



Fungicides and bees: a story of the unexpected

Towards a better understanding of inexplicable bee
colony disorders in the Walloon Region (Belgium)

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*To those believing that anything is possible,
and those making everything possible*

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Summary

For several decades now, an increasing phenomenon of enhanced honey bee colony mortality and morbidity is troubling beekeepers and naturalists. Such negative trends affect also wild pollinators and cast doubt about the sustainability of pollination both of wild plants and crops. These trends are also experienced in Belgium, country showing a level of annual colony losses above 18% in the best years. In addition, Walloon beekeepers describe for many years unspecific symptoms with their colonies like weaken colonies, queen losses, abnormal brood development or full death of the colony without any symptoms of disease.

The aim of my work was to bring light in this situation, which revealed unexpected results. A prevalence case study carried out in the region of Wallonia by myself in collaboration with colleagues of the CRA-Wallonia, pointed at fungicides as the stressing factor linked to honey bee colony disorders. These pesticides considered harmless to bees, we started digging to understand the underlying reasons of our field observations. First we considered if they were just markers of intensive agriculture, not really having any toxicological effects on bees. Indeed, we found that colonies surrounded by conventionally-farmed crops were more likely to be contaminated by pesticides, in special fungicides. In addition, the colonies in these crop areas were more prone to show disorders. We also found that this contamination does not necessarily come from treated crops, but from wild plants or catch/cover crops in the surroundings or succeeding treated

crops within a surface of at least 3 km radius. Then, I tested the direct toxicological effects fungicides on different colony individuals. I started by evaluating the effect of the most frequently found fungicide, boscalid, on bee larvae, which did not show to induce increased mortality of immature bee stages. Then I studied the impact of chronic exposure of adult bees. Finally I suggest further research lines to better understand the role of fungicides in honey bee colony disorders, specially when fungicides are in combination with other stressors, and on the indirect effects of fungicides on bee nutrition and microbiology. Finally, I suggest action lines to improve the existing field situation.

List of abbreviations

2CNA	2-Chloronicotinic acid
6CNA	6-Chloronicotinic acid
ABPV	Acute Bee Paralysis Virus
ABV	Arkansas bee virus
AICc	Akaike Information Criterion corrected
AIV	Apis iridescent virus
ALPV	Aphid lethal paralysis virus
AmFV	Apis mellifera filamentous virus
ANOVA	Analyses of Variance
APBV	Acute Bee Paralysis Virus
ATP	Adenosine Triphosphate
AUC	Area Under Curve
AUROC	Area Under the Receiver Operating Curve
BBPV	Berkeley bee virus
BSRV	Big Sioux River virus
BVX	Bee virus X
BVY	Bee virus Y
CARI	Centre Apicole de Recherche et d'Information
CBPV	Chronic Bee Paralysis Virus
CI	Confidence Intervals
COLOSS	Prevention of honey bee COlony LOSSes
CPBV	Chronic Bee Paralysis Virus
CRA-W	Centre de Recherches Agronomiques de Wallonie
CREA-API	Centro di Ricerca Agronomica – Apicoltura

Ct or CT	Cycle Threshold
CWV	Cloudy wing virus
DNA	Desoxyribonucleic Acid
D _{rn}	delta R _n
DT ₅₀	Degradation Time 50
DWV	Deformed Wing Virus
EBV	Egypt bee virus
EDTA	Ethylene Diamine Triacetic Acid
GC-MS	Gas Chromatography coupled with Mass Spectrometry
GC-MS/MS	Gas Chromatography coupled with tandem Mass Spectrometry
GITC	Guanidinium-Isothiocyanat (protein denaturant)
GLM	Generalised Linear Modelling
IAPV	Israeli acute paralysis virus
IGR	Insect Growth Regulator
KBV	Kashmir bee virus
KV	Kakugo virus
LC-MS/MS	Liquid Chromatography coupled with tandem Mass Spectrometry
LC ₅₀	Lethal Concentration 50
LCD ₅₀	Lethal Cumulative Dose 50
LD ₅₀	Lethal Dose 50
LDD ₅₀	Lethal Dietary dose 50
LOD	Limit of Detection
LOQ	Limit of Quantification
LPIS or SIGEC	Land Parcel Identification Systematic
LR	Likelihood Ratio
LSV-1	Lake Sinai virus-1
LSV-2	Lake Sinai virus-2
LT ₅₀	Lethal Time 50
MMLV	Moloney Murine Leukemia Virus
MRM	Multiple Reaction Monitor
MSPD	Matrix Solid Phase Dispersion
NOED	No Observed Effect Dose
OECD	Organisation for Economic and Development
PER	Proboscis Extension Reflex
PSA	Primary and Secondary Amine
PVC	Polyvinyl Chloride
QBCV	Queen Black Cell Virus

QPCR	Quantitative Polymerase Chain Reaction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RH	Relative Humidity
RNA	Ribonucleic Acid
ROX	6-carboxy-X-rhodamine
RT-PCR	Real Time Polymerase Chain Reaction
CBPSV	Chronic Bee Paralysis Satellite Virus
SBPV	Slow bee paralysis virus
SBV	Sacbrood Virus
SDS	Sodium Dodecyl Sulphat
SE	Standard Error
SD	Standard Deviation
TSBV	Thai Sacbrood virus
TNA	Total Nucleic Acid
TS	Toxic Standard
UPLC-MS/MS	Ultra Pressure Liquid Chromatography coupled with tandem Mass Spectrometry
VdV-1	Varroa destructor virus-1

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Chapter 1

Introduction

1.1 POLLINATION, THE FERTILITY OF WORLD

Until recently, the world agricultural sector regarded the entomophilous pollination of plants (the movement of pollen from female flowers to male flowers to ensure reproduction carried out by insects) as a free service provided by nature. In this frame of mind, few farmers and policy makers were concerned about the conditions under which food production was conducted. Today, however, the loss of biodiversity, including the disappearance of pollinating insects, is challenging this way of thinking. Production system is experiencing a decline in food resources and raising questions about public health.

The economic benefit presented by pollinators of entomophilous plants at the global level is no longer questionable. A report by the United Nations Environment Program (UNEP) in 2010 estimated that the contribution of pollinators to the production of crops used directly for food worldwide is around € 153 billion (\$202 billions at the exchange rate at the time of the study (1.322 \$ per €¹)), 9.5% of the total value of human food production worldwide (Gallai *et al.*, 2009 in Kluser *et al.*, 2010). This figure raised to \$235-577 billion US\$ in later study (Lautenbach *et al.*, 2012). Without food crop pollinators, dietary practices would be subject to a sinister revolution as most fruits (apricots, almonds, cherries, strawberries, peaches, pears, apples, plums) would disappear, as well as vegetables such as courgette, tomato or apple (Such as artichoke, cabbage, fennel, onion, parsley, leek), oilseeds such as rapeseed and sunflower and forage crops (clover, alfalfa). In total, 76% of food production in Europe (Williams, 1996) and 87.5% of plant species depend on insect pollination globally (Ollerton *et al.*, 2011). Nearly 90% of the wild plant species worldwide depend to a certain extent on animal pollination. In Wallonia, the main entomophilous crops before the beginning of the present study were rapeseed with 10,000 hectares in 2006-2007, vegetable crops of peas and beans with 16,000 hectares in 2006, fruit production (apple, pear, cherry, plum, strawberry, redcurrant, raspberries), representing 1,600 hectares in 2003. In 2015, rapeseed represented 11,917 ha, peas and beans 14,018 ha and fruit production (including nurseries) were 2,062 ha (SPW Economie, 2017). There are also horticultural crops requiring pollination grown under glass (tomatoes, courgettes, aubergines, peppers).

Among all animals contributing to pollination, bees play a dominant role due to their biological characteristics, behaviour and ecology. Worldwide exist around 20.000 different species of wild and managed bees, contributing all of them to a higher or lesser extent to plant pollination. Flies occupy a second post as flower visitors and other animals as butterflies, moths, beetles, birds, bats or vertebrates. In Wallonia, 347 species of wild bees and bumble bees are known (Terzo and Rasmont, 2011). With their cousin, the honeybee, they provide most of the pollination

¹ <http://www.xe.com/currencytables/?from=EUR&date=2009-04-01> Accessed 4 August 2017

work.

While visiting flowers to collect nectar and pollen, pollen grains get attached the hairy body of bees and is transported from one flower to another. The fact that bees stick to one plant species whenever they found an interesting source of food, enables the movement of pollen among flowers of the same plant species. The pollination of wild plants, but also that of cultivated ones, ensures the maintenance of biodiversity, considered the immune system of the world, but also our food and non-food productions (fibre, oil) (Klein *et al.*, 2007; Lautenbach *et al.*, 2012).

Human beings have coevolved with bees and learned to use bees or their products for the production of other goods for food, pharmacological, scientific or technological uses (Banskota *et al.*, 2001; Jull *et al.*, 2008; Srinivasan, 2011). In doing so, bees became part of our culture (eg. culinary, recreational, etc.), and have long contributed and continue doing so to the dynamism of rural and urban areas (Bradber, 1990). They also provide a source of inspiration and well-being for many humans, like myself (UNEP, 2005).

1.2 TRENDS IN POLLINATORS AND FIELD OBSERVATIONS

For some years now, despite of the positive trend in numbers of bee colonies (Faostat, 2017), a global trend of increasing colony mortality and morbidity threatens honey bee populations and thereby pollination in agriculture worldwide (Cox-Foster *et al.*, 2007; Kluser and Peduzzi 2007; Lefebvre and Bruneau, 2004; Maxim and van der Sluijs, 2010; UNEP, 2005; van der Zee *et al.*, 2015, 2012; vanEngelsdorp and Meixner, 2010; Yamada *et al.*, 2012). Beekeeping efforts to maintain the colony demography at a certain level goes however with a cost (IPBES, 2016). Mortality patterns remain often restricted to specific regions/areas (Afssa,

2008; Brodschneier *et al.*, 2016; Genersch *et al.*, 2010; Taniguchi *et al.*, 2012; van der Zee *et al.*, 2015; Yamada, *et al.*, 2012). *Apis laboriosa*, the Himalayan bee, seem to follow this negative evolution, colony numbers showing a decrease over the latest 15 years (FAO, 2008).

Similarly, long-term declines have been observed in wild pollinator populations around the world (Berenbaum *et al.*, 2007; Biesmeijer *et al.*, 2006; Carvalheiro *et al.*, 2013; Dicks *et al.* 2002; Goulson *et al.*, 2008; Holden *et al.*, 2006, Potts *et al.*, 2010; Settele *et al.*, 2010). With wild pollinators I refer here any species that is not domesticated or managed by humans – Hymenoptera (bumble bees and solitary bees), Lepidoptera (butterflies and moths), Coleoptera (beetles) and Diptera (flies)). Bumble bee populations seem to follow negative trends in absolute terms (Grixti *et al.*, 2009; Rasmont and Iserbyt, 2012), with a number of species going extinct or being threatened to extinction (Goulson *et al.*, 2008; Kosior *et al.*, 2007). Other studies have registered shifts in community composition in parallel to decreasing overall numbers (Bartomeus *et al.*, 2013; Bommarco *et al.*, 2011). It is worthwhile noting that bumble bees, specially *Bombus terrestris* and *Bombus impatiens* are increasingly being cultured and are subject of global trade for pollination purposes. Solitary bees like *Megachile rotundata* or *Osmia rufa* are also being managed for pollination or testing purposes. This may have an impact in the trends of these species and those of other species, given the potential risk to wild bees through competition, introgression and pathogen spillover (Goulson *et al.*, 2008).

In parallel, wild solitary bees and hoverflies share these negative trends: a decline in species richness, while a minority of species cover the niches gaps left, all in all leading to a uniformity in the composition of the entomofauna and a homogenization of the environment (Bartomeus *et al.*, 2013; Biesmeijer *et al.*, 2006; Carvalheiro *et al.*, 2013). Rare bee species seem to be the most affected, the contrary being observed for species better tolerating human disturbance of habitats. Between 1990 and 2011, populations of European grassland butterflies are reported to have declined by 50% in abundance (van Swaay *et al.*, 2013). Beekeepers of stingless bees (*Melipona beecheii*) in Mexico have also noted a sharp decrease in

colony numbers in the last twelve years (FAO, 2008). Recently, bees inventories carried out at EU level revealed worrying results: from the 44% of European species for which data is available, 9% are considered threatened with extinction, while 7.7% species showed declining population, 12.6% are relatively stable and 0.7% are increasing (Nieto *et al.*, 2014). In 2017, Hallmann *et al.*, 2017, describes a seasonal decline of 76%, and mid-summer decline of 82% in flying insect biomass over the 27 years of study. This overall decline cannot be explained neither by the habitat characteristics, nor changes in weather or the land use and the authors suggest that agricultural intensification, including pesticide usage, year-round tillage, increased use of fertilizers and frequency of agronomic measures, could contribute to the negative trends.

All these trends invite to think that the reasons behind the problem may be common to all these species. That is why in my research work I focus myself not on the individual species, but on the environment and habitat where these species live. This thesis provides some answers regarding the impact of the environment and habitat on honey bees.

1.3 HONEY BEES, BIOINDICATOR OF THE QUALITY OF THE ENVIRONMENT AND FOOD SYSTEMS

Honey bee colonies (*Apis mellifera* L.) are eusocial animals composed of several castes (individual participating to reproduction: drones and queen, and sterile females) and classes (in-hive, foragers) of bees. Many describe the honey bee colony as a super-organism, an indivisible whole integrating multiple individuals. A honey bee colony would equal a cow, while the individual bees conforming it would be the different organs or cells of the cow. A group of in-hive bees, the nurses, have the function to take care of the immature stages of bees, called the brood, and the queen,

while other components of the hive deal with other functions within the colony as food processing and storage or participate to its structural development by producing wax and distributing it where needed. Finally, the older bees are those who leave the hive to forage water, gums and/or food sources like nectar, honeydew or pollen. Different generations live together in one colony where all the components are half brothers or sister of the same mother (the queen). She mates with several males whose sperm is kept during her lifetime and is allocated or not to the different eggs from which the future individuals of the population will emerge. This genetic diversity is a strategy of honey bees to increase its survival chances. Fecundated eggs (diploid) will originate sterile females, while non fecundated eggs (haploid) will originate males.

In addition to the role played in the pollination of certain plant species, honey bees act as the canary in the coal mine identifying environmental areas of concern. In parallel with other bioindicators like lichens, mosses or snails, honey bees can be considered as living organisms able to contribute to the assessment of the quality of the environment under the influence of anthropization (Holt and Miller, 2011). The foragers of a single honey bee colony visits and samples numerous plants within a wide surface every day. Techniques like waggle dance decoding have been used to determine the direction and distance flown by bees to find food resources (Couvillon *et al.*, 2012, 2014). The waggle dance is one of the tools, together with odour cues (Grüter and Farina, 2009), used by bees to communicate with their fellow colleagues where a good source of food can be found, which aims at recruiting foragers to exploit the found source to a maximum.

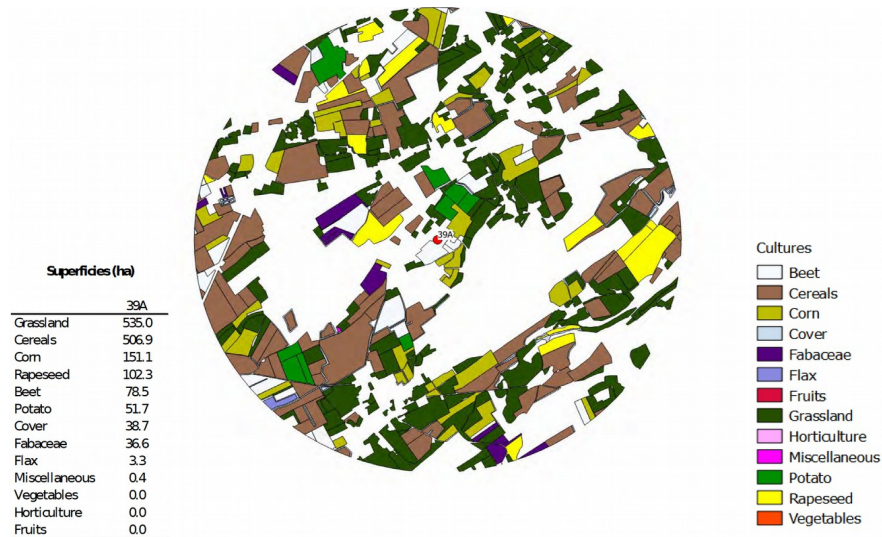


Figure 1.1 – Example of land use in a circumference of 3 km radius around an apiary (red dot)

From the several studies found in literature describing foraging distances we know nowadays that bees visit flowers either close to their colonies up to 15 km away (Beekman and Ratnieks, 2000; Couvillon *et al.*, 2014; Steffan-Dewenter and Kuhn, 2003; Visscher and Seeley, 1982). These distances depend on characteristics like the genetic and physiological status of the colony, the sugar content of nectar, the weather, or the time in the year (Couvillon *et al.*, 2014) with bees running over an average of 2 km in the summers of temperate regions, while they fly 500 m on average in spring or around 1.3 km on average in autumns. Figure 1.1 shows the agricultural land use 3 km around an apiary (red dot) as an illustration of the diversity of crop plants that could potentially be foraged by the bees of this apiary. On top of these surfaces, wild plants, surface water like puddles, streams, plant gums from these buffer zones (the circles of 3 km radius) can as well be visited by bees.

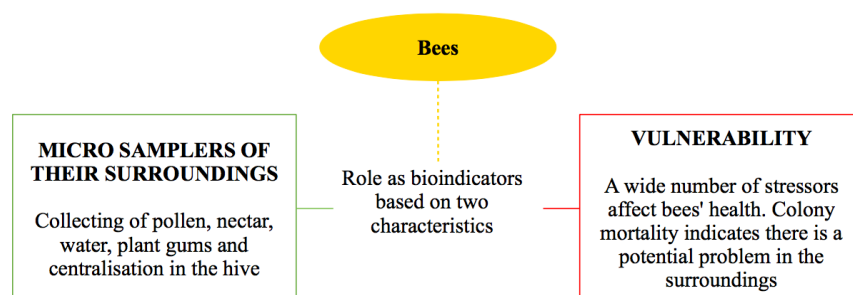


Figure 1.2 - Scheme on the logic of using honey bees and their matrices as bioindicators of the quality of the environment

While foraging around, they also unintentionally collect airborne particles or substances diluted in the air (Girolami *et al.*, 2012). Analyses of their products or bees themselves can therefore reveal the pollutants present in a wide area. Furthermore, honey bee colony management is well known and the health of *A. mellifera* is one of the most studied subjects nowadays. Colony mortality or morbidity has been correlated with landscapes containing many pollutants (Oomen *et al.*, 2001; Smart *et al.*, 2016a, 2016b), little nutritional resources diversity (Sgolastra *et al.*, 2017; Woodcock *et al.*, 2017) or a combination of both like geographical areas of intensive agriculture. Therefore, colony status can as well be used as a bioindicator of the quality of the environment surrounding it. This has lead to using honey bees, a species often used as a model, and beekeeping products as biological indicators for environmental monitoring (Accorti and Persano Oddo, 1986; Al Nagggar *et al.*, 2013; Anderson and Wojtas, 1986; Balayiannis and Balayiannis, 2008; Balestra *et al.*, 1992; Barišić *et al.*, 2002; Bromenshenk *et al.*, 1991; Celli *et al.*, 1988; Celli and Maccagnani, 2003; Celli and Porrini, 1987; Chauzat *et al.*, 2011; Ciemniak *et al.*, 2013; Dobrinas *et al.*, 2008; Driss *et al.*, 1994; Fakhimzadeh *et al.*, 2000; Fernandez-Muino *et al.*, 1995; Fredes and Montenegro, 2006; Ghini *et al.*,

2004; Holt and Miller, 2011; Jan and Černe, 1993; Kevan, 1999; Kump *et al.*, 1996; Lambert *et al.*, 2012a, 2012b; López *et al.*, 2014; Mercuri and Porrini, 1991; Moret *et al.*, 2010; Odoux *et al.*, 2014; Perugini *et al.*, 2011, 2009, Porrini *et al.*, 2014, 2003a, 2003b, 2002, 1998, Stein and Umland, 1987, 1986; Tonelli *et al.*, 1990; Voget, 1989; Zacharis *et al.*, 2012; Zhelyazkova, 2012). Monitoring of exposure to various environmental contaminants has already been carried out, these contaminants include heavy metals (Stein and Umland, 1987, 1986), plant protection products (hereafter pesticides) (Celli and Porrini 1987; Celli *et al.*, 1988; Odoux *et al.*, 2014; Porrini *et al.*, 2003a, 2002, 1998), polycyclic aromatic hydrocarbons (Ciemniak *et al.*, 2013; Dobrinas *et al.*, 2008; Lambert *et al.*, 2012; Moret *et al.*, 2010; Perugini *et al.*, 2009) and radioactivity (Tonelli *et al.*, 1999). Unfortunately, it is often not possible to identify the specific sources of contamination. We will see that Chapter 3 aims specifically at elucidating sources of pollen contamination.

1.3.1 Pollution with a focus on pesticides

From all the pollutants afore mentioned, pesticides are the ones showing more links with honey bee mortality and morbidity. According to European legislation (Regulation (EC) 1107/2009), pesticides are commercialised products supplied to the user, consisting of or containing active substances (chemical substances or micro-organisms, including viruses, having general or specific action against harmful organisms or on plants, parts of plants or plant products), safeners (substances avoiding phytotoxic effects) or synergists (provide enhanced activity to active substances), intended for one of the following uses:

- (a) **protecting** plants or plant products against all harmful organisms or preventing the action of such organisms, unless the main purpose of these products is not hygiene,
- (b) **influencing** the life processes of plants, such as substances influencing their growth, other than as a nutrient (not fertilisers),
- (c) **preserving** plant products, in so far as such substances or products

are not subject to special provisions on preservatives,

(d) **destroying** undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants,

(e) **checking or preventing** undesired growth of plants, except algae unless the products are applied on soil or water to protect plants (European Parliament and Council 2009a).

As a result, products with different target organisms are included under the term pesticides as insecticides (to protect plants from insects), fungicides (to protect plants from fungi), herbicides (to destroy undesired plants or parts of plants), molluscicides, rodenticides, plant growth regulators, adjuvants (enhance the effectiveness of other pesticidal properties like solvents, repellents, emulsifiers, etc.), safeners, synergists, and co-formulants (substances added to commercialised products which are neither safeners, nor synergists, nor adjuvants).

Chemical substances can be natural, semi-synthetic or synthetic. The use of the latter has increased hand in hand with the development of industrialised agriculture. The installation of industrial permanent cultures (e.g. orchards, grape yards, olive yards, etc) or monocultures increases the productivity and rationalised production, but favours the appraisal of pests. Modern industrial agriculture control the pest pressure through the use of pesticides, despite of the fact that, at least in Europe, their use is supposed to be made sustainable by incorporating pesticides in a holistic strategy integrating also agronomical, genetic and managerial tools (European Parliament and Council 2009b).

1.3.2 Beekeeping matrices as tools for environment monitoring

The beekeeping matrices with potential to be used in monitoring for pollutants are the following: bees, pollen, beebread (processed and fermented pollen stored in comb cells which contains a little quantity of nectar/honey) nectar, honey (processed nectar stored in comb cells), propolis (processed plant gums distributed everywhere in the colony) and comb wax.

While bees fly around, particles transported by the air get glued to their hairy body. The analyses of these bees revealed large amount of highly toxic pesticides that had been released to the environment following the seeding of pesticide-coated seeds with pneumatic sowers (Girolami *et al.*, 2012, 2013; Marzaro *et al.*, 2011; Tapparo *et al.*, 2012). These findings gave an explanation to the large episodes of colony losses described by beekeepers ever since coated seeds with systemic insecticides like neonicotinoids or fipronil were commercialised (Bortolotti *et al.*, 2009; Maxim and van der Sluijs, 2013; Pistorius *et al.*, 2009). Foragers can also carry contaminated nectar collected from flowers or water in the honey crop back to the hive. Literature describe values of water consumption of 20-40 l/colony/year and during summer up to 20 l/week/colony or 2.9 l/d/colony (EFSA, 2012). Specific considerations about nectar and honey have been included in section 1.3.2.2. Foragers may carry contaminated pollen packed into pellets or plant gums attached in their hinder legs. Considerations about these two matrices are included in sections 1.3.2.3 and 1.3.2.4, respectively.

However, in-hive bees and larvae do also exposed to pollutants (Mullin *et al.*, 2010). Several reviews have been carried lately showing the wide range of pesticides residues that can be found in different beekeeping matrices (Bonmatin *et al.*, 2014; EFSA, 2012; Johnson *et al.*, 2010; Mullin *et al.*, 2010) and annually at several new publications come to enrich this data base (Botías *et al.*, 2015; David *et al.*, 2016, 2015; Hladik *et al.*, 2016;

Kasiotis *et al.*, 2014; McArt *et al.*, 2017; Odoux *et al.*, 2014; Oliveira *et al.*, 2016; Sanchez-Hernandez *et al.*, 2016; Smart *et al.*, 2016a). From the matrices collected at the colony for monitoring purposes, pollutants, and specially pesticides, tend to be found in larger diversity and concentrations in wax, beebread and pollen (Bonmatin *et al.*, 2014; Johnson *et al.*, 2010; Kasiotis *et al.*, 2014; Mullin *et al.*, 2010; Rissato *et al.*, 2004; Wu *et al.*, 2011).

1.3.2.1 Considerations about beeswax

The chemical composition of beeswax consists of a blend of more than 300 compounds including hydrocarbons (14%), monoesters (35%), diesters (14%), hydroxy polyesters (8%) and free acids (12%) (Callow 1963; Tulloch 1980). As a result, compounds with high partition coefficient octanol/water ($\log P$ or K_{ow}) will tend to accumulate and stabilise there. It is worthwhile acknowledging that beeswax is secreted by wax glands located in the ventral part of the abdomen of bees and not collected from a potentially contaminated environment (Winston, 1991). After being secreted, liquid wax solidifies into scales that are brought to the mandibles by the legs and, with the help of the forelegs, are manipulated to produced combs.

Low values of K_{ow} (eg. less than 1) may be considered hydrophilic, while higher values (eg. greater than 4) are considered hydrophobic. The temperatures at which beeswax is handled for beekeeping purposes like the production of wax foundation may not degrade the pollutants present there (66°C; FAO, undated). Residues of pollutants in wax may migrate within wax in a time frame of weeks or months (Wu *et al.*, 2011) and bees can help their transfer by moving food stores around (Zeggane *et al.*, 2005). Given that beebread and honey are stored in wax cels, a transfer of pollutants among in-hive beekeeping matrices is possible. Studies done with antibiotics showed that the larger the amount of residues in wax, the larger the amount of residues transferred to honey, despite of the fact that the transfer rate varies depending on the concentration studied and ranged between 15.6% and 56.9% (Reybroeck *et al.*, 2010). The level of transfer

seems to be linked to the K_{ow} which determinates the affinity to water. The transfer from nectar to wax was very little (3%). With a similar approach, Jan and Černe (1993) fed colonies with various organochlorine compounds and followed their transfer to wax and honey stored by bees. As in Reybroeck *et al.*, 2010, larger concentrations of pollutants resulted in larger concentrations in both wax and honey. Recovery of the different compounds ranged between 1% and 53% in wax and between 0.2% and 3% in honey.

1.3.2.2 Considerations about honey

One honey bee colony requires between 35 and 80 kg of honey per year to cover its needs (Camazine, 1993; Seeley, 1995 and Weippl, 1928; Rosov, 1944; Seeley 1985 in Winston, 1991). The range is wide because these values depend on the different context the colony may have. Honey is produced from nectar or honey dew collected by foragers from flowers or sap-sucking insects.

Honey is an oversaturated sugar solution that constitutes the source of energy for bees. It is mainly composed of sugars, but it contains in smaller amounts some other constituents like enzymes, amino acids, organic acids, carotenoids, vitamins, minerals and aromatic substances. Da Silva *et al.*, 2016, have done a good review of the state of the knowledge as regards the chemical composition of honey. It is produced by bees after a transformation of bee-collected nectar, which is a phloem sap derivative produced by plant nectaries, which can be either part of flowers (floral nectaries) or elsewhere on the plant (extrafloral nectaries). This transformation, or ripening, involves the drying of nectar and the addition of enzymes that modify the sugar composition of nectar (Crane, 1975).

As we could already see, it is not unusual to find pollutant residues in nectar/honey samples. We have seen that these residues can come from transfer from comb wax. Nectar and honey contain pollen grains in different proportions (von der Ohe, 2004) which could as well be contaminated with pollutants. But floral nectar can readily be contaminated with pesticides as

well (Bonmatin *et al.*, 2015; Botías *et al.*, 2015; EFSA, 2012). Nectar foragers visit a large amount of flowers daily (Winston, 1991). They can be exposed to pollutants during the nectar intake by contact with the mouth parts, the tongue, the oesophagus and the crop. The floral nectar collected by foraging workers is brought back to the colony in the honey stomach (or honey crop) and transferred to the mouth of an in-hive bees for processing. This procedure is called trophallaxis. Salivary secretions containing enzymes (invertase, the α - and β -glucosidase, catalase, acid phosphatase, diastase, and glucose oxidase) are mixed with the nectar where a sugar break down starts (Sak-Bosnar and Sakac, 2012; Won *et al.*, 2008). The water content of nectar is reduced by evaporation happening at the tongue of the worker, who processes the honey during 15-20 minutes and then deposits the mixture in wax comb cells. The humidity of this processed nectar is further reduced through evaporation favoured by thermoregulation and fanning. After the completion of the enzymatic activity and the reduction of the water content is reduced to 18% or less, the nectar is considered ripened and the resulting product is called honey (Winston, 1991).

It is maybe due to this intense processing by bees that honey often contains low concentrations of pollutants. Bees could operate as filters, on the one hand by metabolising them or favouring this metabolism through the enzymes added to nectar (Bogdanov, 2006), and on the other hands as serving as “living barrier” of toxic compounds (should a high concentration of pollutant be present, exposed bees would have died). Bees reduce the initially high pesticide nectar concentration by a factor of about 1000 in the final honey (Schur and Wallner, 2000, 1998 in EFSA, 2012). Finally, nectar/honey are hydro-soluble which involves a lesser tendency for pollutants to accumulate in them as have already been explained. The annual monitoring of residues in foodstuffs developed by the European Food Safety Agency (EFSA, 2016) revealed some residues of pesticides found in honey, some of them originating from plant treatments while others originated from veterinary products used for sanitary control. None of the residues found in honey posed a human safety risk according to established Maximum Residue Levels (MRLs) and honey was not found

among the list of foodstuffs most frequently containing pesticide residues. All in all, these are good news for us humans eating honey, but may not be as good for the bees themselves.

1.3.2.3 Considerations about pollen and bee bread

Honey bee colonies may collect between 10–26 kg of pollen per year (Crailsheim *et al.*, 1992; Jeffree and Allen, 1957; Seeley, 1985) and they tend to keep a reserve of approximately one kg of bee bread stocked in the hive (Jeffree and Allen, 1957; Seeley, 1985).

Environmental monitoring studies have mainly used these pollen pellets as analytical matrix as they provide at the same time information about its botanical origin and pollutant content and its collection is non-invasive, economic and simple. Floral pollen is collected both actively through legs movements and proboscis scraping the anthers of flowers, as well as passively through pollen grains getting glued to their hairy body and then being groomed by the legs. Pollen grains are then packed together by adding nectar or honey regurgitated from the honey crop and mixed with glandular secretions to produce pollen pellets. Loaded into receptacles on their hind legs called corbiculae (van Praagh & Brinkschmidt, 1987), pollen pellets are brought back to the colony. In the hive, the foragers deposit the pollen pellets from their corbiculae in wax comb cells to be conditioned by workers, who are the pollen processors (Seeley, 1995). During this conditioning workers add more glandular secretions that are assumed to contain beneficial microorganisms, enzymes, a small amount of nectar/honey, and seal the mixture with a drop of honey (Lukoschus and Keularts, 1968; Vasquez and Olofsson, 2009). Pollen collected, stored and mixed with the mentioned substances in the hive follows a fermentation process that enables its preservation for several months (Loper *et al.*, 1980). Pollen contains nutrients largely varying in content which can be found in Table 1.1.

Floral pollen has shown to be contaminated by pesticides already in

origin (Bonmatin *et al.*, 2003; Botías *et al.*, 2015; David *et al.*, 2015; Larson *et al.*, 2015). It is then transported to the hive and stored in comb wax cells where eventually will be mixed with pollen from different origins and fermented into bee bread. Similar to what happens between beeswax and honey, pesticide residues could migrate among wax and pollen/bee bread matrices. Indeed, considering the composition of the latter matrices in comparison with honey, this migration might be more important than that happening for honey. Unfortunately despite of the intensive literature research carried out, no study could be found showing this. Most likely because of the limited use of beebread as human foodstuff. All in all, though, the migration of pollutants within the hive remains yet to be researched.

Table 1.1 - Nutritional composition of bee collected pollen (pollen pellets) and bee stored pollen (bee bread) as described in literature.

<i>Nutrient</i>	<i>Bee-collected pollen (pollen pellets)</i>	<i>Bee-stored pollen (bee bread)</i>	<i>Reference</i>
Water	22-30%	19-28% (5.91% commercial bee bread)	Herbert and Shimanuki, 1978; Campos et al., 2008 (review); Bobis et al. 2010
Protein	7-40% ^d	19-27% ^d	Todd and Bretherick, 1942; Herbert and Shimanuki, 1978; Roulston and Cane, 2000; Somerville and Nicol, 2006; Campos et al., 2008 (review)
Lipids*	0.43-14.4% ^d	0.67-8% ^d	Todd and Bretherick, 1942; Herbert and Shimanuki, 1978; Somerville, 2005; Campos et al. 2008, (review); Barene et al., 2014; Bobis et al., 2010
Carbo-hydrates**	13-55% ^d	30.85-50% (simple sugars)	Campos et al., 2008 (review); Anderson et al., 2011; Bobis et al., 2010
Crude fibres	0.3-20% ^d		Bell et al., 1983, in Roulston and Cane, 2000
Ash	0.91-6.36% ^d		Todd and Bretherick, 1942; Herbert and Shimanuki, 1978; Campos et al. 2008 (review)

* lipid content results are dependent from the extraction methodology used (Somerville, 2005)

** Carbohydrates after determination of protein and lipids

^d in dry matter

1.3.2.4 Considerations about propolis

Propolis is produced from plant resins from buds and exudates that bees bite off with their mandibles and placed into the corbicula of the hind leg of the forager to be transported back to the colony. Upon her return, the propolis is unloaded by the mandibles of another worker and pressed or distributed into the area that needs filling in (Winston, 1991). Propolis is a lipophilic material, hard and brittle when cold but soft, pliable, and very sticky when warm. Its chemical composition includes waxes, resins (containing alcohols, aldehydes, aliphatic acids and esters, amino acids, aromatic acids and esters, chalcones, flavones, terpenoids, steroids and sugars), balsams, aromatic and ethereal oils, pollen and other organic matter (Ghisalberti *et al.*, 1978; Marcucci, 1995; Mercan *et al.*, 2006; Moreira *et al.*, 2008) in proportions that may vary depending on origin and time of collection (Bankova *et al.* 2002). Its antiviral, antibacterial, antifungal, antiprotozoan and cytotoxic activity are well known (Marcucci, 1995) reason why it has been used for pharmaceutical purposes from the times of the Egyptians (Abd El Hady and Hegazi, 2002). Propolis makes part to the battery of tools bees have in order to keep in good health. Beeswax is covered by a thin layer of propolis and it is used to seal cracks and holes of the hive that bees find necessary. Even feral colonies have shown to create a “propolis envelope” by covering the entire interior of the cavities they occupy (Seeley and Morse, 1976). Bearing in mind the origin, composition and wide in-hive distribution of propolis, a high transfer of pesticides is to be expected. Unfortunately, due to the difficulty linked to the analysis of residues in this complex matrix, there is limited publications showing contamination of propolis.

1.3.2.5 Considerations for human consumption

Considering the human dimension of honey bees and their products, pesticide contamination becomes not only important for bees, but also for us. Honey can be eaten in Belgium at a rate of 15 Kg head/year (heavy consumer) and pollen has become a food complement for many (Bruneau,

pers. Comm.). Propolis or royal jelly are often used for pharmacological purposes, while beeswax can be consumed as “comb honey” or as food additive E90137. Maximum Residue Levels (MRLs) of pesticides are set up to ensure safety of consumers and are ruled by Regulation 396/2005/EC (European Parliament and Council of the European Union, 2005). However, they are described only for honey, the consumption of other beekeeping products possibly posing a risk to human health (Wilmart *et al.*, 2016).

1.3.3 Profile of pesticide active ingredients present in beekeeping matrices

With difference, synthetic acaricides like amitraz, tau-fluvalinate, coumaphos, flumethrin or chlorfenvinphos, are among the active ingredients most frequently found in beekeeping matrices within the hive like comb wax. They are formulated in veterinary products used by beekeepers for varroa control, as if left unattended, the infestation of these acari often lead to the death of the colony in a certain time. Even commercial wax foundation contains residues of these substances, and some others (Reybroeck *et al.*, 2010).

In addition, literature describes a large amount of fungicides and herbicides found in beekeeping matrices which is not surprising bearing in mind that farmers do not need to put in place any specific bee-related risk mitigation measures in their use. In general terms these active ingredients are of low toxicity for bees in acute terms (LD50 - in µg/bee) so they can be sprayed even during the active foraging of bees. Furthermore, fungicides and herbicides are the largest pesticides sold, and presumably used, in Europe in 2015, the former representing 42% of the pesticide sales (161,730,468 kg), and the later representing 34% (131,370,281 kg; Eurostat, 2017). In Belgium, the percentages are 40% and 39%, respectively (Eurostat, 2017). If these products are persistent in the environment, with systemic properties (able to be absorbed by the plant and move within the plant) or water soluble, the potential for contamination of bees food, water

or gums sources becomes extremely important.

Pesticides used for insect control, like insect growth regulators or insecticides, have shown high toxicity towards bee during their pre-authorisation risk assessment. As a result, farmers should not use them during the day, while bees are foraging. They are used in much less quantities than fungicides and herbicides, the sales of insecticides and acaricides representing 5% at EU level and 9% in Belgium in 2015 (Eurostat, 2017). In some countries or regions, it is also recommended to identify the fields that are treated with insecticides (PAN-Europe *et al.*, 2017) and to warn local beekeepers about a spaying event happening in the area. Similarly as what happens with fungicides and herbicides, the physico-chemical characteristics of the active ingredients like persistence, water solubility or systemic properties are going to determine the possibility of bees to get exposed to these insecticides. Furthermore, for sanitary reasons residue analyses in honey bee matrices have often specifically focus on elucidating the exposure of bees to insecticides and less often considered the residues of pesticides with other target species.

1.4 IMPACT OF POLLUTION OF THE HONEY BEE COLONY AND ITS LARGER BEE HEALTH CONTEXT

1.4.1 Direct effects of pesticides on honey bee colonies

The problem of bee poisoning is not new, as it was not new already back in 1881, from when we have reports about colony intoxications linked to copper- and arsenic-containing insecticides and fungicides (Shaw, 1941). Colony intoxications have been described for almost all, if not all, different generations of insecticides and modes of actions, both natural, as rotenone from the 30's, as synthetic compounds, as organochlorines, organophosphates and carbamates from the 40's, pyrethroids from the 70's, neonicotinoids from the 90's (Greig-Smith *et al.*, 1994; Martinello *et al.*, 2017; Maxim and van der Sluijs, 2010; Oomen, 2000; Pistorius *et al.*, 2009; Porrini *et al.*, 2003; Shaw, 1941; Stoner *et al.*, 1979; van der Geest, 2012). We are yet to see what will happen with the latest generation of chemicals with insecticidal activities, the diamides like chlorantraniliprole or cyantraniliproles. In occasions fungicides or herbicides have been implicated in bee poisonings (Greig-Smith *et al.*, 1994; Aubee and Lieu, 2010), but literature is scarce. Many more cases of intoxication might have occurred without the possibility to make any link to the toxicant that induced them, due to the fast degradation that some pesticides undergo within the bees and the durations of the observed intoxication symptoms (Schott *et al.* 2017).

These intoxications are often linked to spraying or powdering of pesticides in certain areas. With the arrival of neonicotinoids-treated seeds to the market, authorities, field practitioners and industry thought to have solved the problem of bee intoxications. However, beekeepers started soon

to experience intoxication symptoms at their apiaries at moments of the year where no spray/powdering was carried out, at the moment of seed sowing (Maxim and van der Sluijs, 2013; Pistorius *et al.*, 2009; Sgolastra *et al.*, 2017). In 2003, more than 10 years after the introduction in the market of these seed treatments, Greatti *et al.* described the release of neonicotinoid-contaminated dust while sowing treated seeds with pneumatic machines (Greatti *et al.*, 2003). Years later it was shown that the exposure to this dust was toxic to bees (Marzaro *et al.*, 2011) and that humidity was a major parameter determining toxicity (Girolami *et al.*, 2012). While flying around, bees come across airborne particles, which may get glued to their hairy body (which is favoured by humidity). In a different way of exposure, bees could inhale volatile active ingredients or substances which are diluted in the air.

Chemicals have proved to be highly mobile in the environment or the food chain, especially if they are systemic, persistent and water soluble like those of the family of neonicotinoids, or those more lipophilic or with potential for bioaccumulation, like fipronil, organochlorines or alpha-cypermethrin. These properties are concluded from the individual physicochemical characteristics of these compounds that involve a different mobility and exposure profile for each active ingredient. Pesticides can affect bees in many ways depending on their mode of action and level/duration of exposure. Nowadays there is a large bibliography showing either lethal effects from adults (reproductive or non reproductive) or immature stages or subtle effects the impact of which remains uncertain (see reviews like Belzunces *et al.*, 2012; Desneux *et al.*, 2007; Pisa *et al.*, 2015; van der Sluijs *et al.*, 2013).

Honey bees being a social insect, it is not strange to first consider insecticides as an important source of risk for their health. Commercialised insecticides are often targeting the nervous system of insects, which may lead to a disfunction of eusociality. Intoxication of the honey bee with certain insecticides leads to the rapid onset of neurotoxicity symptoms, with consequences on (a) cognitive functions, including learning and memory, habituation, olfaction and gustation, navigation and orientation; (b)

behaviour, including foraging and (c) physiological functions, including thermoregulation, locomotion and muscle activity (Belzunces *et al.*, 2012). Some have shown to compromise the immune competence of bees (Brandt *et al.*, 2017, 2016; Di Prisco *et al.*, 2013) as well as to affect the reproduction capacity of honey bee colonies by reducing the fecundity of honey bee queens (Williams *et al.*, 2015) and quality of drone sperm (Kairo *et al.*, 2016).

It is well established that insecticides can interact with pathogens and either increase pathogen burden on both immature stages of bees and adults (Di Prisco *et al.*, 2013; Pettis *et al.*, 2012; Wu *et al.*, 2012) or increase bee mortality (Alaux *et al.*, 2010; Doublet *et al.*, 2015; Vidau *et al.*, 2011), these interactions seem to occur among others by compromising the immune system of bees (Brandt *et al.*, 2016; Di Prisco *et al.*, 2013; see section 1.4.2). However, other substances (fungicides, herbicides) also deserve an analysis of their specific toxicity, taken individually or in synergy with other substances (Fischer and Wachendorff-Neumann, 2004; Fisher *et al.*, 2017; Meled *et al.*, 1998; Mullin *et al.*, 2015; Pilling and Jepson, 1993; Zhu *et al.*, 2017a, 2017b) or pathogens (DeGrandi-Hoffman *et al.*, 2015; Pettis *et al.*, 2013). Increased larval mortality has been described following chronic exposure to tau-fluvalinate, coumaphos (acaricides), chlorpyrifos (insecticide) and chlorotalonyl (fungicide) or some of their combinations (Zhu *et al.*, 2014). Given the low acute toxicity of these compounds on bees, no restrictions are established to their uses which involves a potential higher exposure of bees to compounds with these modes of action. Indeed, residue analyses show that these substances are found in hives, even though the crops in which they have been applied do not suggest direct bee exposure (Bogdanov, 2006; Chauzat *et al.*, 2011; Simon-Delso *et al.*, 2017).

Some direct toxic effects on bees either by the fungicides formulations, active ingredients or their metabolites have been reported in the past (DeGrandi-Hoffmann *et al.*, 2013). For example, boscalid shows low acute toxicity to bees (IUPAC, 2014) despite the fact that beekeepers in the USA have already reported losses and adverse effects on bee brood development related to the foliar application of this systemic active ingredient (Aubee

and Lieu, 2010). Incidents were often linked to the use of a co-formulation boscalid – pyraclostrobin, mixture that showed to increase the toxicity of acaricides when bees were co-exposed (Johnson *et al.*, 2013). Metabolites can be of toxicological concern for bees. One metabolite of boscalid, for example, the 2-chloronicotinic acid (2-CNA), is similar to the 6-chloronicotinic acid (6-CNA), a metabolite of imidacloprid. The latter has proved to be lethal to bees at low concentrations (0.1 mg/L) following chronic exposure (Suchail *et al.*, 2001). Boscalid has proved toxic to other aquatic invertebrates, reducing daphnid fecundity and *Chironomid* emergence (Aubee and Lieu, 2010). The fungicide captan has been shown to induce effects on growth and development of larval honeybees (Mussen *et al.*, 2004; Thompson, 2003).

1.4.2 Pesticide interaction with other factors affecting bee health

As any other living being, each honey bee colony has its own genetic potential which is translated into different phenotypes depending on characteristics present within the colonies, as pathogens or parasites, or external ones, as food availability and quality, or weather conditions. Honey bee colonies have historically been managed by humans for the obtention of products like wax and honey. Feral colonies also exist out of human control. Beekeeping management contribute also to the colony status and development. So far studies have not achieved to deliver a global picture of the exposure to these stressors and their interactions remain fairly unknown. This is most likely due to the different context of honey bees in the different regions and the large investment needed to get information about some parameters. I introduce a review of state of the knowledge about these interactions and I discuss some of them.

Indeed, considering the different parameters possibly affecting honey bee colony's health, it is difficult, and maybe meaningless, to identify the main cause behind colony losses worldwide, as the prevalence of factors

contributing to the losses and interacting among themselves is different in different places (Hendriks *et al.*, 2009). In this case a *causal web* determines the fate of the colony (Vineis and Kriebel, 2006). Rothman and Greenland, 2005, proposed the “pie” model, involving that a combination of several component causes conform a causal complex (a pie). Following this model, the colony losses happen only if and when the pie is completed. Goulson *et al.*, 2015, for example, propose a combination of parasites, pesticides and lack of nutrition to explain the bee declines observed lately. With this logic, several mathematical models aim to predict the impact of the combinations of several factors on honey bee health (Becher *et al.*, 2014, Betti *et al.*, 2017, Henry *et al.*, 2017). Unfortunately, this multi-factor approach is extremely data consuming and hardly ever a data set is available integrating all relevant parameters. All in all, this approach integrating as much as possible factors and their interactions is increasingly applied in bee research. The following paragraphs show the state of the knowledge on the interactions between pesticides and other potential factors affecting bee health.

The list of factors so far identified inducing stress to colony health are enumerated here (Figure 1.3): (1) stressing factors inherent to bee colonies: (a) infestation of colonies by parasites as the *Varroa destructor* mite; (b) viruses and diseases specific to *Apis mellifera* in Europe (*Nosema ceranae*, etc.); (c) loss of genetic diversity (queen selection); (2) stressing factors external to bee colonies: (a) anthropization of the environment (increased urbanization, intensive agriculture, fragmentation of the environment, natural habitat, use of pesticides (sometimes acting in synergy)), which leads to a decrease in biodiversity and pollution of bee food resources; (b) poor quality of food (inadequate feeding syrups in conjunction with depletion of honey plant resources); (c) weather conditions and the impact of climate change; (d) invasive species (in Europe two may be considered to date: *Vespa velutina* and *Aethina tumida*); (e) beekeeping management; (f) other stressors put forward but explored to a lesser extend: electromagnetic waves, radiation, etc.

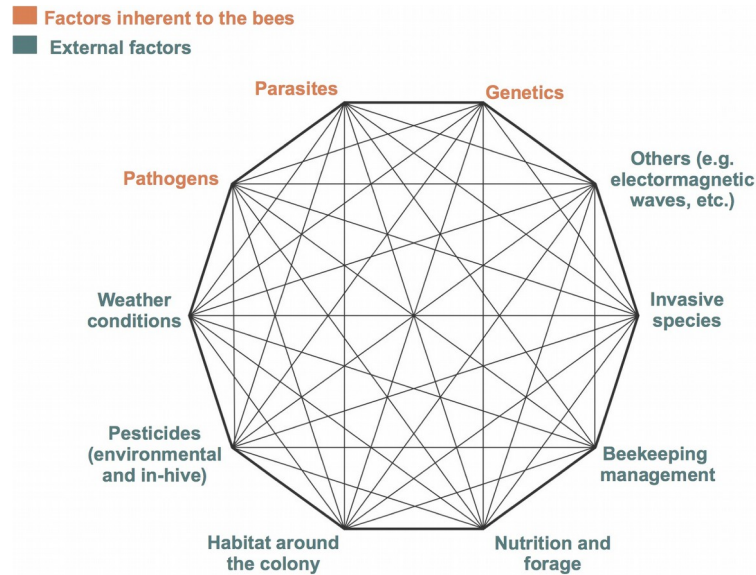


Figure 1.3 - Multi-stressors with capacity to affect bee health and the possible interactions among them. Orange = factors inherent to the bees. Turquoise = external factors

1.4.2.1 Pesticides and genetic diversity

It is well known that polyandry, a form of polygamy in which the female (the queen) mates with a variable number of males (drones, from 4 to 24 (Tarpy and Page, 2001), is one of honey bee immune strategies. In doing so, there are a diversity of subfamilies within the colony. This diversity enables a better adaptation of the honey bee colony as population to any situation it may face, leading to greater food storage, comb construction, and population growth (Mattila and Seeley, 2007). These traits and some others may have an impact on the potential exposure and effects of pesticides on the colony, but also inter-colony variability in detoxification potential and resistance to pollutants, as well as intra-

seasonal (different periods during the same beekeeping season) and inter-seasonal variability (Smirle and Winston, 1987). Neumann and Blacqui re, 2015, hypothesise, however, that the lack of diversity already existing within the honey bee colonies after years of, in their view, intensive selection of wrong traits in terms of bee health, may be reducing their resistance. Unfortunately these are just speculations not supported by any data and should be taken carefully.

Bees subspecies can as well be a reasons of difference in toxicity of pesticides. Suchail *et al.*, 2000, developed toxicity tests to determine the LD50 (both oral and contact) of imidacloprid in two subspecies of *Apis mellifera*, *A.m. caucasica* and *A.m. mellifera*. The dose-effect curve showed a biphasic shape, increasing toxicity as low doses and at high doses. The curve of *A. m. caucasica* was more pronounced than that of *mellifera*, which also determined an LD50 of 14 ng/bee in *A.m. caucasica*, instead of 24 ng/bee for *mellifera*. In the same line, Ladas, 1972, shows that *A. m. caucasica* is less susceptible to DDT and trichlorfon than *A.m. ligustica* and *A.m.carnica*. With a very limited number of repetitions, Ladas did not observe any impact of inbreeding on the susceptibility of bees to toxicants.

1.4.2.2 Pesticides, pathogens and parasites

Pathogens and the parasitic mite *Varroa destructor* are well known stressors of honey bee health. The list of pathogens with pathogenic relevance to honey bees can be found in figure 1.4. However only a short list of those have been identified to pose a real threat.

The infestation of honey bee colonies by varroa can be alone a cause of health problems. Originally an invasive species in Europe, this asian acari lives from sucking the nutrients from the hemolymph of bees, reproducing itself in the brood cells of honey bees (mainly in those of male larvae). Not only is *V. destructor* a source of weakening of parasitized bees, but also it constitutes a vector for transmission of bee viruses among them (Tentcheva *et al.*, 2004). Indeed, populations free from varroa but containing viruses

seem to have experienced less colony losses (Roberts *et al.*, 2017). An excessive population of varroa can weaken young bees and allow the multiplication of viruses (see later). For this reason, beekeepers have put in place strategies to control the level of varroa in their colonies and a number of managerial technics and veterinary products are available in the European market.

PROTOZOA <i>Nosema apis</i> <i>N. ceranae</i> <i>Malpighamoeba mellificae</i> <i>Crithidia</i> sp.	VIRUS CPBV, APBV, DWV, KBV, IAPV, BQCV, ALPV, BSRV, KV, VDV-1, EBV, SBV, TSBV, SBPV, CBPVA, CWV, BVX, BVY, LSV-1, LSV-2, ABV, BBPV, AmFV, AIV	NON-PARASITIC ARTHROPODS <i>Galleria mellonella</i> <i>Aethina tumida</i> <i>Formica rufa</i> <i>Iridomyrmex</i> sp. <i>Vespa cacro</i> <i>V. velutina</i> <i>Acherontia atropos</i> <i>Chelifer</i> sp., <i>Cheridium</i> sp.	VERTEBRATES <i>Merops apiaster</i> <i>Mus musculus</i> <i>Apodemus sylvaticus</i>
FUNGI <i>Ascosphaera apis</i> <i>Aspergillus</i> sp.	BACTERIA <i>Paenibacillus larvae</i> <i>Melissococcus pluton</i> <i>Lactobacillus eurydice</i> <i>Brevibacillus laterosporus</i> <i>Enterococcus faecalis</i> <i>P. alvei</i> , <i>P. apiarius</i> <i>Bacillus coagulans</i>	PARASITIC ARTHROPODS <i>Varroa destructor</i> <i>Acarapis woodi</i> <i>Senotainia tricuspis</i> <i>Braula coeca</i>	

Figure 1.4 – Pathogens, parasites, predators or other animals that have been describe to affect or interact with honey bees' health. Based on Puerta Puerta *et al.*, 2001)

Note: AIV (*Apis Iridescent Virus*), ALPV (*Aphid Lethal Paralysis Virus*), AmFV (*Apis mellifera Filamentous Virus*), APBV (*Acute Bee Paralyzes Virus*), BBPV (*Berkeley bee virus*), BSRV (*Big Sioux River virus*), BVX (*Bee virus X*), BVY (*Bee virus Y*), CBPV (*Chronic Bee Paralyzes Virus*), CPBV (*Chronic Bee Paralyzes Virus*), CWV (*Cloudy wing virus*), DWV (*Deformed Wing Virus*), EBV (*Egypt bee virus*), IAPV (*Israeli acute paralysis virus*), KBV (*Kashmir bee virus*), KV (*Kakugo virus*), LSV-1 (*Lake Sinai virus-1*), LSV-2 (*Lake Sinai virus-2*), QBCV (*Queen Black Cell Virus*), CBPSV (*Chronic Bee Paralysis Satellite Virus*), SBPV (*Slow bee paralysis virus*), SBV (*Sacbrood Virus*), TSBV (*Thai Sacbrood virus*).

Bee viruses are often cited as a potential cause or indicator of decline in honey bee colonies or are included among multifactorial causes of decline (Cox-Foster *et al.*, 2007). In Europe, at least 12 viruses infecting bees have been recorded (Aubert *et al.*, 2008). Many viruses are commonly found in apparently healthy bee populations (i.e. asymptomatic colonies) (Gauthier *et al.*, 2007). These can remain latent and confined in certain cells or tissues without active replication and without disrupting cellular functioning. Similarly, they can be replicated at low levels in permissive cells, at nonessential sites or at a stage of development that has no obvious symptoms or pathologies (Aubert *et al.*, 2008; Gauthier *et al.*, 2007; Tentcheva *et al.*, 2004). Nevertheless, two viruses, ABPV and DWV, have been related to serious disorders for bees (Cox-Foster *et al.*, 2007). In addition, as previously mentioned they may react in association with *V. destructor* to cause winter losses as observed in Germany (Genersch *et al.*, 2010).

Honey bee adults can as well be affected by microsporidians living in the bees' intestines, like *Nosema apis* or *N. ceranae*. These protozoans colonise the epithelial cells of the ventriculus of adult bees which got infected through the ingestion of spores in the feed, trophallaxis exchanges, or by grooming (OIE, 2008b).

Specific diseases affect honey bee larvae. The main bacterial diseases are American Foulbrood, caused by *Paenibacillus larvae*, a gram-positive spore-forming bacterium, and European Foulbrood, caused by *Mellisococcus plutonius*, gram-positive non-sporulant bacterium (Genersch *et al.*, 2010). Both of them affect the brood of honey bees and mainly affect weakened colonies. In Belgium, both are notifiable diseases involving compulsory declaration should any colony be affected by them and different sanitation measures (OIE 2008a, 2016).

Several studies carried out aimed to know better the pathogen and parasite load in Belgian apiaries, but the most comprehensive and recent is that of Ravoet *et al.*, 2013. In this study, a number of known and new pathogens were found to be present in the territory with different predictive

power for winter mortality, and all in all a significant positive correlation was found between the load of pathogen species and colony losses. However, no consideration of environmental contamination or the landscape of the apiaries was included in this study.

Having said that, the interactions between pesticides and pathogens have already been described. Pioneer studies on pathogen-toxic interactions were first conducted in the seventies to test the interaction of *Nosema (apis)* presumably) and DDT (organochlorine) or trichlorfon (organophosphate) (Ladas, 1972). We know today that bees also become more sensitive when co-exposed to pesticides like neonicotinoids and *Nosema ceranae* (Alaux *et al.*, 2010; Doublet *et al.*, 2015; Pettis *et al.*, 2012) or fipronil and *N. ceranae* (Aufauvre *et al.*, 2012; Vidau *et al.*, 2011). Wu *et al.* 2012 showed that larvae raised in heavily contaminated wax were more sensitive to *Nosema ceranae*. Interactions between different pesticides families like pyrethroids, organophosphates or neonicotinoids and different viruses ((CBPV) Bendahou *et al.*, 1997; (DWV) Di Prisco *et al.*, 2013; (BQCV) Doublet *et al.*, 2015), or varroa (Abbo *et al.*, 2016) have also been described. Papach *et al.*, 2017, recently found a reduction in the emergence rate when bee larvae were co-exposed to American foulbrood (either 4000 spores/larva or 800 spores/larva) and thiamethoxam at 0.6 ng/larva (LD50 thiamethoxam = 5 ng/bee). However, no additive effect of the pesticide was detected on the larvae mortality caused by *P. larvae*. However, co exposure to 400 spores of *P. larvae* and thiamethoxam during larval development resulted in a reduction of the learning capacity during their adulthood. The combination of dimethoate (120 ng/bee) or clothianidin (32 ng/larva) and *P. larvae* spores did also result in increased larva mortality, while the effect was not observed for co-exposure with tau-fluvalinate (480 ng/larva) (López *et al.*, 2017). Interestingly, the authors described as well an immune depletion of the larvae exposed to these combinations.

All in all, the year after the end of my field work, (Chapter 2) the Pan-European epidemiological study on colony losses, the Epilobee study (2012-2014), revealed Belgium as a country with high colony losses, but with very low clinical prevalence of diseases like varroosis, nosemosis,

foulbrood or CBPV (Epilobee Consortium 2016).

1.4.2.3 Pesticides, landscape and nutrition

In occasions, individual crops (i.e. maize) treated with neonicotinoids seemed not to show a correlation with bee mortality (Nguyen *et al.*, 2010). However several studies and field observations have shown the limitation of considering just one crop as source of pesticide exposure, unless the surface of crop considered is wide enough around the apiary (Sgolastra *et al.*, 2017; Tsvetkov *et al.*, 2017; Woodcock *et al.*, 2017). The negative impact on colony survival of both pesticide contamination and land use/crop area around the apiaries have been described in other countries as well, as the Netherlands (van der Zee *et al.*, 2015), France (Odoux *et al.*, 2014) or the US (Smart *et al.* 2016a, 2016b). Similar observations have already been described for wild bees (Cariveau and Winfree, 2015; Rundlöf *et al.*, 2015). However, a large controversy exists around the implication of pesticides and (agricultural) land use on bee colony losses inherent to the variability of both environmental and colony conditions and the fragmented data the results are based on.

Nutrition and foraging for food, both in quantity and quality, are key factors of bee health and colony dynamics as they determine colony productivity and dynamism, bees immunocompetence and ensure important regulatory processes within the colony as brood-rearing, thermoregulation and relative individual composition of the colony (Alaux *et al.*, 2011, 2010; Brodschneider and Crailsheim, 2010; DeGrandi-Hoffmann *et al.*, 2010; Scofield and Mattila, 2015). Pollen diversity is key to shape bee physiology, bees fed with poly-floral pollen (four different pollen origins) living longer and being more resistant to diseases (Di Pasquale *et al.*, 2013) and pesticides (Wahl and Ulm, 1983). Palynological analyses of pollen or bee bread can provide insight about the pollen diversity brought to the colony and establish if a risk of nutritional lacks exists.

1.4.2.4 Pesticides and weather

Weather is determinant for the fate of honey bee colonies as it influences the quality and quantity of food and water sources for pollinators and the possibility of bees to forage, having an impact on the nutritional status of colonies. It is well known that honey bees reduce their foraging activity in rainy days and when temperatures are less than 12°C. It may also determine the fate of the pesticide in the environment through processes as photolysis, wind drift or leaching.

In controlled conditions pesticide toxicity on bees have been shown to vary depending on temperature and hygrometry (Faucon *et al.*, 1985; Ladas, 1972; Medrzycki *et al.*, 2011), some pesticides increasing toxicity at higher temperatures as in organophosphates, carbamates and fipronil, while others decrease as neonicotinoids, DDT and pyrethroids. Girolami *et al.*, 2012, showed increased toxicity of neonicotinoid-powdering linked to relative humidity.

1.4.2.5 Pesticides and beekeeping management

Beekeepers use acaricides to keep the level of varroa infestation under a threshold of concern. In certain regions of the world, antibiotics are allowed to control of microsporidians and foulbroods. Synthetic acaricides and antibiotics leave residues in the bee matrices as has been previously shown (section 1.3). Only until recently, the residues of varroa treatments were considered harmless to bees. However, for some years now there is an increasing awareness of the impact these veterinary products, that in most cases are as well authorised for plant protection, may induce to honeybee colonies, alone or in synergy with other factors or active ingredients.

The co-exposure of bees to both acaricides coumaphos (organophosphate) and tau-fluvalinate (pyrethrinoid) has shown to increase the toxicity of both active ingredients (Johnson *et al.*, 2009) due to a hypothesised mechanism of competition between both molecules at

cytochrome P450 monooxygenase enzymes. Later, Johnson *et al.*, 2013, evaluated the toxicological impact of a wide range of pesticide combinations including 6 acaricides (tau-fluvalinate, coumaphos. Fenpyroximate, amitraz, thymol and oxalic acid), 9 fungicides (pyraclostrobin + boscalid, pyraclostrobin, boscalid, chlorothalonil, prochloraz, propiconazole, fenbuconazole, metconazole and myclobutanil) and 3 inhibitors of metabolism (DEM=diethyl maleate, DEF = S,S,S-tributylphosphorotrithioate, PBO = piperonyl butoxide). The largest synergistic effects were observed for combinations of tau-fluvalinate and PBO (Cytochrome P450 Inhibitor) or prochloraz (Sterol Biosynthesis Inhibitor), which increased the mortality of the former by 100 fold. The other combinations revealed either no-interaction or varied in the degree of effect of the interactions. Synergistic interactions result from toxicokinetic interactions at the level of metabolism either through the inhibition of a detoxification enzymes or the inhibition of a transporter which then have toxicodynamic consequences enhancing the toxicity of the mixture (ie. decreasing the LD50).

Being aware of these interactions and the prevalence of pathogens in beeswax, beekeepers know that they need to change the combs of the brood chamber at least once every five years (Lequeux *et al.*, 2009). These combs are then removed from the wax circle.

1.4.2.6 Invasive species

There are other minor stressors in Europe that affect the bee health and may interact with pesticides. Two of them are two invasive species, the asian hornet *Vespa velutina*, and the small hive beetle, *Aethina tumida*. Their presence is restricted to some regions of the continent as can be seen in maps 1.1 and 1.2.



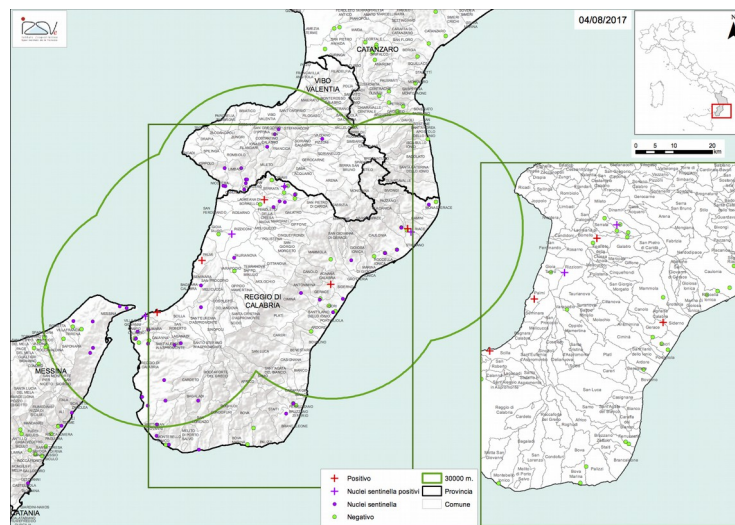
Map 1.1 - Distribution of *Vespa velutina* in Europe as of 14 August 2017. Source: https://www.google.com/maps/d/viewer?mid=1jRfoi4oF6GmiGRgbXuD71Qpbw8s&hl=en_US&ll=45.496136255090015%2C-5.089159244854727&z=6

Many have tried to control *Vespa velutina* either through traps or destroying the nests. Nest destruction often involves the use of permethrin, a pyrethroid with high toxicity to bees. As a result, beekeepers and naturalists have expressed their concern and have requested authorities to look for alternatives to pesticides for nest destruction (UNAF, 2015).

The arrival of *Aethina tumida*, notifiable parasite not present in Europe, to the south of Italy triggered the implementation of eradication programmes in the affected apiaries. As a result, thousands of colonies have been burned and the soil disinfested by spraying pyrethroids. A 1% solution of cypermethrin and tetramethrin was abundantly sprayed at high pressure

(50 l/min) in order to drench the soil after soil ploughing (Mutinelli *et al.*, 2014) to ensure a higher probability of exposure of the beetle. Traps have been developed to be placed in the hive both for surveillance and control, some of them containing pesticides highly toxic to bees, like fipronil (Levot, 2008). In areas of the world where *Aethina* has been present for many years, beekeepers have controlled the populations through beekeeping management and through the use of coumaphos (EFSA, 2015).

The pressure of both invasive species come to increase the stress put on honey bee colonies there where they are present and the proposed control measures, if left without control, may lead to problems of colony intoxications.



Map 1.2 - Distribution of *Aethina tumida* in Europe as of 14 August 2017.
Source: <http://www.izsvenezie.it/documenti/temi/api/aethina-thumida/2017/situazione-epidemiologica/figura-1-zona-protezione-calabria.pdf>

1.5 THE SITUATION IN BELGIUM AND WALLONIA

Belgium is not unscathed from the described trends honey bee colony mortality and morbidity, having shown average colony losses during the winter period of 19.9%, 32.7%, 14.8%, 40%, 12.7% and 25% during the winters of 2008, 2012, 2013, 2014, 2015, 2016 respectively (Nguyen *et al.*, 2010, for 2008; Epilobee consortium, 2016, and Roelandt *et al.*, 2016, for 2012 (Table 1.2), 2013, Keppens, pers. comm. For 2014, Brodschneider *et al.*, 2016, for 2015, Simon-Delso, unpublished data, for 2016). A parallelism can be found as well in the trends of Belgian wild pollinator populations (Rasmont *et al.*, 2005).

The situation of Belgium is somehow special as the mortalities observed in the country are among the largest ones observed throughout Europe. However, recent pan-European epidemiological studies have revealed Belgium as a country with limited prevalence of pathogens and diseases. The history of mortality and disorders is long in the country. I focus here on the experiences carried out in Wallonia, region of the southern part of the country.

Apart from colony mortality, Walloon beekeepers reported since 1999 a number of imprecise morbidity symptoms: colony weakness, mainly in spring, fast renewal of young queens, rapid loss of individuals in the colony, mainly foragers, or slow loss of individuals in the colony. In many cases brood and food remains in the colony. Sometimes a small cluster of bees with the queen survives (Lefebvre et Bruneau, 2004). Some beekeepers described unspecific brood abnormalities not characteristic to any known disease (Baudoin and Lequeux, pers. comm.). By the beginning of the present study, other studies had described similar mortality trends elsewhere (Neumann and Carreck, 2010; van der Zee *et al.*, 2012; vanEngelsdorp *et al.*, 2011) as well as unspecific symptoms: increased colony mortality and/or weakening (Aubert *et al.*, 2008; Genersch *et al.*,

2010; Higes *et al.*, 2009), queen failure (Genersch *et al.*, 2010; Pettis *et al.*, 2013; vanEngelsdorp *et al.*, 2010) or honey yield reduction (Aubert *et al.*, 2008).

A number of studies have been carried out in Belgium aiming to better understand the phenomenon of colony losses. Back in 2003, at the request of the Walloon Region (DGARNE), CARI (Beekeeping Centre of Research and Information) conducted a survey among beekeepers during the 2003-2004 season on the phenomenon of honey bee colony disorders in the region (Lefevbre and Bruneau, 2004). The objective here was to map the apiaries with cases of decline at the regional level, to provide a quantitative estimate of the phenomenon and to describe the environmental conditions in which these declines occurred. Three surveys were carried out to obtain a significant overview of the situation resulting in a collaboration of 15% of the 2658 beekeepers listed in Wallonia. According to the survey, all Walloon regions were affected by honey bee colony declines, at regional level 14% of surveyed colonies died, 15% were weakened and 30% colonies suffered some morbidity symptoms (described before) during the winter 2003-2004. The disappearance of the entire colony (with the exception of a handful of bees dead or alive at the frames) was the most-frequently-described symptom, while no pathological symptoms were observed. Corn fields were generally present in the environment of apiaries with cases of disorders. No correlation could be found between the colony disorders and the veterinary treatments used in varroa control. In those days, regional beekeepers suspected the use of insecticides used in seed coating (with the active ingredient (a.i.) imidacloprid, Gaucho® in particular) to be the cause of increased mortalities as has been shown in France or Italy in crops like sunflower or corn. The study therefore concluded the need for research of the impact of pesticides on pollinators in the region, with special focus on the analysis of foraging areas and their relation with the health status of the colonies and the analyses of the toxic residues in hive products (Lefevbre and Bruneau, 2004).

Table 1.2 - Winter mortality in Belgian honey bee colonies (2012-2013) per province (Source: Roelandt et al., 2016)

Winter mortality in Belgian honey bee colonies (winter 2012–2013)					Weighted colony sample mortality		
Sampling design					WCSM = $\frac{\sum Lw_i \cdot X_{dead_i}}{\sum Lw_i \cdot M_i}$		
Geographical entity	#Apiaries (N) in sampling Frame	#Apiaries (n) selected	#Colonies (Mi) in selected apiaries	#Colonies (mi) selected	Estimate	95% Confid. interval: ‡	
Belgium	2924	149	933	624	32.71%*	26.54%	39.83%
Wallonia*	720	73	473	300	32.84%*	22.93%	42.39%
Flanders*	2197	74	453	317	32.86%*	25.11%	43.02%
Antwerp	448	15	142	76	21.01%	12.49%	33.76%
Brussels	7	2	7	7	14.29%	0.00%	48.09%
Hainaut	189	15	132	73	42.93%	19.26%	66.60%
Liege	137	15	120	70	39.44%	18.14%	59.74%
Limburg	494	15	92	64	45.47%	20.96%	74.12%
Luxembourg	179	15	80	50	13.54%	5.23%	20.31%
Namur	149	15	82	60	33.54%	16.57%	49.30%
East Flanders	509	14	74	54	48.20%	27.29%	69.01%
Flemish Brabant	391	14	89	69	21.54%	5.79%	37.77%
Walloon Brabant	66	14	59	47	22.03%	5.12%	39.71%
West Flanders	355	15	56	54	39.88%	18.55%	60.46%

Note: Brussels (2 apiaries/7 colonies) is only included in the estimate for Belgium and not in the estimates for Flanders or Wallonia.

‡95% confidence intervals are calculated on the weighted and linearized estimate and with a two-stage sampling variance Source: (Ardilly, 2006; Deville, 1999) apiary.

*Brussels is not included in calculations for regions but is included for Belgium; WCSM: Weighted Colony Sample Mortality; $\sum$: sum of; w_i : apiary-specific weight; X_{dead_i} : number of dead colonies in the i th apiary; M_i : total number of colonies in the i th apiary.

In 2004, following the previous results a study including some of the factors involved in honey bee health (varroa infestation, virus load and pesticide exposure (bees, beeswax and honey)) was carried out at the initiative again of the Walloon Region. The research conducted concluded no relation between corn resulting from imidacloprid-treated seeds and colony losses (Nguyen *et al.*, 2009). This study has been strongly criticized and its conclusions undermined (Maini *et al.*, 2010) showing that the methodology used was not fit for purpose and the contextualisation of the study was insufficient. Indeed, Nguyen *et al.*, 2009, results are contrary to the conclusions of other previous and/or coetaneous research programs conducted in Europe, France and Italy in particular, on the sub-lethal effects of neonicotinoid systemic insecticides, including imidacloprid (Bonmatin *et al.*, 2005; Bortolotti *et al.*, 2003; Colin *et al.*, 2004)(sub-lethal dose evaluated between 1 and 20 µg kg⁻¹ or less).

Later, a monitoring of the honey bee colony losses was put in place in Belgium, between 2008 and 2010, in network of an international network of bee researchers called COLOSS (www.coloss.org). National annual averages have been mentioned already. Other studies developed in the region concluded the main factors favouring mortality were the *Varroa destructor* infestation level and viruses (Nguyen *et al.*, 2011), while allowed to discard some factors of stress especially *Nosema* spp and American foulbrood (Nguyen *et al.*, 2011). No environmental considerations were taken into account in these studies.

My project started in June 2011, in a context of high winter losses, no relationship with beekeeping matrices contamination and little consideration of the environmental context of honey bee colonies. The added value of my work is that I consider honey bees in their environment, bearing in mind their landscape context, the diversity of nutrition they may have collected and the weather conditions.

We achieved to give answer to some questions raised by beekeepers regarding the relationship between honey bee colony mortality and morbidity and landscape, namely intensive crop production is incompatible

with good bee health. The results of my field work were both frustrating and worrying for beekeepers and authorities, for we did not identified the insecticides recognised as number one in the ranking of honey bee risks in my study. In contrast, we introduced unexpected factors of stress affecting honey bee health instead: fungicides, products to control fungi infections and considered of low risk to bees. Furthermore, we established that contamination may come from crops that are not visited by bees like cereals, sugar beet or potato, because the pesticides are more mobile in the environment as expected. Finally, I started exploring the direct impacts that some of the fungicides found may have on bees.

The following chapters describe studies performed from 2011 until 2015. From a field observational study with unexpected results: fungicides as a factor of stress for honey bee colonies, we explore the different hypothesis possibly elucidating the reasons behind field observations: (1) fungicides, markers of intensive agriculture; (2) direct toxicity of fungicides to bees; (3) indirect effects of fungicides on bees. For this purpose, I performed experiences in laboratory under controlled conditions. Following to these chapters, I conclude with the outcome of my research and the perspectives I see for future improvement of the situation.

1.6 ABOUT MY THESIS: OBJECTIVES AND RESEARCH QUESTIONS

The picture of spread pollinator declines or increased colony disorders and knowing that the pathogens/parasites affecting honey bees are often restricted to this species invites to point at environmental factors as main source of stress leading to a problem. The fact that honey bees have an economic value to a group of the population, beekeepers, helps to have a close follow up of what is happening with them. Therefore, honey bee colonies mortality and morbidity serves as a parameter to identify areas of

concern. In the previous pages I describe how bees come across a wide number of pollutants due to their biology, behaviour and morphology. This contamination needs to be put in context to understand the consequences to colony's health. Pesticides in particular have proved to act either alone, triggering acute intoxications, or interact in different ways with other factors to weaken them (Farooqui, 2013).

My research started in June 2011, in a context of high winter losses. In collaboration with Louis Hautier and Gilles San Martin, researchers of the Centre de Recherches Agronomiques de Wallonie (CRA-W), I worked on a field monitoring study in order to figure out what was the real situation of colony losses throughout the region. It was a prevalence case-control study with asymptomatic colonies of known pathological diseases, in which the beekeeping management did not result in a bad impact on honey bee health. In these cases we analysed for viruses and pesticides aiming to understand their contribution to colony disorders. For the first time, we contextualised the colonies health by considering a wide range of environmental and habitat factors to which our “patients” (the honey bee colonies) were exposed: their landscape context, the diversity of nutrition they may have collected and the weather conditions. Regional beekeepers were extremely collaborative and they are one key pillar of my study.

This field work was both frustrating and worrying for beekeepers and authorities, for we did not identified the insecticides recognised as number one in the ranking of honey bee risks in my study. In contrast, we introduced unexpected factors of stress affecting honey bee health instead: fungicides, products to control fungi infections and considered of low risk to bees.

The impact of fungicides on bee health is not widely researched. We propose different hypothesis to explain the link between fungicides and colony disorders and mortality: (1) fungicides have nothing to do with colonies' health, but they are an indicator of intensive agricultural farming where bees can get exposed to other factors or products with higher impact on their health that were either not included in the analyses or the analyses

were not sensitive enough; (2) fungicides have a higher toxicity to honey bees than expected; (3) fungicides affect colonies indirectly by compromising the internal microbial homeostasis which can lead to weakness and nutritional modifications.

Hypothesis number (1) was already answered thanks to the field trial (Chapter 2), which was followed by a more thorough evaluation of the origin of pesticide contamination (Chapter 3). In order to test hypothesis (2) I proceed in my research by performing toxicity tests in controlled conditions (laboratory tests) first on immature bees (Chapter 4), and then on adult bees (Chapter 5). The third hypothesis is not part of this PhD thesis and remains a needed follow up approach for future research together with other ones including the interactions between pesticides or between pesticides and other stress factors (Chapter 6).

Since the beginning of my studies, research on honey bees, bee ecotoxicology and the interactions between pesticides and other stress factors like nutrition, weather or pathogens/parasites has boomed worldwide (see section 1.4) and we have more data today to understand the situation in the field. I am happy that the research performed during these years, included in this thesis, has contributed to it.

1.6.1. Objectives of my thesis

- Understand the real situation of colony losses throughout the region and the contribution of pesticides and viruses to the appraisal of unspecific morbidity symptoms or mortality of honey bee colonies.
- Understand the origin of contamination of honey bee colonies by pesticides.
- Understand the role of fungicides on honey bee colony disorders – fungicides as indicators of intensive agricultural farming where bees can get exposed to other factors or products with higher impact on their health that are either not included in the analyses or the analyses are not sensitive enough.

- Understand the role of fungicides on honey bee colony disorders – study the direct impact of fungicides on mortality of immature stages of honey bees.
- Understand the role of fungicides on honey bee colony disorders – study the direct impact on adult bees of chronic exposure to fungicides.

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Chapter 2

Field surveillance and exploration of factors of stress leading to colony mortality and morbidity

FOREWORD

The present chapter summarizes the field experience carried out from summer 2011 until spring 2012. The objectives of the study were to (1) follow up honey bee colonies in field conditions and gather real data; to (2) explore the possible factors contributing to unexplained cases of colony losses in the region, based on the symptoms previously described by beekeepers (see Introduction, section 1.5), and to (3) evaluate if a correlation exists between the environment of the apiaries and the colonies' fate. This study was developed in collaboration with CRA-W, and in an initial phase also in collaboration with Gembloux AgroBioTech.

This study was published in 2014 at the Journal PLOS One with title: *Honeybee colony disorder in crop areas: the role of pesticides and viruses*.

The text included into this chapter has been slightly modified with respect to the published version.

Honey bee Colony Disorder in Crop Areas: The Role of Pesticides and Viruses

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ABSTRACT

As in many other locations in the world, honey bee colony losses and disorders have increased in Belgium. Some of the symptoms observed rest unspecific and their causes remain unknown. The present study aims to determine the role of both pesticide exposure and virus load on the appraisal of unexplained (imprecise symptoms not typically linked to specific diseases, intoxication or bad management) honey bee colony disorders in field conditions. From July 2011 to May 2012, 330 colonies were monitored. Honey bees, wax, beebread and honey samples were collected. Morbidity and mortality information provided by beekeepers, colony clinical visits and availability of analytical matrix were used to form 2 groups: healthy colonies and colonies with disorders (n=29, n=25, respectively). Disorders included: (1) dead colonies or colonies in which part of the colony appeared dead, or had disappeared; (2) weak colonies; (3) queen loss; (4) problems linked to brood and not related to any known disease. Five common viruses and 99 pesticides (41 fungicides, 39 insecticides and synergist, 14 herbicides, 5 acaricides and metabolites) were quantified in the samples. The main symptoms observed in the group with disorders are linked to brood and queens. The viruses most frequently found are Black Queen Cell Virus, Sac Brood Virus, Deformed Wing Virus. No significant difference in virus load was observed between the two groups. Three acaricides, 5 insecticides and 13 fungicides were detected in the analysed samples. A significant correlation was found between the presence of fungicide residues and honey bee colony disorders. A significant positive link could also be established between the observation of disorder and the abundance of crop surface around the beehive. According to our results, the role of fungicides as a potential stressor for honey bee colonies should be further studied, either by their direct and/or indirect impacts on bees and bee colonies.

KEYWORDS

Apis mellifera; honey bee colony disorders; fungicides; pesticides; virus; mortality; queen failure; crop area; landscape

2.1 INTRODUCTION

The evolution of pollinator populations has been the subject of an increasing number of studies, most of them showing worrying negative trends [1–4]. Furthermore, beekeepers have long been notifying enhanced bee winter losses and disorders [5–8]. Bees contribute to ecosystem services and their decline thus threatens pollination of both wild and cultured plants, endangering biodiversity, food and fibre production [9,10]. This decline may also have an impact on the production of other goods with pharmacological uses (e.g. honey, propolis) [11,12], and scientific and technological inspiration (e.g. development of visual guided flight robotics) [13]. Bees are also part of our culture (e.g. culinary, hobby occupation, etc.), contributing to the dynamism of rural and urban areas [14] and providing a source of inspiration and well-being for many [15].

Belgium shares the trends observed worldwide both in terms of wild and reared pollinators [8,16], with enhanced winter mortality observed from 1999 [8,17,18]. However, apart from colony mortality, Walloon beekeepers have reported a number of imprecise symptoms: colony weakness, mainly in spring; fast renewal of young queens; rapid loss of individuals in the colony, mainly foragers, or slow loss of individuals in the colony. In many cases brood and food remains in the colony. Sometime a small cluster of bees with the queen survives [17]. Some beekeepers described unspecific brood abnormalities not characteristic to any known disease (Baudoin and Lequeux, pers. com.). Other studies describe similar mortality trends [8,19,20] as well as unspecific symptoms: increased colony mortality and/or weakening [21–23], queen failure [21,23–25] or honey yield reduction [21].

Multifarious factors have been proposed to provide a cause to such a phenomenon. Climate change is proposed as one of them, together with a decrease of genetic variability of the bee colonies, electromagnetic radiation, pathogens and parasites or the impact of intensive agricultural systems (nutritional lack, GM plants or pesticides) [26]. Previous studies

developed in the region discard some of these factors as causes of bee losses, specifically *Nosema* spp and American Foulbrood [27]. However, pesticide residues and certain viruses were detected in bee colonies [27,28]. These elements were the most relevant to us.

Countless studies have shown lethal and sub-lethal effects of pesticides on bees [29–31]. Insecticides are often the most studied for obvious reasons. However other substances (fungicides, herbicides) deserve analysis for their specific toxicity, individually or in synergy with other substances [32,33] or pathogens [34–36], or their extensive exposure given their large scale and/or repeated use. Indeed, residue analyses show that these types of substance are found in the hives, even though the crops in which they were applied would suggest no bee exposure [37–40].

Beside pesticides, bee viruses are often mentioned as a putative cause of decline of the colonies, or at least to reside among the presumed multi-factorial causes [41]. In Europe, at least 12 viruses infecting bees have been compiled [21]. Many honey bee viruses commonly occur in seemingly healthy populations that continue to run well. Viruses may remain latent and confined within certain cells or tissues with no active replication and no disruption of cellular function. Likewise, they may be replicating at low level in permissive cells but in non-vital sites or in honey bee life stages that do not exhibit any symptoms or obvious pathology [21,42,43]. Nevertheless, two viruses, ABPV and DWV, are able of inducing serious disorders to honey bees and have been shown to cause -in association with *V. destructor* - winter losses in Germany [23]. The results of the later publication need to be considered cautiously, for serious concerns were raised linked with its independency and skewed approach [44].

In this paper, we first studied the relationship of in-hive viral prevalence as well as the presence of pesticide residues on honey bee colonies' health. As a next step, we focused on the relationship between the environment surrounding the monitored apiaries and the health condition of their colonies.

2.2 MATERIALS AND METHODS

2.2.1 Field work – colony follow up

A group of voluntary beekeepers were requested to participate in the study. All of them shared the following criteria: (1) have a minimum of 5 production colonies per apiary in June 2011; (2) regularly follow up the health status and development of their colonies; and (3) monitor the varroa infestation level and carry out officially recommended varroa treatments (treatment in July- August with veterinary medicaments based on thymol and winter treatment with veterinary medicaments based on oxalic acid). A total of 330 colonies distributed among 66 apiaries (5 colonies/ apiary) in Walloon and Brussels regions (Belgium) were followed. In those apiaries composed of 5 or more colonies, the 5 well-developed colonies at the beginning of the study were selected.

Colonies received three visits along the study, the first one from mid-July to mid-August 2011, the second one from mid-September to mid-October 2011 and the third one from March to April 2012. State official beekeeping technicians specialised in bee health were trained in the framework of the project in order to minimise as far as possible the variability of the results due to handling and observations. However, no analyses of the level of such variability was carried out within the study. For each of the visits, beekeepers were requested to fulfil a form asking information about the health history of the apiary and of the followed-up colonies, as well as about their beekeeping practices. Honey bee colony disorder symptoms were reported in the questionnaire. These include the following symptoms for which no causal agent could obviously be identified:

- dead and disappeared colonies: (1a) death of part or the whole colony, where dead bees can be found close to the hive or inside it. Beekeepers also describe the (1b) disappearance of part or the whole colony, leaving behind food reserves and brood, a

phenomenon similar to the one described in North- American apiaries (Colony Collapse Disorder (CCD) [24];

- weak colonies: weakening of the colony, showing in occasions a slow development in spring under optimal conditions (e.g. optimal weather, low varroa pressure, etc) with as consequence the loss of the spring production, but in occasions showing abnormally small colonies or colonies with low activity;
- queen loss: replacement of young queens sometimes leading to queen-less colonies or interruption of the egg-laying activity of the queen [17];
- problems linked to brood and not related to any known disease.

The form also included requests about other symptoms typically linked to known diseases (e.g. diarrhoea, mummified larvae, varroa presence, presence of deformed wing bees, etc). Each of the questions requested further characterisation of the symptoms (e.g. population size, population dynamics, bees behaviour, etc.).

In addition, a thorough clinical inspection was carried out for each colony in the mentioned period. Special attention was given to the strength of the colony in terms of bee numbers, brood surface and reserves content, the presence of the queen and the varroa infestation level. Finally, two different samples were collected before and after the winter: (1) in-hive bees (minimum of 100 bees); (2) a section of the frame containing beebread and honey (about 100 cm²). Samples were collected in hermetic plastic bags, cooled after collection and stored at 220°C.

2.2.2 Case choice – hierarchical sample clustering

Information available from bee colonies comprised field observations, beekeeper answers to the questionnaire and results from the analyses of the samples of beekeeping matrices (honey, beebread, wax and bees). Colonies

for which this information was available were considered for our analyses. Colonies with well identified problems (heavy varroa infestation, lack of food or drone-laying queens) were discarded. After that, two groups were made: a group with disorders and another with healthy colonies. In order to constitute these groups and to limit variations due to potential different bee management, a hierarchical classification was made. The criteria used were the amount of food stores before the winter, the year of colony creation, the subspecies and the age of the queen. The determination of the subspecies was done on the basis of beekeepers opinion and knowledge and no molecular analyses was performed to prove this information. This classification was made by using Ward aggregation method. This method allows to build group with the lower variation within the group and the higher variation between groups [45].

2.2.3 Virus analyses

The viruses under investigation were the Black Queen Cell Virus (BQCV), the Chronic Bee Paralysis Virus (CBPV), the Acute Bee Paralysis Virus (ABPV), the Deformed Wings Virus (DWV), and the Sac Brood Virus (SBV). Viral analyses were conducted on the honey bee workers collected before the winter, during the first and second periods (July–August and September–October). The samples were analysed with a quantitative RT-PCR by the National Bee Unit laboratory, Food and Environment Research Agency (Sand Hutton, York, United-Kingdom).

Total Nucleic acid (TNA) was extracted from 60 bees homogenised for 12 minutes with 20 ml GITC Lysis Buffer (5 M Guandine Thiocynate, 0.05 M Tris base, 0.02 M EDTA, pH 8.0) in a 30 ml bottle containing 3, 7/160 ball bearings. GITC Lysis buffer also contained 17.3 mM SDS buffer (173 mM Sodium dodecyl sulphate (SDS) in 100 ml MGW). The SDS buffer is added prior to use in warmed GITC Lysis Buffer. The homogenate was then incubated at 65uC for 40 minutes and then spun at 6189 g for 5 minutes. Polypropylene 96-deep well plates (DT850301 Elkay Laboratory Products Ltd) were prepared as follows (1 well/extract); plate A: 800 ml extract, and

100 ml MagneSil™ beads (MD1441, Promega); plate B: 1 ml of GITC wash buffer (5 M Guandine Thiocynate, 0.05 M Tris base); plates C, D, E: 1 ml 70%v/v ethanol (E/00665DF/17, Fisher Scientific); plate F: 1 ml 5 M Betaine (B2629, Sigma), plate G, 300 ul 16TE.

Buffer (stock 100X TE (EC-862 National Diagnostics). Plates were loaded onto the Kingfisher Flex and processed as follows: Plate A – Bind 5 mins (fast dual mix), Plate B – Wash 3 mins (fast dual mix), Plate C, D, E – Wash 2 mins (fast dual mix), collect beads at 1 min intervals, Plate F – Wash 20 secs (medium), without releasing beads, Plate G – Mix 1 min then incubate at 65uC for 5 mins with mixing. All steps of the process are looped through twice. TNA was collected from plate G of each reaction and stored at 280uC prior to use in real-time PCR.

Table 2.1 - List of primers used for virus analyses. doi:10.1371/journal.pone.0103073.t001

Target	Primer Name	Sequence (5'-3')
BQCV	BQCV 9195F	GGT GCG GGA GAT GAT ATG GA
	BQCV 8265R	GCC GTC TGA GAT GCA TGA ATA C
	BQCV 8217T	TTT CCA TCT TTA TCG GTA CGC CGC C
SBV	SBV 311F	AAG TTG GAG GCG CGy AAT TG
	SBV 380R	CAA ATG TCT TCT TAC dAG AGG yAA GGA TTG
	SBV 331T (MGB)	CGG AGT GGA AAG AT
CPV	CPV 304F	TCT GGC TCT GTC TTC GCA AA
	CPV 371R	GAT ACC GTC GTC ACC CTC ATG
	CPV 325T	TGC CCA CCA ATA GTT GGC AGT CTG C

Reactions were set up in 96 well reaction plates using Absolute Blue QPCR ROX mix (AB-4139, Thermo Scientific) following the protocols supplied. 0.1 mM of 0.1 M Dithiothreitol (165680250, Arcos Organics) and 0.33 unit of MMLV (EPO441: Fermentas) were added to each reaction. The primers (Table 2.1) were all used at 400 nM and probes at 200 nM final concentration. Total nucleic acid (5 ml) was added to each reaction, giving a final reaction volume of 20 ml. Plates were cycled for 48uC/30 min,

95uC/10 min and 40 cycles of 60uC/1 min, 95uC/15 sec within the 7900 HT Sequence Detection System (Applied Biosystems), using real time data collection. The results were recorded as the cycle threshold (Ct) or cycle number after which a significant accumulation of florescence over the baseline was observed; an average (of duplicate wells) Ct value below 40 was regarded as a positive result with a threshold DRn setting of 0.2. Given the absence of internal standard, we assumed that the extraction method had the same efficiency towards bee DNA/RNA and viral RNA.

Virus primers and probes used for all pathogens tested have been previously described in Chantawannakul *et al.* 2006 [46]. Additional APBV primers are described in Martin *et al.* 2012. [47].

2.2.4 Pesticide analyses

Samples collected before the winter were sent to Eurofins Chemiphar NV, Brugge, Belgium and analysed by SOFIA GmbH Chemisches Labor für Softwareentwicklung und Intelligente Analytik, Berlin, Germany. Two multi-residues methods (SF146 and SF150) were used searching for 99, 93 and 96 pesticides residues in wax (54 samples), beebread (108 samples) and honey (107 samples – one sample did not contain enough matrix) respectively (Table 2.2).

For SF146 method, 10 ml of water was added to 10 g of samples. Methanol was added and the preparation was mixed. The mixture was filtered and centrifuged. Next, for analysis by gas chromatography coupled with mass spectrometry (GC-MS), the filtrate was mixed with sodium chloride and ethyl acetate solution 1:1 (v/v). All was dried with sodium sulfate and filtrated. This filtrate was then concentrated and analysed by GC-MS. For analysis with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), 5 ml of sample were transferred in ChemElut column and eluted with dichloromethane. After concentration, 1 ml of water-methanol 1:1 (v/v) was added and this solution was analysed by LC-MS/MS. For the wax, some modifications were made. For GC-MS,

sodium chloride was replaced by hexane to dissolve wax and the mixture was frozen until a precipitate appears. For LC-MS/MS, 0.2 g of sample were extracted with 30 ml of mixture of naphtha:acetonitrile 1:2 (v/v). 10 ml of acetonitrile phase was isolated and concentrated before the analysis by LC-MS/MS.

For SF150 method, 10 ml of water was added to 10 g of samples as well as methanol and hydrochloric acid. All was mixed, filtrated and centrifuged. A solution of sodium chloride (20%) was added and 5 ml of sample were transferred in ChemElut column and eluted with dichloromethane. After concentration, 1 ml of water-methanol 1:1 (v/v) was added and this solution was analysed by LC-MS/MS.

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Table 2.2 - Active ingredients or metabolite included in the multi-residue analyses by beekeeping matrix (A = Acaricide; F = Fungicide; H = Herbicide; I = Insecticide; S = Synergist). doi:10.1371/journal.pone.0103073.t002

Active ingredient or metabolite*	Class	LOQ (µg/kg)			LD50 µg/bee	LD50 µg/kg
		Wax	Honey	Beebread		
2,4-D	H	100	10		94	940.000
Abamectine	I	100	5	100	0,002	20
Acetamiprid	I	100	5	100	8,09	80.900
Aldicarb	I	100	5	100	0,16	1.600
Alpha-cypermethrin	I	100	5	100	0,033	330
Amitraz	A	100	10	100	50	500.000
Azoxystrobin	F	100	5	100	>25	250.000
Bentazone	H	200	20	-	>200	2.000.000
Benthiavalicarb	F	100	5	100	>100	1.000.000
Betacyfluthrin	I	100	10	100	0,001	10
Bifenthrin	I	100	5	100	0,015	150
Boscalid	F	100	5	100	100	1.000.000
Captane	F	100	10	100	>100	1.000.000
Carbaryl	I	100	5	100	0,14	1.400
Chlorothalonil	F	100	5	100	>40	400.000
Chlorpyrifos	I	100	5	100	0,059	590
Chlorpyrifos-methyl	I	100	5	100	0,15	1.500
Clothianidin	I	100	10	100	0,004	40
Coumaphos	A	100	-	100	14,39	143.900
Coumaphos oxon*	A	100	-	100	-	-
Coumaphos phenolic*	A	100	-	100	-	-
Cyazofamid	F	200	20	200	>100	1.000.000
Cyfluthrin	I	100	10	100	0,001	10
Cymoxanil	F	100	10	100	>85.3	853.000
Cypermethrin	I	100	5	100	0,023	230
Cyproconazole	F	100	5	100	112,5	1.125.000
Cyprodinil	F	100	10	100	113	1.130.000
DDT	I	100	5	100	0,54	5.400
Deltamethrin	I	100	5	100	0,0015	15
Dichlorprop-P	H	100	10	-	>200	2.000.000
Difenoconazole	F	100	5	100	>100	1.000.000
Diiflubenzuron	I	200	20	200	>25	250.000
Dimethenamide-P	H	100	5	100	118,4	1.184.000
Dimethoate	I	100	5	100	0,1	1.000
Dimethomorph	F	100	10	100	>32.4	324.000
Dimoxystrobin	F	100	5	100	>79.4	794.000
Epoxyconazole	F	100	5	100	>83	830.000
Esfenvalerate	I	100	5	100	0,06	600
Ethofumesate	H	500	50	0,5	>50	500.000
Famoxadone	F	100	10	100	>1	10.000
Fenhexamid	F	100	5	100	>102.07	1.020.700
Fenoxycarb	F	100	5	100	>204	2.040.000
Fenpropiidin	F	100	10	100	>10	100.000
Fenpropimorphe	F	100	5	100	>95.6	956.000
Fipronil and*	I	100	10	100	0,00417	42
Flonicamid	I	200	200	200.000	>51100	511.000.000
Fluazinam	F	100	10	100	>100	1.000.000
Flufenacet	H	100	10	100	>170	1.700.000
Fluopicolide	F	100	10	100	>100	1.000.000
Flusilazole	F	100	5	100	33,8	338.000

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Table 2.2 - Continue

Active ingredient or metabolite*	Class	LOQ (µg/kg)			LD50 µg/bee	LD50 µg/kg
		Wax	Honey	Beebread		
Heptenophos	I	100	10	100	>0.53**	5.300
Imidacloprid and*	I	100	5	100	0,0037	37
Indoxacarb	I	100	5	100	0,094	940
Iprodione	F	100	5	100	>25	250.000
Kresoxim-methyl	I	100	5	100	>100	1.000.000
Lambda-cyhalothrin	I	100	5	100	0,038	380
Lindane	I	100	5	100	0,011	110
Linuron	H	100	5	100	>120.9	1.209.000
MCPA	H	100	10	-	>200	2.000.000
Mecoprop-P	H	100	10	-	>100	1.000.000
Metalaxyl-M	F	100	5	100	>97.3	973.000
Metamitron	H	100	5	100	>97.2	972.000
Metconazole	F	200	20	200	85	850.000
Methiocarb	I	100	5	100	0,23	2.300
Methoxyfenozide	F	100	5	100	>100	1.000.000
Metribuzin	H	100	5	100	166	1.660.000
Penconazole	F	100	5	100	>30	300.000
Pendimethalin	H	100	5	100	100	1.000.000
Permethrin	I	200	20	200	0,29	2.900
Picoxystrobin	F	100	5	100	>200	2.000.000
Piperonyl butoxide	S	100	10	100	294	2.940.000
Pirimicarb	I	100	5	100	4	40.000
Prochloraz	F	100	5	100	>101	1.010.000
Propamocarbe	F	100	5	100	-	-
Propiconazole	F	100	10	100	>100	1.000.000
Propyzamide	F	100	10	100	>136	1.360.000
Pymetrozine	I	100	5	100	>117	1.170.000
Pyraclostrobin	F	100	5	100	>73.1	731.000
Pyrethrin	I	200	20	200	-	-
Pyrimethanil	F	100	5	100	>100	1.000.000
Rotenone	I	100	10	100	-	-
Spinosad	I	100	-	100	-	-
Spirodiclofen	I	100	10	100	>196	1.960.000
Spirotetramat	F	100	10	100	>100	1.000.000
Spiroxamine	F	100	5	100	4,2	42.000
Sulcotrione	H	100	10	-	50	500.000
Tau-fluvalinate	IA	100	10	100	12	120.000
Tebuconazole	F	100	5	100	>83.05	830.500
Tebufozide	I	100	5	100	>234	2.340.000
Tefluthrin	I	100	5	100	0,28	2.800
Terbutylazine	H	100	10	100	>22.6	226.000
Tetraconazole	F	100	10	100	63	630.000
Thiabendazole	F	100	5	100	>4	40.000
Thiacloprid	I	100	5	100	17,32	173.200
Thiamethoxam	I	100	5	100	0,005	50
Thiophanate-methyl	F	100	5	100	>100	1.000.000
Trifloxystrobin	F	100	10	100	>200	2.000.000
Zeta-cypermethrin	I	100	5	100	0,002	20
Zoxamide	F	100	10	100	>100	1.000.000

*Note. * Metabolites of Fipronil: sulfide, sulfone, Ethiprol, desulfinyl; Metabolites of Imidacloprid: 6-chloronicotinic acide, oléfine, 4/5-hydroxy-imidacloprid, 4,5-dihydroxy-imidacloprid; ** Bombus terrestris; The transformation of LD50 in terms of µg/bee to µg/kg is based on the average weight of Apis mellifera, estimated to be 0.1 g.*

2.2.5 Descriptive analyses

A descriptive analysis of virus prevalence and pesticide residues was first carried out. The relationship between disorder and these stressing factors was then assessed. For each virus type, a linear model (two way ANOVA) was used, with the cycle threshold value as dependent variable and the visit (first or second), group (with disorder or healthy) and their interaction as explanatory variables. For the pesticides residues, we used a similar model with the total number of residues as dependent variable and type (fungicide or insecticide/acaricide), group (with disorder or healthy) and their interaction as explanatory variable. Based on these models, we constructed a contrast matrix to test explicit post-hoc hypotheses (e.g. compare virus load between groups within each visit, etc.). As several dependent variables had a strongly asymmetric distribution (i.e. non-normal), we computed all p-values by permutation ($n = 1000$). We applied a Bonferoni correction on the post-hoc tests p-values to take into account the multiplicity of the tests.

2.2.6 Relationship between bee colony disorders and potential stressors or surrounding environment of the apiary

We used two separate generalized linear mixed models with a binomial distribution and logit link function. In both models, the “group” (with disorder or healthy) was used as dependent binary variable and the apiary was used as random effect (grouping factor) to take into account the non-independence between colonies from the same apiary and allow the intercept to vary between apiaries [48].

With the first model, we explored the relationship between the probability of disorder in a colony and potential stress factors, i.e. pesticides or viruses total load into the colonies. We used as explanatory variables the

total number of fungicides residues, the total number of insecticides or acaricides (pooled together) residues, the total number of viruses detected for both visits and all first level interactions between these three explanatory variables. The insecticides and acaricides were pooled together because the most frequently found active ingredient, namely tau-fluvalinate, is authorized in Belgium for both purposes.

With the second model, we investigated the relationship between the probability of disorder in a colony and the structure of the agricultural landscape around the beehive. The surfaces of all different kinds of agricultural soil occupancy in a circle with a radius of 1500 m around each apiary were calculated according to the information provided by the farmers to the Walloon administration (Land Parcel Identification System). The different soil occupancy categories were pooled into two categories defined according to the potential frequency of plant protection product use: (1) grasslands (low pesticides use), (2) crops *sensu lato* (potentially higher pesticides use), including major crops (mainly: cereals, potatoes, beet, oilseed rape, maize, flax, etc.) but also surfaces dedicated to fruit or vegetables production, fodder production (mainly legumes) and to horticulture. The surfaces of these two land use options, grasslands and crops (*sensu lato*), were used as explanatory variables in the model. The 1500 m radius was chosen accordingly to the mean pollen and nectar collecting distance for the honey bee [49,50]. However it is well known that the foraging distances of bees are highly variable and can reach to distances larger than 10 km [51]. As the agricultural soil occupancy data was available only for Wallonia, we eliminated from the analysis the apiaries whose buffer had less than 70% of their surface within the administrative boundaries of Wallonia (n=2).

We used Likelihood Ratio (LR) Tests to evaluate the significance of the explanatory variables [48,52]. We respected the marginality rules, i.e. all main effects were tested after removing from the model the interactions in which they are involved [53].

All analyses were performed with R (R Core Team 2013) and the mixed

models were fitted with the package lme4 [54].

2.3. RESULTS

2.3.1 Description of honey bee colony disorders

We gathered all sources of information, i.e. questionnaires, analytical matrices and information from clinical visits, for 173 colonies. After the clustering, 54 colonies coming from 21 apiaries (Figure 2.1) were considered for the study: 25 presenting bee disorders and 29 not presenting them, defined as healthy group. Their data and samples were collected by 8 beekeeping technicians.

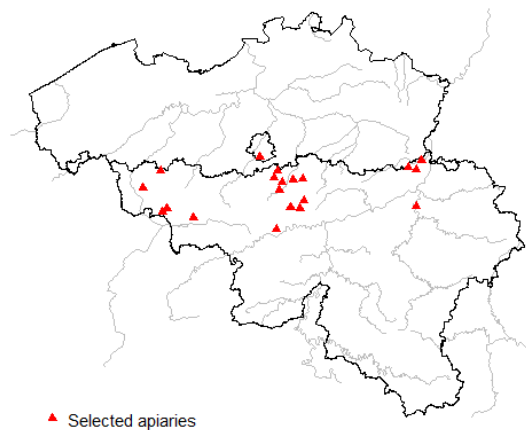


Figure 2.1 - Spatial distribution of selected apiaries
doi:10.1371/journal.pone.0103073.g001

Chapter 2 – Colony Disorders in Crops Areas: Role of Pesticides and Virus

In the group presenting disorders, 6 colonies were dead or unviable -with only a handful of bees remaining with the queen- at the spring visit (Table 2.3). Pre-wintering weakness, winter worker losses, late-season re-queening were reported in these cases. One colony was dead due to queen failure without re-queening in spring. Furthermore, a number of colonies were weak in spring. They were characterised by a low number of individuals and a slow development. In total, nine colonies lost their queens during the study, five of them leading to queenless colonies. These were considered as “disorder colonies” in the framework of the study. Finally, brood abnormalities not linked to known disease were observed in 10 colonies, most of them both before and after the winter.

Symptoms of other diseases or bee parasite observation remained low within the selected colonies. Wax moths were observed in two cases, one in each group. Varroa destructor was present in all colonies of the study. All of them were treated according to official veterinary advice. Diarrhoea was observed in one of the colonies showing brood abnormalities. Anatomical modifications (i.e. deformed or undeveloped wings, small abdomens) were observed in 6 colonies, half of them being healthy and half of them showing disorders. No symptoms of any of the foulbroods were observed.

Table 2.3 - *Symptoms observed in the group with disorders*

Symptoms of disorder	Frequency
Mortality	2*
Weakening	3
Queen failure	5
Brood problems	9
Mortality + Weakening	2
Mortality + Weakening + Queen failure	2
Mortality + Queen failure	1
Queen failure + Brood problems	1
Total	25

* One of these colonies not considered in the model due to a lack of viral results

2.3.2 Virus content

One bees sample from the “disorder” group could not be analysed due to a lack of sufficient matrix. The three most abundantly found viruses were the Deformed Wings Virus (DWV), the Black Queen Cell Virus (BQCV) and the Sac Brood Virus (SBV) (Figure 2.2). The Acute Bee Paralysis Virus (ABPV) and the Chronic Bee Paralysis Virus (CBPV) were found only in a very limited number of samples and did not allowed particular statistical analyses on these two viruses.

There was no significant difference in viral content between the group with disorders or the healthy one, and independently of the visit for any of the three most abundant viruses (2-way ANOVA tested by permutations, DWV: $p = 0.731$, BQCV: $p = 0.373$, SBV: $p = 0.54$). We observed a decrease of virus content from summer to fall 2011, independently of the group, for BQCV ($p = 0.001$) and SBV ($p = 0.001$) but not for DWV ($p = 0.271$). For BQCV only, we observed a significant ($p = 0.036$) group $\times$ visit interaction indicating that the decrease of the virus abundance from visit 1 to visit 2 was higher in the group “with disorder” than in the group “healthy”. Overall, the number of virus changed between visit 1 and visit 2 and a significant decrease was observed in the group with disorders ($p = 0.001$) (Figure 2.3)

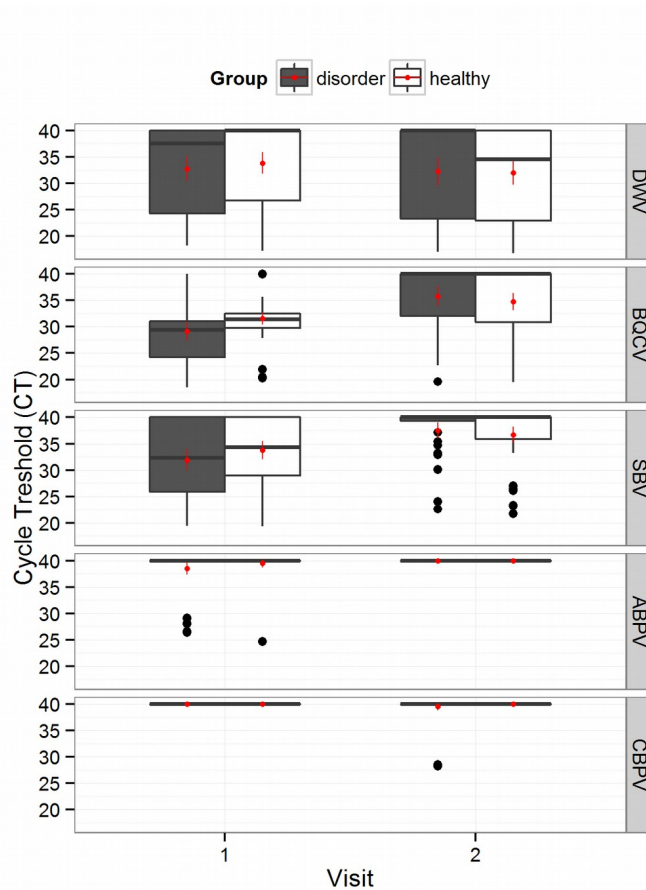


Figure 2.2 - Virus content according to the Cycle Threshold (CT) for the groups “with disorder” and “healthy”. Boxplot of Cycle Threshold for the first and second visits (visit 1 - mid-July to mid-August and visit 2 - mid-September to mid-October) and the group with disorders (grey, $n = 24$ colonies) and the healthy one (white, $n = 29$ colonies). Deformed Wings Virus (DWV), Black Queen Cell Virus (BQCV), SacBrood Virus (SBV), Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV). In red, mean with confidence interval estimated by bootstrap method. Note: CT values below 40 were regarded as positive results and the lowest CT values correspond to the higher virus contents. doi:10.1371/journal.pone.0103073.g002

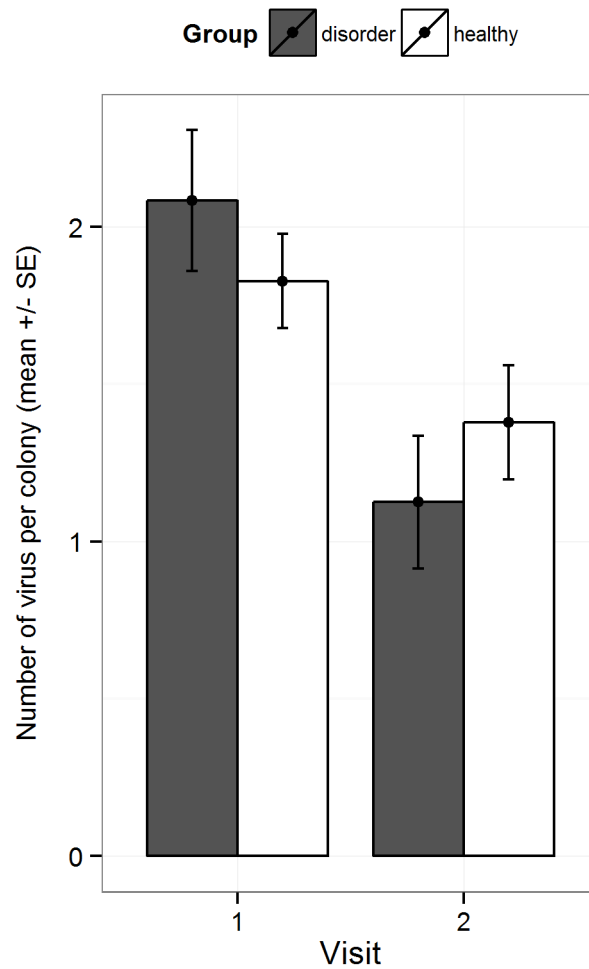


Figure 2.3 - Average number of different viruses per colony. Data shown for the first and second visits (visit 1 - mid-July to mid-August and visit 2 - mid-September to mid-October) and the group with disorders (grey, $n=24$ colonies) and the healthy one (white, $n=29$ colonies). Whiskers show the standard error (SE). doi:10.1371/journal.pone.0103073.g003

2.3.3 Pesticide analysis

172 agrochemical residues of 23 different active ingredients were detected in 94 out of 269 samples. Wax was the most contaminated matrix: 109 residues of 15 different active ingredients; while 39 and 24 residues of 10 and 8 substances were detected in beebread and honey, respectively (Figures 2.4 & 2.5).

Residue levels contained in wax and beebread were higher (0.21– 3.1 mg/kg) than those in honey (0.001–0.058 mg/kg).

For the subsequent statistical analysis, the results obtained for insecticide and acaricides residues were pooled together because the most frequently found active ingredient, tau-fluvalinate (n=46), can be used in Belgium as an acaricide against varroa mite and as an insecticide to control *Meligethes aeneus* in rape. The second most frequently found residue was the coumaphos (n=35), followed by two fungicides, boscalid (n=33), iprodione (n=13) (Table 2.4). Some residues of neonicotinoid insecticides, synergist and herbicide were also detected: thiacloprid (n=3), piperonyl butoxide (n=6), terbuthylazine (n=1). The highest residue level concerned captan with 3.1 and 1.9 mg/kg in the wax and bee bread of the same colony, respectively. Despite of being the most frequently found in matrices, tau-fluvalinate and coumaphos residues never exceeded 0.71 and 0.58 mg/kg, respectively. Boscalid, the most commonly found fungicide, ranged from 0.005 to 1.3 mg/kg.

There was a significant difference ($p = 0.01$) in terms of number of fungicide substances found between the “disorder” group (mean = 2.0) and the “healthy” one (mean = 0.7) as presented in Figure 2.6. The total number of insecticides/acaricides residues was slightly higher in the disorder group (mean = 2.1) than in the healthy group (mean = 1.64) but this difference is not statistically significant ($p = 0.79$). The most frequent fungicides in the group with disorders were boscalid and iprodione, detected in the three investigated matrices (Figure 2.5). However, for single active substances, no significant difference of number of residues was observed between the

groups “with disorder” and “healthy”.

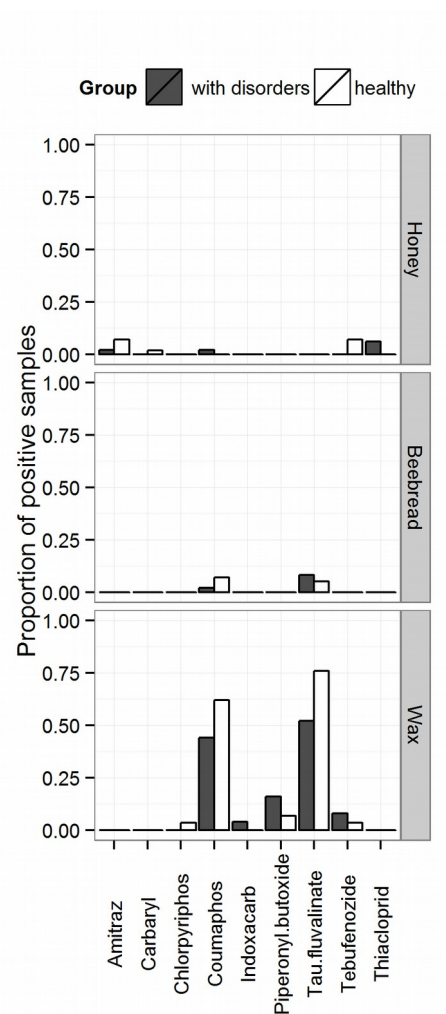


Figure 2.4 - Proportion of samples containing residues of acaricides/insecticides in the different beekeeping matrices (honey, beebread and wax). Data shown for the group with disorders (grey, n=25 colonies) and the healthy one (white, n=29 colonies). doi:10.1371/journal.pone.0103073.g004

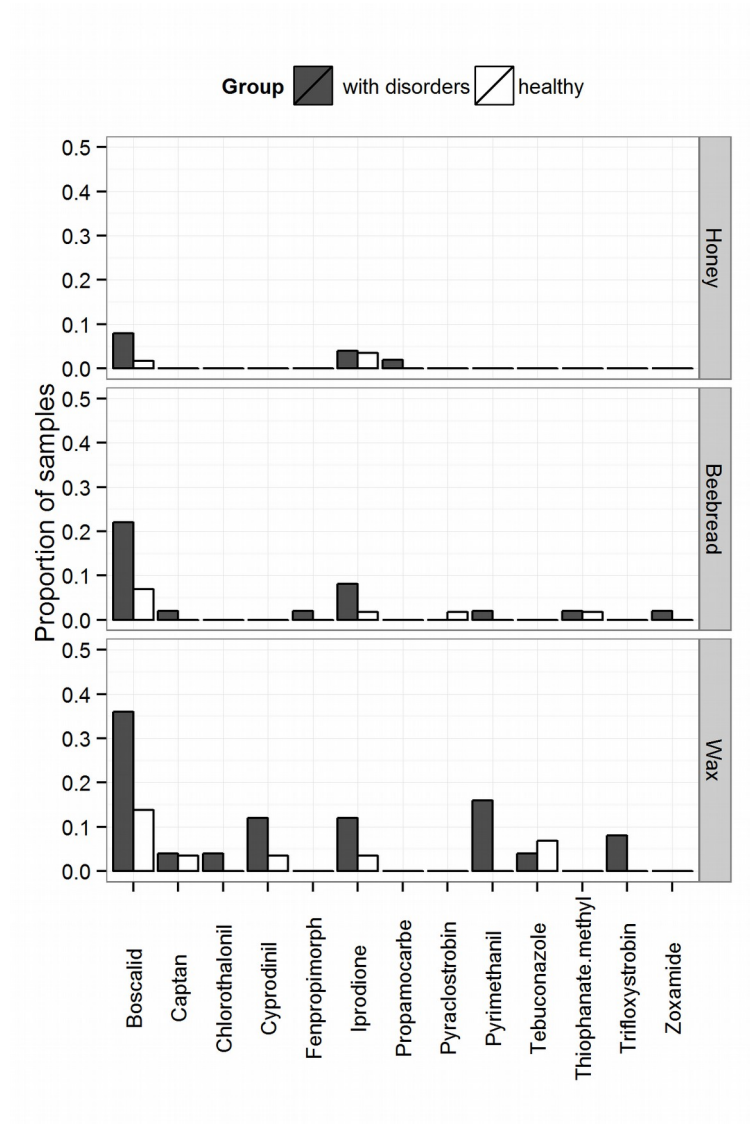


Figure 2.5 - Proportion of samples containing residues of fungicides in the different beekeeping matrices (honey, beebread and wax). Data shown for the group with disorders (grey, $n = 25$ colonies) and the healthy one (white, $n = 29$ colonies). doi:10.1371/journal.pone.0103073.g005

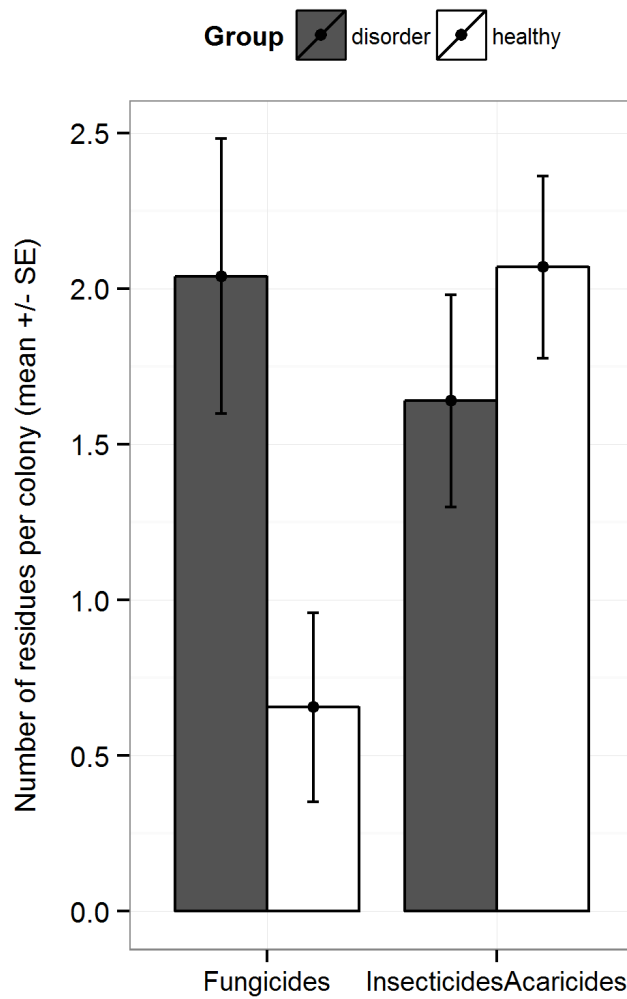


Figure 2.6 - Average number of residues per colony. Data shown for the group with disorders (grey, $n = 25$ colonies) and the healthy ones (white, $n = 29$ colonies). Whiskers show the standard error (SE)
doi:10.1371/journal.pone.0103073.g006

Table 2.4 - Residues of active ingredients found in wax, beebread and honey samples from colonies with disorders (Group D, n=25 colonies) and healthy ones (Group H, n=29 colonies). doi:10.1371/journal.pone.0103073.t004

Active ingredient	Group	LOQ (µg/kg)	Wax						LOQ (µg/kg)	Bee bread						LOQ (µg/kg)	Honey					
			Number of samples (n=54)			Residues amount (mg/kg)				Number of samples (n=108)			Residues amount (mg/kg)				Number of samples (n=107)			Residues amount (mg/kg)		
			<LOD	Detected	Quantified	Range	Mean	SD		<LOD	Detected	Quantified	Range	Mean	SD		<LOD	Detected	Quantified	Range	Mean	SD
Amitraz	D	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-	0,01	49	-	1	0,022	0,02	-
	H		-	-	-	-	-	-		-	-	-	-	-	-		53	-	4	0,01-0,028	0,02	0,01
Boscalid	D	0,01	16	8	1	0,29	0,29	-	0,01	39	7	4	0,4-1,3	0,68	0,42	0,005	46	-	4	0,005-0,026	0,01	0,01
	H		25	4	-	-	-	-		54	4	-	-	-	-		56	-	1	0,056	0,06	-
Captan	D	0,01	24	-	1	3,1	3,1	-	0,01	48	-	1	1,9	1,9	-	0,01	-	-	-	-	-	-
	H		18	1	-	-	-	-		58	-	-	-	-	-		-	-	-	-	-	-
Carbaryl	D	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-	0,005	50	-	-	-	-	-
	H		-	-	-	-	-	-		-	-	-	-	-	-		56	-	1	0,02	0,02	-
Chlorpyrifos	D	0,01	25	-	-	-	-	-	0,01	-	-	-	-	-	-	0,005	-	-	-	-	-	-
	H		28	1	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-
Chlorothalonil	D	0,01	24	1	-	-	-	-	0,01	-	-	-	-	-	-	0,005	-	-	-	-	-	-
	H		29	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-
Coumaphos	D	0,01	14	9	2	0,32-0,34	0,33	0,01	0,01	48	1	-	-	-	-	0,05	49	-	1	0,012	0,01	-
	H		11	10	8	0,23-0,58	0,37	0,01		54	4	-	-	-	-		57	-	-	-	-	-
Cyprodinil	D	0,01	22	3	-	-	-	-	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-
	H		28	1	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-
Fenpropimorph	D	0,01	-	-	-	-	-	-	0,01	49	1	-	-	-	-	0,005	-	-	-	-	-	-
	H		-	-	-	-	-	-		58	-	-	-	-	-		-	-	-	-	-	-
Indoxcarb	D	0,01	24	1	-	-	-	-	0,01	-	-	-	-	-	-	0,005	-	-	-	-	-	-
	H		29	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-
Iprodione	D	0,01	22	1	2	0,24-1,5	0,87	0,89	0,01	45	-	4	0,34-1,5	0,9	-	0,005	48	-	2	0,017-0,022	0,02	0,01
	H		28	1	-	-	-	-		57	1	-	-	-	-		55	-	2	0,022-0,04	0,03	0,01
Piperonyl butoxide	D	0,01	21	4	-	-	-	-	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-
	H		27	2	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-

Table 2.4 - continues

Active ingredient	Group	LOQ (µg/kg)	Wax							LOQ (µg/kg)	Bee bread							LOQ (µg/kg)	Honey						
			Number of samples (n=54)			Residues amount (mg/kg)					Number of samples (n=108)			Residues amount (mg/kg)					Number of samples (n=107)			Residues amount (mg/kg)			
			<LOD	Detected	Quantified	Range	Mean	SD	<LOD		Detected	Quantified	Range	Mean	SD	<LOD	Detected		Quantified	Range	Mean	SD			
Propamocarbe	D	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-	0,005	49	-	1	0,008	0,008	-			
	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	57	-	-	-	-	-			
Pyraclostrobin	D	0,01	-	-	-	-	-	-	0,01	50	-	-	-	-	-	0,005	-	-	-	-	-	-			
	H	-	-	-	-	-	-	-	-	57	1	-	-	-	-	-	-	-	-	-	-	-			
Pyrimethanil	D	0,01	21	4	-	-	-	-	0,01	49	1	-	-	-	-	0,005	-	-	-	-	-	-			
	H	-	29	-	-	-	-	-	-	58	-	-	-	-	-	-	-	-	-	-	-	-			
Tau-fluvalinate	D	0,01	12	9	4	0.29-0.46	0,4	0,08	0,01	45	4	-	-	-	-	0,01	50	-	-	-	-	-			
	H	-	7	13	9	0.21-0.71	0,5	0,08	-	55	3	-	-	-	-	-	53	-	4	0.011-0.02	0,02	0,002			
Tebuconazole	D	0,01	24	1	-	-	-	-	0,01	-	-	-	-	-	-	0,005	-	-	-	-	-	-			
	H	-	27	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Tebufozide	D	0,01	23	2	-	-	-	-	0,01	-	-	-	-	-	-	0,005	-	-	-	-	-	-			
	H	-	28	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Terbutylazine	D	0,01	24	1	-	-	-	-	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-			
	H	-	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Thiacloprid	D	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-	0,005	47	-	3	0.009-0.013	0,01	0,002			
	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	57	-	-	-	-	-			
Thiophanate-methyl	D	0,01	-	-	-	-	-	-	0,01	49	1	-	-	-	-	0,005	-	-	-	-	-	-			
	H	-	-	-	-	-	-	-	-	57	-	1	0,38	0,38	-	-	-	-	-	-	-	-			
Trifloxystrobin	D	0,01	23	2	-	-	-	-	0,01	-	-	-	-	-	-	0,01	-	-	-	-	-	-			
	H	-	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Zoxamide	D	0,01	-	-	-	-	-	-	0,01	49	1	-	-	-	-	0,01	-	-	-	-	-	-			
	H	-	-	-	-	-	-	-	-	58	-	-	-	-	-	-	-	-	-	-	-	-			

2.3.4 Probability of disorders in relation to potential stressors

There is clearly a significant positive relationship between the probability of a colony showing disorders and the total number of fungicides (Figure 2.7, LR = 7.128, df = 1, p = 0.008). The estimated probability for a colony to be in the “with disorder” group is 0.26 without fungicides residues, 0.60 with 2 fungicides residues and 0.88 with 4 fungicides residues when the insecticides-acaricides residues number and virus load are fixed to their observed mean value (Figure 2.7). None of the other variables (total number of viruses, total number of insecticides-acaricides) and none of the first level interactions seem to have any explanatory power on the probability of disorders in a colony (Table 2.5). No direct link could be established between bee colony disorders and the amount (in mg/kg) of residues found in the matrices.

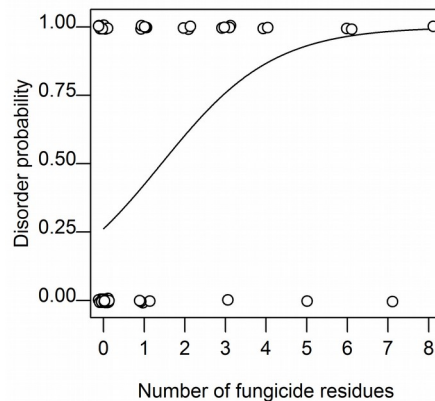


Figure 2.7 - Probability of honey bee colony disorders depending on the number of fungicide residues detected. Model based on averaged coefficients and median value both for the number of insecticides-acaricides residues and total number of virus detected for both visits (n = 53 colonies).
doi:10.1371/journal.pone.0103073.g007

2.3.5 Probability of disorders and crop/grassland surface

Our data clearly show a significant increase in disorder probability with the increase of crop surfaces (*sensu lato*, i.e. including fruit, vegetables, fodder production and horticulture) in the surrounding of the apiary (Figure 2.8, LR = 8.052, df = 1, p = 0.0045). The predicted probability of disorders is close to 0.1 for a crop surface of 0 ha in the radius of 1500 m and increases up to 0.8 for a surface of 500 ha of crops when fixing the grasslands surface to its observed mean.

On the contrary, the probability of disorders strongly decreases when the grassland surfaces increases (Figure 2.8, LR = 14.527, df = 1, p = 0.0001) after controlling for the crop surface. The predicted probability of disorders is close to 1 for a grassland surface of 0 ha in a radius of 1500 m and drops to approx. 0.1 for a surface of 150 ha of grasslands when fixing the crops surface to its observed mean.

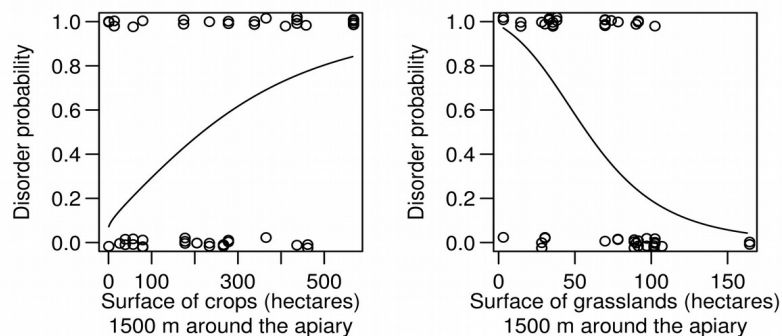


Figure 2.8 - Probability of honey bee colony disorders depending on the apiary's environment. Consideration of crop surface vs. grassland surface in a radius of 1500 m around the apiary. Crops include fruit, vegetables, fodder production and horticulture. For each graph, the value of the variable not displayed is fixed to its observed mean (n = 53 colonies). doi:10.1371/journal.pone.0103073.g008

Very similar results were found with a 3000 m buffer around the apiary (results in Supplementary material Fig. S2.1).

2.4 DISCUSSION

The bee disorders observed after the winter in the Walloon region happened despite of normal climatic conditions. The autumn of 2011 was dry (140.4 mm rainfall water from October to December, average being 219.9 mm), sunny and warm for Belgian conditions (12.4°C on average, which is normally 10.9°C for this period), followed by average winter and spring 2012 in terms of temperature, rainfall, with the exception of a cold wave lasting twelve days in February [55]. These conditions would not induce, a priori, a risk for honey bee colonies. Furthermore, based on studies carried out on the palynological diversity of the pollens collected by these colonies before the winter (Table S1), the hypothesis of nutritional lack is unlikely. All samples analysed contained at least 8 different botanic sources of pollen, including rich protein content as oilseed rape, ivy and Phacelia.

Viruses infections have often been mentioned as a source of stress for honey bee colonies [7]. However no significant difference in quantity or occurrence was observed between the healthy and the “with disorder” groups. DWV is one of the most commonly detected virus in *A. mellifera*. The prevalence of this virus is even more important at colonies infested by *Varroa destructor*, a well- established vector of this virus [7]. In accordance with the present study, further studies run on Belgian apiaries show DWV, BQCV and SBV as the most frequently found viruses [56]. Unlike DWV, we observed that the amount of SBV and BQCV has dropped significantly between the first and second visits for each group. In the case of BQCV, this is of no surprise as the cycle of incidence of this virus has been shown to increase in late winter, with a peak in May or June followed by a rapid decline [57]. The observed decrease of SBV has also been reported in other

studies [42] in which the authors suggested that bees could develop a molecular defensive mechanism to reduce virus multiplication, or that the change in bee susceptibility to the virus could result from environmental conditions such as the quality of pollen.

Table 2.5 - Analysis of deviance table for generalized linear binomial models describing the relationships between the colony disorder probability and three variables: the total number of (1) fungicide residues (*totfungicides*), (2) insecticide-acaricide residues (*totinsaca*) and (3) virus detected for both visits (*totvirus*).

	LR	df	p(>Chisq)
<i>totfungicides</i>	7.13	1	0.008
<i>totinsaca</i>	0.005	1	0.943
<i>totvirus</i>	0.136	1	0.712
<i>totfungicides:totvirus</i>	2.222	1	0.136
<i>totinsaca:totvirus</i>	0.975	1	0.323
<i>totfungicides:totinsaca</i>	0.901	1	0.342

LR = likelihood ratio. doi:10.1371/journal.pone.0103073.t005

We cannot prove any causal relationship between any of these viruses analysed and bee disorders, nor does the interaction between these two factors. Cox-Foster *et al.*, 2007 [41] found no clear correlation between a variety of pathogens, including *Nosema* spp., DWV, CBPV, ABPV, BQCV, *Mellisococcus pluton* and *Paenibacillus larvae* ssp and CCD. No specific spore counts were carried out in our study. However, no signs of nosemosis or foulbrood could be linked to colonies presenting bee disorders. It is noteworthy that a monitoring run at Belgian level found *Nosema* spp spores in one out of four colonies [58]. Nevertheless, Cox-Foster *et al.*, 2007 [41], show a positive correlation between IAPV and CCD, which a priori would not be relevant in our conditions given the low prevalence of IAPV in this country [27,56]. A recent publication [36] shows a positive correlation between the presence of fungicides in pollen loads and the presence of spores of *Nosema ceranae*. We do not exclude the potential involvement of *Nosema* spp. in the case of bee disorders. However, in the framework of our

study, *N. ceranae* seems either to play a role in the development of this weakening, while remaining asymptomatic, or not to play a decisive role in it.

When considering pesticide residues, no direct link could be established between bee colony disorders and the amount (in mg/kg) of residues found in the matrices. Neither could we identify any specific molecule as cause of bee disorders. However, it must be highlighted that the sensitivity of multiresidue analyses was not good enough mainly for insecticides (Table 2.2). This involves that insecticides could be present in the matrices at levels lower than the limits of quantification, but at toxicologically relevant quantities. Nevertheless, the study of the residue load of pesticides, specifically fungicides, opens new avenues for a better understanding of honey bee colony disorders. Wax was the most contaminated matrix, followed by beebread and honey. The matrix in which pesticides are conveyed to the bees seem to determine the impact of those on bees. Yamada *et al.* 2015 [59] for example, showed a 5-fold higher toxicity of the insecticide dinotefuran when bee colonies were exposed through pollen paste than when they were through sucrose syrup.

Even if insecticides/acaricides were the most numerous residues detected in hives mainly in wax, no significant difference in the number of accumulated residues was observed between colonies with disorders and the healthy ones. Indeed the two most abundant active ingredients, tau-fluvalinate and coumaphos, came most probably from varroa control measures even if tau-fluvalinate is used as an insecticide (Mavrik 2F) against *Meligethes aeneus* in oilseed rape. These active ingredients seem to be a frequent outcome of residue analyses studies [38,39,40,60,61,62]. Synergistic effects have been shown between acaricides and other molecules [33,63]. Nevertheless, our modelling did not suggest any synergistic effects in the appearance of bee disorders occurring between residues of fungicides and insecticides-acaricides. Residues of synergist, piperonyl butoxide, were also detected that indicates a prior exposure to synthetic or natural pyrethroids even though residues were not found in the analysed matrix, probably due to a fast degradation of these active

ingredients [64]. A non-authorised active ingredient was also detected: carbaryl, forbidden in Belgium since 2007, indicating an illegal agricultural or gardening use.

The only neonicotinoid found, thiacloprid, was detected in honey during the sampling period of July-August in two colonies. However, the limits of detection achieved in our study do not allow to determine the presence of neonicotinoids -with the exception of acetamiprid- or other highly toxic products like fipronil at levels in the range of the acute toxicity of these substances (30–40 µg/kg, Table 2.2). Residues of these substances could be present at lower levels and thus an exposure to these substances cannot be excluded. Indeed, Charpentier *et al.* 2014 [65] showed toxicological effects of imidacloprid in *Drosophila melanogaster* at levels of picograms per kg arguing for the need to reduce the analytical levels of quantification and detection.

According to our results, the number of fungicide residues seems to plays a role in the appearance of honey bee colony disorder.

Significantly, more fungicides residues were detected in colonies with disorders than in the healthy ones. Mainly four active ingredients, frequently used in plant protection, were detected: boscalid, iprodione, pyrimethanil and cyprodinil. Fungicides are often considered safe for honey bees based on their acute toxicity. However some direct toxic effects on bees either by the mother compounds or their metabolites have been reported in the past [66]. For example, boscalid shows low acute toxicity to bees [67], despite the fact that beekeepers in the USA have already reported losses and adverse effects on bee brood development related to the foliar application of this systemic active ingredient [68]. Incidents were often linked to the use of a co-formulation boscalid – pyraclostrobin. However, both molecules appeared above detection levels in only two colonies of our study and could not explain the general trend. Interestingly, one metabolite of boscalid, the 2-chloronicotinic acid, is similar to the 6-chloronicotinic acid, a metabolite of imidacloprid. The latter has proved to be lethal to bees at low concentrations (0.1 mg/L) following chronic exposure [69]. Further

research should be carried out in order to clarify the mechanism of bee toxicity of boscalid. Indeed, boscalid has proved toxic to other aquatic invertebrates, reducing daphnid fecundity and Chironomid emergence [68]. In addition, synergism with other active ingredients like insecticides are possible and increase the toxicity for honey bee [32,70,71]. Two other fungicides were also detected at very high levels in wax and beebread: iprodione and captan. The former is known for its synergistic effects in collaboration with insecticidal compounds [33]. The latter has been shown to induce effects on growth and development of larval honey bees [72,73]. Chronic and larval toxicity studies would be interesting at this stage in order to evaluate possible direct toxic effects on bee individuals and their behaviour. Indeed, a recent study showed increased larval mortality following chronic exposure to tau-fluvalinate, coumaphos, chlorpyrifos and chlorotalonyl or some of their combinations [74]. All these substances were found in our study. Increased mortality rates in the fall may compromise the size and age structure of the wintering cluster, which could lead to winter losses.

The indirect effects of fungicides on bees or on bee colonies are relatively little-known to date.

Fungicides may have an impact on the colony by modifying the existing microflora present in the food stores or in the bee intestinal tract [75]. Studies have already shown the possible modification of microbial composition both at beebread level [66,76] and at intestinal level [77]. This modification in the composition of microbiota may lead to dysbiosis [78]. The impact that such an unbalance in the bee gut microflora may have on bee health has already been considered. The link between the unspecific symptoms observed in our study and a possible microbial alteration could be subject of further research.

In parallel, the potential impact of microbial modification on digestibility and availability of nutrients should be a target for further research. Indeed, the content of essential amino acids might be altered when beebread is contaminated with fungicides (DeGrandi-Hoffman, 2013, pers

com.). Given the importance of nutrition, especially pollen, in the good development of the colony [79] alterations in composition or lack of essential nutrients would put the homeostasis of the colony at stake. Some studies have already shown the impact of nutritional lack on bee development and health [80]. Provided that pollen is the unique source of amino acids for honey bees, royal jelly production could also be affected [81,82] with unexpected potential consequences for its main consumers, larvae and the queen. A poor nutrition of the queen, could have as a result an impact in its activity. Likewise, a poor nutrition of the larvae has been shown to impact their development [80]. As a result, the presence of fungicides on beebread and honey may have both a direct effect on their health, but also an indirect one on the colony development.

Fungicides are widely used in agriculture and are broadly present in bee matrices, sometimes at high concentrations at levels of mg/kg [38,61, 83,84]. Boscalid, cyprodinil, iprodione are used to control a broad range of fungal pathogens including *Botrytis* spp., *Alternaria* spp. and *Sclerotinia* spp. on a wide range of crops including fruits, vegetables and ornamentals. Pyrimethanil is more specifically used to control grey mould on fruits, vegetables and ornamentals, and leaf scab on apple trees [85]. These active ingredients were already reported in bee matrices in Europe and in the USA [23,39,66]. Their frequent presence in bee matrices might be an indication of chemical plant protection intensity in all agricultural landscapes. Fungicides could also be markers of exposure to other active ingredients with higher toxicity to bees. Mixes of products like fungicides – insecticides are often applied to reduce the number of spray applications. As a result, other pesticides often used in combination with the fungicides found or applied in the same crops could have been at the origin of the effects observed in this study. However, the sensibility of the residue analyses used in our study might explain the lack of detection of such components. Further intensive monitoring and a thorough record of the agricultural practices on pesticide application in tank mixes would help clarifying this alternative explanation.

Factors different from fungicides are most likely involved in the

development of bee disorders. According to our model, the disorder probability was not absent when fungicides residues were not detected in presence of insecticide residues and virus. Non identified pathogens, chemicals or factors of different nature could be operating as silent stressors. Ravoet and colleagues (2013) [86] reported the presence of new pathogens in Belgium that were not taken into consideration in our analyses (i.e. *Crithidia mellificae* or the Lake Sinai Virus (LSV)). Other stressing factors could be also linked to the intensive agriculture. In fact, we observed an increase of colony disorders in the area with high density of crops in comparison with areas with grassland. Some studies and beekeeper claims go in line with our outcome regarding the concentration of bee problems in areas with intensive agriculture [87–90]. In the region of the study, these areas represent a large surface of cereals, which can be a source of honeydew in certain occasions. Unfortunately, exposure of bees to pesticides through this matrix remain highly understudied. Furthermore, the same negative trends on pollinators and biodiversity in areas with intensive agriculture have already been described as the result of as increased pesticide use, decreased landscape heterogeneity, loss and fragmentation of natural habitat [91,92].

In conclusion, the five virus studied (ABPV, CBPV, QBCV, SBV, DWV) do not seem determinant in the appearance of bee disorders in our study. These disorders seem clearly linked to the environment of the apiaries and were observed mainly in agricultural crop areas. We observed also that the number of fungicide residues appears as the main potential stress factor linked to bee disorder. However other stressing factors could be acting or interacting at the same time: insecticides exposure, a lack of amino acids and oligo-elements, etc. Our results open new avenues for future research in order to better understand the side effects of fungicides on the bee colony and questions the sustainability of intensive agriculture model and its impact on bees. Specific toxicological studies on both adult bees and larvae would be recommendable in order to better characterise the toxicity of fungicides.

2.5 SUPPORTING INFORMATION

Table S2.1 - Diversity of pollens collected in the apiaries before the winter.

	Apiary																
	15A	1A	21A	21B	30A	30B	34A	40A	40B	40C	41A	43A	45A	49A	49B	4A	56A
<i>Anacardiaceae</i>	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Apiaceae</i>	1	1	1	1	0	1	1	1	1	0	0	1	0	0	0	0	1
<i>Asteraceae</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
<i>Balsaminaceae</i>	1	0	0	0	0	0	0	0	1	1	1	0	1	1	1	0	0
<i>Boraginaceae</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Brassicaceae</i>	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
<i>Campanulaceae</i>	1	0	1	0	1	1	0	0	1	1	0	0	0	1	0	1	0
<i>Centaurea</i>	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
<i>Chenopodiaceae</i>	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0
<i>Cistaceae</i>	1	1	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0
<i>Epilobium spp.</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
<i>Ericaceae</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
<i>Helianthus spp.</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Hedera spp.</i>	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
<i>Lamiaceae</i>	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Oleaceae</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Phacelia tanacetifo</i>	0	0	1	0	0	1	0	1	1	1	1	1	0	0	0	0	0
<i>Plantago spp.</i>	0	1	0	1	0	1	1	1	0	0	0	0	1	0	0	1	1
<i>Poaceae</i>	0	1	1	1	1	1	0	0	1	1	0	0	1	0	1	0	1
<i>Ranunculus spp.</i>	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
<i>Rhamnaceae</i>	1	0	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1
<i>Rosaceae</i>	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	0	1
<i>Rubus fruticosus</i>	1	0	1	0	0	1	1	1	0	0	1	1	1	0	1	0	1
<i>Rumex spp.</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Sedum spp.</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Taraxacum spp.</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
<i>Trifolium spp.</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
Undertermined	1	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0
<i>Vicia spp.</i>	0	0	1	0	1	1	1	1	0	1	1	0	0	0	0	0	0
Total	16	8	14	13	12	16	14	14	11	13	10	10	16	6	6	7	14

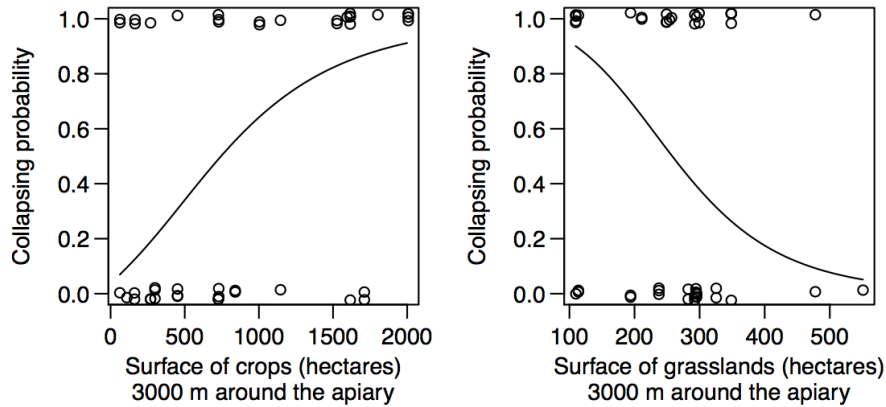


Figure S2.1 - Probability of honey bee colony disorders depending on the apiary's environment. Consideration of crop surface vs. grassland surface in a radius of 3000 m around the apiary. Crops include fruit, vegetables, fodder production and horticulture. For each graph, the value of the variable not displayed is fixed to its observed mean ($n = 53$ colonies).

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2.7 AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: NS LH EB. Performed the experiments: NS LH CM. Analysed the data: GSM LH. Wrote the paper: LH NS LAM GSM.

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Chapter 3

Determination of the possible sources of pesticide exposure through pollen

FOREWORD

From the previous chapter we learn that the colonies showing disorders contained also the largest amount of fungicides. Similarly, the occurrence of bee colony disorders was correlated with the surface of arable crops in the environment of the apiary, while no correlation could be found for viruses. In the present chapter we aimed to understand the source of exposure of bees to the pesticides found during the field observations by considering the landscape and pesticide use around the apiary. Correlations are explored between the environment of the apiaries, bees' food sources and their potential contamination with residues of pesticides. In doing so, it can be established if certain environments are more prone to specific botanical origin and sources of contamination. This study was developed in collaboration with CRA-W.

This study was published in 2017 at the Journal Scientific Reports with

title: *The challenges of predicting pesticide exposure of honey bees at landscape level*. The text included into this chapter has been slightly modified with respect to the published version.

The challenges of predicting pesticide exposure of honey bees at landscape level

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ABSTRACT

To evaluate the risks of pesticides for pollinators, we must not only evaluate their toxicity but also understand how pollinators are exposed to these xenobiotics in the field. We focused on this last point and modelled honey bee exposure to pesticides at the landscape level. Pollen pellet samples (n=60) from 40 Belgian apiaries were collected from late July to October 2011 and underwent palynological and pesticide residue analyses. Areas of various crops around each apiary were measured at 4 spatial scales. The most frequently detected pesticides were the fungicides boscalid (n=19, 31.7%) and pyrimethanil (n=10, 16.7%) and the insecticide dimethoate (n=10, 16.7%). We were able to predict exposure probability for boscalid and dimethoate by using broad indicators of cropping intensity, but it remained difficult to identify the precise source of contamination (e.g. specific crops in which the use of the pesticide is authorized). For pyrimethanil, we were not able to build any convincing landscape model that could explain the contamination. Our results, combined with the late sampling period, strongly suggest that pesticides applied to crops unattractive to pollinators, and therefore considered of no risk for them, may be sources of exposure through weeds, drift to neighbouring plants, or succeeding crops.

KEYWORDS

Apis mellifera; bioindicator; risk assessment models; plant protection product; pollen contamination; exposure

3.1 INTRODUCTION

Pollinators like bees cover very large areas every day, visiting numerous plants for nectar, pollen, or gum collection and water sources. So doing, they also unintentionally collect airborne particles or substances diluted in the air. This has led to using honey bees, a species often used as a model, and beekeeping products as biological indicators for environmental monitoring^{1–18}. Monitoring of exposure to various environmental contaminants has already been carried out; these contaminants include heavy metals^{2,5,14,15,17}, pesticides^{3,4,11–13}, polycyclic aromatic hydrocarbons^{6,7,9,10,18} and radioactivity¹⁶. Unfortunately, it is often not possible to identify the specific sources of contamination.

The exposure of honey bees to pesticides has been linked to increased probability of colony disorders and losses^{19–21}, alone or in combination with other stress-creating factors like poor nutrition or pathogen and parasite loads^{22–24}. For this reason, it is crucial to understand the possible exposure pathways of honey bees to pesticides once they are released in the environment. Pesticide risk assessment is not just about the evaluation of the toxicity of the products. Ideally, we should also be able to accurately estimate how living organisms will be exposed to these products in the environment.

Efforts to model the exposure of bees to pesticides have been carried out recently for risk assessment purposes. Some models aim to estimate direct contact exposure for spray applications²⁵, while others have focused on contact exposure through dust²⁶ or on estimating pesticide intake^{27–29}. Several routes of exposure are today aggregated for a more comprehensive estimation of the exposure of the honey bee colony³⁰. However, more quantitative data on residue levels and their impacts on bee and colony are still needed³¹.

On the other hand, models of honey bee colony dynamics already

integrate a number of stressors, and are a promising tool for impact evaluation of land management or stressors like pesticides at the landscape level^{32,33}. The quality of these models will depend on their capacity to predict the sources of contamination. The aim of this study is to test if it is feasible to identify the contamination origin by modelling the exposure probability at the landscape level.

We study the relationship between pesticide contamination of pollen pellets and both the botanical origin of the pollen and the areas of grasslands and different crops at four spatial scales around the apiaries ($n=40$). Three pesticides with different physic-chemical properties are examined in detail as case studies. The results are interpreted relative to the authorized uses of these pesticides in the different crops present in the potential foraging area of honey bees. The aim here is therefore not to evaluate the toxicity of these pesticides or the consequence of the contamination on honey bee health, but to explore the contamination pathways and to evaluate the methodology on these case studies.

3.2 METHODS

3.2.1 Field work – sample collection

A group of voluntary beekeepers were requested to participate in the study, with a total of 40 apiaries. Pollen samples ($n=80$) were taken from two random colonies per apiary with the help of a PVC pollen trap (Nicot®), placed during one or two days to collect a minimum of 20 g of pollen pellets. Samples from both colonies were pooled together. The samples were collected once, twice or four times per month from mid-July to mid-October 2011. Most of the pollen samples were collected in August and September 2011 ($n=32$ and 36 respectively). Two pollen samples were collected in July and ten samples in October. The July samples were taken on 24 and 30 July, and were similar to the August samples in terms of botanical origin of the pollen (Supplementary information 2 at

<https://figshare.com/s/86785808b5709331aa1c>). One sample collected in April 2012 was removed from the dataset before analysis because this unique sample had a completely different pollen composition and was not comparable to the other samples. Samples were placed in hermetic plastic bags and stored at -20°C until analysis.

3.2.2 Sample processing

Samples collected from the same apiary during the same month were pooled together and thoroughly mixed. One gram of the blend was sampled for palynological analysis. Whenever the sample quantity allowed it, at least 42 grams of the monthly blend were shipped for pesticide residue analysis (n = 60). Frozen samples were sent for pesticide analysis in dry ice.

3.2.3 Pesticide analyses

The monthly samples of pollen pellets were sent to Floramo Corporation, Italy. A multi-residue analysis was used based on the methodology described by Wiest *et al.*⁴⁸, and 45 pesticides/metabolites were analyzed in pollen pellets (Supplementary information 1 Table S3.1). The extraction method was based on a modified “QuEChERS method”: two-step Solid/Liquid extraction with solvent and MSPD (Matrix Solid Phase Dispersion) purification as follows: 10 g of the pollen sample extracted with acetonitrile/water followed by liquid/liquid purification with hexane and combined with MSPD purification on PSA and salts. Finally, the purified extract was concentrated below 100 µl and injected into UPLC-MS/MS (Ultra Pressure Liquid Chromatography coupled with tandem mass spectrometry) and gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) programmed in MRM (Multiple Reaction Monitor) mode with two transitions/a.i.. Pesticide analysis was possible for 28 samples from August and September each, 1 sample from July and 3 samples from October.

3.2.4 Palynological analyses

The extraction and homogenization method was inspired by the harmonized method of pollen analysis with acetolysis developed by Erdtman⁴⁹. A minimum of 1000 pollen grains were counted and identified per slide⁵⁰ at 500x microscopic magnification as described by von der Ohe *et al.*⁵¹. Pollen grains were generally identified up to their taxonomical family due to the difficulty of differentiating plant species. In a few easy cases, identification was performed up to genus level (*Taraxacum* spp., *Trifolium* spp., etc.) and to species level for ivy (*Hedera helix*) and lacy phacelia (*Phacelia tanacetifolia*).

3.2.5 Landscape data

We measured the areas of different kinds of detailed agricultural land use (i.e. different crops and grasslands) in a circle (buffer) with a radius of 500, 1000, 1500 and 3000 m around the 40 apiaries. We used the official Land Parcel Identification System (SIGEC) used by the Walloon administration to distribute agricultural subsidies to the farmers. This land use information was not available for one of the apiaries located outside the Walloon region. These detailed land use categories (n >50) were pooled into thirteen more general categories ("crops") according to their agronomic similarities (see Supplementary information 1 Table S3.2b). For each of the detailed land use categories, we determined whether the three most frequent pesticides observed in this study could be used by checking the official pesticide use authorizations in Belgium in 2011. This allowed us to calculate the areas of authorized crops for each of these pesticides around the apiaries (see Supplementary information 1 Table S3.2a).

3.2.6 Statistical analyses

All analyses were performed in R⁵². All raw datasets and R scripts are provided as supplementary information (Supplementary information 2 at

<https://figshare.com/s/86785808b5709331aa1c>). For confidentiality reasons, we are not allowed to share the exact location of the apiaries nor the agricultural land use geodata.

We considered three main datasets for the analyses: pesticides, landscape (areas of different crops and grasslands) and pollen (taxonomical origin). For the pesticides we used only presence/absence information in each pollen sample. The pollen samples had a variable number of total pollen grains (around 1000). To make direct comparison possible, the number of pollen grain (x) was standardized as $x*1000/N$ (N = total grain number in the sample) and rounded to units.

3.2.7 Predicting bees' exposure to different pesticides with crops and grassland areas

As crop areas are only available at the apiary level, we used binomial GLMs with the proportion of positive samples in a given apiary as the response variable for each pesticide. Preliminary analyses showed that the crop areas are strongly correlated, causing multicollinearity problems when they are used as predictors in multiple regression approaches. Grouping correlated predictors was not an option here because we wanted to keep them separated to interpret the results relative to the authorizations of pesticide use for each crop. Consequently, we decided to use binomial GLMs with only one explanatory variable at a time. We built separate univariate models for each grouped land use surface and for each of the four spatial scales. We also used two additional predictors (at four scales): 1) all crops: sum of the crop areas i.e. without grasslands and without taking into account the product authorizations; 2) authorized crops: sum of the crop areas for which the product is authorized. A "null model" was also built with no explanatory variable, i.e. this model estimates the mean proportion of samples contaminated in the dataset. All areas were square root transformed, because this improved the quality of the models (i.e. linearity and homogeneity of the residuals). At the lowest spatial scales (500 m and

1000 m) some of the minor crops were totally absent from all apiaries and the corresponding models were therefore not estimated.

These models were compared in terms of AICc and AICc weights₅₃ between each other and more particularly with the "null model". Models with lower AICc are considered to be better (good fit but not overly complex to allow extrapolation to other datasets), and a difference of AIC lower than 2 is often considered as negligible. In addition we computed for each single model a likelihood ratio test which compares the model to the null model. We also computed the Area Under the Receiver Operating Curve (AUC or AUROC) as a descriptive statistic of the capacity of each model to discriminate (in this dataset) between apiaries with the pesticide (frequency in the samples >0) or without it (frequency = 0). An AUC = 1 indicates a perfect discrimination capability (all predicted presences are effective and none of the predicted presences are absences). If AUC=0.5, the model predictions are as good as pure chance.

We checked the spatial correlation of the best model residuals for each pesticide with a spline correlogram. Spatial correlation was always low and not significantly different from 0.

3.2.8 Predicting bees' exposure to different pesticides with pollen composition

The pollen data are available at the sample level and there were no multicollinearity problems with these data. Consequently, we computed binomial GLMs with the presence/absence of the pesticide in the sample as response variable and the ten most common pollen types as explanatory variables. We also added the period of the year (July-August or September-October) as explanatory variable. The pollen data were $\log(x+1)$ transformed because this clearly improved the model fit. With 59 pollen samples from 39 apiaries, some pollen samples came from the same apiary and were therefore not independent. We first tried to use binomial

Generalized Linear Mixed Models with the apiary as random effect to take this pseudo-replication into account, but most of these models did not converge, probably because most of the apiaries in the sample had only one or two replicates (rarely three). Our results with the simple GLMs are therefore probably slightly anti-conservative. We removed the explanatory variable Balsaminaceae because none of the pollen samples containing grains of this family were contaminated with dimethoate. This lack of variability posed problems in the statistical analyses.

For each pesticide, we computed models for all possible combinations of explanatory variables (2048 models) along with their AICc and AICc model weight. The AICc model weight was used to compute shrinkage model averaged coefficients, unconditional standard errors and AICc variable weights⁶¹. The AICc model weight is a measure of model selection uncertainty (probability that a model will have the lowest AICc if we resample the data, given a set of models). The AICc variable weights allow us to compare the relative importance of the explanatory variables (it gives the probability that a given variable will be in the best - lowest AICc - model if we resample the data). We interpreted only the explanatory variables with an AICc variable weight > 0.6. The model averaged regression coefficients are shrunk toward 0 when the corresponding explanatory variable is present only in "bad" models.

3.3 RESULTS

We consider three main data sets for the analyses: (1) pesticide load of pollen; (2) botanical origin of pollen and (3) landscape around the apiary.

3.3.1 Pesticides

About half of the analysed samples contained at least one pesticide (n=28/60). The most frequent were two fungicides, boscalid (n=19, 31.7%)

and pyrimethanil (n=10, 16.7%), and one insecticide, dimethoate (n=10, 16.7%). Boscalid residues ranged from 0.70 to 512 µg/kg, pyrimethanil residues ranged from 0.60 to 21.70 µg/kg and dimethoate residues ranged from 0.21 to 1.4 µg/kg (Supplementary information 1 Table S3.1b). Four other active ingredients (a.i.) were detected with lower frequency (n=1): trifloxystrobin, kresoxim-methyl, cyprodinil (fungicides) and thiamethoxam (a neonicotinoid insecticide) (Fig. 3.1). Eleven samples (18.3%) contained two or more a.i. simultaneously, reaching a maximum of three a.i. per sample and five a.i. per apiary. Pollen samples collected in July-August were more frequently contaminated than those from September-October (n=18/29 and n=10/31 respectively, binomial GLM, Likelihood Ratio (LR)= 5.7, df=1, p=0.017, Fig. 3.1). However, despite the small number of October samples analysed, we were surprised to detect boscalid contaminations as late as 14 October.

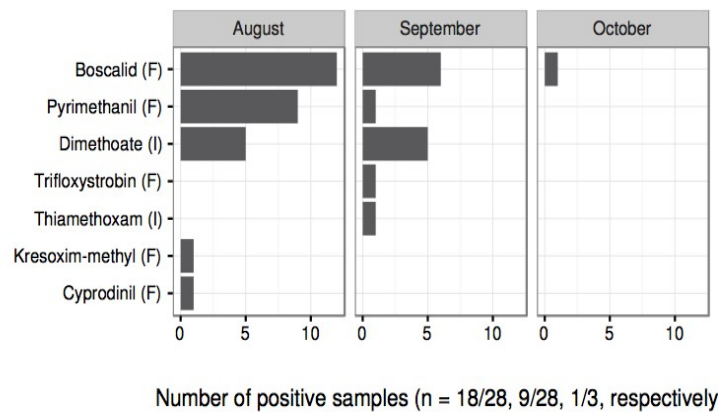


Figure 3.1 - Frequency of pollen contamination per month and for each pesticide. Two samples from July are not shown. No pesticides were detected in these two samples. Note. I= Insecticide, F= Fungicide

3.3.2 Pollen botanical origin

During the period considered (late July-October) the most abundantly collected pollen grains belonged to Brassicaceae, *Hedera elix* (ivy), *Trifolium* spp., *Phacelia tanacetifolia*, Rosaceae and Asteraceae (Supplementary information 1 Fig. S3.2b). The August samples were characterized by more diversified pollen resources with predominance of *Trifolium* spp., Rosaceae and Asteraceae (including *Taraxacum* spp.) pollen. The pollen collected in September and October was less diversified and characterized by a more intensive presence of ivy and *P. tanacetifolia* pollen (Supplementary information 1 Fig. S3.2a). Brassicaceae pollen was found to be used during the whole period under review. These most abundant pollen types were also collected by 35/40 (87.5%) of the apiaries with the exception of Rosaceae (24/40, 60%) and *P. tanacetifolia* (12/40, 30%). This indicates that *P. tanacetifolia* fields are less present around the apiaries, but that they are massively visited by bees when they are present.

3.3.3 Landscape description

A high correlation exists between different crop areas (including grasslands), especially at the highest spatial scale (3000 m radius buffers) (Supplementary information 1 Fig. S3.1c). These correlations are particularly high (0.78 - 0.93) for predictors cereals, beet, potato and vegetables (Supplementary information 1 Table S3.2a). Grassland areas are negatively correlated with most crop areas. The exploratory analysis showed a gradient in landscape composition around the apiaries: from landscapes dominated by crops to landscapes dominated by grasslands or with very little agricultural land use (urban or forest zones). The samples contaminated with pesticides are clearly more frequent in landscapes dominated by crops (Fig. 3.2). Cereals are present around all apiaries in 3000 m buffers. However, zooming into a radius of 500 m around the apiaries, one can divide the landscape into three groups: (1) dominance of cereals, beets and potatoes corresponding to the most intensive agricultural landscape; (2) dominance of cereals and grasslands (without beet and potato

crops) corresponding to more extensive crop landscapes; and (3) areas dominated by grasslands and without cereals, beets and potatoes (clustering with heat map - see Supplementary information 1 Figs. S3.1a and S3.1b).

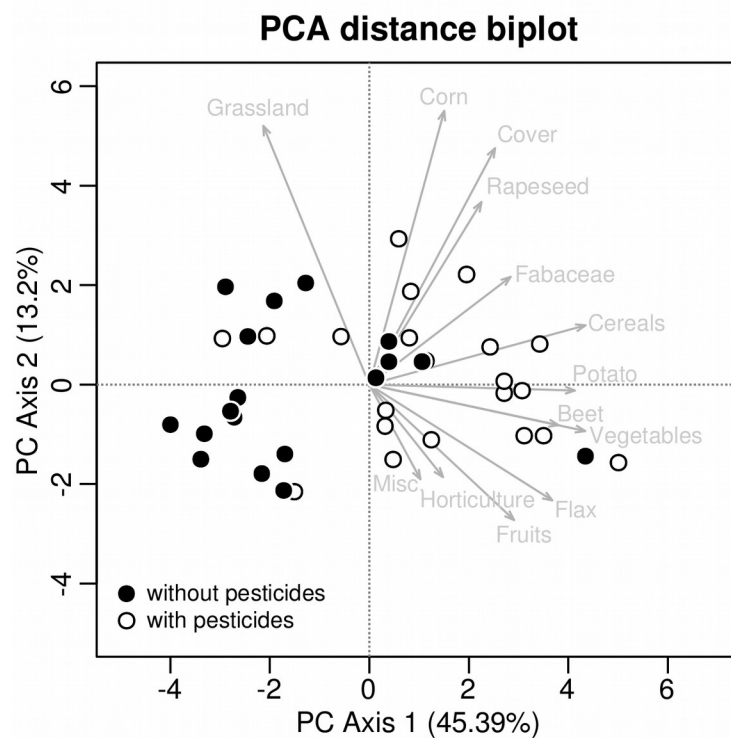


Figure 3.2 - Principal Component Analysis distance biplot of the areas of crops and grasslands 3000 m around the apiaries. The areas were square root transformed and standardized before the analysis.

Case study 1 – Predicting bees' exposure to boscalid.

Boscalid is a carboxamid fungicide³⁴ with systemic properties (octanol-water partition coefficient, log Kow = 2.96). In addition, this active substance is persistent in soil (DT50 typical = 200 days³⁵. In 2011, it was authorized in Belgium for the treatment of cereals, potatoes, rapeseed, many vegetables, fruits and horticulture.

We aimed to identify specific sources of boscalid contamination for bees. However, all crops have a predictive power with the exception of variables corn, rapeseed, cover crops and horticulture (AICc lower than the null model or with a difference <2, Table 3.1). The ten best models include as predictors: all crops and authorized crops areas at 3000 m, 1500 m and 1000 m and beet, potato and cereals at 3000 m (AICc differences > 18; Area Under the Receiver Operating Curve (AUC) 0.83-0.92). The models using 3000 m buffer data systematically have a better predictive performance, while the models using 500 m buffers systematically have the lowest one. A beet area in a 3000 m buffer is the best predictor of boscalid contamination despite the fact that the use of boscalid is not authorized in this crop. This predictor is four times better (ratio of AICc weights) than the second, using all crops as variable (i.e. areas of all crops combined) and more than 15 times better than the third, including potato and cereals areas in 3000 m (i.e. crops for which boscalid is authorized).

The predicted probability of boscalid contamination is close to 0 when no boscalid-authorized crops are present in a radius of 3000 m around the apiary and rises to 0.9 for areas of boscalid-authorized crops of 1500 ha (Fig. 3.3).

As for the botanical origin, Rosaceae and *P. tanacetifolia* pollen are systematically the most important predictors of boscalid contamination (AICc variable weight > 0.71- Table 3.2, positive relationship). There is no difference in boscalid contamination between July-August and September-October (AICc variable weight = 0.267).

Chapter 3 – Predicting pesticide exposure of honey bees at landscape level

Table 3.1. Results of univariate Binomial GLMs modelling the probability that a pollen sample would be contaminated by a given pesticide vs the areas of different (groups of) crops and grasslands at different spatial scales (Buffer column, in meters). Only the ten best models (lowest AICc) are shown along with the null model. LRT = Likelihood Ratio Test statistic (degrees of freedom = 1 for all models). Full tables available in the Supplementary information 1.

ID	Buffer	Variable	Boscalid		AICc w	AUC	Slope	LRT	p
			AICc	Δ AICc					
1	3000	Beet	40.01	0	0.604	0.894	0.33	29.14	<0.0001
2	3000	All Crops	42.81	2.798	0.149	0.894	0.18	26.34	<0.0001
3	3000	Authorized Crops	44.76	4.75	0.056	0.883	0.19	24.39	<0.0001
4	3000	Potato	45.24	5.231	0.044	0.920	0.36	23.90	<0.0001
5	3000	Cereals	45.74	5.734	0.034	0.863	0.21	23.40	<0.0001
6	1000	All Crops	46.06	6.054	0.029	0.846	0.47	23.08	<0.0001
7	1500	All Crops	46.4	6.386	0.025	0.851	0.31	22.75	<0.0001
8	1500	Authorized Crops	47.03	7.016	0.018	0.854	0.34	22.12	<0.0001
9	1000	Authorized Crops	47.35	7.344	0.015	0.84	0.53	21.79	<0.0001
10	500	All Crops	48.79	8.779	0.007	0.834	0.7	20.36	<0.0001
11-41				(...)					
42	-	NULL MODEL	66.92	26.91	0	0.5	-	-	-
43-55				(...)					

ID	Buffer	Variable	Pyrimethanil		AICc w	AUC	Slope	LRT	p
			AICc	Δ AICc					
1	3000	Rapeseed	42.18	0	0.287	0.735	0.39	8.55	0.00346
2	1000	Rapeseed	43.69	1.515	0.134	0.756	0.61	7.03	0.00801
3	1500	Rapeseed	44.25	2.074	0.102	0.731	0.45	6.47	0.01095
4	3000	Flax	46.15	3.97	0.039	0.793	0.24	4.58	0.03240
5	3000	Horticulture	46.82	4.64	0.028	0.641	-1.93	3.91	0.04807
6	500	Beet	47.09	4.908	0.025	0.683	0.47	3.64	0.05643
7	3000	Cover	47.36	5.178	0.022	0.63	0.48	3.37	0.06643
8	500	Potato	47.36	5.184	0.021	0.659	0.46	3.36	0.06666
9	1000	Fabaceae	47.66	5.482	0.018	0.619	0.52	3.07	0.07999
10	3000	Cereals	47.78	5.599	0.017	0.719	0.07	2.95	0.08600
11-12				(...)					
13-55	-	NULL MODEL	48.5	6.322	0.012	0.5	-	-	-
14-55				(...)					

Table 3.1 – Continue

ID	Buffer	Variable	Dimethoate			AUC	Slope	LRT	p
			AICc	ΔAICc	AICc w				
1	1000	Cereals	16.52	0	0.989	0.991	3.32	37.55	<0.0001
2	1000	All Crops	26.93	10.4	0.005	0.955	0.93	27.15	<0.0001
3	1500	Beet	29.61	13.08	0.001	0.942	0.77	24.47	<0.0001
4	1000	Beet	30.29	13.76	0.001	0.946	0.97	23.79	<0.0001
5	1500	Cereals	30.68	14.15	0.001	0.906	0.62	23.40	<0.0001
6	1500	Authorized Crops	31.37	14.85	0.001	0.92	0.67	22.70	<0.0001
7	1500	All Crops	31.4	14.88	0.001	0.929	0.46	22.67	<0.0001
8	1000	Authorized Crops	33.26	16.73	0	0.924	0.72	20.82	<0.0001
9	3000	Beet	33.73	17.21	0	0.915	0.36	20.34	<0.0001
10	3000	Authorized Crops	34.36	17.84	0	0.924	0.3	19.71	<0.0001
11-37				(...)					
38	NA	NULL MODEL	51.85	35.32	0	0.5	-		-
39-55				(...)					

Table 3.2 - Results of the model selection for the GLMs modelling the presence of pesticides in the pollen vs the abundance of different pollen taxa. "w" = AICc variable weight, "coef" = models averaged coefficient, "se" = unconditional standard error. We interpreted only the explanatory variables with $w > 0.60$ (in bold). Intcpt = model intercept and SepOct = binary explanatory variable corresponding to the period: July/August or September/October. Abbreviation of the pollen types: api = Apiaceae, ast = Asteraceae, bal = Balsaminaceae, bra = Brassicaceae, ivy = Hedera elix, pha = Phacelia tanacetifolia, ros = Rosaceae, tar = Taraxacum spp., tri = Trifolium

Boscalid				Pyrimethanil				Dimethoate			
	w	coef	se		w	coef	se		w	coef	se
Intcpt	1	-2.760	1.456	Intcpt	1	-4.179	2.274	Intcpt	1	-3.413	1.614
ros	0.834	0.323	0.158	SepOct	0.959	-3.282	1.288	vic	0.996	0.822	0.269
pha	0.715	0.206	0.119	bra	0.952	0.747	0.341	pha	0.768	0.296	0.160
tar	0.538	0.173	0.132	ivy	0.25	0.002	0.064	ros	0.388	0.109	0.116
bal	0.433	-0.108	0.102	tar	0.248	0.028	0.071	tar	0.28	0.054	0.087
vic	0.395	0.103	0.103	api	0.244	-0.021	0.067	SepOct	0.263	-0.124	0.374
ivy	0.35	0.062	0.071	pha	0.242	-0.014	0.045	api	0.261	-0.043	0.092
bra	0.332	0.056	0.066	ast	0.24	0.017	0.072	ivy	0.26	-0.027	0.061
api	0.273	0.039	0.067	tri	0.24	-0.016	0.065	tri	0.25	0.03	0.071
SepOct	0.267	-0.019	0.321	ros	0.237	0.004	0.048	ast	0.246	-0.029	0.084
ast	0.243	-0.004	0.058	vic	0.232	-0.011	0.068	bra	0.23	0.007	0.055
tri	0.231	0.002	0.042								

Case study 2 – Predicting bees' exposure to pyrimethanil.

The second most frequently detected pesticide is pyrimethanil, a fungicide with systemic properties ($\log K_{ow} = 2.84$) and moderate persistence in soil (typical $DT_{50} = 55$ days)³⁶. In 2011 it was authorized in Belgium for fruit production and horticulture (including plant nurseries and Christmas trees).

It was impossible to predict pyrimethanil contamination based on its authorized uses (Table 3.1). We even found contaminated pollen samples coming from apiaries with no crops in a radius of 3000 m for which pyrimethanil use is authorized (Fig. 3.3). The only crop that could predict the frequency of pyrimethanil contamination to a certain extent was rapeseed, for which pyrimethanil use was not authorized in Belgium in 2011 (AICc difference with the null model: 6.3-4.4). However, the AUCs of these rapeseed models are quite low (0.73 - 0.75), meaning that their discrimination capability is not very good with this dataset and should be even lower with a different testing dataset. Areas of flax and horticulture in a radius of 3000 m are weak predictors of pyrimethanil contamination (AICc difference 3.3 and 1.7, respectively). Pyrimethanil use is authorized for horticulture, but the model slope is negative, which means that we tend to observe less pyrimethanil contamination when the horticulture areas increase.

Among the models considering the pollen types, both the sampling period and the Brassicaceae pollen are good predictors of the presence of pyrimethanil (AICc variable weight >0.95, Table 3.2). The contamination is significantly more frequent in July-August than in September-October. After controlling for the period, we find a strong positive relationship between pyrimethanil and the abundance of Brassicaceae pollen. This pollen probably comes from wild plants or cover crops like mustard, since rapeseed does not bloom at this time of the year in Belgium. Hence these pollen results (pyrimethanil-Brassicaceae) do not particularly support the

landscape results (pyrimethanil-rapeseed).

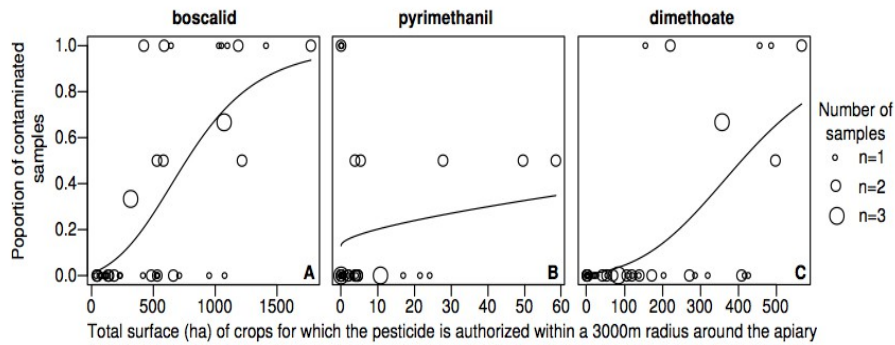


Figure 3.3 - Observed proportion of samples contaminated for each pesticide and the corresponding predicted value (binomial GLM) relative to the areas of authorized crops around the apiary.

Case study 3 – Predicting bees' exposure to dimethoate.

Dimethoate is an organophosphorous insecticide, systemic ($\log K_{ow} = 0.704$), and non-persistent in soil (typical $DT_{50} = 2.6$ days)³⁷. It was authorized in 2011 in Belgium to control insect pests in beets, peas, multi-annual fruit production, many vegetables, horticulture (incl. plant nurseries and Christmas trees), and until 2010 for potatoes.

Cereals, beet, all crops and authorized crops areas were the best predictors of pollen contamination by dimethoate (ten best models with differences of AICc relative to the null model between 35 and 17, AUC between 0.92 and 0.99, Table 3.1). Many other crop areas also had a good predictive power: vegetables, flax, grassland, horticulture, or potato. The models that consider the cropped areas at 1000 m and at 1500 m systematically had better predictive power, while the 500 m spatial scale systematically provided the worst models. Cereal areas in a 1000 m buffer

were by far the best predictors of dimethoate contamination (AICc difference = 10.40), having a very high explanatory power (AUC = 0.99; AUC = 0.955 for the second best model).

As for the botanical origin, the probability of pollen contamination by dimethoate increases when pollen samples contain more *Vicia* spp. or *P. tanacetifolia* (AICc variable weight >0.76, Table 3.2). There is no evidence for a difference of contamination between July-August and September-October.

3.4 DISCUSSION

Contamination of pollen pellets provide a representative image of flower contamination at a moment in time in contrast with the pollen that is stored in the beehive as beebread. The variety and frequency of pesticides detected in pollen pellets of our study is surprising for two reasons. Firstly, we did not expect pollen pellets to be contaminated with such a variety of pesticides at the latitude of Belgium and at this period of the year (late July-October) because few pesticides are applied so late in the season. Secondly, most of the crops with authorized uses for the detected pesticides do not bloom at this time of the year, which is confirmed by the botanical origin of pollen samples, containing mainly pollen from wild flowers and crops grown as cover or catch crops. As a result, the current approach of pesticide risk assessment and authorization based on the attractiveness to bees of different crops proves to be erroneous. Our results entail that pesticides applied to crops unattractive to bees as food sources like cereals or sugar beets can in fact be a source of exposure, either through weed contamination, drift or by the mobilization of residues of systemic/persistent products by succeeding crops. These results provide strong evidence that the concept of “crop not attractive to bees” is irrelevant to evaluating the risk of pesticide exposure. This conclusion is further supported by our landscape analyses. Our results add support to an increasing body of evidence indicating that pesticides applied to a crop are

much more mobile than expected. Contamination of pollen pellets collected by bees late in the season, outside the period of pesticide application, has also been observed in other studies^{21,38,39}, and contamination of wild flowers of the field margins has also been described^{40–42}.

Simon-Delso *et al.* 2014¹⁹ described pesticides found in beebread collected during the same period of the year as the present study in 21 apiaries. Boscalid, the most frequently detected molecule in pollen pellets (present study), was observed in beebread as the third most frequent residue. Pyrimethanil was reported in ten samples in pollen pellets, and was the sixth most common contaminant in beebread, after the fungicide iprodione and the synergist piperonyl butoxide. The insecticide dimethoate was detected in pollen pellets but not in beebread. Of the a.i. detected only once, only trifloxystrobin was also found in beebread. These different contamination profiles indicate the value of analysing different beekeeping matrices. Pesticides with low persistence like dimethoate are more likely to be found in pollen pellets than in beebread because they are collected at the beehive entrance, while beebread is pollen processed and stored within the colony during longer periods. However, this also indicates how difficult it is to characterize the whole range of pathways of pesticide exposure for honey bees.

There are long debates about the foraging radius of honey bees around their colony, most likely due to the variability of and dependence on the resources available in the surroundings, the weather conditions and the needs of the colony⁴³. In our study, based on pollen samples collected from August to October, the models with best predictive value for pollen contamination were almost systematically those that considered crops located in a radius of more than 500 m, and up to 3000 m from the colony. Future work could test if differences in foraging range are translated into differences of pesticide exposure. However, the higher predictive power of 3000 m models may be independent from the foraging distance of bees, as crop surfaces are an indicator of agricultural practices in the surroundings of the apiary (crops rotations, more/less intensive agriculture, etc.; see below).

We aimed to identify potential contemporaneous boscalid uses to explain direct pollen contamination. Boscalid-based products are authorized for a wide range of crops. They can still be used in orchards in August-September, a few weeks before the fruit harvest. They can also be used in August on beans. Consequently, direct exposure to treated crops like vegetables or orchards should be considered. However, the best predictor of boscalid contamination in pollen samples is sugar beet area in a radius of 3000 m, despite the fact that boscalid use is not authorized for this crop. Sugar beet is typically included in a crop rotation scheme with cereals and potatoes (sugar beet or potato/wheat/barley), in the most intensive agricultural areas of Belgium (Sandy-Loam Region). In contrast with cereals that are widely farmed even in less intensive areas, sugar beet may be a better indicator of cereals farmed in a more intensive way and hence potentially receiving more pesticides. After the beet model, all crops is the model with best predictive value and the most frequently found among the ten most predictive models (at all four landscape scales), followed by authorized crops. This, together with the fact that individual crops (i.e. potatoes and cereals) complete the list of the ten most predictive models, indicates that direct exposure to treated crops is unlikely to be the only source of contamination, as pesticides containing boscalid are typically applied to these crops much earlier in the season. Furthermore, with the exception of beans, none of the crops for which boscalid is authorized are attractive to honey bees for food collection at this time of the year. The high persistence of the molecule could possibly explain contamination later in the year or even during the next year. This may lead to the contamination of wild flowers or of succeeding crops like cover/catch crops. Contamination linked to cover/catch crops is supported by the positive relationship between boscalid presence and the abundance of *P. tanacetifolia* pollen observed in this study. *P. tanacetifolia* is not native in Belgium and is only used as a catch crop and rarely in gardens. Other persistent and systemic pesticides (e.g. neonicotinoids) can contaminate wild flowers in field margins^{41,42} and succeeding crops⁴⁴⁻⁴⁷.

The case of pyrimethanil pollen contamination is difficult, because none of the authorized uses predict its presence. In addition, we found a

number of positive samples ($n=4$) with no crops for which this pesticide was authorized in a radius of 3000 m around the corresponding apiary. At this point, we considered the following hypotheses: (1) the contamination came from further than 3000 m, which could be very possible given that at these time of the year the radius of foraging seems to be larger than 3 km in similar latitudes⁴⁴; (2) the notification of crop areas for which this fungicide is authorized is not complete (e.g. Christmas trees, some horticultural or vegetable crops); (3) there is an illegal use of pyrimethanil in rapeseed earlier in the season (rapeseed is already harvested at this time of the year but the product is moderately persistent). The later hypothesis seems unlikely because there are many other efficient fungicides authorized for rapeseed and the models' discriminatory power was quite low for this crop. Pyrimethanil-based products are also used in orchards at the beginning of the season, but we found no support for a pyrimethanil-Fruit areas relationship. These products could also be used for the production of some specific vegetables like peas, beans, and other legumes. However, in the year of the study, 2011, these uses were not authorized in Belgium. Pea fields that are typically harvested in July could be followed by mustard as cover/catch crop. This would match the link we found between the residues of pyrimethanil and the pollen of Brassicaceae in August. Models specifically using areas of cultivated peas and broad beans (instead of the grouped category fabaceae) showed better predictive value at 1500 m and 1000 m (difference of AICc = 3.60 and 2.85, Likelihood Ratio Test statistic (LRT p values = 0.016 and 0.024), but their discrimination power was not very high (AUC = 0.67 and 0.68 - see Supplementary information 1 Table S3.6 for details). As a result, this hypothesis remains only a putative scenario that should be tested by specific sampling on peas in the field.

Dimethoate is not a persistent pesticide. We can therefore assume that pollen contamination came from an application during August-September. The best predictors of the presence of dimethoate in pollen were cereals (non-authorized use and crop already harvested at this time of the year), beet (authorized use, but unlikely to be applied because there are no insect pests at this time of the year), and all crops. At this time of the year, vegetables (e.g. carrots and Brussels sprouts) are the only crops possibly

being treated with dimethoate for which the area can be used to predict the frequency of dimethoate in our models. As a result, the fact that cereals or sugar beets are the best predictors for dimethoate contamination could be because these large arable crops are good indicators of intensive, large scale, vegetable production, which may be included in crop rotation schemes: there is a strong correlation ($R>0.77$) between vegetables and beet, cereals and potato areas (Supplementary information 1 Fig. S3.1c). However, none of these vegetable crops are in bloom at the sampled period (except occasionally carrots), which made us wonder about the pertinence of this hypothesis. The palynological results show that pollen from *Vicia* spp. and *P. tanacetifolia* are positively linked with the presence of dimethoate. It is common agricultural practice in the region to include flowering strips in the borders of vegetable fields. Therefore, a possible explanation could be that *Vicia* spp. occur in field margins or that *P. tanacetifolia* is planted in flowering strips and that their flowers get contaminated by drift with dimethoate applied on the field. The abundance of *Vicia* spp. pollen is positively correlated to vegetables, beet, potato and cereals areas (see Supplementary information 1 Fig. S3.3).

In conclusion, our findings show that the highest spatial scales (3 km) provide the best predictive power for pollen contamination. Pesticides applied to “non-bee-attractive” crops like cereals or sugar beets, generally considered of negligible risk for bees, can in fact be a source of exposure through weeds, through drift to neighbouring plants or through succeeding crops. These results imply that the concept of “bee-attractive crop” (i.e. a crop visited by bees for nectar and/or pollen collection) is irrelevant for risk assessment and should not be used as a criterion for pesticide authorization. At the landscape level, honey bee exposure to pesticides depends on pesticide use level, physicochemical characteristics, period of the year and landscape composition. Our findings show that the task of modelling the exposure of bees to pesticides once released in the environment may be more complicated than expected. We were able to efficiently predict exposure for two pesticides by using very broad indicators of cropping intensity, but it remains difficult to track the direct source of contamination in the landscape. For the third pesticide, we were not able to find any

convincing landscape model that could explain the contamination. On the other hand, our results have consequences for policies and agricultural practices intended to promote the multiplication of nutritional resources for pollinators, like flowering strips, buffer zones, catch crops with melliferous flowers, etc. These should be designed and applied in parallel to policies and practices leading to pesticide use reduction i.e. integrated pest management, organic farming or agro-ecological practices, precision farming and favouring non-persistent/non-systemic pesticide active ingredients. Without such considerations, instead of favouring pollinators through habitat improvement or food availability, we may transform these areas into highly risky zones or even ecological traps for pollinators.

3.5 SUPPORTING INFORMATION

The following tables and figures have been extracted from the Supplementary Information of the article Simon-Delso, Noa, Gilles San Martin, Etienne Bruneau, Christine Delcourt, and Louis Hautier. 2017. ‘The Challenges of Predicting Pesticide Exposure of Honey Bees at Landscape Level’. *Scientific Reports* 7 (1): 3801 for facilitation of comprehension of the chapter. However, extensive supplementary information have been made available and can be consulted at <http://www.nature.com/articles/s41598-017-03467-5>.

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Table S3.1a - Pesticides analysed + LOQ. Summary of all pesticides (and some products of degradation) that have been looked for into the trap pollen samples + LOQ values in µg/kg (10⁻³ mg/kg)

fullname	type	LOQ_Pollen
Amitraz	A	5
Beta-cyfluthrin	I	5
Bifenthrin	I	3
Boscalid	F	7.5
Captan	F	7.5
Carbaryl	I	0.75
Chlorpyrifos	I	5
Clothianidin	I	0.75
Coumaphos	A	5
Cyfluthrin	I	5
Cyprodinil	F	5
Deltamethrine	I	5
Dimethoate	I	0.75
Esfenvalerate	I	7.5
Fenpropimorph	F	7.5
Fipronil	I	1
Heptenophos	I	7.5
Imidacloprid	I	0.75
Indoxacarb	I	7.5
Iprodione	F	7.5
Kresoxim-methyl	F	7.5
Lambda-cyhalothrin	I	7.2
Piperonyl butoxide	S	7.5
Pirimicarb	I	5
Propamocarb	F	5
Pyraclostrobin	F	5
Pyrimethanil	F	5
Tau-fluvalinate	IA	7.5
Tebuconazole	F	7.5
Tebufenozide	I	7.5
Terbuthylazine	H	7.5
Thiacloprid	I	0.75
Thiamethoxam	I	0.75
Thiophanate-methyl	F	5
Trifloxystrobin	F	7.5

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Zoxamide	F	7.5
Fipronil carboxamide	I	7.5
Fipronil desulfinil	I	7.5
Fipronil sulfone	I	7.5
5-OH Imidacloprid	I	7.5
Desnitroimidacloprid	I	7.5
6-Cloronicotinc acid	I	7.5
Olefin	I	7.5
Urea Derivate	I	7.5

Table S3.1b - Summary table of the pesticides found in pollen samples. Type : F = Fungicide, I = Insecticide. Nb = number of positive samples. NbTot = total number of samples analysed. The columns Min, Max, Median, Mean and SD (Standard deviation) provide descriptive statistics of the quantities (mg/kg) observed in the positive samples only.

Pesticide	Type	Nb	NbTot	Min	Max	Median	Mean	SD
Boscalid	F	19	60	0.70	512.00	4.10	37.93	116.50
Pyrimethanil	F	10	60	0.60	21.70	1.90	4.93	6.60
Dimethoate	I	10	60	0.21	1.40	0.40	0.53	0.40
Trifloxystrobin	F	1	60			15.40		
Thiamethoxam	I	1	60			1.60		
Kresoxim-methyl	F	1	60			2.40		
Cyprodinil	F	1	60			1.50		

Table S3.2a - Landscape land use categories used. In the following table we show how we have used the different agricultural land use informations that were available :

- *Agri*: is the detailed land use types provided by the SIGEC (agricultural aids administration). The name are in French but most of them have been translated in the paper.
- *Agri_group*: we have grouped the detailed *Agri* into these more general denominations to reduce the number of categories
Areas : sum of the areas (ha) in the 3000 m buffers for all the apiaries
- *Dime*, *Pyri*, *Bosc*: is the use of dimethoate, pyrimethanil, boscalid authorized at least partially for each detailed culture type? When we have calculated the areas of cultures authorized for each pesticide, we used the detailed *Agri* codes before grouping. For example for dimethoate, the authorized *Fabaceae* areas are only using the *Peas* areas, not the other detailed cultures that have been grouped under the name of *Fabaceae* for other analyses.

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Agri	Agri_group	Areas	Bosc	Dime	Pyri
Betterave sucrière	Beet	4578	yes		
Chicorée à inuline	Beet	675	yes		
Betterave fourragère	Beet	63.7	yes		
Chicorée à café	Beet	3.4	yes		
Froment d'hiver	Cereals	12986			yes
Orge d'hiver	Cereals	3004			yes
Epeautre	Cereals	600			yes
Froment de printemps ou froment alternatif	Cereals	287.5			yes
Avoine	Cereals	151.4			yes
Triticale	Cereals	101.5			yes
Orge de printemps	Cereals	74			yes
Autres grains (p.e mélanges)	Cereals	62.7			
Orge de brasserie	Cereals	26.8			yes
Autres céréales (Sorgho, millet, alpiste et blé dur)	Cereals	8.6			
Seigle d'hiver	Cereals	0.9			yes
Maïs ensilage	Corn	3976			
Maïs grain	Corn	696.2			
Tournière enherbée	Cover	565.5			
Autres couvertures (Moutarde, Phacélie, etc.)	Cover	47			
Autres couvertures dont "mélange certifié"	Cover	18.6			
Pois (autres que récoltés secs)	Fabaceae	390.2	yes		
Luzerne	Fabaceae	161.2			
Mélange protéagineux (culture principale) + céréales	Fabaceae	95.2			
Fèves et féveroles (sec)	Fabaceae	30			
Mélange graminées et légumineuses	Fabaceae	25.4			
Trèfle	Fabaceae	24			
Pois (sec)	Fabaceae	22			
Légumineuses	Fabaceae	0.1			
Lin textile	Flax	599.1			
Lin oléagineux	Flax	4			
Cultures fruitières pluriannuelles	Fruits	212.8	yes	yes	yes
Cultures fruitières annuelles	Fruits	11.7		yes	yes
Vignes	Fruits	0.9		yes	yes
Prairie permanente	Grassland	15902			
Prairie temporaire	Grassland	1371			
Pâturage à statut particulier	Grassland	52.5			
Cultures horticoles non comestibles	Horticulture	15	yes	yes	yes
Sapins de Noël	Horticulture	5	yes	yes	

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Pépinières	Horticulture	4.9	yes	yes
Autres fourrages	Miscellaneous	428.2		
Couvert naturel	Miscellaneous	37		
Boisement	Miscellaneous	11.6		
Miscanthus	Miscellaneous	7.4		
Terres retirées de la production	Miscellaneous	7.3		
Sarrasin	Miscellaneous	1.8		
Chanvre non-alimentaire autre que chanvre textile	Miscellaneous	0.1		
Pomme de terre de consommation	Potato	2734		yes
Pomme de terre (Plants)	Potato	114.6		yes
Pomme de terre féculière	Potato	31.7		yes
Pomme de terre (arrachage avant le 1er aout)	Potato	8.8		yes
Pomme de terre (primeur, arrachage avant le 20 juin)	Potato	0.2		yes
Colza et navette d'hiver	Rapeseed	941.5		yes
Colza et navette de printemps	Rapeseed	11.5		yes
Cultures maraîchères	Vegetables	587.7	yes	yes
Cultures maraîchères sous verre	Vegetables	1.8		
Plantes aromatiques	Vegetables	0.1		yes

Table S3.2b - Total areas (ha) for each grouped agricultural land use category in the 3000 m buffers around the apiaries and the areas of authorized crops for each pesticide. The small differences between the areas for a same grouped land use are due to the fact that we considered the authorizations of each product relative to the detailed land uses.

Grouped land use	Total	Bosc	Pyri	Dime	Detailed land uses
Cereals	16695	16632	0	0	wheat, barley, spelt, oat, triticale, etc.
Grassland	15993	0	0	0	permanent and temporary grasslands
Beet	5190	0	0	5190	sugar and fodder beet, root chicory
Corn	4211	0	0	0	grain and fodder corn
Potato	2711	2711	0	0	mainly potato for food
Rapeseed	947	947	0	0	rapeseed (mainly sown before the winter)
Fabaceae	727	0	0	386	peas, alfalfa, mixed protein crops, etc.
Cover	611	0	0	0	cover crops and set-aside field borders
Flax	593	0	0	0	flax
Vegetables	521	520	0	520	vegetables (including glasshouses)
Miscellaneous	456	0	0	0	misc. land uses, mainly "other fodder"
Fruits	225	225	225	213	perennial and annual fruits, vineyards
Horticulture	25	15	25	25	plant and tree nurseries, Christmas trees

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Table S3.6 - Pyrimethanil vs peas crops models. Here we examine the hypothesis that pyrimethanil treatments of peas could explain pyrimethanil contaminations (in the previous analysis, peas are in the grouped culture Fabaceae with other crops from this family) The peas models performs better than the null model and that the previous Fabaceae models however these models are not very good (difference of AICc low and AUC low).

ID	Product	Buffer	Variable	AICc	AICc.delta	AICc.w	AUC	slope	LRT	p
1	pyri	1500	peas	44.9	0	0.436	0.665	0.51	5.82	0.01582
2	pyri	1000	peas	45.66	0.752	0.299	0.676	0.66	5.07	0.02433
3	pyri	3000	peas	46.85	1.942	0.165	0.739	0.27	3.88	0.04884
4	pyri	NA	NULL MODEL	48.5	3.598	0.072	0.5	NA	NA	NA
5	pyri	500	peas	50.37	5.47	0.028	0.481	-0.61	0.35	0.5526

Fig. S3.1a and S3.1b - Heatmaps of the landscape dataset. Method: The row dendrogram is based on Euclidean distance. The column dendrogram is based directly on the correlation matrix as similarity matrix. Both row and columns dendrogram use the Ward agglomerative algorithm. The blue coloured band close to the row dendrogram represent the number of different pesticides for each apiary (white = 0, deepest blue = 5). The numbers represent the surface in hectares.

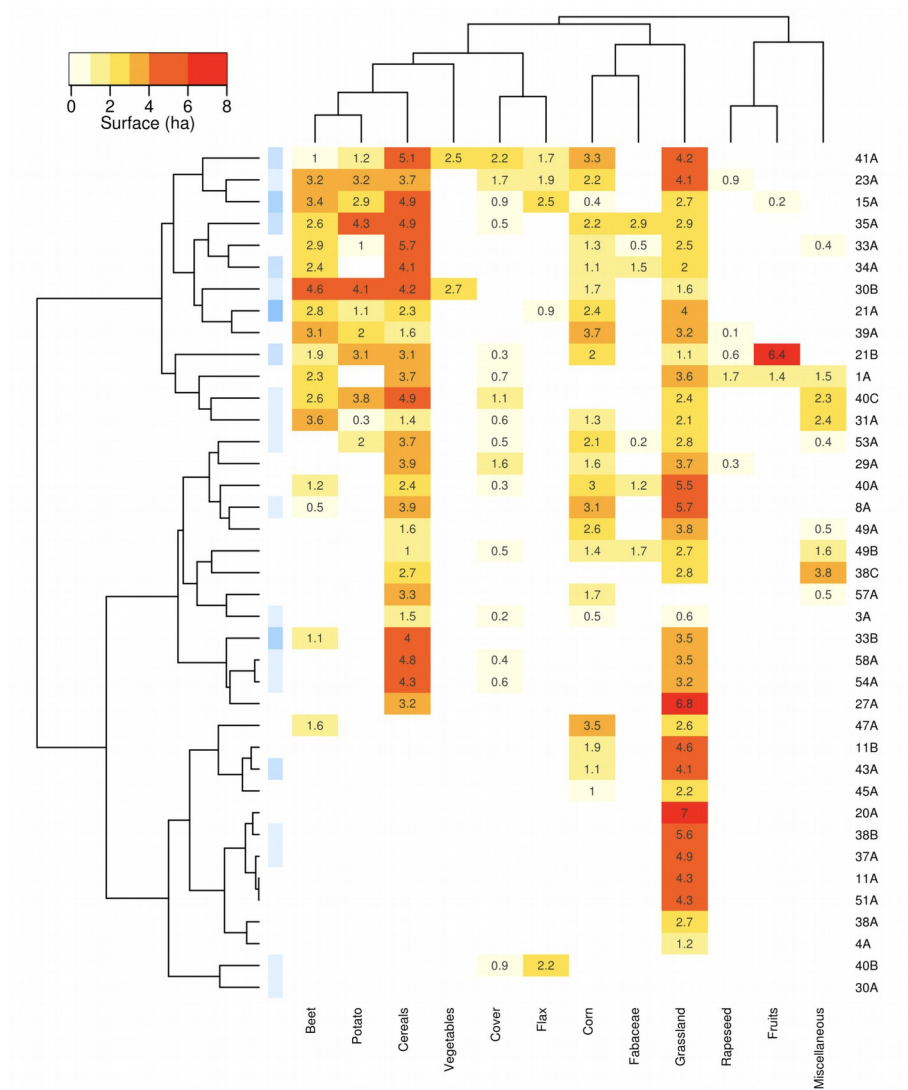


Fig. S3.1a - 500 m buffer heatmap. The 500 m buffer heatmap is quite informative. One can clearly see that there are 3 groups of sites. One group with beet and potato cultures (on top), one group without beet and potato cultures but with cereals (in the middle) and one group with very few crops and generally more grasslands (at the bottom + 2 sites without major crops and without grasslands probably more urbanized areas)

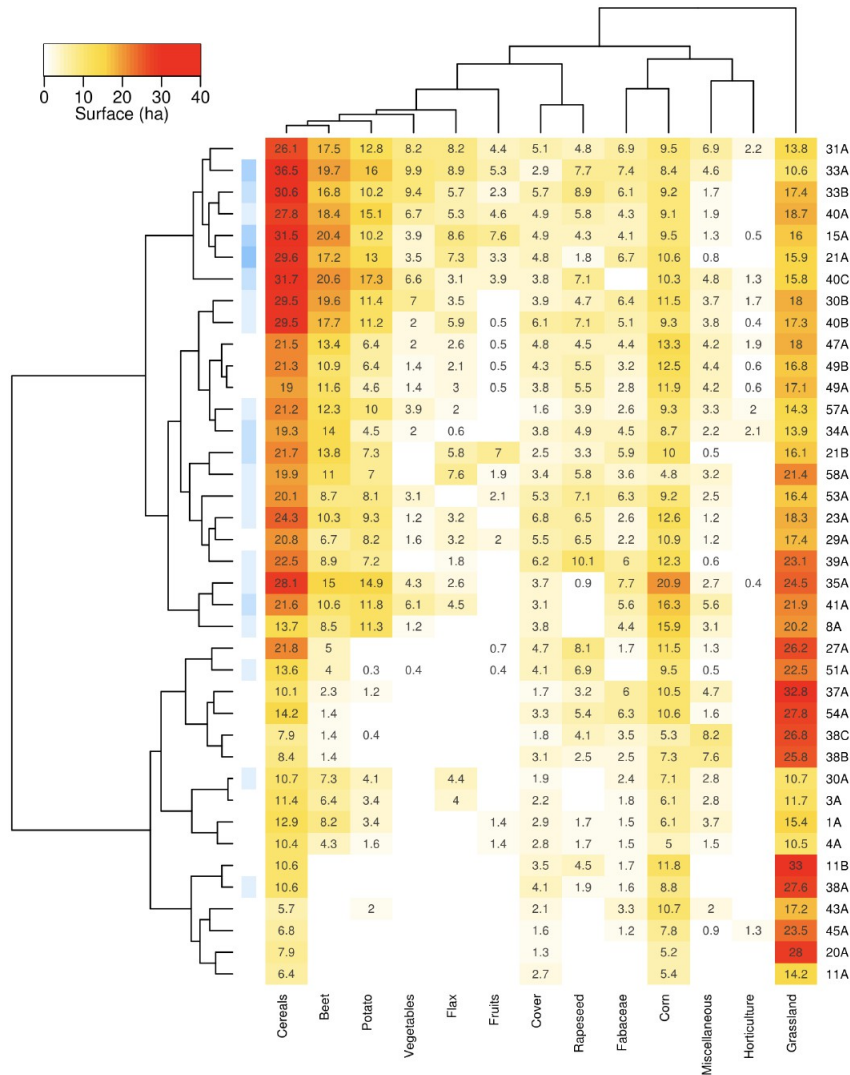


Fig. S3.1b - 3000 m buffer heatmap. The 3000 m heatmap is less structured. One can see 2 clear groups: one with few big crops at the bottom (with or without grasslands) and one with lots of crops on top, particularly beet, potatoes, vegetables, etc. In fact there is a clear gradient in the dataset from sites with a lot of crops toward sites with few crops (either with grasslands or with non agricultural land uses). Samples with pesticides are clearly in the second group.

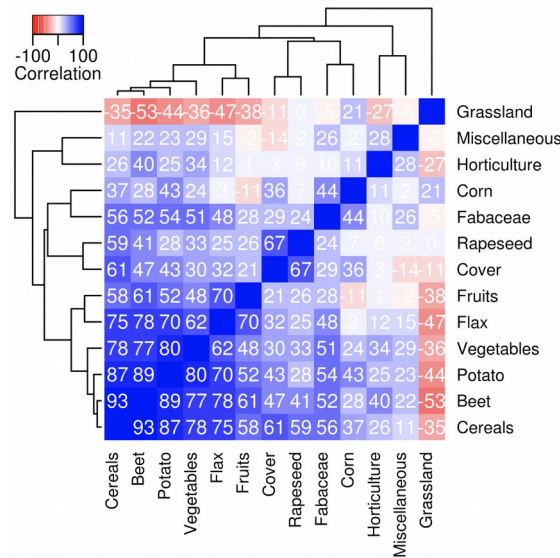
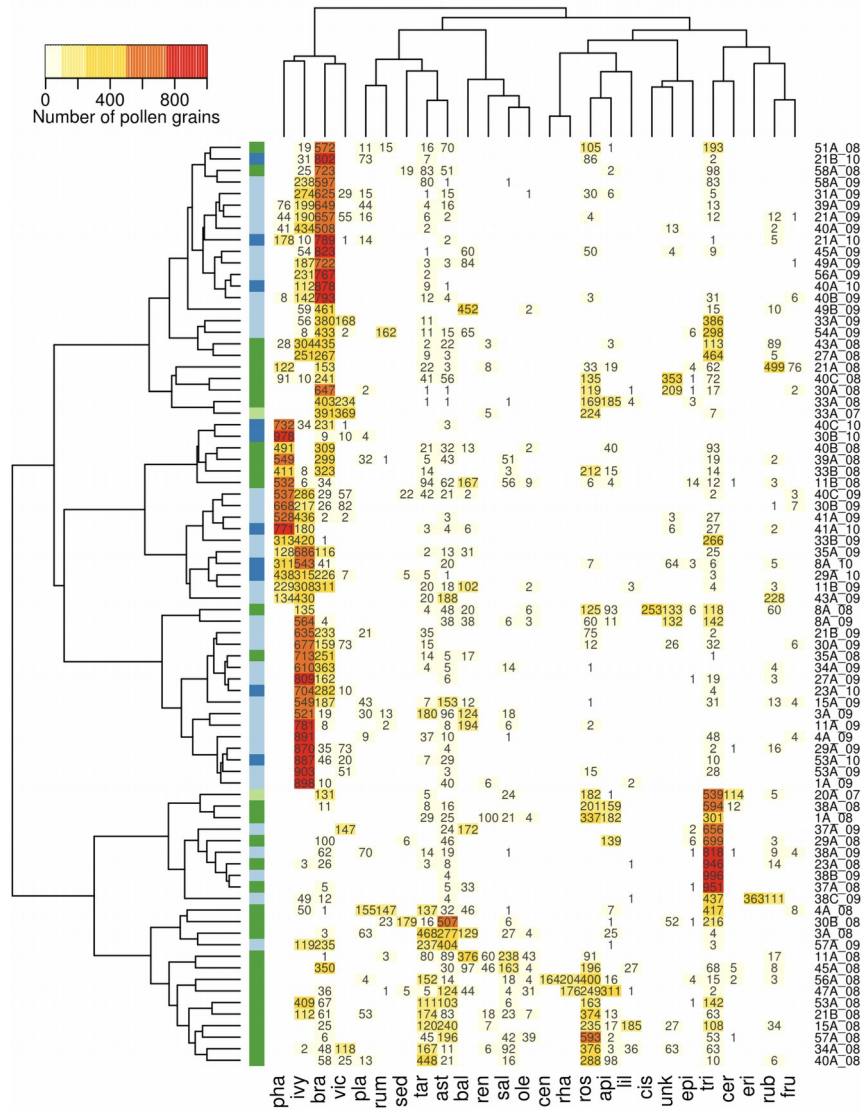


Fig S3.1c - Heatmap of the correlation matrix of the areas of crops and grasslands 3000 m around the apiaries. The numbers represent the Pearson correlation * 100. The dendrograms are build with a ward algorithm on the correlation matrix transformed into distance matrix.

Fig. S3.2a (next page)- Heatmap of the pollen dataset. Methods & description: Ward dendrogram on the rows on Hellinger distances. Correlation on the columns after Hellinger transformation (Legendre & Legendre 2012). The blue and green column on the left represent the sampling date: light green = July, green = August, light blue = September, dark blue = October. The row names are composed of the Apiary ID and the number of the month (08 = August etc.) corresponding to the sample. We took only the 25 most abundant pollen types. Interpretation: There are two main groups of samples (rows). The bottom group is mainly composed of samples from August (green). This group is itself composed of two groups: one group has massively exploited clover (*Trifolium*), the other group has exploited a very wide variety of pollen resources including *Rosaceae*, *Taraxacum*, Other *Asteraceae*, *Plantaginaceae*, *Trifolium*, etc. The top group is itself divided into 3 groups characterized by the massive exploitation of *Phacelia* or *Ivy* or *Brassicaceae* respectively.

Chapter 3 – Predicting pesticide exposure of honey bees at landscape level



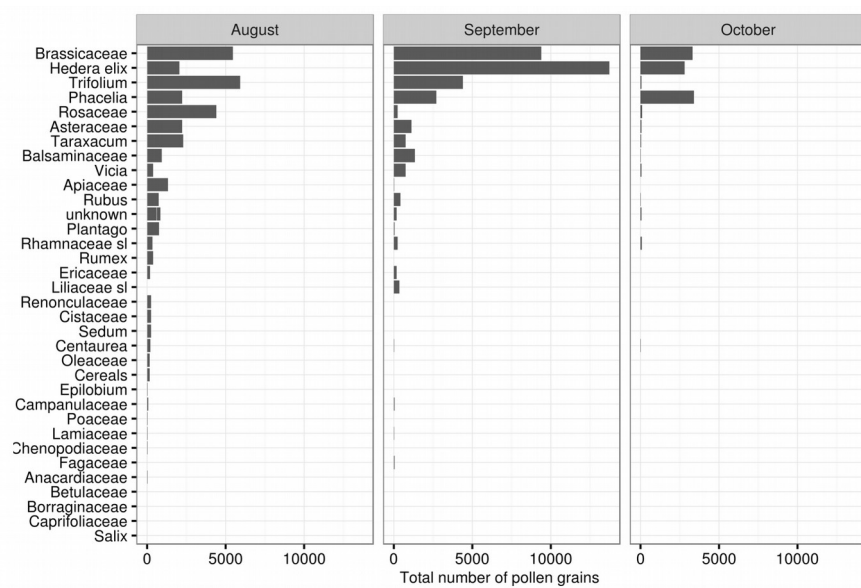


Fig. S3.2b - Total number of pollen grains from different botanical origin per month. The two samples from end July are not shown but have a composition very similar to the August samples (see Supplementary 2 at <https://figshare.com/s/86785808b5709331aalc>)

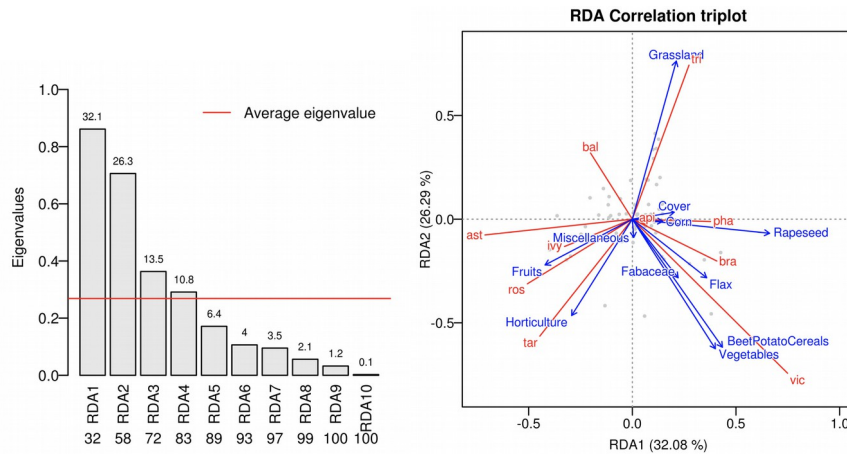


Fig. S3.3 - Pollen vs landscape RDA triplot

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3.7 AUTHOR CONTRIBUTIONS

NS and GSM wrote the main manuscript text. GSM performed the analyses with the help of LH and NS. NS, LH and EB designed the experiment and NS collected the samples in the field. CD performed the palynological analyses. All authors reviewed the manuscript.

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Chapter 4

Understanding the possible mode of
action of fungicides as factors of bee
colony disorders.

Direct chronic toxicity on immature
stages of bees

FOREWORD

The results of the first chapter opened new research avenues to understand the mode of action of fungicides on bees and bee colonies. A number of hypothesis are brought forward in order to explain such a mode of action: (1) direct toxicity of fungicides on bees, either adults or larvae; (2) indirect toxicity of fungicides on bee colonies, by affecting the microflora in bees' guts or that responsible of beebread formation; (3) fungicides as tracers of exposure to other molecules. In the present chapter we aimed to understand possible modes of action of fungicides on bees and bee colonies by exploring direct toxic effects of fungicides on immature stages of bees. We explore potential lethal and sub-lethal effects following a

prolonged exposure to contaminated food through the development of chronic toxicity tests on immature stages of bees. The active substances tested include the fungicide boscalid. This study was developed in collaboration with CRA-W and the Italian CREA-API.

This study was published in 2017 at the Journal Bulletin of insectology with title: *Toxicity assessment on honey bee larvae of a repeated exposition of a systemic fungicide, boscalid*. The text included into this chapter has been slightly modified with respect to the published version.

Toxicity assessment on honey bee larvae of a repeated exposition of a systemic fungicide, boscalid

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Louis Hautier, Piotr Medrzycki

ABSTRACT

Bees are key participators to the fertility of plants and yet they are suffering from losses and disorders. In other studies, double the amount of fungicides were found in colonies showing disorders than in healthy ones, with boscalid among the most frequently detected residues. Boscalid was mainly found in bee bread, main ingredient of larvae food and consequently exposing larvae to the compound in the long run. Here, we wanted to understand if boscalid could be toxic for immature stages of honey bees. Honey bee larvae were administered food to a range of doses from 0.04 to 40.25 µg boscalid/larva over 3 days. The NOED and LD50 at D8 were 4.025 µg a.i./larva and 86.786 µg a.i./larva, respectively; at D15 were 40.25 µg a.i./larva and 78.782 µg/larva, respectively; and at D22 were 40.25 µg/larva and 75.191 µg/larva, respectively. Worst-case field observed doses are 26 ppm in pollen and 1.43 ppm in nectar. Calculated NOEC of boscalid for larvae would be 741 ppm for pollen and 27 ppm for nectar. However, our results with the active ingredient could be different than those observed for queen larvae, those obtained with the formulated product containing boscalid or in the field considering the exposure of bee hives to multiple pesticide contaminants.

KEYWORDS

Honey bees; larvae; boscalid; fungicide; toxicity assessment; repeated exposure

4.1 INTRODUCTION

The honey bee *Apis mellifera* L. is a bee species present worldwide, serving humans and nature with regulating ecosystem services like pollination, but also providing food and pharmaceutical products and many other socio-economic ecosystem services (Kremen *et al.*, 2007; MEA, 2005; Van Der Sluijs *et al.*, 2013). Unfortunately a wide spread phenomenon of increasing honey bee colony losses and disorders has been settled in most of the countries where the subject has been a matter of study (Lu *et al.*, 2014; Pistorius *et al.*, 2015; Tosi *et al.*, 2016; Van der Zee *et al.*, 2015, 2012; Yamada *et al.*, 2012). These trends are part of the general framework of pollinators decline described in Europe and other countries for several years now (Bartomeus *et al.*, 2013; Biesmeijer *et al.*, 2006; Bommarco *et al.*, 2011; Carvalheiro *et al.*, 2013; Goulson *et al.*, 2008; Kosior *et al.*, 2007; Maini *et al.*, 2010; Nieto *et al.*, 2014; Renzi *et al.*, 2016; Van Dyck *et al.*, 2009). The current situation threatens the continuity of pollinators role and the balance in nature (Garibaldi *et al.*, 2014; IPBES, 2016). Possible stressing factors proposed to explain such trends are pathogens, habitat loss, pesticide exposure, nutrition (or lack of it) and climate change, and in the case of managed bees, management practices, all of them intervening with different relative weight depending on the context (Goulson *et al.*, 2015).

In the southern part of Belgium, a field case study was carried out by Simon-Delso *et al.*, 2014, in 2011-2012 aiming to clarify the phenomenon of unexplained winter colony losses beekeepers were reporting since the 2000s. Most of the mentioned stressors were included in the study. They found that honey bee colony disorders during the winter were strongly correlated with the presence of fungicides in the beekeeping matrices collected in July and September and with the area of arable crops around the apiary. The main pesticide residue detected in that study was boscalid, a systemic, fat-soluble and persistent carboxamide fungicide (Fungicide Resistance Action Committee -FRAC- Group 7) authorized to be used in Belgium in many crops attractive or not for bees, i.e. cereals, potatoes,

oilseed rape, fruit trees, berries, aromatic and ornamental plants, vegetables, hops, vine plants (Fytoweb, 2015). Boscalid is a pesticide molecule widely used in agriculture and, as a result, widely present in beekeeping matrices. Numerous publications have found levels of contamination of beekeeping matrices like bees, pollen, bee bread, honey or wax, between some parts per billion (ppb, $\mu\text{g/kg}$) levels and up to 26.2 parts per million (ppm, mg/kg) (Aubee and Lieu, 2010; Johnson *et al.*, 2010; Mullin *et al.*, 2010; Simon-Delso *et al.*, 2014; Stoner and Eitzer, 2013; Wallner, 2010; Wu *et al.*, 2011), which corresponds to the largest amount found in pollen loads (Wallner, 2010). Nectar residues have been described up to 1.43 ppm (Wallner, 2010) and wax amounts ranged from 1-13 ppm (Aubee and Lieu, 2010; Ravoet *et al.*, 2015, respectively). These concentrations can be translated into dose per larva basing on food consumption data (U.S. Environmental Protection Agency *et al.*, 2014), the total amount of pollen, nectar and royal jelly ingested by a worked larva being 216.3 mg. This would translate into a nominal dose of 5.6 $\mu\text{g/larva}$ should we assume that all these matrices were contaminated at the same level (26 ppm), or 1.19 $\mu\text{g/larva}$ should we assume pollen and royal jelly residues being 26 ppm and nectar ones 1.43 ppm. This reasoning is conservative and the worst case might lead to even higher exposure levels. In fact, due to the lack of knowledge about the fate of this a.i. in the nurse bee body, it is unknown whether its concentration in royal jelly may be similar to the one found in pollen, may be lower or even higher. The latter possibility, if relevant, could be due either to accumulation mechanisms or to the fact that the protein/aminoacid content in pollen pellets (6.31-37.40% of dry matter (Forcone *et al.*, 2011; Liolios *et al.*, 2015; Somerville, 2001; Taha, 2015; Tasei and Aupinel, 2008; Todd and Bretherick, 1942) is lower than in the royal jelly (30-50% of dry matter (Scarselli *et al.*, 2005)), even so if we consider that royal jelly is always contaminated by pollen grains. In other words, in order to produce 1 mg of royal jelly much more pollen is consumed and if all the a.i. contained in this pollen finished in the royal jelly, the relative concentration would be much higher.

Boscalid exhibits its mode of action through the inhibition of the mitochondrial respiration (succinate deshydrogenase) and subsequently

reduction of adenosine triphosphate (ATP) in fungal cells (Stammler *et al.*, 2008). The authorization dossier of boscalid shows a low acute toxicity to honey bees, with an acute LD50 (both oral and contact) of >11 µg/bee (Aubee and Lieu, 2010) and >100 µg/bee (European Commission, 2008). For this reason, no higher tier studies were submitted by the producer, BASF AG, for authorization purposes. Preliminary tests done with boscalid did not induce to consider it as an Insect Growth Regulator (IGR). As a result, no complementary studies were carried out specifically targeting the toxicity of larvae and no toxicity problems were evaluated at colony level. However, recently boscalid was found to be linked to less pollen consumption and digestion in bees, lower ATP concentrations in the thoracic muscle tissue and higher virus titers (DeGrandi-Hoffman *et al.*, 2015).

Following the results found in the field case study, we hypothesize that honey bees could be chronically exposed to boscalid residues from the first life stage. In this study, we aim to evaluate whether the larval toxicity of boscalid is a plausible explanation of the elevated colony mortality by using the new OECD methodology for larva toxicity of repeated exposure.

4.2 MATERIAL AND METHODS

The methodology defined by the OECD Draft Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure was used (OECD, 2014). A schematic representation of the most important steps of the larval repeated exposure test can be found in figure 4.1. Accordingly, the queen bees of three different queen-right healthy colonies were caged for around 30 hours, then released and three days later, the caged frames brought to the lab. A minimum of 144 first instar (L1) larvae were grafted per colony with the help of a paintbrush and deposited into crystal polystyrene grafting cells placed at sterile polystyrene 48-well tissue culture plates with flat bottom, treated by vacuum gas plasma (Falcon®), containing an uncontaminated diet. On the third day (D3) apparently dead larvae were removed and the living ones were randomly allocated to the different test

plates ensuring that at least 12 larvae per colony were present in each plate. There were no deviations from the official OECD protocol. Temperature and relative humidity conditions during the test were monitored and are presented in supplemental material (table S4.1).

4.2.1 Diets and test solutions

The diet composition as well as the test solutions administered and larvae used per treatment are specified in supplemental material table S4.2 and S4.3, respectively. Boscalid has a solubility in water of 4 mg/L and 180 g/L in acetone. To enable full solution, pure boscalid (Sigma-Aldrich, batch number SZBC180XV, 99.9% purity) was diluted into acetone and the test diet contamination was carried out into a cold chamber at 4 °C to reduce solvent evaporation. Dimethoate (Sigma-Aldrich, batch number SZBC243XV, 99.5% purity) was used as toxic standard and the respective diet was prepared as described above. Seven polystyrene plates were used, one for each treatment, namely one water control, one solvent control, 4 boscalid doses and the toxic standard.

Given that boscalid is supposed to be no toxic to bees, nor to be an insect growth regulator and because, to our knowledge, there are no previous studies carried out with this active ingredient on honey bee larvae, a range-finding test was used for the determination of NOED (No Observed Effect Dose) and LD50. Depending on the results, a subsequent finer test could be envisaged, but according to our results it was not necessary. A geometric ratio of 10 was used, starting at a high concentration of 57.5 mg/mL (261.4 mg a.i./kg diet) and reaching to 0.0575 mg/mL. Ten µL of each boscalid solution were added to the different diets. Dimethoate was dosed at 1.082 mg/mL (49.2 mg a.i./kg diet) in demineralized water and 100 µL were added to the diets (prepared in vials of 2 mL). Pesticide concentrations in the different diets and dose received per larvae can be found in supplemental material (table S3).

Both boscalid and dimethoate are described as stable and non-volatile

(IUPAC, 2014a, 2014b). For this reason, the diets were contaminated and homogenised at the beginning of the assay and kept in a cold chamber (4 °C) until the end of the test (22 days). Before administration respective diets were warmed to the temperature of the incubator (34.5 ± 0.5 °C).

4.2.2 Effect observations

Individual survival was tested based on the development capacity of larvae or on the reaction of individual larvae to stimulus (gentle touch with a paintbrush). NOED determination was based on survival. Therefore a non-developed larva or a larva that did not react to stimulus were considered as dead. Results were registered and plates were photographed at days 4 (D4), 5 (D5), 6 (D6), 7 (D7), 8 (D8), 15 (D15) and 22 (D22) of the test (supplemental material, figure S4.1). On D8, the end of the exposure phase, it was recorded if larvae had consumed or not the diet provided, which was assessed following visual inspection. Non-consumed diet is presented as a thin layer of gel in the bottom of the cells. Cells were not weighted in order to establish food consumption as it would be difficult to discern the food weight and the larva one. On D22 the non-emerged adults were counted, recorded and photographed. The number of deformed bees was also recorded.

Plates were always processed from the control up to those containing the highest dose and toxic in order to avoid contamination. The paintbrush was thoroughly cleaned with water after each evaluation.

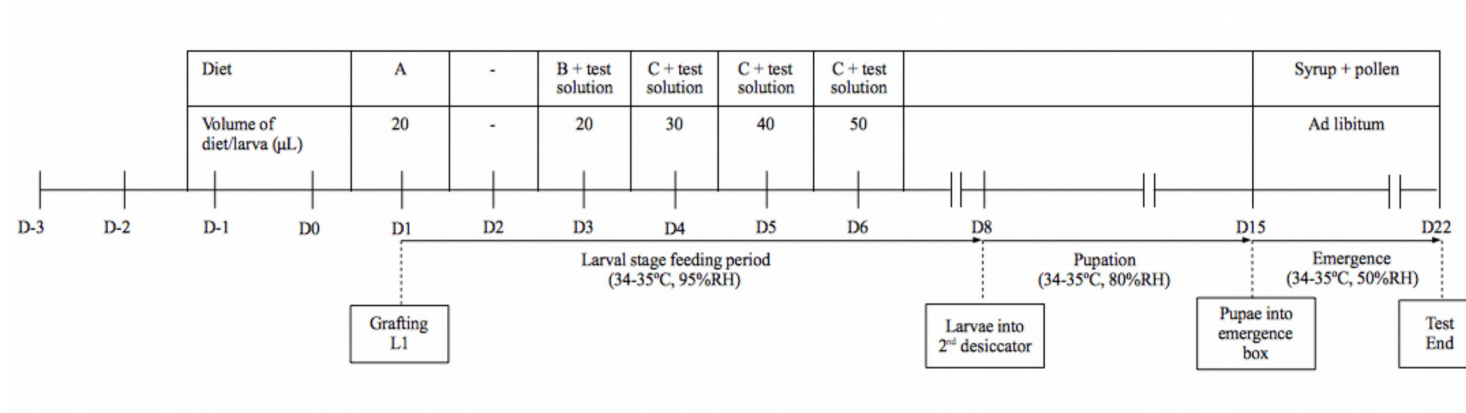


Figure 4.1 - Schematic representation of the important steps of the larval repeated exposure toxicity test (D = day; RH = relative humidity). Adapted from (OECD, 2014) OECD (2015).

4.2.3 Statistical analyses

The numbers of dead larvae (at D8), pupae (at D15) and non-emerged adults (at D22), in respect of the total amount of active ingredient (a.i.) consumed by the individuals, were processed using the software ToxRat Professional, version 3.2.1 – 2015. Fisher's Exact Binomial Test was carried out in order to compare the mortality in water and solvent control. The LD50 values and their confidence limits (at 95%), both expressed in μg a.i./larva, were estimated using the Spearman-Kärber method (Carter, 1994) (table 4.1).

The NOED expressed in μg a.i./larva, was calculated with χ^2 2×2 test with sequential Holm-Bonferroni correction for D8 and with Step-down Cochran-Armitage test for D15 and D22 accordingly to the structure of data.

4.3 RESULTS

The difference in larval mortality in the control and control solvent (0.5% acetone) was not significant at D8 (two tailed Fisher's Exact Binomial Test, $p = 0.23$), D15 ($p = 1.00$), D22 ($p = 0.3$). The two control groups showed a cumulative larval mortality at D8 of 9% ($n = 44$) and 2.5% ($n = 40$), respectively (figure 4.2). This seems within the accepted range of control mortal toxicity previously observed in in-vitro larval rearing (Aupinel *et al.*, 2007; Crailsheim *et al.*, 2013). Thus, accordingly to OECD GD 54 (OECD, 2006), for those two assessment periods the data were pooled in a unique control group. The treatment response was corrected by the control response using Abbott's formula (Abbott, 1925).

At the key test dates of D8, D15 and D22, LD50 was 86.78 μg a.i./larva (CI: 65.75 - 114.55), 78.78 μg a.i./larva (CI: 58.60 - 105.92), 75.19 μg a.i./larva (CI: 54.99 - 102.82), respectively. NOED values were 4.025 μg a.i./larva for D8, and higher or equal to 40.25 μg a.i./larva for D15 and D22

(table 4.1). Raw data of absolute mortality in each treatment and at each assessment time are reported in table 4.2.

It must be stressed that during the experiment the 50% relative mortality was never reached in the boscalid treated plates. Thus the LD50 values are higher than the highest tested dose, being a product of mathematical extrapolation. This situation results in relatively high Confidence Limit range. Nevertheless it is possible to assert with an acceptable uncertainty level (5%) that the LD50 of boscalid is not higher than 115 µg a.i./larva but the toxic effects may be observed above 4 µg a.i./larva (NOED at D8).

On D22, 9 dead emerged bees out of 31 surviving larvae were found in the control, while in the other groups dead emerged bees ranged between 0 and 2. These results have no impact on the validity of the control according to OECD standards. Some deformed emerged bees (i.e. abnormal abdomen shape, humpbacks, n = 4) were observed in the boscalid 57.5 mg/mL group, while the number of deformed emerged bees in the other groups was between 0 and 2.

Table 4.1 - Toxicological endpoints and statistical analyses used in D8, D15 and D22.

Assessment Day	D8 µg a.i./larva	D15 µg a.i./larva	D22 µg a.i./larva
NOED	4.025*	40.25*	4.025*
Test	Chi2 2x2 test with Bonferroni correction	Step-down Cochran.Armitage	Step-down Cochran.Armitage
LD50	86.78	78.78	54.53
(range CL 95%)	(65.75–114.55)	(58.60-105.92)	(36.72-80.97)
Test	Trimmed Spearman-Karber	Trimmed Spearman-Karber	Trimmed Spearman-Karber

* Control and solvent control data are pooled

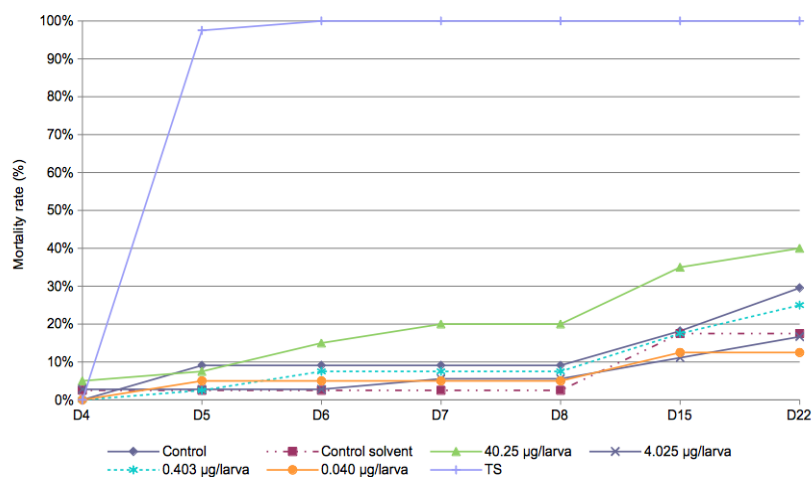


Figure 4.2 - Evolution of mortality after an oral exposition of a diet alone (control), a diet with solvent (control solvent), a diet with four boscalid concentrations or one dimethoate concentration (TS) from day 4 (D4) to day 22 (D22).

Table 4.2 - Cumulated mortality and effects observed in larvae during the 22 days of test.

Treatments	Number of larvae at D3		D4	D5	D6	D7	D8	D15	D22	Dead larvae	Dead pupae	Total mortality
Control	44	Dead	0	4	4	4	4	8	13	9.09%	20.45%	29.55%
		Affec.	7	1	0	0	0	0	9			
Control acetone	40	Dead	1	1	1	1	1	7	7	2.50%	15.00%	17.50%
		Affec.	8	0	0	0	0	0	1			
Solution 1	40	Dead	2	3	6	8	8	14	16	20.00%	20.00%	40.00%
		Affec.	6	2	4	0	0	0	2			
Solution 2	36	Dead	1	1	1	2	2	4	6	5.56%	11.11%	16.67%
		Affec.	6	0	0	0	0	0	1			
Solution 3	40	Dead	0	1	3	3	3	7	10	7.50%	17.50%	25.00%
		Affec.	12	0	0	0	0	0	0			
Solution 4	40	Dead	0	2	2	2	2	5	5	5.00%	7.50%	12.50%
		Affec.	5	0	0	0	0	0	1			
Toxic standard	40	Dead	0	39	40	40	40	40	40	100.00%	0.00%	100.00%
		Affec.	40	0	0	0	0	0	0			

Note. Solution X – boscalid; Toxic standard - dimethoate

4.4 DISCUSSION

Larval mortality on D22 of the control water was just below the validity criteria accepted in tests carried out for regulatory purposes (30%) mentioned in OECD guidelines. However, the solvent control showed acceptable mortality rates and indicate that larvae used for the test were in good conditions. The source of a higher mortality in the water control could have been the fact that this plate was always processed first, possibly serving as a training plate. This could be the reason behind the larger number of dead emerged bees from the control plate. Operators observing recurrently these effects could foresee to include an extra training control water plate to be handled always at the beginning of the manipulations/observations.

The number of deformed emerged bees remained low and was mainly linked to abnormal abdomen shape (humpbacks). These malformations have been previously described and could be an artefact of the methodology used as described by Riessberger-Gallé *et al.*, 2008. In real conditions, larvae are reared on vertical frames, while the plates in our test remained always horizontally. Riessberger-Gallé *et al.*, 2008, proposed solutions to this issue.

Larvae mortality was observed to be 97.5 and 100% in the toxic standard (dimethoate) group after two and three days of exposure, respectively, all larvae seeming to have been affected following the first day of exposure. This may indicate an acute toxic dose of dimethoate. Aupinel *et al.*, 2007, show a weak mortality dynamics at doses lower than 40 mg/kg dimethoate. It could be recommended to reduce the concentration of dimethoate in the toxic standard to mimic chronic toxicity dynamics.

The consumption of pollen and nectar during the larval development (5 days for worker larvae and 6.5 days for male ones) has been already estimated to: (1) 59.4 - 98.2 mg sugar in nectar for workers and male larvae, respectively (Rortais *et al.*, 2005); and (2) 5.4 mg pollen for worker larvae (Babendreier *et al.*, 2004). Based on these findings, the residues of boscalid

found in beekeeping matrices and our results, the calculated NOEC of boscalid for larvae would be 741 ppm for pollen and 27 ppm for nectar. Our results show that the active ingredient boscalid alone does not seem to be toxic to worker larval development and survival at the concentrations mentioned in literature for the beekeeping matrices. In particular, Simon-Delso *et al.*, 2014, found boscalid residues in bee bread both morbid ($n = 24$) and healthy colonies ($n = 10$), with concentrations between 0.005 and 1.3 ppm in the former, while boscalid residues remained lower to 0.058 ppm in the latter. However, assuming that toxicity of boscalid on queen larvae is similar to that of worker bees and following our reasoning in terms of residue content in royal jelly, queen larvae would be exposed to doses just above the NOED (4.56 μg boscalid/larva). These estimations might be far from reality. This is why it would be worthwhile investing future research efforts in clarifying the amount of toxicants ending up in royal jelly. Indeed, Yamada *et al.*, 2015, showed a 5-fold increase of toxicity of the insecticide dinotefuran on full honey bee colonies when the exposure was done through pollen, in comparison to when the bees consumed contaminated syrup. The authors argue that the queen and larvae may be more affected in the long-run, leading to the failure of the colony.

The systemic properties and persistence of boscalid increases its potential to be found in nature over long periods of time. Indeed, pollen pellets collected by bees have proved to be contaminated with boscalid over periods of three months at least (personal observation). The presence of boscalid in bee bread samples collected in July-August indicates even previous exposure to this compound. This involves chronic exposure of several generations of bees within the colony and a plausible exposure of the queen to the same chemical over months. As a result, not only larvae could be exposed over their complete developmental period, but also after emergence and during their life as in-hive bees. Future laboratory experiences could envisage to evaluate the impact of a life time exposure, including larval development, on bees. This would be specifically interesting for compounds with similar physicochemical characteristics as boscalid.

However, our results may not be generalised to all active ingredients with fungicide action. Mussen *et al.*, 2004, revealed toxic effects of fungicides on larvae like captan, ziram or iprodione, while others active ingredients, i.e. cyprodinil, myclobutanil, trifloxystrobin, fenhexamid, and azoxystrobin did not show a larvicidal effect. Iprodione and captan were also found by Simon-Delso *et al.*, 2014, iprodione being the second most-frequently observed fungicide (n = 13). Specifically, iprodione residues were detected in beekeeping matrices of morbid colonies (n = 9) and in healthy ones (n = 4) with levels between 0.017 and 1.5 ppm and 0.022-0.04 ppm, respectively. Zhu *et al.*, 2014, showed larva toxicity of in hive levels of several pesticides including chlorothalonil. Furthermore, Chen *et al.*, 2015, carried out a PER (proboscis extension reflex) test following the exposure during the larvae development with triadimefon and found that bees performed significantly worse at 0.4 ppm. The effects may be linked to the mode of action of the different active ingredients. Triadimefon is ergosterol biosynthesis inhibitor resulting in cell membrane disruption in fungi (IUPAC, 2015), while boscalid affects the cell metabolism by inhibiting the enzyme succinate dehydrogenase (Stammler *et al.*, 2008). Captan is a dicarboximide and disrupts the interactions with the sulfur moiety of glutathione, inhibiting the cell respiration (Roberts and Hutson, 1999). Iprodione is also a dicarboximide may additionally inhibit protein kinases affecting intracellular communication (Roberts and Hutson, 1999).

In field conditions, a co-exposure to different active ingredients or co-formulants exists. A number of studies showed the importance of this co-exposure in larval development (DeLorenzo and Serrano, 2003; Johnson and Percel, 2013; Zhu *et al.*, 2014). Specifically on fungicide formulations, Mullin, 2015, describes an increase by up to 26.000-fold the toxicity for bees with regards to that of the active substance alone. A similar observation might be true for larval toxicity as well. In the present study we did not take this into account, but in the future or for risk assessment purposes, it may be interesting to study this hypothesis and test in parallel at least the active ingredient together with a number of formulated products or other active ingredients most frequently found in beekeeping matrices.

In conclusion, the active ingredient boscalid, at the level of exposure observed in field conditions, seems not to be lethal to worker honey bee larvae. Based on the study carried out, however, we cannot pronounce ourselves about potential effects on the adult bees emerged from these exposed larvae or about the potential impact on queen larvae. Furthermore, it would be necessary to evaluate the potential impact of boscalid alone or in combination with other products on adult bees (Renzi *et al.*, 2016). Considering the persistency of boscalid in the field, an interesting approach would be to combine both methodologies: chronic exposure during larval and emerged-adult bees. However, the present results need to be considered in light of the latest findings of increased toxicity of pesticide formulations with regards to the active ingredients alone or pesticide mixtures.

4.5 SUPPORTING INFORMATIONS

Table S4.1 - Temperature and relative humidity conditions during the test

Days of the test	Temperature	RH
	°C (SD)	% (SD)
D1-D8	34.34 (0.28)	96.29 (2.75)
D8-D15	34.42 (0.17)	81.43 (9.39)
D15-D22	34.19 (0.33)	59.94 (13.38)

Note. Temperature and HR followed the recommendations of the OECD Guidelines

Table S4.2 - Composition of the different diets components used during the experiment

Diet	Royal jelly	Aqueous Solution			Total weight	Diet weight
		Yeast extract	Glucose	Fructose		
	g	g	g	g	g	g
A	9.00	2.00	12.02	12.03	9.09	18.09
B	10.00	1.50	7.51	7.52	10.00	20.00
C	27.83	2.00	9.01	9.10	27.83	55.66

Table S4.3 - Pesticide concentrations in the different diets and dose received per larvae

Treatments	Amount of active ingredient (μg) per diet				Total a.i. consumed $\mu\text{g/larva}$	Total diet provided mL/larva	Remaining food on D8 ni / ntotal
	D3	D4	D5	D6			
Amount of diet provided	20	30	40	50		140	
Control	0	0	0	0	0	140	0/44
Control acetone	0	0	0	0	0	140	0/40
Solution 1 (Boscalid)	5.75	8.625	11.5	14.375	40.25	140	0/40
Solution 2 (Boscalid)	0.575	0.863	1.15	1.438	4.025	140	0/36
Solution 3 (Boscalid)	0.058	0.086	0.115	0.144	0.403	140	1/40
Solution 4 (Boscalid)	0.006	0.009	0.012	0.014	0.04	140	1/40
Toxic standard (Dimethoate)	1.082	1.623	2.164	2.705	7.574	140	40/40

Figure S4.1 - Photos of well-plates Control and Solution 1 ($40.25 \mu\text{g/larva}$) at D8

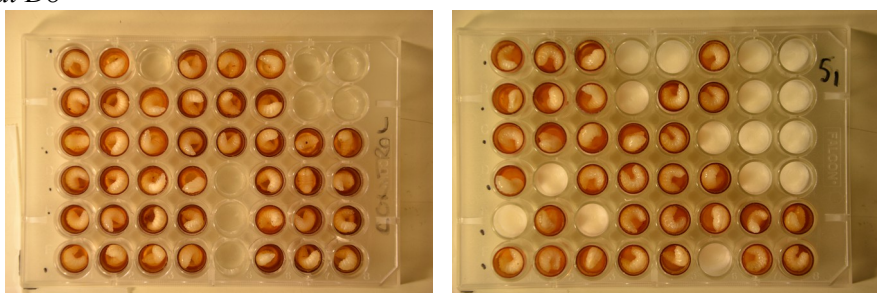
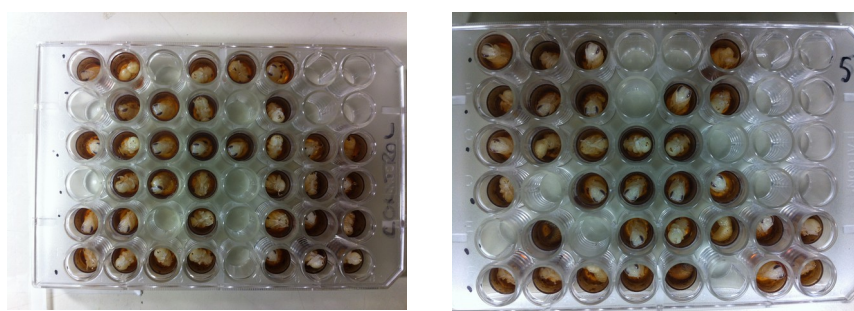


Figure S4.2 - Photos of well-plates Control and Solution 1 ($40.25 \mu\text{g/larva}$) at D15



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Chapter 5

Understanding the possible mode of
action of fungicides as factors of bee
colony disorders.

Direct chronic toxicity on immature
stages of bees

FOREWORD

The results of the first chapter opened new research avenues to understand the mode of action of fungicides on bees and bee colonies. A number of hypothesis are brought forward in order to explain such a mode of action: (1) direct toxicity of fungicides on bees, either adults or larvae; (2) indirect toxicity of fungicides on bee colonies, by affecting the microflora in bees' guts or that responsible of beebread formation; (3) fungicides as tracers of exposure to other molecules. In the present chapter we aimed to understand possible modes of action of fungicides on bees and bee colonies by exploring direct toxic effects of fungicides on immature stages of bees. We explore potential lethal and sub-lethal effects following a

prolonged exposure to contaminated food through the development of chronic toxicity tests on emerging bees and we propose a new methodology for chronic toxicity testing of pollutants on adult honey bees. The active substances tested include the fungicide boscalid in its formulated. This study was developed in collaboration with CRA-W.

The text included in this chapter is the basis of a publication currently submitted to the Journal Scientific Reports with tentative title: *Chronic and cumulative toxicity for honey bees of the fungicide boscalid revealed with a test longer than the standard 10 days trial.*

Chronic and cumulative toxicity for
honey bees of the fungicide boscalid
revealed with a test longer than the
standard 10 days trial

Noa Simon-Delso, Gilles San Martin Etienne Bruneau, Louis Hautier

ABSTRACT

Synthetic fungicides are active ingredients widely used in agriculture to control phytopathogenic fungi. Bees are frequently exposed to them when visiting flowers, flying around or collecting water or gums. The systemicity, persistency and intense application of some of these fungicides, like boscalid, leads to long periods of exposure of honey bees via contaminated pollen and nectar. We exposed in the lab adult honey bees to food contaminated with boscalid for 33 days instead of the standard 10 days test. Most of the toxic effects were observed after 10 days. The median time to death (LT_{50}) ranged from 24.9 days (lowest concentration) to 7.1 days (highest concentration) and were all significantly shorter than the control (32.0 days). The concentration and dietary doses of boscalid inducing 50% mortality (LC_{50} , LDD_{50} , respectively) decreased strongly with the time of exposure: $LC_{50} = 14,729$ and $1,174$ mg/l and $LDD_{50} = 0.318$ and 0.0301 mg bee⁻¹day⁻¹ at days 8 and 25, respectively. Using different statistical approaches, we found evidence of reinforced toxicity when exposure is prolonged but with an unusual pattern: no cumulative toxicity is observed until 17-18 days, then, the relationship between the lethal dose and the time of exposure shows a point of inflexion suggesting that the capacity of the bees to deal with the toxicant might be reduced. Our results show the importance of time to death experiments rather than fixed duration studies to evaluate chronic toxicity.

KEYWORDS

Apis mellifera; pesticides; 10-day chronic toxicity; time to death; metabolites; Haber's rule; bioaccumulation

5.1 INTRODUCTION

Insect pollinators are essential for plant fertility and dissemination, the majority of the food produced worldwide depends on them¹⁻³. It is for this reason that the current negative trends in pollinator populations⁴ threatens the quality and quantity of the food in our diets and global health¹. For many they add an aesthetic value to the landscape^{5,6} and specific species like the honey bee, *Apis mellifera*, provide us with jobs, food (i.e. pollen, honey and even bees or larvae)⁴, pharmaceutical (i.e. honey, venom, propolis)⁷⁻⁹ and technical products (i.e. wax).

A recent field study revealed a significant positive correlation between honey bee colony disorders over the winter period and the presence of fungicides residues in the bee hives in summer and autumn¹⁰. Among the most frequently found active ingredients, the first four were fungicides: boscalid, pyrimethanil, iprodione and cyprodinil. Fungicide and bactericide products were indeed the most widely used plant protection products in 2015 in Europe (41.76%), followed by herbicides (33.92%)¹¹. Thus, it is not surprising to find residues in the environment and our food: in water even over long periods¹²⁻¹⁴, in air¹⁵, in fruits^{16,17}, in legumes^{16,17}, or in processed food¹⁸. Fungicide contamination have also been found in flower matrices like nectar^{19,20}, or pollen^{20,21}. As a result, bees can get in contact with them either when flying around, when collecting water, nectar or pollen or when consuming those afterwards.

Boscalid, specifically, is authorised for a wide variety of uses. It is systemic (Kow= 2.96) and persistent in the soil (DT₅₀ = 246 days)²². Described levels of boscalid contamination in beekeeping matrices range between a few µg/kg (ppb) and up to 26.2 mg/kg (ppm), with pollen containing the largest amounts^{10,20,23-34}. Simon-Delso et al.^{10,21} found that boscalid was the most frequent fungicide within the beehives and in pollen pellets and contaminated samples of these pollen were found from July to October, ranging from 0.9 to 512 µg/kg. David *et al.* 2016³³ described the

presence of boscalid residues both in oilseed rape pollen (OSR, up to 25 µg/kg) and pollen from field margins (up to 38 µg/kg), and proved that bees readily collect these pollens and bring the residues to the hive, during and after the bloom of OSR (up to 21 and 17 µg/kg, respectively). As a result, drift, run-off of residues from the treated fields or their remanence in soil or water seem to be a reality. These results open new questions regarding the possible long term impact of fungicide exposure on bees, which was not evaluated before 2014 within the pesticide authorisation procedure (Regulation EU 283/2013)³⁵.

Boscalid inhibits mitochondrial respiration (succinate dehydrogenase) and blocks ATP production which affects cell respiration³⁶. In mammals, it increases liver enzymes (alanine aminotransferase), gamma-glutamyl transferase, and is proved to induce thyroid adenomas although the responses were considered adaptive and reversible¹³. Boscalid shows a low acute toxicity to honey bees, with an oral LD₅₀ >11 µg/bee²⁴ and 100 µg/bee³⁷ and a contact LD₅₀ >100 µg/bee³⁷. To our knowledge, the biochemical effects on insects have not yet been described, but recently boscalid (in co-formulation with pyraclostrobin) was found to be linked to decreased pollen consumption and digestion in bees, as well as lower ATP concentrations in the thoracic muscle tissue and higher virus titers³⁸. In addition, it was shown to interact with neonicotinoid insecticides like thiamethoxam and clothianidin, whose toxicity to honey bees nearly doubled³⁴. Disruptive effects on nest recognition of solitary bees *O. lignaria* and *Megachile rotundata* (Fabricius) have been described following the exposure to field doses of fungicides, including boscalid³⁹. Around 60% of bumble bee queens died when exposed to treated blueberry plant material with Pristine® (pyraclostrobin 12.8% and boscalid 25.2%) at a rate of 1.6 kg/ha⁴⁰. As for chronic toxicity, hardly any of the described interactions of boscalid with other chemicals were considered when boscalid was first authorised.

Considering the length of the exposure to boscalid through bee bread and corbicular pollen (at least 4 months^{10,21}), the present study aims to detect potential effects of a chronic exposure to boscalid over the lifetime may

have on newly emerged bees.

5.2 MATERIALS AND METHODS

Test substances

We tested five concentrations of boscalid from 18,000 mg a.i./l to 1,125 mg a.i./l (two-fold dilutions). The test substances were dissolved in a 50% w/v sucrose solution provided as food to the bees. We used the commercial formulation Cantus® (BASF, 500 g/kg solid boscalid) because the pure active substance has a low water solubility (4.6 mg/l) and a tendency to flocculate. The concentrations were chosen based on previous studies and the potential concentrations used in the field. Application rates in the field range from 250 to 1,880 g a.i./ha³⁷. Based on the maximum application rate and on the typical spray rate per hectare in Belgium (100-200 l/ha), a concentration of 18,800 – 9,400 mg/l would be in the upper range of concentration expected to be found in the field, while 1,250 mg/l would be in the lower range of application. Dimethoate (Perfektion®, BASF, 400 g a.i./l dimethoate) was used as toxic standard at 1.5 mg/l.

Bees

Three frames containing capped cells with emerging bees were collected from three healthy queen-right colonies with queens of different origin. They were left in an incubator at $32.8^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $60\% \pm 20\%$ relative humidity. One-day old worker bees were transferred without anaesthesia into cages by groups of 10 bees per cage.

Experimental conditions

Bees were kept in cardboard cages with a mobile plastic window, in darkness, within an incubator at $32.8^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $60\% \pm 20\%$ relative humidity for the duration of the test. They were fed *ad libitum* with a syrup (sucrose solution with different concentrations of test substances) provided through 2 ml plastic syringes to which the tip was removed.

Treatments and data gathering

At the beginning of the test, each treatment group was composed of three replicates each of them containing 10 bees. In one cage one of the 10 bees died before the start of the experiment (i.e. before the contaminated syrup was provided). The initial number of bees was considered to be 9, instead of 10, in this cage. Bee mortality was recorded every day at approximately the same time as the syringes were changed. Dead bees were removed at each assessment. Syringes containing freshly prepared syrup were replaced every day and weighted just before administration (t0) and again the following day (t1), to estimate the quantity of syrup consumed by the bees. The test lasted until the mortality in the control group reached 50%, instead of stopping the test after 10 days as recommended by OECD⁴¹.

Data analysis

All analyses were performed with R⁴² mainly with drc package⁴³ for dose response curves (Weibull models) and toxicological endpoints estimations, lme4 package⁴⁴ for mixed models and multcomp package⁴⁵ for post-hoc multiple comparisons. All datasets, R code and detailed data analyses are provided as a public figshare repository (<https://figshare.com/s/865e87feaad34c095bbd>).

Data analysis – Syrup consumption

We first compared the daily syrup consumption per bee between the different treatments (different Cantus® concentrations) without taking the time into account. We used a gaussian mixed model with the cage as random effect and the treatment as fixed effect. In a second time we analysed how syrup consumption changes across time for each treatment. We used graphical tools like loess (locally weighted polynomial regression) to visualize the trends in the noisy data and used mixed models to check if the observed patterns were statistically significant. We used the cage as random effect and the treatment, time (second order polynomial) and their interactions as fixed effects. The evaporation of syrup was also measured for a subset of the days in cages without bees or with only dead bees (see Supplementary material, section S5.1).

Data analysis – Toxicological endpoints

We computed 4 toxicological endpoints (and their 95% confidence intervals): Lethal Time (LTx), Lethal Concentration (LCx), Lethal Dietary Dose (LDDx) and Lethal Cumulative Dose (LCDx)(table 5.1). The LTx was computed for each concentration and the LCx, LDDx and LCDx were computed for each day of the test between day 8 and day 25 (outside this range, the mortality is either too low or too high). All "x" levels of mortality between 10% and 90% in 10% increments were calculated.

To estimate these toxicological endpoints, we choose a 3 parameters Weibull 2 model (*sensu Ritz*⁴⁶) to model the relationship between uncorrected mortality and time, concentration or dose. This 3-parameters-sigmoid model fixes the higher asymptote (i.e. the mortality is 100% for an infinite dose) while it estimates the lower asymptote (i.e. the mortality in the control). The toxicological endpoints are then calculated relative to these two asymptotes. The LCx, LDDx and LCDx estimates are therefore corrected for mortality in the control. For LTx we used the same approach but the lower asymptote in that case estimates the mortality at D0 which is zero. Hence, the LTx values as we calculate them here are not corrected for mortality in the control. See Supplementary material, section S5.3, for more details.

Only the Weibull 2 model results are reported here because this model provided the best compromise between goodness of fit and stability. However, three other types of models have been fitted and compared: logistic, log-logistic and Weibull 1 (*sensu Ritz*⁴⁶), all with 3 parameters. See Supplementary material, section S5.2, for a detailed comparison of these 4 types of models for the determination of LCx. We also provide in the Supplementary Material of the publication ("raw_output" directory) all toxicological endpoint values for the 4 types of models at each day, each concentration and for all levels of mortality between 10% and 90%.

Table 5.1 – Definitions and abbreviations. Weibull 2 models are 3 parameters sigmoid dose response curves⁴⁶

Variable	Abbreviation	Definition	Units	Model
Concentration	C	Quantity of test product within the syrup provided as food to the bees	mg a.i./ l syrup	
Dose	D	Quantity of test product really consumed by the living bees through their food (syrup) on a given day. The calculations are based on syrup consumption, concentration of the test product in the syrup, syrup density (1.23 g/ml) and the number of bees still alive	mg a.i./ (bee*day)	
Daily Dietary Dose	DD	Average of the doses consumed during a given day and all preceding days	average mg a.i./ (bee*day)	
Cumulative dose	CD	Sum of the doses consumed during a given day and all preceding days	total mg a.i./ bee	
Lethal Time	LTx	Number of days of exposure to cause x% mortality at a given concentration	days	Based on the Weibull 2 model: Mortality vs Time
Lethal Concentration	LCx	Concentration needed to cause x % mortality after a given period of exposure	mg a.i./ l syrup	Based on the Weibull 2 model: Mortality vs Concentration
Lethal Dietary Dose	LDDx	Average daily dose consumed per bee needed to cause x% mortality after a given period of exposure	average mg a.i./ (bee*day)	Based on the Weibull 2 model: Mortality vs Daily Dietary Dose
Lethal Cumulative Dose	LCDx	Cumulative doses consumed per bee from day 0 needed to cause x% mortality	total mg a.i./ bee	Based on the Weibull 2 model: Mortality vs Cumulative Dose

Data analyses – Cumulative toxicity estimation

Most of the methods used to evaluate the potential of cumulative toxicity (or time reinforced toxicity) are based on Haber's rule⁴⁷ which states that the product of exposure concentration (C) and exposure duration (t) leads to a constant toxic effect. In other words, if there is no cumulative toxicity, when the concentration (or dose) is divided by 2 the time of exposure to reach the same level of mortality should be doubled. Haber's rule is a specific case of the Druckrey-Küpfmüller model, for which the exponent (b) has an absolute value of 1⁴⁸.

$$C \times t^b = \text{constant (Eq. 1)}$$

We used two different approaches to test if Cantus® induces time reinforced toxicity: (1) log-log linear regression between concentration or dose and time to estimate the slope b from equation (Eq.) 1^{49,50}; (2) comparing between treatments the cumulative dose needed to reach 50% of mortality as proposed by the European Food Safety Authority (EFSA)⁵¹.

For the first approach, we estimated the slope (and 95% Confidence Intervals) of 3 types of simple linear regressions: (1) $\log(\text{Concentration})$ vs $\log(\text{LTx})$; (2) $\log(\text{LCx})$ vs $\log(\text{Time})$; (3) $\log(\text{LDx})$ vs $\log(\text{Time})$. Should cumulative toxicity exist, these slopes would be significantly smaller than -1. This was repeated for each level of mortality between 10% and 90% in 10% increments.

We could also estimate the slope of the linear regression $\log(\text{LCDx})$ vs $\log(\text{Time})$ however for this cumulative dose the expected slope under Haber's rule is not -1, but 0, and we used the cumulative dose as used in the second approach detailed below in a slightly different way.

For the second approach, the EFSA⁵¹ proposed to evaluate the LC_{50} at 48h and then launch a test with two treatments: one corresponding to the estimated LC_{50} value and another with $\frac{1}{4}$ of this concentration. The cumulative dose needed to reach each 50% mortality in each treatment is then compared. These cumulative doses should be equal if there is no

cumulative toxicity.

We did not observe mortality in any treatment at 48h, but we used a similar idea by comparing the cumulative dose needed to reach 50% of mortality in our 5 concentrations (with two-fold dilutions). We used a one way ANOVA (each cage in a dose is a replicate) and all pair-wise post-hoc comparisons (multcomp package⁴⁵) to test for differences. For this part only we used corrected mortalities based on the Abbott's formula⁵².

5.3 RESULTS

Food consumption and duration of the trial

The average daily food consumption per bee over the duration of the trial (33 days) was 39 ± 1.7 mg/bee/day and no significant difference was found in the individual consumption among the different treatments (Gaussian Mixed model with cage as random effect: $F_{5,12,2} = 2.289$, $p = 0.1104$).

However, syrup consumption was not constant over time and showed clear differences between treatments once the time is taken into account. Firstly, we observe a strong increase of individual food consumption in most cages when there were only 1 to 3 living bees left, with extreme values above 80 mg syrup bee⁻¹ day⁻¹ (Figs. 5.1 and 5.2). These extreme values may be partially due to the measurement error induced by syrup evaporation. The difference of syrup weight due to evaporation is divided by the number of living bees. Consequently, the relative importance of the evaporation increases when there are only a few living bees left. Nevertheless, after correcting consumption for evaporation (Supplementary material S1) we still obtained some large peaks of consumption when there are only a few living bees left. Secondly, when we remove these outliers by considering only the consumption data while a minimum of 5 bees remained per cage, we observe that the consumption is still not stable over the duration of the test (Fig. 5.3).

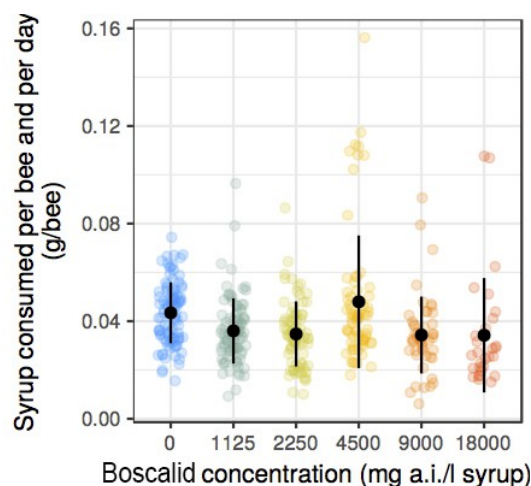


Figure 5.1 – Daily syrup consumption at different boscalid concentrations
The black dots represent the average value and the bars are the standard deviation. Each transparent coloured point represents the consumption per bee during one day in one cage. There is no significant difference of average consumption between treatments (gaussian mixed model with cage as random effect: $F = 2.289_{5, 12.2}$, $p = 0.1104$).

An inverted “bell” shape is observed in the control, with individual consumption increasing to reach a maximum around days 17-18 of the test and then decreasing slowly until the end of the observation period (33 days). A similar pattern in consumption is observed for the bees at the different concentrations but with a different kinetics: the higher the concentration, the sooner the maximum is reached and the steeper the increase and decrease are (Fig. 5.3). At a given point in time, bees tend to significantly reduce their consumption when Cantus® contaminates the food, the reduction being greater at the higher concentrations (These visual differences are supported by statistical testing with a polynomial mixed model, Supplementary material section S5.1) These differences in overall consumption among treatments are visible only when bee consumption until 50% mortality per cage is considered. Otherwise, the variability in individual and daily consumption masks the differences.

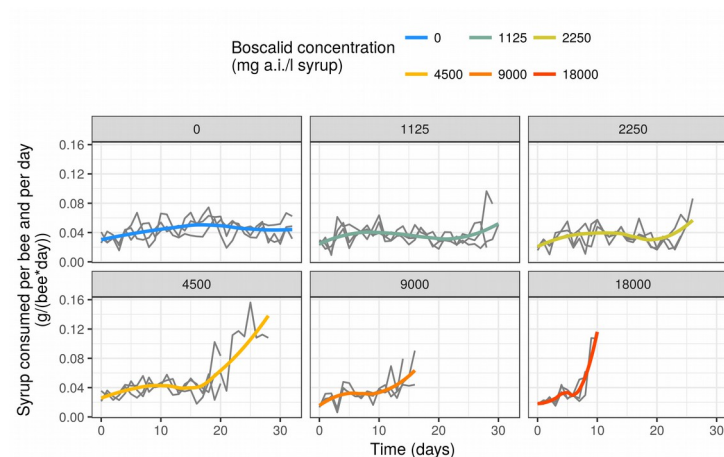


Figure 5.2 – Evolution of syrup consumption per bee in each replicate (in grey) and the general trend (loess - locally weighted polynomial regression, red line) along the duration of the test in the control and the different treatments: 1,125, 2,250, 4,500, 9,000, 18,000 mg a.i./l. Note: all bees in the toxic standard had died by day 5. The consumption of the bees in the toxic standard varied daily and followed a slight negative trend until 2 or 3 bees were left per cage, when the individual consumption rocketed same as was observed for the other boscalid treatments.

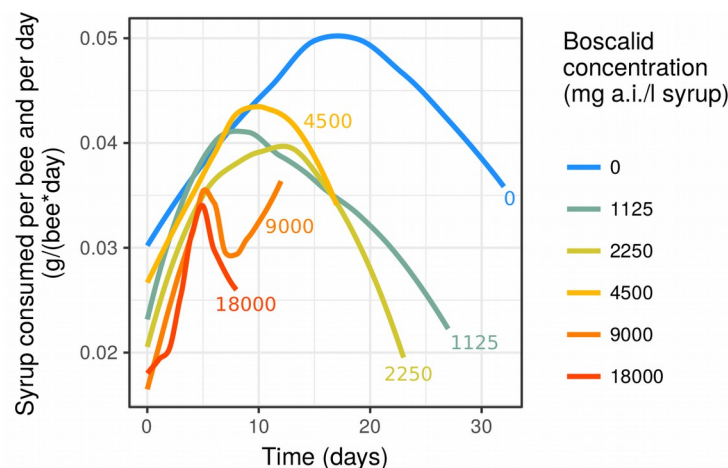


Figure 5.3 – Daily syrup consumption over time until 50% mortality is reached in each cage.

The lines for each boscalid concentration are loess trends (locally weighted polynomial regression). The model show highly significant time x concentration and main concentration effects: the kinetics of the consumption is different between treatments and the average consumption on a given day is also different.

Mortality rate and toxicological endpoints

The mortality rates were in agreement with the validity criteria established by OECD draft test guidelines for the evaluation of the toxicity of pesticides over 10-days⁴⁷: (1) the average mortality for the controls was lower than 15 % at day 10 of exposure and remained so up to day 20; (2) the average mortality in the toxic standard was higher than 50 % at day 10 (Fig. 5.4).

Before day 10 - i.e. the normal duration of a chronic test in standard guidelines - the observed mortality was low. A mortality higher than 50% was only observed at the highest Cantus® concentration (18,000 mg boscalid/l) from day 8 onwards. Before this date it is not possible to correctly estimate any LC_{50} or LDD_{50} values. On day 10 the mortality had

reached almost 100% at the highest concentration while all the other treatments remained below 50% and most of them close to 0%. On day 20, when the mortality in the control had reached 15%, the mortality in the 2 highest doses was 100%. On day 31, the mortality of all Cantus® treatments had reached 100% while the control was just below 50%

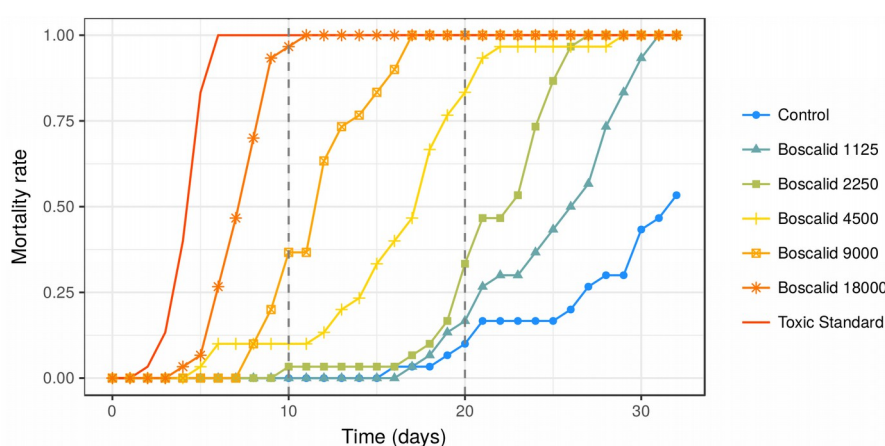


Figure 5.4 – Observed mortality rate over the duration of the test ($n=30$ bees per treatment, except in boscalid 9000 mg/l, $n=29$).

Dashed lines indicate 10 days, i.e. duration of the test currently proposed by international standards, and 20 days, i.e. time at which the mortality in the control reached 15%. Current international standards (10 day) propose as validity criteria up to 15% mortality in the control group.

The Weibull 2 and logistic models performed most of the time better (better fit, lower AIC) than the log-logistic and Weibull 1 models. The Weibull 2 model was more stable than the logistic model for which the standard errors were often impossible to compute. Both models provided very similar estimates of ecotoxicological endpoints in any cases.

The median lethal time (LT_{50}) decreases when the concentration increases (Fig. 5.5A, Table 5.2): 7 days (95% CI: 6.89-7.37) at the largest concentration (18,000 mg/l) and 25 days (24.38-25.47) at the lowest tested concentration (1,125 mg/l, Fig. 5.5A). The LT_{50} of the latter group was significantly lower than the 32 days (29.84-34.16) of the control group. The LT_{50} of the toxic standard was 4 days (3.87, 4.4).

Table 5.3 summarises the LC_{50} , LDD_{50} and LCD_{50} values from day 8 to 25 of the trial (Figs. 5.5 B, C and D, respectively). LC_x and LDD_x decreased over time. LC_{50} started at 14,728.73 mg/l (12,055.50-17,401.95) on day 8 and was 1,174.25 mg/l (-150.51-2,499.01) on day 25. In terms of individual dose, LDD_{50} was 0.32 mg boscalid bee⁻¹ day⁻¹ (0.27-0.36) on day 8, and decreased up to 0.03 mg boscalid bee⁻¹ day⁻¹ (-0,004-0.064) on day 25.

The median lethal cumulative dose (LCD_{50}) showed a plateau around 2.4 mg boscalid bee⁻¹ until day 17, when LCD_{50} started to decrease to reach 0.76 mg boscalid bee⁻¹ (0.42-1.480) on day 25 (Fig. 5.5D).

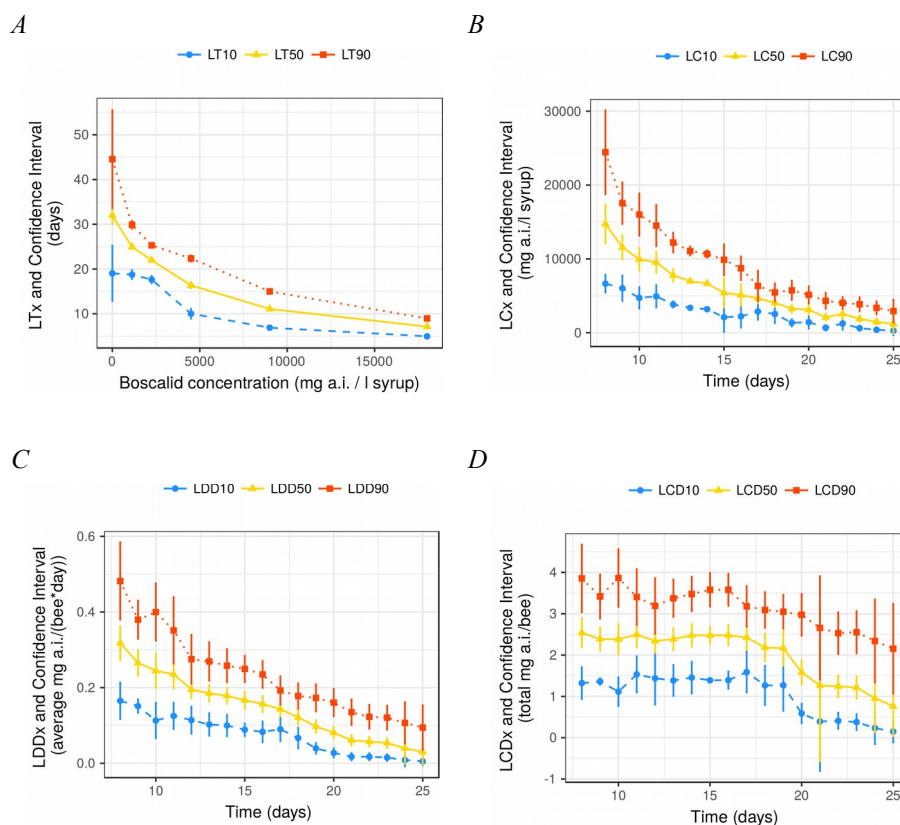


Figure 5.5 – Representations of LTx vs. Concentration (A), LDx vs. Time (B), LDDx vs. Time (C) and LCDx vs. time (D).

All graphs represent the toxic endpoint for 10%, 50% and 90% mortality estimated with 3 parameters Weibull 2 models. See table 5.1 for the meaning of the abbreviations.

Table 5.2 - Estimated values of LT_{10} , LT_{50} and LT_{90} for the different concentrations of the test

Treatment group	LT10 (days)				LT50 (days)				LT90 (days)			
	Estimate	SE	Lower CI	Upper CI	Estimate	SE	Lower CI	Upper CI	Estimate	SE	Lower CI	Upper CI
Control	19,04	3,26	12,65	25,43	32,00	1,10	29,84	34,16	44,56	5,69	33,41	55,71
1125	18,76	0,61	17,56	19,95	24,93	0,28	24,38	25,47	29,88	0,55	28,80	30,96
2250	17,65	0,52	16,63	18,66	21,99	0,26	21,48	22,50	25,30	0,34	24,64	25,97
4500	10,02	0,66	8,72	11,32	16,36	0,43	15,52	17,21	22,36	0,45	21,47	23,26
9000	6,90	-	-	-	11,09	0,13	10,83	11,35	15,00	-	-	-
18000	4,97	0,03	4,90	5,04	7,13	0,12	6,89	7,37	8,97	0,21	8,55	9,39
TS	2,87	0,22	2,44	3,29	4,14	0,14	3,87	4,40	5,22	0,19	4,84	5,60

Note. When “-” appears, the model is unable to estimate the values; LT_x calculated based on a model Mortality vs. Time. SE = Standard Error; CI = Confidence Interval.

Table 5.3 - Estimated values of LC_{50} , LDD_{50} and LCD_{50} from day 8 to day 25 of the test

Time (days)	LC_{50} (mg/l)				LDD_{50} (mg bee ⁻¹ day ⁻¹)				LCD_{50} (mg bee ⁻¹)			
	Estimate	SE	Lower CI	Upper CI	Estimate	SE	Lower CI	Upper CI	Estimate	SE	Lower CI	Upper CI
8	14,728.73	1,363.91	12,055.50	17,401.95	0.3177	0.0226	0.2734	0.3620	2.5418	0.1808	2.1875	2.8961
9	11,571.99	835.23	9,934.97	13,209.01	0.2653	0.0094	0.2469	0.2836	2.3875	0.0842	2.2225	2.5525
10	9,949.15	778.86	8,422.60	11,475.69	0.2446	0.0232	0.1991	0.2900	2.3822	0.1875	2.0147	2.7498
11	9,533.63	731.20	8,100.51	10,966.75	0.2352	0.0192	0.1976	0.2728	2.4968	0.1602	2.1828	2.8109
12	7,775.04	430.79	6,930.70	8,619.37	0.1954	0.0120	0.1719	0.2188	2.3397	0.1452	2.0551	2.6243
13	6,971.13	6.80	6,957.81	6,984.45	0.1850	0.0115	0.1625	0.2075	2.3864	0.1380	2.1159	2.6569
14	6,668.31	-	-	-	0.1784	0.0108	0.1572	0.1996	2.4767	0.1417	2.1989	2.7544
15	5,410.47	1,003.79	3,443.07	7,377.86	0.1668	0.0105	0.1463	0.1873	2.4770	0.1185	2.2449	2.7092
16	5,121.77	747.35	3,656.99	6,586.54	0.1568	0.0113	0.1347	0.1789	2.4786	0.1298	2.2241	2.7331
17	4,665.58	344.38	3,990.61	5,340.55	0.1431	0.0097	0.1242	0.1621	2.4264	0.1534	2.1257	2.7271
18	4,059.89	286.03	3,499.29	4,620.49	0.1215	0.0099	0.1021	0.1408	2.1840	0.1714	1.8481	2.5198
19	3,261.72	278.23	2,716.41	3,807.04	0.0973	0.0084	0.0809	0.1137	2.1683	0.2152	1.7464	2.5901
20	3,126.91	362.59	2,416.25	3,837.57	0.0808	0.0081	0.0650	0.0966	1.5826	0.1467	1.2950	1.8701
21	2,095.29	225.02	1,654.26	2,536.32	0.0608	0.0072	0.0467	0.0750	1.2638	0.9318	-0.5625	3.0901
22	2,559.12	366.78	1,840.24	3,278.00	0.0573	0.0066	0.0443	0.0702	1.2441	0.1347	0.9801	1.5080
23	1,887.04	207.70	1,479.95	2,294.13	0.0538	0.0066	0.0408	0.0668	1.2147	0.1386	0.9430	1.4864
24	1,471.58	206.09	1,067.65	1,875.51	0.0400	0.0207	-0.0006	0.0805	0.9547	0.4193	0.1330	1.7765
25	1,174.25	675.91	-150.51	2,499.01	0.0301	0.0175	-0.0043	0.0645	0.7621	0.3668	0.0433	1.4810

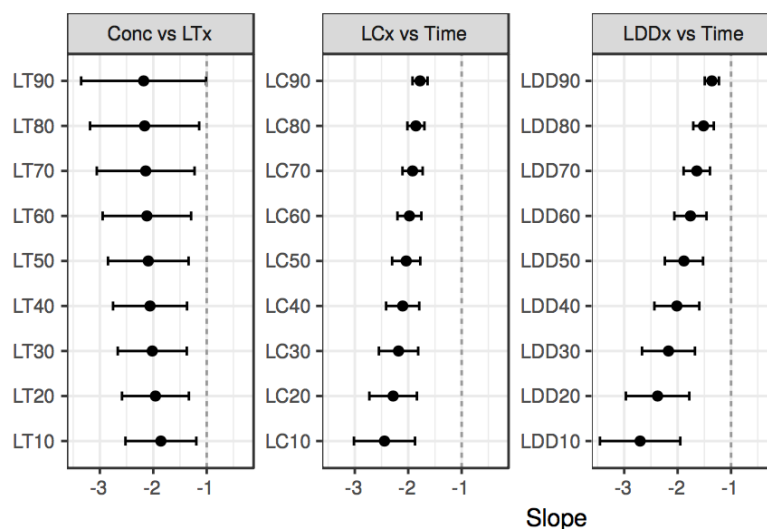


Figure 5.6 – Estimates of the slopes (black dots) and their confidence intervals of the models for the different log-log linear regressions. The dashed vertical lines indicates a slope equal to -1, which is the value expected by the Haber's law if there is no cumulative toxicity. All LTx, LCx and LDDx slopes values are significantly lower than -1. Lower precision (larger Confidence Intervals (CI)) was observed for the log(Concentration) vs. log(LTx) models because they are based on 5 points (one for each concentration tested).

Cumulative toxicity - first approach: log-log linear regressions

The slope of all log-log linear regressions are significantly lower than -1 (Figure 5.6) except for the slope of the regression between log(concentration) and log(LT90) that is only borderline significant (CI: -3.370 - -0.995). The time-concentration relationships diverge clearly from Haber's law and point toward an increased toxicity for long time exposure.

However the slope is only a rough summary of this relationship. When plotting the data it appears that the log-log relationship is not always linear and show interesting patterns (Figure 5.7).

More precisely, the $\log(\text{LDDx})$ vs $\log(\text{Time})$ relationship seems to follow closely Haber's rule up to day 17-18 with a slope of around -1. At day 17-18 the slope abruptly decreases and the relationship clearly deviates from Haber's rule. This pattern is more marked for lower levels of mortality (LDD_{10} , LDD_{20} , etc) and tend to disappear at higher levels of mortality (LDD_{90}). For the LDD_{90} the points are almost perfectly aligned on a straight line without any inflection point. However even for the LDD_{90} were the observed regression line seems to be close to the theoretic Haber's line, the slope is significantly smaller than the expected -1 (estimate = -1.359 with a 95% confidence interval of [-1.492,-1.226]).

This pattern is less marked on the $\log(\text{LCx})$ vs $\log(\text{Time})$ graph (Supplementary material, Fig. S5.7) and not visible on the $\log(\text{LTx})$ vs $\log(\text{Concentration})$ graphs (Supplementary material Fig. S5.8, but these graphs are based only on 5 points which are probably not enough to visualise this non linear relationship).

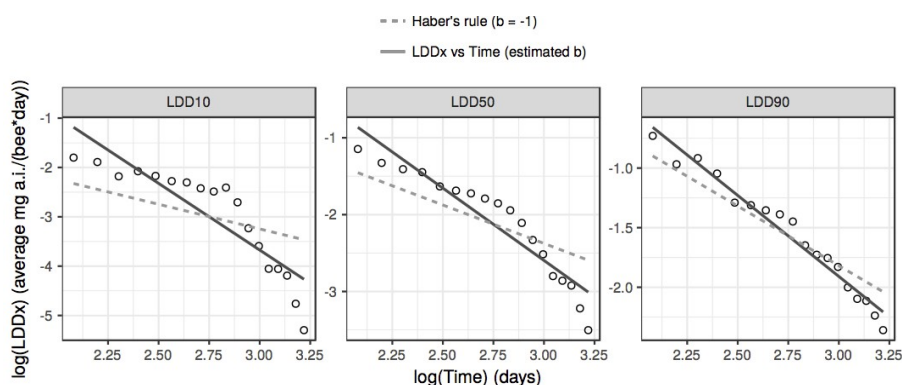


Figure 5.7 – Log-log regression of LDDx vs. Time compared to the regression line expected in the absence of cumulative toxicity

The dashed lines show regression lines with a fixed -1 slope (the models estimate only the intercepts). This is the expected relationship under Haber's law i.e. if there were no cumulative toxicity. The slopes of the observed linear regression are significantly lower than -1, which implies some level of cumulative toxicity. However the log-log relationship is clearly not linear at least for the lowest mortality rates. The representation of the log-log relationship of the other parameters can be found in Supplementary material, section S5.4.

Cumulative toxicity - second approach: cumulative doses

The cumulative dose necessary to reach 50% of mortality is similar (approximately 2.5 mg a.i./bee) for the three highest concentration treatments (18,000, 9,000 and 4,500 mg a.i./l syrup - see Figure 5.8). The same level of mortality is reached with significantly lower cumulative doses for the two lowest concentration treatments: 1.38 mg a.i./bee for a concentration of 2,250 mg a.i./l syrup and 0.84 mg a.i./bee or a concentration of 1125 mg a.i./l syrup (Figure 5.8). Figure 5.9 shows another way to look at the same data. While the time to reach 50% of mortality (black dots - LT_{50}) is longer for the lowest concentrations, the cumulative dose necessary is much lower than what we observe for higher concentration treatments.

Without cumulative toxicity (under Haber's rule) we would expect that the bees should be exposed to the same cumulative dose to reach the same level of mortality. Again these results point toward an increased toxicity for long term exposure to Cantus ®.

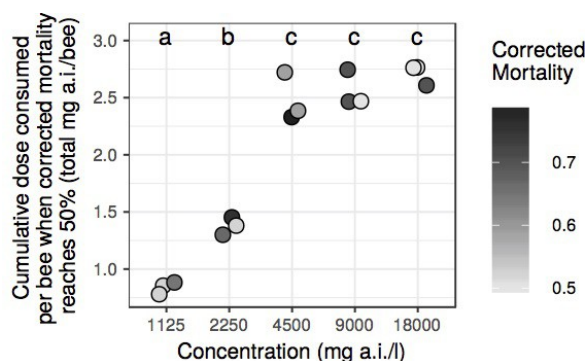


Figure 5.8 – Cumulative dose consumed by the bees when they reach 50% of corrected mortality. Each circle represent a replicate, i.e. a cage. Some horizontal noise has been added to avoid overlapping. In some cages the exact 50% corrected mortality was never observed. We took the first value above the 50% threshold. The corrected mortality is mapped on the graph with a continuous grey colour scale.

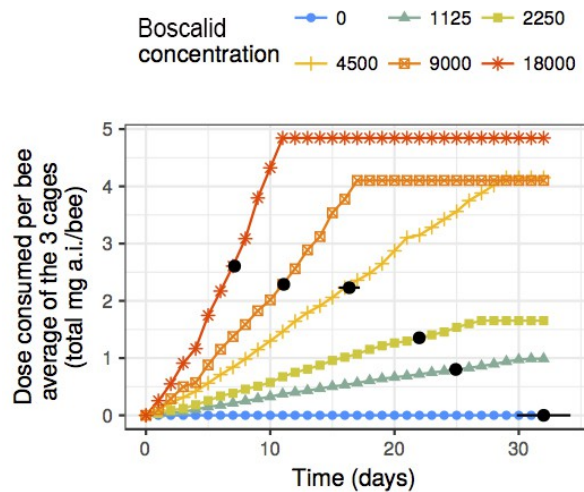


Figure 5.9 – Cumulative dose consumed per bee for each treatment (concentration) over time.

The black dots indicate the estimated values of LT_{50} (with 95% confidence intervals) for each concentration. Without cumulative toxicity, the black dots should appear horizontally at approximately the same level of cumulative dose or aligned vertically above the control LT_{50} for the two lowest concentrations.

5.4 DISCUSSION

Syrup consumption

Despite having used a standardised methodology, syrup consumption was highly variable within the cages, between the cages and over time. We observed a non-constant individual daily syrup consumption over the duration of the trial, with high consumption values when only a few bees were left in the cage (1 to 3 bees). These extreme values may be in part due to syrup evaporation, reason why it would be worthwhile to include this parameter as a measurement to be performed during chronic toxicity testing. In contrast to our results, Arnold^{5 3} observed smaller levels of consumption when bees were isolated. We also clearly observed a non

constant consumption level over time and a difference between the treatments both in terms of kinetics (the first peak of consumption appears at a different time) and in term of absolute consumption (the food intake is lower when the concentration is higher whatever the time). In contrast to our observations, a constant daily consumption per bee and no treatment effect was reported in a similar experiment testing chronic mortality of imidacloprid and deltamethrin⁵⁴. This would have involve a more even exposure of bees to pollutants, even if the distribution of food within the bees of the same cage is known to be variable^{55,56}. However our results show that real consumption differences can be masked when the data are analysed with simple statistical approaches because of complex non linear consumption vs time relationships and differences in kinetics (time x treatment interactions). The variation of syrup consumption over the trial and food distribution among bees are realities whose impact needs to be considered during the interpretation of toxicity results. After all, they will have an impact on dose calculation, but also on the implementation of methodologies proposed for regulatory purposes, i.e. cumulative toxicity⁵¹.

Toxicity of Cantus® on honey bee

The mortality of the bees in the Cantus® treatments was significantly higher than that of the control. Chronic toxicity of this pesticide in terms of concentration (LC₅₀) was 1,174 mg boscalid/l on day 25. This concentration is far from the highest residue value described in literature for boscalid in pollen (26 mg/kg)²⁶, but would be in the lower range of concentrations applied in the fields. In addition, the effects measured here are mortalities and we could expect sublethal effects at even lower concentrations. Both LC₅₀ and LDD₅₀ decreased in time, their values being reduced by approximately 90% from day 8 to day 25. Therefore, we found a reinforcement of the toxicity following long-term exposure of bees to a fungicide, the same as other studies found mainly with insecticides^{54,56-65,52-59}. The main differences between our study and others were the level of toxicity described and the order of magnitude of the concentrations tested. These tested concentrations are a reflection of the amount of residues bees are exposed to in real conditions.

Standard methodologies currently prescribe to test chronic toxicity over a period of 10 day⁴¹. These standard methodologies are themselves inspired by previous toxicological studies evaluating chronic effects on bees after 10-14 days exposure^{57,63,64}. This duration seems adequate for testing pesticides with high to moderate acute toxicity on bees. The effects we observed here, however, would not have been detected over a duration of 10 days. Pesticide exposure longer than 10 days is not an unrealistic case for bees, given that boscalid was found to contaminate food resources over months²¹. This should not be surprising considering that boscalid is systemic, persistent and is authorised for a large list of uses, even during the flowering period of many crops⁶⁶. For this reason, we think that a time-to-death approach might be more adequate to evaluate the risk of chronic exposure to a pesticide. The estimation of the LT_x as toxicological endpoint is useful to compare the effect of chronic exposure to different doses and allows to evaluate the impact of stressors on the lifespan of bees. In our case, even the lowest concentration tested significantly reduced the time to reach 50% of mortality. An additional advantage of such an approach is that it can help detect a potential cumulative toxicity.

Cumulative toxicity

A first easy indication about the existence of cumulative toxicity of Cantus® can be inferred from the relationship between the Lethal Cumulative Dose (LCD_x) and Time. The plateau observed in figure 4d until day 17-18 indicates that a certain amount of bees manage to detoxify the ingested pesticide: whatever the duration of repeated exposure, the same cumulated amount of pesticide is necessary to kill 50% of the bees. From days 17-18 onwards, a smaller and smaller total amount of pesticide is sufficient to kill the same amount of bees: their detoxifying capacity seems to be overwhelmed, or the accumulation in the bee bodies is enough to trigger toxic effects, and then toxicity increases with time. This time increased toxicity and sudden change in toxicity around 17-18 days is confirmed by several other statistical approaches: the log-log regressions to test if the relationships between concentration or dose and time follow Haber's rule⁶⁷ and the so-called EFSA protocol (comparison of cumulative doses between concentrations)⁵¹.

Moncharmont *et al.*⁵⁴ observed a delayed toxicity of the insecticide imidacloprid on bees and argued that the effects could be linked to a different sensitivity of bees linked to age or accumulation of the compound in the bees. The dynamic of syrup consumption of the control also suggests a naturally occurring change in the physiology of the bees even without consumption of Cantus®. Indeed, at days 18-19 of their life, bees in the control achieve a maximum syrup consumption while the mortality slowly increases. The sudden change of toxicity around 17-18 days could then be explained by a higher sensitivity due to the natural ageing process, by a physiological fatigue due to the repeated exposure to the pesticide during their life or by a combination of both processes. The similar shapes in food consumption observed for the different concentrations, but with maximums at earlier dates for higher concentrations, might indicate a faster ageing of bees when exposed to the pesticide and suggest that ageing alone is not a sufficient hypothesis to explain the patterns observed. The effects of xenobiotics on honey bees involve responses of detoxification, oxidative and general stress leading to an increase in the insect's energetic metabolism⁶⁸. These may lead to a physiological wear-out and reduction of vitellogenin production, thus shortening the lifespan and the immunocompetence of bees^{69,70}. We did not supply any dietary protein during the test. Therefore, the already limited detoxification capacity of bees⁷¹ would have been even more restricted when the bees exhausted their capacity of protein synthesis due to a lack of essential aminoacids⁷².

The toxicity and the sudden change in toxicity could also be explained by other mechanisms than ageing and physiological fatigue. For example, considering that boscalid is moderately lipophilic ($K_{ow} = 2.96$)²², it could have a certain tendency to be accumulated in the bees' fatty tissue (i.e. bioconcentration). The products contained in Cantus® or their metabolites could accumulate in the bees' fatty bodies from the onset of the exposure up to a level of saturation, when these chemicals could target other tissues, accelerating the toxic effect of these compounds if exposure is prolonged. Unfortunately we did not evaluate the residues of boscalid and metabolites on dead bees. This would have clarified the level of accumulation happening in bees⁵⁷. The lower food intake when the syrup is contaminated

by the pesticide may also contribute to the weakening of the bees and decrease their capacity to handle the toxicity on the long term. Finally, it must be stressed that the toxicity observed here might be induced by boscalid itself, but also by the co-formulants present in Cantus® or interactions between all these xenobiotics^{39,73}.

Cumulative toxicity

The time to death approach used here generates a large amount of data that allowed us to explore different methodologies to test for cumulative toxicity. The main advantage of the protocol proposed by EFSA is that it is the less costly approach: after an acute toxicity test of 48h, one must run a new test and monitor the mortality for only two concentrations (the LC_{50} and $\frac{1}{4}$ of this concentration) up to 50 % of mortality and then test whether the cumulative lethal doses are equal. However in our case the toxicity after 48h was too low to estimate the LC_{50} (no acute toxicity). After 10 days, the estimated LC_{50} was approximately 10,000 mg/l and could have been compared with a 2,500 mg/l treatment. Instead of running a new test, we monitored the mortality for all our initial concentrations until the control reached 50% of mortality. Using these data, we could show that the cumulative dose to kill 50% of the bees at a concentration of 9,000 mg/l (the concentration closest to the LC_{50}) and 2,250 mg/l are significantly different, supporting the hypothesis of cumulative toxicity with an approach close to the simple EFSA protocol. However using only two concentrations would mask some of the most interesting patterns observed in this study that could help to understand the toxicological effect of the pesticide. Indeed, using the log-log regression between the Lethal Dose and Time revealed a clear breaking point in the toxicological effect after 17-18 days that would remain unnoticed with simpler approaches. The log-log regression between the Concentration and the Lethal Time⁵⁰ are also less interesting for the same reason: the lower number of points (i.e. the number of different concentrations tested) makes such subtle patterns difficult to spot. We also showed that simply estimating the slope of the log-log regression might be useful but it is only a rough summary and plotting the data is necessary to check if we have a simple, constant, deviation from Haber's rule or a more complex relationship between toxicity and time. The

Lethal Dose should also be preferred to the Lethal Concentration in the log-log regressions because the later does not take into account the differences in food intake between treatments. Lethal Concentration estimates are however still useful to compare the lab results to the concentrations observed in beehive matrices or applied on the field by farmers.

In conclusion, we showed that at field application rates, Cantus® (500 g boscalid/kg) leads to a chronic toxicity in honey bees that would have remained undetected with current proposed methodologies for pesticide risk assessment. This fungicide significantly reduces the lethal time for all concentrations tested. Furthermore, a cumulative toxicity potential was detected particularly after 17-18 days of exposure. All in all, we recommend a time-to-death approach rather than fixed duration studies to explore the chronic toxicity effects of pollutants that are present over long periods in field conditions.

5.5 SUPPLEMENTARY MATERIAL

S5.1. Syrup consumption and evaporation

S5.1.1 Evaporation data

We collected partial data about the difference of syrup weight that might be due to simple evaporation by adding syringes with syrup into cages without bees or with 10 dead bees during some days of the experiment and measuring differences in their weight as described for syrup consumption.

One rather extreme data seems to be a transcription or measurement error (Fig S5.1A). Excluding this outlier, it appears that the weight loss due to evaporation is between 0.04 and 0.1 g per cage and per day. There is clear variation from day to day (Fig S5.1B). Therefore it seems that a daily measurement would be better to correct for evaporation on each day. The presence of dead bees does not seem to affect the values. However the presence of living bees might change the evaporation rate. The effect of evaporation is mainly important in the evaluation of the daily consumption when there are only 1 or 2 living bees left. However the impact of the evaporation (as measured here, ie not very precisely) on the overall results (kinetic of the toxicity and kinetic of the consumption during most of the test) seem minor.

We compared the results with and without correction for evaporation (using the average evaporation value after excluding the outlier). The impact of this correction on the daily consumption is discussed in section S5.1.2. This correction did not significantly affect the rest of the analyses (i.e. LCx, LDDx, LCDx, LTx and accumulation toxicity, results not shown here). As a result, the results described in the document do not take into consideration evaporation. Anyway, only recently evaporation was taken into account when performing chronic toxicity studies. Baring in mind the

importance of evaporation, it would be recommendable to perform specific measurements to evaluate the impact of syrup evaporation along chronic toxicity trials to improve the estimation of the dose-effect statistics and reduce the day to day measured variability in consumption.

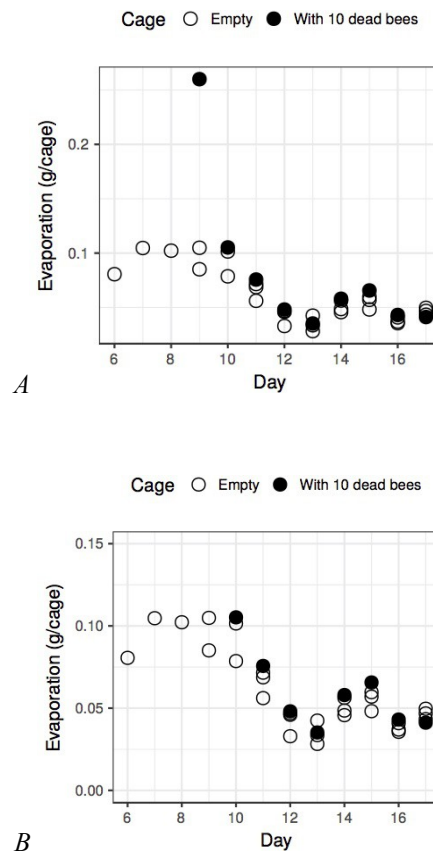


Figure S5.1 – Values of evaporation (g/cage) on the days evaluated, including the outlier (A) and excluding the outlier (B)

Note: since syrup consumption is generally measured as “consumption per bee”, the measurement error caused by evaporation will have a higher impact when the mortality per cage is high (i.e. when there are fewer living bees).

S5.1.2 Consumption of syrup between doses and over time: kinetic of the consumption with evaporation correction

The most important results have been included in the main text. Here we just add some complementary information on the impact of the correction for evaporation (Figs. S5.2A and B).

If we use the consumption data corrected for evaporation, most of the high values of consumption described at the end of the test soften. However there are still a few cases of peaking consumption associated with low number of bees, notably at the doses 4,500 mg/l and 18,000 mg/l. Therefore, it seems that the observed peaks might be due to a combination of measurement error due to evaporation (more important when there are few bees) and the mere fact that the bees are in low number.

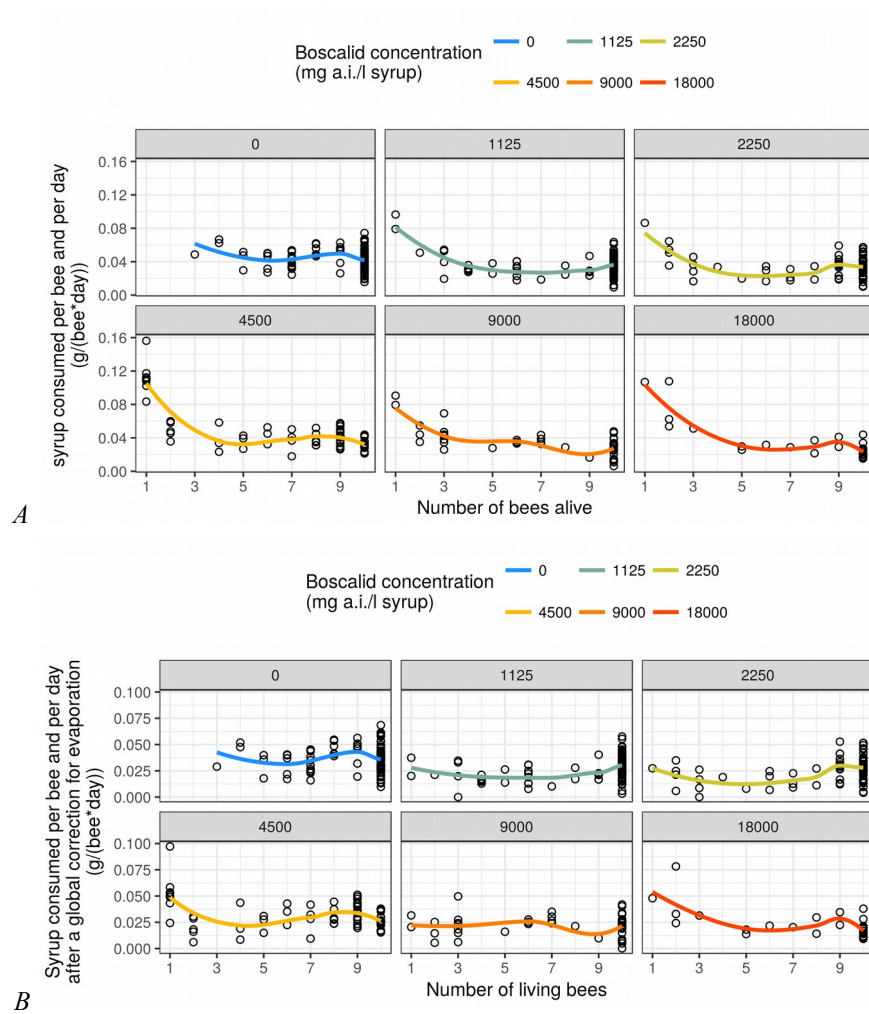


Figure S5.2 - Syrup consumed per bee and per day vs. the amount of living bees per cage, without correction for evaporation (A) and with correction (B). The figures show the influence of the number of bees in the cage on the sirup consumption.

S5.1.3 Kinetic of the consumption up to 50% of mortality

The question of the difference of consumption in time and between dose is mainly important up to 50% mortality (e.g. for application of the accumulation toxicity test proposed by EFSA, for ED₅₀ calculations, etc.). We can look at the consumption data after removing the data corresponding to more than 50% of mortality. The results are almost identical with or without correction for evaporation. As a result, we show the data without evaporation correction.

A number of data and figures have been included in the main text. Here we include only some complementary information.

Likelihood ratio test (Type II) - The Time x Concentration interactions are significant meaning that the kinetic of the consumption (consumption vs time) is different between concentration. The fact that the main Concentration effect is highly significant means that even if we do not take into account the interactions (type II test) the consumption at the mean day (~12.5 days) is different between the treatments (concentrations).

Table S5.1 – Results of the Likelihood Ration (RT) test

	LR	df	p(>Chisq)	
Conc	30.11	5	1.4e-05	***
Time	1.194	1	0.275	
I(Time^2)	68.47	1	1.11e-16	***
Conc:Time	33.48	5	3.02e-06	***
Conc:I(Time^2)	11.87	5	0.0366	*

Note: a model without centring the time still shows clear differences of consumption at day 0 (i.e. the intercept for non centred data) at least between the control and the 2 highest concentrations.

S5.1.4 Average consumption (without taking the time into account)

In most bee tests described in literature, the average consumption between the treatments is compared without taking into account the time/kinetic. We can test if there is a significant difference between the treatments with a gaussian mixed model with the concentration as fixed effect and the cage as random effect (Figs. S5.3 A and B). The main results have been in the text.

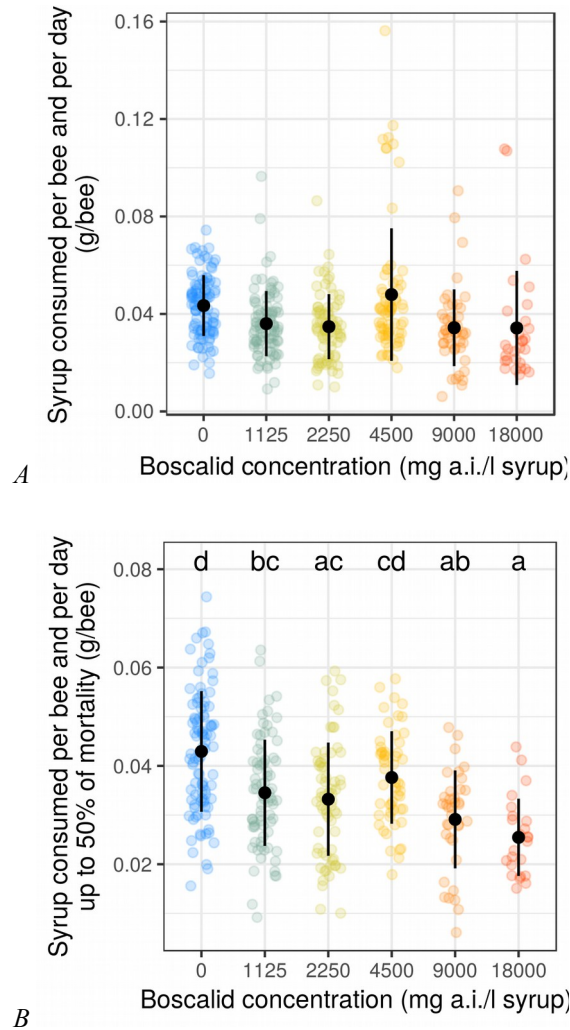


Figure S5.3 – Average syrup consumption (without taking into consideration the time), including the consumption of all bees in the cage (A) or including only consumption up to 50% mortality in the cage (up to 5 dead bees per cage, B). Note: the black dots represent the mean and the bars their standard deviation. The significant differences are displayed on the graph with letters (concentrations sharing the same letter are not significantly different). Daily consumption of syrup with different Cantus® concentrations expressed in boscalid concentration.

S5.2 Comparison of the 4 types of models

The aim of this section is to explain why we choose a Weibull 2 model in all the analyses shown at the manuscript. The Supplementary material of this publication includes can be found online and includes the complete statistical approach followed. However, here I just included the estimation of one of the toxicological endpoints, de Lcx as an example.

In most of the studies the type of model is chosen *a priori* and without justification. We compare 4 types of dose response curves (logistic, loglogistic, weibull 1 and weibull 2) for each day between D8 and D25 and for each type of effect: LC, LDD, LCD. A similar approach is used to compute the LT (using the data from all days). The aim is to choose the model with the best fit (most of the time) and best statistical properties.

We compare the quality of the fit of each model by extracting the AIC value and computing the difference between the best model (with lowest AIC) and the other AIC values for a given time (for LC, LDD, LCD) or a given concentration (for LT) (Tables S5.2 and S5.3). We have also computed a goodness of fit test (modelFit function from drc package in R²) for each model (available in the raw outputs). We also compare graphically the differences of LCx, LDDx, LCDx and LTx computed with each model. Here we will show only the results for the LC.

Here are the formulas of the 4 models³:

3 parameters Logistic Model (with d = 1)

$$f(x) = c + \frac{d - c}{1 + \exp(b(x - e))}$$

3 parameters Weibull 1 Model (with d = 1)

$$f(x) = c + (d - c) \exp(-\exp(b(\log(x) - \log(e))))$$

3 parameters Log-Logistic Model (with d = 1)

$$f(x) = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(e)))}$$

3 parameters Weibull 2 Model (with d = 1)

$$f(x) = c + (d - c) \left(1 - \exp \left(- \exp \left(b(\log(x) - \log(e)) \right) \right) \right)$$

Lethal concentrations (LCx) at each time

The first lines of the AIC and goodness of fit results can be found in Table S5.2, and the first lines of the LCx estimates results in Table S5.3.

Table S5.2- First lines of the AIC and goodness of fit results

Time	Model	df	Chisq	p	AIC	DeltaAIC	ED50	LowerCI	UpperCI
8	Logistic	12	11.92	0.452	35.72	0	14818	NA	NA
8	Weibull2	9	11.46	0.245	36.5	0.78	14729	12056	17402
8	LogLogistic	12	12.38	0.416	37.6	1.882	14452	12115	16789
8	Weibull1	9	13.68	0.134	40.9	5.187	14787	11397	18177
9	Logistic	9	7.485	0.587	29.78	0	11556	NA	NA
9	Weibull2	9	8.076	0.527	30.27	0.496	11572	9935	13209
9	LogLogistic	12	10.72	0.553	33.47	3.69	10886	9207	12566
9	Weibull1	9	14.28	0.113	40.15	10.37	10114	8040	12187
10	Logistic	12	6.099	0.911	29.43	0	10030	NA	NA
10	Weibull2	15	7.135	0.954	29.54	0.106	9949	8423	11476
10	LogLogistic	15	10.3	0.8	32.87	3.444	9527	7872	11183
10	Weibull1	15	13.92	0.531	37.88	8.448	8823	7195	10452

Table S5.3 - First lines of the LCx results

Time	PctMort	Model	Estimate	Std..Error	Lower	Upper
8	10	Logistic	6546	NA	NA	NA
8	20	Logistic	9599	NA	NA	NA
8	30	Logistic	11628	NA	NA	NA
8	40	Logistic	13292	318	12669	13915
8	50	Logistic	14818	NA	NA	NA
8	60	Logistic	16345	NA	NA	NA
8	70	Logistic	18008	NA	NA	NA
8	80	Logistic	20038	NA	NA	NA
8	90	Logistic	23091	NA	NA	NA
8	10	LogLogistic	6569	535.5	5519	7618
8	20	LogLogistic	8788	720.3	7376	10199
8	30	LogLogistic	10663	876.6	8945	12381
8	40	LogLogistic	12495	1029	10478	14512
8	50	LogLogistic	14452	1192	12115	16789
8	60	LogLogistic	16716	1381	14009	19423

The comparison of LCx estimates for each rate (LC10, LC20, etc) for the 4 types of models show that the estimates of the Weibull2 model and Logistic models are very close to each other. For the LC₅₀ all four models provide similar estimates. The Logistic is however almost always the best model or close to be the best model. Nevertheless, from day 8 to 15 the standard error of the LC₅₀ could not be estimated by the model-. The Weibull2 model has generally an AIC very close to the logistic model excepted at days >15 where it regularly peaks far away from the best model. The Weibull1 and LogLogistic models can deviate quite strongly from the others particularly for the estimates of LC₁₀, LC₂₀, LC₈₀ and LC₉₀ (Fig. S5.4).

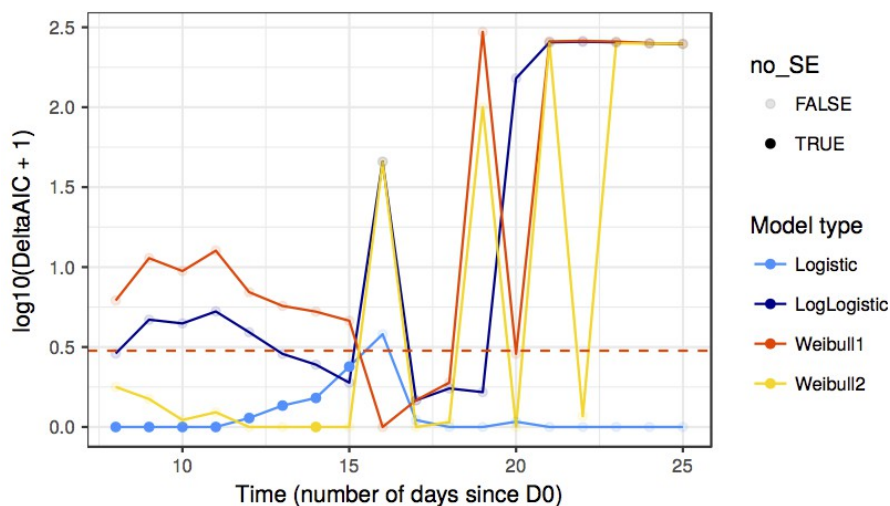


Figure S5.4 – Comparison of the delta AIC values (on a $\log_{10}(x+1)$ scale) for the 4 types of models at each day after treatment (between 8 and 25). The horizontal dotted red line is the classical threshold of difference of AIC = 2 considered to be significant. When a dot is plotted (grey or black), it means that the LC50 was estimated at that day but no standard error (and hence no confidence interval) could be estimated by the model).

The Weibull2 model seem to be a good compromise that can be used to estimates LC50 and other LCx at all days between 8 and 25. The confidence interval is not available on day 14 and on day 13 it is abnormally small. However the estimates at these dates are quite similar to the estimates of other models and within the range of the confidence interval of the next best model (Weibull1).

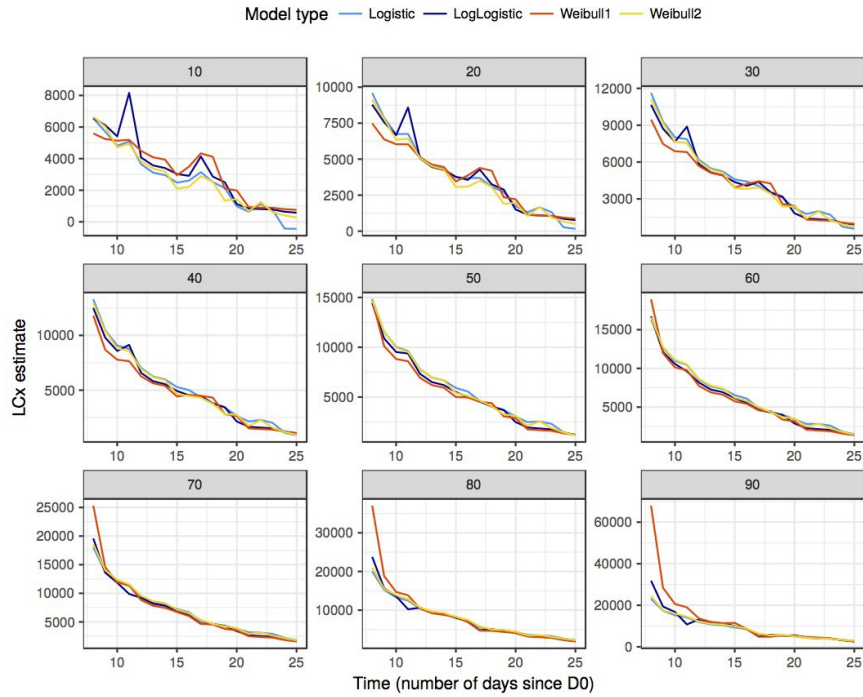


Figure S5.5 – Comparison of the standard errors of 3 of the model types for the LCx estimates.

Even after day 15 where the Weibull2 models had some times AIC clearly higher than the Logistic model, both the estimates and their confidence intervals are comparable (but confidence intervals are not available for logistic models for days 8-15).

S5.3 Comparison of models with 2 or 3 parameters using corrected mortality or not

In the previous analyses we used 3 parameters models and uncorrected mortalities. The results of such models are identical or very similar to estimates of more classical 2 parameters models based on corrected mortalities. Here, we compare the results based on these two approaches.

We have used the corrected mortalities (Abbott's formula⁴ on the % of mortality) and performed 2 parameters models. This approach is not ideal for at least two reasons: (1) the corrected mortality is no more a ratio of to integers (ie because of the unbalance in the design); and (2) the total number of individuals used in the model (as weights) remains constant while it should decrease when the correction is higher. However this should have mainly an impact on the standard errors and not on the estimate itself which is our main interest here.

It could be possible to correct directly the number of dead bees and the total and then compute the corrected ratio. This means however that we have to group the data from the 3 replicates, slightly change the way the consumption is calculated and the correction will be unfair for the doses with a different initial number of bees.

The comparison of the 2 approaches shows that the estimate are very close to each other particularly for the Weibull2 model used in the previous analyses.

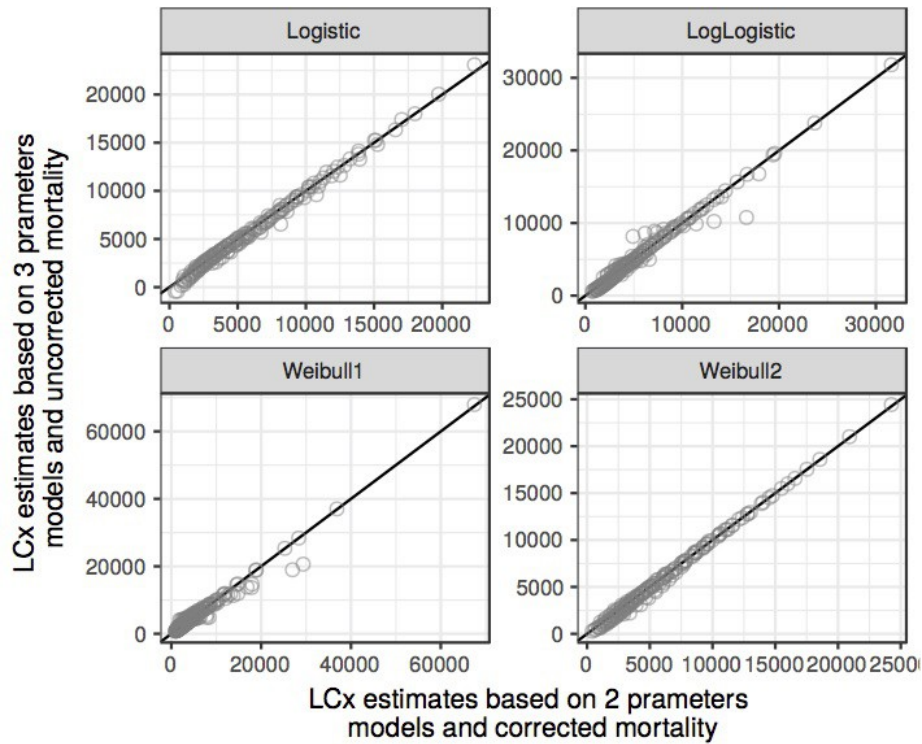


Figure S5.6 – Comparison of the approach including 2 and 3 parameters models and corrected and uncorrected mortality, respectively.

Note: the black line shows a line with slope = 1 and intercept = 0 (ie the line showing the perfect match). The few points that deviates in the LogLogistic models and Weibull1 models are due to 2 parameters models with a clear lack of fit and very large standard errors.

S5.4 Representation of the log-log relationship of the other parameters

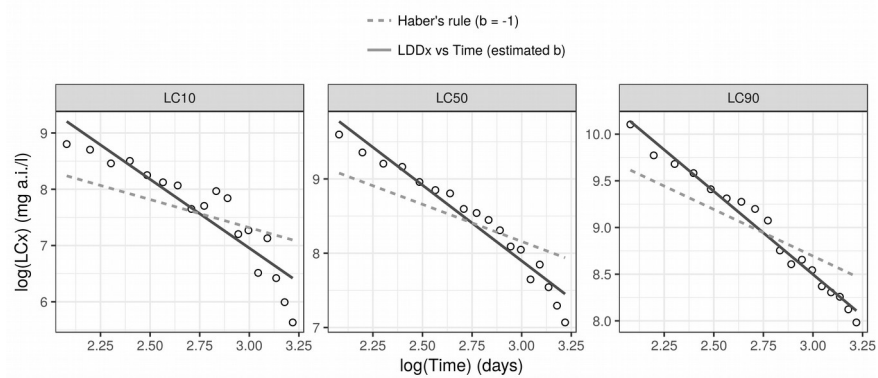


Figure S5.7 – Log-log relationship LC_x vs Time

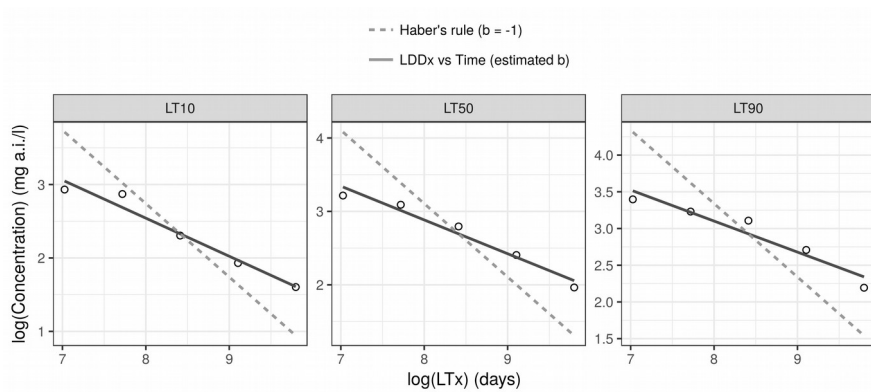


Figure S5.8 – Log-log relationship Concentration vs LT_x . Sanchez-Bayo et al., 2009, show this relationship expressed as $\log(\text{LT}_x)$ vs $\log(\text{Concentration})$. The trend in both graphics is however the same, indicating a deviation of the observations vis-a-vis the expected values obtained if the Haber's law applies.

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5.7 AUTHOR CONTRIBUTIONS

NS performed the experimental part and GSM performed the analyses with the help of NS and LH. NS and GSM wrote the main manuscript text. All authors reviewed the manuscript.

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Chapter 6

General discussion and perspectives

6.1. GENERAL DISCUSSION

The aim of this work was to understand the nature of the relationship between newly recognised bee health stressing factors, fungicides, and colony mortality and morbidity. Together with my colleagues of CRA-W, I started to evaluate the field situation by performing a prevalence case study of colony losses and disorders and different stress factors involved among apiaries distributed along the region. Then I proceeded to carrying out *in vitro* experiments with larvae in an attempt to reduce the amount of the interfering factors and elucidate the potential impacts on the diverse individuals of the colony. Both approaches (field and laboratory) are necessary to provide an answer to the observations described by beekeepers. Finally, we aimed at investigating the origin of pesticide contamination of pollen.

The present discussion is not structured in the same order as in the

chapters of objectives and results, but rather under the form of main outcomes and “take-home” messages.

OUTCOME 1 – Fungicides and arable crops linked to honey bee colony mortality and morbidity

The epidemiological case study carried out and included in Chapter 2 revealed that beekeeping matrices contain a wide range of pollutants coming from agriculture or beekeeping management, wax, beebread and pollen being the matrices most frequently contaminated. It concluded that neither the five virus studied (ABPV, CBPV, QBCV, SBV, DWV) nor the acaricides/insecticides found seemed determinant in the appearance of bee disorders in our study. These disorders seemed clearly linked to the environment of the apiaries and were observed mainly in agricultural crop areas. We observed also that the number of fungicide residues appeared as the main potential stress factor linked to bee disorder. However other stressing factors could be acting or interacting at the same time: insecticides exposure, a lack of amino acids and oligo-elements, etc.

Our results lead to three explanatory hypothesis: (1) fungicides have nothing to do with colonies' health, but they are an indicator of intensive/conventional agricultural farming; (2) fungicides have a higher toxicity to honey bees than expected; (3) fungicides affect colonies indirectly by compromising the internal microbial homeostasis which can lead to weakness and nutritional modifications.

The answer to the first hypothesis “*fungicides as indicator of intensive agricultural farming*” can be intuited through the results obtained during the prevalence case study (Chapter 2). Here, we clearly showed increased probability of observing morbidity and mortality of honey bees during the winter time in areas of arable cropping. On the contrary, the probability of disorders strongly decreases when the grassland surfaces increases. As previously mentioned, we also observed a positive correlation between morbidity and mortality and the amount of fungicides in bee matrices (wax, beebread and honey). The fungicides found in the study (boscalid, captan,

chlorothalonil, cyprodinil, fenpropimorph, iprodione, propamocarbe, pyraclostrobin, pyrimethail, tebuconazole, thiophanate-methyl, trifloxystrobin, zoxamide) are not registered for organic production in Belgium (Phytoweb, 2016). Therefore, it is logic to assume that they originate from a conventionally managed farm. Furthermore, the study aimed at finding the sources of contamination (Chapter 3) indicate a larger probability for the bees to collect contaminated pollen the larger the crop area they have in the surroundings (Fig. 3.3). Therefore, we can conclude that in our study, residues in fungicides in beekeeping matrices are an indicator of intensive/conventional agricultural farming. Indeed, the level of distribution of organic farming in Belgium is rather minimal, as it represents 0.02% of farms and 2.1% of the agricultural area (Eurostat, 2017). As a result, these conclusions lead to question the sustainability of intensive agriculture model and its impact on bees and other productions, like the organic one.

Our results posed doubts about the side effects of fungicides on the bee colony. Further studies need to be developed to improve our understanding of these effects. Their results may show that we need to reconsider the risk mitigation measures imposed for fungicide application in agriculture and avoid the contact of bees with fungicides and the spreading of these products in the environment to a maximum.

OUTCOME 2 – Non-bee-attractive crops as a source of pesticides for bees

The findings of Chapter 3 show that the highest spatial scales considered (3 km) provided the best predictive power for pollen contamination in our study. Pesticides applied to “non-bee-attractive” crops like cereals or sugar beets, generally considered of negligible risk for bees because bees collect neither pollen nor nectar from them, can in fact be a source of exposure through weeds, drift to neighbouring plants or subsequent or succeeding crops. These results imply that the concept of “bee-attractive crop” (i.e. a crop visited by bees for nectar and/or pollen collection) is irrelevant for risk assessment and should not be used as a criterion for decision making for pesticide authorization or use. At the landscape level, honey bee exposure to pesticides depends on pesticide use level, physicochemical characteristics, of the pesticides, period of the year when it is used and landscape composition.

We also showed that the task of modelling the exposure of bees to pesticides once released in the environment may be more complicated than expected. We were able to predict exposure for two pesticides, the fungicide boscalid and the insecticide dimethoate, by considering the surface of different crops cultured in the surrounding of the apiaries, the typical agricultural practices in the region and the list of pesticides authorised per crop. However, it remains difficult to track the direct source of contamination in the landscape. For the third pesticide, the fungicide pyrimethanil, we were not able to find any convincing landscape model (in terms of the composition of crops existing in a radius of 3 km around the apiary) that could explain the contamination.

The results we show in Chapter 3 have consequences for policies and agricultural practices intended to promote the multiplication of nutritional resources for pollinators, like flowering strips, buffer zones, catch crops with melliferous flowers, etc. as they have large probability to increase the

exposure of bees to potentially damaging pollutants. Therefore, these policies and agricultural practices should be designed and applied in parallel to policies and practices leading to pesticide use reduction i.e. integrated pest management, organic farming or agro-ecological practices, precision farming and favouring non-persistent/non-systemic pesticide active ingredients. Without such considerations, instead of favouring pollinators through habitat improvement or food availability, we may transform these areas into highly risky zones or even ecological traps for pollinators.

OUTCOME 3 – Boscalid alone does not show chronic toxicity in immature bee individuals

The active ingredient boscalid, widely used in Belgium and frequently detected in our field experience, seems not to be lethal to worker honey bee larvae at the level of exposure we observed in honey, beebread, wax or pollen. Based on the study carried out *in vitro*, however, the potential effects on the adult bees emerged from these exposed larvae or the potential impact on queen larvae remain unknown. Furthermore, it would be necessary to evaluate the potential impact of boscalid alone or in combination with other active ingredients or co-formulants both on larvae and on adult bees (Renzi *et al.*, 2016). Considering the persistency of boscalid in the field, an interesting approach would be to combine both methodologies: chronic exposure during larval stage and evaluation of the lifespan and performance of emerged-adult bees. The results obtained in Chapter 4 need to be considered in light of the latest findings of increased toxicity of pesticide formulations and combinations with regards to the active ingredients alone or pesticide mixtures. Indeed, Johnson *et al.*, 2013, showed increased toxicity of the acaricide tau-fluvalinate, frequently found in beeswax due to previous veterinary treatments, when caged adult bees were co-exposed to pyraclostrobin and boscalid, compounds formulated in Pristine® (pyraclostrobin 12.8% boscalid 25.2%). The larval studies testing combination of stressors need to be multiplied.

OUTCOME 4 – Boscalid, in its commercial formulation Cantus®, shows chronic toxicity in adult bee individuals

The commercial formulation Cantus® (500 g/l boscalid) was tested at the level of field applications through a time-to-death experiment. Officially, the chronic toxicity of pesticides to bees is assessed through an experience involving 10 days of exposure (European Commission, 2013a; European Commission, 2013b). Bearing in mind the length of the exposure to boscalid observed in field conditions (over 4 months, see Chapter 3) I triplicated the duration of the test in my experiment in order to resemble as much as possible field conditions. The exposure to the contaminated diets significantly increased chronic toxicity of bees in laboratory conditions. The LT_{50} (median lethal time) of the bees in the cages receiving boscalid were significantly shorter than that of the control. Furthermore, both the median lethal concentration (LC_{50}) and the median dietary dose per day per bee (LDD_{50}) decreased along the duration of the test, the effect of the product becoming more important with time.

Different methods used for evaluating the cumulative toxicity of toxicants revealed boscalid, its co-formulants in Cantus® or any of their possible metabolites occurring in bees, increase its toxicity when exposure is prolonged, mainly from days 17-18. Since I did not evaluate the level of toxicant clearance by the bees (the amount of residues in their tissues), I can only speculate about the mechanisms underlying this cumulative mortality. Until the mentioned testing days, cumulative toxicity was not observed, but then the mortality curve showed a point on inflexion (Fig. 5.7). Around these dates, the control group showed a maximum of food consumption. This dynamic of syrup consumption of the control could indicate a naturally occurring impact of ageing. A similar dynamic in food consumption was observed in all treatment groups, but the maximums in consumption were reached earlier as the Cantus® concentration increased. This may indicate that there is a modification happening in the physiology of bees exposed to

the pesticide. Faster ageing could lead to a wear-out of detoxification factors and reduction of vitellogenin production, thus shortening the lifespan and the immunocompetence of bees (Amdam *et al.*, 2004; Corona *et al.*, 2007). I did not supply any dietary protein during the test. Therefore, the already limited detoxification capacity of bees (Claudianos *et al.*, 2006) would have been even more restricted when the bees exhausted their capacity of protein synthesis due to a lack of essential aminoacids (Groot, 1978).

These results show the importance of time-to-death experiments rather than fixed duration studies to explore chronic toxicity effects, mainly for low toxic compounds in acute terms (high median lethal dose, LD₅₀) present over long periods in field conditions.

6.2 PERSPECTIVES

The findings presented through this thesis provide crucial information regarding an unexpected observation: the appraisal of fungicides as factors of stress for bee health. The understanding of the role of fungicides in honey bee colony mortality or morbidity is at its infancy in comparison with other stress factors like insecticides, pathogens or parasites. The work developed so far contributes to the lean state of knowledge on the subject. There is a large list of possibilities to continue elucidating “what fungicides have to do with colony disorders”. I include here a non-exhaustive list of suggestions.

6.2.1 Direct effects of fungicides on bees

6.2.1.1 *In vitro* research

In vitro toxicological studies on both larvae and adult bees were the logic follow up of the field observations in order to better characterise the toxicity of fungicides. The advantages of *in vitro* studies with honey bees are that they allow to isolate the multiple stress factors that permanently interact to determine the fate of honey bee health. On the contrary, their main disadvantage and reason leading most of the outcomes obtained with these experiences not be considered relevant, is the limitation to extrapolate the results to colony real conditions.

Unlike for insecticides, little is known about the impact of long term exposure of bees to pesticides with other mode of action or target groups like fungicides or herbicides. In the future this should change because the set of data requirements needed for pesticide authorisation in Europe includes the development of acute and chronic toxicity tests on adult bees and on larvae and on the evaluation of sublethal effects (European Commission, 2013a, 2013b). Furthermore, there has been a revolution in bee ecotoxicology lately, which has led to giant steps in methodological

development. Since the beginning of the present thesis, we count now with standardised methodologies for *in vitro* larvae rearing with repeated exposure to pesticides (OECD, 2014a), and methodologies to evaluate chronic exposure effects on adult bees (OECD, 2014b). A number of publications have lately proposed methodologies for the impact of pesticides on the reproductive individuals of the colonies, the queen and the drones, some of them combining both *in vitro* and *in vivo* approaches (Brandt *et al.*, 2017; DeGrandi-Hoffman *et al.*, 2013; Kairo *et al.*, 2016; Williams *et al.*, 2015). Given the availability of methodologies and the wide exposure to fungicides readily described, I would suggest to start testing the impact of long term exposure to fungicides on bees of different castes. This testing should be done at environmental relevant doses.

In the future, the objective should be the understanding of the toxicokinetics (behaviour of a toxicant to enter the body and once in the body) and toxicodynamics (interactions of a toxicant with a biological target and its biological effects) of fungicides. For this purpose, the development of molecular markers analysed in different bee body compartments (head, thorax, abdomen) can provide insight about the consequences of this exposure on bees immunocompetence, detoxification capacity or organ development (De Smet *et al.*, 2017; Di Pasquale *et al.*, 2013; Smart *et al.*, 2016). Biomolecular technology like transcriptomics, proteomics or metabolomics allows to describe the physiological status of bees (Münch *et al.*, 2008), while morphological and physiological markers provide information about their functional potential (Hatjina *et al.*, 2013). Fungicides alone or in combination with other chemicals, biological or environmental factors, can affect the biomarker profile, in doing so helping to understand the reactions triggered by them. The egg-yolk protein vitellogenin, for example, is nutrition regulated (Alaux *et al.*, 2011), is involved in ageing delay (Seehuus *et al.*, 2006) and immune regulation (Amdam *et al.*, 2004) and proved to be a good predictor for winter survival (Smart *et al.*, 2016). Other biomarkers have been proposed related to different functions of bee biology (De Smet *et al.*, 2017).

6.2.1.2 *In vivo* research

Working with little or full-size colonies provide a more holistic perspective of the colony as living being and enables the testing of for example, behavioural or developmental traits. *In vitro* rearing tests may show if a product or its combinations with other factors are dangerous for larvae development, but a test developed with the full colony enables to understand the interface queen-nurse-larve: the viability of the eggs laid by the queen, the capacity of nurse bees to feed larvae and the viability of the emerged bees. However, often new questions appear that lead back to *in vitro* studies.

An approach with promising potential may be the use of specific biomarkers to know the physiological status of colony bees or larvae. By combining an *in vitro* and *in vivo* approach, the use of biomarkers could provide a way to translate individual responses after exposure to stress factors obtained *in vitro* into colony level impact. With this logic, the impact of stress factors alone and the possible combinations among them could be described in biomarker terms. This could provide a diagnostic tool of the health status of bee colonies and the factors that could be affecting it. Actually, it is towards this approach that trends in bee research are leading, but we are far from having a ready-to-use tool yet.

6.2.2 Indirect effects of fungicides on bees

Fungicides may have an impact on the colony by modifying the existing microflora present in the food stores or in the bee intestinal tract (Batra *et al.*, 1973). Studies have already shown the possible modification of microbial composition both at beebread level (DeGrandi-Hoffman *et al.*, 2013; Yoder *et al.*, 2013) and at intestinal level (Anderson *et al.*, 2011). This modification in the composition of microbiota may lead to dysbiosis (Sartor, 2008). The impact that such an unbalance in the bee gut microflora may have on bee health has already been considered. The link between the

colony morbidity observed in our study and a possible microbial alteration could be subject of further research. Again here, the impact of fungicides alone or in combination with other factors could be studied from a perspective of describing the microbial imbalance, its impact on bees immunocompetence and possible favouring of pathogens, like *Nosema* spp (Pettis *et al.*, 2013).

In parallel, the potential impact of microbial modification on digestibility and availability of nutrients should be a target for further research. Indeed, the content of essential amino acids might be altered when beebread is contaminated with fungicides (DeGrandi-Hoffman, 2013, pers com.). Given the importance of nutrition, especially pollen, in the good development of the colony (Di Pasquale *et al.*, 2013) alterations in composition or lack of essential nutrients would put the homeostasis of the colony at stake. Some studies have already shown the impact of nutritional lack on bee development and health (Brodschneider and Crailsheim, 2010). Provided that pollen is the unique source of amino acids for honeybees, royal jelly production could also be affected (Liming *et al.*, 2009; Standifer, 1967) with unexpected potential consequences for its main consumers, larvae and the queen. A poor nutrition of the queen, could have as a result an impact in its activity. Likewise, a poor nutrition of the larvae has been shown to impact their development (Brodschneider and Crailsheim, 2010). As a result, the presence of fungicides on beebread and honey may have both a direct effect on their health, but also an indirect one on the colony development.

6.2.3 Final reflections: Working for the future

The problem of the exposure of bees to pesticides is not new and unfortunately its end does not seem to be close by. The research on the impact of fungicides, or pesticides in general, on bee health or their

interactions with other stress factors will most likely last forever. This is positive in the way that we will understand far better the physio-pathological underlying mechanisms involved and we will increase our knowledge on honey bee biology.

However, thinking bigger and positively into the future, the only way forward to improve the current pollinator crisis is that we, human beings, reduce our negative footprint on nature. Different concepts have been proposed historically to ground the need to protect nature due either its value to us humans or its inherent value, as are for example the conservation movement, which later evolved into ecosystems services approach. What seems evident is that, if we understand the level of dependance of the planet on pollination, we need to find ways to more efficiently feed the world population in a way that pollination potential is not hampered.

The development of intensive agriculture production has gone hand-in-hand with an alienation of food production from nature and a deterioration of water, air and soil quality. The negative externalities of this activity are now well described (Bourguet and Guillemaud, 2016; Pimentel and Burgess, 2014) and reveal a complicated problem that involves many stakeholders and economic interests. This means that the solutions are not straight forward because every stakeholder has a biased view of the problem. In this context, the only solution I can think of is to bring the people together with the objective to jointly look for workable solutions in the long-run. Nothing will change to improve the situation of bees in terms of pollutant exposure if farmers do not understand the impact of their practices and if they do not have applicable, affordable and sustainable alternatives.

As for recommendations about pesticide use, with a special focus on fungicides, the objective would be to minimise their presence in the environment in terms of quantity and time. Implementation and research on non-pesticidal alternatives should be favoured, as well as the use of non-persistent pesticides should alternatives not be available. Honey bees have

enough to manage without we human beings putting extra burden on their lives.

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