MS-MS	gas chromatography/tandem mass spectroscopy
GC-MSD	gas chromatography-mass selective detector
LD_{50}	Acute median lethal dose after 48 h of exposition = is a statistically derived single dose of a substance that can cause death in 50% of animals when administered by the oral route/by contact. The LD_{50} value is expressed in μg of test substance per bee. For pesticides, the test substance may be either an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient (OECD, 2017)
LOD	Limit of detection
LOQ	Limit of quantification
MRL	Maximum Residue Limit
PPDB	Pesticides properties DataBase
QuEChERS	Quick Easy Cheap Effective Rugged Safe
ROC	Receiver operating characteristic
VSDB	Veterinary Substances DataBase

CRediT authorship contribution statement

Noëmie El Agrebi: Conceptualization, Methodology, Software, Data curation, Writing - original draft, Visualization, Investigation. **Kirsten Traynor:** Writing - review & editing. **Olivier Wilmart:** Writing - review & editing, Data curation, Validation. **Simone Tosi:** Writing - review & editing. **Laurent Leinartz:** Software, Visualization. **Ellen Danneels:** Writing - review & editing. **Dirk C. de Graaf:** Writing - review & editing. **Claude Saegerman:** Conceptualization, Methodology, Software, Data curation, Visualization, Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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